Effects of feeding a calf starter on molecular adaptations in the ruminal epithelium and liver of Holstein dairy calves

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ABSTRACT

The objective of this study was to elucidate the effect of feeding a calf starter on the volatile fatty acid (VFA) profile in the rumen and on expression of genes involved in epithelial intracellular pH regulation, butyrate metabolism, and hepatic urea cycle during the weaning transition. Twenty Holstein bull calves were fed either milk replacer and hay (MR) or milk replacer, hay, and a commercial texturized calf starter (MR+S) in a randomized complete block design. All calves were fed 750 g/d of milk replacer as the basal diet. Calves on the MR+S treatment were also fed starter *ad libitum*, and the energy intake of calves within blocks was maintained by supplementing the MR group with extra milk replacer that was equivalent to the energy intake from calf starter. Calves were killed 3 d after they consumed 680 g/d of calf starter for 3 consecutive days. Calves fed MR+S had higher VFA concentrations in the rumen (99.1 ± 8.1 vs. 64.6 ± 8.6 mM) and a higher molar proportion of butyrate (15.6 ± 1.7 vs. $7.9 \pm 1.9\%$) than calves fed MR. Relative abundance of mRNA for monocarboxylate transporter isoform 1 was higher (1.45 vs. 0.53), and that of Na^+/H^+ exchanger isoform 3 (0.37 vs. 0.82) and 3-hydroxy-3-methylglutaryl synthase isoform 1 (0.40 vs. 0.94) lower for the MR+S treatment compared with the MR treatment. In the liver, relative mRNA abundances of argininosuccinate synthetase isoform 1 (2.67 vs. 1.56), argininosuccinate lyase (1.44 vs. 0.99), and arginase isoform 1 (3.21 vs. 1.74) were greater for MR+S than for MR calves. Calf starter consumption appeared to increase fermentation in the rumen and affected expression of genes involved in cholesterol synthesis and intracellular pH regulation in ruminal epithelium, and those involved in urea cycle in the liver.

Key words: calf starter, rumen epithelium, gene expression

INTRODUCTION

Neonatal calves do not have a functioning rumen. When calves begin to consume solid feed, the weight of the rumen as a fraction of live weight increases (Walker and Walker, 1961). Fermentation of calf starter increases ruminal concentrations of VFA, especially propionate and butyrate (Warner et al., 1956; Stobo et al., 1966), which most potently stimulate papillae development in the rumen (Sander et al., 1959). To foster rumen development, it is recommended that calves maximize intake of easily fermentable calf starter (NRC, 2001), which promotes ruminal papillae development (Stobo et al., 1966). Rapid ruminal fermentation can decrease rumen pH, which is associated with detrimental health effects in mature ruminants (Nocek, 1997). However, feeding calf starter does not appear to affect rumen pH in dairy calves (Laarman and Oba, 2011), which may indicate that the rumen epithelium adapts to calf starter fermentation. The specific adaptations to increasing fermentation in the rumen, however, are not well understood.

The neonatal rumen epithelium relies heavily on glucose as a metabolic fuel, but the mature rumen epithelium derives most of its energy from VFA oxidation (Rémond et al., 1995), and it is around the time of weaning that major changes in preferred metabolic substrates occur (Baldwin and Jesse, 1992). Although the effects of calf starter fermentation on morphological development of the rumen are well accepted (Warner et al., 1956), effects of calf starter consumption on metabolic adaptations of ruminal epithelium are not well understood. Mature cows absorb 60 to 80% of all VFA and approximately 50% of protons across the rumen wall (Allen, 1997), and the VFA transport capacity needs to increase to accommodate greater fermentation in the rumen. The rumen epithelium does not only transport VFA, but also metabolizes some of it, especially butyrate. Between 75 and 90% of absorbed butyrate is metabolized in the ruminal epithelium, and 83% of metabolized butyrate is converted into ketone bodies (Rémond et al., 1995). The neonatal rumen, however,

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is nonketogenic (Lane et al., 2002); thus, the ketogenic capacity of the ruminal epithelium develops over time.

Furthermore, calf starter fermentation in the rumen increases feed protein degradation to ammonia, and absorbed ammonia needs to be detoxified via the hepatic urea cycle. During weaning in calves, urine urea concentrations have been shown to increase following increased concentrate consumption (Hugi and Blum, 1997), suggesting changes in urea cycle activity. Although age has shown to affect activity and mRNA abundance of hepatic enzymes involved in the urea cycle (Takagi et al., 2008), the effect of feeding calf starter on these genes is not well understood.

Good understanding of metabolic changes, including those at the molecular level, is important to develop proper nutritional management programs for calves during the weaning transition. However, the metabolic adaptations of the rumen epithelium and liver during the weaning transition have not been well investigated. Moreover, the role of diet, energy intake, and age in metabolic adaptations is unclear. The objective of this study was to examine expression of genes during the weaning transition and distinguish those primarily affected by consumption of calf starter and those primarily affected by age or energy intake. We hypothesized that the calf starter fermentation in the rumen would affect the expression of genes involved in butyrate metabolism and regulation of intracellular pH (pH_i) in ruminal epithelium and the urea cycle in the liver.

MATERIALS AND METHODS

Animals and Diets

All procedures for the animal study were approved by the Livestock Animal Care and Use Committee of the University of Alberta, using the guidelines of the Canadian Council for Animal Care (Ottawa, ON) and carried out as described previously (Laarman and Oba, 2011). Twenty Holstein bull calves were obtained in October 2008 ($n = 10$) and May 2009 ($n = 10$), from a commercial dairy farm, at 46.7 ± 1.0 kg of BW and 13.1 ± 0.4 d of age. Prior to arrival, calves were individually housed and given up to 4 L of whole milk daily but had no access to calf starter. After calves were moved to the Metabolic Unit at the University of Alberta Edmonton Research Station, all animals were housed in 3.05×3.66 -m individual pens with wood shavings for bedding. All animals had ad libitum access to water and chopped hay throughout the study. Calves obtained in October 2008 were fed chopped timothy hay (7.7% CP, 65.6% NDF), whereas calves obtained in May 2009 were fed chopped mixed legume hay (15.0% CP, 56.8% NDF). Calves were blocked into pairs using starting date of

the study and BW as blocking criteria. In each pair-fed block, one calf was assigned to milk replacer (MR) treatment and the other calf was assigned to milk replacer and starter (MR+S) treatment.

All calves received milk replacer (22% CP, 17% fat, and 45% lactose; High Performance Milk Replacer, Grober Nutrition, Cambridge, ON, Canada) at 750 g/d (12.5% wt/vol) as the basal diet. The MR+S calves were also offered a commercial texturized calf starter containing canola meal, soybean meal, wheat, barley, corn, peas, vitamins, and minerals with 23% CP and 4% ether extract (EE) as the guaranteed minimum (19.5% NDF, 27.1% starch; Wetaskiwin Co-Op Country Junction, Wetaskiwin, AB, Canada) ad libitum. The ME of the milk replacer and starter, respectively, were calculated using the formulas (NRC, 2001) below:

$$\text{ME} = 0.93 \times (0.057 \times \% \text{ CP} + 0.092 \times \% \text{ fat} + 0.0395 \times \% \text{ lactose}),$$

$$\text{ME} = [(1.01 \times \text{DE}) - 0.45] + [0.0046 \times (\% \text{ EE} - 3)],$$

$$\text{where digestible energy (DE)} = 0.7$$

$$\times [(0.057 \times \% \text{ CP}) + (0.092 \times \% \text{ EE}) + (0.0415 \times \% \text{ carbohydrates})],$$

$$\text{Carbohydrate content} = 100 - \text{EE} - \text{CP} - \text{ash}.$$

For the MR+S calves, starter intake was recorded daily, and ME intake from starter was calculated. The MR calves were fed additional milk replacer to supply ME equivalent to that of calves consuming starter in the same block. Therefore, calves within each block were expected to have similar ME intake, attenuating the confounding effect of energy intake within blocks on results. Milk replacer was offered twice daily in 2 equal portions at 0515 and 1515 h, and starter was offered once daily at 0515 h. All animals were weighed twice weekly and blood was sampled once weekly at 0930 h. Blood was collected from the jugular vein into a Vacutainer containing heparin (Becton Dickinson, Franklin Lakes, NJ) and immediately centrifuged at $3,000 \times g$ for 20 min and plasma was harvested and stored at -20°C until analysis.

When a calf on the MR+S treatment was ready to wean, as indicated by consumption of starter at 680 g/d for 3 consecutive days, a small ruminant rumen pH sensor system (Dascor, Escondido, CA) was orally dosed to a pair of calves (MR+S and MR calves), rumen pH was monitored continuously for 3 d (Laarman and Oba, 2011), and the calves were killed. The 3-d data collection period allowed for calculation of representative

average rumen pH accounting for daily variation while minimizing the risk of pH sensor failure. A plasma sample was taken as described above at 0930 h and then both calves within the same block were harvested at 1100 and 1200 h by stunning by captive bolt gun followed by exsanguination. The order of treatment at harvest was alternated each time; if an MR+S calf was killed first on one day, an MR calf was killed first on the next harvest day. A sample of liver tissue was taken from the caudate lobe of the liver and snap frozen in liquid nitrogen. Ruminal epithelial tissue was harvested from the ventral sac of the rumen; the epithelium was manually peeled off from the muscle layer, rinsed in PBS (pH 7.4), and snap-frozen in liquid nitrogen and kept on dry ice. Then, rumen contents were strained through a layer of porous material (Peetex, pore size = 355 μ m; Sefar Canada Inc., Scarborough, ON, Canada) and rumen fluid was aliquoted in a scintillation vial, snap frozen in liquid nitrogen, and kept on dry ice. Liver tissue, rumen tissue, and rumen fluid were kept at -80°C until analysis.

Sample Analysis

Calf starter was sampled at the arrival date of each group of calves, dried at 55°C for 48 h, ground through a 1-mm screen using a Wiley mill (Thomas-Wiley, Philadelphia, PA), and analyzed for chemical composition. Dry matter content was determined by drying samples at 135°C for 2 h (AOAC, 2002; method 930.15) and OM was analyzed following a 2-h combustion at 600°C (AOAC, 2002; method 942.05). Crude protein content was analyzed using flash combustion (TruSpec analyzer, Leco Corp., St. Joseph, MI; Wrolstad et al., 2004). Starch content was analyzed using an enzymatic method (Karkalas, 1985). Concentration of NDF was analyzed using heat-stable amylase and sodium sulfite as described previously (Van Soest et al., 1991). Ether extract content was analyzed using a Goldfish extractor (Labconco, Kansas City, MO; Wrolstad et al., 2004).

Plasma samples were analyzed for concentrations of BHBA, insulin, and glucose. Plasma BHBA was analyzed using a β -hydroxybutyrate dehydrogenase enzyme (Roche, Mannheim, Germany), and glucose was analyzed using a glucosidase/peroxidase enzyme reaction (Sigma-Aldrich Canada, Oakville, ON, Canada) as described previously (Penner et al., 2009). The BHBA and glucose assays were conducted using a plate reader (SpectraMax, Molecular Devices Inc., Sunnyvale, CA). Insulin concentration was analyzed using a commercial radioimmunoassay kit (Siemens Medical Diagnostics, Los Angeles, CA). The IGF-1 concentration was measured by competitive solid-phase immunoassay based on the method described by Sugino et al. (2004) using hu-

man IGF-1 (Novozymes Gropep, Adelaide, Australia), Eu-labeled human IGF-1, polystyrene microtiter strips coated with anti-rabbit γ -globulin, and anti-human IGF-1 rabbit serum (1:60,000; Novozymes Gropep). The IGF-1 was extracted from plasma by addition of 1 mL of 1 *N* acetic acid (pH 2.0) to 1 mL of plasma. Plasma protein was precipitated by addition of 4 mL of acetone. After centrifugation ($1,500 \times g$ for 20 min at 4°C), the supernatant was evaporated and resuspended in assay buffer (50 mM Tris-HCl, 140 mM NaCl, 0.5% BSA, 0.05% γ -globulins, 0.00078% diethylene-triamine-pentaacetic acid, 0.05% sodium azide, and 0.01% Tween 40, pH 7.8) with 10 kIU/mL of aprotinin. The mean recovery of IGF-1 from bovine plasma, confirmed by dose-response displacement of the IGF-1 tracer by the addition of bovine plasma, was 98.2%. Intra- and interassay CV were 6.9 and 5.5%, respectively, and the least detectable dose was 0.053 ng/mL.

Rumen fluid samples were thawed and centrifuged at $15,000 \times g$ at 4°C to obtain clear supernatant. Concentration of VFA was determined using isocaproic acid as an internal standard with gas chromatography (Varian, Walnut Creek, CA; Penner et al., 2009).

Real-Time PCR

The RNA was extracted using a Trizol-chloroform (Life Technologies, Burlington, ON, Canada) extraction method (Chomczynski and Sacchi, 1987). After the purity of the RNA was measured with a spectrophotometer (ND-1000, Nanodrop Technologies, Wilmington, DE), 1.6 μ g of RNA was treated with DNase (Life Technologies) and then reverse-transcribed using Superscript II (Life Technologies) and Oligo-d(T) (Life Technologies). Real-time PCR was performed using Taqman Fast Universal Master Mix (Life Technologies). Primers (Life Technologies) and probes (Life Technologies) were designed using Primer Express v.3.0 (Life Technologies; Table 1) and analyzed by BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) to verify primer specificity. In addition, reverse transcription PCR was performed to verify that each set of primers produced only 1 specific amplicon with the desired size. The target genes were 3-hydroxy-3-methylglutaryl-CoA synthase isoform 1 (*HMGCS-1*), 3-hydroxy-3-methylglutaryl-CoA lyase (*HMGCL*), β -hydroxybutyrate dehydrogenase, isoforms 1 (*BDH-1*) and 2 (*BDH-2*), all of which are enzymes involved in butyrate metabolism. The target genes involved in pH_i were monocarboxylate transporter isoform 1 (*MCT-1*), putative anion transporter isoform 1 (*PAT-1*), downregulated in adenoma (*DRA*), and Na^+/H^+ exchanger isoforms 1 (*NHE-1*), 2 (*NHE-2*), and 3 (*NHE-3*). In the liver, the target genes for the urea cycle were argininosuc-

Table 1. Gene names, primer and probe sequences, and accession numbers (National Center for Biotechnology Information) for genes used in real-time PCR analysis

Gene name	Primer and probe sequence	Amplicon size, bp	Accession number
Monocarboxylate transporter, isoform 1 (<i>MCT-1</i>)	Forward: ATC TAC GCG GGA TTC TTT GGA T Reverse: AAG GTC CAT CAG CGT TTC AAA C Probe: TGC TTT TGG GTG GCT C	72	NM_001037319
Sodium/proton exchanger, isoform 1 (<i>NHE-1</i>)	Forward: GAA AGA CAA GCT CAA CCG GTT T Reverse: GGA GCG CTC ACC GGC TAT Probe: AAG TAC GTG AAG AAG TGT CT	67	NM_174833
Sodium/proton exchanger, isoform 2 (<i>NHE-2</i>)	Forward: TTG TGC GAT GAC CAT GAA TAA GT Reverse: TGA TGG TCG TGT Probe: AGG ATT TCT GA	65	XM_604493
Sodium/proton exchanger, isoform 3 (<i>NHE-3</i>)	Forward: AGC CTT CGT GCT CCT GAC A Reverse: TGA CCC CTA TGG CCC TGT AC Probe: TGC TCT TCA TCT CCG	56	AJ131764
Downregulated in adenoma (<i>DRA</i>)	Forward: TGC ACA AAG GGC CAA GAA A Reverse: GCT GGC AAC CAA GAT GCT ATG Probe: TGC CTT CTC CTC CTT C	60	NM_001083676
Putative anion transporter, isoform 1 (<i>PAT-1</i>)	Forward: GGG CAC TTC TTC GAT GCT TCT Reverse: GTC GTG GAC CGA GGC AAA Probe: TCA CCA AGC AGC ACC T	57	BC_123616
3-Hydroxy-3-methylglutaryl-CoA synthase, isoform 1 (<i>HMGCS-1</i>)	Forward: AGG ATA CTC ATC ACT Reverse: TGG CCA ACT Probe: CAT GTT CCT TCG AAG AGG GAA T	67	AY581197
3-Hydroxy-3-methylglutaryl-CoA lyase (<i>HMGCL</i>)	Forward: TGC AGA TGG GAG TGA GTG TCA Reverse: GAC GCC CCC TGT GCA TAG Probe: TGG CAG GAC TGG GAG	73	NM_001075132
β -Hydroxybutyrate dehydrogenase, isoform 1 (<i>BDH-1</i>)	Forward: GAC TGC CAC CAC TCC CTA CAC Reverse: TCC GCA GCC ACC AGT AGT AGT Probe: CGC TAC CAT CCC ATG	59	NM_001034600
β -hydroxybutyrate dehydrogenase, isoform 2 (<i>BDH-2</i>)	Forward: CTG TGG CTT CCA GCA TCA AA Reverse: CGC CTT GGT TGT GCT GTA CA Probe: CGT TGT GAA CAG GTG C	59	NM_001034488
Carbamoyl phosphate synthetase, isoform 1 (<i>CPS-1</i>)	Forward: TCA ATG TCG TTC GCC ACT TG Reverse: TTG AGG TAG GAT GAA GGG CAA A Probe: ATT GTG GGT GAA TGC AA	69	XM_587645
Ornithine transcarbamoylase (<i>OTC</i>)	Forward: GCC AAG GAG AAC GGT ACC AA Reverse: CAC AAG CTG CTT CCA ATG GA Probe: CTG TCG CTG ACA AAT	58	NM_177487
Argininosuccinate synthase, isoform 1 (<i>ASS-1</i>)	Forward: GCC AAA GCC CCC AAC AG Reverse: GGA CCC CTT TCT TGA ACT CGA T Probe: CCG GAC ATG CTC G	55	NM_173892
Argininosuccinate lyase (<i>ASL</i>)	Forward: GTG GCC ACT GGT GTC ATC TCT Reverse: GAG CCC GTC CCA TGT TCT C Probe: CCT GCA GAT TCA CCG	58	NM_001034428
Arginase, isoform 1 (<i>ARG-1</i>)	Forward: GAT GCC TGA GGT CCC AGG AT Reverse: ACA ATG TCT TTG GCA GAT ATG CA Probe: CTA CTG GGT GGC TCC	60	NM_001046154
Glyceraldehyde-3-phosphate dehydrogenase (<i>GAPDH</i>)	Forward: TGC CGC CTG GAG AAA CC Reverse: CGC CTG CTT CAC CAC CTT Probe: CCA AGT ATG ATG AGA TCA A	57	NM_001034034
β -Actin (<i>ACTB</i>)	Forward: TCA CGG AGC GTG GCT ACA G Reverse: TTG ATG TCA CGG ACG ATT TCC Probe: CAC CAC CAC GGC CG	61	NM_173979
Ribosomal protein, large P0 (<i>RPLP0</i>)	Forward: AGG GCG TCC GCA ATG TT Reverse: CGA CGG TTG GGT AAC CAA TC Probe: CCA GCG TGT GCC TG	54	NM_001012682

cinase synthase isoform 1 (***ASS-1***), argininosuccinate lyase (***ASL***), arginase isoform 1 (***ARG-1***), carbamoyl phosphate synthetase isoform 1 (***CPS-1***) and ornithine transcarbamoylase (***OTC***). The program used for real-

time PCR (StepOnePlus Thermocycler, Life Technologies) was 40 cycles of 95°C for 1 s and 60°C for 20 s.

Abundance of mRNA for hepatic target genes was calculated using ribosomal protein large P0 (***RPLP0***)

as an internal control gene because of its stable expression. For ruminal target genes, as *RPLP0* was not sufficiently stably expressed, mRNA abundance was normalized to 3 reference genes: *RPLP0*, *GAPDH*, and β -actin as described previously (Vandesompele et al., 2002). Briefly, all samples from both treatments were analyzed on the same plate for one gene. For each sample, the cycle threshold (C_t) was calculated and then subtracted from the minimum C_t in the treatment. Then, the relative abundance of mRNA and the normalized mRNA abundance (Q_n) were calculated, respectively, by the formulas

$$Q = \text{Amplification efficiency}^{\Delta C_t}$$

$$Q_n = Q/NF,$$

where NF is the normalization factor, calculated as the geometric average of the Q values for *GAPDH*, β -actin, and *RPLP0* for each sample.

Statistics

Average daily gain was calculated via regression of weekly BW using PROC REG (v. 9.1; SAS Institute Inc., Cary, NC). Data were tested for normality using PROC GLM of SAS. Non-normally distributed data (i.e., BW at harvest) were analyzed by signed rank test. The other data were analyzed as randomized complete block design using PROC MIXED of SAS. Data were expressed as least squares means $\pm$ standard errors. The statistical model used for gene expression and VFA data was

$$Y_{ij} = \mu + \text{Block}_i + \text{Treatment}_j + e_{ij},$$

where Y_{ij} is a response variable, μ is the overall mean, Block_i is the effect of blocking by age and starting date, Treatment_j is the effect of MR or MR+S treatment, and e_{ij} is the residual error.

Hay intake was originally included as a covariate, but was found to have no effect on the outcome, so it was dropped from the model. For weekly blood plasma metabolites, the statistical model was

$$Y_{ijk} = \mu + \text{Block}_i + \text{Treatment}_j + \text{Week}_k \\ + \text{Treatment} \times \text{Week}_{jk} + e_{ijk},$$

where Y_{ijk} is a response variable, μ is the overall mean, Block_i is the effect of block by age and starting date, Treatment_j is the effect of MR or MR+S treatment, Week_k is the effect of week in age as a repeated measure, $\text{Treatment} \times \text{Week}_{jk}$ is the treatment by week

interaction, and e_{ijk} is the residual error. Significance was declared at $P < 0.05$ and tendency of treatment effects were declared at $P < 0.10$. Due to a recurring respiratory illness, one block of animals had to be removed from the trial and was not included in statistical analysis.

RESULTS

Growth and Blood Metabolites

Age of calves at slaughter did not differ between MR and MR+S (49.6 vs. 50.0 d; $P = 0.45$; Table 2). Average daily gain did not differ between MR and MR+S (0.80 vs. 0.86 kg/d; $P = 0.45$), and treatment had no effect on final BW ($P = 0.30$). Plasma insulin concentration was also not affected by treatment ($P = 0.23$), although plasma insulin increased between 6 and 8 wk of life, increasing from 25.2 to 92.2 $\mu\text{IU/mL}$ ($P < 0.01$; Figure 1). No treatment by time interaction existed for plasma insulin ($P = 0.16$). Plasma BHBA concentration was not affected by treatment ($P = 0.53$) or by time ($P = 0.65$; Figure 2), and we observed no treatment by time interaction ($P = 0.62$). Treatments did not differ in plasma glucose ($P = 0.38$), although plasma glucose increased between 6 and 8 wk of life ($P < 0.01$; Figure 3); no treatment by time interaction was found ($P = 0.86$). Plasma IGF-1 concentrations did not differ between treatments ($P = 0.40$; Figure 4), but differed with time ($P < 0.01$). At harvest, plasma glucose and plasma BHBA did not differ with treatment, but plasma insulin was higher in the MR calves (146.4 vs. 47.9 $\mu\text{IU/mL}$; $P = 0.02$; Table 3) than in the MR+S calves.

Rumen Fermentation

The MR+S treatment altered the VFA profile in the rumen fluid (Table 3). The MR+S calves had a lower molar proportion of acetate (57.6 vs. 69.3%; $P < 0.01$), a higher molar proportion of butyrate (15.6 vs. 7.9%; $P = 0.03$), and a higher total VFA concentration (99.1 vs. 64.6 mM; $P < 0.01$) than MR calves.

Rumen Epithelium and Liver Gene Expression

Abundance of mRNA was calculated as a relative value with arbitrary units. The mRNA abundance was higher for *MCT-1* (1.45 vs. 0.53; $P < 0.01$; Table 4) and lower for *NHE-3* (0.37 vs. 0.82; $P < 0.01$) and *HMGCS-1* (0.40 vs. 0.94; $P = 0.01$) in the MR+S calves compared with the MR calves (Table 4). Relative mRNA abundance of *DRA*, *NHE-1*, *NHE-2*, *HMGCL*, *BDH-1*, and *BDH-2* were not affected by treatment.

Figure 1. Plasma insulin concentrations between 3 and 8 wk of age in Holstein bull calves fed milk replacer and hay without calf starter (MR) or with calf starter ad libitum (MR+S). Treatment did not affect plasma insulin levels over time ($P = 0.23$) and no interaction was observed between treatment and week of life ($P = 0.16$). Letters denote significant differences between weeks ($P < 0.01$).

Figure 2. Plasma BHBA concentrations between 3 and 8 wk of age in Holstein bull calves fed milk replacer and hay without calf starter (MR) or with calf starter ad libitum (MR+S). Treatment did not affect plasma BHBA concentrations ($P = 0.53$). Between 3 and 8 wk of life, no statistical difference in average weekly plasma BHBA concentrations was found ($P = 0.65$) and no treatment $\times$ week interaction was found ($P = 0.62$).

In the livers of calves fed calf starter, genes encoding *ASS-1*, *ASL*, and *ARG-1* had higher mRNA abundance than in the MR treatment (Table 5).

DISCUSSION

Calf Starter-Dependent Adaptations

Rumen. Greater VFA concentrations in MR+S than in MR calves is consistent with expected greater fermentation in the rumen of MR+S calves. Although leakage of milk from the esophageal groove may cause some fermentation of milk in the rumen, it is unlikely to be a major contributor to total rumen fermentation,

as esophageal groove failure has been shown to be in the order of several milliliters of milk per feeding (Wise and Anderson, 1939). As such, responses in mRNA abundance in the rumen epithelium and the liver are likely attributed to fermentation of calf starter in the rumen of MR+S calves.

One particular gene of interest is apical *NHE-3*, an apical membrane sodium-proton anti-porter involved in regulation of intracellular pH (Malo and Fliegel, 2006). The expression of *NHE-3* is up-regulated by butyrate (Kiela et al., 2007), whereas long-term regulation of *NHE-3* is primarily accomplished through changes in transcription (Zachos et al., 2005). In disagreement

Table 2. Average daily gain and BW at harvest and average plasma metabolite concentrations of Holstein bull calves fed calf starter ad libitum with milk replacer and hay (MR+S), and those fed milk replacer and hay only (MR)

Item	MR	MR+S	P-value
Final age, d	49.6 $\pm$ 3.3	50.0 $\pm$ 3.3	0.45
ADG, kg/d	0.86 $\pm$ 0.07	0.80 $\pm$ 0.08	0.45
BW, kg	78.4 $\pm$ 3.0	76.8 $\pm$ 3.5	0.30
Plasma insulin, ¹ μ IU/mL	57.6 $\pm$ 14.7	31.1 $\pm$ 14.7	0.23
Plasma glucose, ¹ mg/dL	106.6 $\pm$ 7.0	98.5 $\pm$ 7.0	0.38
Plasma BHBA, ¹ mg/dL	6.2 $\pm$ 0.1	6.1 $\pm$ 0.2	0.53
Plasma IGF-1, ¹ ng/mL	80.0 $\pm$ 8.4	76.2 $\pm$ 7.9	0.74
PUN, ^{1,2} mg/dL	4.3 $\pm$ 0.3	5.6 $\pm$ 0.3	0.01

¹Average concentration between wk 3 and 8 of age.

²Plasma urea nitrogen.

Figure 3. Plasma glucose concentrations between 3 and 8 wk of age in Holstein bull calves fed milk replacer and hay without calf starter (MR) or with calf starter ad libitum (MR+S). Plasma glucose was not affected by treatment ($P = 0.38$). Average plasma glucose did increase significantly between wk 6 and 7 of life ($P < 0.01$), but no treatment $\times$ week interaction ($P = 0.86$) was observed. Weekly averages with different letters are significantly different.

Figure 4. Plasma IGF-1 concentrations between 3 and 8 wk of age in Holstein bull calves fed milk replacer and hay without calf starter (MR) or with calf starter ad libitum (MR+S). Plasma glucose was not affected by treatment ($P = 0.74$). Average plasma IGF-1 did increase significantly between wk 6 and 7 of life ($P < 0.01$), but no treatment $\times$ week interaction ($P = 0.40$) was observed. Weekly averages with different letters are significantly different.

with findings of Kiela et al. (2007), abundance of *NHE-3* mRNA was lower in the MR+S treatment despite a higher butyrate concentration in rumen fluid. The reason for lower *NHE-3* mRNA abundance in rumen epithelia from the MR+S calves is not known, but lower *NHE-3* expression may indicate decreased proton recycling into the lumen and greater net proton uptake by ruminal epithelium for the MR+S calves. Lower

proton recycling into the rumen (and thus greater net proton uptake) in the MR+S treatment may explain why rumen pH did not differ (Laarman and Oba, 2011) in spite of greater VFA concentrations for MR+S treatment.

Increased expression of *MCT-1*, located on the basolateral membrane (Connor et al., 2009), in MR+S supports the idea that net proton absorption may be

Table 3. Rumen VFA profile¹ and blood plasma metabolites² on the day of harvest in Holstein bull calves fed milk and hay only (MR) or calf starter ad libitum, milk, and hay (MR+S) at the start of weaning

Item	MR	MR+S	P-value
Total VFA, mM	64.6 $\pm$ 8.6	99.1 $\pm$ 8.1	<0.01
Acetate, mol/100 mol of VFA	69.3 $\pm$ 1.9	57.6 $\pm$ 1.9	<0.01
Propionate, mol/100 mol of VFA	18.7 $\pm$ 1.3	21.3 $\pm$ 1.2	0.32
Butyrate, mol/100 mol of VFA	7.9 $\pm$ 1.9	15.6 $\pm$ 1.7	0.03
Plasma glucose, mg/dL	84.5 $\pm$ 5.5	84.2 $\pm$ 5.5	0.97
Plasma BHBA, mg/dL	7.6 $\pm$ 0.4	7.1 $\pm$ 0.4	0.33
Plasma insulin, μ IU/mL	146.4 $\pm$ 26.7	47.9 $\pm$ 28.6	0.02
Plasma IGF-1, ng/mL	246.3 $\pm$ 25.2	216.5 $\pm$ 23.6	0.36
NH ₃ -N, mg/dL	11.7 $\pm$ 2.1	4.9 $\pm$ 2.1	0.06
Log(PUN) ^{3,4} , mg/dL	0.33 $\pm$ 0.08	0.73 $\pm$ 0.08	<0.01
PUN ^{3,5} , mg/dL	2.14 $\pm$ 1.20	5.37 $\pm$ 1.20	NA

¹Sampled 6 h after morning feeding.

²Sampled 4 h after morning feeding.

³Plasma urea nitrogen.

⁴Statistical analyses were performed on log-transformed, normally distributed data.

⁵Reverse-transformed values are shown for reader convenience.

Table 4. Abundance of mRNA^{1,2} involved in intracellular pH regulation and butyrate metabolism in the rumen epithelium of Holstein bull calves fed milk replacer and hay only (MR) or milk replacer, hay, and ad libitum calf starter (MR+S)

Gene	MR	MR+S	P-value	E ³ (%)	R ²
Monocarboxylate transporter, isoform 1 (<i>MCT-1</i>)	0.53 ± 0.23	1.45 ± 0.22	<0.01	104.1	1.00
Na ⁺ /H ⁺ exchanger, isoform 1 (<i>NHE-1</i>)	0.84 ± 0.16	0.86 ± 0.17	0.92	112.9	0.99
Na ⁺ /H ⁺ exchanger, isoform 2 (<i>NHE-2</i>)	0.68 ± 0.16	0.81 ± 0.16	0.36	103.7	0.98
Na ⁺ /H ⁺ exchanger, isoform 3 (<i>NHE-3</i>)	0.82 ± 0.12	0.37 ± 0.12	<0.01	97.5	0.99
Down-regulated in adenoma (<i>DRA</i>)	0.37 ± 0.14	0.11 ± 0.15	0.23	96.8	1.00
Putative anion transporter, isoform 1 (<i>PAT-1</i>)	0.89 ± 0.11	1.14 ± 0.11	0.22	102.9	1.00
3-Hydroxy-3-methylglutaryl-CoA lyase (<i>HMGCL</i>)	1.15 ± 0.16	1.26 ± 0.16	0.36	115.5	1.00
3-Hydroxy-3-methylglutaryl-CoA synthase, isoform 1 (<i>HMGCS-1</i>)	0.94 ± 0.16	0.40 ± 0.17	0.01	100.1	0.98
β-Hydroxybutyrate dehydrogenase, isoform 1 (<i>BDH-1</i>)	0.85 ± 0.20	0.93 ± 0.20	0.78	144.5	0.99
β-Hydroxybutyrate dehydrogenase, isoform 2 (<i>BDH-2</i>)	0.94 ± 0.14	0.92 ± 0.13	0.91	106.2	0.99

¹Relative to the sample with the highest abundance (lowest cycle threshold); values were normalized by geometric means of non-normalized mRNA abundance of 3 reference genes (glyceraldehyde-3-phosphate dehydrogenase, β-actin, and ribosomal protein large P0).

²n = 18.

³Efficiency of amplification.

higher in MR+S. Because *MCT-1* is a monocarboxylate/H⁺ co-transporter, it contributes to proton removal from the ruminal epithelium to the blood circulation (Meredith and Christian, 2008). The increased expression of *MCT-1* appears to complement the decreased expression of *NHE-3* and may increase net proton removal from the rumen.

Cholesterol synthesis also appears to be affected by diet. Specifically, *HMGCS-1* has been shown to be down-regulated in mature nonlactating dairy cows switched to a high-grain diet from a high forage diet, possibly to mitigate excess cellular cholesterol (Steele et al., 2011). Further, increased supply of VFA in vitro caused the downregulation of 9 genes involved in cholesterol synthesis in a Caco-2/TC-7 cell line (Alvaro et al., 2008). In our study, the MR+S treatment was associated with lower mRNA abundance of *HMGCS-1*, suggesting that provision of calf starter might down-regulate cholesterol synthesis at the mRNA level.

Liver. Of all genes involved in the urea cycle, mRNA abundance of *CPS-1* was not different between treatments and that of *OTC* tended to be higher for the MR+S treatment. In growing Holstein calves, *CPS-1* mRNA abundance did not increase between 2 and 19

wk of age, but carbamoyl phosphate synthetase activity did (Takagi et al., 2008), suggesting that activity of *CPS-1* may not be regulated by transcription. The remaining genes in the urea cycle, *ASL*, *ASS-1*, and *ARG-1*, had greater abundance of mRNA in MR+S than in MR calves, which is consistent with greater plasma urea N concentration for calves fed a calf starter. Previous work showed that the mRNA abundance of *ASL* and *ARG-1* decreased, whereas activity of *OTC*, *ASL*, and *ASS-1* increased with age in Holstein calves (Takagi et al., 2008). Urea production has been shown to be dependent on nitrogen intake in steers across a range of high-forage and high-concentrate diets (Huntington and Archibeque, 2000). As such, greater plasma urea N for the MR+S calves might be also attributed to their higher N intake compared with the MR calves.

Calf Starter-Independent Adaptations

Plasma IGF-1, insulin, BHBA, and glucose concentrations appeared to be not related to calf starter fermentation as none of these parameters were affected by treatment between wk 3 and 8. Although plasma glucose did not differ between treatments, plasma glucose

Table 5. Abundance of mRNA^{1,2} involved in the urea cycle in the liver of Holstein bull calves fed milk replacer and hay only (MR) or milk replacer, hay, and ad libitum calf starter (MR+S)

Gene	MR	MR+S	P-value	E ³ (%)	R ²
Carbamoyl phosphate synthetase (<i>CPS</i>)	8.67 ± 1.10	9.90 ± 1.10	0.45	98.1	0.99
Ornithine transcarbamoylase (<i>OTC</i>)	1.34 ± 0.11	1.64 ± 0.11	0.08	101.3	0.99
Arginosuccinate synthetase, isoform 1 (<i>ASS-1</i>)	1.56 ± 0.29	2.67 ± 0.29	0.03	99.8	0.99
Arginosuccinate lyase (<i>ASL</i>)	0.99 ± 0.10	1.44 ± 0.10	0.02	98.6	0.99
Arginase, isoform 1 (<i>ARG-1</i>)	1.74 ± 0.32	3.21 ± 0.32	0.01	99.7	0.99

¹Relative to the sample with the highest abundance (lowest cycle threshold); values were normalized by geometric means of non-normalized mRNA abundance of ribosomal protein large P0.

²n = 18.

³Efficiency of amplification.

increased between 6 and 7 wk of age to approximately 110 mg/dL, regardless of treatment, which is nearly double the concentration found in lactating dairy cows (Silveira et al., 2007). We observed a significant effect of age on plasma IGF-1, insulin, and glucose, but not BHBA, concentrations. The lack of change in plasma BHBA concentrations over time agrees with previous findings that plasma BHBA concentration is not dependent on diet or energy intake (Lane et al., 2000). Previous studies have shown plasma BHBA concentrations in lambs to be low (Baldwin and Jesse, 1992) or nondetectable (Lane et al., 2000) until 42 d of age, at which point plasma BHBA concentrations increase. In our study, increases in plasma concentrations were not detected for BHBA, but were detected for IGF-1, glucose, and insulin. Although energy intake increased steadily from wk 3 to 8 of life, plasma IGF-1, glucose, and insulin concentrations spiked between 6 and 8 wk of life, suggesting that plasma IGF-1, plasma glucose, and plasma insulin, before weaning, appear to be affected by age or energy intake rather than diet type.

Implications for Nutritional Management

The conventional recommendation in nutritional management for dairy calves is to wean calves as soon as starter intake is above 680 g/d, as early as 4 wk of age (NRC, 2001). The increased mRNA abundance of hepatic urea cycle enzymes in MR+S shows that provision of calf starter may help enhance the urea cycle before weaning. Furthermore, changes in regulation of *MCT-1* and *NHE-3* in the rumen epithelium suggest that the rumen epithelium may be adapting to increase VFA absorption, which is the primary mode of acid removal from the rumen (Allen, 1997). Provision of calf starter has been shown to have substantial effects on the rumen epithelium and the liver and may be important in metabolically preparing a calf for weaning. It is important to note, however, that gene expression does not imply protein expression or activity, which limits the scope of the current study.

As for age of weaning, our results showed that plasma IGF-1 and insulin concentrations did not increase until 7 wk of age, and thus very early weaning may not be beneficial if calves are not metabolically ready for weaning. When weaning is initiated at 3 wk of age, calves are not able to compensate for decreased milk intake by increasing starter intake (Sweeney et al., 2010), resulting in decreased intake of digestible energy. Delays in weaning until 12 to 13 wk of age effectively eliminate the decreases in digestible energy intake that are typical in calves weaned early (de Passillé et al., 2011), suggesting that weaning age affects the metabolic capability of calves to adapt to solid feed. The lack of treatment

effect on the plasma metabolites measured in the current study indicated that some metabolic adaptations might not be induced by consumption of solid feed per se. As such, calf starter intake may not be used as the sole indicator to determine whether calves are ready for weaning.

CONCLUSIONS

Calf starter fermentation in preweaned Holstein calves alters expression of genes involved in pH_i regulation and *HMGCS-1* in the rumen epithelium, and mRNA abundance of urea cycle enzymes in the liver. Although the liver and rumen epithelium responded to calf starter feeding at the level of gene transcription, plasma concentrations of IGF-1, glucose, insulin, and BHBA did not appear to be affected by calf starter fermentation. Plasma IGF-1, glucose, and insulin concentrations may be influenced by age or energy intake to a greater extent than diet type.

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