Reticulo-rumen mass, epithelium gene expression, and systemic biomarkers of metabolism and inflammation in Holstein dairy cows fed a high-energy diet

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ABSTRACT

Feeding a higher-energy diet by increasing cereal grains at the expense of forage during the last 3 to 4 wk prepartum is a traditional approach to help the rumen "adapt" to the traditional diets fed at the onset of lactation. Increasing grain/concentrate in the diet changes ruminal fermentation and in sheep and goats elicits marked changes in mRNA expression of immune-related genes in ruminal epithelium. Whether such changes at the epithelial and systemic levels occur in dairy cows when the dietary energy content increases at a fixed level of concentrate is unknown. Fourteen non-pregnant, nonlactating Holstein cows were fed a control lower-energy (CON, 1.30 Mcal/kg of dry matter) diet to meet 100% of estimated nutrient requirements for 3 wk, after which half of the cows were assigned to a higher-energy diet (OVE, 1.60 Mcal/kg of dry matter) and half of the cows continued on CON for 6 wk. Levels of forage and concentrate for CON and OVE were 80 and 79% and 20 and 21%, respectively. Plasma samples were collected 1 d before slaughter to examine biomarkers of metabolism, liver function, inflammation, and oxidative stress. The reticulo-rumen mass was recorded at slaughter, and samples of epithelium were harvested from all cows. The expression of 29 genes associated with tight junctions, immune function, and nutrient transport (volatile fatty acids, urea, and trace minerals) was examined. Overfeeding energy led to consistently greater dry matter intake over time, and lowered plasma concentrations of haptoglobin, paraoxonase, bilirubin, fatty acids, and myeloperoxidase (secreted by neutrophils). In contrast, OVE resulted in greater hydroxybutyrate and cholesterol concentrations. A greater reticulo-rumen mass in cows fed OVE did not alter genes associated with tight junctions (*CDLN1, CDNL4, OCLN, TJP1*), immune function (*IL1B, IL10, NFKB1, TLR2, TLR4, TNF*), oxidative stress (*SOD1, SOD2*), or most nutrient transporters. However, feeding OVE upregulated the acute-phase protein SAA3 by 3.5-fold and downregulated a volatile fatty acid transporter (*SLC16A1*) and a Fe and Cu transporter (*SLC11A2*). The lack of effect on mRNA expression along with lower plasma concentrations of inflammation biomarkers indicates that long-term intake of a higher-energy diet ad libitum was not detrimental to ruminal epithelium integrity. In that context, a protective function of SAA3 could be envisioned with a role in opsonizing gram-negative bacteria that produce endotoxins. The long-term control of volatile fatty acid absorption and trace minerals from the rumen in cows overfed energy does not seem to be controlled at the gene transcription level. The relevance of these findings to the nutritional management of pregnant dry cows merits further research.

Key words: nutrition, gene expression, ruminal epithelium

INTRODUCTION

The rumen contains microorganisms which under "normal" conditions ferment carbohydrates and protein from the feed to gas (CO_2 and CH_4), short-chain fatty acids, and ammonia. It is well established that ruminal epithelium expresses transporters for short-chain fatty acids that allow for efficient absorption (Penner et al., 2009, 2011). Recent data, however, have underscored that feeding incremental levels of cereal grains induces the production of toxic, inflammatory, and unnatural compounds in the rumen (Saleem et al., 2012). The degree to which the ruminal epithelium responds under such conditions is unclear. Evidence indicates that ruminal epithelium could play an "immune role" in the

context of preventing the translocation of commensal bacteria or its metabolites that could induce a systemic immune response (Trevisi et al., 2014).

To date, most studies of molecular mechanisms in ruminal epithelium have focused on the morphological and molecular adaptations induced by differences in total dietary concentrate (Penner et al., 2011; Minuti et al., 2015). However, there is a lack of research evaluating the molecular changes that occur in the rumen epithelium of cows when the level of dietary energy is increased while maintaining a similar level of dietary concentrate. Besides the focus on genes associated with energy metabolism and VFA transport under such dietary conditions, little information exists regarding the expression of transporters for trace minerals (Fe, Zn, Cu, and Mn) and immune-related genes. That information is important because increasing dietary energy content through the use of higher-fermentable feedstuffs can induce production of immune-related compounds that potentially could trigger a response by the ruminal epithelium (Trevisi et al., 2014).

The hypothesis in the present study was that increasing dietary energy content and intake through inclusion of rapidly fermentable carbohydrate while maintaining a similar level of concentrate would alter mRNA expression of genes associated with inflammation, permeability, immunity, and transport of trace minerals. Furthermore, changes in metabolism, immune, and inflammatory responses also would be altered in response to long-term energy overfeeding. Thus, transcriptome and systemic biomarker analyses were used to address the hypothesis and objectives.

MATERIALS AND METHODS

Animals and Treatments

All procedures were conducted under protocols approved by the University of Illinois Institutional Animal Care and Use Committee (protocol #12134). Fourteen nonlactating, nonpregnant multiparous (had completed 3.3 ± 0.8 lactations; mean $\pm$ SD) Holstein cows (initial BW = 717 ± 39 kg; initial BCS = 3.31 ± 0.14) were enrolled in the experiment and were fed for 3 wk a control diet (lower-energy, **CON**; NE_L = 1.30 Mcal/kg of DM) designed to meet 100% of NRC (2001) requirements. During this time, it was estimated that all cows consumed an average of $110 \pm 5\%$ of NRC requirements. At the end of the 3 wk, half of the cows were randomly assigned to a higher-energy diet (**OVE**; NE_L = 1.60 Mcal/kg of DM) and half of the cows continued on CON for 6 wk (Table 1). The diets fed as a TMR were designed to maintain a similar level of forage (~80%) and concentrate (~20%), while total

energy content was varied. Cows were offered feed once daily at 0600 h and had unlimited access to fresh water. Cows in CON were feed restricted to consume only 100% of NRC requirements, whereas cows in OVE had ad libitum access to feed. It was estimated that OVE cows consumed nutrients at ~180% of NRC requirements. Cows were housed in ventilated indoor pens (10 m × 15 m; photoperiod of 8 h light and 16 h dark) equipped with individual electronic transmission gates and transponders (American Calan, Northwood, NH) for access to feed. Each pen had 10 sand-bedded free stalls with at least 1 stall per cow.

Sample Collection

Blood samples were collected before the morning feeding from the coccygeal vein or artery the day before slaughter. Samples were collected into evacuated

Table 1. Ingredient and analyzed nutrient composition of the control (CON) and higher-energy (OVE) diet fed to dry and nonpregnant Holstein cows for a period of 6 wk

Item	Diet	
	CON	OVE
Ingredient, % of DM		
Alfalfa hay	2.00	5.97
Alfalfa silage	8.88	13.61
Ground shelled corn	4.04	12.56
Corn silage	33.21	54.08
Dicalcium phosphate	0.79	0.70
Limestone	0.82	0.84
Magnesium chloride	0.46	0.70
Magnesium oxide	0.40	0.38
Magnesium sulfate	0.99	1.05
Mineral-vitamin premix[1]	0.20	0.21
Salt	0.20	0.14
Soybean meal, 48% CP	11.56	4.35
Urea	0.20	0.19
Vitamin A[2]	0.01	0.01
Vitamin D[3]	0.01	0.01
Vitamin E[4]	0.26	0.24
Wheat straw	35.97	—
Whole cottonseeds	—	4.98
Total forage, % of DM	80.06	78.64
Total concentrate, % of DM	19.94	21.38
Chemical analysis		
NE_L,[5] Mcal/kg	1.30	1.60
CP, % of DM	14.08	14.45
ADF, % of DM	34.40	26.30
NDF, % of DM	50.40	38.30

[1]Contained a minimum of 5% Mg, 10% S, 7.5% K, 2.0% Fe, 3.0% Zn, 3.0% Mn, 5,000 mg/kg of Cu, 250 mg/kg of I, 40 mg/kg of Co, 150 mg/kg of Se, 2,200 IU/kg of vitamin A, 660 IU/kg of vitamin D_3, and 7,700 IU/kg of vitamin E.

[2]Contained 30,000 kIU/kg.

[3]Contained 5,009 kIU/kg.

[4]Contained 44,000 IU/kg.

[5]Calculated using the Dairy Cattle NRC (2001) model. Inputs were 8.5 kg of DMI for CON or 14.4 kg of DMI for OVE. A BW of 717 kg was the input for both diets.

tubes (Vacutainer, Becton Dickinson and Co., Franklin Lakes, NJ) containing clot activator or lithium heparin. After blood collection, tubes containing lithium heparin were placed on ice, whereas the tubes with clot activator were kept ~30 min at 21°C until centrifugation. Serum and plasma were obtained by centrifugation of clot activator and lithium heparin tubes, respectively, at 1,900 × g for 15 min at 4°C and frozen at −80°C until later analysis.

Cows were euthanized at the College of Veterinary Medicine diagnostic facilities (University of Illinois) via captive bolt stunning followed by exsanguination. Immediately after death, rumen tissue from the ventral blind sac was quickly excised and washed with sterile 0.01 M PBS (pH 6.8). The papillae were scraped to remove attached feed particles and rinsed 3 times to remove the nonadherent bacteria. Epithelium for extracting RNA was separated from the muscle layers and transferred into liquid N within 5 min of exsanguination and stored at −80°C.

Blood Analyses

Biomarkers were analyzed in lithium heparin samples at 37°C following the procedures previously described by Jacometo et al. (2015) in a clinical auto-analyzer (ILAB 600, Instrumentation Laboratory, Lexington, MA).

RNA Isolation and cDNA Synthesis

Total RNA was extracted from frozen rumen epithelial tissue (100–200 mg) using QIAZol Lysis Reagent with the miRNeasy Mini Kit (catalog no. 217004, Qiagen, Hilden, Germany). Tissue was completely homogenized in 1 mL of QIAZol Reagent in 2 mL of RNase/DNase Free tubes with an O-ring. One bead (catalog no. 69989, 5 mm, Qiagen) per tube was added using the Qiagen bead dispenser. The tubes were loaded into a semi-automated homogenizer and the samples homogenized twice for 30 s with a 1 min incubation time on ice. Subsequently, 200 µL of chloroform was added to each sample and placed at room temperature for 3 min after shaking vigorously for 15 s. The upper phase was transferred into a new collection tube without disturbing the mid and lower phase after centrifugation at 12,000 × g at 4°C for 15 min. Subsequently, 750 µL of 100% ethanol was added to each sample and mixed well. Total RNA plus miRNA extraction was performed following the procedure recommended by Qiagen. Eighty microliters of DNase I digestion mix (catalog no. 79254, Qiagen) was added to each column to remove genomic DNA. Lastly, 50 µL of RNase free water was added to each sample to elute total RNA. The

RNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). The purity of RNA was assessed by ratio of optical density at 260/280 nm ($OD_{260/280}$), which was above 2.0 for all samples. The RNA integrity was evaluated via electrophoretic analysis of 28S and 18S rRNA subunits using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). The average RNA integrity number value for samples was 7.2 ± 0.5. Total RNA yield averaged 1,179 ± 759 ng/µL (or 58.9 ± 38 µg of total RNA per sample).

Quantitative PCR, Design, and Evaluation of Primers

For quantitative PCR (**qPCR**), cDNA was synthesized using 100 ng of RNA, 1 µg of dT18 (Operon Biotechnologies, Huntsville, AL), 1 µL of 10 mmol/L dNTP mix (Invitrogen Corp., Carlsbad, CA), 1 µL of random primer p(dN)6 (Roche catalog no. 11 034 731 001, Roche Diagnostics GmbH, Mannheim, Germany), and 10 µL of DNase/RNase free water. The mixture was incubated at 65°C for 5 min and kept on ice for 3 min. A total of 6 µL of master mix composed of 4.5 µL of 5× first-strand buffer, 1 µL of 0.1 M dithiothreitol, 0.25 µL (50 U) of SuperScript III RT (Invitrogen Corp.), and 0.25 µL of RNase Inhibitor (10 U; Promega, Madison, WI) was added. The reaction was performed in an Eppendorf Mastercycler Gradient (Eppendorf, Hamburg, Germany) using the following temperature program: 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. The cDNA was then diluted 1:4 (vol:vol) with DNase/RNase free water.

Quantitative PCR was performed using 4 µL of diluted cDNA (dilution 1:4) combined with 6 µL of a mixture composed of 5 µL of 1 µL SYBR Green master mix (Applied Biosystems, Waltham, MA), 0.4 µL each of 10 µM forward and reverse primers, and 0.2 µL of DNase/RNase free water in a MicroAmp Optical 384-Well Reaction Plate (Applied Biosystems). Each sample was run in triplicate and a 6-point relative standard curve plus the nontemplate control was used (User Bulletin #2, Applied Biosystems). The reactions were performed in an ABI Prism 7900 HT SDS instrument (Applied Biosystems) using the following conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C (denaturation), and 1 min at 60°C (annealing and extension). The presence of a single PCR product was verified by the dissociation protocol using incremental temperatures to 95°C for 15 s plus 65°C for 15 s. Data were calculated with the 7900 HT Sequence Detection Systems Software (version 2.2.1, Applied Biosystems). The final data were normalized using the geometric mean of CKLF-like MARVEL transmembrane domain containing 6 (*CMTM6*), dystrobrevin binding protein 1

(*DBNDD2*), and DEAD (Asp-Glu-Ala-Asp) box polypeptide 54 (*DDX54*). These genes were previously validated in our laboratory for use with ruminal epithelium (Naeem et al., 2012).

Primer sequences for genes evaluated in this study are reported in Supplemental Table S1 (https://doi.org/10.3168/jds.2017-12866). Primers were designed using Primer Express 3.0 with minimum amplicon size of 80 bp (when possible amplicons of 100–150 bp were chosen) and limited 3′ G+C (Applied Biosystems). When possible, primers were designed to fall across exon–exon junctions. Primers were aligned against publicly available databases using BLASTN (https://blast.ncbi.nlm.nih.gov/Blast.cgi) at National Center for Biotechnology Information and the University of California Santa Cruz Cow (*Bos taurus*) Genome Browser gateway (https://genome.ucsc.edu/index.html). Prior to qPCR, primers were tested in a 20-µL PCR reaction using the same protocol described for qPCR except for the final dissociation protocol. For primer testing we used a universal reference cDNA (RNA mixture from 5 different bovine tissues) to ensure identification of desired genes. Five microliters of the PCR product was run in a 2% agarose gel stained with ethidium bromide (2 µL). The remaining 15 µL was cleaned using QIAquick PCR Purification Kit (Qiagen) and sequenced at the Core DNA Sequencing Facility of the Roy J. Carver Biotechnology Center at the University of Illinois, Urbana-Champaign. Only those primers that did not present primer-dimer, a single band at the expected size in the gel, and had the right amplification product (verified by sequencing; Supplemental Table S2; https://doi.org/10.3168/jds.2017-12866) were used for qPCR. The accuracy of a primer pair also was evaluated by the presence of a unique peak during the dissociation step at the end of qPCR. Efficiency of PCR amplification for each gene was calculated using the standard curve method [E = $10^{(-1/\text{slope})}$] (Supplemental Table S3; https://doi.org/10.3168/jds.2017-12866). Efficiency cutoff during qPCR was between 1.80 and 2.1.

Statistical Analysis

All data were analyzed with the PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC) with each animal as the experimental unit. After normalization with the geometric mean of the 3 internal control genes, the triplicate qPCR data for each gene were averaged and then $\log_2$ transformed before statistical analysis. For mRNA expression, reticulo-rumen mass, and blood biomarkers, the fixed effect in the model was diet, and cow within diet was designated as the random effect. For DMI data, the fixed effects in the model were diet, day on experiment, and diet × day. In this case a repeated statement was used, with first-order autoregressive [AR(1)] as the covariate structure used because it resulted in the lowest Bayesian information criterion. In both models the Kenward-Roger statement was used for computing the denominator degrees of freedom.

RESULTS AND DISCUSSION

Blood concentrations of biomarkers are reported in Table 2. The greater ($P < 0.05$) concentrations of cholesterol (3.90 vs. 2.64 mmol/L) and BHB (0.43 vs. 0.22 mmol/L) as well as lower ($P < 0.05$) fatty acids (0.07 vs. 0.17 mmol/L) in cows fed OVE agree with previous research reporting a positive energy balance in cows fed dietary energy in excess of requirements (Danicke et al., 2014). Regarding liver function, a greater ($P < 0.05$) concentration of aspartate transaminase (**AST**; 78.8 vs. 64.3 U/L) was observed, whereas bilirubin was lower ($P < 0.05$) in cows fed OVE (0.89 vs. 1.49 µg/mol). The higher level of AST and the greater cholesterol concentration (3.90 vs. 2.64 mmol/L) could be attributed to the greater liver mass (1.42 vs. 1.10 as % of BW; data not shown) in the OVE cows, and the associated increase in liver workload (Abeni et al., 2012) including lipoprotein metabolism and breakdown of heme (both important functions of the liver).

Inflammatory biomarkers in OVE cows were within the normal range, with lower concentrations of paraoxonase ($P = 0.05$; 92.1 vs. 113 U/mL; Bionaz et al., 2007) and haptoglobin ($P = 0.08$; 0.14 vs. 0.27 g/L; Bertoni and Trevisi, 2013). Although AST was greater than reference values for cows with optimal liver function (Bertoni and Trevisi, 2013), these data indicate that despite the greater workload of the liver induced by the marked overfeeding, cows did not experience a state of inflammation or oxidative stress. Furthermore, the lack of systemic inflammatory response based on biomarker analyses suggest that the greater energy intake did not induce detrimental effects on the ruminal epithelium. An increase in ruminal epithelium permeability could cause a systemic inflammatory response (Minuti et al., 2014).

Out of the 29 target genes evaluated (Table 3), only *IL6* was not expressed (i.e., cycle threshold value > 30), which agrees with our previous work with ruminal epithelium in peripartal Holstein cows (Minuti et al., 2015). The absence of differences in the expression of genes encoding for cell adhesion and tight-junction (**TJ**) proteins in cows fed OVE (*CDLN1*, *CDLN4*, *OCLN*, *TJP1*) indicates that the much greater level of dietary energy intake (Figure 1) did not impair the integrity of the ruminal epithelium. Tight junctions lo-

Table 2. Concentrations of biomarkers of metabolism, liver function, inflammation, and oxidative stress in plasma from dry and nonpregnant Holstein cows fed a control (CON; 1.30 Mcal/kg of DM) or higher-energy (OVE; 1.60 Mcal/kg of DM) diet for a period of 6 wk

Item	Diet		SEM	P-value
	CON	OVE		
Metabolism				
Glucose, mmol/L	4.52	4.69	0.09	0.20
Cholesterol, mmol/L	2.64	3.90	0.24	0.004
Fatty acids, mmol/L	0.17	0.07	0.02	0.002
BHB, mmol/L	0.22	0.43	0.03	0.0002
Urea, mmol/L	4.49	4.36	0.36	0.81
Liver function				
Aspartate aminotransferase, U/L	64.3	78.8	4.37	0.04
γ-Glutamyl transpeptidase, U/L	23.9	23.0	1.47	0.67
Bilirubin, μmol/L	1.49	0.89	0.13	0.005
Total protein, g/L	76.9	75.1	1.47	0.40
Inflammation				
Ceruloplasmin, μmol/L	3.30	3.16	0.28	0.73
Haptoglobin, g/L	0.27	0.14	0.07	0.08
Albumin, g/L	34.3	35.4	0.82	0.36
Globulin, g/L	42.6	39.7	1.77	0.27
Paraoxonase, U/mL	113	92.1	7.43	0.05
Myeloperoxidase, U/L	460	411	22	0.14
Oxidative stress				
Reactive oxygen metabolites, mg of H_2O_2/100 mL	14.0	14.0	0.71	0.98

cated in the middle layers of ruminal epithelium play a key role in maintaining permeability and preventing the translocation of LPS and other toxins (Penner et al., 2011). Feeding a 65% high-grain diet to goats decreased mRNA expression of *CDLN4*, *OCLN*, and *TJP1* and increased *CDLN1* in addition to causing profound morphological alterations of the ruminal epithelium, and a deterioration of ruminal function as a consequence of a sharp decrease in pH and a marked increase in ruminal LPS concentration (Liu et al., 2013). Although it was not feasible to measure ruminal parameters of digestion in the present study, the sustained increase in DMI throughout the whole experimental period for OVE cows (Figure 1) further supports the idea that there was no impairment of ruminal function.

Among the few genes that were affected by feeding OVE, a ~3.6-fold increase ($P = 0.07$) was observed in the mRNA expression for the acute-phase protein serum amyloid A3 (*SAA3*; Table 2). Although the precise biological function of SAA in ruminants is not clear, it could play a role in metabolism, inflammatory processes, and opsonization (Ceciliani et al., 2012). For instance, Reigstad et al. (2009) detected greater mRNA expression of *SAA3* in mouse colonic epithelial cells as a result of microbial colonization or cultivation of the cells with LPS, suggesting it could play a bactericidal role.

Although the reticulo-rumen mass was greater ($P < 0.05$; 15.5 vs. 13.3 kg) in OVE cows (Figure 1), ex-

pression of urea and VFA transporters (*SLC14A1* and *SLC16A3*) did not differ ($P > 0.10$) and feeding OVE led to lower ($P < 0.05$) mRNA expression of *SLC16A1*, a transporter catalyzing movement of lactate and pyruvate across plasma membranes. The lack of change in urea transporter expression agrees with the similar concentration of plasma urea (Table 2). However, the lower expression of *SLC16A1* seems unexpected given that OVE cows consumed an average of 8 kg more DM per day (Figure 1) and although not measured in the present study likely had greater rates of fermentation and production of VFA (Bauman et al., 1971; Sutton et al., 2003). The 2-fold greater BHB concentration in plasma of OVE cows supports the idea of greater VFA production (i.e., greater butyrate production likely accounted for the BHB response). It could be possible that abundance of SLC16A1 protein was affected by OVE but it was not measured in the present study.

Most previous studies addressing the expression of genes associated with VFA absorption in the ruminal epithelium have focused on the effect of higher diet fermentability (via the inclusion of concentrates) resulting in a greater production of VFA, changes in their proportions, and a possible decrease in ruminal pH. In the short term, the ruminal epithelium responds mainly through cellular functional adaptations associated with an increase in gene expression and activity of proteins involved in the transport of VFA (Schurmann et al., 2014). In the long term, however, the response appears

mostly related to morphological adaptations [e.g., hyperplasia and hypertrophy of the ruminal epithelium to increase the absorptive surface (Penner et al., 2011)].

Previous research studying the response in *SLC16A1* to higher dietary energy in dairy cows detected either no change (Steele et al., 2012; Schurmann et al., 2014) or an increase in expression (Kuzinski and Rontgen, 2011; Yan et al., 2014). Expression of this transporter seems to be sensitive to dietary concentrate level because Metzler-Zebeli et al. (2013) detected an increase in *SLC16A1* in growing goats only when dietary concentrate reached 60% of total diet, with expression being unaffected at 0% and 30% of dietary concentrate. In general, the studies reporting greater expression of *SLC16A1* fed a high proportion of concentrate resulting in a noticeable decrease in ruminal pH and were of short experimental duration (Kuzinski and Rontgen, 2011; Yan et al., 2014; Zebeli et al., 2015). The present study is the first to detect a lower expression of *SLC16A1* in cows chronically overfed energy; thus, we

speculate that this response represents an adaptive mechanism to the long-term feeding as suggested by Penner et al. (2011). The fact that cows overfed energy had a greater reticulo-rumen mass (Figure 1) could be taken as an indication of greater absorptive surface to efficiently handle production of VFA. Thus, this type of long-term adaptation would make it unnecessary for the epithelium to upregulate the *SLC16A1* transport system.

Regarding trace mineral transporters, to our knowledge, no studies have been published on their expression in the ruminal epithelium. Those that were the focus of the present study are involved in the inflammatory response (Gruys et al., 2005). No differences ($P > 0.10$) in the expression of the SLC30 and SLC39 Zn family of transporters or the SLC31A1 Cu transporter were observed. However, there was a lower expression ($P = 0.06$) in OVE cows of the divalent metal transporter *SLC11A2*. This gene is widely expressed in various tissues, especially in duodenal enterocytes, and plays a

Table 3. mRNA expression (log$_2$ back-transformed LSM) among genes in ruminal epithelium of dry and nonpregnant Holstein cows fed a control (CON; 1.30 Mcal/kg of DM) or higher-energy (OVE; 1.60 Mcal/kg of DM) diet for a period of 6 wk

Gene	Diet		SEM	*P*-value
	CON	OVE		
Tight junctions				
CDLN1	1.11	0.91	0.13	0.15
CDLN4	1.16	1.07	0.14	0.57
OCLN	0.98	0.89	0.11	0.38
TJP1	1.04	1.00	0.05	0.52
Immune function and inflammation				
CD45	0.88	1.09	0.15	0.17
IL1B	0.14	0.27	0.76	0.38
IL10	0.52	0.80	0.42	0.31
IRAK1	0.97	0.88	0.09	0.28
NFKB1	1.07	0.95	0.10	0.27
NFKBIA	0.95	0.91	0.07	0.47
SAA3	0.21	0.75	0.66	0.07
STAT3	0.87	0.94	0.07	0.24
TLR2	0.94	1.00	0.19	0.19
TLR4	0.95	1.08	0.10	0.21
TNF	0.93	1.03	0.15	0.47
TOLLIP	0.81	0.79	0.08	0.75
Oxidative stress				
SOD1	0.99	0.94	0.10	0.62
SOD2	0.82	0.87	0.08	0.52
Monocarboxylate and urea transport				
SLC14A1	0.78	0.69	0.13	0.35
SLC16A1	0.83	0.69	0.08	0.04
SLC16A3	0.90	0.92	0.08	0.82
Trace mineral transport				
SLC11A2	0.96	0.81	0.09	0.06
SLC30A1	0.90	0.79	0.20	0.50
SLC30A10	0.96	0.85	0.35	0.74
SLC31A1	0.98	0.88	0.15	0.49
SLC39A1	1.04	1.00	0.08	0.58
SLC39A3	1.08	0.97	0.13	0.41
Hydroxycarboxylic acid receptor				
HCAR2	0.86	0.92	0.32	0.85

crucial role in the absorption of Fe and other divalent transition metals (Garrick et al., 2003). Iron is an essential element for many vital functions such as oxidative metabolism, erythropoietic function, and immune cell response. However, in excess Fe is toxic; hence, its circulating levels need to be maintained at a very narrow range. As there is no active excretion mechanism of Fe, its homeostasis depends on the recycling in erythrocyte hemoglobin and intestinal absorption.

The present study underscores the potential contribution of ruminal epithelium (along with intestinal absorption) in the maintenance of Fe homeostasis. The lower expression of this gene due to feeding OVE is consistent with the work of Hansen et al. (2010) who

Figure 1. Weekly DMI (top panel) and reticulo-rumen mass (bottom panel) at slaughter in dry and nonpregnant Holstein cows fed a control (CON; 1.30 Mcal/kg of DM) or higher-energy (OVE; 1.60 Mcal/kg of DM) diet for a period of 6 wk. *Diet × time, $P < 0.05$; [a,b]Means differ, $P < 0.05$. Values are means, with SE represented by vertical bars.

detected a trend for lower expression of the divalent transporter 1 but a lack of change in mRNA in duodenal tissue of calves fed higher levels of Fe. Therefore, the present data indicate a possible excess of Fe intake as a result of the overfeeding, which elicited a decrease in its absorption to maintain adequate levels. The well-documented interaction between Fe absorption with other metals, mainly Cu, Zn, and Mn, should also be kept in mind (Arredondo et al., 2003; Hansen et al., 2010). Therefore, the interaction between Fe and Cu could be one of the causes for the observed difference in expression of *SLC11A2*. Both SLC11A2 and SLC31A1 are Cu-specific transporters that play important roles in absorption as shown by the work of Arredondo et al. (2003) in which culturing Caco-2 cells with increasing concentrations of Cu decreased Fe uptake. Therefore, Fe can interfere with the absorption of Cu in 2 ways (i.e., by decreasing *SLC11A2* gene expression and competing for the transport protein).

Most studies that have examined the effect of supplementation with Fe on Cu status have been performed with high concentrations of Fe, but Bremner et al. (1987) observed a decrease in the amount of hepatic Cu concentration in calves only when supplemented with 250 mg of Fe/kg of DM in the diet. In the present study, the calculated concentrations of Fe in the CON and OVE diets, taking only into account the mineral premix and dicalcium phosphate [~10,000 mg of Fe/kg (Kerr et al., 2008)] was ~120 mg Fe/kg of DM, which does not seem excessively high. However, the average calculated daily intakes were 952 and 1,851 mg/d for CON and OVE, which far exceed current recommendations (NRC, 2001), especially in the OVE treatment. In addition, the contributions of Fe-rich feeds like alfalfa were not taken into account. Another indirect sign that could be indicative of an interference in Cu absorption in OVE cows is the 22% lower ($P < 0.05$) concentration of plasma paraoxonase.

We speculate a biological link between Cu and paraoxonase in OVE cows based on data from rats, where animals fed a diet deficient in Cu (not in Zn) had very low liver Cu concentration and a 22% decrease in paraoxonase concentration (Klevay, 2004). It is noteworthy that ceruloplasmin concentrations in plasma, an indicator of Cu status (Blakley and Hamilton, 1985), were within a normal range and unaffected ($P = 0.73$) in OVE cows. Thus, although this response supports the view of an absence of inflammation (Gruys et al., 2005), it does not seem to support the idea that Cu status was affected by feeding OVE.

In conclusion, the lack of effect on the mRNA expression of key genes related to TJ, inflammation, and oxidative stress along with a lower plasma concentration of inflammation biomarkers indicates that long-term
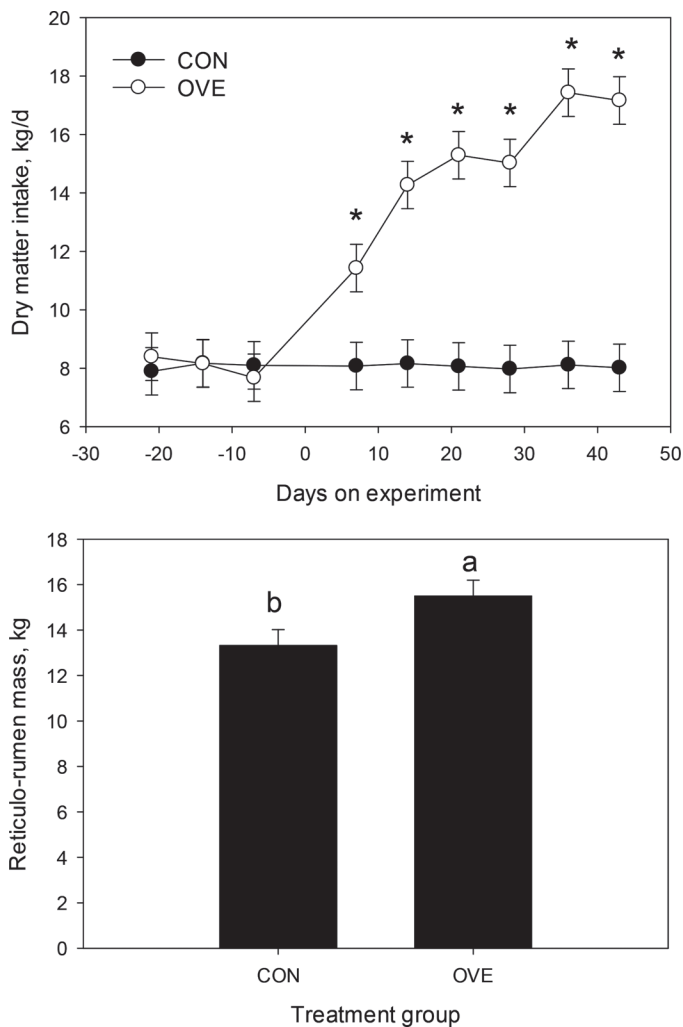
consumption of higher-energy diets ad libitum was not detrimental to the integrity of ruminal epithelium. The marked increase in DMI over time and the greater plasma concentrations of BHB when cows were overfed energy indicate normal rumen function, absorption, and metabolism of VFA. The long-term control of VFA and trace mineral absorption from the rumen in cows chronically overfed energy does not seem to be controlled at the gene transcription level. The relevance of these findings to the nutritional management of pregnant dry cows merits further research.

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