# Variation in the risk of developing sub-acute ruminal acidosis in lactating dairy cows

by

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## Abstract

Sub-acute ruminal acidosis (SARA) is a common metabolic disorder in high-producing dairy herds. Diet formulation strategies can be implemented to decrease the incidence of SARA, but some cows in a herd may still experience SARA. The overall objectives of this research were to evaluate if variation exists in the risk of developing SARA among lactating dairy cows and to determine factors associated with this variation. In study 1, as expected, variation was detected in the risk of developing SARA among lactating dairy cows when they were fed the same high-grain diet. Cows with lower risk of SARA (LS) sorted to a less extent but also had less chewing time compared with cows with higher risk of SARA (HS). In addition, higher milk urea nitrogen (MUN) concentration was found for LS cows compared with HS cows. Therefore, in study 2, it was further evaluated if MUN and milk fat content could be used as non-invasive indicators to identify cows with higher or lower risk of SARA. Results showed that minimum and mean rumen pH was higher, and duration of rumen pH below 5.8 was shorter for cows identified presumably with lower risk of SARA compared with ones identified presumably with higher risk. In study 3, other factors that might potentially relate to the variation in the risk of SARA among lactating cows were evaluated. Results showed that the microbial enzymatic activity for carbohydrate digestion in the rumen was not different between LS and HS cows. In addition, no difference was found in volatile fatty acid (VFA) absorption rate between LS and HS cows. But differences in relative mRNA abundance of genes involved in VFA metabolism in rumen epithelium among cows might be related to the variation in the risk of SARA. In addition, in study 3, it was found that HS cows identified in mid-lactation consistently had lower rumen pH in late-

lactation than LS cows. Feeding different types of carbohydrates may have a different effect on VFA absorption rate, which may play an important role in affecting the risk of SARA. Therefore, in study 4, effects of increasing dietary non-fibre carbohydrate content with starch, sucrose or lactose on VFA absorption, rumen fermentation, and milk production of mid-lactating dairy cows were evaluated. It was found that feeding high disaccharide diets to lactating dairy cows increased dry matter intake, milk fat and protein yields, and energy corrected milk yields compared with a high starch diet even though rumen pH was lower for cows fed high disaccharide diets. But, not as expected, the treatment effects on rumen pH could not be attributed to VFA absorption rate. In summary, substantial variation exists in the risk of developing SARA among lactating dairy cows fed the same high-grain diet. This variation may not be caused by chewing activity, microbial enzymatic activities and VFA absorption rate, but it may be associated with differences in sorting behaviour and expression of genes involved in VFA metabolism in rumen epithelium among the cows.

## Preface

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X. Gao involved in experimental designs, conducted animal experiments, lab work, data analysis and draft of manuscripts; M. Oba designed experiments, contributed to data analysis and manuscript writing.

All the experimental protocols (Chapters 2, 3, 4, 5) were reviewed and approved by the University of Alberta Animal Care and Use Committee for Livestock (protocol ID AUP00000748 for Chapter 2, 3, 4, and protocol ID AUP00000580 for Chapter 5), and all the procedures were conducted according to the guidelines of the Canadian Council of Animal Care (Ottawa, Ontario, Canada).

## Dedication

I dedicate this thesis to my parents for supporting and encouraging me throughout this degree.

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## Table of contents

## List of tables

## List of figures

## List of abbreviations

| | |
|---|---|
| ACAA | Acetyl-CoA acyltransferase |
| ACAT | Acetoacetyl-CoA thiolase |
| Acetyl-CoAs | Acetyl-CoA synthetase 2 |
| Acyl-CoAs | Acyl-CoA synthetase short-chain family member 1 |
| ACTB | β-Actin |
| AE | Anion exchanger |
| BCS | Body condition score |
| BDH | β-Hydroxybutyrate dehydrogenase |
| BW | Body weight |
| BHBA | β-hydroxy-butyric acid |
| CA | Carbonic anhydrase |
| CLDN | Claudin |
| CP | Crude protein |
| DIM | Days in milk |
| DM | Dry matter |
| DMI | Dry matter intake |
| DRA | Down-regulated in adenoma |
| ECM | Energy corrected milk |
| FDPS | Farnesyl diphosphate synthase |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| HMGCL | 3-hydroxy-3-methylglutaryl-CoA lyase |
| HMGCR | 3-hydroxy-3-methylglutaryl-CoA reductase |

| | |
|---|---|
| HMGCS | 3-hydroxy-3-methylglutaryl-CoA Synthase |
| HP | Haptoglobin |
| HS | Higher risk of sub-acute ruminal acidosis |
| JAM | Junctional adhesion molecule |
| LPS | Lipopolysaccharide |
| LS | Lower risk of sub-acute ruminal acidosis |
| LSS | Lanosterol synthase |
| MCT | Monocarboxylate transporter |
| MUN | Milk urea nitrogen |
| $Na^+/K^+$ ATPase | Sodium/Potassium transporting ATPase alpha 1 polypeptide |
| NDF | Neutral detergent fibre |
| NEFA | Non-esterified fatty acids |
| NFC | Non-fibre carbohydrate |
| NHE | Sodium hydrogen exchanger |
| NSC | Nonstructural carbohydrate |
| OCLN | Occludin |
| OM | Organic matter |
| PAT | Putative anion transporter |
| peNDF | Physically effective neutral detergent fibre |
| RPLP0 | Ribosomal protein, large P0 |
| SAA | Serum amyloid A |
| SARA | Sub-acute ruminal acidosis |
| SREBP | Sterol regulatory element-binding protein |

| | |
|---|---|
| TLR | Toll-like receptor |
| TMR | Total mixed ration |
| VFA | Volatile fatty acid |
| $vH^+$ATPase | Vacuolar $H^+$ ATPase subunit B |
| ZO | Zonula occluden |

# Chapter 1. Literature review

## 1.1 General review of sub-acute ruminal acidosis

Milk production of dairy cows has increased over three times in the last decades. In order to maximize milk production, highly fermentable diets are often fed to high producing cows. Volatile fatty acids (VFA) from dietary carbohydrate fermentation in the rumen are the primary energy substrate for cows (Bergman, 1990). However, excessive consumption of highly fermentable carbohydrates and lack of physically effective fibre can cause accumulation of acids in the rumen (Owens et al., 1998; Stone, 2004), which increases the risk of the metabolic disorder - ruminal acidosis. When acid production rate becomes higher than acid removal from the rumen, ruminal acidosis occurs (Allen, 1997). Depending on the severity of rumen pH decrease, ruminal acidosis can be classified as either acute or sub-acute ruminal acidosis.

### 1.1.1 Definition

Acute ruminal acidosis is defined as the condition whereby rumen pH is lower than 5.2 (Owens et al., 1998). It typically occurs when a ruminant consumes excess rapidly fermentable carbohydrates, resulting in a sudden drop in rumen pH (Stone, 2004). Increased lactic acid concentration is associated with acute ruminal acidosis, which further decreases rumen pH (Owens et al., 1998). Clinical signs of acute ruminal acidosis include anorexia, diarrhea, systemic acidosis, and possibly death (Owens et al., 1998).

Definition of sub-acute ruminal acidosis (SARA) varies among researchers, but pH thresholds commonly used for SARA range between 5.5 and 5.8 (Krause and Oetzel, 2006; Penner et al., 2007) and include certain duration below the threshold, such as rumen pH < 5.6 for more than 3 h (Gozho et al., 2005). Zebeli and Metzler-Zebeli (2012)

recently suggested that SARA could be defined as rumen pH lower than 5.8 for at least 6 hours per day because this threshold resulted in a decrease in fibre digestibility and an elevation in plasma acute phase proteins. Unlike acute acidosis, SARA is resulted from the accumulation of VFA rather than lactic acid, and ruminants experiencing SARA often do not have clinical signs (Krause and Oetzel, 2006). Acute acidosis does not often occur on modern dairy farms, whereas the prevalence of SARA is widespread. It was shown that SARA occurred in 19% early-lactation cows and 26% mid-lactation cows on farms in the United States (Garret et al., 1997). Another survery suggested that 13.8% of lactating cows across all stages of lactation experienced SARA on 18 dairy farms in Netherlands (Kleen et al., 2009). Also, O'Grady et al. (2008) indicated that almost half cows had moderate to severe SARA from 12 grazing herds in Ireland. Rumenocentesis technique was used in these filed studis to collect rumen fluid samples for measuring rumen pH, and rumen pH 5.5 was used as the threshold of diagnosis SARA. SARA can cause large economic losses for the dairy industry, because it is related to decreased dry matter intake (DMI) (Kleen et al., 2003), decreased fibre digestion (Russell and Wilson, 1996), milk fat depression (Kleen et al., 2003), diarrhea, and laminitis (Nocek, 1997). Stone (1999) estimated that US$400 was lost per affected cow per year due to SARA, which was about US$1.12 loss per affected cow per day (Enemark, 2009). The calculation of financial loss was based on 2.7 kg/d milk production loss, 0.3% milk fat content loss and 0.12% milk protein content loss (Stone, 1999; Plaizier et al., 2008). Therefore, methods to prevent and manage SARA are important for dairy producers to improve feeding efficiency and health for dairy cows.

### 1.1.2 SARA and acid base balance

Ruminants use VFA as the primary energy source, and 50 to 85% of VFA produced in the rumen is absorbed into rumen epithelial cells (Bergman, 1990; Kristensen et al., 1998). Then VFA and its metabolites can be transported from the rumen epithelial cells into the blood. When VFA get into the blood, there are a few systems coordinated to maintain the acid-base balance of the animal, as acid-base balance is critical to maintain blood pH and the animal's health state (Fetmann 2007). The chemical buffering system, $HCO_3^-$: $H_2CO_3$, can respond immediately to neutralize the acid. Also, the animal can increase its respiration rate to exhale $CO_2$ to increase blood pH. In addition, the kidney can regulate the amount of excreted acid and reabsorbed $HCO_3^-$ to affect the blood pH. Finally, the animal can resorb carbonate and phosphate buffers stored in the bones into the blood to improve the existing chemical buffer system (Houpt 1993).

Volatile fatty acids accumulated in the rumen result in SARA. Normally only acetate can reach the peripheral blood stream, because approximately 90% of butyrate is metabolized to beta-hydroxybutyrate (BHBA) (Bergman, 1990) after it is absorbed into the rumen epithelial cells, and most propionate is converted to glucose in the liver. Therefore, it is assumed that VFA do not accumulate in blood sufficiently to cause the decrease of blood pH (Owens et al., 1998). Li et al. (2012) indicated that blood pressure of $CO_2$ was increased but blood pH was not affected by SARA challenges. Similar response of blood $CO_2$ pressure to SARA challenge was detected in another study (Gianesella et al., 2010). The increased blood pressure of $CO_2$ might not be high enough to cause systemic acidosis, and it was suggested that SARA might not change blood homeostasis although some minor changes related to systemic acid base status might be associated with SARA (Li et al., 2012). However, Goad et al. (1998) and Brown et al.

(2000) found that blood pH and $H_2CO_3$ concentration was reduced in grain-challenged beef steers. A decrease in blood pH can occur if there is an increase in lactate concentration in the blood (Owens et al. 1998; Brown et al. 2000). Therefore, Owens et al. (1998) suggested that systemic acidosis could be caused by acute ruminal acidosis, mainly due to the increased rumen lactic acid moving into blood and thus overwhelming the buffering system.

### 1.1.3 SARA and acute phase response, large intestine acidosis

Inflammation can occur in different organs and tissues when dairy cows experience SARA, such as kidney and heart inflammation, and liver abscesses (Oetzel, 2000; Kleen et al., 2003). The reasons behind this inflammation are believed due to VFA accumulation in the rumen and low rumen pH during SARA, leading to a parakeratosis of the rumen epithelium (Nocek, 1997; Kleen et al., 2003). Thus, damage occurs to the barrier function of rumen epithelium, which enables endotoxins or microbes to move into the blood from the rumen and then spread to the liver and other organs in the body, resulting in a systemic immune response (Nordlund et al., 1995; Nagaraja and Titgemeyer, 2007).

Previous studies have shown that SARA induced by high-grain diets was consistently associated with the increases of the endotoxin - lipopolysaccharide (LPS) in the rumen of dairy cows (Khafipour et al., 2009a; Zebeli and Ametaj, 2009). Cell wall of gram-negative bacteria contains LPS (Plaizier et al., 2008). When these bacteria die or grow rapidly, LPS is released or shed into the rumen (Andersen et al., 2000; Plaizier et al., 2009). Moreover, grain-induced SARA can also cause an increase in blood acute phase proteins, such as LPS-binding protein (LBP) (Khafipour et al., 2009a; Zebeli and Ametaj,

2009), haptoglobin (Hp) and serum amyloid A (SAA) (Khafipour et al., 2006; Gozho et al., 2007; Khafipour et al., 2009a). Acute phase proteins increase in blood when animals are injured or during infection, stress, and inflammation, all of which are indicators of a systemic immune and inflammatory response (Baumann and Gauldie, 1994; Cray et al., 2009). Thus, the increases of acute phase protein during SARA may indicate that LPS is translocated from the digestive tract into blood (Khafipour et al., 2009a; Zebeli and Ametaj, 2009). Khafipour et al., (2009c) found that grain induced SARA caused an increase of the abundance of *Escherichia coli* in rumen fluid, and they suggested that LPS released by *Escherichia coli* may mainly contribute to the LPS pool and inflammation. In addition, it should be noted that other toxins in rumen fluid, such as biological amines, might also contribute to initiation of the immune response (Plaizier et al., 2008).

Khafipour et al. (2009a,b) administrated two acidosis challenge models in lactating dairy cows, and found that both models caused an increase in free LPS in the rumen. However, SARA induced by substituting alfalfa hay with alfalfa pellets did not increase blood SAA and Hp, while both of them were increased during SARA induced by substituting alfalfa hay with wheat-barley pellets. Thus, they suggested that the systemic inflammation associated with SARA is probably due to the translocation of endotoxins produced by intestinal bacteria rather than that produced by rumen bacteria (Khafipour et al., 2009a,b). Li et al. (2012) found that cecal concentration of LPS only increased in response to grain induced SARA, although concentration of LPS in the rumen increased in both alfalfa pellets induced and grain induced SARA. They proposed that the translocation of LPS from the large intestine might account for the responses of acute phase proteins for cows with grain induced SARA.

The large intestine is also a location that carbohydrate fermentation happens. Large intestine fermentation accounts for small part of total-tract nutrient digestion (5 to 10% of dietary energy) (Gressley et al., 2011). But during ruminal acidosis, increased flow of fermentable carbohydrates from the rumen can cause excessive carbohydrate fermentation and acid accumulation in the large intestine (Hall, 2002; Plaizier et al., 2008), which decreases pH, increases VFA and LPS, and causes damage to the intestinal mucosa (Dijkstra et al., 2012; Li et al., 2012). There are four layers of cells in rumen epithelium, in which two middle layers have the structure of tight junctions; in contrast, there is only one layer of cells in the large intestine epithelium (Graham and Simmons, 2005). Due to these differences in epithelium structure, increased VFA and LPS may cause damage to the barrier function more easily for the epithelium of large intestine than that of the rumen (Li et al., 2012). In addition, rumen has the supply of saliva to buffer the acids whereas large intestine does not, which may also make the large intestinal mucosa more susceptible to the damage (Gressley et al., 2011). However, two recent studies did not detect responses of acute phase proteins in the blood when corn starch and oligofructose were infused into abomasum to induce increased hindgut fermentation in nonlactating cows and steers (Bissell and Hall, 2010; Mainardi et al., 2011). The authors suggested that lack of response in acute phase proteins might be due to the relatively short challenge periods.

### 1.1.4 SARA and rumen microorganisms

The rumen hosts a large and varied population of microorganisms, mainly consisting of four categories: bacteria, protozoa, fungi and archaea. These microbes live interdependently and involve multiple metabolic pathways (Brulc et al., 2009; Zebeli and

Metzler-Zebeli, 2012). Rumen pH is one critical factor affecting these microbes. A decrease in rumen pH during SARA can cause changes in the microbial population and shifts in the microbial profile, such as an increase in the population of gram-positive bacteria and reduce in the population of gram-negative bacteria (Nagaraja and Titgemeyer, 2007; Chen et al., 2012).

Fibre digestion usually decreases during SARA, as bacteria digesting fibre are sensitive to the change of rumen pH (Shi and Weimer, 2002). *In vitro* studies have shown that the population of main cellulolytic bacteria decline rapidly as pH decreased below 6.0 (Russell and Wilson, 1996). In addition, Fernando et al. (2010) found that the population of two primary fibre digesters, *Butyrivibrio fibrisolvens* and *Fibrobacter succinogenes*, reduced when beef steers were adapted to high grain diets. Low rumen pH also inhibits the activity of cellulolytic bacteria in the rumen (Mouriño et al., 2001; Zebeli and Metzler-Zebeli, 2012). In contrast, the proportion of bacteria that ferment starch and sugar can increase up to 90 to 95% of the total cultural bacteria in grain-fed ruminants (Leedle and Hespell, 1980). Goad et al. (1998) detected that total number of amylolytic bacteria was higher for steers fed high concentrate diet compared with steers fed alfalfa hay. Mao et al. (2013) also found more abundance of *Firmicutes* at the phylum level for dairy cow induced with SARA compared with the control group. The phylum of *Firmicutes* is mainly consisted of gram-positive bacteria, which may indicate that SARA inducement increased the bacterial species that metabolize fermentable carbohydrates (Mao et al., 2013). In addition, the population of bacteria that produce lactic acid, such as *Streptococcus bovis*, also increased during SARA. But majority of the lactic acid can be metabolized by lactate-utilising bacteria, such as *Megasphaera elsdenii* and *Selenomonas*

*ruminantium* in the rumen (Stone, 2004; Fernando et al., 2010). Therefore, it is commonly suggested that lactate does not accumulate in the rumen fluid during SARA (Goad et al., 1998).

Similar to fibre digesting bacteria, ciliated protozoa are also sensitive to fluctuations and decreases in rumen pH (Nagaraja and Titgemeyer, 2007). As such, there is a significant reduction in the numbers of ciliated protozoa during SARA (Nagaraja and Towne, 1990; Braun et al., 1992; Goad et al., 1998). As summarized by Zebeli and Metzler-Zebeli (2012), ciliated protozoa are able to moderate starch digestion (Nagaraja et al., 1992; Brossard et al., 2004), because they can engulf starch granules and sugars to isolate them from bacteria in the rumen, which decreases starch fermentation rate in the rumen and shifts starch to the lower part of digestive tract (Mendoza et al., 1993). In addition, ciliate protozoa can also engulf bacteria to reduce the population and activity of bacteria in the rumen, which moderates rumen fermentation rate (Nagaraja et al., 1992; Brossard et al., 2004). Therefore, ciliated protozoa have a "buffering" effect on rumen pH (Nagaraja and Titgemeyer, 2007). Rumen fungi are also sensitive to low pH (Orpin and Joblin, 1997; Zebeli and Metzler-Zebeli, 2012). Therefore, it is believed that the population of both protozoa and fungi would reduce during SARA.

Individual variation exists in the severity of ruminal acidosis among ruminants (Brown et al., 2000; Penner et al., 2009; Schlau et al., 2012) when animals were fed the same diet. Carbohydrate fermentation in the rumen is accomplished through the enzymes produced by the microbes. Therefore, it is possible that differences in the population and profile of rumen microbes that exists among animals may be related to the variation in the risk of ruminal acidosis. Chen et al. (2012) found that beef steers with lower risk of

SARA had lower bacterial density and diversity in rumen digesta than higher risk cattle. The lower bacterial density might indicate less active microbial fermentation, thus less VFA produced in the rumen of steers with lower risk of SARA compared with higher risk cattle (Chen et al., 2012). In addition, diversity of rumen epimural bacteria were also different between steers with lower and higher risk of SARA (Chen et al., 2012). Rumen epithelial epimural bacteria have a variety of functions necessary for the host, such as protection of rumen wall and facilitation of absorption (Freter, 1970). However, Mohammed et al. (2012) reported that the change of bacterial community composition before and after calving was not associated with the variation in the severity of acidosis among primiparous cows. Palmonari et al. (2010) also found that ruminal bacterial community composition was similar among dairy cows even though variation existed in rumen pH profiles when they were fed one diet. But Khafipour et al., (2009c) found that dairy cows with different responses to grain induced SARA had different phylum profiles in rumen microbiota composition. Phylum *Firmicutes* increased while phylum *Bacteroidetes* decreased for cows with severe risk of SARA during the SARA challenge compared with cows with mild risk. In addition, *Streptococcus bovis* and *Escherichia coli* were dominant for cows with severe risk of SARA, while *Megasphaera elsdenii* was dominant for cows with mild risk. The inconsistent findings in rumen microbiota composition might be partly related to different challenge models, and future study is warranted to fully understand if variation in the risk of SARA among ruminants is associated with differences in rumen microbial population and profile.

### 1.2 SARA and dietary carbohydrate source

Ruminal acidosis is the consequence of excessive carbohydrate fermentation in the rumen and inadequate rumen buffering by the lack of physically effective fibre (Oetzel, 2006), thus source of carbohydrates in the diet directly affects the risk of ruminal acidosis for ruminants. Nonstructural carbohydrates (NSC) and structural carbohydrates are broadly the two classifications of plant carbohydrates (NRC, 2001). The composition of carbohydrates is shown in figure 1.1 (adapted from NRC, 2001; Ishler and Varga, 2001).

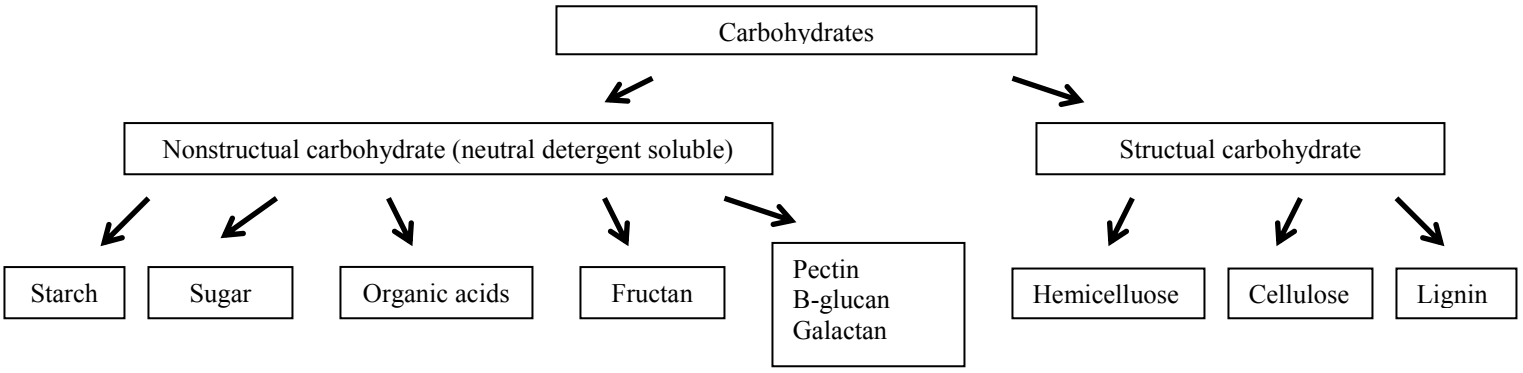


Figure 1.1 Composition of carbohydrates

### 1.2.1 Nonstructural Carbohydrates

Nonstructural carbohydrates are comprised of starch, sugar, fructans and organic acids (NRC, 2001). Pectins, B-glucans and galactans are in plant cell walls, but they are highly and rapidly fermented in the rumen, thus they are treated as NSC (Ishler and Varga, 2001). NSC is generally more digestible and ferments faster in the rumen than structural carbohydrates (NRC, 2001). Increasing NSC in the ration usually results in higher yields of VFA and microbial protein than structural carbohydrates, thus milk yield increases. However, excessive consumption of highly fermentable NSC can cause accumulated acid production and increase the risk of ruminal acidosis.

Starch is the major source of NSC in the diets of lactating cows (NRC, 2001) due

to its high energy density and relatively low cost. However, the cost of cereal grains has increased rapidly over the last decade, thus including more byproducts with high sugar content in the dairy ration to replace part of the starch has become an approach to control feed costs while maintaining milk production. Sugars are soluble in water, and can be classified as monosaccharides and disaccharides. Monosaccharides consist of glucose, fructose and galactose, and disaccharides consist of sucrose, maltose and lactose (Oba, 2011). In a review, Oba (2011) extensively discussed the effects of feeding sugars on rumen fermentation and productivity of dairy cows. In general, partially replacing dietary starch with sugars could increase DMI and milk fat yield for lactating dairy cows (Broderick et al., 2008; Penner and Oba, 2009). One recent study also detected an increased milk fat yield and energy corrected milk yield for lactating cows when sucrose was used to replace part of the corn grain in the diet (Razzaghi et al., 2016).

Sugars are fermented rapidly in the rumen (Oba, 2011). The hydrolysis rates were between 248 - 1404 %/h for sugars (Weisbjerg et al., 1998). In addition, the fermentation rates varied from 264 to 738 %/h (Weisbjerg et al., 1998) for sugars, which is more rapid than starch fermentation rate (between 15 to 40%/h). Therefore, it was expected that rumen pH would be lower with feeding sugar than starch. However, previous studies showed that rumen pH did not decrease when high sugar diets were fed to lactating cows (DeFrain et al., 2004; Broderick et al., 2008; Chibisa et al., 2015). Oba (2011) summarized a few possible explanations, but the reasons are still not fully understood. *In vitro* studies have indicated that feeding sugar increases butyrate production (Vallimont et al., 2004; Ribeiro et al, 2005), but the results were not consistent under *in vivo* conditions (Oba, 2011). In a recent study, Oba et al (2015) found that ruminally dosed sucrose or

lactose increased molar proportion of butyrate in rumen fluid within 3 h after dosing compared with dosing starch, which suggested that feeding sugars might increase butyrate production. Chibisa et al (2015) also found an increased ruminal molar proportion of butyrate when starch was partially replaced with lactose for lactating cows. Butyrate infusion into the rumen was shown to stimulate the growth of rumen epithelia and increase VFA absorption (Malhi et al., 2013). Moreover, Storm et al. (2011) reported that infusion of butyrate into the rumen increased local blood flow to rumen epithelia and net portal flux of propionate, suggesting an increased VFA absorption rate. Indeed, Chibisa et al (2015) has shown that $Cl^-$-competitive absorption rate was increased for acetate and propionate when lactating cows were fed a high lactose diet compared with those fed high starch diets. There was a positive relationship between rumen pH and acetate and butyrate absorption capacity in rumen epithelium (Penner et al. 2009a). Therefore, lack of decrease in rumen pH might be due to the increased VFA absorption rate when partially replacing dietary starch with sugar.

Whey and molasses are byproducts with high sugar content commonly included in the diets for dairy cows, mainly containing lactose and sucrose respectively. Digestion of disaccharides in the rumen starts with the hydrolysis of disaccharides to monosaccharides, and is followed by the fermentation of monosaccharides (Oba, 2011). Hydrolysis and fermentation rates vary greatly among different types of sugar. It has been shown that lactose had slower hydrolysis rate than sucrose (540 vs. 1404 %/h; Weisbjerg et al., 1998). Galactose produced from the hydrolysis of lactose also had slower fermentation rate than glucose and fructose produced from the hydrolysis of sucrose (Sutton, 1968). Consistent with the slower hydrolysis and fermentation rates for lactose than sucrose, Weisbjerg et al.

(1998) reported that sucrose had the lowest pH after a single dose (1.0 kg each) of different types of sugar in the rumen, but lactose had the highest pH after the dose and a slowest decrease in rumen pH. In addition, Oba et al. (2015) also found that sucrose treatment decreased rumen pH to a greater extent than lactose at 3 hour after ruminally dosed sucrose or lactose into the rumen of non-lactating dairy cows. Thus, diets including different types of sugars (lactose or sucrose) may have variable effect on rumen fermentation and milk production for lactating cows. But no study has been conducted to directly compare the responses of milk production and rumen fermentation when sucrose and lactose are fed to lactating dairy cows.

### 1.2.2 Structural Carbohydrates

Measuring neutral detergent fibre (NDF) is considered as the best method to separate structural and nonstructural carbohydrates (NRC, 2001). Structural carbohydrates are in the plant cell walls, which are required in ruminant diets to optimize production and rumen health by maintaining a stable rumen environment (Allen, 1997). Fibre has the capability to simulate chewing activity, which can promote the saliva buffer secretion to neutralize the acids in the rumen, thus elevating rumen pH (Stone, 2004). In addition, dietary fibre may also affect VFA absorption by increasing gut fill, which stimulates rumen motility, thus exposing more VFA to the rumen wall (Allen, 1997).

Due to different physical and chemical traits, fibre from different sources has different capability to stimulate chewing (Mertens, 1997). Therefore, physical effective NDF (peNDF) was proposed by Mertens (1997) to better describe the capability of fibre to stimulate chewing by considering both physical (particle size) and chemical (NDF content) characteristics of fibre. The Penn State Particle Separator is widely used on farm

to determine peNDF due to its convenience and efficiency in measuring dietary particle size (Lammers et al., 1996). Yang and Beauchemin (2006b) evaluated a few different methods, and suggested that the sum of the proportion on the 19 mm and 8 mm sieves multiplied by the dietary NDF content was the most appropriate approach to calculate peNDF in rations (Lammers et al., 1996).

Increasing dietary peNDF can reduce the risk of SARA. Beauchemin et al. (2003) showed that peNDF intake was positively correlated with rumination time and negatively correlated with the area of rumen pH below 5.8. Yang and Beauchemin (2007) found a strong correlation between duration of rumen pH below 5.8 with intake of particles on 19 mm screen and the sum of intake of particles on the 19 mm and the 8 mm screens. There are two approaches to increase dietary peNDF content: increasing the proportion of forage in the diet (elevate NDF content) and/or increasing the particle length of forage (Beauchemin and Yang, 2005). Compared with increasing the particle length, elevating dietary NDF content can also decrease the rumen fermentation rate by reducing dietary starch content (Beauchemin and Penner, 2009). Thus, Yang and Beauchemin (2007) suggested that it is more effective to reduce the risk SARA by elevating dietary NDF content compared with increasing particle length of forage. In addition, peNDF content in the basal diet also affects the responses of chewing activity and rumen pH to increased peNDF content. Beauchemin (2007) suggested that up to 7 h/d of chewing time was increased by 1kg increase of peNDF intake for diets with low peNDF content (below 10% DM), but 0 to 2 h/d of chewing time was increased by 1kg increase of peNDF intake for diets with high peNDF content (over 20% DM). Therefore, increasing peNDF content can be more effective in increasing chewing time for low forage diets than for diets already

having enough forage. Plaizier et al (2008) also suggested that when dietary peNDF is greater than 16.5% DM basis, the effect of increasing in peNDF content of diets on rumen pH might be absent.

Increasing dietary peNDF content can increase chewing time, but daily saliva production is not always increased to the expected extent (Beauchemin, 2007). The reason is that increased saliva secrection during chewing reduces resting saliva secrection (Beauchemin, 2007), thus saliva production decreases during resting although increased chewing time can increase saliva production. Therefore, Yang and Beauchemin (2007) suggested that increased rumen pH by increased intake of peNDF is not necessarily due to the increased chewing time. As Beauchemin and Penner (2009) summarized, increased peNDF intake may also decrease meal size and eating rate (Beauchemin, 2007), cause starch digestion to move from rumen to the lower part of the digestive tract (Yang and Beauchemin, 2006b), and increase rumen contractions, thus stimulating VFA absorption as well as VFA passage from the rumen (Yang and Beauchemin, 2007). All of these factors may also help to reduce the risk of SARA.

### 1. 3 Variation in the risk of developing SARA among ruminants

Even though diet formulation strategies have been extensively studied to reduce the incidence of SARA, SARA may still occur for some cows in a herd. It has been shown that there is large variation in the risk of developing ruminal acidosis among sheep (Penner et al., 2009), primiparous dry cows (Penner et al., 2007) and beef steers (Brown et al., 2000; Schlau et al., 2012) when animals within a study were fed the same diet. Brown et al. (2000) dosed steam-flaked corn into the rumen of 5 beef steers, and found the range of mean rumen pH was between 4.26 and 5.63. In addition, Penner et al. (2009)

found that area below rumen pH 5.8 ranged from 0.0 to 84.0 pH×min/d among 24 sheep when the sheep were orally drenched the same dose of glucose. Schlau et al. (2012) also found that the range of mean rumen pH was from 5.44 to 6.13 among 17 beef steers when they were fed a diet with 85% grain. These studies clearly indicated that substantial individual variation exists in the risk of developing SARA among ruminants even when they were fed the same diet.

### 1.4 Factors regulating rumen pH

Balance between acid production and acid removal determines rumen pH (Allen, 1997). When the balance is not achieved (production > removal), SARA may occur. The uptake through rumen epithelium, neutralization by buffers, and passage from rumen to lower part of digestive tract are the three primary pathways of acid removal (Allen, 1997). Therefore, the variation in the risk of developing SARA among ruminants can be related to one or more of these factors (Penner et al., 2009a).

#### 1.4.1 VFA production

Monogastric animals obtain most of their energy from glucose absorbed directly in the intestines. However, for ruminants, VFA are the primary energy source, which are produced by microbial fermentation of organic matter (OM) in the rumen. In addition, OM fermentation also provides energy for rumen microbial maintenance and growth. The primary VFA produced in the rumen are acetate, propionate and butyrate. The range of molar proportion is from 45 to 70% for acetate, 15 to 40% for propionate and 5 to 20% for butyrate in the rumen fluid (Bergman, 1990; Kristensen et al., 1996; Udén, 2010). These three VFA generally account for more than 95% of all fermentation acids, and the total concentration ranges from 60 to 150 mmol/L in the rumen fluid (Bergman, 1990).

Concentration is determined by production and removal, and due to the variation in buffer neutralization and absorption in the rumen, the relationship between rumen pH and concentration of VFA in rumen fluid is not strong ($r^2 = 0.13$, $P < 0.001$; Allen, 1997).

Isotopic tracer technique is the major approach for measuring fermentation acid production in the rumen (Martin et al., 2001; Sutton et al., 2003). But this method has some limitations, such as possible estimate error resulting from carbon sequestration in rumen microbes (Kristensen, 2001) and microbial carbon interconversion of VFA (Sutton et al., 2003). Using the isotopic tracer technique, rate of VFA production in sheep was measured between 8.8 and 14.8 mol/d when they were fed at 45% and 95% ad libitum intake, respectively (Martin et al., 2001). Sutton et al. (2003) measured total VFA production for Holstein dairy cows fed moderate (40%) and high (90%) concentrate diets at approximately DMI of 13 kg/d, and found that total net VFA production was 79.8 and 90.0 mol/d, respectively. Even though each individual VFA can release one $H^+$, the quantity of acid produced from fermenting one unit of hexose can be different due to the different types of VFA produced from fermentation (Penner et al., 2011). It was indicated that fermentation of 1 mol of glucose could produce 2 mol of acetate, 2 mol of propionate, or 1 mol of butyrate (Bannink et al., 2006). Therefore, fermentation into butyrate rather than acetate or propionate may produce less acid (Penner et al., 2011).

Feed intake and dietary composition, such as carbohydrate fermentability, carbohydrate digestion rate and proportion of NDF and forage NDF, affects total VFA production and the molar proportions of individual VFA (Allen, 1997; Stone, 2004; Bannink et al., 2008). The extent of carbohydrate fermentation in the rumen is variable among feedstuffs. Allen (1997) suggested that this variation is only partially due to feed

characteristics, but it is highly influenced by interactions among the diet, the animal, and rumen microbes, which eventually affects microbial activity and the retention time in the rumen. Indeed, degradation and metabolism of carbohydrates in the rumen is accomplished through the action of enzymes produced by the microbes. Also, previous studies have shown that passage rate of digesta affects feed degradability (Robinson et al., 1987) and microbial efficiency (Sniffen and Robinson, 1987). Orskov and McDonald (1979) suggested that higher passage rate leads to less degradability. Therefore, rumen microbial activity and digesta passage rate from the rumen directly affect carbohydrate fermentation and VFA production in the rumen. In addition, efficiency of microbial fermentation (microbial yield/ kg of ruminally fermented OM) also affects the amount of acid production (Allen, 1997). Allen (1997) suggested that diets formulated to maximize microbial yield should decrease the acid production and might reduce the incidence of ruminal acidosis.

Sorting feed against long particles and for short particles is commonly found for dairy cows even when a TMR is fed (Kononoff et al., 2003; Leonardi and Armentano, 2003; DeVries et al., 2007). The consequence of sorting is that cows consume more highly fermentable carbohydrates and less peNDF compared to the expected intake, thus may increase the risk of SARA due to the possible increased VFA production (Cook et al., 2004; Stone, 2004; DeVries et al., 2008). It has been shown that lower rumen pH was associated with more sorting for medium and short particles when a diet with 45% forage was fed to early-lactation cows (DeVries et al., 2008). On the other hand, ruminal acidosis can cause cows to alter their sorting behaviour. It has been suggested that dairy cows would choose feeds with high buffering capacity (long forage particles) to alleviate the

effects of very low rumen pH (Beauchemin and Yang, 2005; Yang and Beauchemin, 2006). Keunen et al. (2002) also showed that lactating dairy cows induced with SARA increased their preference for long alfalfa hay rather than pelleted alfalfa. In addition, a few studies have shown that individual variation also exists in sorting behaviour among dairy cows even fed the same diet (Leonardi and Armentano, 2003; Leonardi et al., 2005; Leonardi and Armentano, 2007). Therefore, it is possible that variation in sorting behaviour among cows is one of the factors that cause the variation in rumen pH and severity of SARA.

Acid production in the rumen is a primary factor affecting rumen pH. Chen et al. (2012) suggested that lower bacterial density in the rumen digesta for steers with lower risk of SARA might indicate less VFA produced in the rumen compared with higher risk ones. Therefore, differences in acid production from rumen OM fermentation might be related to the variation in the severity of SARA among steers. Factors related to VFA production in the rumen, such as feed intake, microbial enzyme activity, rumen digesta passage rate, microbial yield and sorting behaviour, warrant further research to understand if VFA production contributes the individual variation in the risk of SARA among ruminants.

### 1.4.2 VFA absorption

Absorption of VFA across the rumen wall plays a critical role in removing the acid from the rumen and buffering rumen pH to maintain the optimal rumen environment. Allen (1997) estimated that VFA absorption contributes approximately 53% of the total proton removal from the rumen. There are two key pathways of VFA absorption: passive diffusion and protein-mediated transport. Passive diffusion is the main absorption

pathway for undissociated VFA (Gäbel et al., 2002), while the dissociated VFA absorption is mainly via the transport protein mediated pathway (Aschenbach et al, 2011). In addition, there are two different protein-mediated transport pathways, bicarbonate-dependent and bicarbonate-independent but sensitive to nitrate (Penner et al., 2009a). Bicarbonate-dependent pathway is the main pathway, which could contribute up to 50% of VFA absorption (Penner et al., 2009a). Bicarbonate-dependent transport includes an anion exchange system in rumen epithelial cells whereby dissociated VFA are exchanged for bicarbonate $HCO_3^-$, providing a source of bicarbonate to the rumen environment where it can neutralize protons and increase rumen pH (Aschenbach et al., 2009). The amount of VFA absorption that occurs via these different mechanisms depends on the type of VFA. As Penner (2014) summarized, 42 to 57% of acetate and 24 to 46 % of butyrate was absorbed via bicarbonate-dependent transport *in vitro*, while 29 to 59% of acetate and 25 to 76% of butyrate was absorbed via passive diffusion (Penner et al., 2009a; Schurmann, 2013). Gäbel et al. (1991) suggested that the bicarbonate-dependent mechanism accounts for approximately 52% of the total acetate uptake. As butyrate is more lipophilic than propionate and acetate (Sehested et al., 1999), butyrate has the greater potential for passive diffusion through epithelial cell membrane (Walter and Gutknecht, 1986). Therefore, protein-mediated pathways contribute more to acetate uptake than the passive diffusion pathway (Aschenbach et al., 2009; Penner et al., 2009a), while more butyrate is absorbed via passive diffusion compared with protein-mediated pathways (Penner et al., 2009a). In addition, similar to acetate, propionate is expected to have higher proportion of absorption via bicarbonate-dependent transport than the passive diffusion (Aschenbach et al., 2009).

When undissociated VFA molecules are absorbed into epithelial cells, they are dissociated to $VFA^-$ and $H^+$ in the cytosol (Aschenbach et al., 2011). The dissociated VFA can be oxidized within the cell as an energy source for the epithelial cell. They can also be metabolized into ketone bodies (Rémond et al., 1995) or cholesterol (Steele et al., 2011). But the proportion of metabolism is not the same among different types of VFA. It was estimated that 30% of acetate, 50% of propionate and 90% of butyrate is metabolized in rumen epithelial cells (Bergman, 1990). Metabolism of butyrate is mainly via ketogenesis pathway into BHBA (Rémond et al., 1995). Dissociated VFA and the metabolites, such as lactate and BHBA, in the epithelial cells can cross the basolateral membrane into the bloodstream, and are subsequently metabolized by the liver or peripheral tissues (Bergman, 1990). As for the $H^+$ released in the epithelial cells, some are neutralized by $HCO_3^-$ (Gäbel and Sehested, 1997). In addition, to maintain intracellular pH, excess protons are recycled back to the rumen or transported to extracellular space (Aschenbach et al., 2011). Therefore, when undissociated VFA is absorbed into the rumen epithelial cells via passive diffusion, if the proton is exported back into the rumen, there would be no net proton removal from the rumen.

Absorption of VFA into rumen epithelial cells and regulation of intracellular pH involves a variety of ion exchangers and transporters on both apical and basolateral membrane, such as $Na^+$ (Gäbel et al., 1991; Etschmann et al., 2009), $K^+$ (Leonhard and Marek et al., 2007), $HCO_3^-$ (Aschenbach et al., 2009; Penner et al., 2009a). When undissociated VFA is absorbed into rumen epithelial cells, the released proton stimulates $Na^+$ transport into the cells via $Na^+/H^+$ exchange (NHE) proteins (Gäbel et al., 1991). Three isoforms of NHE proteins have been identified in the rumen epithelial tissue:

NHE1, NHE2, and NHE3 (Graham et al., 2007). These exchange proteins together regulate $Na^+$ transport and intracellular pH (Gäbel et al., 2002; Graham et al., 2007). In addition, there is also $Na^+$ and $K^+$ exchange on the basolateral membrane of the rumen epithelial cells via the $Na^+/K^+$-ATPase pump (Aschenbach et al., 2011). When dissociated VFA is transported into rumen epithelial cells, $HCO_3^-$ can be secreted into the rumen. This process is regulated via $VFA^-/HCO_3^-$ exchange proteins, including putative anion transporter 1 (PAT1) and downregulated in adenoma (DRA) on the apical membrane, and anion exchanger 2 (AE2) on the basolateral membrane (Bilk et al., 2005). In addition, after VFA is absorbed, active VFA metabolism occurs in the epithelial cells, which is regulated by a variety of different enzymes, such as 3-hydroxy, 3-methylglutaryl CoA synthase (HMGCS) (Baldwin, 1998). VFA can be converted to 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) by HMGCS1 in the cytoplasm, and then goes into the pathway of cholesterol biosynthesis (Steele et al., 2012). In the mitochondria, VFA can be converted to HMG-CoA by HMGCS2, and then goes into the pathway of ketogenesis (Penner et al., 2011; Steele et al., 2012). Increased VFA absorption into the rumen epithelial cells can cause changes in the expression of genes of the enzymes that relate to VFA metabolism (Penner et al., 2011). Current understanding of ion transporters associated with VFA absorption and intracellular pH regulation, and enzymes related toVFA metabolism at mRNA and protein expression levels has been summarized in a few published review papers (Connor et al., 2010; Aschenbach et al., 2011; Penner et al., 2011).

Figure 1.2 shows a summary of VFA transportation and metabolism in ruminal epithelium that is described above.

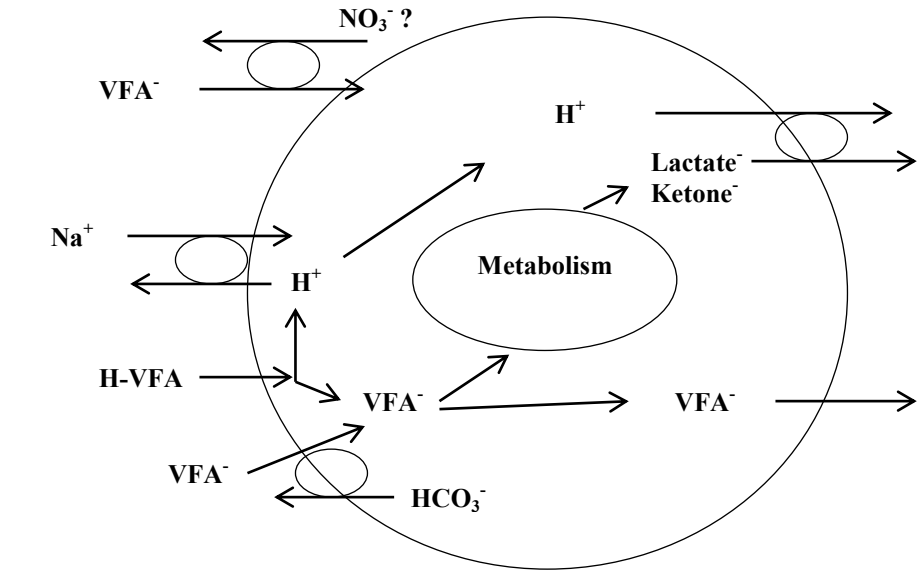


**Figure 1.2** Pathways of VFA transportation in ruminal epithelium (adaped from Aschenbach et al., 2011)

When SARA occurs, VFA production increases and pH decreases in the rumen, leading to an increased VFA gradient across the rumen epithelium. As rumen pH decreases, proportion of the undissociated form of VFA increases. It has been shown that undissociated VFA across the rumen epithelium increased via passive diffusion when rumen pH decreased in sheep and bovine (Gabel and Sehested, 1997; Schurmann et al., 2014). In addition, diets with higher fermentability can cause morphological changes in the rumen epithelium. Previous studies have reported that increasing dietary fermentability enhanced the rate of cellular proliferation and papillae growth, thereby increasing surface area of absorption (Dirksen and Mayer, 1985; Shen et al., 2004) and further increasing absorption capacity. Among the different types of VFA, butyrate has been reported to be the most potent one in stimulating epithelial proliferation (Blottière et al., 2003). Absorption activity of rumen epithelial cells also changes when high grain diets are fed (Penner et al., 2011). Increases in gene expression have been observed for

transporters and enzymes associated with VFA absorption, VFA metabolism and intracellular pH regulation in ruminal epithelium when highly fermentable diets are fed (Penner et al., 2009b; Penner et al., 2011; Steele et al., 2012). In addition, changes in absorption activity for the rumen epithelium occur more rapidly than the morphological adaptation (Etschmann et al., 2009; Penner et al., 2011). Together, feeding high grain diets can lead to an increase in rumen surface area of absorption (Aschenbach et al., 2011; Penner et al., 2011), an elevation of rumen epithelial absorption activity (Etschmann et al., 2009), and a decrease in rumen pH (Dijkstra et al., 1993), all of these can further result in increased VFA absorption rate. However, excessive exposure of the rumen epithelium to acidic condition can also reduce the capacity of absorption (Schwaiger et al., 2013; Wilson et al., 2012) and damage the barrier function (Penner et al., 2010; Wilson et al., 2012) for ruminal epithelium.

It was suggested that VFA absorption contributes roughly 53% of the total proton removal from the rumen in dairy cows (Allen, 1997). Thus, it is possible that difference in VFA absorption rate exists among the cows even when they are fed the same diet, which may cause the variation in the risk of SARA. Penner et al. (2009a) orally administered glucose to sheep to induce SARA, and measured the capacity of VFA absorption across the ruminal epithelia by using Ussing chambers. They found that sheep with lower risk of SARA had greater capacity of bicarbonate independent uptake of butyrate and tended to have greater capacity of bicarbonate dependent uptake of acetate. Schlau et al. (2012) showed that the mRNA abundance of sodium hydrogen exchanger, isoforms 3 (NHE3) in rumen epithelial cells was greater for steers with lower risk of SARA than those with higher risk, which suggested that lower risk steers might have higher VFA absorption rate

through the pathway of passive diffusion. Therefore, difference in VFA absorption rate may be one of the key factors causing the individual variation in the risk of SARA among ruminants.

### 1.4.3 Neutralization

Lactating dairy cows produce large amount of saliva, approximately 98 to 190 L/d (Bailey, 1961). Saliva is an important mechanism to neutralize acids produced from rumen fermentation, because it is rich in $HCO_3^-$ and $HPO_4^{2-}$ (Church, 1988; Van Soest, 1994). Owens et al. (1998) suggested that saliva contributed approximately half of the bicarbonate into the rumen. Allen (1997) suggested that salivary buffer neutralization accounts for approximately 37% of the total proton removal from the rumen. The concentration of $HCO_3^-$ and $HPO_4^{2-}$ was measured at 126 and 26 meq/L saliva, respectively, when saliva was collected from four dry Shorthorn cows (Bailey and Balch, 1961). Therefore, saliva $HCO_3^-$ has greater buffering capacity and is more important than $HPO_4^{2-}$ for the cow (Bailey and Balch, 1961).

As saliva composition does not change much by diet or secretion rate (Bailey and Balch, 1961), amount of saliva secreted determines the saliva buffering capacity. Saliva secretion rates vary with different activities, and Allen (1997) suggested that the amount of total saliva secretion should be evaluated by measuring the time spent resting, eating, and ruminating as well as their respective rates of secretion. The secretion rate of saliva ranges from 0.166 to 0.253 L/min during eating (Cassida and Stokes, 1986 and Maekawa et al., 2002a,b), 0.088 to 0.173 L/min during resting (Cassida and Stokes, 1986 and Maekawa et al., 2002a,b), and 0.272 L/min during ruminating (Cassida and Stokes, 1986). Beauchemin et al. (2008) showed that the rate of saliva secretion (g/min) did not change

during eating when cows were fed different types of forage, such as barley silage, alfalfa silage, long-stemmed alfalfa hay, and barley straw. But type of forage did change the rate of eating and eating time, which affected the amount of saliva secreted per unit of DMI. Therefore, factors related to the time spent eating and ruminating, such as particle size, forage to concentrate ratio and NDF content of forages (Bailey, 1961; Beauchemin et al., 2008), directly affect secretion amount of saliva buffers (Church, 1988).

As discussed in the section of VFA absorption, one pathway of VFA absorption is bicarbonate dependent protein mediated transport. This pathway involves exchange of $SCFA^-/ HCO_3^-$, which provides $HCO_3^-$ into the rumen, thus neutralize the $H^+$ to $CO_2$ and water (Aschenbach et al., 2009, 2011). Gäbel et al. (1991) estimated that 0.53 mole of $HCO_3^-$ is released when 1 mole of VFA is absorbed via this pathway. Aschenbach et al. (2011) suggested that $HCO_3^-$ secretion by rumen epithelium contributes approximately equal amount of $HCO_3^-$ from saliva secretion to the rumen in high producing dairy cows. Therefore, $HCO_3^-$ buffer secreted by rumen epithelial cells also plays a critical role in neutralizing protons in the rumen and regulating rumen pH.

Besides $HCO_3^-$ and $HPO_4^{2-}$, there are other minor buffers, such as ammonia and the endogenous buffering capacity of feeds (Allen, 1997). Penner et al. (2011) suggested that due to the high pKa value (9.21), $NH_3$ could immediately bind $H^+$ to form $NH_4^+$ in the rumen content. If $NH_4^+$ leave the rumen and move into the blood, this would finally remove protons from the rumen.

### 1.4.4 Passage to lower digestive tract

Volatile fatty acids and protons are soluble in water, and it has been suggested that 15 to 50 % of VFA in the liquid phase passes to the lower part of the digestive tract from

the rumen (Penner et al., 2011). But the fractional passage rate of VFA is not equal to the passage of protons, as the majority of VFA in the rumen is in the dissociated form. Penner et al. (2011) indicated that only a small fraction of the VFA carries protons passing into the lower part of the digestive tract. Allen (1997) suggested that approximately 15 % of the total acid removed from the rumen was through the passage. Therefore, passage to the lower part of the digestive tract contributes relatively small amount of the total acid removal from the rumen compared with acid absorption and neutralization.

### 1.5 SARA and milk composition

#### 1.5.1 Milk fat content

Milk fat depression commonly occurs with SARA (Kleen et al., 2003; Oetzel, 2003; Stone, 2004). Stone (1999) conducted a field study on a large dairy farm and found that milk fat content decreased by 0.3% when SARA occurred. Also, a positive relationship was suggested between rumen pH and milk fat content (Allen, 1997; Enemark et al., 2004). Therefore, decrease in milk fat content is commonly used as a sign to indicate SARA on dairy farms (Mertens, 1997). However, milk fat depression is not simply a consequence of SARA (Kleen et al., 2003). Some studies showed that low rumen pH or grain induced SARA did not change milk fat concentration (Keunen et al., 2002; Rustomo et al. 2006; Gozho et al. 2007). In addition, Oetzel (2007) indicated that some dairy herds did not have milk fat depression when rumen pH was substantially depressed. These findings indicated that low rumen pH may need to interact with some other factors, such as dietary fat content and fatty acid composition, before milk fat depression occurs (Oetzel, 2007).

Bauman and Griinari (2003) suggested a theory to explain milk fat depression.

Specific dietary factors can cause alternative pathways of fatty acid biohydrogenation in the rumen. Some intermediates produced from the alternative pathways pass the rumen and are absorbed in the small intestine. When these intermediates are transported to the mammary gland, they can decrease the expression of lipogenic enzymes and induce substantial decreases in milk fat. Trans-10, cis-12 conjugated linoleic acid was confirmed first as an inhibitor of fatty acid synthesis in the mammary gland (Baumgard et al. 2000). Later, two additional intermediates, trans-9, cis-11 CLA (Perfield II et al., 2007) and cis-10, trans-12 CLA (Saebo et al., 2005), were identified as possible inhibitors. But these two intermediates were only evaluated in one study, and more studies are needed to confirm their effect on milk fat synthesis (Bauman et al., 2011). Lock et al. (2008) indicated that milk fat depression requires two conditions. The first one is changes in the microbial population due to altered rumen fermentation, such as decreased rumen pH. The second one is the presence of polyunsaturated fatty acids in the rumen. AlZahal et al. (2009) found that rumen pH was decreased by diets with moderate level of forage and low in polyunsaturated fatty acids content, but milk fat depression did not occur. However, greater extent of milk fat depression was detected for cows fed a moderate forage diet and infused with soybean oil compared with cows fed a high forage diet and infused with soybean oil (AlZahal et al., 2010). Although milk fat content does not have a strong relationship with SARA, a few studies have been conducted to monitor milk fatty acid profile and link the specific fatty acids to the risk of SARA. Increased concentration of trans-10 C18:1 fatty acid in milk was observed in some studies when SARA occured, thus it was suggested that milk trans-10 C18:1 fatty acid may have the potential in the diagnosis of SARA (Enjalbert et al., 2008; Fievez et al., 2012). In addition, Colman et al.

(2010) found that some odd- and branched-chain fatty acids in milk, such as iso C13:0, iso C16:0 and C18:2 cis-9,trans-11, were more effective than milk fat content and trans-10 C18:1 fatty acid as indicators to detect SARA. But more studies are needed to confirm if specific milk fatty acids could be used to identify cows with the high risk of ruminal acidosis.

### 1.5.2 Milk urea nitrogen

Milk urea nitrogen (MUN) is commonly measured for bulk tank milk and DHI analysis, and it has been proposed as an indicator of the efficiency of feed N utilization for lactating dairy cow (Broderick and Clayton, 1997). Wattiaux et al. (2011) summarizd data from eight experiments and found the positive relationships between dietary CP concentration with MUN and urinary N excretion. When the amount of available N in the rumen is more than the microbial needs, N in the form of $NH_4^+$ is absorbed by the rumen wall and converted to urea in the liver (Parker et al., 1995). In addition, if absorbed amino acids and peptides are more than the animal's requirement, they are also converted to urea in the liver. Urea then ends up in blood, urine, and milk. Concentrations of urea in milk and blood are closely correlated in lactating cow (Broderick and Clayton, 1997). MUN is also highly correlated with urinary N excretion (Jonker et al., 1998; Kauffman and St-Pierre, 2001). Besides being used as an indicator of N utilization efficiency, Enemark (2009) suggested that MUN might be one potential indicator in the milk that could be related to SARA. The possible model of action is that more rumen fermentable carbohydrate increases microbial protein synthesis in the rumen, which results in decreased rumen $NH_3$ and decreased urea in the liver, thus less N excreted in milk. But research data is limited to validate MUN as a monitoring tool of ruminal acidosis

(Enemark, 2009).

Nutritional factors can influence MUN, such as the ratio of dietary CP to energy (Oltner and Wiktorsson, 1983), the extent of CP degradation in the rumen (Roseler et al., 1993), the amount of ammonia exceeding the microbial N requirement (Hof et al., 1997), and the protein or energy intake relative to the animal's requirement (Carlsson and Pehrson, 1994). Dietary CP concentration is the most important dietary factor affecting MUN concentration (Broderick and Clayton, 1997; Nousiainen et al., 2004). Because the excretion of N is determined by the balance between N intake and N stored in body and milk protein, higher MUN values may indicate that excess CP is fed, or rumen degradable protein is not balanced with dietary rumen fermentable carbohydrate. Less rumen digestible OM can decrease microbial protein synthesis, thus decrease N utilization and increase N excretion. In addition, some non-nutritional factors also have an effect on MUN value, such as production level, sampling type (AM or PM milking), season, breed, DIM (Carlsson et al., 1995; Broderick and Clayton, 1997), and animal genetic factors (Wattiaux et al., 2005; Cyriac et al., 2008; Rius et al., 2010). Moreover, carbohydrate fermentation has been suggested to stimulate urea-N recycling back to the rumen through changing the permeability of the rumen epithelium due to the production of VFA and $CO_2$ (Abdoun et al., 2006). When feeding a rapidly fermentable diet, increased amount of urea across the rumen wall allows the animal to capture and use available carbohydrates by supplying N for microbial protein synthesis (Hall and Huntington, 2008), which can also affect MUN. More urea-N recycling back to the rumen may decrease the amount of urea excreted in urine and milk.

**1.6 Summary**

Highly fermentable diets are often fed to high producing cows to maximize milk production. However, excessive consumption of highly fermentable carbohydrates and lack of physically effective fibre can increase the risk of ruminal acidosis and milk fat depression. Previous studies on beef steers, primiparous dry cows and sheep have indicated that variations exist in the severity of ruminal acidosis among animals when they are fed the same diet. But limited data is available for lactating dairy cows. Therefore, further study is needed to determine if variation in the risk of developing SARA exists among lactating dairy cows when fed a high-grain diet. The balance between acid production in the rumen and acid removal from the rumen determines rumen pH. Absorption through rumen epithelium, neutralization by buffers, and passage from rumen to the lower part of digestive tract are the three primary pathways of acid removal. Therefore, variation in any of these factors can be associated with the variation in the risk of developing SARA among the cows. Further research is warranted to determine specific mechanisms causing this variation. In addition, if such a variation exists among lactating dairy cows, it is not practical to measure rumen pH for all cows to identify those with higher risk of SARA on commercial farms. Therefore, it is necessary to determine a noninvasive indicator to identify the cows with higher and lower risk of SARA.

Absorption of VFA contributes to the largest amount of the total proton removal from the rumen, and affects the risk of SARA. Even though the hydrolysis and fermentation rate of sugar is more rapid than starch in the rumen, substitution of starch with sugar usually did not decrease rumen pH. Butyrate production is usually increased in the rumen when high sugar diets are fed, and butyrate can stimulate rumen epithelia growth and increase VFA absorption capacity. Thus, no decrease in rumen pH for high

sugar diets may be due to the increased VFA absorption rate, but further research is needed to evaluate this theory.

Hydrolysis and fermentation rates are different among different types of sugar, thus diets including different types of sugar may have variable effects on rumen fermentation and milk production for lactating cows. Whey and molasses are byproducts with high sugar content, and sometimes included in the diets for dairy cows. Therefore, in order to use these high disaccharide byproducts efficiently, it is necessary to understand the specific effects of feeding sucrose or lactose on rumen fermentation and productive performance for lactating dairy cows.

The overall objectives of this research were to evaluate if variation exists in the risk of developing SARA among lactating dairy cows when they are fed the same diet, and to determine specific mechanisms associated with this variation. It was hypothesized that there would be variation in the risk of SARA among lactating dairy cows when they are fed the same diet, and this variation would be associated with VFA procution, absorption, neutralization, passage, and any other factors related to these four factors.

**1.7 References**

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# Chapter 2. Relationship of severity of sub-acute ruminal acidosis to rumen fermentation, chewing activities, sorting behaviour and milk production in lactating dairy cows fed a high grain diet

## 2.1 Introduction

Sub-acute ruminal acidosis (SARA) is a prevalent metabolic disorder found in high producing dairy cows, and mainly caused by feeding excessively fermentable diets. One field survey in the United States indicated that incidences of SARA were 19% of early lactation dairy cows and 26% of mid-lactation cows (Garret et al., 1997). Substantial economic losses are caused by SARA due to its association with decreased feed intake, liver abscesses (Nagaraja and Lechtenberg, 2007), milk fat depression (Kleen et al., 2003), diarrhea, laminitis (Nocek, 1997), increased bacterial endotoxin and inflammation (Khafipour et al., 2009). Diet formulation strategies to decrease the incidence of SARA have been extensively studied, however, some cows in a herd still experience SARA even if these strategies are implemented. Previous studies indicated that large variations exist in the extent of severity of ruminal acidosis among beef steers (Brown et al. 2000; Schlau et al. 2012), primiparous dry cows (Penner et al, 2007) and sheep (Penner et al, 2009a) fed identical diets. But, to our knowledge, similar data have not been demonstrated for lactating dairy cows.

Balance between rumen acid production and removal determines rumen pH (Allen, 1997). Pathways of acid removal include absorption through rumen wall, buffer neutralization, and passage to the lower part of digestive tracts (Allen, 1997). Therefore,

variations in any of these factors could be associated with the variation in the risk of SARA among the ruminants (Penner et al. 2009). Penner et al. (2009) found that capacity of acetate and butyrate absorption by rumen epithelium was higher for acidosis resistant sheep than the susceptible ones *in vitro*. Schlau et al. (2012) found lower total VFA concentration in the rumen for steers with lower risk of SARA than higher risk ones, and they suggested that lower rumen pH for higher risk steers might be attributed to greater VFA production, lower VFA absorption rate, or both. Besides VFA production and absorption, neutralization is another main factor contributing to the regulation of rumen pH. Chewing activities are expected to stimulate salivary secretion (Church, 1988), and Allen (1997) suggested that saliva buffers could neutralize approximately 37% of the total protons removed from the rumen. Therefore, it was hypothesized that cows with lower risk of SARA would have more chewing time than cows with higher risk of SARA. In addition, dairy cows have been suggested sorting for short particles and against long particles (Kononoff et al., 2003; Leonardi and Armentano, 2003; DeVries et al., 2007). Sorting can lead to less consumption of physically effective fibre and more consumption of highly fermentable carbohydrates, which may decrease chewing time and increase rumen fermentation (Cook et al., 2004; DeVries et al., 2008), thus increase the risk of SARA. Therefore, I hypothesized that cows with lower risk of SARA would sort feed to a less extent than cows with higher risk of SARA.

It has been suggested that milk fat depression is commonly associated with SARA (Kleen et al., 2003; Oetzel, 2003; Stone, 2004). A field study on a large dairy farm found that SARA reduced milk fat production by 0.3% (Stone, 1999). In addition, experimentally induced SARA, either by adding grains to the diet or by replacing alfalfa

hay with alfalfa pellets, reduced milk fat concentration (Fairfield et al., 2007; Khafipoor et al., 2007). Moreover, Allen (1997) and Enemark et al. (2004) reported a positive relationship between milk fat concentration and ruminal pH ($r^2$ = 0.39 and 0.31 for each study, respectively). Therefore, I hypothesized that cows with lower risk of SARA would have higher milk fat content compared to the cows with higher risk, and expected that milk fat content might be a non-invasive indicator to identify cows with higher and lower risk of SARA on farms.

The objectives of this study were to evaluate the variation in the severity of SARA among lactating dairy cows fed a high-grain diet, and to determine if chewing, sorting and milk fat content could characterize cows with higher and lower risk of SARA.

## 2.2 Materials and methods

All experimental procedures used in this study were approved by the University of Alberta Research Centre Animal Care Committee and conducted according to the guidelines of the Canadian Council of Animal Care (Ottawa, Ontario, Canada).

### *2.2.1 Animals, Diets, and Experimental Design*

Sixteen (8 primiparous and 8 multiparous) ruminally cannulated lactating Holstein cows (DIM = 282 ± 33.8; BW = 601 ± 75.9 kg; mean ± SD) were used in this study. Cows were fed a diet containing 35% forage and 65% concentrate mix (Table 2.7.1) ad libitum for 21 d, consisting of a 17-d diet adaptation period and a 4-d data and sample collection period.

Cows were housed individually in tie stalls bedded with wood shavings, and fed the experimental diet as a TMR once daily at 0900 h and had free access to water. Feed

was offered at 105 to 110% of actual feed intake of the previous day. Samples of TMR and feed ingredients were collected daily during sample collection period. The weight of feed offered and refused was recorded daily on d 19, 20, and 21 of the study, and 12.5% of the total daily refusal from each cow was composited to yield one sample per cow per period.

The DM concentration of barley silage and alfalfa hay was determined twice weekly and diet formulation was adjusted if necessary.

Cows were weighed after the morning milking on 2 consecutive days immediately before the start of experiment. Cows were milked twice daily at 0400 and 1500 h. Milk was sampled from both a.m. and p.m. milkings on d 19, 20, and 21 of the study.

### *2.2.2 Rumen pH and Rumen Fermentation*

Ruminal pH was measured in the ventral sac every 30 s continuously for 72 h (d 19 - 21) using the pH measurement system developed by Penner et al. (2006). Minimum, mean, and maximum pH, duration and area below pH 5.8 were determined for each cow daily, and averaged over 3-d periods. These data were used to determine acidosis index (area under pH 5.8 divided by DMI; Penner et al., 2009a) to assess the severity of SARA normalized for a feed consumption level.

Rumen fluid was collected from cranial, ventral, and caudal sacs, then combined and strained through a perforated screen (Peetex, Sefar Canada Inc., Scar- borough, ON, Canada; pore size = 355 μm) every 9 h over a 72-h period. The samples were centrifuged at 3,000 × g at 4°C for 20 min immediately after collection, and the supernatants were stored at −20°C until analysis. Rumen fluid samples were composited to yield one sample per cow for further analysis.

Ruminal fluid samples were analyzed for VFA profile by gas chromatography according to the method described by Khorasani et al. (1996). Rumen ammonia-N concentration was determined as described by Fawcett and Scott (1960) using a plate reader (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA).

### *2.2.3 Chewing Activity and Sorting Behaviour*

Chewing activities were directly monitored for 24 h on d 18. Eating and ruminating activities were recorded every 5 min and each activity was assumed to last for the entire 5-min interval between observations as described previously (Beauchemin et al., 2003; Krause et al., 2003; Zhang et al., 2010). Total chewing time was calculated as the sum of eating time and ruminating time.

Particle size distribution of the TMR and orts was determined by using Penn State Particle Separator with 3 sieves (aperture size of 19, 8, and 1.18 mm). Sorting index was calculated as the ratio of actual intake to predicted intake for particles retained on each sieve of the separator (Leonardi and Armentano, 2003). A sorting index of 100, greater than 100, and less than 100 indicate no sorting, selective consumption, and selective refusals, respectively. Physically effective factor was determined as the proportion of particles retained on 19- and 8-mm sieves (Lammers et al., 1996).

### *2.2.4 Blood Collection*

Blood samples were collected every 18 h over a 72-h (d 19 – 21) period from the coccygeal vessels into tubes containing sodium heparin (Fisher Scientific Company; Nepean, Ontario, Canada). 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 composited to yield one sample per cow for further 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 by a plate reader (SpectraMax 190) at a wavelength of 450 nm. Plasma BHBA concentration was measured by the enzymatic oxidation of BHBA to acetoacetate using 3-hydroxybutyrate dehydrogenase (Roche, Mississauga, ON, Canada) followed by determination of reduction of $NAD^+$ to NADH at a wavelength of 340 nm. Commercial kits were used to determine concentrations of plasma NEFA (Wako Chemicals USA, Inc., Richmond, VA) and insulin (Coat-a-Count kit, Diagnostic Products Corp., Los Angeles, CA).

### *2.2.5 Milk composition*

Milk samples were analyzed for milk fat, CP, lactose, and MUN by infrared spectroscopy (AOAC, 2002; method 972.16; MilkoScan 605, Foss North America, Brampton, ON) at the Alberta Central Milk Testing Laboratory (Edmonton, AB).

### *2.2.6 Statistical Analysis*

Effect of parity was originally tested by using the PROC TTEST procedure of SAS (version 9.2 SAS Institute Incorporated; Cary, NC), but it was not included in the final model as significant parity effects were not observed for ruminal pH variables (Table 2, $P > 0.05$). Values of mean ± 0.5 × SD of acidosis index were used as criteria to identify groups of extreme animals (i.e., animals with higher and lower risk of SARA), and all response variables were evaluated for the group effect using PROC TTEST. In addition, sorting index data were tested to determine if they are different from 100 by using PROC TTEST. PROC REG procedure was used to determine the relationships between sorting index vs. minimum ruminal pH, sorting index vs. acidosis index, and

MUN vs. acidosis index. Significance was declared at $P < 0.05$ and tendency was declared at $0.05 < P < 0.10$.

## 2.3 Results

No differences were observed in minimum, mean and maximum ruminal pH between primiparous cows and multiparous cows as well as duration of pH below 5.8 and area of pH below 5.8 ($P > 0.10$; Table 2.7.2). Although there was a tendency of higher DMI for multiparous cows, acidosis index was not different between primiparous cows and multiparous cows.

Among all cows, minimum ruminal pH, mean pH, duration and area pH below 5.8 ranged from 5.16 to 6.04, 5.94 to 6.57, 0 to 606 min/d, and from 0 to 193 pH×min/d, respectively. The acidosis index ranged from 0.0 to 10.9 pH×min/kg DMI. Acidosis index of 6 cows were lower than the value of mean - 0.5×SD, and that of 4 cows were higher than the value of mean + 0.5×SD, and they were classified as animals with lower SARA risk (LS) and higher SARA risk (HS), respectively.

### *2.3.1 Ruminal pH and VFA Profile*

No differences were observed in BW and DMI between LS and HS animals ($P > 0.10$; Table 2.7.3). However, the minimum (5.83 vs. 5.22; $P < 0.01$) and mean ruminal pH (6.47 vs. 6.02; $P < 0.01$) was higher for LS animals compared with HS animals, whereas maximum pH values were not different between the groups. Duration (10.1 vs. 556 min/d; $P < 0.01$) and area that pH was below 5.8 (0.86 vs. 140 pH×min/d; $P < 0.01$) were lower in LS animals. Acidosis index was lower in LS animals (0.04 vs.7.67 pH×min/kg; $P < 0.01$). Total VFA concentration, and molar proportion of acetate, propionate and butyrate

were not different between the groups ($P > 0.10$; Table 2.7.4), while molar proportion of isobutyrate (1.07 vs. 0.65 mol/100mol; $P < 0.01$) and isovalerate (1.97 vs. 1.26 mol/100mol; $P = 0.03$) were higher for LS cows than HS ones. In addition, the concentration of rumen $NH_3$-N tended to be higher for LS cows ($P = 0.06$).

### *2.3.2 Sorting Behaviour and Chewing Activity*

Both groups sorted for short particles, but HS animals sorted to a greater extent (sorting index: 105 vs. 102; $P = 0.05$; Table 2.7.5). Moreover, HS animals sorted against long particles whereas LS animals did not (sorting index: 87.6 vs. 97.9; $P = 0.05$).

Eating, ruminating and total chewing time (min/d) were not different between LS and HS animals ($P > 0.10$; Table 2.7.6). However, the LS cows had shorter ruminating time per unit of DMI (25.4 vs. 33.2 min/kg DMI; $P = 0.05$) and total chewing time per unit of DMI (35.8 vs. 45.1 min/kg DMI; $P < 0.05$).

### *2.3.3 Milk Production*

No differences were observed in milk yield and milk component yields between LS and HS cows ($P > 0.10$; Table 2.7.7). In addition, concentrations of milk fat, protein and lactose did not differ between the groups. However, concentration of MUN was higher for LS animals compared with HS animals (12.8 vs. 8.6 mg/dl; $P < 0.05$),

### *2.3.4 Plasma Metabolites and Hormones*

Plasma glucose, insulin, BHBA, and NEFA concentrations were not different between LS and HS cows ($P > 0.10$; Table 2.7.8).

## 2.4 Discussion

Sub-acute ruminal acidosis is a common metabolic disorder for high producing dairy cows. The risk of SARA is greater for early- and mid-lactation cows compared with late-lactation cows due to feeding highly fermentable diets and also greater feed intake for mid-lactation cows. Garret et al., (1997) indicated that the incidences of SARA were 19% for early lactation cows and 26% for mid-lactation cows. However, late-lactation cows were used in the current study due to the animal availability. Although early- or mid-lactation cows would be the better model for the current study, a substantial variation in the severity of SARA was detected among late-lactating cows fed the same high-grain diet, which is consistent with previous studies using different types of animals. Brown et al. (2000) found that, when 5 steers were intraruminally dosed with steam-flaked corn, average ruminal pH ranged from 4.26 to 5.63. In another study by Schlau et al. (2012), 17 beef steers were force-fed the same diet consisting of 85% grain through rumen cannulas, and the acidosis index ranged from 4.0 to 96.5 pH×min/kg among the animals. In addition, when Penner et al. (2007) provided additional concentrate to primiparous cows during the periparturient period, they found high SEM for ruminal pH variables within the treatment; for example, SEM value was 30.7% of the mean for the area < pH 5.8 (mean ± SEM: 766 ± 235 pH×min). The high SEM values indicated that some cows within a treatment were able to cope with diet challenge better than the others. Another experiment was conducted to induce SARA in sheep through oral glucose drench by Penner et al. (2009a). Although the dose of glucose was same for all sheep, mean rumen pH was higher for resistant animals compared with susceptible ones (5.97 vs. 5.57). These individual variations among animals within the treatment clearly demonstrate that ruminants markedly vary in

the extent of tolerance to dietary factors that predispose them to acidosis. However, as the type and intensity of acidosis challenge was not same for the studies mentioned above, it is not possible to compare variations in rumen pH response and severity of SARA among different type of animals.

The second objective of this study was to identify factors that are related to cows with higher or lower risk of SARA, and I found that LS cows sorted feed to a less extent than HS ones. It has been shown that cows generally sort for short particles and against long particles even when they are fed a TMR (Kononoff et al., 2003; Leonardi and Armentano, 2003; DeVries et al., 2007). The majority of previous studies evaluating sorting behaviour of dairy cows focused on management factors such as effects of feeding frequency and stocking density, and dietary factors such as effects of DM content, forage content, and particle size of TMR. However, there seems to be a substantial individual variation in sorting behaviour among cows even fed the same diet. Leonardi and Armentano (2003) indicated that although all cows generally sorted against long particles (retained on a sieve of 26.9-mm apertures), intake of long particles as a percentage of predicted intake was < 70% for 4 cows, between 71 and 80% for 11 cows, between 81 and 90% for 5 cows, and between 91 and 100% for 2 cows. One extreme cow even did not consume any of the long particles of TMR. Leonardi et al. (2005) found similar animal variation in sorting; sorting index of long particles (retained on a sieve of 26.9-mm apertures) ranged from approximately 10 to 100 when dry TMR (89.9 % DM) was offered. In another study, Leonardi and Armentano (2007) detected that sorting index of the long particles were from 40 to 100 among 29 cows when fed a diet containing 68% DM. In the current study, I also found that sorting index of long particles (retained on a

sieve of 19-mm apertures) ranged from 76.1 to 103.6 even though all cows were fed the same diet, and cows with higher risk of SARA sorted against long particles while the lower risk cows did not. The DM content of the experimental diet was 60.8% due to the high concentrate content while the typical TMR given to high-producing dairy cows is from 40 to 60% DM (Eastridge, 2006). The dry TMR used in the current study may have increased sorting behaviour for cows, but the effect of DM content on sorting is not conclusive. It is commonly believed that it would be more difficult for cows to sort when water is added to the dry TMR, because water could make particles stick together (Miller-Cushon and DeVries, 2009). Leonardi et al. (2005) added water to a dry TMR (DM reduced from 81 to 64%) and found a decrease in the extent of sorting against long particles. However, Miller-Cushon and DeVries (2009) found that when adding water to a TMR (reducing DM content from 58 to 48%), sorting was increased. The differences in diet DM content and composition between these two studies may explain the inconsistency of the results (Miller-Cushon and DeVries, 2009). Felton and DeVries (2010) also found that greater amounts of water added to the TMR (DM of diets were 56.3, 50.8, and 44.1% DM) resulted in greater sorting against long particle diets. Therefore, it is not clear whether sorting behaviour of cows fed a relatively dry TMR in the current study would be different from cows fed TMR with less DM content.

Cows sorting of a TMR can cause overconsumption of highly fermentable carbohydrates and refusal of physically effective fibre compared with the expected intake, which may increase VFA production and decrease acid neutralization by reduced chewing (Cook et al., 2004; DeVries et al., 2008). Therefore, sorting behaviour may be one of the reasons that increase the risk of SARA. DeVries et al. (2008) found that when early

lactation cows were fed a low forage diet (45% forage), their sorting activity was related to rumen pH; the more cows sorted for medium and short particles, the lower their minimum, mean, and maximum ruminal pH were. In the current study, LS cows tended to consume more long particles (> 19.0 mm) than HS cows (Table 2.7.5). In addition, sorting index of long particles was positively correlated with minimum ruminal pH (r = 0.60, $P = 0.01$; Figure 2.7.9) and negatively correlated with acidosis index (r = -0.64, $P < 0.01$). Therefore, variation in the risk of SARA among the cows might be related to the variation in the sorting against long particles, and further work needs to be done to confirm these findings.

The current study showed the relationship between sorting behaviour and rumen pH, but there were no differences in rumen pH in some of the previous studies where significant sorting was found (Kononoff and Heinrichs, 2003; Leonardi et al., 2005; Maulfair et al., 2010). In addition, Maulfair et al. (2010) detected that rumen pH tended to increase quadratically (P = 0.07) with increased sorting against long particles. Moreover, a few studies indicated that cows sorted for long particles as an attempt to meet physically effective fibre requirement when they experience low rumen pH (Keunen et al., 2002; Beauchemin and Yang, 2005; DeVries et al., 2008), which suggested that cows may change their sorting behaviour to attenuate the effects of acidosis. Therefore, the speculation that ration sorting decreases rumen pH is not conclusively supported, and the reasons are not clear. However, the effects of sorting on rumen pH might have been confounded by dietary (treatment) effects, and the extent of sorting in these studies might not be severe enough to pose the potential effect on rumen pH. DeVries et al. (2011)

speculated that the risk of SARA would be much greater when sorting against long particles is more substantial (i.e., 20-30% refusal of long particles).

I found that LS cows sorted feed to a less extent than HS cows, and as such I expected that LS cows would chew more. It has been suggested that chewing time is a good indicator of rumen health because chewing stimulates salivary buffer secretion (Allen, 1997), which helps neutralization of acids produced from rumen fermentation. Dietary particle length and chemical NDF content can affect chewing activity (Mertens, 1997; Zebeli et al., 2008). In order to minimize the confounding effects of different feed intake, Balch (1971) proposed using total time spent chewing per kg of DMI to indicate dietary physical property. In the current study, I expected that LS cows would have longer chewing time per unit of DMI than HS cows. However, I found the opposite results. Total chewing time was 768 min/d and total chewing time / NDF intake was 140 min/kg for LS cows, while total chewing time was 830 min/d and total chewing time / NDF intake was 176 min/kg for HS cows. Chewing time measured in the current study was longer, regardless of the group, than that reported in the previous studies; Yang and Beauchemin (2007) reported total chewing was 655 min/d and 101.3 min/kg of NDF intake when diet forage to concentrate ratio was 35 to 65. In another study that they conducted later (Yang and Beauchemin, 2009), similar total chewing time was found (657 min/d and 102 min/kg of NDF intake) for cows fed the diet with similar forage to concentrate ratio. Longer chewing time for the current study might be partly due to the different methods of chewing activity monitoring (visual observation vs. automated data collection). In addition, in this study, the data collection period for chewing behaviour was limited to a relatively short period (1 d). Dado and Allen (1994) indicated that there is considerable

day-to-day variation in feeding behaviour data within cows. As such, the differences observed in chewing activities between two groups need to be interpreted with caution. Nonetheless, our findings provided no evidence to attribute higher rumen pH of LS cows to the difference in chewing activity.

There are a couple of possible explanations for our observation that LS cows chewed less than HS cows. First, it may be possible that cows possess an adaptive response to the reduction in rumen pH. Previous studies have shown that when rumen pH was low, cows tended to increase the amount of rumination needed per unit of NDF (Beauchemin, 1991; Beauchemin et al., 1994), likewise, chewing time per unit of NDF intake was less for high NDF than for low NDF diets (Oba and Allen, 2000; Yang et al., 2001; Maulfair and Heinrichs, 2013). These findings suggest that forage stimulates chewing more effectively when rumen pH becomes lower. In the current study, HS cows had lower rumen pH. Therefore, chewing time per unit of DMI might have increased for HS cows, which could attenuate the decrease in rumen pH by increasing saliva secretion or movement of particulate and fluid from the rumen (Krause et al., 2002). DeVries et al. (2009) dosed 4 kg of ground barley/wheat into the rumen to induce ruminal acidosis before feeding TMR (45:55 of forage to concentrate ratio), and found that rumination time was longer for animals experiencing more severe SARA as a result of the grain dosage. Therefore, although the particle length and chemical NDF concentration of the diets influence chewing activity, additional metabolic mechanisms regulating chewing activity need to be identified (Oba and Allen, 2000).

The second possibility for greater chewing time per unit of DMI for HS cows is that lower rumen pH decreased fibre digestibility in the rumen (Russell and Wilson, 1996;

Beauchemin, 2000) and increased the retention time of ruminal digesta for HS cows. Thus, expected greater digesta mass in the rumen of HS cows may have stimulated chewing activity. Grant et al. (1995) found that total chewing time per kg of NDF intake was lower for cows fed brown midrib sorghum silage compared with those fed normal sorghum silage. Brown midrib sorghum silage was greater in NDF degradability than normal sorghum silage. Therefore, possible higher NDF degradability for LS cows could lead to faster digesta disappearance rate in the rumen, which might have decreased the physical effectiveness of forage in stimulating chewing. Oba and Allen (2000) also suggested that forage NDF degradability might affect chewing activities unless a critical amount digesta in the rumen is maintained. In the current study, rate of fibre digestion and digesta mass in the rumen were not determined, but the possibility that low rumen pH increased chewing activities via a greater rumen fill cannot be excluded as a reason for greater chewing activities for HS cows.

Diets are often formulated for a group of cows on dairy farms, thus the variation in the risk of developing SARA among cows can be a potential probelm, as SARA may occur for HS cows while not for other ones (Penner et al., 2009). Therefore, identifying LS and HS cows and adjusting nutritional management accordingly may reduce this nutritional disorder. In the current study, ruminally canulated cows were used, and LS and HS cows were identified by measuring rumen pH. However, it is not practical to measure rumen pH for numbers of cows on farms. Therefore, it is necessary to evaluate an easy indicator of rumen pH to identify LS and HS cows. I expected that milk fat content might be the non-invasive indicator, but milk fat content did not differ between the two groups in the current study. Some previous studies also reported no effect of rumen pH on milk

fat concentration and indicated that milk fat depression does not always occur with SARA (Keunen et al., 2002; Cottee et al., 2004; Gozho et al., 2007). The duration of SARA may explain the inconsistent response in milk fat content when SARA was experimentally induced, and it was suggested that short durations of SARA might not affect milk fat content (Krause and Oetzel, 2005). Therefore, multiple acidosis challenges may be needed to change the ruminal biohydrogenation and thus cause the milk fat depression due to the possible slow response of microbes to acidosis challenges (Oetzel, 2007). However, in the current study, cows were fed the high-grain diet for 21 d. As such, the duration of SARA is expected to be long enough, and may not be a possible explanation for this case. Oetzel (2007) suggested that the relationship is not consistent between SARA and milk fat depression, and many cows and herds do not have milk fat depression when rumen pH decreased. In the current study, I observed a large numerical difference in milk fat concentration between the two groups (3.22 vs. 2.73), but I could not detect this as a significant difference due to a substantial variation within groups (SE = 0.33). This indicates that other unidentified factors besides rumen pH affected milk fat content, and that milk fat content may not be a sensitive indicator to identify cows with higher or lower risk of SARA. In addition, fat content of LS cows was 3.59% immediately prior to the current study when cows were fed a high forage diet (60 : 40 forage to concentrate ratio), which indicated that the high-grain diet fed during the current study decreased milk fat content to some extent even for LS cows.

However, I found that LS cows had higher MUN concentration than HS ones. In addition, there was a negative correlation between MUN and acidosis index (r = -0.64, $P$ = 0.01; Figure 2.7.10). Milk urea nitrogen concentration did not differ between the two

groups before the start of experiment when all cows were fed a diet containing 60% forage on a DM basis. Therefore, MUN might be potentially used as an indicator to identify LS and HS cows fed high-grain diets. Concentration of MUN is a good predictor of urinary N excretion and the efficiency of protein utilization in dairy cows (Gustafsson and Palmquist, 1993; Kohn et al., 2002). It has been indicated that MUN is affected by nutritional factors such as dietary CP content, ruminally fermentable OM, the ratio of dietary CP to energy, and the extent of CP degradation in the rumen (Carlsson et al., 1995; Hof et al., 1997; NRC, 2001). In addition, it is affected by non-nutritional factors, such as DIM, parity, season and milking frequency (Carlsson et al., 1995; Hof et al., 1997). Also, MUN concentration is affected by unidentified animal factors (Wattiaux et al., 2005; Cyriac et al., 2008; Rius et al., 2010). In the current study, all cows were fed the same diet and most likely they were in positive balance for all nutrients including energy and protein. Due to the numerical difference in DMI, LS cows had 445 g more CP intake, and higher MUN and rumen $NH_3$ may appear to be attributable to the difference in CP intake. However, the difference in CP intake between the groups was not significant. In addition, before the start of experiment when cows were fed a 60%-forage diet, LS and HS cows had similar ruminal $NH_3$ concentration (9.37 vs. 10.9 mg/dL, respectively; $P = 0.56$) and MUN concentration (12.5 vs. 11.6 mg/dL, respectively; $P = 0.54$) although DMI was numerically greater for the LS cows (23.9 vs. 19.6 kg/d; $P = 0.14$). Therefore, the numerical difference in CP intake may not be the exclusive reason that made LS cows had higher MUN and rumen $NH_3$ when cows were fed the high-grain diet. Schlau et al. (2012) suggested that higher rumen pH for acidosis resistant steers was partly due to lower VFA production. Greater MUN and ruminal $NH_3$ concentrations for LS cows in the current

study may indicate that OM fermentation is lower for LS cows even if the same diet was fed. However, I did not measure the rate of VFA production or OM fermented in the rumen in this study. Another possibility is that the proteolysis in the rumen was different between LS and HS cows even though CP intake was the same. The molar proportion of isobutyrate (1.07 vs. 0.65 mol/100mol; $P < 0.01$) and isovalerate (1.97 vs. 1.26 mol/100mol; $P = 0.03$) were higher for LS cows than HS ones. These ruminal branched-chain VFA are primarily from the ruminal deamination and decarboxylation of branch chain amino acids substrates (Tedeschi et al., 2000). The bacteria responsible for deamination and production of $NH_3$ are pH sensitive, and Rychlik and Russell (2000) showed that cattles fed hay had greater amount of hyper ammonia producing bacteria than the ones fed mostly grain. Therefore, in this study, higher pH for LS cows might cause higher proteolysis and deamination in the rumen than HS cows, which might also explain the greater MUN and ruminal $NH_3$ concentrations for LS cows. Further research is warranted to confirm this preliminary finding and identify if MUN could be used as a non-invasive indicator to identify cows with lower and higher risk of SARA on farms.

## 2.5 Conclusion

A substantial variation exists in the severity of SARA among lactating dairy cows when fed the same high-grain diet. Cows with lower risk of SARA sorted to a less extent compared with higher risk ones. However, cows with lower risk of SARA may not necessarily have longer chewing time than higher risk cows. In addition, MUN concentration might be potentially used as a non-invasive indicator to identify cows with higher and lower risk of SARA on farms.

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## 2.7 Figures and tables

**Table 2.7.1.** Ingredient, chemical composition and particle size distribution of the diet

| Item | |
|---|---|
| Ingredient, % DM | |
| Barley silage | 30.0 |
| Barley grain, dry rolled | 25.0 |
| Corn grain, ground | 20.0 |
| Canola meal | 7.35 |
| Corn gluten meal | 5.26 |
| Alfalfa hay | 5.0 |
| Beet pulp | 3.96 |
| Vegetable oil | 1.0 |
| Mineral and vitamin mix[1] | 2.43 |
| Nutrient Composition | |
| DM | 60.8 |
| Ash | 7.95 |
| CP | 15.9 |
| NDF | 25.6 |
| Starch | 31.1 |
| Ether extract | 4.0 |
| NFC | 49.8 |
| Forage NDF | 14.3 |
| Particle size distribution, % as fed | |
| >19 mm | 20.2 |
| 19–8 mm | 24.3 |
| 1.18-8 mm | 39.4 |
| <1.18 mm | 16.1 |
| Physical effectiveness factor[2] | 44.5 |

[1]Contained 15.7% Ca, 3.32% P, 14.1% Na, 21.8% Cl, 5.70% Mg, 0.23% S, 0.06% K, 2867.4 mg/kg Fe, 468.7 mg/kg Cu, 902.8 mg/kg Mn, 11.2 mg/kg Co, 718.0 mg/kg Zn, 7.08 mg/kg Se, 21.0 mg/kg I, 442.8 kIU/kg vitamin A, 45.0 kIU/kg vitamin D, 1449.9 kIU/kg vitamin E

[2] Determined as the proportion of particles retained on 19- and 8-mm sieves on as-fed basis (Lammers et al., 1996).

**Table 2.7.2** Comparison of BW, DMI, and pH measurements between primiparous and multiparous cows

| Variable | Primiparous | Multiparous | SE | *P* |
|---|---|---|---|---|
| BW, kg | 564 | 639 | 23.9 | 0.04 |
| DMI, kg/d | 19.4 | 22.1 | 1.02 | 0.08 |
| Ruminal pH | | | | |
| Nadir | 5.48 | 5.61 | 0.10 | 0.38 |
| Mean | 6.20 | 6.34 | 0.07 | 0.19 |
| Maximum | 6.85 | 6.97 | 0.05 | 0.15 |
| Duration < pH 5.8, min/d | 298 | 154 | 78.3 | 0.21 |
| Area < pH 5.8, pH × min/d | 68.1 | 27.7 | 20.4 | 0.18 |
| Acidosis index, pH × min/kg | 3.84 | 1.21 | 1.12 | 0.12 |

**Table 2.7.3** Comparison of BW, DMI, and pH measurements between LS and HS cows

| **Variable** | **LS (n=6)** | **HS (n=4)** | **SE** | ***P*** |
|---|---|---|---|---|
| BW, kg | 622 | 566 | 36.2 | 0.31 |
| DMI, kg/d | 21.6 | 18.8 | 1.31 | 0.17 |
| Ruminal pH | | | | |
| Nadir | 5.83 | 5.22 | 0.06 | < 0.01 |
| Mean | 6.47 | 6.02 | 0.04 | < 0.01 |
| Maximum | 6.98 | 6.88 | 0.08 | 0.38 |
| Duration < pH 5.8, min/d | 10.1 | 556 | 23.3 | < 0.01 |
| Area < pH 5.8, pH × min/d | 0.855 | 140 | 9.92 | < 0.01 |
| Acidosis index, pH × min/kg | 0.037 | 7.67 | 0.67 | < 0.01 |

**Table 2.7.4** Comparison of ruminal VFA profile and rumen NH3-N between LS and HS cows

| **Variable** | **LS (n=6)** | **HS (n=4)** | **SE** | ***P*** |
|---|---|---|---|---|
| Total VFA, m*M* | 126 | 131 | 7.82 | 0.66 |
| Acetate, mol/100mol | 54.2 | 53.5 | 1.91 | 0.81 |
| Propionate, mol/100mol | 26.4 | 29.3 | 2.08 | 0.36 |
| Isobutyrate, mol/100mol | 1.07 | 0.65 | 0.04 | <0.01 |
| Butyrate, mol/100mol | 13.8 | 11.5 | 1.25 | 0.24 |
| Isovalerate, mol/100mol | 1.97 | 1.26 | 0.18 | 0.03 |
| Valerate, mol/100mol | 2.08 | 2.36 | 0.19 | 0.33 |
| Caproate, mol/100mol | 0.56 | 1.48 | 0.46 | 0.20 |
| Acetate:Propionate | 2.13 | 1.89 | 0.23 | 0.48 |
| Rumen $NH_3$-N (mg/dL) | 9.38 | 4.66 | 1.51 | 0.06 |

**Table 2.7.5** Feed refusal and sorting index between LS and HS cows

| | **LS (n=6)** | **HS (n=4)** | **SE** | ***P*** |
|---|---|---|---|---|
| Feed refusal, kg/d | 3.7 | 4.1 | 0.49 | 0.61 |
| Sorting index[1] | | | | |
| >19.0mm | 97.9 | 87.6* | 3.19 | 0.05 |
| 19.0 to 8.0 mm | 97.3* | 98.8 | 1.17 | 0.39 |
| 8.0 to 1.18 mm | 102* | 105* | 0.91 | 0.05 |
| <1.18 mm | 101 | 104* | 1.02 | 0.06 |
| DMI, kg/d | | | | |
| >19.0mm | 4.29 | 3.33 | 0.32 | 0.07 |
| 19.0 to 8.0 mm | 5.12 | 4.51 | 0.32 | 0.23 |
| 8.0 to 1.18 mm | 8.73 | 7.79 | 0.48 | 0.22 |
| <1.18 mm | 3.51 | 3.14 | 0.21 | 0.26 |

[1]Sorting index was calculated as the ratio of actual intake to predicted intake for particles retained on each sieve of the separator. A sorting index above 100 indicates sorting for particles, and a sorting index below 100 indicates sorting against particles (Leonardi and Armentano, 2003).

* Different from 100 ($P < 0.05$).

**Table 2.7.6** Comparison of chewing activity between LS and HS cows

| **Variable** | **LS (n=6)** | **HS (n=4)** | **SE** | ***P*** |
|---|---|---|---|---|
| Time, min/d | | | | |
| Eating | 223 | 220 | 13.7 | 0.87 |
| Ruminating | 544 | 610 | 33.7 | 0.21 |
| Total chewing[1] | 768 | 830 | 32.8 | 0.22 |
| Time, min/kg of DMI | | | | |
| Eating | 10.3 | 12.0 | 0.70 | 0.14 |
| Ruminating | 25.4 | 33.2 | 2.35 | 0.05 |
| Total chewing | 35.8 | 45.1 | 2.65 | 0.04 |
| Time, min/kg of NDF | | | | |
| Eating | 40.3 | 46.7 | 2.72 | 0.14 |
| Ruminating | 99.4 | 130 | 9.05 | 0.05 |
| Total chewing | 140 | 176 | 10.1 | 0.04 |

[1]The sum of eating time and ruminating time.

**Table 2.7.7** Comparisons of milk yield and milk composition between LS and HS cows

| **Variable** | **LS (n=6)** | **HS (n=4)** | **SE** | ***P*** |
|---|---|---|---|---|
| Yield, kg/d | | | | |
| Milk | 28.6 | 24.2 | 3.58 | 0.41 |
| Fat | 0.93 | 0.67 | 0.16 | 0.30 |
| CP | 1.03 | 0.87 | 0.11 | 0.32 |
| Lactose | 1.26 | 1.11 | 0.16 | 0.54 |
| Milk composition | | | | |
| Fat, % | 3.22 | 2.73 | 0.33 | 0.33 |
| CP, % | 3.64 | 3.60 | 0.14 | 0.84 |
| Lactose, % | 4.42 | 4.59 | 0.13 | 0.40 |
| MUN, mg/dL | 12.8 | 8.60 | 0.97 | 0.02 |

**Table 2.7.8** Comparison of plasma blood metabolite and hormone concentration between LS and HS cows

| **Variable** | **LS (n=6)** | **HS (n=4)** | **SE** | ***P*** |
|---|---|---|---|---|
| Glucose, mg/dL | 70.2 | 67.0 | 3.86 | 0.57 |
| BHBA, mg/dL | 7.73 | 9.37 | 1.73 | 0.52 |
| NEFA, mEq/L | 64.9 | 74.9 | 4.01 | 0.12 |
| Insulin, μIU/dL | 21.2 | 19.8 | 5.04 | 0.85 |

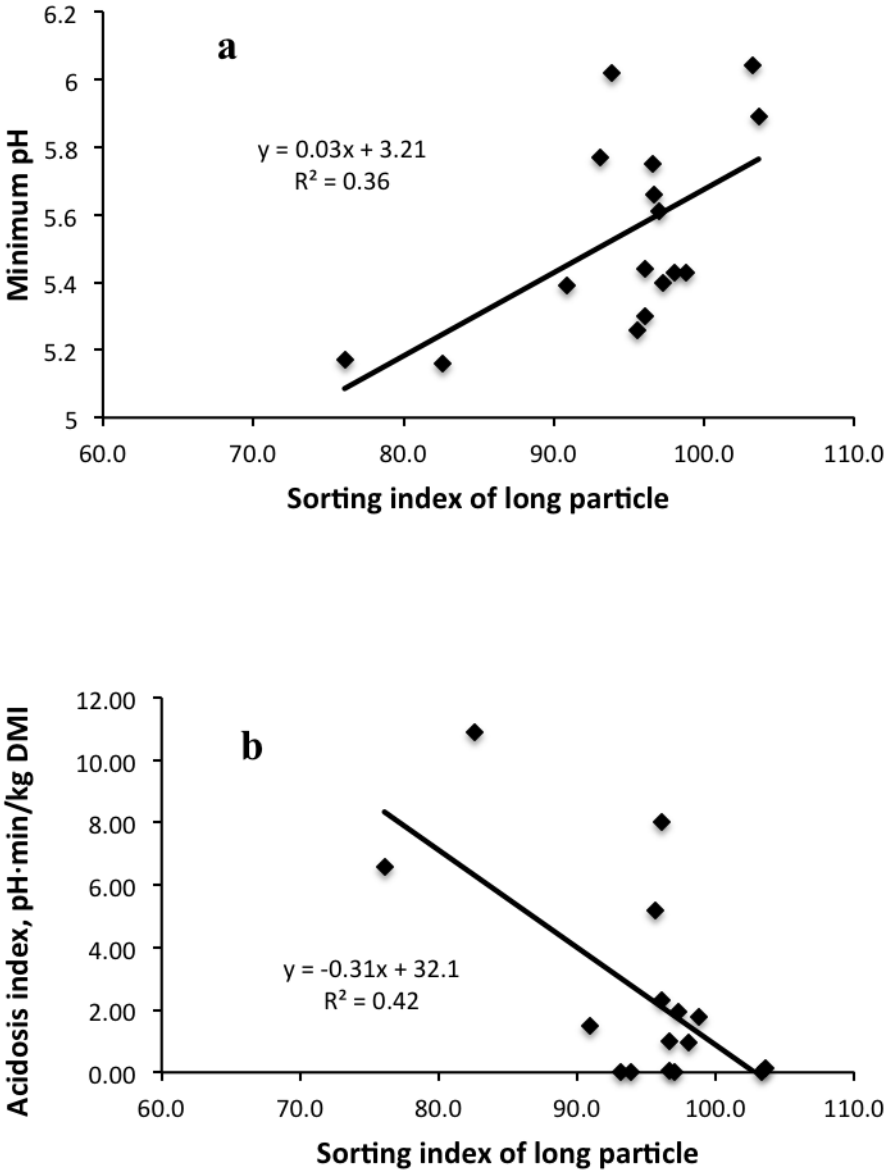


**Figure 2.7.9** Relationship between sorting index of long particles (retained on a sieve of 19-mm apertures) with (a) minimum ruminal pH ($P = 0.01$), and (b) acidosis index ($P < 0.01$).

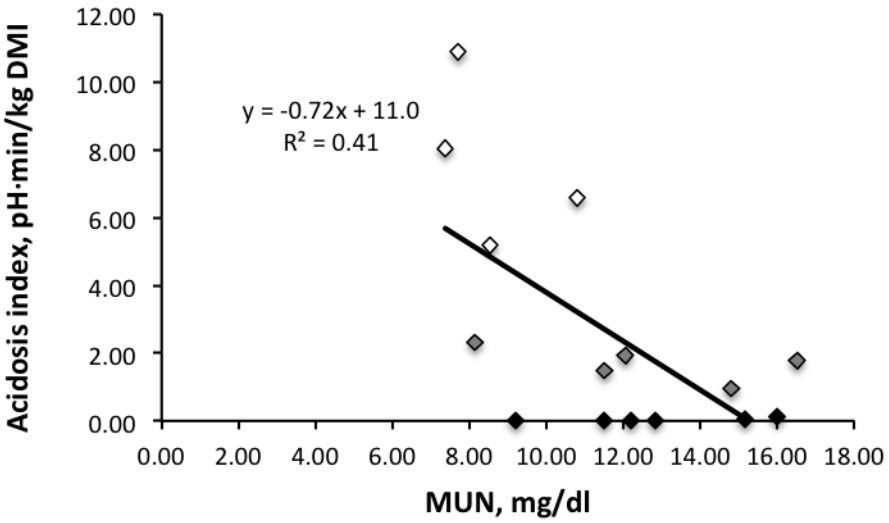


**Figure 2.7.10** Relationship between acidosis index with milk urea nitrogen (MUN) concentration ($P = 0.01$). Cows identified with higher risk of SARA (white diamond), and cows identified with lower risk of SARA (black diamond).

# Chapter 3. Non-invasive indicators to identify lactating dairy cows with the greater risk of sub-acute ruminal acidosis

## 3.1 Introduction

Sub-acute ruminal acidosis (SARA) is a metabolic disorder particularly prevalent in high-producing dairy cows. Better understanding of diet formulation could reduce the incidence of SARA, but SARA may still occur for some cows in a herd. It has been indicated that variations exist in the risk of developing SARA among primiparous dry cows (Penner et al., 2007), sheep (Penner et al., 2009), beef steers (Brown et al., 2000; Schlau et al., 2012), and lactating dairy cows (Gao and Oba, 2014) when they were fed a same diet. Although the reasons are not fully understood, this variation could be associated with variations in acid production in the rumen or acid removal from the rumen through uptake via rumen wall, neutralization by buffers, and passage from rumen (Penner et al., 2009). Rations are often formulated for a group of cows on dairy farms, thus due to this variation in the risk of SARA, the susceptible cows may experience SARA whereas others do not even they are fed the same diet (Penner et al., 2009). Therefore, it is necessary to identify cows that have higher risk of SARA and adjust the nutritional management accordingly for these cows (Gao and Oba, 2014). However, it is not practical to measure rumen pH of all cows on commercial dairies, and a non-invasive indicator is needed. Milk fat depression is commonly related to SARA (Oetzel, 2003; Stone, 2004). However, in our previous study (Gao and Oba, 2014), I found that cows with higher risk of SARA had lower MUN compared with those with lower risk of SARA

although there was no difference in milk fat concentration. Therefore, the objective of the current study was to evaluate if MUN and milk fat content could be used as the non-invasive indicator to identify cows with high and low risk of SARA.

## 3.2 Materials and methods

The experiment was conducted in accordance with the guidelines of the Canadian Council of Animal Care (Ottawa, Ontario, Canada). All the experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock.

Thirty-five late-lactating Holstein cows (DIM = 250 ± 71.1; BW = 601 ± 45.4 kg; mean ± SD) were used in the first study (screening study). Their average milk yield was 28.8 kg/d. Cows were fed a high-grain diet consisting of 35% forage and 65% concentrates ad libitum for 21 d to induce SARA (Table 3.6.1). Cows were housed individually in tie stalls bedded with wood shavings, fed the experimental diet as a TMR once daily at 0800 h, and had free access to water. Feed was offered at 105 to 110% of actual feed intake of the previous day. The weight of feed offered and refused was recorded daily. The DM concentration of barley silage was determined twice weekly and diet formulation was adjusted if necessary. Cows were weighed after the morning milking on 2 consecutive days immediately before the start of experiment. Cows were milked twice daily at 0400 and 1500 h. After a 18-d diet adaptation period, milk samples were collected for 3 consecutive days. Milk was sampled from both a.m. and p.m. milkings on d 19, 20, and 21. Milk samples were analyzed for milk fat, CP, lactose, and MUN by infrared spectroscopy (AOAC International, 2002; method 972.16; MilkoScan 605, Foss

North America, Brampton, ON, Canada) at the Alberta Central Milk Testing Laboratory (Edmonton, AB, Canada). Concentration of MUN and milk fat ranged from 5.7 to 13.9 mg/dL and from 2.32 to 4.84%, respectively. As the average milk fat content was 3.5%, five cows with highest MUN concentrations with milk fat content higher than 3.5% were selected as animals that presumably had low risk of SARA (**LS**), and 5 cows with lowest MUN concentrations with milk fat less than 3.5% were selected as animals that presumably had high risk of SARA (**HS**) (Figure 3.6.3). Concentrations of milk fat (4.10 ± 0.31 vs. 2.95 ± 0.31 %; $P < 0.01$) and MUN (12.9 ± 0.64 vs. 8.03 ± 1.39 mg/dL; $P < 0.01$) were higher for LS compared with HS cows. These 10 animals were ruminally cannulated during the subsequent dry period.

The second study (rumen pH monitoring study) was conducted during the following mid-lactation. As one LS cow was culled due to fatty liver, five HS and four LS ruminally cannulated cows (DIM = 122 ± 33.2; BW = 615 ± 49.1 kg; mean ± SD) were used. All cows were fed a high grain diet in TMR consisting of 35% forage and 65% concentrates ad libitum for 21 d to induce SARA (Table 3.6.1). The daily cow management was similar to the first study, and described above. The pH measurement system developed by Penner et al. (2006) was used to measure ruminal pH in the ventral sac every 30 s continuously for 72 h (d 19–21). Minimum, mean, and maximum pH, as well as duration and area below pH 5.8 were determined for each cow daily, and averaged over the 3-d period as described in Chapter 2. Then acidosis index (area under pH 5.8 divided by DMI; Penner et al., 2009) was calculated to assess the severity of SARA normalized for DMI. Milk samples were collected from both a.m. and p.m.

milkings on d 19, 20, and 21 of the study as well. Milk samples were analyzed for milk fat, protein and MUN as described above.

All response variables were evaluated for the group effect using the PROC TTEST procedure of SAS (version 9.2, SAS Institute Inc., Cary, NC). Significance was declared at $P < 0.05$, and tendency was declared at $0.05 < P < 0.10$.

### 3.3 Results and discussion

In the rumen pH monitoring study, there was no difference in DIM, BW and DMI between LS and HS animals ($P > 0.10$; Table 3.6.2). However, the minimum (5.75 vs. 5.30; $P = 0.02$) and mean ruminal pH (6.35 vs. 6.04; $P = 0.02$) was higher for LS animals compared with HS animals. In addition, duration of rumen pH below 5.8 was shorter in LS animals (52.5 vs. 395 min/d; $P = 0.04$). Although there were large numerical differences, area of rumen pH below 5.8 and acidosis index were not different between the groups, which is possibly due to relatively low number of animals in the study; I had 25% chance to detect the differences in area below pH 5.8 and acidosis index between the groups according to a power analysis. In the current study, LS cows identified in the screening study had higher rumen pH than the HS cows in the rumen pH monitoring study, which is conducted in the subsequent lactation, but it is important to note that the risk of SARA can change during the life of cows. Dohme et al (2008) has shown that the severity of acidosis increased for cows with repeated acidosis challenges, which suggests that feeding history can affect SARA risk within the same cows.

Milk yield was lower for LS cows compared with HS cows in the rumen pH monitoring study (33.8 vs. 40.7 kg/d; $P = 0.05$). However, no difference was observed in

milk yield between LS and HS cows in our previous study (Gao and Oba, 2014). Therefore, I cannot suggest that there is a relationship between milk yield and risk of SARA. In addition, there were no differences in yields or concentration of milk components between LS and HS groups in the rumen pH monitoring study.

Milk fat depression commonly occurs with SARA (Oetzel, 2003; Stone, 2004). Due to low rumen pH, biohydrogenation intermediates are produced in the rumen from the alternative pathways of ruminal fatty acid biohydrogenation, and these intermediates can be transported to the mammary gland and result in the decrease in milk fat content and yield (Bauman and Griinari, 2003). However, milk fat content did not differ between LS and HS cows in the rumen pH monitoring study, which was not consistent with the screening study, but it is consistent with our previous study (Gao and Oba, 2014). In that study, a large numerical difference was found in milk fat concentration between the two groups (3.22 vs. 2.73%), but it was not detected as statistical significance due to the large variation within groups (SE = 0.33). Similarly, there was a large numerical difference in milk fat concentration between the two groups (3.61 vs. 3.08%) in the current rumen pH monitoring study, and the difference was not significant as well due to the substantial variation within groups (SE = 0.29). Oetzel (2007) also suggested that milk fat depression does not occur for many cows and herds with depressed rumen pH. Therefore, as summarized in our previous study (Gao and Oba, 2014), it is not sensitive to use milk fat content alone to identify cows with high or low risk of SARA, because besides rumen pH, milk fat content is also affectd by other factors.

The current results suggest that MUN content along with milk fat content can be useful indicators to identify cows with high or low risk of SARA. MUN is generally used

to predict the efficiency of protein usage and excretion of urinary N in dairy cows (Gustafsson and Palmquist, 1993; Kohn et al., 2002). Nutritional factors can have an effect on MUN concentration, such as the extent of CP degradation in the rumen (Roseler et al., 1993) and the ratio of dietary energy to CP (Oltner and Wiktorsson, 1983). In addition, Abdoun et al. (2006) suggested that carbohydrate fermentation in the rumen can alter the permeability of the ruminal epithelial cell and the extent of urea recycled back to the rumen, which could affect MUN concentration; the more urea-N is recycled back to the rumen, the less would be excreted in urine and milk. Non-nutritional factors, such as sampling time (a.m. vs. p.m. milking), season, breed, DIM and level of production also affect MUN concentration (Carlsson et al., 1995; Broderick and Clayton, 1997).

Significant difference in MUN concentration was observed between cows that differ in SARA risk in our previous study (Gao and Oba, 2014) as well as in this screening study, in which late-lactation cows were used. Greater MUN concentration for LS cows may be attributed to lower amount of OM fermentation in the rumen than HS cows, even if the same diet was fed, potentially resulting in lower microbial protein synthesis (Nocek and Russell, 1988) and more N secretion via MUN. Schlau et al. (2012) showed that acidosis-resistant steers had higher rumen pH and lower VFA concentration compared with cows prone to SARA. However, I used mid-lactation cows in the current rumen pH monitoring study, and MUN concentration did not differ between LS and HS cows (11.8 vs. 10.9 mg/dL, respectively; $P = 0.56$). Rumen pH is expected to be generally lower for mid-lactation cows than late-lactation cows due to greater DMI. Although I used a different set of animals, mean rumen pH was 6.47 for LS cows in the previous study that used late-lactation cows (Gao and Oba, 2014) while mean rumen pH

of LS cows was 6.35 in the current rumen pH monitoring study, where mid-lactation cows were used. Abdoun et al. (2010) found that urea transport rates increased as mucosal pH decreased from 7.0 to 6.2. Therefore, lower rumen pH for LS cows used in the current rumen pH monitoring study, compared with the screening study, might have increased urea recycling to the rumen and then decreased the amount of urea excreted in urine or milk, reducing the difference in MUN concentration between LS and HS cows and making it undetectable with statistical significance. Further research is warranted to explain why differences in MUN concentration between LS and HS cows were not consistent for different stage of lactation.

## 3.4 Conclusions

Results from our study suggest that MUN and milk fat content in late-lactating cows fed a high grain diet may be used to identify cows that have higher or lower risk of SARA. However, further research using a greater number of animals is warranted. In addition, it is necessary to determine whether differences in MUN concentration between LS and HS cows are affected by stage of lactation.

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## 3.6 Tables and figures

**Table 3.6.1** Ingredient and nutrient composition of the experimental diets

| Item | Screening study | Rumen pH monitoring study |
|---|---|---|
| Ingredient, % DM | | |
| Barley silage | 35.6 [1] | 35.0 [2] |
| Barley grain, rolled | 23.5 | 23.6 |
| Corn grain, rolled | 22.2 | 17.5 |
| Canola meal | 5.13 | 13.5 |
| Corn gluten meal | 4.35 | 4.36 |
| Amino plus | 5.26 | - |
| Beet pulp | - | 2.73 |
| Vegetable oil | 1.52 | 0.84 |
| Sucrose | 0.57 | - |
| Mineral and vitamin mix | 1.87 [3] | 2.40 [4] |
| Nutrient composition | | |
| DM | 57.3 | 54.4 |
| Ash, %DM | 7.15 | 6.95 |
| CP, %DM | 16.5 | 16.4 |
| NDF, %DM | 25.8 | 28.2 |
| Starch, %DM | 32.0 | 29.0 |
| Ether extract, %DM | 4.56 | 4.10 |
| NFC, %DM | 46.1 | 46.6 |
| Forage NDF, %DM | 16.7 | 16.2 |

[1] 31.0% DM, 12.4% CP, 48.1% NDF, and12.1% starch

[2] 32.1% DM, 11.3% CP, 51.7% NDF, and11.6% starch

[3]Contained 11.6% Ca, 3.4% P, 18.0% Na, 27.8% Cl, 6.8% Mg, 0.24% S, 0.04% K, 2447 mg/kg Fe, 551 mg/kg Cu, 1054 mg/kg Mn, 13 mg/kg Co, 842 mg/kg Zn, 8.23 mg/kg Se, 24.7 mg/kg I, 504 kIU/kg vitamin A, 51.3 kIU/kg vitamin D, 1651 IU/kg vitamin E

[4]Contained 15.3% Ca, 2.8% P, 12.4% Na, 25.0% Cl, 5.4% Mg, 0.29% S, 6.5% K, 3270 mg/kg Fe, 665 mg/kg Cu, 1246 mg/kg Mn, 14.9 mg/kg Co, 1010 mg/kg Zn, 9.95 mg/kg Se, 32.4 mg/kg I, 510 kIU/kg vitamin A, 51.7 kIU/kg vitamin D, 1668 IU/kg vitamin E

**Table 3.6.2** Comparison of DIM, BW, DMI, and pH measurements between LS [1] and HS [2] cows in the rumen pH monitoring study

| | LS (n = 4) | | HS (n = 5) | | |
|---|---|---|---|---|---|
| **Variable** | **LSM** | **SE** | **LSM** | **SE** | ***P*** |
| DIM | 116 | 17.5 | 123 | 15.7 | 0.66 |
| BW, kg | 637 | 23.9 | 598 | 21.4 | 0.27 |
| DMI, kg | 26.7 | 1.25 | 26.5 | 1.12 | 0.90 |
| Ruminal pH | | | | | |
| Nadir | 5.75 | 0.11 | 5.30 | 0.09 | 0.02 |
| Mean | 6.35 | 0.07 | 6.04 | 0.06 | 0.01 |
| Maximum | 6.93 | 0.07 | 6.81 | 0.07 | 0.27 |
| Duration < pH 5.8, min | 52.5 | 103 | 395 | 91.9 | 0.04 |
| Area < pH 5.8, pH × min | 7.68 | 45.8 | 107 | 41.0 | 0.15 |
| Acidosis index, pH × min/kg | 0.29 | 1.83 | 4.26 | 1.64 | 0.15 |

[1] LS: cows with highest MUN concentrations with milk fat higher than 3.5% in the screening study; selected as animals that presumably have low risk of SARA.

[2] HS: cows with lowest MUN concentrations with milk fat less than 3.5% in the screening study; selected as animals that presumably have high risk of SARA.

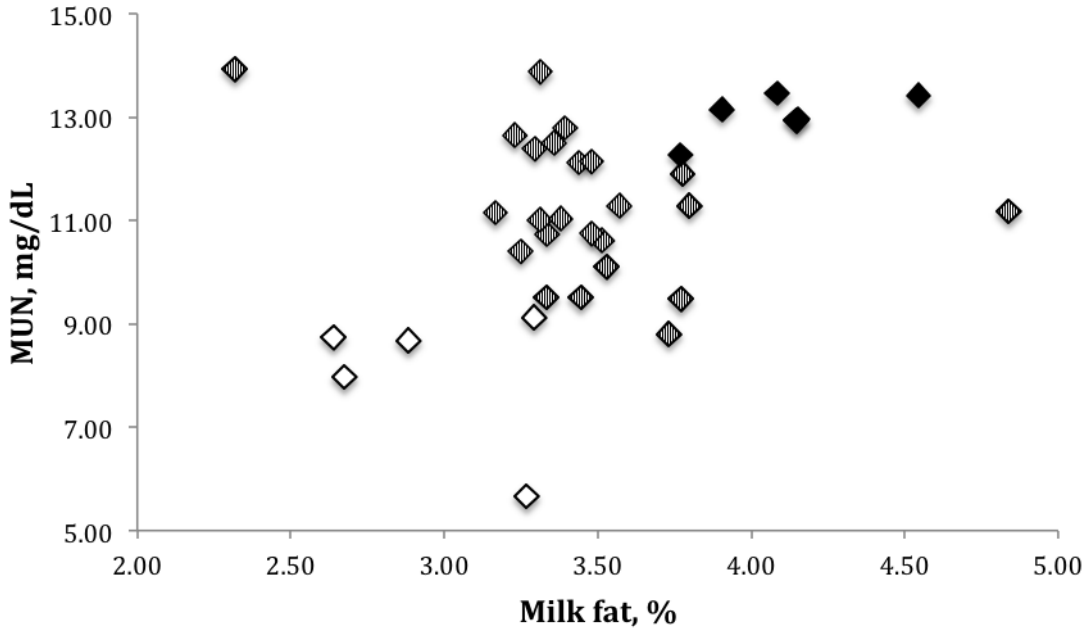


**Figure 3.6.3** Variation of MUN and milk fat content among 35 animals in the screening study: cows with presumably high risk of SARA (white diamond) and cows with presumably low risk of SARA (black diamond). Striped diamonds represent the other 25 cows used in the screening study that were not selected for the rumen pH-monitoring study.

# Chapter 4. Characteristics of dairy cows with a greater or lower risk of sub-acute ruminal acidosis: volatile fatty acid absorption, rumen digestion and consistency of the risk

## 4.1 Introduction

Highly fermentable diets are often fed to high-producing lactating cows to achieve high level of milk production. However, excessive fermentable diets can increase the risk of sub-acute ruminal acidosis (SARA). Although NRC (2001) recommends the maximum NFC and minimum NDF inclusion levels (% of DM) in the rations to decrease the occurance of SARA, some animals in a herd may still have SARA. Previous studies on primiparous dry cows (Penner et al., 2007), beef steers (Brown et al., 2000; Schlau et al., 2012), sheep (Penner et al., 2009a) and lactating dairy cows (Gao and Oba, 2014) suggested that variation exists in the severity of SARA among animals even when they were fed the same diet. The reasons of this variation are not fully understood. Rumen pH is the difference between acid production in the rumen and acid removal from the rumen (Allen, 1997). There are three primary pathways of acid removal: absorption by rumen epithelium, neutralization by buffers, and passage from the rumen to the lower part of digestive tracts (Allen, 1997). Therefore, variation in the risk of SARA among ruminants could be related to at least one of these factors (Penner et al. 2009a). Absorption of VFA contributes approximately 53% of the total rumen proton removal (Allen, 1997), and Penner et al., (2009a) found that *in vitro* absorption rates of acetate and butyrate by rumen epithelial tissue were higher for sheep with a lower risk of SARA than those with

a higher risk, which suggested that differences in the VFA absorption rate might be related to the variation in the severity of SARA among the animals in vivo. Therefore, I hypothesized that lactating dairy cows with lower risk of SARA would have faster VFA absorption rate compared with cows with higher risk of SARA. In addition, besides VFA and proton transportation, the process of VFA absorption also involves the exchange of other ions, such as $Na^+$ and $HCO_3^-$, and the metabolism of VFA in the epithelial tissue, which can regulate the intracellular pH and VFA absorption (Aschenbach et al., 2011; Penner et al., 2011). Therefore, investigation is needed for the relationship between variation in the severity of SARA among cows and expression of genes associated with ion exchanges, intracellular pH regulation and VFA metabolism in the rumen epithelial tissue.

Besides VFA absorption, acid production from OM fermentation is another main factor affecting rumen pH. Chen et al. (2012) found that steers with lower risk of SARA had lower bacterial density from rumen digesta than higher risk ones, which might indicate that less VFA produced in the rumen of lower risk steers compared with higher risk ones. Therefore, differences in acid production from OM fermentation in the rumen might be related to variation of severity of SARA among lactating cows, and I hypothesized that cows with greater risk of SARA would have greater in situ carbohydrate digestibility in the rumen than cows with lower risk of SARA. In addition, factors causing variation of the risk in developing SARA among cows are related to the animal itself, but it is not known if risk categories (higher or lower) of SARA are consistent throughout the lactation within animals.

The objectives of this study were to examine if lactating dairy cows with a greater or lower risk of SARA have differences in VFA absorption rate, expression of genes involved in VFA metabolism and intracellular pH regulation in rumen epithelial cells, and in situ carbohydrate digestibility in the rumen, and determine if cows identified to have a greater risk of SARA in mid-lactation consistently have lower rumen pH in late-lactation than those identified to have a lower risk of SARA.

## 4.2 Materials and methods

The experiments were conducted at the Dairy Research and Technology Center of University of Alberta (Edmonton, AB, Canada), and they were performed in accordance to the guidelines of the Canadian Council of Animal Care (Ottawa, Ontario, Canada). All experimental procedures were preapproved by the Animal Care and Use Committee of University of Alberta.

### *4.2.1 Experiment 1*

#### *4.2.1.1 Animals, Diets, and Experimental Design*

Fourteen multiparous ruminally cannulated mid-lactating Holstein cows (DIM = 119 ± 47.2; BW = 640 ± 47.9 kg; mean ± SD) were used in the first study. Cows were fed a high-grain diet containing 30% forage and 70% concentrate mix (Table 4.7.1) ad libitum for 25 d with a 18-d diet adaptation period and a 7-d data and sample collection period. Cows were housed in tie stalls individually bedded with wood shavings, and had free access to water. The experimental diet as a TMR was fed to cows once daily at 0800h, and was offered at 105 to 110% of actual feed intake of the previous day. The

amounts of feed offered and refused were recorded daily during the study, and samples of feed ingredients were collected daily during the sample collection period. The DM concentration of barley silage was measured twice weekly by drying samples in an air-forced oven at 55°C for 48 h, and as-fed proportions of feed ingredients were adjusted if necessary. Cows were weighed after the morning milking on 2 consecutive days immediately before the start of experiment as described in Chapter 2. Cows were milked twice daily at 0400 and 1500 h.

#### *4.2.1.2 Rumen pH and Rumen Fermentation*

The pH measurement system developed by Penner et al. (2006) was used to measure rumen pH in the ventral sac every 30 s continuously for 72 h (d 19 - 21) as described in Chapter 2. Minimum, mean, and maximum pH was determined for each cow daily, and averaged over 3-d periods. Daily duration and area below pH 5.8 was also calculated for each cow, and averaged over 3-d periods. DMI was recorded daily for each cow on d 19, 20 and 21, and averaged over 72-h periods. Acidosis index (area under pH 5.8 divided by DMI; Penner et al., 2009b) was calculated to evaluate the severity of SARA normalized for DMI. Values of mean ± 0.5 × SD of acidosis index were used as thresholds to identify groups of extreme animals.

Rumen fluid samples were collected every 9 h over a 72-h period on d 19, 20, and 21. Samples were collected in equal proportions from the cranial, ventral, and caudal sacs, and combined to form one sample. Then samples were strained through a perforated screen (Peetex, Sefar Canada Inc., Scarborough, ON, Canada; pore size = 355 μm). Immediately after collection, rumen fluid samples were centrifuged at 3,000 × g at 4°C

for 20 min, and the supernatants were stored at −20°C until analysis. Samples were composited to yield one sample for each cow for further analysis.

Gas chromatography was used to analyze VFA profile for rumen fluid samples according to the method described by Khorasani et al. (1996). In addition, rumen ammonia-N concentration was determined as described by Fawcett and Scott (1960).

#### *4.2.1.3 Blood Collection*

Blood samples were collected every 18 h on d 19 - 21 from the coccygeal vessels into tubes containing sodium heparin (Fisher Scientific Company; Nepean, Ontario, Canada) as described in Chapter 2. Immediately following collection, plasma was harvested by centrifuging blood samples at 3,000 × g at 4ºC for 20 min. Plasma samples were stored at -20ºC until analysis. Samples were composited to yield one sample per cow for further analysis.

Plasma samples were analyzed for glucose, BHBA, NEFA and insulin concentrations. Plasma glucose concentration was measured using a glucose oxidase/peroxidase enzyme (Sigma; St. Louis, MO) and dianisidine dihydrochloride (Sigma) procedure followed by determination of absorbance at a wavelength of 450 nm. Plasma BHBA concentration was measured by the enzymatic oxidation of BHBA to acetoacetate using 3-hydroxybutyrate dehydrogenase (Roche, Mississauga, ON, Canada), and the reduction of $NAD^+$ to NADH was measured by a plate reader at a wavelength of 340 nm. Commercial kits were used to measure concentrations of plasma NEFA (Wako Chemicals USA, Inc., Richmond, VA) and insulin (Coat-a-Count kit, Diagnostic Products Corp., Los Angeles, CA).

#### *4.2.1.4 Milk composition*

Milk samples were collected from both a.m. and p.m. milkings on d 19, 20, and 21 of the study. Milk samples were analyzed for milk fat, CP, lactose, and MUN by mid-infrared spectroscopy (AOAC, 2002; method 972.16; MilkoScan FTIR 6000, Foss North America, Brampton, ON) at the Alberta Central Milk Testing Laboratory (Edmonton, AB) as described in Chapter 2. Milk yield and milk composition data from both a.m. and p.m. milking were used to calculate the daily milk composition, and daily data were averaged over 3-d periods for one value per cow.

#### *4.2.1.5 In situ starch and NDF digestibility*

Samples of feed ingredients (barley silage and concentrate mix) were collected and dried at 55°C for 48 h in a forced air oven (V-31 STD, Style II, Despatch Industries Inc., Nashua, Mississauga, Ontario, Canada). Then they were ground through a 1-mm screen using a Wiley mill (Thomas Wiley, Philadelphia, PA). After grinding, they were totally mixed according to the DM proportion of TMR (30% barley silage and 70% concentrate mix). Bags were prepared as that 2 g of the mixed sample was placed into one nitrogen free polyester bag (5 × 10 cm, pore size = 50 μm; R510, Ankom Technology, Macedon, NY). Then bags with ten replicates (5 for starch and 5 for NDF digestibility) were placed into the rumen through rumen cannula for each cow on d 22. After 7h incubation, 5 bags were taken out of the rumen. The other 5 bags were removed from the rumen after 30h incubation. Bags were washed with cold water, and then placed in the oven and analyzed for concentrations of analytical DM (AOAC, 2002; method 930.15). Then samples with 7-h incubation were analyzed for starch concentration (Hall, 2009), and samples with 30-h incubation were analyzed for NDF concentration (Van Soest et al., 1991, method A) by Cumberland Valley Analytical Services (Hagerstown, MD). Mixed

feed ingredient samples prior to incubation were analyzed for DM, and both starch and NDF concentration. The 7h starch and 30h NDF digestibility was calculated as (1 – amount of starch or NDF remaining after rumen incubation / amount of starch or NDF prior to rumen incubation) × 100, respectively.

#### *4.2.1.6 Rumen VFA absorption*

Rumen VFA absorption rate was determined on d 24 according to the method described by Penner et al. (2009b). Co-EDTA was used as a marker of fluid passage (Uden et al., 1980), and n-valeric acid was used as an indicator of VFA clearance (absorption and passage) from the rumen (Resende Júnior et al., 2006). Therefore, the fractional rate of VFA absorption is the n-valeric acid clearance rate subtracted from Co clearance rate. At 4 h after feeding, 2.5-L solution containing 125 g of Co-EDTA buffered to pH 6.2 with NaOH and 300 g of n-valeric acid (VWR International, Edmonton, AB, Canada) was dosed into the rumen for each cow. The solution was mixed thoroughly with the rumen contents by hand. Immediately before the marker dose, an initial rumen sample was collected (time 0). Subsequent rumen fluid samples were collected at 30, 60, 120, 180, 360, 720, and 1,080 min after the dose. Valeric acid concentration was determined for the individual rumen fluid samples as described above for analyzing VFA profile. Co concentration was determined for each sample by using atomic absorption (AA240FS, Varian, Palo Alto, CA).

Co concentration at time 0 was used as the baseline to correct concentrations measured at other time points (i.e., 30, 60, 120, 180, 360, 720, and 1,080 min relative to dosing). Because markers were dosed at 4 h after feeding, concentration of n-valeric acid at time 0 was not necessarily lowest. Therefore, instead of concentration at time 0, the

lowest concentration was used as the baseline concentration of n-valeric acid to correct concentrations measured at other individual time points. As described by Resende Júnior et al. (2006), the exponential decay rate of rumen n-valeric acid concentration is the VFA clearance rate, and the exponential decay rate of ruminal Co concentration is the VFA passage rate. Then the exponential decay rate for the ratio of rumen n-valeric acid and Co concentration were calculated to determine the VFA absorption rate, using the nonlinear model procedure of JMP (version 11.0, SAS Institute Inc., Cary, NC) with the equation $Y_t = Y_0 \times e^{-kt}$, where $Y_t$ = concentration at a given time, $Y_0$ = baseline concentration, k = fractional rate of absorption, t = sampling time, h.

#### *4.2.1.7 Ruminal Papillae Collection*

Ruminal papillae were biopsied from the ventral sac of the rumen 3h after feeding on d 24 from each cow. The papillae were rinsed with phosphate buffered saline (pH 7.4), snap-frozen in liquid nitrogen, and stored at -80ºC in RNA-Later (Ambion, Incorporated; Foster City, CA) solution until analysis.

#### *4.2.1.8 RNA Extraction and Reverse Transcription*

Total RNA was extracted from the ruminal papillae with Trizol reagent (Life Technologies, Burlington, ON, Canada) as described by Chomczynski and Sacchi (1987). Then RNA concentration was determined using a spectrophotometer (NanoDrop 2000 Spectrophotometer, NanoDrop Technologies; Wilmington, DE). The quality and integrity of the total RNA extracted were verified by TapeStation (Agilent Technologies, Waldbronn, Germany), and the value of RIN was higher than 8.2 for all samples.

RNA was treated with DNase (QIAGEN®) and then equal amounts of RNA (1µg) was reverse-transcribed by MultiScribe™ reverse transcription (Life Technologies; Carlsbad, CA) and random primers (Life Technologies; Carlsbad, CA).

#### *4.2.1.9 Real Time Quantitative PCR*

Real time PCR was performed by two methods in this study. First batch of target genes were analyzed by using Taqman kits, because these target genes had been evaluated in previous studies in our lab by using Taqman kits (Penner et al., 2009b; Schlau et al., 2012). Thus, Taqman kits were available for these target genes, and they were evaluated first in this study. Later, second batch of target genes were selected for the real time PCR analysis. Most of these genes had been evaluated in previous studies by using Fast SYBR® Green kits (Steele et al., 2011; Chen et al., 2012; Malmuthuge et al., 2013; Yan et al., 2014), and Fast SYBR® Green kits are less expensive than Taqman kits. Therefore, Fast SYBR® Green kits were used and evaluated for the second batch of target genes.

Real time PCR was performed as described previously (Laarman et al., 2012) using Taqman Fast Universal Master Mix (Life Technologies; Carlsbad, CA) with The StepOne Plus Real-Time PCR System (Life Technologies; Carlsbad, CA) for target genes 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 ATPase, $Na^+/K^+$ transporting, α 1 polypeptide ($Na^+/K^+$ ATPase), which are involved in VFA absorption and intracellular pH regulation; target genes 3-hydroxy-3-methylglutaryl-CoA Synthase 1 and 2 (HMGCS-1, HMGCS-2), 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL) and β-

Hydroxybutyrate dehydrogenase, isoform 1 and 2 (BDH1, BDH2), which are involved in VFA metabolism and ketogenesis. Primers and probes (Table 4.7.2) were used as described in the previous published literature (Penner et al., 2009b; Schlau et al., 2012). The primers' efficiency was determined using standard curve method (with at least four serial dilutions) in a pool of all samples. All samples were analyzed on one plate per gene in triplicate.

Real-time RT-PCR assays were performed with a StepOnePlus Real-Time PCR System (Life Technologies, Carlsbad, CA) using Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions for target genes monocarboxylate cotransporter, isoform 4 (MCT4), vacuolar $H^+$ ATPase subunit B ($vH^+$ ATPase), anion exchanger 2 (AE2), carbonic anhydrase 2 (CA2), which are involved in VFA absorption and intracellular pH regulation; target genes acyl-CoA synthetase short-chain family member 1 (Acyl-CoAs), acetyl-CoA synthetase 2 (Acetyl-CoAs), acetyl-CoA acyltransferase 1 (ACAA1), which are involved in VFA metabolism and ketogenesis; target genes acetoacetyl-CoA thiolase 2 (ACAT2), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), farnesyl diphosphate synthase (FDPS), sterol regulatory element-binding protein 1 and 2 (SREBP1, SREBP2), lanosterol synthase (LSS), which are involved in cholesterol synthesis; target genes of toll-like receptor 2 and 4 (TLR2, TLR4), claudin 1 (CLDN1), junctional adhesion molecule 2 (JAM2), occludin (OCLN), zonula occluden 1 (ZO1), which are involved in barrier function and tight junction. The primers for MCT4, CA2, JAM2 and ZO1 (Table 4.7.2) were designed using Primer Express v.3.0 (Applied Biosystems; Foster City, CA) and analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify the primer specificity. Other primers

were used as described in the previous published literature (Penner et al., 2009b; Steele et al., 2011; Chen et al., 2012; Schlau et al., 2012; Malmuthuge et al., 2013; Yan et al., 2014). The primers' efficiency was determined using standard curve method (with at least four serial dilutions) in a pool of all samples. The program was 40 cycles of 3 s at 95ºC and 30 s at 60ºC. Melting curve analyses were performed for each qPCR sample at the end of each run to ensure the specificity of the amplification. All investigated PCR products showed only single melting peak. All samples were analyzed on one plate per gene in triplicate.

The mRNA abundance of target genes was evaluated using the comparative cycle threshold (Ct) method, and then normalized using the geometric mean of three housekeeping genes, ribosomal protein large, P0 (RPLP0), β-actin (ACTB), and glyceraldehyde-3-phosphate dehydrogenase (GADPH), as described previously (Vandesompele et al., 2002; Laarman et al., 2012; Salehi et al., 2016). Briefly, samples from all 14 cows were analyzed on the same plate for one gene. For each sample, Ct value of the gene was calculated, and it was subtracted from the minimum Ct among all 14 samples to obtain ΔCt value of the gene. Then, the relative abundance of mRNA for the gene was calculated for each sample by using the formula: Q = Amplification efficiency$^{\Delta Ct}$. The normalized mRNA abundance for the gene was calculated for each sample by using the formula: $Q_n = Q/NF$, where NF is the normalization factor, calculated as the geometric average of the Q values for RPLP0, β-actin and GAPDH for each sample. $Q_n$ value of the gene for each cow was used to evaluate if there was difference in the relative abundance of mRNA between cows with higher and lower risk of SARA.

### *4.2.2 Experiment 2*

Nine extreme cows (4 higher risk and 5 lower risk) identified from the experiment 1 were used in this study when they were at later stage of lactation (DIM = 243 ± 19.6; BW = 703 ± 48.8 kg; mean ± SD). Cows were fed the same high-grain diet as experiment 1 containing 30% forage and 70% concentrate mix (Table 4.7.1) ad libitum for 21 d, consisting of a 18-d diet adaptation period and a 3-d data and sample collection period. The daily cow management was similar to the experiment 1, and described above. After 18-d diet adaptation, rumen pH was measured in the ventral sac every 30 s continuously for 72 h (d 19–21) using the pH measurement system evaluated by Penner et al. (2006).

### *4.2.3 Statistical Analysis*

In experiment 1, all response variables were evaluated using the fit Y by X procedure of JMP (version 11.0, SAS Institute Inc., Cary, NC) to compare the group effect, and relationships between mRNA abundance of slelcted genes and minimum ruminal pH were analyzed by using the fit Line procedure of JMP.

In experiment 2, data were analyzed using the fit model procedure of JMP according to the following model:

$$Y_{ijk} = \mu + C_i + S_j + CS_{ij} + e_{ij},$$

where $Y_{ijk}$ is the dependent variable, $\mu$ is overall mean, $C_i$ is fixed effect of category, $S_j$ is fixed effect of stage of lactation, $CS_{ij}$ is interaction of category and stage of lactation, and $e_{ij}$ is the residual. For response variables with a significant category × stage of lactation interaction, a Student's t-test was used to separate treatment means.

Significance was declared at $P < 0.05$ and tendency was declared at $0.05 < P < 0.10$.

## 4.3 Results

### *4.3.1 Experiment 1*

Among all cows, minimum rumen pH, mean rumen pH, duration and area pH below 5.8 ranged from 5.13 to 6.03, 5.90 to 6.45, 0.17 to 633 min/d, and from 0.0 to 111 pH × min/d, respectively. The acidosis index ranged from 0.0 to 4.35 pH × min/kg DMI. Acidosis index of 8 cows were lower than the value of mean - 0.5 × SD, and that of 5 cows were higher than the value of mean + 0.5 × SD, and they were categorized as animals with lower SARA risk (LS) or higher SARA risk (HS), respectively.

#### *4.3.1.1 Rumen pH, VFA Profile and VFA absorption rate*

No differences were observed in BW and DMI between LS and HS animals ($P > 0.10$; Table 4.7.3). However, the minimum (5.75 vs. 5.33; $P < 0.01$) and mean ruminal pH (6.33 vs. 5.98; $P < 0.01$) was higher for LS animals compared with HS animals, whereas maximum pH values were not different between the groups. Duration (24.9 vs. 481 min/d; $P < 0.01$) and area (2.94 vs. 102 pH × min/d; $P < 0.01$) that pH was below 5.8 were lower in LS animals. Acidosis index was lower in LS animals (0.10 vs. 3.72 pH × min/kg DMI; $P < 0.01$). Total VFA concentration, VFA profiles, concentration of rumen $NH_3$-N and VFA absorption rate were not different between the groups ($P > 0.10$; Table 4.7.4).

#### *4.3.1.2 In situ starch and NDF digestibility*

In situ 7h starch and 30h NDF digestibility were not different between LS and HS animals ($P > 0.10$; Table 4.7.4).

#### *4.3.1.3 Milk Production*

No differences were observed in milk yield and milk component yields between LS and HS cows ($P > 0.10$; Table 4.7.5). In addition, concentrations of milk protein, lactose and MUN did not differ between the groups. However, concentration of milk fat tended to be higher for LS animals compared with HS animals (3.36 vs. 2.93 %; $P = 0.07$).

#### *4.3.1.4 Plasma Metabolites and Hormones*

Plasma glucose, BHBA, NEFA and insulin concentrations were not different between LS and HS cows ($P > 0.10$; Table 4.7.6).

#### *4.3.1.5 Rumen Epithelium Gene Expression*

Relative mRNA abundance of genes associated with VFA absorption and intracellular pH regulation were not different between LS and HS cows ($P > 0.10$; Table 4.7.7). But relative mRNA abundance of NHE1 tended to be positively correlated with minimum rumen pH ($r = 0.47$, $P = 0.08$; Figure 4.7.9). Gene encoding LSS had higher mRNA abundance for LS cows than HS cows ($P = 0.03$). In addition, the mRNA abundance of HMGCS1 tended to be higher for LS cows compared with HS cows ($P = 0.08$). Relative mRNA abundance of the other genes associated with epithelial cell VFA metabolism was not different between LS and HS cows ($P > 0.10$). But mRNA abundance of BDH2 tended to be positively correlated with minimum rumen pH ($r = 0.50$, $P = 0.07$). Relative mRNA abundance of genes associated with epithelial barrier function and tight junction were not different between LS and HS cows ($P > 0.10$).

### *4.3.2 Experiment 2*

No difference was observed in DMI between LS and HS animals ($P > 0.10$; Table 4.7.8), but DMI was higher for cows during mid-lactation than late-lactation ($P = 0.01$).

Minimum ($P < 0.01$) and mean rumen pH ($P < 0.01$) was higher for LS animals compared with HS animals, and there was no significant effect of stage of lactation and interaction between category and stage of lactation on rumen pH. Duration ($P < 0.01$) and area ($P < 0.01$) that pH was below 5.8 were lower in LS animals than HS animals. In addition, cows during mid-lactation had longer duration ($P = 0.03$) and tended to have higher area ($P = 0.08$) that pH was below 5.8 than late-lactation. Interactions were detected between category and stage of lactation for duration and area that pH was below 5.8. HS cows during mid-lactation had longer duration and higher area that pH was below 5.8 than late-lactation (488 vs. 304 min/d, $P < 0.01$; 103 vs. 67.0 pH × min/d, $P < 0.01$, respectively). However, LS cows did not have differences in duration and area that pH was below 5.8 between mid- and late-lactation. Acidosis index was lower in LS animals than HS cows ($P < 0.01$). There was no difference of acidosis index for cows between mid- and late-lactation. But tendency of interaction between category and stage of lactation was detected for acidosis index ($P = 0.07$).

## 4.4 Discussion

Sub-acute ruminal acidosis is a metabolic disorder commonly found in high producing dairy cows. Diet formulation strategies could decrease the incidence of SARA, but it still occurs for some cows in a herd. Previous study has shown that a substantial variation exists in the severity of SARA among late-lactating dairy cows when fed a same high-grain diet (Gao and Oba, 2014), and I also detected the variation of rumen pH and severity of SARA among mid-lactating cows in the current study. VFA absorption is a major factor that regulates rumen pH, and it was suggested that roughly 53% of the

proton removal (largest proportion) from the rumen is via VFA absorption through rumen epithelial cells in lactating dairy cows (Allen, 1997). In addition, Schlau et al. (2012) showed that LS steers had lower total rumen VFA concentration and higher expression of NHE3 gene in ruminal epithelial cells compared with HS ones. NHE3 gene is located on the apical side of ruminal epithelial cells, which takes up $Na^+$ into epithelial cells and exports $H^+$ to the rumen (Connor et al., 2010). Higher expression of NHE3 may increase the release of $H^+$ to the rumen (Schlau et al., 2012), leading to the decrease of local pH and increase of the undissociated form of VFA near the epithelial cells, and these effects may increase VFA absorption via simple diffusion (Graham et al., 2007; Connor et al., 2010; Schlau et al., 2012). Therefore, responses of rumen VFA concentration and expression of NHE3 gene suggested that higher rumen pH for LS steers might be partly due to the higher VFA absorption rate than HS steers (Schlau et al., 2012). In the current study, I hypothesized that VFA absorption might be one of the main factors causing the variation in the risk of SARA for lactating dairy cows. However, I did not detect the difference in VFA absorption rate between LS and HS cows.

There are a couple of possibilities for my observation that the fractional rate of VFA absorption did not differ between LS and HS cows. One is that variation in the risk of SARA among cows was not large enough in the current study, thus LS and HS cows selected were not extreme enough, and differences in VFA absorption rate was not large enough to be detected between LS and HS cows. In previous beef steer study, acidosis index was 13.5 pH × min/kg DMI for LS steers and 61.7 pH × min/kg DMI for HS steers (Schlau et al., 2012). But in the current study, acidosis index was 0.10 pH × min/kg DMI for LS cows and 3.72 pH × min/kg DMI for HS cows. Therefore, animals selected in the

current study were less extreme. Smaller variation in the risk of SARA in the current study might be due to decreased genetic diversity and increased inbreeding coefficient for Holstein dairy cows. Inbreeding levels have increased in the past decades in Holstein breed, and it was shown that the average inbreeding coefficient for Holstein cows was between 6-7% in Canada in 2008 (Stachowicz et al., 2011). Increased inbreeding levels could cause significant loss of genetic diversity for Holsteins (Sørensen et al., 2005), and Stachowicz et al. (2011) suggested that about 6.5% of genetic diversity in Holsteins was lost since the founder generations. On our research dairy farm, the herd is closed, and artificial insemination is implemented with frozen semen from limited number of sires. But in that beef steer study, crossbred of Angus and Charolais beef steers were used. Crossbreeding has the opposite genetic effects compared with inbreeding, which can decrease inbreeding coefficients (Schaeffer et al., 2011). Therefore, Holstein cows used in the current study may have higher inbreeding coefficients compared with the crossbred steers used in the previous study. Less genetic variation for Holstein cows might explain the smaller variation in the risk of SARA in the current study than that of the crossbred steers in the previous study. But inbreeding coefficient data are not available to direcly compare the inbreeding levels between these two groups of animals.

The second possibility is that the intensity of acidosis challenge in the current study might not be severe enough to cause different VFA absorption responses between LS and HS cows. In this study, the challenge was feeding cows a TMR ration with 44.4 % grain on DM basis. But in the beef steer study, Schlau et al. (2012) challenged steers by feeding a diet consisting of 85% grain on DM basis to induce SARA. Mean rumen pH in the current study was 6.33 for LS and 5.98 for HS cows, while mean rumen pH in beef

steer study was 6.01 for LS and 5.51 for HS steers. These indicated that the challenge intensity in beef steer study might be stronger to the rumen epithelial tissue compared with the current study. Therefore, if there is a potential of variation in the capacity of VFA absorption among animals, stronger challenge might have larger chance to stimulate the different responses for animals compared with the milder challenge, which may explain why no difference was detected in VFA absorption rate between LS and HS cows in this study.

Increasing absorption and metabolism of VFA in rumen epithelial cells has been proposed as one potential mechanism to decrease the risk of ruminal acidosis (Gäbel et al., 2002). Metabolism of VFA could create a concentration gradient between the cytosol and rumen, which may promote the absorption of VFA (Gäbel et al., 2002; Penner et al., 2011). In this study, I did not detect significant difference in the fractional rate of VFA absorption between LS and HS cows, but I found that the mRNA abundance of LSS was higher for LS cows than HS ones, and mRNA abundance of HMGCS1 tended to be higher for LS cows compared with HS cows. Lanosterol synthase is the enzyme catalyzing the synthesis of lanosterol, which is the key intermediate in the biosynthesis of cholesterol (Dempsey, 1974; Huff and Telford, 2005). The enzyme HMGCS1 also catalyzes butyrate metabolism in the cytosol for cholesterol synthesis (Hegardt, 1999; Penner et al., 2011). Cholesterol biosynthesis is an alternative pathway of VFA metabolism in the rumen epithelial cells besides ketogenesis (Steele et al., 2011). Steele et al. (2011) has shown that genes involved in cholesterol biosynthesis were upregulated in the rumen epithelial cells during the first week of high grain diet challenge, but then downregulated in the third week. High cholesterol concentration in the cell is associated

with cellular inflammation, oxidative stress, proliferation and membrane permeability (Kleemann and Kooistra, 2005; Steele et al., 2011), thus it was proposed that upregulation of genes related to VFA metabolism during the first week was due to the increased amount of VFA substrate for cholesterol biosynthesis in the rumen epithelial cells during a high grain diet challenge, which might increase the intracellular cholesterol concentration and cause the possible abnormalities in cholesterol homeostasis and inflammation (Steele et al., 2011). Therefore, genes associated with cholesterol biosynthesis were downregulated from the first to third weeks to compensate for the increased intracellular cholesterol concentration and maintain the intracellular cholesterol homeostasis (Steele et al., 2011). In this study, LS cows had higher mRNA abundance of genes related to cholesterol biosynthesis than HS cows, which suggested that LS cows might have higher capacity to cope with the elevated cholesterol levels in rumen epithelial cells or higher capacity to clear the cholesterol from rumen epithelial cells to blood. In addition, ketogenesis is the primary pathway of VFA metabolism in the ruminal epithelial cells (Penner et al., 2011). However, I did not detect significant differences in mRNA abundance of genes associated with VFA metabolism via ketogenesis pathways between LS and HS cows. Consistently, Schlau et al. (2012) did not find differences in the expression of genes related to ketogenesis between LS and HS steers. But I found that mRNA abundance of BDH2 tended to be positively correlated with minimum rumen pH. BDH2 catalyzes the pathway of cytosolic ketogenesis, and the ketone body could be further used as precursors for lipid and sterol synthesis (Guo et al., 2006). Together, in this study, variation in the severity of SARA among cows might be partly due to differences in VFA metabolism in rumen epithelial cells. Penner et al. (2009b) also

reported the negative correlations between the expression of genes associated with VFA metabolism and the severity of SARA for dairy cows. But it needs to be noted that even though I detected the changes in expression of genes, the protein abundance or activity was not measured in this study, and the differences of mRNA abundance do not necessarily mean the expression of similar level of functional protein.

In the current study, I did not find significant differences in mRNA abundance of genes related to tranpsotation of ions and VFA, and intracellular pH regulation. Also, there is no siginificant relationship between VFA absorption rate and expression of these genes in this study (data not shown). Schlau et al. (2012) found that the mRNA abundance of NHE3 in ruminal epithelial cells was greater for LS compared with HS beef steers in the previuos study. Ruminal papillae samples were collected at 2-h, 4-h and 6-h after force-feeding in that study, while samples were biopsied 3h after feeding in this study. Although the numerical difference in expression of NHE3 between LS and HS steers was highest at the 2-h time point, there was no significant interaction between the group effect and the time of sampling for NHE3 gene expression (Schlau, 2012). Therefore, sampling time may not be the key factor to explain the different responses in NHE3 expression between these two studies. As discussed above, genetic variation and the intensity of acidosis challenge may be the possible reasons. In addition, although there was no difference between the two groups, I found a tendency for the relative mRNA abundance of NHE1 to be positively correlated with minimum rumen pH. Both NHE1 and NHE3 are located on the apical side of ruminal epithelial cells, and they can absorb $Na^+$ into epithelial cells and release $H^+$ to the rumen (Connor et al., 2010). But there was no relationship between relative mRNA abundance of NHE1 with mean rumen

pH and acidosis index, which indicated that the intensity of acidosis challenge might not be severe enough to cause different responses on expression of NHE genes between LS and HS cows.

Rumen pH is the balance between acid production and removal (Allen, 1997). Therefore, acid produced from rumen OM fermentation is another main factor affecting rumen pH. Schlau et al. (2012) suggested that higher rumen pH for LS steers might be partly due to the lower VFA production. Carbohydrates normally account for approximately 65 - 70% DM basis in the diets for lactating dairy cow (NRC, 2001), and starch and fiber are the main carbohydrate sources. Therefore, I had expected that LS cows would have less in situ rumen starch and NDF digestibility than HS cows. However, I did not detect differences in in situ 7-h starch or 30-h NDF digestibility between LS and HS cows. Factors of substrate, microbes and retention time in the rumen affect the amount of rumen carbohydrate fermentation. Measurement of in situ carbohydrate digestibility removes the possible confounding factors of substrate and retention time in the rumen, which indicates the capacity of microbes to ferment carbohydrates in the rumen. Carbohydrates are digested and metabolized by the action of microbial enzymes in the rumen (Wang and McAllister, 2002). Also, the attachment and contact is necessary for the occurance of enzymatic hydrolysis and efficient digestion in the rumen (McAllister et al., 1994; Wang and McAllister, 2002). Therefore, both the process of adhesion and the activity of microbial enzymes determine the extent and capacity of rumen microbial fermentation. No difference in in situ carbohydrate digestibility between LS and HS cows in this study indicated that the capacity of rumen microbes to ferment carbohydrates might not be related to the variation in the risk of SARA among cows. But

I could not exclude the possibility of VFA production as a factor causing this variation, because other factors, such as rumen digesta passage rate, also have an effect on rumen fermentation and VFA production. Previous studies have shown that passage rate of digesta affects feed degradability (Robinson et al., 1987) and microbial efficiency (Sniffen & Robinson, 1987). Orskov and McDonald (1979) suggested that higher passage rates lead to less degradability. Therefore, it is possible that rumen digesta passage rate was variable among cows, which might cause the differences in VFA production, even though the in situ starch and NDF digestibility is the same between LS and HS cows. Therefore, further research on differences in rumen digesta passage rate and net rumen VFA production between LS and HS cows is needed to determine if the extent of rumen fermentation and VFA production is potentially related to the variation in the risk of SARA among the cows.

The variation in the risk of developing SARA has been indicated among late-lactating dairy cows (Gao and Oba, 2014) and mid-lactating cows in the current study (experiment 1). In these studies, cows were fed the same high-grain diet with studies, so factors causing this variation are likely due to the animal itself. Therefore, I hypothesized that the identified risk category of SARA for cows would not change at different stage of lactation. Indeed, I found that LS cows consistently had higher rumen pH and lower severity of SARA than HS cows at both mid- and late-lactation. However, significant interaction existed on duration and area of rumen pH below 5.8 between category and stage of lactation. Duration and area of rumen pH below 5.8 for HS cows decreased in late-lactation compared with mid-lactation, probably due to the decreased DMI. Because acidosis index, severity of SARA normalized for DMI, was not different for HS cows

between mid- and late-lactation. But for LS cows, duration and area of rumen pH below 5.8 did not decrease from mid- to late-lactation. Although LS cows consistently had higher rumen pH and lower severity of SARA than HS cows during the mid- and late-lactation, the possibility could not be excluded that risk of SARA for some cows may change during the lifetime. Dohme et al (2008) has reported that cows increased the severity of acidosis with repeated acidosis challenges, which suggested that the risk of SARA within the same cow could be influenced by previous exposure to diet challenges. But it is not known about the length of time that cows need to recover the capability to cope with the subsequent challenge of SARA (Dohme et al, 2008). Therefore, further studies are needed to evaluate if identified risk category of SARA for LS cows would change during the lifetime, particularly when there are multiple acidosis challenges.

## 4.5 Conclusions

A substantial variation exists in the risk of developing SARA among lactating dairy cows fed a high grain diet; however, this variation may not be attributed to differences in rumen microbial capacity to ferment carbohydrates, as in situ carbohydrate digestibility in the rumen was not different between cows with higher and lower risk of SARA. Although expression of some genes involved in VFA metabolism in rumen epithelium may be related to the variation in the risk of SARA, VFA absorption rate may not be responsible for the difference of rumen pH between cows with higher and lower risk of SARA for the current study. In addition, cows identified to have a greater risk of SARA in mid-lactation consistently have lower rumen pH in late-lactation than cows with a lower risk of SARA.

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## 4.7 Figures and tables

**Table 4.7.1** Ingredient and nutrient composition of the experimental diet

| **Item** | |
|---|---|
| Ingredient, % DM | |
| Barley silage | 30.0 |
| Barley grain, rolled | 25.5 |
| Corn grain, rolled | 18.9 |
| Canola meal | 14.5 |
| Corn gluten meal | 4.70 |
| Beet pulp | 2.94 |
| Mineral and vitamin mix[1] | 2.60 |
| Vegetable oil | 0.90 |
| Nutrient Composition, % DM | |
| DM | 54.6 |
| CP | 16.9 |
| NDF | 26.8 |
| Starch | 31.8 |
| Ether extract | 4.10 |
| NFC | 47.5 |
| Forage NDF | 13.9 |

[1]Contained 15.4% Ca, 2.83% P, 12.4% Na, 25.0% Cl, 5.53% Mg, 0.28% S, 6.43% K, 3274 mg/kg Fe, 617 mg/kg Cu, 1160 mg/kg Mn, 13.9 mg/kg Co, 938 mg/kg Zn, 9.23 mg/kg Se, 30.3 mg/kg I, 473 kIU/kg vitamin A, 48.0 kIU/kg vitamin D, 1548 IU/kg vitamin E.

**Table 4.7.2** Gene names, primer and probe (for Taqman) sequences for real-time PCR analysis (qPCR)

| Gene name | Category | Primer and probe sequences | Source |
|---|---|---|---|
| **Genes with Taqman kits** | | | |
| Monocarboxylate cotransporter, isoform 1 (MCT1) | VFA absorption | Forward: CGC GGG ATT CTT TGG ATT T<br>Reverse: GTC CAT CAG CGT TTC AAA CAG TAC<br>Probe: TTT TGG GTG GCT CAG C | Schlau et al., 2012 |
| Sodium/hydrogen exchanger, isoform 1 (NHE1) | Intracellular pH regulation | Forward: GAA AGA CAA GCT CAA CCG GTT T<br>Reverse: GGA GCG CTC ACC GGC TAT<br>Probe: AAG TAC GTG AAG AAG TGT CT | Schlau et al., 2012 |
| Sodium/hydrogen exchanger, isoform 2 (NHE2) | Intracellular pH regulation | Forward: TTG TGC GAT GAC CAT GAA TAA GT<br>Reverse: TGA TGG TCG TGT AGG ATT TCT GA<br>Probe: CGT GGA AGA GAA CGT G | Schlau et al., 2012 |
| Sodium/hydrogen exchanger, isoform 3 (NHE3) | Intracellular pH regulation | Forward: AGC CTT CGT GCT CCT GAC A<br>Reverse: TGA CCC CTA TGG CCC TGT AC<br>Probe: TGC TCT TCA TCT CCG | Schlau et al., 2012 |
| 3-hydroxy-3-methylglutaryl-CoA | Cholesterol synthesis | Forward: AGG ATA CTC ATC ACT TGG CCA | Schlau et al., |

| | | | |
|---|---|---|---|
| Synthase 1 (HMGCS-1) | | ACT<br>Reverse: CAT GTT CCT TCG AAG AGG GAA T<br>Probe: CAT TCC CCA GAG TTC CA | 2012 |
| 3-hydroxy-3-methylglutaryl-CoA Synthase 2 (HMGCS-2) | Ketogenesis | Forward: CCT GCT GCA ATC ACT GTC ATG<br>Reverse: TCT GTC CCG CCA CCT CTT C<br>Probe: TTG CAG AGC CCT TTC | Schlau et al., 2012 |
| 3-hydroxy-3-methylglutaryl-CoA lyase (HMGCL) | Ketogenesis | Forward: TGC AGA TGG GAG TGA GTG TCA<br>Reverse: GAC GCC CCC TGT GCA TAG<br>Probe: TGG CAG GAC TGG GAG | Penner et al., 2009b |
| β-Hydroxybutyrate dehydrogenase, isoform 1 (BDH1) | Ketogenesis | Forward: GAC TGC CAC CAC TCC CTA CAC<br>Reverse: TCC GCA GCC ACC AGT AGT AGT<br>Probe: CGC TAC CAT CCC ATG | Penner et al., 2009b |
| β-hydroxybutyrate dehydrogenase, isoform 2 (BDH2) | Ketogenesis | Forward: CTG TGG CTT CCA GCA TCA AA<br>Reverse: CGC CTT GGT TGT GCT GTA CA<br>Probe: CGT TGT GAA CAG GTG C | Penner et al., 2009b |
| ATPase, $Na^+/K^+$ transporting, α 1 | Intracellular pH | Forward: CAT CTT CCT CAT CGG CAT CA<br>Reverse: ACG GTG GCC AGC AAA CC | Schlau et al., 2012 |

| | | | |
|---|---|---|---|
| polypeptide ($Na^+/K^+$ ATPase) | regulation | Probe: TGT AGC CAA CGT GCC AG | |
| Putative anion transporter, isoform 1 (PAT1) | VFA absorption | Forward: GGG CAC TTC TTC GAT GCT TCT<br>Reverse: GTC GTG GAC CGA GGC AAA<br>Probe: TCA CCA AGC AGC ACC T | Schlau et al., 2012 |
| Downregulated in adenoma (DRA) | VFA absorption | Forward: TGC ACA AAG GGC CAA GAA A<br>Reverse: GCT GGC AAC CAA GAT GCT ATG<br>Probe: TGC CTT CTC CTC CTT C | Schlau et al., 2012 |
| Ribosomal protein large, P0 (RPLP0) | Housekeeping gene | Forward: AGG GCG TCC GCA ATG TT<br>Reverse: CGA CGG TTG GGT AAC CAA TC<br>Probe: CCA GCG TGT GCC TG | Schlau et al., 2012 |
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | Housekeeping gene | Forward: TGC CGC CTG GAG AAA CC<br>Reverse: CGC CTG CTT CAC CAC CTT<br>Probe: CCA AGC GTG TGC CTG | Schlau et al., 2012 |
| β-Actin (ACTB) | Housekeeping gene | Forward: CCT GCG GCA TTC ACG AA<br>Reverse: GCG GAT GTC GAC GTC ACA<br>Probe: CTA CCT TCA ATT CCA TCA TG | Schlau et al., 2012 |

**Genes with SYBR Green kits**

| | | | |
|---|---|---|---|
| Monocarboxylate cotransporter, isoform 4 (MCT4) | VFA absorption | Forward: TCA GCG TCT TCT TCA AGG AAC<br>Reverse: CAG CCA CGA TTC ACA C | |
| Vacuolar $H^+$ ATPase subunit B ($vH^+$ATPase) | Intracellular pH regulation | Forward: TTT TAT TGA ACA AGA AGC CAA TGA<br>Reverse: GAT TCA TCA AAT TGG ACA TCT GAA | Yan et al., 2014 |
| Anion exchanger 2 (AE2) | Intracellular pH regulation | Forward: AGC AGC AAC AAC CTG GAG T<br>Reverse: GGT GAA ACG GGA GAC GAA | Yan et al., 2014 |
| Carbonic anhydrase 2 (CA2) | Intracellular pH regulation | Forward: AAG GTT CTG AGC ATA CTG TGG<br>Reverse: CTG TTC CAA AGT CCC CGT AC | |
| Acyl-CoA synthetase short-chain family member 1 (Acyl-CoAs) | VFA metabolism activation | Forward: CCG ATC AGG TCC TGG TAG TGA<br>Reverse: GCC TCC GCA TGA CTT TTC C | Penner et al., 2009b |
| Acetyl-CoA synthetase 2 (Acetyl-CoAs) | VFA metabolism activation | Forward: GCT CTC ACT GAG GAG CTC AAG AA<br>Reverse: AAT CCG GTG TGG CAA TGG | Penner et al., 2009b |
| Acetyl-CoA acyltransferase 1 (ACAA1) | Ketogenesis | Forward: CAC TGG CTT CCC AGC AAA A<br>Reverse: ACG ATC TCG GCC TGG AAA C | Penner et al., 2009b |

| | | | |
|---|---|---|---|
| Acetoacetyl-CoA thiolase 2 (ACAT2) | Cholesterol synthesis | Forward: CCG CTG GCT GAC AGT ATA CTT TG<br>Reverse: TGG CCA CAT TTT CAG CTG TAA T | Steele et al., 2011 |
| 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) | Cholesterol synthesis | Forward: GAG TGG CAG GAC CTC TGT GC<br>Reverse: GCA CCT CCA CCA AGG CCT AT | Steele et al., 2011 |
| Farnesyl diphosphate synthase (FDPS) | Cholesterol synthesis | Forward: GGT ATC AGA AGC CAG GCA TAG G<br>Reverse: TAG GGC TGC TCC CGA CAG TA | Steele et al., 2011 |
| Sterol regulatory element-binding protein 1 (SREBP1) | Cholesterol synthesis | Forward: CCA GCT GAC AGC TCC ATT GA<br>Reverse: TGC GCG CCA CAA GGA | Steele et al., 2011 |
| Sterol regulatory element-binding protein 2 (SREBP2) | Cholesterol synthesis | Forward: CTG GCT CCA GGG AGA TGA C<br>Reverse: GCT CTG CAG GTG TGG AAG AC | Steele et al., 2011 |
| Lanosterol synthase (LSS) | Cholesterol synthesis | Forward: TGC AGG CGC TGA AGA CTT T<br>Reverse: GCT CAA GGG TCT CCC TGA TCT | Steele et al., 2011 |
| Toll-like receptor 2 (TLR2) | Barrier function | Forward: CTG TGT GCG TCT TCC TCA GA<br>Reverse: TCA GGG AGC AGA GTA ACC AGA | Chen et al., 2012 |
| Toll-like receptor 4 (TLR4) | Barrier function | Forward: GGT TTC CAC AAA AGC CGT AA<br>Reverse: AGG ACG ATG AAG ATG ATG CC | Chen et al., 2012 |
| Claudin 1 (CLDN1) | Tight junction | Forward: GCG CTG CCC CAG TGG AAA GT | Malmuthuge |

| | | | |
|---|---|---|---|
| | | Reverse: GGA TCT GCC CGG TGC TCT GC | et al., 2013 |
| Junctional adhesion molecule 2 | Tight junction | Forward: CCC CAT CGG AAC AAG GTC AA | |
| (JAM2) | | Reverse: GAC ATC GCA GCT CTA CCA CA | |
| Occludin (OCLN) | Tight junction | Forward: ACG CAG GAA GTG CCT TTG GTA GC | Malmuthuge |
| | | Reverse: GCA GCC ATG GCC AGC AGG AA | et al., 2013 |
| Zonula occluden-1 (ZO1) | Tight junction | Forward: AAT GCA TCC TGA CCA CCA GG | |
| | | Reverse: GAT GGT GCC GGG TTT GTT TC | |
| Ribosomal protein large, P0 (RPLP0) | Housekeeping gene | Forward: AGG GCG TCC GCA ATG TT | Schlau et al., |
| | | Reverse: CGA CGG TTG GGT AAC CAA TC | 2012 |
| Glyceraldehyde-3-phosphate | Housekeeping gene | Forward: TGC CGC CTG GAG AAA CC | Schlau et al., |
| dehydrogenase (GAPDH) | | Reverse: CGC CTG CTT CAC CAC CTT | 2012 |
| β-Actin (ACTB) | Housekeeping gene | Forward: CCT GCG GCA TTC ACG AA | Schlau et al., |
| | | Reverse: GCG GAT GTC GAC GTC ACA | 2012 |

**Table 4.7.3** Comparision of BW, DMI and pH measurements between LS[1] and HS[2] cows (experiment 1)

| | LS (n=8) | | HS (n=5) | | |
|---|---|---|---|---|---|
| **Variable** | **LSM** | **SE** | **LSM** | **SE** | ***P*** |
| BW, kg | 658 | 16.2 | 612 | 20.5 | 0.11 |
| DMI, kg | 29.2 | 1.18 | 28.1 | 1.50 | 0.56 |
| Ruminal pH | | | | | |
| Minimum | 5.75 | 0.05 | 5.33 | 0.06 | < 0.01 |
| Mean | 6.33 | 0.03 | 5.98 | 0.04 | < 0.01 |
| Maximum | 6.90 | 0.04 | 6.81 | 0.05 | 0.20 |
| Duration < pH 5.8, min | 24.9 | 20.5 | 481 | 26.0 | < 0.01 |
| Area < pH 5.8, pH × min | 2.94 | 2.18 | 102 | 2.76 | < 0.01 |
| Acidosis index, pH × min/kg | 0.10 | 0.15 | 3.72 | 0.19 | < 0.01 |
| DMI | | | | | |

[1] LS: cows identified as animals that have low risk of SARA.

[2] HS: cows identified as animals that have high risk of SARA.

**Table 4.7.4** Comparison of rumen VFA profile, $NH_3$-N concentration, VFA absorption rate, and in situ digestibility between LS[1] and HS[2] cows (experiment 1)

| | LS (n=8) | | HS (n=5) | | |
|---|---|---|---|---|---|
| **Variable** | **LSM** | **SE** | **LSM** | **SE** | ***P*** |
| VFA profile | | | | | |
| Total VFA, m*M* | 129 | 2.88 | 129 | 3.64 | 0.93 |
| Acetate, mol/100mol | 63.0 | 1.34 | 64.0 | 1.69 | 0.67 |
| Propionate, mol/100mol | 19.7 | 0.53 | 20.4 | 0.67 | 0.48 |
| Butyrate, mol/100mol | 13.5 | 0.99 | 11.9 | 1.25 | 0.32 |
| Acetate: Propionate | 3.21 | 0.14 | 3.17 | 0.17 | 0.85 |
| Rumen $NH_3$-N, mg/dL | 11.2 | 0.84 | 11.8 | 1.06 | 0.69 |
| VFA absorption rate, %/h | 29.9 | 2.19 | 30.8 | 2.90 | 0.80 |
| 7h starch digestibility, % | 74.9 | 1.32 | 73.1 | 1.67 | 0.41 |
| 30h NDF digestibility, % | 53.2 | 1.78 | 50.2 | 2.25 | 0.32 |

[1]LS: cows identified as animals that have low risk of SARA.

[2]HS: cows identified as animals that have high risk of SARA.

**Table 4.7.5** Comparisons of milk yield and milk composition between LS[1] and HS[2] cows (experiment 1)

| | LS (n=8) | | HS (n=5) | | |
|---|---|---|---|---|---|
| **Variable** | **LSM** | **SE** | **LSM** | **SE** | ***P*** |
| Yield, kg/d | | | | | |
| Milk | 37.9 | 2.04 | 40.4 | 2.59 | 0.46 |
| Fat | 1.27 | 0.08 | 1.18 | 0.10 | 0.50 |
| CP | 1.19 | 0.06 | 1.23 | 0.07 | 0.65 |
| Lactose | 1.75 | 0.11 | 1.85 | 0.14 | 0.58 |
| Milk composition | | | | | |
| Fat, % | 3.36 | 0.14 | 2.93 | 0.17 | 0.07 |
| CP, % | 3.17 | 0.07 | 3.06 | 0.09 | 0.40 |
| Lactose, % | 4.60 | 0.06 | 4.57 | 0.08 | 0.77 |
| MUN, mg/dL | 12.9 | 0.58 | 12.2 | 0.74 | 0.49 |

[1] LS: cows identified as animals that have low risk of SARA.

[2] HS: cows identified as animals that have high risk of SARA.

**Table 4.7.6** Comparison of plasma blood metabolite and hormone concentrations between LS[1] and HS[2] cows (experiment 1)

| | LS (n=8) | | HS (n=5) | | |
|---|---|---|---|---|---|
| **Variable** | **LSM** | **SE** | **LSM** | **SE** | ***P*** |
| Glucose, mg/dL | 64.1 | 0.79 | 61.9 | 1.00 | 0.12 |
| BHBA, mg/dL | 10.3 | 0.84 | 11.3 | 1.07 | 0.48 |
| NEFA, mEq/L | 84.0 | 7.95 | 92.1 | 10.1 | 0.54 |
| Insulin, µIU/mL | 8.57 | 0.57 | 7.66 | 0.72 | 0.34 |

[1] LS: cows identified as animals that have low risk of SARA.

[2] HS: cows identified as animals that have high risk of SARA.

**Table 4.7.7** Comparison of mRNA abundance of genes involved in intracellular pH regulation, VFA metabolism and tight junction between LS[1] and HS[2] cows (experiment 1)

| | LS (n=8) | | HS (n=5) | | | |
|---|---|---|---|---|---|---|
| **Variable** | **LSM** | **SE** | **LSM** | **SE** | ***P*** | **E**[3] (%) |
| MCT1 | 1.09 | 0.08 | 0.99 | 0.10 | 0.46 | 97.9 |
| NHE1 | 1.15 | 0.05 | 1.07 | 0.06 | 0.38 | 98.4 |
| NHE2 | 1.07 | 0.03 | 0.99 | 0.04 | 0.18 | 93.0 |
| NHE3 | 0.94 | 0.09 | 0.80 | 0.11 | 0.33 | 101 |
| HMGCS 1 | 0.91 | 0.05 | 0.77 | 0.06 | 0.08 | 92.2 |
| HMGCS 2 | 1.15 | 0.08 | 1.17 | 0.10 | 0.87 | 95.9 |
| HMGL | 1.13 | 0.05 | 1.09 | 0.06 | 0.68 | 89.3 |
| BDH1 | 0.99 | 0.06 | 0.99 | 0.08 | 0.93 | 101 |
| BDH2 | 1.19 | 0.05 | 1.10 | 0.06 | 0.25 | 97.7 |
| $Na^+$/K ATPase | 1.03 | 0.05 | 0.93 | 0.06 | 0.22 | 97.0 |
| PAT1 | 1.01 | 0.05 | 0.96 | 0.07 | 0.61 | 101 |
| DRA | 0.93 | 0.10 | 0.80 | 0.12 | 0.41 | 98.3 |
| MCT4 | 0.84 | 0.05 | 0.92 | 0.06 | 0.35 | 93.3 |
| $VH^+$ATPase | 0.95 | 0.03 | 0.94 | 0.04 | 0.78 | 99.9 |
| AE2 | 0.97 | 0.04 | 1.02 | 0.05 | 0.46 | 94.8 |
| CA2 | 0.84 | 0.06 | 0.88 | 0.07 | 0.70 | 104 |
| Acyl-CoAs | 0.92 | 0.05 | 0.96 | 0.07 | 0.67 | 101 |
| Acetyl-CoAs | 0.95 | 0.05 | 1.03 | 0.07 | 0.35 | 101 |

| | | | | | | |
|---|---|---|---|---|---|---|
| ACAA1 | 1.01 | 0.03 | 0.97 | 0.04 | 0.52 | 95.7 |
| ACAT2 | 1.04 | 0.04 | 1.09 | 0.04 | 0.44 | 96.0 |
| HMGCR | 0.93 | 0.03 | 0.86 | 0.04 | 0.29 | 94.9 |
| FDPS | 0.91 | 0.05 | 0.84 | 0.07 | 0.42 | 97.7 |
| SREBP1 | 0.88 | 0.05 | 0.89 | 0.07 | 0.87 | 96.5 |
| SREBP2 | 0.94 | 0.02 | 0.97 | 0.03 | 0.38 | 97.4 |
| LSS | 0.91 | 0.04 | 0.75 | 0.05 | 0.03 | 95.6 |
| TLR2 | 0.66 | 0.06 | 0.80 | 0.08 | 0.22 | 103 |
| TLR4 | 0.79 | 0.08 | 0.85 | 0.10 | 0.62 | 104 |
| CLDN1 | 0.89 | 0.05 | 0.82 | 0.06 | 0.39 | 96.9 |
| JAM2 | 0.89 | 0.06 | 1.03 | 0.08 | 0.21 | 104 |
| OCLN | 0.99 | 0.04 | 1.01 | 0.05 | 0.76 | 98.3 |
| ZO1 | 1.04 | 0.05 | 1.05 | 0.06 | 0.89 | 95.5 |

[1] LS: cows identified as animals that have low risk of SARA.

[2] HS: cows identified as animals that have high risk of SARA.

[3]Efficiency of amplification.

**Table 4.7.8** Effect of stage of lactation (mid- and late-lactation) on DMI and pH measurements between LS[1] (n = 5) and HS[2] (n = 4) cows (experiment 2)

| | Mid-lactation | | | | Late-lactation | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | LS | | HS | | LS | | HS | | *P*[*] | | |
| **Variable** | **LSM** | **SE** | **LSM** | **SE** | **LSM** | **SE** | **LSM** | **SE** | ***1*** | ***2*** | ***3*** |
| DIM | 138 | 9.37 | 137 | 10.5 | 244 | 9.37 | 243 | 10.5 | 0.91 | 0.01 | 0.99 |
| DMI, kg/d | 30.7 | 1.54 | 27.9 | 1.72 | 24.0 | 1.54 | 24.2 | 1.72 | 0.44 | 0.01 | 0.36 |
| Ruminal pH | | | | | | | | | | | |
| Minimum | 5.74 | 0.07 | 5.32 | 0.08 | 5.73 | 0.07 | 5.32 | 0.08 | < 0.01 | 0.96 | 0.91 |
| Mean | 6.36 | 0.04 | 5.97 | 0.05 | 6.32 | 0.04 | 6.07 | 0.05 | < 0.01 | 0.50 | 0.14 |
| Maximum | 6.92 | 0.06 | 6.78 | 0.07 | 6.84 | 0.06 | 6.87 | 0.07 | 0.37 | 0.90 | 0.17 |
| Duration < pH 5.8, min/d | 8.43[c] | 27.1 | 488[a] | 30.3 | 57.0[c] | 27.1 | 304[b] | 30.3 | < 0.01 | 0.03 | < 0.01 |
| Area < pH 5.8, pH × min/d | 1.72[c] | 6.57 | 103[a] | 7.34 | 10.7[c] | 6.57 | 67.0[b] | 7.34 | < 0.01 | 0.08 | < 0.01 |
| Acidosis index, pH × min/kg DMI | 0.06[b] | 0.32 | 3.77[a] | 0.36 | 0.43[b] | 0.32 | 2.82[a] | 0.36 | < 0.01 | 0.41 | 0.07 |

[1] LS: cows identified as animals that have low risk of SARA

[2] HS: cows identified as animals that have high risk of SARA

[*] 1. Category: LS vs. HS cows

2. Stage of lactation: mid- vs. late-lactation

3. Interaction between category and stage of lactation

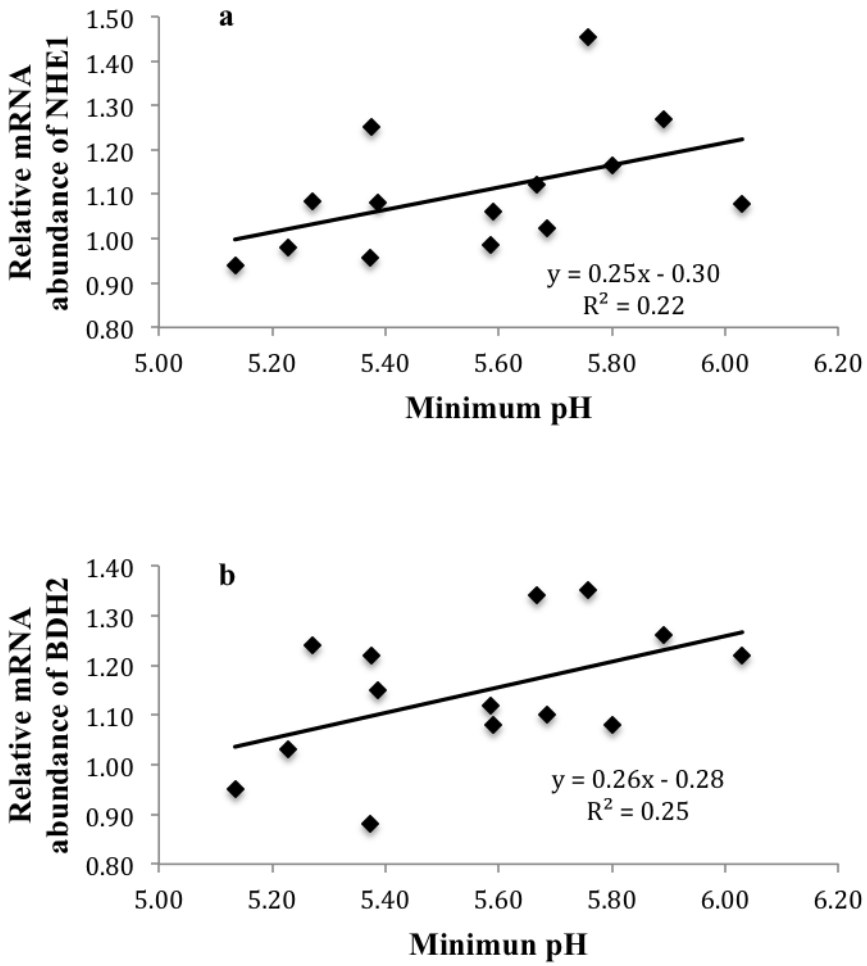


**Figure 4.7.9** Relationship between minimum rumen pH with (a) relative mRNA abundance of NHE1 ($P = 0.08$) and (b) relative mRNA abundance of BDH 2 ($P = 0.07$).

# Chapter 5. Effect of increasing dietary non-fibre carbohydrate with starch, sucrose or lactose on rumen fermentation and productivity of lactating dairy cows

## 5.1 Introduction

Increasing dietary non-fibre carbohydrate (NFC) is a common diet formulation approach to maximize milk production of high-producing dairy cows. Starch is the primary source of NFC in the diets of lactating dairy cows (NRC, 2001), but feeding more starch in dairy diets may also increase the risk of ruminal acidosis, which is related to decreased feed intake, liver abscesses (Nagaraja and Lechtenberg, 2007), milk fat depression (Kleen et al., 2003) and laminitis (Nocek, 1997). As the demand and cost of cereal grains has increased in the last decade, partial replacement of grains with high-sugar byproducts becomes a diet formulation approach to decrease feed costs while maintaining high milk yield. Sugars are water-soluble carbohydrates, which can be fermented easily and quickly in the rumen (Oba, 2011), consisting of monosaccharides (glucose, fructose and galactose) and disaccharides (sucrose, maltose and lactose). Previous research indicated that partial substitution of dietary starch with sugars increased DMI (Broderick and Radloff, 2004; Broderick et al., 2008; Penner and Oba, 2009) and milk fat yield (Broderick et al., 2008; Penner and Oba, 2009). Therefore, increasing dietary NFC content by feeding more sugars can be a viable option to maximize energy intake and productivity of lactating dairy cows. In addition, a recent review (Oba, 2011) showed that rumen pH did not decrease (McCormick et al., 2001; DeFrain et al., 2004; Broderick et al., 2008), but even increased (Chamberlain et al., 1993; Heldt et al., 1999)

when sugar was used to replace a part of dietary starch even though the hydrolysis and fermentation rate of sugar in the rumen is faster than starch (Sniffen et al., 1992). *In vitro* studies indicated that feeding sugar could result in increased molar proportion of butyrate in the rumen (Vallimont et al., 2004; Ribeiro et al., 2005), and Malhi et al. (2013) reported that ruminal butyrate infusion increased the ruminal epithelia growth and absorption capacity of VFA in the rumen. The increased capacity of ruminal epithelia to uptake acetate and butyrate is positively related to rumen pH (Penner et al. 2009a). As such, I hypothesized that increasing dietary NFC by feeding more disaccharide instead of starch would not decrease rumen pH by increasing the rate of VFA absorption.

Whey and molasses are high sugar byproducts commonly included in diets for dairy cows. Whey is a byproduct of cheese manufacturing containing about 70% lactose on a DM basis (Oba, 2011) while molasses is a byproduct of refining sugarcane or sugar beets into sugar containing mostly sucrose. In order to use these high disaccharide byproducts efficiently, it is necessary to understand specific effects of feeding sucrose or lactose on rumen fermentation and animal performance. Weisbjerg et al. (1998) reported that the hydrolysis rate of sucrose is faster than that of lactose, and Sutton (1968) showed that glucose and fructose ferment faster than galactose. Therefore, effects of feeding sucrose and lactose on rumen fermentation and animal performance are expected to be different. Previous studies evaluated effects of partial replacement of dietary starch with sucrose (Broderick et al., 2008) or lactose (DeFrain et al., 2004) on rumen fermentation and animal performance. However, due to the different basal dietary ingredients, forage to concentrate ratio, and sugar inclusion rate, it is difficult to compare specific effects of

feeding sucrose and lactose on rumen fermentation and animal productivity for those previous studies.

The objective of this study was to investigate the effect of increasing dietary NFC content with starch, sucrose or lactose on rumen fermentation, VFA absorption, and milk production of lactating dairy cows.

## 5.2 Materials and methods

The study was conducted according to the guidelines of the Canadian Council of Animal Care (Ottawa, Ontario, Canada). All experimental procedures were approved by the University of Alberta Animal Care and Use Committee.

### *5.2.1 Animals, Diets, and Experimental Design*

Twenty-eight multiparous mid-lactating Holstein cows (141 ± 50 DIM; 614 ± 53 kg of BW; mean ± SD) including 8 ruminally cannulated cows were used in this study. Cows were randomly assigned to one of four dietary treatments in a replicated 4 × 4 Latin square design balanced for carryover effects. Each period consisted of a 17-d diet adaptation period and a 4-d data and sample collection period. The treatments were control (CON; 27% starch and 4% sugar on a DM basis; Table 5.7.1), a high NFC diet in which dietary starch content was increased by replacing beet pulp in CON diet with corn grain (STA; 32% starch and 4% sugar on a DM basis), and two more high NFC diets in which dietary sugar content was increased by replacing beet pulp in CON diet with 5.5% of sucrose (SUC; 27% starch and 9% sugar on a DM basis) or lactose (LAC; 27% starch and 9% sugar on a DM basis). All experimental diets were formulated according to NRC

(2001) to meet or exceed the nutritional requirements for a 650-kg cow producing 40 kg/d of milk with 3.5% milk fat and 3.2% milk protein, and formulated for similar CP concentrations.

Cows were housed individually in tie-stalls bedded with wood shavings, and fed the experimental diets as a TMR once daily at 0800 h and had free access to water. Feed was offered at 105 to 110% of actual feed intake of the previous day. The amounts of feed offered and refused were recorded daily during sample collection periods. Samples of feed ingredients and orts were collected daily during sample collection periods and composited by period for feed ingredients, and by period and cow for orts. The DM concentration of barley silage was determined, by drying samples in a forced-air oven at 55°C for 48 h, twice weekly and as-fed proportions of feed ingredients were adjusted if necessary. Cows were weighed after the morning milking but before the feeding on two consecutive days immediately before the start of the experiment. Cows were milked twice daily at 0400 and 1500 h. Milk was sampled from both a.m. and p.m. milkings on d 18, 19, and 20 of each period of the study.

### *5.2.2 Rumen pH and Rumen Fermentation*

Rumen pH was measured by using the pH measurement system developed by Penner et al. (2006) in the ventral sac every 30 s continuously for 72 h (d 18, 19 and 20) in each experimental period. Minimum, mean, and maximum pH was determined, and duration and area below pH 5.8 were calculated for each cow daily, and averaged over 72-h periods as described in Chapter 2. DMI was determined daily for each cow on d 18, 19 and 20, and averaged over 72-h periods. These data were used to calculate acidosis

index (area under pH 5.8 divided by DMI; Penner et al., 2009c) to assess the severity of SARA normalized for DMI.

Rumen fluid samples were collected from cranial, ventral, and caudal sacs, then combined and strained through a perforated screen (Peetex, Sefar Canada Inc., Scarborough, ON, Canada; pore size = 355 μm) every 9 h over a 72-h period on d 18, 19, and 20 of each experimental period. The samples were centrifuged at 3,000 × g at 4°C for 20 min immediately after collection, and the supernatants were stored at −20°C until analysis as described in Chapter 2. Rumen fluid samples were composited to yield one sample per cow per period for further analysis.

Ruminal fluid samples were analyzed for VFA profile by gas chromatography according to the method described by Khorasani et al. (1996). Rumen $NH_3$-N concentration was determined as described by Fawcett and Scott (1960) using a plate reader (SpectraMax 190, Molecular Devices Corp., Sunnyvale, CA).

### *5.2.3 Ruminal VFA absorption*

Ruminal VFA absorption rate was estimated on d 21 of each experimental period using Co-EDTA as a fluid passage marker (Uden et al., 1980), and n-valeric acid as an indicator of VFA clearance from the rumen (Resende Júnior et al., 2006). At 4 h after feeding, each cannulated cow received a 2.5-L solution containing 300 g of n-valeric acid (VWR International, Edmonton, AB, Canada), and 125 g of Co-EDTA buffered to pH 6.2 with NaOH as described in Chapter 4. The Co-EDTA/n-valeric acid solution was thoroughly mixed by hand into the ruminal contents. An initial rumen fluid sample was collected immediately before the marker dose (time 0), and subsequent samples were collected at 30, 60, 120, 180, 360, 720, and 1,080 min after the dose. Individual samples

of ruminal fluid were analyzed for valeric acid concentration as described previously, and for Co concentration using atomic absorption (AA240FS, Varian, Palo Alto, CA).

Time 0 was used as baseline concentration of Co to correct concentrations measured at each individual time point (i.e., 30, 60, 120, 180, 360, 720, and 1,080 min relative to dosing) as described in Chapter 4. Because the dosing time was 4 h after feeding, concentration of n-valeric acid at time 0 was not necessarily lowest. Therefore, the lowest concentration rather than concentration at time 0 was used as the baseline concentration of n-valeric acid to correct concentrations measured at other time points. The exponential rate of decay for the ratio of n-valeric acid and Co were calculated as described by Resende Júnior et al. (2006) using the nonlinear model procedure of JMP (version 11.0, SAS Institute Inc., Cary, NC) using the equation $Y_t = Y_0 \times e^{-kt}$, where $Y_t$ = concentration at a given time, $Y_0$ = baseline concentration, k = fractional rate of clearance, t = sampling time, h.

### *5.2.4 Apparent Total Tract Digestibility*

Fecal samples were collected from the rectum every 9 h over a 72-h period on d 18, 19, and 20 of each experimental period (at the same time as rumen fluid collection). Samples were composited by cow and period. Period composites of feed ingredients, orts, and feces were dried in a forced-air oven for 72 h at 55°C to determine DM concentrations. Dried samples were then ground to pass through a 1-mm screen using a Wiley mill (Thomas-Wiley, Philadelphia, PA) for chemical composition analysis. The samples were analyzed for concentrations of DM (AOAC, 2002; method 930.15), OM (AOAC, 2002; method 942.05), NDF (Van Soest et al., 1991), starch (Hall, 2009), sugar (Dubois et al., 1956) and CP (AOAC, 2000; method 990.03) by Cumberland Valley

Analytical Services (Hagerstown, MD). Indigestible NDF was used as an internal marker to calculate the apparent total-tract digestibility (Cochran et al., 1986). The indigestible NDF concentration was determined for ingredient, orts, and fecal samples by placing 2 g of each sample into a nitrogen free polyester bag (5 × 10 cm, pore size = 50 μm; R510, Ankom Technology, Macedon, NY). Bags were incubated in triplicate in the rumen of a non-lactating cow fed a 100%-forage diet for 120 h.

### *5.2.5 Blood Collection*

Blood samples were collected every 18 h over a 72-h period (d 18, 19 and 20) in each experimental period from the coccygeal vessels into tubes containing sodium heparin (Fisher Scientific Company; Nepean, Ontario, Canada). 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 as described in Chapter 2. Plasma samples were composited to yield one sample per cow per period for further analysis.

Plasma samples were analyzed for glucose concentration using a glucose oxidase/peroxidase enzyme (Sigma; St. Louis, MO) and dianisidine dihydrochloride (Sigma) procedure. Plasma BHBA concentration was measured by the enzymatic oxidation of BHBA to acetoacetate using 3-hydroxybutyrate dehydrogenase (Roche, Mississauga, ON, Canada) followed by determination of reduction of $NAD^+$ to NADH at a wavelength of 340 nm. Commercial kits were used to determine concentrations of plasma NEFA (Wako Chemicals USA, Inc., Richmond, VA) and insulin (Coat-a-Count kit, Diagnostic Products Corp., Los Angeles, CA).

### *5.2.6 Milk composition*

Milk samples were analyzed for milk fat, CP, lactose, and MUN by mid-infrared spectroscopy (AOAC, 2002; method 972.16; MilkoScan FTIR 6000, Foss North America, Brampton, ON) at the Alberta Central Milk Testing Laboratory (Edmonton, AB) as described in Chapter 2. Milk yield and milk composition data from a.m. and p.m. milking were used to calculate the daily milk composition for d 18, 19 and 20, and the data were averaged for one value per cow per period. The ECM yield was calculated according to the equation described by Tyrrell and Reid (1965): ECM = [0.327 × milk yield (kg) + 12.95 × fat yield (kg) + 7.2 × protein yield].

### *5.2.7 Statistical Analysis*

Data were analyzed using the fit model procedure of JMP (version 11.0, SAS Institute Inc., Cary, NC) according to the following model:

$$Y_{ijk} = \mu + P_i + T_j + C_k + e_{ijk},$$

where $Y_{ijk}$ is the dependent variable, $\mu$ is overall mean, $P_i$ is fixed effect of period, $T_j$ is fixed effect of treatment, $C_k$ is the random effect of cows, and $e_{ijk}$ is the residual. Preplanned orthogonal contrasts were used to compare treatment means of high NFC diets (STA, SUC, and LAC) vs. CON, high disaccharide diets (SUC and LAC) vs. STA, and SUC vs. LAC. Treatment effects were declared significant at $P \leq 0.05$, and a tendency was declared at $0.05 < P \leq 0.10$.

## 5.3 Results

### *5.3.1 Feed intake and Milk Production*

Dry matter intake was higher ($P = 0.04$) for cows fed the high NFC diets than those fed CON diet (Table 5.7.2). In addition, cows fed high disaccharide diets had higher

DMI than those fed STA diet ($P < 0.01$), but there was no difference between cows fed SUC and LAC diets.

Milk yield was not different between cows fed CON diet and high NFC diets, but yield ($P = 0.06$) and concentration ($P = 0.07$) of milk fat tended to be lower for cows fed high NFC diets than those fed CON diet. The concentration of milk protein was higher ($P = 0.02$) for cows fed high NFC diets compared with those fed CON diet, whereas there was no difference in the yields of milk protein. In addition, cows fed high NFC diets had lower MUN concentration than those fed CON diet ($P < 0.01$). Cows fed high disaccharide diets tended to have higher ($P = 0.06$) milk yield compared with those fed STA diet, and had higher ($P < 0.01$) ECM yield than those fed STA diet. Cows fed high disaccharide diets had greater milk fat yield ($P = 0.01$), protein yield ($P = 0.02$) and tended to have greater milk protein concentration ($P = 0.09$) compared with those fed STA diet. Milk and milk component yields did not differ between cows fed SUC diet and LAC diet, but cows fed LAC diet tended to have higher milk fat concentration ($P = 0.06$) than those fed SUC diet.

### *5.3.2 Ruminal pH, VFA Profile and VFA absorption*

There was no difference in rumen pH between cows fed CON and high NFC diets (Table 5.7.3). Daily minimum ($P = 0.03$) and mean rumen pH ($P = 0.02$) were lower for cows fed the high disaccharide diets than for those fed STA diet. Moreover, the duration that rumen pH was below 5.8 was longer ($P = 0.05$), and the area that rumen pH was below 5.8 tended to be greater ($P = 0.09$) for cows fed the high disaccharide diets compared with those fed STA diet. But there was no difference in rumen pH between cows fed SUC and LAC diets. In addition, acidosis index was similar across treatments.

The molar proportion of acetate was lower ($P < 0.01$), but that of butyrate was higher ($P < 0.01$) for cows fed high NFC diets compared with those fed CON diet. In addition, cows fed high NFC diets had lower rumen $NH_3$-N concentration ($P < 0.01$) and ratio of acetate to propionate ($P = 0.02$) than CON diet. Cows fed high disaccharide diets had lower molar proportion of acetate ($P = 0.03$), but higher molar proportion of butyrate ($P < 0.01$) compared with those fed STA diet. The molar proportion of butyrate was higher for cows fed LAC diet than those fed SUC diet ($P = 0.01$). Total VFA concentration, the molar proportion of propionate and total VFA absorption rate were not different among all treatments.

### *5.3.3 Digestibility and Plasma Metabolites*

The apparent total-tract digestibility of DM ($P < 0.01$), OM ($P < 0.01$), starch ($P = 0.02$) and NDF ($P < 0.01$) were lower for cows fed high NFC diets than those fed CON diet (Table 5.7.4). In addition, compared with cows fed STA diet, cows fed high disaccharide diets had lower DM ($P = 0.01$), OM ($P = 0.01$) and starch ($P = 0.03$) digestibilities, but higher sugar digestibility ($P < 0.01$). When comparing cows fed SUC and LAC diets, cows fed the LAC diet had higher total tract digestibility of DM ($P = 0.01$), OM ($P = 0.01$), starch ($P = 0.02$) and CP ($P < 0.01$), but the digestibility of NDF and sugar was not different between cows fed the SUC and LAC diets.

Plasma glucose, NEFA, BHBA and insulin concentrations were not different among all treatments (Table 5.7.5).

## 5.4 Discussion

Dietary NFC content is often increased in diets for high-producing cows to increase energy intake and maximize milk yield. However, greater rumen fermentation from feeding more NFC may also increase the risk of ruminal acidosis, which potentially can have negative impacts on feed intake (Krajcarski-Hunt et al., 2002), health and production performance (Broderick, 2003; Ferraretto et al., 2013). In the current study, cows were fed low forage diets (35% forage and 15.6% forage NDF on DM basis) for all treatments, and dietary NFC contents were further increased by replacing beet pulp with corn grain, sucrose or lactose, respectively for STA, SUC, and LAC treatments. I found that rumen $NH_3$-N and MUN concentrations were lower for cows fed high NFC diets even if dietary CP content was similar for all experimental diets. These results might indicate more OM fermentation and microbial protein synthesis in the rumen (Nocek and Russell, 1988; Russell et al., 1992) when dietary NFC content increased, resulting in less $NH_3$-N concentration in the rumen and less urea excretion in milk. Even though beet pulp in CON diet was expected to be less fermentable than corn grain or disaccharides in the rumen, I observed no differences in rumen pH between CON and high NFC diets. Consistent with our findings, Voelker and Allen (2003) did not detect the difference in rumen pH when they replaced high moisture corn grain with up to 24% beet pulp in the diet, which decreased dietary starch content from 34.6 to 18.4% (DM basis). In addition, it should be noted that the responses in rumen pH were variable within the high NFC treatments (STA, SUC and LAC diets) in the current study. This is probably due to the different fermentation characteristics among NFC sources (Sniffen et al., 1992), and it is

necessary to investigate effects of feeding different types of NFC on rumen fermentation and animal production.

***Starch vs. Disaccharides***

Although rates of hydrolysis (248 - 1404 %/h) and fermentation (264 – 738 %/h) of sugars were reported to be very rapid (Weisbjerg et al., 1998), previous studies showed that replacing dietary starch with either sucrose (Broderick and Radloff, 2004; Broderick et al., 2008) or lactose (DeFrain et al., 2004) did not decrease rumen pH in lactating cows. *In vitro* studies have indicated that feeding sugar could increase molar proportion of butyrate in the rumen (Vallimont et al. 2004; Ribeiro et al, 2005), which was suggested as one possible explanation of no decrease in rumen pH for animals fed diets in which starch was partially replaced with sugar (Oba, 2011). Because 1 mole of hexose is fermented to 1 mole of butyrate, but to 2 moles of propionate or acetate, increased butyrate production could reduce proton production compared with acetate or propionate (Oba, 2011). I found higher butyrate molar proportion for high disaccharide diets in this study, however, rumen pH was lower for cows fed high disaccharide diets compared with those fed STA diet. Lower rumen pH for high disaccharide treatments might be partly due to greater DMI; for 8 ruminally cannulated cows, high disaccharide treatments increased DMI by more than 2 kg/d compared with STA treatment. In addition, low forage diets (35% forage and 15.6% forage NDF on DM basis) were fed to the animals in this study. Therefore, it is possible that the increased DMI of the highly fermentable diets may cause more OM fermentation in the rumen for cows fed high disaccharide diets, leading to the lower rumen pH compared with STA diet. Acidosis index, which indicated the severity of ruminal acidosis normalized for DMI, did not differ between high disaccharide diets and STA diet.

However, the *P* value for acidosis index was 0.11, which is close to the tendency, and the lower rumen pH for high disaccharide treatments may not be exclusively attributed to greater DMI.

Milk yield tended to be greater for cows fed high disaccharide diets than STA diet in this study, leading to the higher milk protein, milk fat and ECM yield for high disaccharide diets. Previous studies (Broderick et al., 2008; Penner and Oba, 2009) also found greater milk fat yield for cows fed high sugar diets. Therefore, although rumen pH was decreased for high sugar diets in this study, partially replacing dietary starch with disaccharide did not have negative effects on feed intake and milk production, but in fact increased DMI, milk and milk component yields for lactating cows.

Both *in vitro* (Vallimont et al., 2004) and *in vivo* (DeFrain et al., 2004) studies have shown that feeding high-sugar diets is often associated with greater butyrate concentration in the rumen. It was suggested that butyrate stimulated ruminal papillae growth and proliferation (Mentschel et al., 2001), and may be a modulator of functional changes in ruminal epithelial tissue (Penner et al., 2011). Malhi et al. (2013) reported that the ruminal epithelia growth and absorption capacity of VFA was increased when butyrate was ruminally infused in goats. In the current study, I found that high disaccharide diets increased the molar proportion of butyrate in the rumen compared with STA diet. However, I did not detect a difference in rumen VFA absorption rate across the treatments. One possibility accounting for the lack of treatment effects on VFA absorption is insufficient sensitivity of the method used to measure rate of VFA absorption in the current study. Penner et al. (2009c) has summarized a few limitations associated with this method, such as temporal increase of rumen osmolarity right after

dosing and different fractional rate of absorption between n-valeric acid and other VFA. Another possibility is that 21-d periods in this study might be too short for the diets to exert significant effects on the ruminal papillae growth and their metabolism to affect VFA absorption. When cows are exposed to a dietary change, 4 to 8 weeks adaptation period was suggested for maximal increases in the ruminal papillae number and size (Dirksen et al., 1985; Bannink et al., 2008). In addition, it is possible that increasing dietary sugar content affected relative proportions of VFA absorption pathways (i.e., passive diffusion or bicarbonate-dependent transport) without affecting overall VFA absorption rate. Chibisa et al. (2015) found that total VFA absorption rate was not different, but $Cl^-$-competitive absorption (bicarbonate-dependent transport) of acetate and propionate was increased by partial replacement of dietary starch with lactose. However, this speculation could not be assessed with the methodology in the current study. Therefore, further investigation is needed to improve the understanding of VFA absorption mechanism, particularly for specific impacts of sugar fermentation.

Apparent total tract digestibilities of DM and OM were lower for cows fed high disaccharide diets than STA diet. This may be attributed to the higher DMI for the high disaccharide diets, and subsequent greater solid or liquid passage rates (Khalili and Huhtanen, 1991; Sutoh et al., 1996). Greater DMI associated with faster passage rates would generally decrease nutrient digestibility (Tyrrell and Moe, 1975; Colucci et al., 1982). In addition, lower rumen pH for high disaccharide diets could affect the growth of cellulolytic bacteria and decrease fibre digestibility in the rumen (Nagaraja and Titgemeyer, 2007). However, our results on apparent OM digestibility may not be associated with fibre digestibility because NDF digestibility was similar between STA

and high disaccharide diets. Other studies reported that substituting corn grain with sucrose at 4.4 to 4.8% of dietary DM did not affect total tract digestibility of any nutrients in lactating dairy cows (Penner and Oba 2009; Penner et al., 2009b). Reasons for the discrepancy are not clear.

Although apparent total tract digestibility of sugar and starch were found lower for cows fed high disaccharide diets than STA diet, the values reported in this study were generally lower than the expected values regardless of treatment. It was noted that contents of sugar (1.29 ± 0.62 %, mean ± SD) and starch (10.8 ± 4.86 %, mean ± SD) in the fecal samples were relatively high. High starch diets were fed in the current study, and Owens et al. (1986) suggested that the extent of starch digestion decreased when the activity of enzymes, such as amylase and maltase, is limited in the small intestine. However, some previous studies detected over 90% total tract starch digestibility when feeding diets with similar starch content as the current study (Yang and Beauchemin, 2005; Silveira et al., 2007). Yang et al. (2000) and Beauchemin et al. (2001) showed that total tract starch digestibility increased as the extent of processing increased for steam-rolled and temper-rolled barley, respectively. Therefore, it is possible that rolled barley grain, the main dietary starch source used in this study, was not processed to the optimal extent, resulting in relatively lower total tract starch digestibility regardless of treatment. In addition, the current study used indigestible NDF as an internal maker to estimate nutrient digestibility, and its accuracy to predict digestibility is affected by diet type (Chochran et al., 1986), which might be another possible reason for the relative low values of nutrient digestibility in this study.

***Sucrose vs. Lactose***

Due to the faster hydrolysis and fermentation rate, previous studies found that ruminal dose of sucrose decreased rumen pH to a greater extent than lactose (Weisbjerg et al., 1998; Oba et al., 2015). Therefore, I had expected that cows fed SUC diet would have lower rumen pH compared with those fed LAC diet, but this was not observed. One possible reason is that feeding sugars in TMR may have different effects on rumen fermentation and rumen pH compared with rapid fermentation of sugars from a single dose. Feeding sugars in TMR would sustain their effects over a whole day while a single dose would have strong short-term effect immediately after the dose.

Due to the expected lower rumen pH for SUC, I had also expected lower milk fat content for SUC than LAC treatment. In fact, I found that SUC treatment tended to decrease milk fat concentration compared with LAC diet, but this cannot be attributed to effects of rumen pH because rumen pH was not lower for SUC treatment. Tendency of lower milk fat concentration for SUC treatment might be related to the lower butyrate concentration in the rumen because butyrate is a substrate for de novo fatty acid synthesis (Van Soest, 1994). In addition, I found that cows fed SUC diet decreased apparent total tract digestibility of DM, OM, starch and CP than LAC diet. However, I did not find any differences in DMI, milk and milk component yields between SUC and LAC treatments, which suggested that the differences in rumen fermentation and nutrient digestibility in this study might not be large enough to cause significant effects on milk production.

## 5.5 Conclusions

Feeding high disaccharide diets to lactating dairy cows increased DMI, milk fat, milk protein, and ECM yields compared with high starch diet even though rumen pH was

lower for cows fed high disaccharide diets. The treatment effects on rumen pH cannot be attributed to VFA absorption rate. For effects of sugar types, some differences in nutrient digestibility and rumen fermentation were observed between cows fed sucrose and lactose, but the differences might not be large enough to affect rumen pH, DMI, and milk yield.

## 5.6 References


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## 5.7 Tables

**Table 5.7.1** Ingredient and analyzed chemical composition of the experiment diets

| Item | CON | STA | SUC | LAC |
|---|---|---|---|---|
| Ingredient, % DM | | | | |
| Barley grain, rolled | 23.5 | 23.5 | 23.5 | 23.5 |
| Barley silage[1] | 35.6 | 35.6 | 35.7 | 35.7 |
| Canola meal | 5.31 | 5.13 | 6.65 | 6.65 |
| Corn grain, rolled | 15.2 | 22.2 | 15.5 | 15.5 |
| Corn gluten meal | 4.35 | 4.35 | 4.35 | 4.35 |
| Heat-processed soybean meal [2] | 5.26 | 5.26 | 5.26 | 5.26 |
| Mineral and vitamin mix[3] | 1.87 | 1.87 | 1.87 | 1.87 |
| Vegetable oil | 1.83 | 1.52 | 1.70 | 1.70 |
| Beet pulp | 7.00 | - | - | - |
| Lactose | - | - | - | 5.52 |
| Sucrose | - | 0.57[4] | 5.52 | - |
| Nutrient Composition | | | | |
| DM | 54.4 | 53.7 | 54.2 | 54.0 |
| CP, % DM | 16.8 | 17.2 | 17.0 | 17.0 |
| NDF, % DM | 28.4 | 26.2 | 26.7 | 26.1 |
| Forage NDF, % DM | 15.6 | 15.6 | 15.6 | 15.6 |
| Starch, % DM | 27.8 | 31.8 | 27.1 | 27.4 |
| Sugar, % DM | 4.60 | 4.50 | 9.15 | 8.60 |

[1] 31.0% DM, 12.4% CP, 48.1% NDF, and 12.1% starch

[2]AminoPlus (AGP, Omaha, NE)

[3]Contained 11.6% Ca, 3.39% P, 18.0% Na, 27.8% Cl, 6.77% Mg, 0.24% S, 0.04% K, 2447 mg/kg Fe, 551 mg/kg Cu, 1054 mg/kg Mn, 13.0 mg/kg Co, 842 mg/kg Zn, 8.23 mg/kg Se, 24.7 mg/kg I, 504 kIU/kg vitamin A, 51.3 kIU/kg vitamin D, 1651 IU/kg vitamin E

[4]Sucrose was added to the STA diet to make dietary sugar content similar between CON and STA diets.

**Table 5.7.2** Effect of increasing dietary non-fibre carbohydrate with starch, sucrose or lactose for lactating dairy cows on DMI, milk production and milk composition (n = 28)

| | Diet | | | | | *P* values | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **CON** | **STA** | **SUC** | **LAC** | **SEM** | **1** | **2** | **3** |
| DMI, kg/d | 26.3 | 26.2 | 27.5 | 27.5 | 0.70 | 0.04 | < 0.01 | 0.94 |
| Yield, kg/d | | | | | | | | |
| Milk | 37.5 | 36.9 | 38.1 | 37.7 | 1.79 | 0.97 | 0.06 | 0.54 |
| ECM | 39.5 | 38.0 | 39.5 | 39.6 | 1.59 | 0.37 | < 0.01 | 0.94 |
| Fat | 1.35 | 1.26 | 1.31 | 1.33 | 0.05 | 0.06 | 0.01 | 0.43 |
| CP | 1.28 | 1.26 | 1.32 | 1.30 | 0.05 | 0.59 | 0.02 | 0.45 |
| Lactose | 1.71 | 1.69 | 1.73 | 1.71 | 0.09 | 0.85 | 0.19 | 0.56 |
| Milk composition | | | | | | | | |
| Fat, % | 3.65 | 3.51 | 3.54 | 3.63 | 0.10 | 0.07 | 0.31 | 0.06 |
| CP, % | 3.46 | 3.48 | 3.51 | 3.50 | 0.05 | 0.02 | 0.09 | 0.55 |
| Lactose, % | 4.52 | 4.54 | 4.52 | 4.51 | 0.04 | 0.91 | 0.18 | 0.68 |
| MUN, mg/dL | 14.5 | 13.2 | 12.9 | 13.3 | 0.38 | < 0.01 | 0.67 | 0.20 |

1: high NFC diets (STA, SUC, and LAC) vs. CON

2: high disaccharide diets (SUC and LAC) vs. STA

3: SUC vs. LAC

**Table 5.7.3** Effect of increasing dietary non-fibre carbohydrate with starch, sucrose or lactose for lactating dairy cows on rumen pH, rumen fermentation and VFA absorption rate (n = 8 ruminally cannulated cows)

| | Diet | | | | | *P* values | | |
|---|---|---|---|---|---|---|---|---|
| **Item** | **CON** | **STA** | **SUC** | **LAC** | **SEM** | **1** | **2** | **3** |
| DMI, kg/d | 23.5 | 22.4 | 25.1 | 24.5 | 0.98 | 0.42 | < 0.01 | 0.52 |
| Ruminal pH | | | | | | | | |
| Nadir | 5.62 | 5.74 | 5.57 | 5.52 | 0.09 | 0.93 | 0.03 | 0.57 |
| Mean | 6.20 | 6.32 | 6.21 | 6.17 | 0.05 | 0.55 | 0.02 | 0.46 |
| Maximum | 6.77 | 6.83 | 6.81 | 6.80 | 0.05 | 0.37 | 0.58 | 0.85 |
| Duration < pH 5.8, min/d | 97.2 | 46.9 | 101 | 183 | 43.9 | 0.77 | 0.05 | 0.13 |
| Area < pH 5.8, pH × min/d | 20.8 | 7.1 | 18.3 | 45.4 | 13.4 | 0.84 | 0.09 | 0.11 |
| Acidosis index, pH × min/kg DMI[1] | 0.99 | 0.31 | 0.81 | 1.97 | 0.63 | 0.95 | 0.11 | 0.12 |
| VFA profile | | | | | | | | |
| Total VFA, m*M* | 96.9 | 92.1 | 94.2 | 94.1 | 2.47 | 0.20 | 0.46 | 0.95 |
| Acetate, mol/100mol | 57.7 | 55.8 | 54.6 | 54.3 | 0.78 | < 0.01 | 0.03 | 0.68 |
| Propionate, mol/100mol | 24.0 | 25.4 | 26.3 | 24.7 | 0.90 | 0.11 | 0.88 | 0.15 |
| Butyrate, mol/100mol | 13.3 | 13.7 | 14.4 | 15.9 | 0.39 | < 0.01 | < 0.01 | 0.01 |
| Acetate: Propionate | 2.42 | 2.22 | 2.11 | 2.23 | 0.10 | 0.02 | 0.58 | 0.26 |
| Rumen $NH_3$-N, mg/dL | 13.0 | 10.3 | 9.62 | 10.3 | 1.00 | < 0.01 | 0.69 | 0.48 |
| Total VFA absorption, %/h | 30.1 | 34.4 | 33.6 | 34.1 | 2.21 | 0.17 | 0.86 | 0.88 |

[1] Acidosis index: area under pH 5.8 divided by DMI (Penner et al., 2009a)

1: high NFC diets (STA, SUC, and LAC) vs. CON

2: high disaccharide diets (SUC and LAC) vs. STA

3: SUC vs. LAC

**Table 5.7.4** Effect of increasing dietary non-fibre carbohydrate with starch, sucrose or lactose for lactating dairy cows on apparent total-tract nutrient digestibility (n = 8 ruminally cannulated cows)

| | Diet | | | | | *P* values | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **CON** | **STA** | **SUC** | **LAC** | **SEM** | **1** | **2** | **3** |
| Digestibility, % | | | | | | | | |
| DM | 67.9 | 64.9 | 57.8 | 63.0 | 1.45 | < 0.01 | 0.01 | 0.01 |
| OM | 69.1 | 66.2 | 59.7 | 64.4 | 1.41 | < 0.01 | 0.01 | 0.01 |
| Starch | 89.1 | 86.0 | 73.7 | 83.2 | 2.70 | 0.02 | 0.03 | 0.02 |
| CP | 64.1 | 66.9 | 59.7 | 68.0 | 2.00 | 0.73 | 0.22 | < 0.01 |
| NDF | 43.7 | 35.3 | 34.8 | 32.4 | 2.03 | < 0.01 | 0.50 | 0.41 |
| Sugar | 92.2 | 89.0 | 93.8 | 95.7 | 1.31 | 0.64 | < 0.01 | 0.30 |

1: high NFC diets (STA, SUC, and LAC) vs. CON

2: high disaccharide diets (SUC and LAC) vs. STA

3: SUC vs. LAC

**Table 5.7.5** Effect of increasing dietary non-fibre carbohydrate with starch, sucrose or lactose for lactating dairy cows on plasma metabolite concentrations (n = 8 ruminally cannulated cows)

| | Diet | | | | | *P values* | | |
|---|---|---|---|---|---|---|---|---|
| **Variable** | **CON** | **STA** | **SUC** | **LAC** | **SEM** | **1** | **2** | **3** |
| Glucose, mg/dL | 48.6 | 51.3 | 49.0 | 49.9 | 1.35 | 0.35 | 0.26 | 0.66 |
| NEFA, mEq/L | 37.9 | 36.0 | 42.6 | 41.0 | 3.80 | 0.67 | 0.25 | 0.77 |
| BHBA, mg/dL | 10.7 | 9.69 | 10.4 | 11.1 | 1.16 | 0.84 | 0.44 | 0.68 |
| Insulin, uIU/mL | 7.36 | 9.49 | 9.88 | 8.53 | 1.16 | 0.13 | 0.83 | 0.37 |

1: high NFC diets (STA, SUC, and LAC) vs. CON

2: high disaccharide diets (SUC and LAC) vs. STA

3: SUC vs. LAC

# 6. General discussion

## 6.1 Summary

Similar to other species, Chapter 2 suggested that a substantial variation exists in the severity of developing SARA among lactating dairy cows. Because the experimental diet and daily management were the same among all cows, factors causing this variation are expected to relate to the animals themselves. In Chapter 2, it was shown that HS cows sorted against long particles, whereas LS cows did not. In addition, sorting index of long particles was positively correlated with minimum rumen pH and negatively correlated with acidosis index. However, HS cows had longer total chewing time and chewing time per unit of NDF intake than LS cows. In addition, Chapter 4 indicated that in situ starch and NDF digestibility was not different between LS and HS cows. Also, there was no difference in VFA absorption rate between LS and HS cows. But I found that the mRNA abundance of LSS gene was significantly higher for LS cows than HS ones, and the mRNA abundance of HMGCS1 gene tended to be higher for LS cows compared with HS cows, which suggested that variation in the severity of SARA among cows might be partly due to differences in VFA metabolism in rumen epithelial cells.

In Chapter 2, rumen pH was determined to identify cows with higher or lower risk of SARA. But it is not practical to measure rumen pH for all cows on farms. Therefore, Chapter 3 was conducted to determine if MUN and milk fat content could be used as non-invasive indicators to identify cows with higher and lower risk of SARA. Results showed that minimum and mean ruminal pH was higher, and duration of rumen pH below 5.8 was shorter for cows identified presumably with lower risk of SARA, based on MUN and milk fat content, compared with ones identified presumably with higher risk. In addition,

Chapter 4 suggested that cows identified with lower risk in mid-lactation consistently had higher rumen pH and lower severity of SARA than cows with higher risk of SARA at both mid- and late-lactation.

Feeding different types of carbohydrates to dairy cows might have a different effect on VFA absorption rate, and absorption of VFA accounts for more than half of the total proton removal from the rumen (Allen, 1997). Therefore, Chapter 5 evaluated if feeding more disaccharide instead of starch would increase the rate of VFA absorption. Results showed that high disaccharide diets increased the molar proportion of butyrate in the rumen compared with high starch diet, but difference in rumen VFA absorption rate was not detected. As for the effects of different types of disaccharides, no differences were detected in rumen pH, VFA absorption rate, DMI, and milk yield between cows fed sucrose and lactose, although some differences in nutrient digestibility and rumen butyrate concentration were observed.

## 6.2 Industry implication

One TMR ration is often fed to a group of cows on dairy farms, and my studies suggested that there is substantial variation in the severity of SARA among dairy cows when they are fed the same high grain diet. Therefore, cows have different capability to cope with the high grain diets, and some cows in a herd may experience SARA but others do not, even though they are fed the same diet. It would be helpful to understand the mechanisms that contribute to lower risk of SARA, and then management strategies could be implemented to improve the capability of cows to cope with the high grain diets and decrease the risk of SARA. Chapter 2 showed that LS cows sorted to a less extent than HS cows. There are a few factors that affect sorting behaviour, such as DM% of the ration

and particle size of the diet. The DM content ranges from 40 to 60% in the typical TMR fed to high-producing dairy cows (Eastridge, 2006), and ration with over 60% DM content may increase sorting behaviour. In addition, greater forage particle size may increase sorting of the TMR by cows (Leonardi and Armentano, 2003; Kononoff and Heinrichs, 2003; Maulfair and Heinrichs, 2013). Therefore, management practices can be implemented to minimize sorting behaviour of cows, such as controlling DM% of TMR between 40 – 60 % (Eastridge, 2006) and the proportion of long particles in TMR following the guidelines of Penn State Particle Separator (2-8%) (Heinrichs, 2013), which may be helpful to minimize the extent of variation in the risk of SARA among a group of cows and decrease the risk of SARA. In addition, to manage HS cows, they can be grouped together and fed diets with lower fermentability and higher amount of peNDF than the diets fed to LS cows.

Chapter 3 showed the potential of using MUN and milk fat content as indicators to identify LS and HS cows during late-lactation when they were fed a high grain diet. But the application of these indicators on farm may be limited, as there are multiple confounding factors affecting MUN responses, such as parity, stage of lactation, milk production, DMI and milk protein content (Godden et al., 2001; Arunvipas et al., 2003). In Chapter 2 and 3, small number of cows was used, and the potential confounding factors, such as stage of lactation, milk production, DMI and milk protein content, were similar among cows. However, it would become difficult to control these confounding factors on farm. In addition, a high grain diet was fed to late-lactating cows in Chapter 2 and 3. But it is not practical to feed a high grain diet for late-lactaing cows on farm. Therefore, it would be limited to use MUN as an indicator to indentify LS and HS cows on farm.

Moreover, soluble protein content in diets may also affect the difference in MUN concentration between HS and LS cows. As discussed in Chapter 2, lower MUN for HS cows may be due to higher OM fermentation in the rumen than LS cows, so that more $NH_4^+$ may be used by rumen microbes and less would be excreted in the milk for HS cows. If microbes in the rumen of HS cows could still utilize more $NH_4^+$, then increasing dietary soluble protein content may increase the difference in MUN between HS and LS cows because more supply of $NH_4^+$ could be used by microbes in the rumen for HS cows while the increased $NH_4^+$ in the rumen would be excreted in the milk and urine for LS cows. However, if microbes in the rumen of HS cows could not utilize more $NH_4^+$, then more soluble protein in the diet may increase $NH4^+$ concentration in the rumen to the same extent for both HS and LS cows. More $NH4^+$ may be absorbed into the blood and excreted in the milk and urine for both groups, thus difference in MUN between LS and HS cows may be masked and decreased due to the increased MUN concentration for both groups. Therefore, difference in MUN concentration between HS and LS cows may be affected by diets with different soluble protein content.

Chapter 5 showed that partially replacing dietary starch with disaccharides increased DMI, milk fat, milk protein, and ECM yields in mid-lactating dairy cows. Therefore, byproducts with high disaccharide content could be used to replace part of the grain in the ration to increase milk production particularly when cost of cereal grains increases. In addition, sugar content is typically 4-6% DM basis in the ration for lactating cows, but sugar content was about 9% on a DM basis in the rations used in Chapter 5. Therefore, it may be feasible to increase dietary sugar content up to 9% on a DM basis without negatively affecting productive performance for lactating cows. In addition,

Chapter 5 suggested that differences in rumen butyrate concentration and apparent total tract digestibility of DM, OM, starch and CP between cows fed high sucrose and lactose diets were not large enough to affect DMI, milk and milk component yields. Thus, sucrose and lactose may not have different effect on productive performance for lactating dairy cows, and cost may be the key factor affecting the usage of these two different disaccharides in the ration.

## 6.3 Future studies

Volatile fatty acid production from OM fermentation in the rumen is a major factor affecting rumen pH (Allen, 1997). Therefore, it is possible that VFA production is related to the variation in the risk of SARA among lactating cows. Net fermentation acid production in the rumen can be directly measured by using isotopic tracer technique (Martin et al., 2001; Sutton et al., 2003) to evaluate if differences in VFA production exist between LS and HS cows. In addition, amount of rumen fermentation is affected by factors of substrate, microbes and retention time in the rumen. As cows were fed the same diet in my study, substrate factor was removed. Chapter 4 showed that in situ carbohydrate digestibility in the rumen was not different between LS and HS cows, which suggested that the variation in the risk of SARA among cows might not be associated with the capacity of fermenting carbohydrate by rumen microbes. Then I evaluated the rumen retention time by measuring rumen digesta passage rate by dosing Cr-mordanted fibre into the rumen as solid phase marker. However, the results were not reliable due to the sampling error caused by poor mixing of marker with rumen digesta. Therefore, further study can be conducted to measure rumen digesta passage rate by using other techniques, such as rumen evacuation method, to determine if there is difference in rumen

digesta passage rate between LS and HS cows. Method of rumen evacuation assumes that the rumen pool size is steady state and there is no disturbance of normal rumen function (Robinson et al., 1987). This technique has been used in a few studies described by Voelker and Allen (2003) and Kammes and Allen (2012). Brifely, the indigestible NDF is used as an internal marker to calculate duodenal flow of digesta. Ruminal contents need to be evacuated twice, and one time point needs to be close to the minimum value of rumen content, and the other one needs to be close to its maximum value. Then ruminal pool sizes (kg) of nutrients are determined by multiplying the concentration of each component in DM by the ruminal digesta DM mass (kg). Fractional passage rate from the rumen %/h = (Duodenal flow of component / Ruminal pool of component) / 24 *100.

Differences in rumen microbial population and metabolic activity may account for the difference in the risk of SARA among cows, as oganic matter is fermented by the enzymes produced by the microbes in the rumen. Previous findings were inconsistent in the relationship between variation in the risk of SARA among ruminants with differences in rumen microbial population and profile. Chen et al. (2012) found different total bacterial density and diversity in the rumen digesta between LS and HS steers. Khafipour et al. (2009) also found that lactating cows with different responses to grain induced SARA had different phylum profiles in rumen microbiota composition. However, it was reported that ruminal bacterial community composition was not associated with the variation in the severity of acidosis among lactating cows and primiparous cows during transition period (Palmonari et al., 2010; Mohammed et al., 2012). The inconsistent findings in thesis studies might be partly due to the different acidosis challenge intensity. In the two studies that no relationship was detected between bacterial community

composition with variation in the severity of acidosis, lactating cows were fed a TMR diet with 45.5% concentrate on DM basis in one study (Palmonari et al., 2010), and primiparous cows were fed TMR diets and the highest concentrate content was 53.4% on DM basis among all diets in the other study (Mohammed et al., 2012). However, in the two studies that the relationship was detected, lactating cows were fed a TMR diet with 60.0% concentrate on DM basis in one study (Khafipour et al., 2009), and beef steers were force fed a diet with 85% grain on DM basis at 60% of animal's average DMI in the other study (Chen et al., 2012). Therefore, more severe challenge to induce SARA may have larger chance to stimulate the different responses for rumen microbes if there is a potential of variation in rumen microbial population and profile among animals. In these studies, total bacterial population and bacterial composition was determined for LS and HS animals. But compared with data of total bacteria, the information of active microbes may be more helpful to explain the relationship between micrbobes and the risk of SARA, as the active microbes are the ones having an effect on OM fermentation in the rumen. In addition, the function of microbes in different species was not determined in previous studies. Therefore, RNA sequencing technique can be used to measure total RNA of microbes to obtain the population and function of the active microbes in different species to represent their activity, and qPCR technique can be used to validate the RNA sequencing results. Besides microbes living in the rumen content, there are also bacterial populations adherent to the rumen wall. Although the rumen epimural bacterial community is very stable (Petri et al., 2013), Chen et al. (2012) found that the diversity of rumen epimural bacteria was different between LS and HS steers. Rumen epithelial epimural bacteria have necessary functions for the host, such as the facilitation of

absorption (Freter, 1970). Therefore, understading the differences in composition and function of epimural bacterial community that may exist among cows may also help to understand the relationship between the host and micrbobes and its possible effect on the variation in the risk of SARA among cows.

SARA is caused by the accumulation of VFA in the rumen, and lactate usually does not consistently accumulate in the rumen for dairy cows during SARA (Oetzel et al., 1999; Krause and Oetzel, 2006). The reason is that the growth of lactate-utilising bacteria, such as *Megasphaera elsdenii* and *Selenomonas ruminantium*, are able to metabolize the lactate to VFA (Goad et al., 1998). But when rumen lactate concentration is measured frequently, transient increase in the concentration of rumen lactate (up to 20 mM) can be detected (Kennelly et al., 1999; Krause and Oetzel, 2006). Therefore, a possibility could not be excluded that there is difference in rumen lactate concentration betwee LS and HS cows, and future study should determine if rumen lactate concentration differs between LS and HS cows.

As discussed in Chapter 1, there are a few factors contributing to the regulation of rumen pH, which are also possible mechanisms explaining variation in the risk of SARA among cows. In my studies, individual factor was evaluated to determine if it is related to the variation in the risk of SARA among cows. However, it is also possible that a combination of multiple factors contributes to the higher or lower risk of SARA, and this may mask the difference between LS and HS cows when evaluating individual factor. For example, VFA absorption rate may explain the difference in the risk of SARA for some cases, but not for all animals; it is possible that higher rumen pH for some LS cows is due to higher VFA absorption rate, and higher rumen pH for the other LS cows is due to other

factors but not VFA absorption rate. Similarly, lower rumen pH for some HS cows is due to lower VFA absorption rate, and lower rumen pH for the other HS cows is due to other factors but not VFA absorption rate. Then, when VFA absorption rate is compared between LS and HS cows, the difference may not be large enough to be detected. In my studies, due to the limited number of animals, it was not possible to evaluate if the different risk of SARA is caused by combination of multiple factors. It may be difficult to determine if a combination of multiple factors contributes to the risk of SARA for each animal. But it may be possible to evaluate if a combination of multiple factors exists in one risk group. For example, cows in LS group may be divided into three categories by VFA absorption rate (LS cows with high VFA absorption rate (LSH), LS cows with medium VFA absorption rate, and LS cows with low VFA absorption rate (LSL)). If there are differences in acidosis index and VFA absorption rate between HS and LSH cows, while difference in acidosis index exists but no difference in VFA absorption rate is detected between HS and LSL cows, then this may indicate that a combination of multiple factors contributes to the risk of SARA for LS cows.

Although my studies suggested that cows had different risk of SARA when they were fed the same diet, I did not find significant differences in DMI, milk yield and milk component yields between LS and HS cows. This might be due to the relatively low number of cows used in the study and relatively short experimental period. It has been shown that SARA is related to decreased DMI, laminitis (Nocek, 1997), decreased fibre digestibility, and increased inflammation (Khafipour et al., 2009). Therefore, studies with longer experimental period and larger number of cows may be helpful to evaluate if cows with different risk of SARA would have different performance in milk production and

health status.

In Chapter 5, high disaccharide diets increased the molar proportion of butyrate in the rumen compared with high starch diet. However, no difference was detected in rumen VFA absorption rate. One speculation for no difference in VFA absorption rate was the insufficient sensitivity of the measuring method. As Resende Junior et al. (2006) summarized, there are a few methods that have been used to measure VFA absorption rate. First, blood samples can be collected from the portal vein to determine VFA appearance (Huntington et al., 1983), but this technique has limitations due to the VFA metabolism in the rumen wall (Kristensen, 2001). Second, VFA labeled with radioactive or stable isotopes can be infused into the rumen to measure absorption rate (Kristensen, 2001; Sutton et al., 2003), but the cost is high, and the radioactive isotopes is harmful to humans, animals, and the environment, which restricts their usage (Resende Junior et al., 2006). In addition, temporarily isolated and washed reticulorumen technique can be used to measure VFA absorption rate (Care et al.,1984; Kristensen et al., 2000), but this technique also has limitations, because the estimation of absorption is not under typical physiological condition, and the ratio of rumen absorptive surface area to VFA solution is different from what is found in the rumen of a productive animal (Resende Junior et al., 2006). Therefore, in my studies, the technique with valeraic acid and Co-EDTA as markers was used to measure rumen VFA clearance as described by Allen et al. (2000) and Penner et al. (2009). Resende Júnior et al. (2006) evaluated this technique, and showed that results measured by using this technique were similar to the results determined by the stable isotope technique. In addition, this technique has relatively low cost, causes low invasiveness to the animals, and it is easy to operate (Resende Júnior et

al., 2006). But as Penner et al. (2009) summarized, there are also limitations of this technique. Increased ruminal osmolarity was found after dosing the markers, which could decrease the absorption rate and affect the precision of the estimation (Owens et al., 1998; Penner et al., 2009). In addition, this method assumes that the absorption rate of n-valeric acid is similar to other VFA (Allen et al., 2000), and Resende Júnior et al. (2006) confirmed this assumption and showed that the absorption rate of valeraic acid was similar to the absorption rates of acetate, propionate and butyrate estimated by using the stable isotope method. But, when they evaluated this assumption, the conversion of $^{13}C$ among VFA in the rumen was not considered, which might affect the precision of their evaluation (Penner et al., 2009). Therefore, these limitations may affect the sensitivity and precision of the technique of using valeraic acid and Co-EDTA as markers to measure rumen VFA absorption (Penner et al., 2009), which may explain why no difference was detected in rumen VFA absorption rate between high starch and high disaccharide diets in my study.

Another speculation was that increasing dietary sugar content might have an effect on relative proportions of VFA absorption pathways (i.e., passive diffusion or bicarbonate-dependent transport) but not affect overall VFA absorption rate. Chibisa et al. (2015) suggested that bicarbonate-dependent transport of acetate and propionate was increased by partial replacement of dietary starch with lactose, even though total VFA absorption rate was not different between high starch and high lactose diets. In addition, feeding sucrose and lactose may also have a different effect on relative proportions of VFA absorption pathways. Oba et al. (2015) found that ruminal dose of sucrose increased expression of PAT1 gene compared with lactose dose. The PAT1 is a transport protein

that facilitates the transport of dissociated VFA into epithelial cells and export bicarbonate ions back to the rumen (Connor et al., 2010), which involves in the pathway of bicarbonate-dependent transport. Therefore, sucrose treatment might have different relative proportion of VFA absorption via bicarbonate-dependent pathway compared with lactose treatment. But in that study, sugar was dosed into the rumen of cows, and samples were collected within 3 hours after dosing. Compared with a single dose of sugar into the rumen, feeding sugar in TMR may have different effects on rumen fermentation and rumen pH (Chapter 5). Therefore, future study needs to be conducted to determine if there are differences in relative proportions of VFA absorption pathways between cows fed high sucrose and lactose diets by using a different technique, such as the temporarily isolated and washed reticulorumen technique, which has been used in previous studies to measure VFA absorption rate (Kristensen et al., 2000; Storm et al., 2011; Chibasa et al., 2015) and estimate the absorption rate of individual VFA via different pathways (Chibasa et al., 2015).

## 6.4 Conclusions

Lactating dairy cows have different capability to cope with the high grain diets, and they have the variability in the severity of SARA even when they are fed the same high grain diet. Therfore, some cows in the herd may suffer from SARA while others do not, even though they are fed the same diet. Reasons of this variability lie in cows themselves. Although VFA absorption is a major pathway to remove acids from the rumen and regulate rumen pH, the difference in VFA absorption rate did not explain the variation in the severity of SARA among lactating cows in my studies. Chewing activity, another important pathway of acid removal, was not identified as the reason either, in my

study. In fact, responses of chewing activity might be the result of the variation in the severity of SARA among cows. However, sorting behaviour might be a factor causing this variation. Thus, management practices to reduce the sorting behavior may be used to decrease the variation in the risk of SARA among a group of cows, particularly decrease the severity of SARA for cows with high risk. In addition, future work is needed to determine if acid production and rumen microbial population and activity are the causes of the variation, as acid production is another major factor regulating rumen pH.

Various diet formulation strategies have been applied to improve the performance of lactating cows without increasing the risk of SARA. My study suggested that partially replacing dietary starch with sucrose and lactose increased productive performance in mid-lactating cows even though rumen pH was decreased. In addition, the productive performance was not different between feeding sucrose and lactose for lactating cows. Therefore, usage of these two types of disaccharides in the ration may mainly depend on the cost.

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