Characteristics of dairy cows with a greater or lower risk of subacute ruminal acidosis: Volatile fatty acid absorption, rumen digestion, and expression of genes in rumen epithelial cells

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ABSTRACT

The objective of this study was to examine whether lactating dairy cows with a greater or lower risk of subacute ruminal acidosis (SARA) have differences in volatile fatty acid (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. We fed 14 ruminally cannulated mid-lactating dairy cows (119 ± 47.2 d in milk; body weight 640 ± 47.9 kg) a high-grain diet consisting of 30% forage ad libitum, with an 18-d diet adaptation and a 7-d sample and data collection period. Eight cows with the lowest acidosis index [area below pH 5.8 normalized for dry matter intake (DMI); 0.10 ± 0.16 pH $\times$ min/kg of DMI] and 5 with the highest acidosis index (3.72 ± 0.19 pH $\times$ min/kg of DMI) were classified as animals with lower risk (LS) and higher risk (HS) of SARA, respectively. Minimum (5.75 vs. 5.33) and mean rumen pH (6.33 vs. 5.98) were higher for LS than for HS cows. In addition, the duration and area of rumen pH below 5.8 was lower in LS cows (24.9 vs. 481 min/d; 2.94 vs. 102 pH $\times$ min/d). Although DMI, milk yield, and milk component yields did not differ, milk fat concentration tended to be higher for LS cows than for HS cows (3.36 vs. 2.93%). However, we observed no difference in VFA absorption rate between LS and HS cows. In situ starch and neutral detergent fiber digestibility were not different between LS and HS cows, but the relative mRNA abundance of lanosterol synthase (*LSS*) was higher for LS cows than for HS cows. In addition, the mRNA abundance of hydroxy-3-methylglutaryl-CoA synthase 1 (*HMGCS1*) tended to be higher for LS cows than for HS cows. These results suggested that VFA absorption rate might not explain the difference in rumen pH between LS and HS cows in the current study, even though expression of some genes

related to VFA metabolism in rumen epithelium may be associated with variation in the risk of SARA among lactating cows. This variation in the risk of SARA may not be attributed to differences in the capacity of rumen microbes to ferment carbohydrates, because in situ carbohydrate digestibility in the rumen was not different between cows with higher and lower risk of SARA.

Key words: rumen acidosis, volatile fatty acid absorption and metabolism, gene expression, in situ rumen digestibility

INTRODUCTION

Highly fermentable diets are often fed to high-producing lactating cows to increase milk production. However, excessively fermentable diets can increase the risk of SARA. Although the NRC (2001) recommends maximum NFC and minimum NDF levels (% of DM) to decrease the occurrence of SARA, some cows may still develop it. 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) have suggested that variation exists in the severity of SARA among animals even when they are fed the same diet. The reasons for this variation are not fully understood.

Rumen pH is determined by the difference between acid production in the rumen and acid removal from the rumen (Allen, 1997). Acid removal has 3 primary pathways: absorption by rumen epithelium, neutralization by buffers, and passage from the rumen to the lower parts of the digestive tract (Allen, 1997). Variations in risk for SARA among ruminants could be related to at least 1 of these factors (Penner et al. 2009a). Absorption of VFA contributes to approximately 53% of the total proton removal from the rumen (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 at low risk of SARA than for those at high risk, suggesting that differences in VFA absorption rate might be related to variations in the severity of SARA in vivo. We hypothesized that lactat-

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ing dairy cows with lower risk of SARA would have a faster VFA absorption rate than cows with higher risk of SARA.

Besides VFA and proton transportation, the process of VFA absorption involves the exchange of other ions such as Na^+ and HCO_3^- , and the metabolism of VFA in epithelial tissue, which can regulate intracellular pH and VFA absorption (Aschenbach et al., 2011; Penner et al., 2011). Investigation is needed to better understand the relationship between variations in the severity of SARA among cows and the expression of genes associated with ion exchanges, intracellular pH regulation, and VFA metabolism in rumen epithelial tissue.

Acid production from OM fermentation is another major factor that affects rumen pH. Chen et al. (2012) found that steers with a lower risk of SARA had lower bacterial density from rumen digesta than steers with a higher risk, possibly indicating that less VFA are produced in the rumen of lower-risk steers. Differences in acid production from OM fermentation in the rumen may also be related to variations in the severity of SARA among lactating cows, and we hypothesized that cows with a higher risk of SARA would have greater in situ carbohydrate digestibility in the rumen than cows with a lower risk of SARA.

The objective of this study was to examine whether lactating dairy cows with a higher 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.

MATERIALS AND METHODS

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

Animals, Diets, and Experimental Design

Fourteen multiparous ruminally cannulated mid-lactating Holstein cows (DIM 119 ± 47.2 ; BW 640 ± 47.9 kg; mean $\pm$ SD) were used in this study. Cows were fed a high-grain diet containing 30% forage and 70% concentrate mix (Table 1) ad libitum for 25 d, with an 18-d diet adaptation period and a 7-d data and sample collection period. Cows were housed individually in tie stalls, bedded with wood shavings, and had free access to water. The experimental diet was fed as a TMR to cows individually once daily at 0800 h, and was offered

at 105 to 110% of actual feed intake from the previous day. The amounts of feed offered and refused were recorded daily throughout the study, and samples of feed ingredients were collected daily during the sample collection period. Daily samples of each feed ingredient were composited into 1 sample, and then dried in a forced-air oven (Yamato Scientific America Inc., Santa Clara, CA) for 48 h at 55°C to determine DM concentrations. Dried samples were analyzed for NDF (Van Soest et al., 1991, method A), starch (Hall, 2009), fat (AOAC International, 2006; method 2003.05), and CP (AOAC International, 2000; method 990.03) by Cumberland Valley Analytical Services (Hagerstown, MD). The DM concentration of barley silage was measured twice weekly, also by drying samples in a forced-air oven for 48 h at 55°C, 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 the experiment. Cows were milked twice daily at 0400 and 1500 h.

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). Minimum, mean, and maximum pH were determined each day for each cow and averaged over a 3-d period. Daily duration and area below pH 5.8 were also calculated each day for each cow and averaged over a 3-d period. DMI was recorded each day for each cow on d 19, 20, and 21, and averaged over a 72-h period. Acidosis index (area under pH 5.8 divided by DMI; Penner et al.,

Table 1. Ingredient and nutrient composition of the experimental diet

Item	% of DM
Ingredient	
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	54.6
CP	16.9
NDF	26.8
Starch	31.8
Ether extract	4.10
NFC	47.5
Forage NDF	13.9

¹Contained 15.4% Ca, 2.83% P, 12.4% Na, 25.0% Cl, 5.53% Mg, 0.28% S, 6.43% K, 3,274 mg/kg Fe, 617 mg/kg Cu, 1,160 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, 1,548 IU/kg vitamin E.

2009b) was calculated to evaluate the severity of SARA normalized for DMI. Values of mean $\pm$ 0.5 $\times$ SD of the 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 (0500, 1400, and 2300 h on d 19; 0800 and 1700 h on d 20; and 0200, 1100, and 2000 h on d 21). Samples were collected in equal proportions from the cranial, ventral, and caudal sacs, and combined to form 1 sample. Samples were then strained through a perforated screen (Peetex; Sefar Canada Inc., Scarborough, ON, Canada; pore size 355 μ m). Immediately after collection, rumen fluid samples were centrifuged at 3,000 $\times$ g at 4°C for 20 min, and the supernatants were stored at -20°C until analysis. Samples were composited to yield 1 sample per 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).

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, ON, Canada). Immediately following collection, plasma was harvested by centrifuging blood samples at 3,000 $\times$ g at 4°C for 20 min. Plasma samples were stored at -20°C until analysis. Samples were composited to yield 1 sample per cow for further analysis.

Plasma samples were analyzed for glucose, BHB, free fatty acids, 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 BHB concentration was measured by the enzymatic oxidation of BHB 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. We used commercial kits to measure concentrations of plasma free fatty acids (Wako Chemicals USA Inc., Richmond, VA) and insulin (Coat-a-Count kit; Diagnostic Products Corp., Los Angeles, CA).

Milk Composition

Milk samples were collected from morning and evening milkings on d 19, 20, and 21 of the study.

Samples were analyzed for milk fat, CP, lactose, and MUN using mid-infrared spectroscopy (AOAC International, 2002; method 972.16; MilkoScan FTIR 6000; Foss North America, Brampton, ON, Canada) at the Alberta Central Milk Testing Laboratory (Edmonton, AB, Canada). Milk yield and milk composition data from both morning and evening milkings were used to calculate daily milk composition, and daily data were averaged over 3 d to yield 1 value per cow.

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, ON, Canada). Samples were ground through a 1 mm screen using a Wiley mill (Thomas Wiley, Philadelphia, PA). After grinding, they were mixed according to the DM proportion of TMR (30% barley silage and 70% concentrate mix). Bags were prepared so that 2 g of the mixed sample was placed in 1 nitrogen-free polyester bag (5 $\times$ 10 cm, pore size 50 μ m; R510, Ankom Technology, Macedon, NY). Ten replicate bags (5 for starch and 5 for NDF digestibility) were placed in the rumen of each cow via the rumen cannula on d 22. Five bags were removed from the rumen after 7 h of incubation, and the other 5 bags were removed after 30 h of incubation. Bags were washed with cold water, and then placed in the forced-air oven for 48 h at 55°C to analyze DM concentrations. Samples collected after 7 h of incubation were analyzed for starch concentration (Hall, 2009), and samples collected after 30 h incubation were analyzed for NDF concentration (Van Soest et al., 1991, method A) by Cumberland Valley Analytical Services. Mixed feed ingredient samples before incubation were also analyzed for both starch and NDF concentration by Cumberland Valley Analytical Services. The 7-h starch and 30-h NDF digestibility were calculated as (1 – amount of starch or NDF remaining after rumen incubation / amount of starch or NDF before rumen incubation) $\times$ 100, respectively.

Rumen VFA Absorption

Rumen VFA absorption rate was determined on d 24 according to the method described by Penner et al. (2009b). Cobalt-EDTA was used as a marker of fluid passage (Udén 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). At 4 h after feeding, 2.5 L of 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 using atomic absorption (AA240FS; Varian, Palo Alto, CA).

The concentration of Co 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, the concentration of *n*-valeric acid was not necessarily the lowest at time 0. Therefore, instead of using the concentration at time 0, we used the lowest concentration as the baseline concentration of *n*-valeric acid to correct concentrations measured at other 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. We then calculated the exponential decay rate for the ratio of rumen *n*-valeric acid and Co concentration to determine the VFA absorption rate, using the nonlinear model procedure of JMP (version 11.0; SAS Institute Inc., Cary, NC) and 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, and t = sampling time, h.

Ruminal Papillae Collection

Approximately 30 ruminal papillae were biopsied from the ventral sac of the rumen 3 h 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 -80°C in RNA-Later solution (Ambion, Inc., Foster City, CA) until analysis.

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, Wald-

bronn, Germany), and the value of the RNA integrity number was higher than 8.2 for all samples.

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

Real-Time Quantitative PCR

Real-time PCR was performed by 2 methods in this study. First, a batch of target genes was analyzed using Taqman kits, because these target genes had been evaluated in previous studies in our laboratory using these kits (Penner et al., 2009b; Schlau et al., 2012), so they were available for these target genes. Later, a second batch of target genes was selected for real-time PCR analysis. Most of these genes had been evaluated in previous studies using Fast SYBR Green kits (Steele et al., 2011; Chen et al., 2012; Malmuthuge et al., 2013; Yan et al., 2014). Because Fast SYBR Green kits are less expensive than Taqman kits, they 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) for the target genes monocarboxylate cotransporter, isoform 1 (*MCT1*), downregulated in adenoma (*DRA*), putative anion transporter, isoform 1 (*PAT1*), sodium hydrogen exchanger, isoforms 1, 2, and 3 (*NHE1*, *NHE2*, *NHE3*, respectively), and sodium/potassium ATPase pump, α 1 (*ATP1*), which are involved in VFA absorption and intracellular pH regulation; and 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 2) were used as described previously (Penner et al., 2009b; Schlau et al., 2012). The primers' efficiency was determined using a standard curve method (with at least 4 serial dilutions) in a pool of all samples. All samples were analyzed on 1 plate per gene in triplicate.

Real-time PCR was performed with the StepOnePlus Real-Time PCR System (Life Technologies) 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*), and 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*), and 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*), and lanosterol synthase (*LSS*), which are involved in cholesterol synthesis; target genes toll-like receptor 2 and 4 (*TLR2*, *TLR4*), claudin 1 (*CLDN1*), junctional adhesion molecule 2 (*JAM2*), occludin (*OCLN*), and zonula occluden 1 (*ZO1*), which are involved in barrier function and tight junction. The primers for *MCT4*, *CA2*, *JAM2*, and *ZO1* (Table 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 primer specificity. Other primers were used as described previously (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 4 serial dilutions) in a pool of all samples. The program was 40 cycles of 3 s at 95°C and 30 s at 60°C. 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 a single melting peak. All samples were analyzed on 1 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 3 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 1 gene. For each sample, the *Ct* value of the gene was calculated, and it was subtracted from the minimum *Ct* among all 14 samples to obtain a ΔC_t value for the gene. Then, the relative abundance of mRNA for the gene was calculated for each sample by using the formula $Q = \text{amplification efficiency}^{\Delta C_t}$. 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*, *ACTB*, and *GADPH* for each sample. The Q_n value of the gene for each cow was used to evaluate for differences in the relative abundance of mRNA between cows with higher and lower risk of SARA.

Statistical Analysis

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 according to the following model:

$$Y_i = \mu + G_i + e_i,$$

where Y_i is the dependent variable, μ is overall mean, G_i is fixed effect of group, and e_i is the residual. Relationships between the mRNA abundance of genes and minimum ruminal pH were analyzed using the fit line procedure in JMP. Significance was declared at $P < 0.05$ and tendency was declared at $0.05 < P < 0.10$.

RESULTS

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 $\times$ min/d, respectively. The acidosis index ranged from 0.0 to 4.35 pH $\times$ min/kg of DMI. The acidosis index of 8 cows was lower than the threshold of mean $- 0.5 \times SD$, and the acidosis index of 5 cows was higher than the value of mean $+ 0.5 \times SD$; they were categorized as cows with lower SARA risk (**LS**) or higher SARA risk (**HS**), respectively. The acidosis index of 1 cow was between the value of mean $\pm 0.5 \times SD$; data collected from this cow were not used when comparing the group effect.

Rumen pH, VFA Profile, and VFA Absorption Rate

We observed no differences in BW or DMI between LS and HS cows ($P > 0.10$; Table 3). However, the minimum (5.75 vs. 5.33; $P < 0.01$) and mean ruminal pH (6.33 vs. 5.98; $P < 0.01$) were higher for LS cows than for HS cows; maximum pH values were not different between groups. The duration (24.9 vs. 481 min/d; $P < 0.01$) and area (2.94 vs. 102 pH $\times$ min/d; $P < 0.01$) that pH was below 5.8 were lower in LS cows. The acidosis index was lower in LS cows (0.10 vs. 3.72 pH $\times$ min/kg DMI; $P < 0.01$). Total VFA concentration, VFA profiles, concentration of rumen $\text{NH}_3\text{-N}$, VFA absorption rate, and liquid passage rate were not different between groups ($P > 0.10$; Table 4).

In Situ Starch and NDF Digestibility

In situ 7-h starch and 30-h NDF digestibility were not different between LS and HS cows ($P > 0.10$; Table 4).

Table 2. Gene names, primer, and probe (for Taqman) sequences for real-time quantitative PCR analysis (qPCR)

Gene name	Category	Primer and probe sequence	Source
Genes with Taqman kits			
Monocarboxylate cotransporter, isoform 1 (<i>MCT1</i>)	VFA absorption	Forward: CGCGGGATTCTTTGGATTT Reverse: GTCCATCAGCGTTTCAAACAGTAC Probe: TTTTGGGTGGCTCAGC	Schlau et al., 2012
Sodium/hydrogen exchanger, isoform 1 (<i>NHE1</i>)	Intracellular pH regulation	Forward: GAAAGACAAGCTCAACCGGTTT Reverse: GGAGCGCTCACC GGCTAT Probe: AAGTACGTGAAGAAGTGTCT	Schlau et al., 2012
Sodium/hydrogen exchanger, isoform 2 (<i>NHE2</i>)	Intracellular pH regulation	Forward: TTGTGCGATGACCATGAATAAGT Reverse: TGATGGTCGTGTAGGATTTCTGA Probe: CGTGGAAGAGAACGTG	Schlau et al., 2012
Sodium/hydrogen exchanger, isoform 3 (<i>NHE3</i>)	Intracellular pH regulation	Forward: AGCCTTCGTGCTCCTGACA Reverse: TGACCCCTATGGCCCTGTAC Probe: TGCTCTTCATCTCCG	Schlau et al., 2012
3-Hydroxy-3-methylglutaryl-CoA synthase 1 (<i>HMGCS-1</i>)	Cholesterol synthesis	Forward: AGGATACTCATCACTTGGCCAAC Reverse: CATGTTTCCTTCCAAGAGGGAAT Probe: CATCCCCAGAGTTCCA	Schlau et al., 2012
3-Hydroxy-3-methylglutaryl-CoA synthase 2 (<i>HMGCS-2</i>)	Ketogenesis	Forward: CCTGCTGCAATCACTGTTCATG Reverse: TCTGTCCCGCCACCTCTTC Probe: TTGCAGAGCCCTTTC	Schlau et al., 2012
3-Hydroxy-3-methylglutaryl-CoA lyase (<i>HMGCL</i>)	Ketogenesis	Forward: TGCAGATGGGAGTGAGTGTCA Reverse: GACGCCCCCTGTGCATAG Probe: TGGCAGGACTGGGAG	Penner et al., 2009b
β -Hydroxybutyrate dehydrogenase, isoform 1 (<i>BDH1</i>)	Ketogenesis	Forward: GACTGCCACCACTCCCTACAC Reverse: TCCGCAGCCACCACTAGTAGT Probe: CGCTACCATCCCATG	Penner et al., 2009b
β -Hydroxybutyrate dehydrogenase, isoform 2 (<i>BDH2</i>)	Ketogenesis	Forward: CTGTGGCTTCCAGCATCAAA Reverse: CGCCTTGTTGTGCTGTACA Probe: CGTTGTGAACAGGTGC	Penner et al., 2009b
Sodium/potassium ATPase pump, α 1 (<i>ATP1</i>)	Intracellular pH regulation	Forward: CATCTTCCTCATCGGCATCA Reverse: ACGGTGGCCAGCAAACC Probe: TGTAGCCAACGTGCCAG	Schlau et al., 2012
Putative anion transporter, isoform 1 (<i>PAT1</i>)	VFA absorption	Forward: GGGCACTTCTTCGATGCTTCT Reverse: GTCGTGGACCGAGGCAAA Probe: TCACCAAGCAGCACCT	Schlau et al., 2012
Downregulated in adenoma (<i>DRA</i>)	VFA absorption	Forward: TGCACAAAGGGCCAAGAAA Reverse: GCTGGCAACCAAGATGCTATG Probe: TGCCTTCTCCTCCTTC	Schlau et al., 2012
Ribosomal protein large, P0 (<i>RPLP0</i>)	Housekeeping gene	Forward: AGGGCGTCCGCAATGTT Reverse: CGACGGTTGGGTAACCAATC Probe: CCAGCGTGTGCCTG	Schlau et al., 2012
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	Housekeeping gene	Forward: TGCCGCTGGAGAAACC Reverse: CGCCTGCTTCACCACCTT Probe: CCAAGCGTGTGCCTG	Schlau et al., 2012
β -Actin (<i>ACTB</i>)	Housekeeping gene	Forward: CCTGCGGCATTACGAA Reverse: GCGGATGTCGACGTCACA Probe: CTACCTTCAATCCATCATG	Schlau et al., 2012
Genes with SYBR Green kits			
Monocarboxylate cotransporter, isoform 4 (<i>MCT4</i>)	VFA absorption	Forward: TCAGCGTCTTCTTCAAGGAAC Reverse: CAGCCACGATTACAC	Yan et al., 2014
Vacuolar H ⁺ ATPase subunit B (<i>vH⁺ATPase</i>)	Intracellular pH regulation	Forward: TTTTATTGAACAAGAAGCCAATGA Reverse: GATTTCATCAAATTGGACATCTGAA	
Anion exchanger 2 (<i>AE2</i>)	Intracellular pH regulation	Forward: AGCAGCAACAACCTGGAGT Reverse: GGTGAAACGGGAGACGAA	Yan et al., 2014
Carbonic anhydrase 2 (<i>CA2</i>)	Intracellular pH regulation	Forward: AAGTTCTGAGCATACTGTGG Reverse: CTGTTCCAAAGTCCCCGTAC	Penner et al., 2009b
Acyl-CoA synthetase short-chain family member 1 (<i>Acyl-CoAs</i>)	VFA metabolism activation	Forward: CCGATCAGGTCTGCTAGTGA Reverse: GCCTCCGCATGACTTTTCC	
Acetyl-CoA synthetase 2 (<i>Acetyl-CoAs</i>)	VFA metabolism activation	Forward: GCTCTCACTGAGGAGCTCAAGAA Reverse: AATCCGGTGTGGCAATGG	Penner et al., 2009b
Acetyl-CoA acyltransferase 1 (<i>ACAA1</i>)	Ketogenesis	Forward: CACTGGCTTCCCAGCAAAA Reverse: ACGATCTCGGCCTGGAAC	Penner et al., 2009b
Acetoacetyl-CoA thiolase 2 (<i>ACAT2</i>)	Cholesterol synthesis	Forward: CCGCTGGCTGACAGTATACTTTG Reverse: TGGCCACATTTTCAGCTGTAAT	Steele et al., 2011
3-Hydroxy-3-methylglutaryl-CoA reductase (<i>HMGCR</i>)	Cholesterol synthesis	Forward: GAGTGGCAGGACCTCTGTGC Reverse: GCACCTCCACCAAGGCCTAT	Steele et al., 2011
Farnesyl diphosphate synthase (<i>FDPs</i>)	Cholesterol synthesis	Forward: GGTATCAGAAGCCAGGCATAGG Reverse: TAGGGCTGCTCCCGACAGTA	Steele et al., 2011

Continued

Table 2 (Continued). Gene names, primer, and probe (for Taqman) sequences for real-time quantitative PCR analysis (qPCR)

Gene name	Category	Primer and probe sequence	Source
Sterol regulatory element-binding protein 1 (<i>SREBP1</i>)	Cholesterol synthesis	Forward: CCAGCTGACAGCTCCATTGA Reverse: TGCGCGCCACAAGGA	Steele et al., 2011
Sterol regulatory element-binding protein 2 (<i>SREBP2</i>)	Cholesterol synthesis	Forward: CTGGCTCCAGGGAGATGAC Reverse: GCTCTGCAGGTGTGGAAGAC	Steele et al., 2011
Lanosterol synthase (<i>LSS</i>)	Cholesterol synthesis	Forward: TGCAGGCGCTGAAGACTTT Reverse: GCTCAAGGGTCTCCCTGATCT	Steele et al., 2011
Toll-like receptor 2 (<i>TLR2</i>)	Barrier function	Forward: CTGTGTGCGTCTTCCCTCAGA Reverse: TCAGGGAGCAGAGTAACCAGA	Chen et al., 2012
Toll-like receptor 4 (<i>TLR4</i>)	Barrier function	Forward: GGTTTCCACAAAAGCCGTAA Reverse: AGGACGATGAAGATGATGCC	Chen et al., 2012
Claudin 1 (<i>CLDN1</i>)	Tight junction	Forward: GCGCTGCCCCAGTGGAAGT Reverse: GGATCTGCCCGGTCTCTGC	Malmuthuge et al., 2013
Junctional adhesion molecule 2 (<i>JAM2</i>)	Tight junction	Forward: CCCCATCGGAACAAGCTCAA Reverse: GACATCGCAGCTCTACCACA	Malmuthuge et al., 2013
Occludin (<i>OCN</i>)	Tight junction	Forward: ACGCAGGAAGTGCCCTTTGGTAGC Reverse: GCAGCCATGGCCAGCAGGAA	Malmuthuge et al., 2013
Zonula occluden-1 (<i>ZO1</i>)	Tight junction	Forward: AATGCATCCTGACCACCAGG Reverse: GATGGTGCCGGGTTTGTTC	Schlau et al., 2012
Ribosomal protein large, P0 (<i>RPLP0</i>)	Housekeeping gene	Forward: AGGGCGTCCGCAATGTT Reverse: GACGGTTGGGTAACCAATC	Schlau et al., 2012
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	Housekeeping gene	Forward: TGCCGCCTGGAGAAACC Reverse: CGCCTGCTTCACCACCTT	Schlau et al., 2012
β -Actin (<i>ACTB</i>)	Housekeeping gene	Forward: CCTGCGGCATTCACGAA Reverse: GCGGATGTGACGTCACA	Schlau et al., 2012

Milk Production

We observed no differences in milk yield or milk component yields between LS and HS cows ($P > 0.10$; Table 5). Concentrations of milk protein, lactose, and MUN did not differ between the groups. However, concentrations of milk fat tended to be higher for LS cows than for HS cows (3.36 vs. 2.93%; $P = 0.07$).

Plasma Metabolites and Hormones

Plasma glucose, BHB, free fatty acids, and insulin concentrations were not different between LS and HS cows ($P > 0.10$; Table 6).

Rumen Epithelium Gene Expression

The 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 7). However, the relative mRNA abundance of *NHE1* tended to be positively correlated with minimum rumen pH ($r = 0.47$, $P = 0.08$; Figure 1). Gene-encoding *LSS* had higher mRNA abundance for LS cows than for HS cows ($P = 0.03$). In addition, the mRNA abundance of *HMGCS1* tended to be higher for LS cows than for HS cows ($P = 0.08$). The relative mRNA abundance of the other genes associated with epithelial cell VFA metabolism was not different between LS and HS cows.

Table 3. Comparison of BW, DMI, and pH measurements between LS and HS cows¹

Variable	LS (n = 8)		HS (n = 5)		P-value
	LSM	SE	LSM	SE	
BW, kg	658	16.2	612	20.5	0.11
DMI, kg	29.2	1.18	28.1	1.50	0.56
Ruminal pH					
Nadir	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 $\times$ min	2.94	2.18	102	2.76	<0.01
Acidosis index, pH $\times$ min/kg of DMI	0.10	0.15	3.72	0.19	<0.01

¹LS = cows identified as having low risk of SARA; HS = cows identified as having high risk of SARA.

Table 4. Comparison of rumen VFA profile, $\text{NH}_3\text{-N}$ concentration, VFA absorption rate, and in situ digestibility between LS and HS cows¹

Variable	LS (n = 8)		HS (n = 5)		P-value
	LSM	SE	LSM	SE	
VFA profile					
Total VFA, mM	129	2.88	129	3.64	0.93
Acetate, mol/100 mol	63.0	1.34	64.0	1.69	0.67
Propionate, mol/100 mol	19.7	0.53	20.4	0.67	0.48
Butyrate, mol/100 mol	13.5	0.99	11.9	1.25	0.32
Acetate:propionate	3.21	0.14	3.17	0.17	0.85
Rumen $\text{NH}_3\text{-N}$, 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
Liquid passage rate, %/h	13.6	0.92	11.3	1.09	0.13
7-h starch digestibility, %	74.9	1.32	73.1	1.67	0.41
30-h NDF digestibility, %	53.2	1.78	50.2	2.25	0.32

¹LS = cows identified as having low risk of SARA; HS = cows identified as having high risk of SARA.

($P > 0.10$), but the mRNA abundance of *BDH2* tended to be positively correlated with minimum rumen pH ($r = 0.50$, $P = 0.07$). The relative mRNA abundance of genes associated with epithelial barrier function and tight junction were not different between LS and HS cows ($P > 0.10$).

DISCUSSION

Subacute ruminal acidosis is a metabolic disorder commonly found in high-producing dairy cows. Diet formulation strategies can decrease the incidence of SARA, but it still occurs for some cows in a herd. It has been shown that variation in the severity of SARA exists among late-lactation cows when they are fed the same high-grain diet (Gao and Oba, 2014). In the current study, we also detected variations in rumen pH and the severity of SARA among mid-lactation cows. The absorption of VFA is a major factor that regulates rumen pH, and it has been suggested that over 50% of the total protons (largest proportion) in the rumen are removed 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 the *NHE3* gene in ruminal epithelial cells compared with HS steers. The *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 a decrease in local pH and increase of the undissociated form of VFA near the epithelial cells; these effects may increase VFA absorption via simple diffusion (Graham et al., 2007; Connor et al., 2010; Schlau et al., 2012). Responses of rumen VFA concentration and expression of the *NHE3* gene suggested that higher rumen pH for LS steers might be partly due to higher VFA absorption rate than for HS steers (Schlau et al., 2012). In the present study, we hypothesized that VFA absorption might be one of the main factors causing the variation in the risk of SARA for lactating dairy cows, but we did not detect a difference in VFA absorption rate between LS and HS cows.

Table 5. Comparison of milk yield and milk composition between LS and HS cows¹

Variable	LS (n = 8)		HS (n = 5)		P-value
	LSM	SE	LSM	SE	
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

¹LS = cows identified as having low risk of SARA; HS = cows identified as having high risk of SARA.

Table 6. Comparison of plasma blood metabolite and hormone concentrations between LS and HS cows¹

Variable	LS (n = 8)		HS (n = 5)		P-value
	LSM	SE	LSM	SE	
Glucose, mg/dL	64.1	0.79	61.9	1.00	0.12
BHB, mg/dL	10.3	0.84	11.3	1.07	0.48
Free fatty acids, mEq/L	84.0	7.95	92.1	10.1	0.54
Insulin, μ IU/mL	8.57	0.57	7.66	0.72	0.34

¹LS = cows identified as having low risk of SARA; HS = cows identified as having high risk of SARA.

There are a couple of possible explanations for our 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 present study—that the LS and HS cows selected were not extreme enough—and differences in VFA absorption rate were not large enough to be detected between LS and HS cows. In a previous study using crossbred beef steers, the acidosis index was 13.5 pH $\times$ min/kg of DMI for LS steers, but 61.7 pH $\times$ min/kg of DMI for HS steers (Schlau et al., 2012).

In the present study, the acidosis index was 0.10 pH $\times$ min/kg of DMI for LS cows and 3.72 pH $\times$ min/kg of DMI for HS cows, so differences between the animals selected for the present study were less extreme. Smaller variations in the risk of SARA in the present study might be due to decreased genetic diversity and an increased inbreeding coefficient for Holstein dairy cows. Inbreeding levels have increased over the past decades in the Holstein breed, and it has been shown that the average inbreeding coefficient for Holstein cows was between 6 and 7% in Canada in 2008 (Stachowicz et al.,

Table 7. Comparison of mRNA abundance of genes involved in intracellular pH regulation, VFA metabolism, and tight junction between LS and HS cows¹

Gene ²	LS (n = 8)		HS (n = 5)		P-value	Efficiency of amplification, %
	LSM	SE	LSM	SE		
<i>MCT1</i>	1.09	0.08	0.99	0.10	0.46	97.9
<i>NHE1</i>	1.15	0.05	1.07	0.06	0.38	98.4
<i>NHE2</i>	1.07	0.03	0.99	0.04	0.18	93.0
<i>NHE3</i>	0.94	0.09	0.80	0.11	0.33	101
<i>HMGCS-1</i>	0.91	0.05	0.77	0.06	0.08	92.2
<i>HMGCS-2</i>	1.15	0.08	1.17	0.10	0.87	95.9
<i>HMGCL</i>	1.13	0.05	1.09	0.06	0.68	89.3
<i>BDH1</i>	0.99	0.06	0.99	0.08	0.93	101
<i>BDH2</i>	1.19	0.05	1.10	0.06	0.25	97.7
<i>ATP1</i>	1.03	0.05	0.93	0.06	0.22	97.0
<i>PAT1</i>	1.01	0.05	0.96	0.07	0.61	101
<i>DRA</i>	0.93	0.10	0.80	0.12	0.41	98.3
<i>MCT4</i>	0.84	0.05	0.92	0.06	0.35	93.3
<i>vH⁺ATPase</i>	0.95	0.03	0.94	0.04	0.78	99.9
<i>AE2</i>	0.97	0.04	1.02	0.05	0.46	94.8
<i>CA2</i>	0.84	0.06	0.88	0.07	0.70	104
<i>Acyl-CoAs</i>	0.92	0.05	0.96	0.07	0.67	101
<i>Acetyl-CoAs</i>	0.95	0.05	1.03	0.07	0.35	101
<i>ACAA1</i>	1.01	0.03	0.97	0.04	0.52	95.7
<i>ACAT2</i>	1.04	0.04	1.09	0.04	0.44	96.0
<i>HMGCR</i>	0.93	0.03	0.86	0.04	0.29	94.9
<i>FDPS</i>	0.91	0.05	0.84	0.07	0.42	97.7
<i>SREBP1</i>	0.88	0.05	0.89	0.07	0.87	96.5
<i>SREBP2</i>	0.94	0.02	0.97	0.03	0.38	97.4
<i>LSS</i>	0.91	0.04	0.75	0.05	0.03	95.6
<i>TLR2</i>	0.66	0.06	0.80	0.08	0.22	103
<i>TLR4</i>	0.79	0.08	0.85	0.10	0.62	104
<i>CLDN1</i>	0.89	0.05	0.82	0.06	0.39	96.9
<i>JAM2</i>	0.89	0.06	1.03	0.08	0.21	104
<i>OCN</i>	0.99	0.04	1.01	0.05	0.76	98.3
<i>ZO1</i>	1.04	0.05	1.05	0.06	0.89	95.5

¹LS = cows identified as having low risk of SARA; HS = cows identified as having high risk of SARA.

²See Table 2 for full list of gene names.

Figure 1. Relationship between minimum rumen pH with (a) relative mRNA abundance of sodium/hydrogen exchanger, isoform 1 (*NHE1*; $P = 0.08$) and (b) relative mRNA abundance of β -hydroxybutyrate dehydrogenase, isoform 2 (*BDH2*; $P = 0.07$).

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 has been lost since the founder generations. On our research dairy farm, the herd is closed, and artificial insemination is implemented with a limited number of sires. Less genetic variation for Holstein cows used in the present study might explain the smaller variations in the risk of SARA than that of the crossbred steers in the previous study (Schlau et al., 2012).

A second possibility is that the intensity of acidosis challenge in the current study might not have been severe enough to cause different VFA absorption responses between LS and HS cows. In this study, the challenge was feeding cows a TMR with 44.4% grain on a DM basis. 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. The mean rumen pH in the present study was 6.33 for LS cows and 5.98 for HS cows; in the beef steer study, it was 6.01 for LS steers and 5.51 for HS steers. These findings indicate that the intensity of the challenge to the rumen epithelial tissue might have been less sufficient in the present study than in the beef steer study (Schlau

et al., 2012) to explain why no difference was detected in VFA absorption rate between LS and HS cows.

Increasing absorption and metabolism of VFA in rumen epithelial cells has been proposed as a potential mechanism for decreasing the risk of ruminal acidosis (Gäbel et al., 2002). The 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, we did not detect a significant difference in the fractional rate of VFA absorption between LS and HS cows, but we did find that the mRNA abundance of *LSS* was higher for LS cows than HS ones, and the mRNA abundance of *HMGCS1* tended to be higher for LS cows than for 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) showed that genes involved in cholesterol biosynthesis were upregulated in the rumen epithelial cells during the first week of a 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). It was proposed that upregulation of genes related to VFA metabolism during the first week was due to an 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 wk 1 to 3 to compensate for the increased intracellular cholesterol concentration and maintain intracellular cholesterol homeostasis (Steele et al., 2011). In the present study, LS cows had a higher mRNA abundance of genes related to cholesterol biosynthesis than HS cows, suggesting that LS cows might have a higher capacity to cope with elevated cholesterol levels in rumen epithelial cells or a higher capacity to clear the cholesterol from rumen epithelial cells to the blood. In addition, ketogenesis is the primary pathway of VFA metabolism in the ruminal epithelial cells (Penner et al., 2011). However, we did not detect significant differences in the 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. Still, we found that the 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 a precursor for lipid and sterol synthesis (Guo et al., 2006). Together, in this study, variations 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 negative correlations between the expression of genes associated with VFA metabolism and the severity of SARA for dairy cows. But even though we detected the changes in the expression of genes, protein abundance and activity were not measured in this study, and differences in mRNA abundance do not necessarily mean differences in protein activity.

In the present study, we did not find significant differences in the mRNA abundance of genes related to transportation of ions and VFA, and to intracellular pH regulation. Also, we observed no significant 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 than for HS beef steers in a previous study. Ruminal papillae samples were collected at 2, 4, and 6 h after force-feeding in that study, but samples were biopsied at 3 h after feeding in the present study. Although the numerical difference in expression of *NHE3* between LS and HS steers was highest at the 2-h time point, we observed no significant interaction between group effect and time of sampling for *NHE3* gene expression (Schlau, 2012). Therefore, sampling time may not be the key factor for explaining the different responses in *NHE3* expression between these 2 studies. As discussed above, genetic variation and the intensity of acidosis challenge may be the possible reasons. In addition, we found a tendency for the relative mRNA abundance of *NHE1* to be positively correlated with minimum rumen pH, although we observed no difference between LS and HS cows. However, we observed no relationship between the relative mRNA abundance of *NHE1* with mean rumen pH and acidosis index, indicating that the intensity of the acidosis challenge might not have been severe enough to cause different responses in expression of *NHE* genes between LS and HS cows.

Rumen pH is a function of 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 lower

VFA production. Carbohydrates normally account for approximately 65 to 70% of DM in the diets for lactating dairy cows (NRC, 2001), and starch and fiber are the main carbohydrate sources. Therefore, we expected that LS cows would have less ruminal fermentation of starch and NDF than HS cows. Substrate, microbes, and ruminal retention time affect the extent of carbohydrate fermentation in the rumen. Measurement of in situ carbohydrate digestibility removes possible confounding factors caused by differences in substrate and retention time in the rumen, and indicates the capacity of microbes to ferment carbohydrates in the rumen. However, we did not detect differences in in situ 7-h starch or 30-h NDF digestibility between LS and HS cows. This lack of difference indicated that the capacity of rumen microbes to ferment carbohydrates might not be related to variations in risk of SARA among cows. But we could not exclude the possibility of differences in VFA production as a factor causing variations in rumen pH, because other factors (such as rumen digesta passage rate) also have an effect on rumen fermentation and VFA production. Previous studies have shown that the passage rate of digesta affects feed degradability (Robinson et al., 1987) and microbial efficiency (Sniffen and Robinson, 1987). Orskow 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, possibly causing the differences in VFA production, even though in situ starch and NDF digestibility was the same between LS and HS cows. Further research on differences in rumen digesta passage rate and net rumen VFA production between LS and HS cows is needed to determine whether the extent of rumen fermentation and VFA production is related to variation in risk of SARA among cows. In addition, as a pathway of proton removal, passage from the rumen is also a factor regulating rumen pH. Allen (1997) suggested that approximately 15% of total acids were removed from the rumen by passage. In this study, liquid passage rate was not significantly different between LS and HS cows. Therefore, passage from the rumen may not be a factor contributing the variation in the risk of SARA among cows in this study.

CONCLUSIONS

Variation exists in the risk of developing SARA among lactating dairy cows fed a high-grain diet; however, this variation cannot be attributed to differences in rumen microbial capacity to ferment carbohydrates, because in situ carbohydrate digestibility in the rumen was not different between cows with higher and lower risk of SARA. Although the expression of some genes involved

in VFA metabolism in rumen epithelium was related to variation in risk of SARA, VFA absorption rate did not explain the difference in rumen pH between cows with higher and lower risk of SARA in the present study.

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