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Rumen epithelial adaptation to high-grain diets involves the coordinated regulation of genes involved in cholesterol homeostasis

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Steele MA, Vandervoort G, AlZahal O, Hook SE, Matthews JC, McBride BW. Rumen epithelial adaptation to high-grain diets involves the coordinated regulation of genes involved in cholesterol homeostasis. *Physiol Genomics* 43: 308–316, 2011. First published January 18, 2011; doi:10.1152/physiolgenomics.00117.2010.—The molecular mechanisms underlying rumen epithelial adaption to high-grain (HG) diets are unknown. To gain insight into the metabolic mechanisms governing epithelial adaptation, mature nonlactating dairy cattle ($n = 4$) were transitioned from a high-forage diet (HF, 0% grain) to an HG diet (65% grain). After the cattle were fed the HG diet for 3 wk, they returned to the original HF diet, which they were fed for an additional 3 wk. Continuous ruminal pH, ruminal short chain fatty acids, and plasma β-hydroxybutyrate were measured on a weekly basis, and rumen papillae were biopsied from the ventral sac to assess alterations in mRNA expression profiles. The subacute form of ruminal acidosis was diagnosed during the first week of the HG period (4.6 ± 1.6 h/day <pH 5.6), but not during *weeks 2* and *3*, thereby indicating ruminal adaption to the HG diet. Changes in the mRNA expression profile of rumen papillae were initially examined using Bovine Affymetrix microarrays; a total of 521 differentially expressed genes (false discovery rate $P < 0.08$) were uncovered from the first to third week of the HG period. Ingenuity Pathway Analysis of microarray results revealed that enzymes involved in cholesterol synthesis were coordinately downregulated from the first to third week of the HG period. In addition, the LXR/RXR activation pathway was significant and included several genes involved in intracellular cholesterol homeostasis. The differential expression signature of eight genes representing the key regulatory points of cholesterol homeostasis was confirmed by quantitative real-time PCR. Based upon our pathway and network results we propose a model to explain cellular events during rumen epithelial adaptation to HG diets and thus provide molecular targets that may be useful in the treatment and prevention of ruminal acidosis.

ruminal acidosis; rumen epithelium; acidosis; gene expression

IN INTENSIVE RUMINANT LIVESTOCK systems, rapidly fermentable (high grain) diets are commonly utilized to increase energy intake. When ruminants are fed high-grain (HG) diets, short chain fatty acid (SCFA) production can exceed absorption and ruminal pH can be depressed causing ruminal acidosis (8, 62, 66). Subacute ruminal acidosis (SARA), which is diagnosed when ruminal pH falls <5.6 for more than 3 h per day, is common in commercial dairy cows (2, 26). It has been estimated that 20% of all commercial dairy cattle in North America suffer from SARA (64) at a cost of $1.12 US per cow per day (78). SARA is associated with extensive alterations in rumen microflora populations and has a wide variety of clinical manifestations including depressed feed intake and milk production, liver abscesses, diarrhea, and inflammation (70).

The rumen is the first organ to face the insult of grain-induced SARA. Therefore, SARA may elicit an adaptive response by the rumen epithelium to maintain whole animal homeostasis as it responds to increased SCFA production. For example, protruding from the wall of the rumen are papillae, which greatly increase the epithelial surface area for absorption of SCFAs, mainly acetate, propionate, and butyrate (21). When more SCFAs are available for absorption, the rumen epithelium commonly responds by increasing the size of the papillae, thereby increasing the surface area for absorption (22, 63, 72). HG diets also increase the SCFA absorption capacity of individual rumen epithelial cells (21), yet minimal changes in epithelial cell metabolism have been detected (7, 31).

Of the four strata of the rumen epithelium, the basale and spinosum have an enhanced ability to metabolize and thus help clear SCFAs from the rumen milieu (27). Among SCFAs, butyrate is the preferred substrate for rumen epithelial metabolism (46, 47), and it has been estimated that 90% of butyrate produced in the rumen is metabolized by the gut (8). The epithelial uptake of butyrate in the rumen epithelium is likely under the control of moncarboxylate transporters (28, 43, 44). The first step of intraepithelial metabolism encompasses the conversion of SCFAs to acetyl-CoA, thought to be orchestrated by a family of short-chain acyl-CoA synthetases (4). Acetoacetyl-CoA thiolase (*ACAT*) and HMG-CoA synthase (*HMGCS*) convert acetyl-CoA to 3-hydroxy, 3-methylglutaryl CoA (HMG-CoA), a central metabolite of the rumen epithelium (5). Exclusively in the mitochondria, HMG-CoA can be converted by a HMG-CoA lyase (*HMGCL*) to synthesize the ketone bodies acetoacetate and β-hydroxybutyrate (BHBA) (5, 72). In the fed state the rumen epithelium synthesizes more ketone bodies compared with the liver, thereby providing energy to peripheral tissues (49).

In addition to ketogenesis, HMG-CoA may also proceed to cholesterol biosynthesis (16), which is a pathway that has not been characterized in the rumen epithelium. Although cholesterol is an essential component of mammalian cell membranes, hypercholesterolemia is associated with altered membrane permeability, inflammation, and increased cellular proliferation and migration (51). Interestingly, increasing dietary grain has been associated with rumen epithelial permeability, inflammation (26, 41) and proliferation (25). Since the amount of available substrate (SCFAs) for intraepithelial metabolism increases during a grain challenge, it is possible that genes involved SCFA metabolism are regulated to maintain cellular and whole animal homeostasis.

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The first objective of this study was to develop and characterize a nutritional model for inducing SARA. The second objective was to temporally characterize mRNA expression of genes involved in SCFA metabolism at different stages of the grain challenge using a combination of microarray, pathway analysis and quantitative real-time reverse-transcription polymerase chain reaction (qPCR). We hypothesized that mRNA expression of enzymes involved in SCFA metabolism are substrate mediated and therefore would be upregulated during periods when cattle were fed elevated levels of grain.

MATERIALS AND METHODS

Ruminal Acidosis Study: Animals and Management

Prior to this study, it was determined from ruminal pH data that a minimum of four biological replicates were required to detect a 30% difference with an 80% statistical power (9). Therefore, four mature, nonlactating Holstein dairy cattle (760 ± 30 kg, mean ± SD) were utilized for this study. All protocols were approved by the University of Guelph Animal Care Committee in accordance with the Canadian Council on Animal Care. Several years prior to the experiment, the cattle were fitted with rumen cannulas (20). For the months preceding the study, the cattle consumed a high-forage (HF) diet consisting exclusively of chopped hay (90.6% dry matter, 114 g crude protein/kg dry matter, 600 g neutral detergent fiber/kg dry matter, 173 g nonfiber carbohydrate/kg dry matter, 69 g starch/kg dry matter).

Throughout the experimental protocol, the cattle were housed in a tie-stall facility and exercised in an outside lot twice per week. All cattle were provided free-choice access to water and a commercial mineral and vitamin premix was supplemented each day at 0.02% of body weight during the experiment. The experiment was conducted over 7 wk, during which time the animals were fed either the HF diet or a HG diet (35% chopped hay and 65% mixed grain: 88.9% dry matter, 117 g crude protein/kg dry matter, 307 g neutral detergent fiber/kg dry matter, 504 g nonfiber carbohydrate/kg dry matter, 409 g starch/kg dry matter) (Fig. 1). The chopped hay portion of the diet was fed in equal allotments daily at 0800 and 1600. The mixed grain was in pelleted form and consisted of 40% ground wheat, 40% ground barley, and 20% ground corn. To induce ruminal acidosis, the mixed grain was fed at 0800, 1200, and 1600 based upon previously developed nutritional models developed in our laboratory (40). Both diets were fed at 1.4% body weight (HF) and 1.7% body weight (HG) to minimize feed refusals, and both met the metabolizable energy and protein requirements according to the National Research Council (NRC, 2001) (60) for nonlactating cattle. A baseline measurement was taken at the end of *week 0* (Fig. 1) before the transition to the HG diet in gradual increments over 4 days. The HG diet was fed until the end of experimental *week 3* (HG period). After the HG period, the cattle were switched back to the original HF diet and monitored for 3 additional wk (HF period). This feeding regimen was designed to promote the adaption of the rumen to HG and HF diets.

Physiological Measurements

Ruminal pH was measured every minute of the last 2 days of each experimental week with a pH recording system and methodology described previously by our research group (3). The pH electrodes were calibrated every experimental week. To temporally characterize how ruminal SCFA, plasma BHBA, and plasma nonesterified fatty acid (NEFA) concentrations respond to shifts in dietary grain, ruminal fluid samples, and blood samples were collected at 1900 on the last day of each experimental week via the cannula and jugular vein, respectively. Rumen fluid samples were immediately frozen at −80°C, and the blood was put on ice for 10 min, centrifuged at 3,000 *g* at 4°C for 15 min, and frozen at −80°C. The amount of SCFA in rumen fluid samples was quantified in duplicate by gas chromatography (59). The concentration of BHBA in plasma was determined in duplicate using an enzymatic kit (kit 310-A, Sigma) following previously described methods (84). Plasma NEFA concentrations were also determined in duplicate using a colorimetric assay (NEFA-C kit; Wako Chemicals, Richmond, VA) following previously described methodology (38).

Rumen Papillae Biopsies and RNA Collection and Quality Assessment

The ventral sac of the rumen was chosen for a biopsy site because it has the highest capillary blood flow per unit weight mucosa of any location within the rumen (82). Rumen papillae were biopsied from the ruminal ventral sac at the end of experimental *weeks 0, 1, 3, 4,* and *6* based upon previously developed methodology (39). In brief, the reticulorumen contents were partially evacuated to facilitate the retraction of the ventral sac. Rumen papillae were excised (150 mg) with surgical scissors each week from exclusive sites, washed 20 times in ice-cold PBS, snap-frozen in liquid nitrogen, and then stored at −80°C until RNA isolation.

Total RNA was isolated from ~100 mg of tissue using an RNeasy midi kit (Qiagen, Missisauga, Canada) and then treated with DNase (Invitrogen, Burlington, Canada). The quality was then assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and the RNA 6000 Nano kit (Caliper Life Sciences, Mountain View, CA). The RNA integrity number of samples taken for microarray and qPCR analysis was 9.88 ± 0.21 and 8.75 ± 0.20, respectively.

Microarray, Pathway, and Network Analysis

Microarrays were utilized to assess transcriptomic expression patterns of papillae from each cow ($n = 4$) during *weeks 1* and *3* of the HG period (8 microarrays in total). Hybridization of microarrays and data acquisition was carried out in the London Regional Genomics Facility (University of Western Ontario, London, Canada) using the 24K Affymetrix GeneChip Bovine Genome Arrays (Affymetrix, Santa Clara, CA). A complete description of the microarray hybridization protocol can be found in the Supplemental Material 1[1] and the

[1] The online version of this article contains supplemental material.

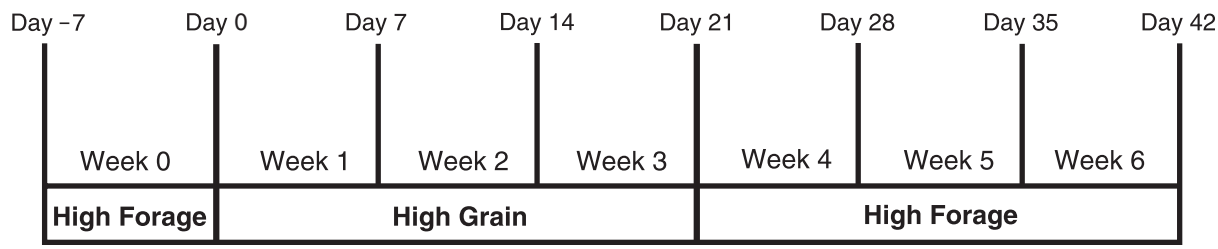

Fig. 1. Time-line of experimental feeding regimen.

Gene Expression Omnibus repository (GSE17849). The effect of dietary treatment on gene expression (relative mRNA content) was evaluated after subjecting the data to the GC Robust Multichip Analysis preprocessor in GeneSpring GX 7.3.1 (Agilent Technologies, Santa Clara, CA; http://www.home.agilent.com) at the University of Guelph Genomics Facility. Partial least squares methodology in the PLS procedure (85) of Statistical Analysis System (SAS) (75) was used to compare between weeks. A subset list of genes was compiled using a variance ratio measure (VIP) threshold of 0.8. The test statistic *P* values for the subset list of genes were adjusted for the number of comparisons using Benjamini and Hochberg's false discovery rate (FDR) following previously described methodology (79). Based upon the distribution of the data, the optimal FDR threshold to minimize the number of false positives was 0.08 (Supplemental Material 1, Fig. 1). Therefore, differences in relative expression between weeks were considered significant at an FDR-adjusted $P < 0.08$.

Pathway and network analysis of differentially expressed genes (DEG) was performed using Ingenuity Pathway Analysis (IPA) V7.5 (Ingenuity, Redwood City, CA; http://www.ingenuity.com/) software licensed by the University of Kentucky. The IPA knowledge base was used for the enrichment analysis of DEG and canonical pathway and network analysis statistical calculations were completed between experimental weeks. The significance value associated with pathways in IPA is a measure of the likelihood that the distribution of DEG was due to chance. Since our objective was to uncover which pathways are responsive in our dataset of DEG, Benjamini and Hochberg's FDR ($P < 0.05$) was employed. In addition, networks of DEG were generated based on their connectivity in IPA and a network score (negative log of *P* value) was assigned to each gene network based upon the hypergeometric distribution.

qPCR

To confirm microarray results of candidate genes discovered through IPA and to assess mRNA expression of rumen papillae once the cattle were switched back to the HF diet (*weeks 4* and *6*), qPCR was performed. After RNA isolation and quality assessment, samples (5 μg each) were reverse transcribed before iTaq SYBR Green (Bio-Rad Laboratories) qPCR analysis in triplicate using an ABI Prism 7000 (Applied Biosystems). When necessary, exon-spanning primers were designed for target genes using Primer Express 3.0 (Applied Biosystems) based upon bovine sequences listed on GenBank [National Center for Biotechnology Information (NCBI), Bethesda, MD]. The amplicons of all primers designed for this study were sequenced and verified using BLASTN in NCBI. The efficiency of qPCR amplification for each gene was calculated using the standard curve method with five dilutions ranging from 1:5 to 1:3,125. The sequences, R^2, standard curve slope, and primer efficiencies of primers are presented in the Supplemental Material 1 (Table 1). Dissociation curves were generated at the end of amplification to verify presence of a single product, and amplicons were validated by sequencing. Three common bovine housekeeping genes (*GAPD*, *ACTB*, and *B2M*) were tested prior to target gene quantification, and the Ct values were evaluated using Best-Keeper software (69). Since *GAPD* displayed a lower standard deviation (0.16, ± Ct) and coefficient of variation (0.80, %Ct) compared with the geometric mean of all three housekeeping genes it was used as the single internal control to validate the microarray results.

Statistical Analysis of Physiological and qPCR Measurements

Values from the 2 days of ruminal pH recordings were averaged for each experimental week before analysis. The mixed-model procedure of SAS (75) was used to account for the repeated measurements on each cow according to methods described elsewhere (83) and to contrast ruminal pH, ruminal SCFAs, and plasma BHBA data between experimental weeks and diets. The baseline week was subtracted from all weeks prior to analysis. The model included the fixed effect of week and the random effect of cow. Best fitting covariance structure for each variable was determined. Candidate genes uncovered from the microarray analysis (methodology described in previous section) were validated by qPCR. To analyze mRNA expression, normalized qPCR data were transformed to obtain a perfect mean of 1.0 at *week 0*. The abundance of genes was calculated using the inverse of qPCR efficiency raised to ΔCt (10). For the temporal gene expression analysis, normalized qPCR data were presented as fold-change relative to the first baseline HF measurement (*week 0*). Gene expression data also were analyzed by the same mixed-model procedure with repeated measures to determine significance. For all statistical analysis, significance was declared when $P < 0.05$.

RESULTS

Physiological Measurements: Ruminal pH, Ruminal SCFAs, and Plasma BHBA

As expected, cows fed the HF diet (*weeks 0, 4, 5, 6*) displayed steady ruminal pH traces with no hours below pH 5.6 and 6.0 (Fig. 2). In contrast, SARA was diagnosed during the first week of the HG period as ruminal pH was depressed below 5.6 for 4.6 ± 1.6 h daily and below 6.0 for 14.9 ± 1.1 h per day (Fig. 2). SARA was not diagnosed during *weeks 2* and *3* of the HG period and ruminal pH was below 6.0 only 4.8 ± 0.6 and 3.4 ± 1.0 h per day, respectively.

In agreement with continuous ruminal pH results, total ruminal SCFAs (acetate, propionate, isobutyrate, butyrate, isovalerate, valerate) and plasma BHBA concentrations were higher ($P < 0.01$) during the HG period compared with the HF period (Fig. 3, *A–C*). The highest concentrations of SCFAs occurred during *week 1* (99.9 ± 5.6 mM) of the HG period when SARA was diagnosed (Fig. 3*A*). The concentrations of total ruminal SCFAs decreased ($P < 0.01$) from *week 1* to *3* of the HG period, which corresponds with the elevation of ruminal pH. Values between baseline and all HF weeks did not

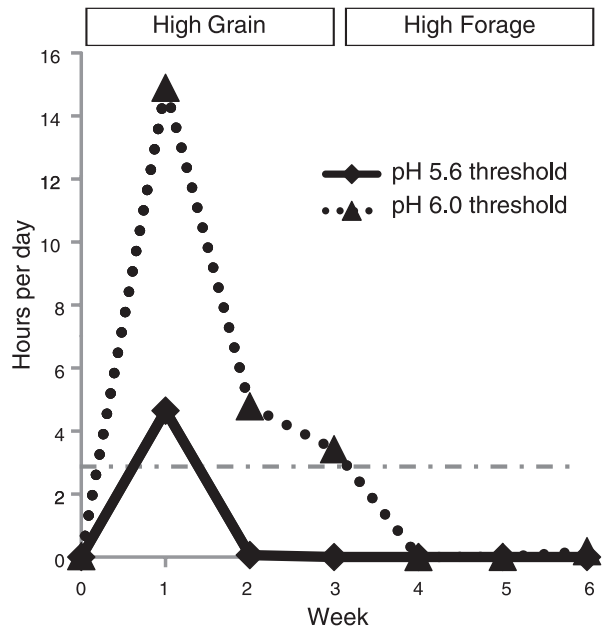

Fig. 2. Summary of daily continuous ruminal pH recordings taken during the last 2 days of every week. Data are presented as the mean ($n = 4$) for h/day below pH threshold 5.6 and 6.0 during each experimental week [*weeks 1, 2, 3*, high-grain (HG) diet; *weeks 4, 5, 6,* high-forage (HF) diet].

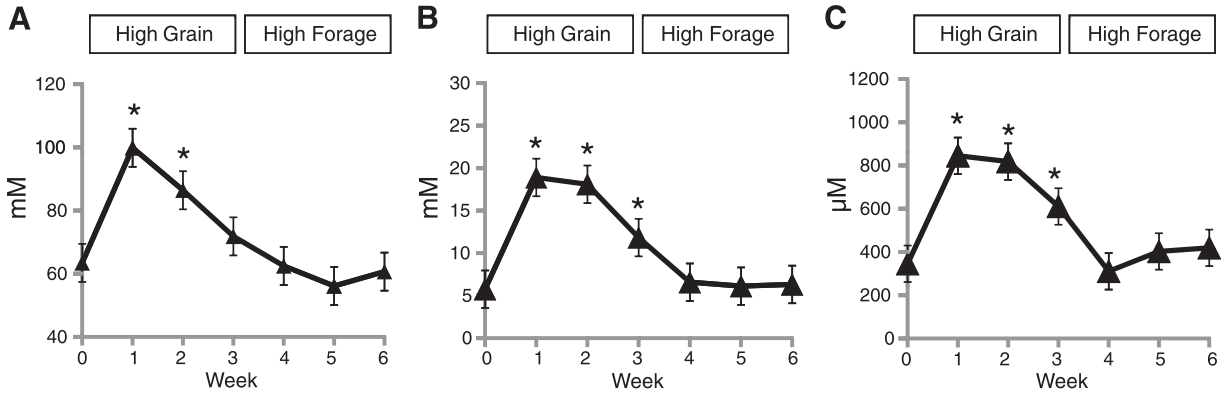

Fig. 3. Mean ($n = 4$) concentrations of total ruminal short chain fatty acids (SCFA, *A*), ruminal butyrate (*B*), and plasma beta-hydroxybutyrate concentrations (*C*) during experimental weeks (*weeks 1, 2, 3,* HG diet; *weeks 4, 5, 6,* HF diet). *Different from *week 0* baseline, $P < 0.05$. Pooled SE for ruminal SCFA concentration, 9.6 mM; ruminal butyrate concentration, 2.2 mM; plasma beta-hydroxybutyrate, 86 μM.

differ from one another (Fig. 3*A*). Of all six SCFAs analyzed, ruminal butyrate had the largest increase (3.3 times) from baseline to *week 1* before declining ($P < 0.01$) during the HG period but still remained elevated compared with baseline (2.1 times during *week 3*) (Fig. 3*B*). Consistent with the concept that ruminal butyrate is the major substrate for epithelial ketogenesis, plasma BHBA concentrations followed a similar pattern (Fig. 3*C*). Plasma BHBAs increased by 2.4 times during the first week of the HG diet. However, only one sample was >1,200 μM, which is the typical threshold for subclinical ketosis in dairy cattle (16). In contrast to BHBA, plasma NEFA concentrations were lower ($P < 0.05$) during the HG diet compared with the HF diet, yet no weeks were significantly different compared with *week 0* (Supplementary Materials 1, Fig. 2).

Microarray and Pathway Analysis

Since our ruminal pH and SCFA results indicate an adaptive response from the first to third week of the HG diet, our transcriptomic analysis was focused upon responsive pathways and networks between *weeks 1* and *3*. A total of 521 DEG were identified (247 upregulated, 274 downregulated; refer to Supplementary Material 2) and used to compare how epithelial mRNA expression is modified from the acute phase of a grain challenge (*week 1*) to the adapted phase (*week 3*). Of the 521 DEG, 376 could be mapped to a known molecular function for canonical pathway and network analysis using IPA.

Canonical pathway analysis revealed two significantly responsive pathways. The biosynthesis of steroids (cholesterol biosynthesis) was the most significant [$-\log(P \text{ value}) = 4.51$] with the highest ratio of 0.25 (ratio defined as the number of molecules meeting criteria by total number of molecules in a pathway). All of the DEG in the cholesterol biosynthesis pathway were downregulated from 1.5- to 2.8-fold. The second significant pathway uncovered was liver X receptor and retinoid X receptor (LXR/RXR) activation [$-\log(P \text{ value}) = 1.34$, ratio = 0.09], which included four upregulated and three downregulated genes. The complete list of the IPA canonical pathways and the expression values for all genes in the biosynthesis of steroids and LXR/RXR activation can be found in Supplemental Material 2.

A total of 22 networks were identified by IPA; 13 of these had a score [$-\log(P \text{ value})$] of 6 or greater with 8–25 "focus" genes (focus genes are defined as DEG that directly interact with other genes in the Ingenuity global molecular network). The first network had a score of 33 with 25 focus genes and revealed links between cell cycle, cancer, cellular growth, and proliferation. The second and third highest network scores involved lipid metabolism, small molecule biochemistry, and vitamin and mineral metabolism. The sterol regulatory element binding proteins (*SREBP1* and *SREBP2*) were dominant transcription factors in the second and third networks, which contained genes involved in cholesterol biosynthesis and LXR/RXR activation pathways. The entire list of significant networks is presented in Supplementary Material 2.

qPCR

Based upon the high ratio and the level of significance of the cholesterol biosynthesis pathway, the relative expression of *ACAT2*, *HMGCS1*, HMG-CoA reductase (*HMGCR*), farnesyl-PP synthase (*FDFT1*), farnesyl diphosphate synthase (*FDPS*) and lanosterol synthase (*LSS*) were validated by qPCR (Fig. 4). Collectively these genes represent the key branch points of cholesterol biosynthesis. *ACAT2* and *FDPS* were upregulated ($P < 0.05$) from the baseline to the first week of the HG diet and *HMGCS1* displayed a trend of upregulation ($P = 0.06$). During *week 3*, *HMGCS1*, *FDPS,* and *LSS* were all downregulated ($P < 0.05$) compared with baseline, and all genes shared a similar expression pattern of being downregulated ($P < 0.05$) 1.4- to 2.2-fold from *weeks 1* to *3*. The results of the microarray and qPCR analysis of genes expressed by *week 3* vs. *week 1* are presented together to illustrate their concurrence in the context of the affected biochemical pathways (see Fig. 5).

qPCR was also performed on RNA collected from papillae at the end of *week 4* and *6* to measure how the expression of genes responded once the diet was switched back to the HF diet after 3 wk of the HG diet (Fig. 4). When the cattle were returned to the HF diet, mean fold changes of cholesterolgenic genes approached baseline values and were not significantly ($P > 0.05$) different from the baseline (*week 0*) by *week 6*.

The expression of genes found in the LXR/RXR activation pathway, including low-density lipoprotein receptor (*LDLR*), ATP-binding cassette, subfamily A, member 1 (*ABCA1*), and *SREBP1* were also validated by qPCR. Both *LDLR* and *SREBP1* followed a similar expression pattern as cholesterol

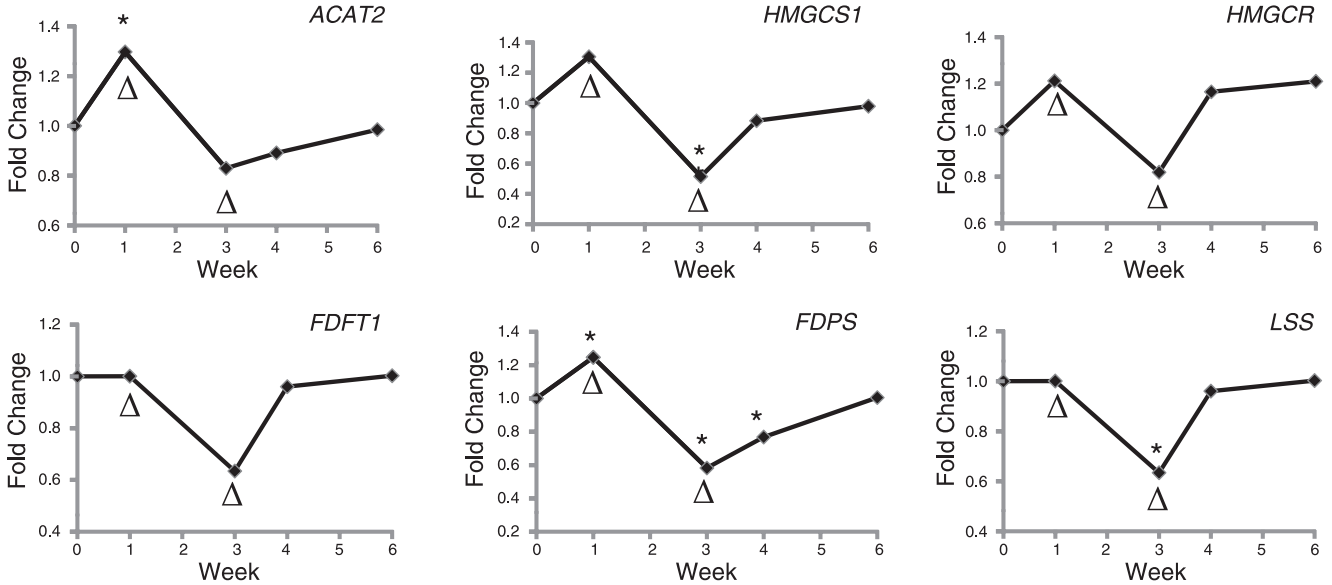

Fig. 4. Rumen papillae mRNA expression of acetyl-coenzyme A acetyltransferase 2 (*ACAT2*), 3-hydroxy-3-methylglutaryl-coenzyme A synthase 1 (*HMGCS1*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGCR*), farnesyl-diphosphate farnesyltransferase 1 (*FDFT1*), farnesyl diphosphate synthase (*FDPS*), and lanosterol synthase (*LSS*) using quantitative real-time PCR (qPCR) in rumen papillae throughout the characterization experiment (baseline, *week 0*; HG feeding, *weeks 1–3*; HF feeding, *weeks 4–6*). Values are means ($n = 4$) expressed as fold-change from the baseline measurement (*week 0*; HF), which was set to 1.0 (*different from *week 0* baseline, Δdifferences between *weeks 1* and *3*; $P < 0.05$). Pooled SE: *ACAT2*, 0.08; *HMGCS1*, 0.10; *HMGCR*, 0.18; *FDFT1*, 0.25; *FDPS*, 0.11; *LSS*, 0.12.

biosynthesis genes and were significantly downregulated ($P < 0.05$) from *week 1* to *week 3* by 1.89 ± 0.12-fold and 1.68 ± 0.11-fold, respectively (Supplementary Material 1, Fig. 3). In contrast, the expression of *ABCA1* increased 1.65 ± 0.10-fold from *week 1* to *3* of the HG diet (Supplementary Material 1, Fig. 3). The mean fold changes for these genes also approached baseline levels once the cattle were transitioned back to the HF diet and were not significantly ($P > 0.05$) different from the baseline (*week 0*) by *week 6*.

DISCUSSION

Physiological Results

The overall goal of this study was to gain insight into how the rumen epithelium adapts to the metabolic challenge produced by consumption of HG diets by identifying putative mechanisms responsible for these changes as evidenced by the alteration of mRNA expression profiles. Therefore, it was imperative to develop a nutritional model that induced a state of ruminal acidosis that could be mitigated using commercially relevant diets. This approach for studying SARA is novel as previous studies induced ruminal acidosis for 1 day (17, 18, 68) or took measurements after an HG diet had been fed for several weeks (67). In this study, SARA was established during the first week of the HG period, yet the cattle recovered during the second week of the HG diet. This experimental model is ideal for elucidating the mechanisms of rumen epithelial metabolic adaptations at different stages of a grain challenge.

Of the SCFAs, ruminal butyrate concentrations displayed the most striking increase, especially during the first week of the HG period. In a recent study, ruminal butyrate concentrations were measured in cattle during the first 4 days of a grain-induced acidosis and an approximate doubling of ruminal butyrate concentration was reported compared with control (13). Most HG vs. HF diet studies report a smaller increase in ruminal butyrate than what we found, presumably because samples were taken several weeks after adaption (67, 76). Our findings suggest that during the early onset of a grain challenge, the relative proportion of butyrate in the rumen fluid may be higher compared with after adaption. Elevated concentrations of butyrate during the early stage of the HG period agree with reports of higher states of rumen epithelial proliferation during the initial stage of a grain challenge (25). It has been shown in sheep and cattle that ruminal butyrate infusions can stimulate rumen epithelial proliferation (57, 74). However, cell culture studies report conflicting results (6, 61). Butyrate's direct and indirect effects on the expression of genes controlling the cell cycle in colonic epithelial cell models have been well characterized, yet the results are also conflicting therefore the exact mechanisms responsible for this regulation remain elusive (29).

It has been proposed that increasing intraepithelial uptake and metabolism is one potential mechanism for mitigating ruminal acidosis (21, 68). Greater intraepithelial metabolism could increase the concentration gradient between the lumen and the cytoplasm, thereby elevating the uptake of SCFAs to decrease ruminal pH (21). In this study, ruminal butyrate and plasma BHBA concentrations were higher ($P < 0.01$) during the HG diet vs. the HF diet. Because the cattle were on a high plane of nutrition during the HG period (1.7× metabolizable energy requirements; NRC), it is reasonable to assume that hepatic ketogenesis would be negligible (49). In addition, NEFA levels were lower during the HG period; therefore, most blood BHBA would be derived from synthesis by the rumen epithelium, yet plasma BHBA still remained below threshold values of subclinical ketonemia (19). Interestingly, ruminal butyrate and plasma BHBA declined ($P < 0.01$) from the first to the third week of the grain challenge. These results suggest

that the increase in ruminal SCFA caused a substrate-mediated equilibrium shift to increased production of BHBA.

Rumen Papillae Gene Expression

The central focus of this characterization study was to test the hypothesis that mRNA expression of pathways involved in SCFA metabolism, in particular ketogenesis and cholesterol biosynthesis, are upregulated in rumen papillae when cattle are fed an HG diet. To ensure candidate pathways were responsive at the mRNA level, a transcriptomic approach was employed before validation of genes of interest by qPCR.

Ketogenesis. The molecular control of ketogenesis by *HMGCS2* has been extensively characterized in the liver and intestine (33, 55), and its mRNA expression is highly correlated with ruminal ketogenesis during rumen development in lambs (15, 48). *HMGCS2* can be regulated by succinylation in the short-term (71) and at the transcriptional level in the long-term (55). The promoter region of the *HMGCS2* gene contains a peroxisome proliferator response element, which is under the transcriptional regulation of peroxisome proliferator-activated receptor alpha (*PPARα*) (56). It is well established that SCFA can influence the expression of genes under the control of PPARs in intestinal tissue (42); therefore, it is possible that a shift in ruminal SCFA during a grain challenge would influence ketogenic gene expression.

In this study the changes in total rumen epithelial BHBA production were not reflected in the differential expression of ketogenic enzymes and *PPARα* in our microarray analysis. We also validated the lack of differential expression ($P > 0.05$) between weeks and diets of ketogenic enzymes (*HMGCS2* and *HMGCL*) by qPCR (Supplementary Materials 1, Fig. 3). These results are in agreement with a recent study that detected no differences between mRNA expression of ketogenic enzymes in the rumen epithelium between cattle fed HG and HF diets (67). Moreover, Harmon et al. (31) detected no differences in the net production of ketone bodies from butyrate and acetate in rumen tissue slices collected from cattle fed HG and HF diets. When these results are considered, it can be assumed that the increase in BHBA production during the HG diet was based upon increased substrate (i.e., SCFA) and not the differential expression and thus activity of enzymes controlling ketogenesis.

Cholesterol homeostasis. While cholesterol is an essential component of mammalian cell membranes, excess cellular cholesterol has been shown to elicit pleiotropic effects in a variety of cell models (51). First and foremost, increasing cellular cholesterol and its metabolites (isoprenoids) is an integral component of initiating the inflammatory response (65), which is a documented response of the rumen epithelium during grain-induced acidosis (70). Secondly, alterations in the levels of isoprenoid intermediates can trigger cellular proliferation, which is also an observed response in the rumen epithelium when dietary grain is increased (25). Furthermore, isoprenoid intermediates are also linked to oxidative stress (45), an event hypothesized to occur in the rumen epithelium during grain feeding (21). Finally, cholesterol is an essential part of cellular membranes and its cellular content can influence permeability (23, 77).

To maintain precise control of cholesterol homeostasis, the synthesis, efflux, and influx of cellular cholesterol are under precise control in mammalian cells. Among homeostatic mechanisms, cholesterol biosynthesis has been extensively characterized; however, it has not been investigated in the rumen epithelium prior to this study. The cholesterol biosynthesis pathway (Fig. 5) occurs in the cytoplasm and is initiated when HMG-CoA is catalyzed by *HMGCR* to mevalonate (commonly termed the mevalonate pathway) (14). *HMGCR* is the rate-limiting enzyme of cholesterol biosynthesis and is considered to be one of the most highly regulated enzymes in nature (24). Mevalonate is then converted to isoprenoid intermediates such as geranylgeranylpyrophosphate and farnesylpyrophosphate (FPP) by *IDI* and *FDFT1*. Isoprenoid intermediates induce cellular proliferation, migration, and oxidative stress by permitting the attachment, subcellular localization and intracellular trafficking of membrane associated signaling proteins (45). The final branch point of cholesterol biosynthesis encompasses FPP being catalyzed to squalene by *FDPS* commonly referred to as squalene synthase. Squalene is then converted to lanosterol, and after a series of reactions is converted to cholesterol (51).

In this study, ruminal SCFAs were elevated during the HG period; therefore, more substrate was available for cholesterol biosynthesis in the rumen epithelium. Because elevated intracellular cholesterol can cause potent pleiotropic effects described above, its biosynthesis must be tightly regulated. It has been shown that cholesterol synthesis in the rat liver and intestine can be suppressed through feeding higher levels of SCFAs (30). In a recent in vitro study, increased SCFAs in intestinal cell media downregulated nine key genes involved in intestinal cholesterol biosynthesis (1). The downregulation of cholesterol biosynthesis genes in this study did not occur until *week 3* of the HG diet. During the first week of the HG period, it can be hypothesized that intracellular cholesterol levels were elevated, thus causing abnormalities in cholesterol homeostasis and possibly inflammation. This hypothesis is in agreement with histological examination of papillae (described in Supplemental Material 1; histology results), which detected higher lesion scores in *week 1* papillae compared with baseline. Therefore, the downregulation of cholesterol biosynthesis genes from the first to third weeks of the HG diet may be a mechanism for compensating for the increased amount of available substrate, which would otherwise synthesize sterols at concentrations that may be associated with an inflammatory response, oxidative stress, proliferation, and alter membrane permeability leading to tissue damage.

Microarray and qPCR results from this study indicate a coordinated downregulation of genes during the HG period yet return to baseline levels when cattle were switched back to the HF diet (Fig. 4). These expression patterns suggest that enzymes involved in cholesterol biosynthesis are responsive to the level of substrate and the stage of ruminal adaptation. Furthermore, the coordinated regulation of this pathway may be controlled by the same transcriptional factor. From the IPA network analysis, it became evident that all DEG in the cholesterol biosynthesis pathway are controlled by *SREBP* transcriptional factors (Fig. 5).

The *SREBP* family of transcription factors are synthesized as inactive precursors bound to the endoplasmic reticulum (ER), known for regulating fat synthesis and cholesterol biosynthesis (35, 36). Recent evidence suggests that *SREBPs* control cholesterolgenic and lipogenic gene expression in the bovine liver

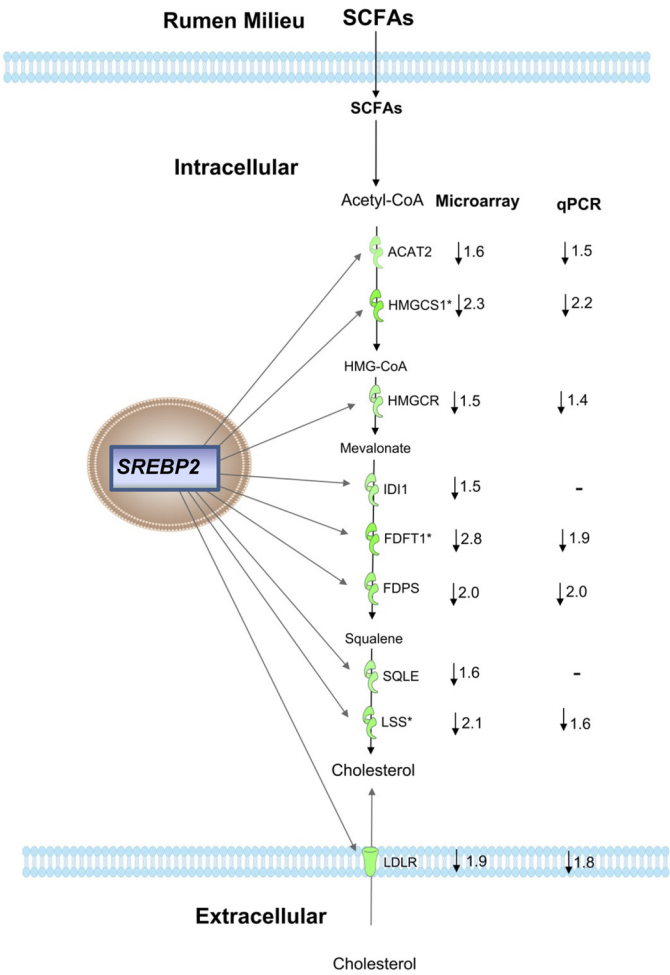

Fig. 5. Illustrative comparison of microarray and quantitative real-time reverse-transcription PCR (qPCR) analyses of the relative difference ($P < 0.05$) in the mRNA content of genes for proteins involved in cholesterol homeostasis isolated from rumen papillae of mature cows ($n = 4$) fed an HG diet for 3 wk vs. 1 wk. Green symbols denote decreased mRNA content, whereas "-" denotes not assayed. Illustration adapted from canonical pathway and network outputs from Ingenuity Pathway Analysis (IPA).

and mammary gland, respectively (32, 52, 81). The bovine genome encodes three *SREBP* isoforms designated *SREBP1a, SREBP1c,* and *SREBP2*. Of these isoforms, *SREBP2* preferentially activates cholesterol biosynthesis (14), including all genes of this pathway in this study. Activation of *SREBPs* requires proteolytic cleavage enabling transmigration of the transcription factor from the ER to the nucleus. Posttranscriptional regulation of *SREBPs* involves the sterol mediated suppression of *SREBP* cleavage on the ER, which blocks nuclear translocation (34, 35). The microarray results we validated by qPCR indicated no differential expression of *SREBP2*; therefore, it is likely that the control is at the posttranscriptional level. It can be speculated that during the unadapted phase of the HG period (*week 1*), the increase in substrate (SCFAs), in combination with increased mRNA expression of genes involved in cholesterol biosynthesis, may cause intracellular levels of cholesterol to increase. Upon adaptation, increased levels of sterols can suppress nuclear translocation of *SREBP2* and thus cholesterol biosynthesis gene expression. These results concur with in vivo models in which mice were administered poloxome 407 (P-407) to induce hypercholesterolemia. During the acute response (24 h after administration of P-407), liver *HMGCR* expression and activity increased yet reduced during prolonged administration (40 and 200 days), likely as an attempt to maintain cholesterol homeostasis or sterol balance (37, 50).

Cholesterol homeostasis is not only controlled by cholesterol biosynthesis via *SREBP2*, but also by the influx and efflux of cholesterol in the cell. The LXR/RXR heterodimer nuclear receptor family of transcription factors (second highest scoring canonical pathway) can influence the expression of immune genes in macrophages, yet it behaves as a sterol sensor and mediates the expression of genes responsible for cholesterol flux in hepatic and colon cells (73). Among candidate genes involved in cholesterol efflux, *ABCA1* is the most highly characterized gene target of LXR/RXR activation (58, 73, 77). *ABCA1* is a membrane associated protein that controls cholesterol efflux from cells, and its expression was upregulated from *week 1* to *3* of the HG diet, further supporting the concept that the rumen epithelium is mitigating an excess of cellular cholesterol during a grain challenge. Furthermore, the influx of cholesterol from low-density lipoproteins from the blood is mediated by *LDLR* (77), which shared a similar expression pattern to the cholesterol biosynthetic genes as it was downregulated from *week 1* to *3* of the grain challenge. Collectively, these mechanisms suggest that prolonged grain feeding requires the synchronized regulation of cholesterol homeostasis through the action of cellular biosynthesis, influx, and efflux via *SREBP2* and the *LXR/RXR* families of transcription factors.

Summary and Conclusions

In the past, the majority of studies investigating rumen epithelial adaptation to HG diets used tissue morphology and rumen metabolite clearance methodologies. These techniques advanced our understanding yet provided limited insight into potential molecular mechanisms controlling rumen epithelial metabolic adaptation to HG diets. Considering the lack of molecular-based research conducted with rumen tissue, the transcriptomic approach is the ideal starting point for elucidating metabolic gene mechanisms and networks.

Our findings during the first week of the grain challenge supported our hypothesis that genes involved in rumen epithelial SCFA metabolism were upregulated. However, a coordinated downregulation of cholesterol biosynthesis and influx genes occurred from the first to third weeks of the HG period, which corresponded with an increase in ruminal pH and decrease in ruminal SCFA and plasma BHBA. The gene expression changes, especially for cholesterol homeostasis, could be linked to well-documented symptoms of ruminal acidosis such as epithelial permeability, inflammation, and proliferation and should be a priority for further investigation. In conclusion, our findings reveal a gene expression signature centralized around the *SREBP2* and LXR/RXR transcriptional factors that may play an important role in the metabolic adaptation of the rumen epithelium to HG diets. Further understanding of the molecular adaptation of the rumen epithelium during grain challenges may aid in the development of technologies that can ultimately attenuate the detrimental impact that acidosis imposes on ruminants.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

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