Form of calf diet and the rumen. II: Impact on volatile fatty acid absorption

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ABSTRACT

Diet is known to affect rumen growth and development. Calves fed an all-liquid diet have smaller and less developed rumens and a decreased ability to absorb volatile fatty acids (VFA) compared to calves fed both liquid and dry feed. However, it is unknown how rumens respond when challenged with a defined concentration of VFA. The objective of this study was to assess the effects of 2 different feeding programs on VFA absorption in preweaned calves. Neonatal Holstein bull calves were individually housed and randomly assigned to 1 of 2 diets. The diets were milk replacer only (MRO; $n = 5$) or milk replacer with starter (MRS; $n = 6$). Diets were isoenergetic (3.87 ± 0.06 Mcal of metabolizable energy per day) and isonitrogenous (0.17 ± 0.003 kg/d of apparent digestible protein). Milk replacer was 22% crude protein, 21.5% fat (dry matter basis). The textured calf starter was 21.5% crude protein (dry matter basis). Feed and ad libitum water intakes were recorded daily. Calves were exposed to a defined concentration of VFA buffer (acetate 143 mM, propionate 100 mM, butyrate 40.5 mM) 6 h before euthanasia on $d 43 \pm 1$. Rumen fluid samples were obtained every 15 to 30 min for 6 h to measure the rate of VFA absorption. Rumen tissues were obtained from the ventral sac region and processed for morphological and immunohistochemical analyses of the VFA transporters monocarboxylate transporter 1 (MCT1) and 4 (MCT4). Body growth did not differ between diets, but empty reticulorumens were heavier in MRS than MRO calves (0.67 vs. 0.39 ± 0.04 kg) and MRS calves had larger papillae areas (0.76 vs. 15 ± 0.08 mm²). We observed no differences between diets in terms of the abundance of MCT1 and MCT4 per unit area. These results indicate that the extrapolated increase in total abundance of MCT1 or MCT4 in MRS calves was not due to increased transporter

density per unit area. Modeled VFA absorption metrics (flux, mmol/h, or 6 h absorbed VFA in mmol) were not different across diets. These results demonstrate that the form of calfhod diet, whether solely MR or MR and starter, does not alter VFA absorption capacity when the rumen is exposed to a defined concentration of VFA at 6 wk of age.

Key words: dairy, calf nutrition, ruminant physiology, short-chain fatty acids, transporter

INTRODUCTION

At birth, the dairy calf has an undeveloped rumen (Tamate et al., 1962; Warner and Flatt, 1965) that must grow and develop to support ruminant metabolism. Both rumen growth (an increase in organ size) and rumen development (a change in function) are stimulated when the calf starts consuming dry feed (i.e., calf starter; Warner et al., 1956; Flatt et al., 1958; Connor et al., 2013). Calves that remain on a liquid-only or primarily liquid diet have underdeveloped rumens. The physical characteristics of underdeveloped rumens include small overall rumen size and low papillae dimensions and mass (Lengemann and Allen, 1959; Harrison et al., 1960; Tamate et al., 1962). Feeding a liquid-only diet limits the growth of the rumen, but it promotes ADG during the preweaning period when calves can consume large amounts of milk replacer (MR) or milk (Jasper and Weary, 2002; Schäff et al., 2016; Frieten et al., 2017). Encouraging ADG during the preweaning period is a common goal among producers and has been shown to increase first lactation milk yield (Soberon et al., 2012; Soberon and Van Amburgh, 2013; Gelsinger et al., 2016), so producers have an incentive to promote ADG by feeding a diet heavy in MR or milk preweaning. Despite limiting dry feed intake and thus limiting rumen growth and development (Warner et al., 1956; Flatt et al., 1958; Connor et al., 2013), we do not know the consequences of these 2 feeding strategies (i.e., promoting liquid or promoting dry feed intake) for the ability of the rumen to absorb VFA in the preweaning calf.

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Most of the existing data on rumen VFA absorption in calves was generated in the 1960s. Although this information is useful, given the advances in calf nutrition and the genetic progress made via selection for high-yielding dairy cows since then, the topic is worthy of reinvestigation because of improvements in the methodologies currently used to measure VFA absorption. Considering the limited volume capacity of the preweaned calf rumen (Tamate et al., 1962; Warner and Flatt, 1965) one might assume that the ability of the rumen to absorb VFA is decreased when compared with a developed calf rumen. However, this assumption has not been empirically tested. This study assessed the “maximal” VFA absorptive ability of the rumen tissue of 6-wk-old calves fed diets designed to generate undeveloped and developed rumens. Our hypothesis was that when exposed to defined concentrations of VFA, calves fed a diet of liquid and solid feed meant to stimulate rumen development would have an enhanced ability to absorb VFA compared to calves fed a liquid-only diet. The findings had the potential to influence the way calves are fed if either of the feeding strategies resulted in more efficient VFA absorption.

MATERIALS AND METHODS

Animals, Treatments, and Experiment

This experiment was approved by the Virginia Tech Institutional Animal Care and Use Committee (protocol #16-165) and conducted from February 2017 to May 2017. Full experimental details are described in a companion paper (Yohe et al., 2019). Briefly, 12 neonatal Holstein bull calves were purchased from a single farm and transported approximately 240 km to Virginia Tech. Calves arrived at Virginia Tech in 1 of 2 arrival periods ($n = 6$ per period): period 1 calves were on site from February 17, 2017, to April 2, 2017, and period 2 calves were on site from April 11, 2017, to May 25, 2017. Initial calf BW averaged 43.67 ± 0.55 kg (mean $\pm$ SEM), and initial calf age averaged 2.5 ± 0.14 d (mean $\pm$ SEM).

As calves arrived at Virginia Tech, a single jugular blood sample was collected for serum IgG determination (to assess passive transfer of immunity), and calf BW, hip height, and withers height were measured. After initial measurements had been obtained, each calf was moved into an individual pen that contained a rubber mat and frequently laundered cotton towels for bedding. Pens were located in a temperature-controlled room at Virginia Tech, where the calves remained for the duration of the experiment. Calves had visual and auditory contact with the other calves, but no physical contact.

Within 24 h of their arrival at Virginia Tech, calves were balanced by age, BW, serum IgG, and dam lactation number and split into 2 dietary treatment groups: milk replacer only (**MRO**; $n = 5$) or milk replacer and calf starter (**MRS**; $n = 6$). A period 2 MRO calf died at 2 wk of age from septicemia and was not replaced; its death was not treatment-related. Both dietary treatment groups were fed MR (22% CP as-fed, 20% fat as-fed; Ledger, Southern States Cooperative Inc., Richmond, VA) twice daily at 0700 and 1900 h; water was available ad libitum. The calf starter offered to MRS calves was a textured feed (18% CP as-fed; Grower, Southern States Cooperative Inc.). The MRO and MRS treatment diets were formulated to be isocaloric (based on ME) and isonitrogenous (based on apparent digestible protein; **ADP**; NRC, 2001) to negate ME or N supply as confounding factors. A detailed description of the diets is presented in a companion paper (Yohe et al., 2019), but a brief description of the diets follows. The MRO calves were offered a static amount of MR for the duration of the trial. In contrast, the MRS calves were fed decreasing amounts of MR throughout the trial to accommodate the planned increase in calf starter intake (Yohe et al., 2019); this design maintained the necessary energy (formulated to be 4.0 Mcal/d of ME throughout the 6 wk trial) and protein (formulated to be 0.198 kg/d of ADP on average throughout the 6-wk trial) to meet isocaloric and isonitrogenous constraints. The nutrient composition of the diets is listed in Table 1.

At 10 d of age, the calves underwent rumen cannulation surgery, performed similarly to that described by Kristensen et al. (2010). The surgically placed rumen cannula (2.75 i.d.) was the same design used in Lesmeister and Heinrichs (2004), Suarez-Mena et al. (2015), and Suarez-Mena et al. (2016). The cannula opening was plugged with a #6 rubber laboratory stopper.

Termination of the trial was completed over 3 consecutive days (d 42 to 44) with 2 calves killed each day (1 per treatment). On the final day of the trial, each calf underwent a washed rumen procedure adapted from Storm et al. (2011). The procedure involved evacuating the rumen using a vacuum-assisted collection device, washing the rumen with warm tap water and warm wash buffer (Table 2), and then filling the rumen with an experimental VFA buffer (Table 2) as described in Yohe et al. (2018). For this, starting at 0 h the experimental VFA buffer was introduced (Table 2), the primer dose of LiCoEDTA (40 mL at 15 mg/mL) was given, an initial baseline sample of rumen fluid was collected, and then continuous LiCoEDTA infusion (0.158 mg/mL) was administered through the rumen cannula. We then collected rumen fluid samples over the next 6 h to assess liquid passage rate (LiCoEDTA) and VFA absorption. The sampling procedure is described in

Table 1. Chemical composition (% of DM unless otherwise noted) of milk replacer and textured calf starter fed to calves throughout the 6 wk trial

Chemical composition	Milk replacer ¹	Textured calf starter ²
DM	96.2	85.8
CP	22.4	21.5
Fat (ether extract for starter)	21.5	3.95
NDF	—	19.0
ADF	—	9.3
Starch	—	27.7
Ash	9.49	9.36
ME ³ (Mcal/kg)	4.74	3.09

¹Milk replacer was fed to both treatments (22% CP and 20% fat, as fed; Ledger, Southern States Cooperative Inc., Richmond, VA) and included milk protein and animal fat sources.

²Textured calf starter was fed to milk replacer and starter (MRS) calves (18% CP, as fed; Grower, Southern States Cooperative Inc.) and contained processed grains from plant protein sources, including cracked corn and oats.

³Both milk replacer and starter ME were calculated using equations from NRC (2001). Milk replacer ME = $(0.057 \times \text{CP DM \%} + 0.092 \times \text{fat DM \%} + 0.0395 \times \text{lactose DM \%}) \times 0.93$; starter ME = $(1.01 \times \text{digestible energy} - 0.45) + 0.0046 \times (\text{ether extract} - 3)$.

detail by Yohe et al. (2018), but briefly, in this study we obtained a total of 19 samples of rumen fluid (~5 to 10 mL per sample) using a 60 mL syringe, drawing fluid from the custom-made sampling device over the 6 h infusion/sampling period (starting with time 0) for VFA analysis and cobalt determination. The initial 5 to 10 mL drawn up by syringe were discarded (sample line dead volume). After the final sample was collected, calves were killed and tissue samples collected for further analyses.

Analytical Procedures

Tissue Collection and Gross Rumen Measurements. Calves were killed on d 43 ± 1 , approximately 7 to 9 h after their last feeding. Although feed had been withheld, calves were undergoing the VFA absorption experiment, in which their rumen was exposed to a buffer containing higher concentrations of VFA than typically found in calves at 6 wk of age. At slaughter, samples were taken for rumen morphometric analyses according to our previously described methods (Yohe et al., 2015). Briefly, the full stomach and reticulorumen were weighed and then evacuated for empty reticulorumen weight determination. Rumen samples intended for gene expression and histological analyses were obtained from the cranial ventral sac region of the rumen using a punch biopsy tool with an internal diameter of 2.54 cm or securing an approximately 2.54×7.62 cm section of tissue to a tongue depressor, respectively. Representative full-thickness rumen biopsy samples were fixed

Table 2. Wash and experimental VFA buffers used throughout the rumen wash, evacuation, and sampling protocol

Chemical composition (mmol/L)	Wash buffer	Experimental VFA buffer
NaCl	106.01	20.01
NaHCO ₃	24.00	24.00
NH ₄ Cl	—	2.50
NaOH ¹	6.50	212.03
KOH	19.99	19.99
K ₂ HPO ₄	2.00	2.00
CaCl ₂	1.51	1.50
MgCl ₂	1.50	1.50
Acetic acid	—	143.02
Propionic acid	—	100.01
Butyric acid	—	40.58
HCl ¹	49.72	—
pH ¹	5.85	5.76

¹pH was adjusted to a target of 5.8.

in 10% neutral buffered formalin for 24 h and then switched to 70% ethanol before being processed and readied for immunohistochemical staining.

Real-Time Quantitative PCR. A more detailed description of the real-time quantitative PCR (qPCR) procedure is provided in the companion paper (Yohe et al., 2019). Briefly, samples from the ventral sac region sample of the rumen were used to assess the relative gene abundance of the rumen epithelial transporters of interest. The list of rumen epithelial transporters and reference genes used during qPCR analysis is shown in Table 3.

Immunohistochemistry. Fixed rumen samples stored in 70% ethanol routinely processed (Leica TP 1020; Leica Microsystems Inc., Buffalo Grove, IL), embedded in paraffin, cut to 5-μm thickness on a microtome (model HM 340 E; Micron International GmbH, Munich, Germany), and mounted onto positively charged glass microscope slides (Yohe et al., 2015). A more detailed description of the immunohistochemistry procedures is provided in the companion paper (Yohe et al., 2019); the procedures were based on previously described methods (Daniels et al., 2009; Tucker et al., 2016). A brief description is provided here. The VFA transporter proteins identified through immunohistochemistry were monocarboxylate transporter 1 and monocarboxylate transporter 4 (**MCT1** and **MCT4**, respectively). Primary antibodies used included anti-MCT1 at 1:3,000 dilution (chicken polyclonal; catalog no. AB1286I; EMD Millipore, Burlington, MA) and anti-MCT4 at 1:250 (rabbit polyclonal; catalog no. AB3314P; EMD Millipore). Tissue sections received 100 μL of the primary antibody or diluent as a negative control and were incubated at 4°C overnight. Primary antibody solutions were removed, sections were washed, and all tissue sections received 100 μL of the fluorescent

secondary antibody solution, which were either goat anti-chicken IgG at 1:200 (catalog no. A11042; Thermo Fisher Scientific, Waltham, MA) for MCT1 or goat anti-rabbit IgG at 1:200 (catalog no. A11037; Thermo Fisher Scientific) for MCT4. Protein abundance and localization of both MCT1 and MCT4 were quantified in the following manner. Fourteen digital images per calf were acquired at 40× magnification using a Nikon Eclipse E600 epi-fluorescence microscope fitted with a Texas Red filter cube and a Nuance FX Multispectral Imaging System (Perkin-Elmer, Waltham, MA). To assess the intensity of the signal without confounding the data within the tissue antigen of interest, each picture was taken at the same wavelength and exposure time (MCT1: 625 nm wavelength, 275 ms exposure time; MCT4: 625 nm wavelength, 375 ms exposure time). Monochrome images were opened with and analyzed in Image-Pro Plus version 7.0 (Media Cybernetics, Rockville, MD). The rumen epithelium was outlined using a freehand drawing tool, designating areas of interest. The sum of the intensity in areas of interest was calculated by an algorithm in Image-Pro Plus (Media Cybernetics). The staining intensity sum was then divided by the measured area of the area of interest for each image to yield measurements expressed as sum of intensity/ μm^2 . This approach was selected because of the non-nuclear and diffuse staining patterns of both MCT1 and MCT4 (Figures 1 and 2).

VFA Absorption and Codetermination. At designated sampling times, approximately 5 to 15 mL of strained rumen fluid were collected and stored at -20°C in pyrolyzed glass screwtop tubes for later VFA

analysis. Volatile fatty acids underwent derivatization and were then analyzed by gas chromatography using a method adapted from Kristensen (2000). Samples were run on a Thermo Electron Polaris Q mass spectrometer (Thermo Fisher Scientific Inc.) in tandem with a Thermo Electron Focus gas chromatograph (Thermo Fisher Scientific, Inc.) using XCalibur software version 1.4 (Thermo Fisher Scientific Inc.). We used a Varian FactorFour capillary column VF-170ms (30 m, 0.25 mm, 0.25 μm). We loaded 1 μL of derivatized sample, with the inlet temperature set to 225°C on a split ratio of 80 running a constant flow of helium carrier gas set to 1.2 mL/min. The gas chromatograph was initiated at 75°C , ramped at $5^\circ\text{C}/\text{min}$ to 135°C , then at $40^\circ\text{C}/\text{min}$ to 225°C . The mass spectrometer was programmed to run in positive selected ion monitoring mode, collecting 3 consecutive segment m/z pairs for acetate (43, 47), propionate (57, 59), and butyrate (71, 73) in that elution order. The processing method used to integrate the area under the curves for each m/z used the International Conference on Computer and Information Science (ICIS) algorithm.

At designated sampling times, approximately 5 to 15 mL of strained rumen fluid were collected and stored at -20°C in plastic tubes for later Co analysis. Initially, samples underwent nitric acid digestion using method 3030H (EPA, 1998) to remove organic matter. After removal of all organic material, samples were resuspended in 50 mL mineral-free water and sent to the Virginia Tech Soil Testing Laboratory (Blacksburg, VA) for Co determination via inductively coupled plasma atomic emission spectroscopy.

Table 3. Real-time quantitative PCR information for select genes

Gene symbol ¹	Primer ²	Primer (5'–3')	Annealing temperature ($^\circ\text{C}$)	Amplicon size (bp)	GenBank accession no.
<i>MCT1</i>	F: 352 R: 480	GTCATTGGAGGTCTTGGGCT GGTAGAGAGGAACACAGGGC	62.6	129	NM_001037319.1
<i>MCT2</i>	F: 300 R: 445	TGGTCTCGGCCTTTACAGT GCCATTGCTACAGGTCGTT	62.4	146	NM_001076366.2
<i>MCT4</i>	F: 173 R: 322	GTGACACAGCCTGGATCTCC AGAAGGACGCAGACACCATG	64.5	150	NM_001109980.1
<i>NHE1</i>	F: 1122 R: 1253	GTCCACACGACCATCAAGT AGGGTGCTGATGACAAACGT	62.0	132	NM_174833.2
<i>NHE2</i>	F: 1602 R: 1730	CGAGCAGCTCTACATCCTGG ATGCCAGCAAACACGTCAAC	63.0	129	XM_604493.6
<i>NHE3</i>	F: 1560 R: 1688	CCTCATGAGAAAGGTGCGCTC GAACGGATGAAAGCCAGGGA	63.0	129	NM_001192154.1
<i>RPS9</i> (reference)	F: 122 R: 229	GTGAGGTCTGGAGGGTCAAA GGGCATTACCTTTCGAACAGA	62.4	108	BC148016.1
<i>RPS15</i> (reference)	F: 183 R: 329	CAAGGCCAAGAAAGATGCGC TCAGGCTTGATTTCCACCTGG	62.4	147	NM_001024541.2
<i>RPS26</i> (reference)	F: 219 R: 347	CTGCGTGAGTTGTGCCATTC TACATGGGCTTTGGTGGAGG	62.4	129	NM_001015561.2

¹MCT = monocarboxylate transporter; NHE = sodium–hydrogen exchanger; RPS = ribosomal protein.

²F = forward; R = reverse.

Figure 1. Representative immunohistochemical images showing the intensity of staining for monocarboxylate transporter 1 (MCT1) protein in the rumen of milk replacer only (MRO) calves and milk replacer and starter (MRS) calves during the 6-wk trial. Green staining indicates MCT1 protein abundance and blue staining indicates nuclei. (A) Negative control of MCT1; (B) MRO staining of MCT1 protein; (C) MRS staining of MCT1 protein; (D) image showing red outline of epithelial area used to calculate the intensity per unit area to assess the level of protein abundance of MCT1 in rumen epithelium. Scale bars represent 50 μ m.

Statistical Analyses

Intake, growth, qPCR, and IHC data were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). All models included the fixed effects of treatment, with repeated measures data (intake and growth) including week and the interaction of treatment and week. Calf nested within treatment was the random effect in all models. Period was initially included in all models, but was found to be nonsignificant and was removed. All analyses included the best-fit covariance structure when appropriate; denominator degrees of freedom were not specified. Best-fit covariance structures as determined by lowest Akaike information criterion were as follows: ME and ADP intake measurements used autoregressive, and BW used Toeplitz.

Data for qPCR were not normally distributed (significant Shapiro–Wilk test) and were log₁₀ transformed and reanalyzed. Untransformed data are presented.

Absorption rates for each VFA and fluid passage rates were derived by fitting modeled VFA or Co concentrations to either the measured VFA concentration data or the measured Co concentration using the FME package of R (version 3.4.0; Soetaert and Petzoldt, 2010). The models used 2 pools: one for the substrate of interest (VFA or Co) and one for the volume of fluid in the rumen. Input to the fluid volume pool was assumed to be 40 mL/h based on the applied infusion rates, and the initial volume was equal to the volume of buffer solution used for that animal. Input to the Co pool was 1.04 mg/h based on the rate of Co infusion, and the initial mass of Co was assumed to be 0. The

flux of water out of the rumen was represented by mass action kinetics, and the rate of Co removal was directly proportional to the rate of fluid removal. After Co concentrations were used to determine fluid passage, the fluid passage rate was applied to the VFA models. For each VFA, input was assumed to be 0; the initial mass was equal to the quantity of VFA in the buffer solution. The VFA pool was subject to draining by fluid passage (proportional to fluid exit rate) and absorption, which followed mass action kinetics and was fit during the derivation process. A fourth-order Runge Kutta integration algorithm was used to integrate the differential equations describing the Co, VFA, and fluid pools, and the fitting of the fractional passage or absorption rates was done by minimizing the weighted residuals of the model using a Nelder-mead optimization algorithm. In

all cases, significance was declared when $P \leq 0.05$ and a tendency declared when $0.10 \geq P \geq 0.05$.

RESULTS AND DISCUSSION

Intake and Growth

The dietary treatments were formulated to be both isocaloric and isonitrogenous (NRC, 2001) to prevent any confounding factors influencing rumen growth and development. As planned, ME and ADP intakes did not differ for the interaction of treatment by week ($P = 0.78$ and $P = 0.52$, respectively) or by treatment alone ($P = 0.94$ and $P = 0.30$, respectively). Actual ME and ADP intakes for MRO calves were 3.96 ± 0.06 Mcal/d and 0.17 ± 0.01 kg/d, respectively. For MRS

Figure 2. Representative immunohistochemical images showing the intensity of staining for monocarboxylate transporter 4 (MCT4) protein in the rumen of milk replacer only (MRO) calves and milk replacer and starter (MRS) calves during the 6-wk trial. Green staining indicates MCT4 protein abundance and blue staining indicates nuclei. (A) Negative control of MCT4; (B) MRO staining of MCT4 protein; (C) MRS staining of MCT4 protein; (D) image showing red outline of epithelial area used to calculate intensity per unit area to assess the level of protein abundance of MCT4 in rumen epithelium. Scale bars represent 50 μ m.

Table 4. Relative mRNA abundance (expressed as 2^{-ΔCt} values; higher values equate to more mRNA) of selected genes in rumen epithelial tissue relevant to VFA transport in calves fed 2 differing diets at 6 wk of age

Gene ¹	Treatment ²		SEM	Test of fixed effects, <i>P</i> -value ³
	MRO	MRS		
<i>MCT1</i>	2.4 × 10 ⁻²	5.4 × 10 ⁻²	1.1 × 10 ⁻²	0.06
<i>MCT2</i>	9.8 × 10 ⁻⁴	5.1 × 10 ⁻⁴	2.0 × 10 ⁻⁴	0.13
<i>MCT4</i>	2.9 × 10 ⁻⁴	3.0 × 10 ⁻⁴	1.2 × 10 ⁻⁴	0.47
<i>NHE1</i>	3.1 × 10 ⁻⁴	2.6 × 10 ⁻⁴	1.2 × 10 ⁻⁴	0.50
<i>NHE2</i>	1.6 × 10 ⁻³	3.3 × 10 ⁻³	1.6 × 10 ⁻³	0.61
<i>NHE3</i>	9.1 × 10 ⁻⁵	3.3 × 10 ⁻⁵	2.8 × 10 ⁻⁵	0.10

¹MCT = monocarboxylate transporters; NHE = sodium–hydrogen exchangers; primer efficiencies: *MCT1* = 2.18; *MCT2* = 2.36; *MCT4* = 2.22; *NHE1* = 2.18; *NHE2* = 2.21; *NHE3* = 2.47.

²MRO = milk replacer only, MRS = milk replacer with starter; diets were formulated to be isocaloric and isonitrogenous.

³Significance declared at *P* ≤ 0.05.

calves, these were 3.95 ± 0.06 Mcal/d and 0.18 ± 0.01 kg/d, respectively. At the end of the trial, MRO calves weighed 63.10 ± 1.16 kg and MRS calves weighed 62.90 ± 1.05 kg (*P* = 0.99). Also, ADG did not differ between MRO and MRS calves (0.46 ± 0.03 kg and 0.46 ± 0.03 kg, respectively; *P* = 0.94).

Rumen Epithelial qPCR

The only difference between treatment for any of the VFA transport-related genes was a trend for an increase in *MCT1* expression for MRS compared with MRO calves (*P* = 0.06; Table 4). This trending increase in expression of *MCT1* is interesting because of the implication of *MCT1* in transporting VFA from the epithelium to the interstitial fluid/portal blood circulation (Graham et al., 2007). Laarman et al. (2012a) noted a decrease in *MCT1* mRNA abundance in calves fed only MR and hay compared with MR, hay, and starter around 50 d of age, but no treatment in that study was MR only, and diets were not formulated to be isocaloric or isonitrogenous. The other 2 VFA transporter genes (*MCT2* and *MCT4*) did not exhibit any differences between treatments, which may reflect the experimental VFA buffer used, causing a uniformly increased expression of VFA transporters into the rumen epithelium (*MCT4*), as well as between the rumen epithelial cell layers (*MCT2*) based on their cellular localization (Kirat et al., 2007, 2013). The other genes examined (*NHE1*, *NHE2*, and *NHE3*) are relevant to VFA transport because of their ability to help regulate intracellular pH (Müller et al., 2000). We found no differences observed for the NHE genes, which also may be explained by the experimental VFA buffer that was given to both treatments. The reasoning behind this may be that the exposure of each rumen to the same pH controlled the concentration gradients of H⁺, Na⁺, K⁺, and other ions that may play a role in controlling

the convoluted VFA transport/intracellular pH relationship (Connor et al., 2010). The lack of change in gene expression that we noted may also have been due to the isocaloric and isonitrogenous nature of the diets, which supplied the rumen epithelium with equal “nutrients,” even if they were coming mainly from opposite sides (i.e., rumen lumen vs. blood). How these gene expression results compare with protein abundance as well as activity (i.e., VFA absorption) are discussed, but the expression of a gene does not necessarily equate with protein abundance or protein activity.

Rumen Immunohistochemistry

We expected enhanced rumen growth in the calves fed a fermentable starter diet compared with calves fed a solely liquid diet; however, we were interested primarily in whether the rumen function of these calves differed. Assessing VFA absorption involved using immunohistochemistry to measure the abundance of the VFA transporters *MCT1* and *MCT4*, which have previously been identified in bovine rumen (Kirat et al., 2005, 2007). Images depicting the staining of both *MCT1* and *MCT4* in rumen tissue are shown in Figures 2 and 3. The pattern of staining of *MCT1* is similar to that shown previously by Kirat et al. (2005), Graham et al. (2007), and Flaga et al. (2015) and that hypothesized by Connor et al. (2010) in the basale layer of the stratified rumen epithelium, where it is important for the movement of VFA from the epithelial tissue to the extracellular space (i.e., interstitial fluid and blood). We found no difference in the abundance of *MCT1* between treatments based on the intensity measures per area of rumen epithelial tissue (*P* = 0.19), which suggests a lack of difference in the ability of the tissue to absorb VFA from a facilitated transport mode of action via protein transporter. The staining pattern of *MCT4* was similar to that seen by Kirat et al. (2007), but did not

Table 5. Mean fluid exit, VFA absorption rate, and total 6-h absorption of VFA after being exposed to a defined concentration of VFA in calves fed 2 differing forms of diet for 6 wk

Item	Treatment ¹		SEM	Test of fixed effects, <i>P</i> -value ²
	MRO	MRS		
Fluid exit (%/h)	15.70	10.70	2.85	0.14
VFA absorption (%/h)				
Acetate	20.90	35.30	7.73	0.20
Propionate	27.40	33.70	7.93	0.57
Butyrate	41.10	42.00	10.50	0.95
Total 6-h VFA flux (mmol)				
Acetate	176.70	165.70	22.80	0.73
Propionate	74.80	70.50	10.30	0.76
Butyrate	78.80	75.90	11.80	0.83

¹MRO = milk replacer only, MRS = milk replacer with starter; diets were formulated to be isocaloric and isonitrogenous.

²Significance declared at $P \leq 0.05$.

seem to fit the hypothesized location by Connor et al. (2010) in the upper layers of the rumen epithelium (i.e., stratum granulosum and stratum corneum). The diffuse staining of MCT4 throughout the rumen epithelial cell layers questioned the idea that MCT4 is important mainly for transporting VFA from the ruminal lumen into the rumen epithelial cell layers. Although we considered that artifacts might have explained this staining pattern, the localization of MCT4 observed in the current study was consistent with that observed by Kirat et al. (2007), and the lack of staining in the negative control (Figure 2) seemed valid. We found no difference in the intensity of MCT4 staining between treatments ($P = 0.60$), suggesting a lack of difference in the ability of the tissue to absorb VFA from a facilitated transport mode of action via protein transporter from the rumen. Taken together, the staining results for MCT1 and MCT4 suggest that even though MRO calves were given only a liquid diet, their rumens were still just as capable of absorbing VFA as the rumens of MRS calves when both were exposed to a defined concentration of VFA. These transporter immunohistochemical results did not quantitatively assess VFA absorption, leaving open the possibility that passive diffusion could still play a role in VFA transport.

VFA Absorption

To assess VFA absorption, we recorded the concentration of VFA over a 6-h period on the last day of the trial, and we measured the liquid flow rate out of the rumen. Results in Table 5 show no differences in fluid exit rate to the omasum ($P = 0.14$), or VFA absorption rates for acetate, propionate, or butyrate ($P = 0.20$, $P = 0.57$, and $P = 0.95$, respectively) between the MRO and MRS calves. Also, total 6-h fluxes of acetate, propionate, or butyrate were not different between the

MRO and MRS calves ($P = 0.73$, $P = 0.76$, and $P = 0.83$, respectively; Table 5). When the modeled VFA absorption rates are taken together with the gene expression and transporter immunohistochemistry, they indicate that diet did not alter the ability of the rumen to absorb VFA when tested with the washed rumen technique.

Sutton et al. (1963) performed a similar study and showed that calves fed only milk had a decreased maximal absorption rate of acetate from wk 1 to 34 (stayed constant around 30 mg of acetate/100 mL of solution/h) compared with calves fed milk, grain, and hay that increased from wk 1 (about 30 mg of acetate/100 mL of solution/h) to 34 (about 210 mg of acetate/100 mL of solution/h). The results in the current study go against these earlier findings and instead support those of Khouri (1969), who noted no differ-

Figure 3. Mean ($\pm$ SEM) immunohistochemical measurements of intensity/epithelial area for VFA transporter proteins monocarboxylate transporter (MCT)1 and MCT4 in the rumen of milk replacer only (MRO) calves and milk replacer and starter (MRS) calves during the 6-wk trial. We observed no differences for MCT1 ($P = 0.19$) or MCT4 ($P = 0.60$).

Table 6. Mean covariate effects (*P*-values) on VFA absorption rate and total VFA absorbed over 6 h after being exposed to a defined concentration of VFA in calves fed 2 differing forms of diet for 6 wk

Covariate	VFA absorption rate ¹			Total VFA absorbed ¹		
	Acetate	Propionate	Butyrate	Acetate	Propionate	Butyrate
Monocarboxylate transporter (MCT) 1 abundance	0.81	0.90	0.90	0.60	0.20	0.84
MCT4 abundance	0.28	0.42	0.22	0.04	0.10	0.15
Empty reticulorumen weight	0.73	0.55	0.43	0.42	0.56	0.40
Reticulorumen 2-dimensional area	0.59	0.70	0.61	0.91	0.50	0.65
Reticulorumen density	0.55	0.50	0.45	0.41	0.71	0.22
Total biopsy weight	0.10	0.23	0.06	0.75	0.70	0.77
Epithelium weight	<0.01	0.01	0.01	0.55	0.67	0.19
Epithelium density	<0.01	<0.01	0.08	0.19	0.28	0.07

¹Significance declared at $P \leq 0.05$.

ence in VFA absorption between calves fed only milk or fed milk and hay around 40 to 44 d of age. Rumen VFA absorption via protein-mediated transport might not explain the differences observed between the current results and those of Sutton et al. (1963), but the difference may be explained by passive diffusion of VFA because of buffer pH differences. Sutton et al. (1963) used a buffer pH of 6.5, whereas the current study used a pH of 5.85. We know that pH has an effect on VFA absorption in the rumen, with lower pH resulting in enhanced passive diffusion of protonated VFA (Danielli et al., 1945; Dijkstra et al., 1993; Aschenbach and Gabel, 2000; Penner et al., 2011). The pH of 5.85 used in the current study was selected to reflect the rumen pH of 6-wk-old calves (Laarman et al., 2012b; Yohe et al., 2015). Considering that a 0.7 difference in pH is rather large, it is conceivable that the lower pH may have facilitated an increase in passive diffusion across the rumen epithelial membrane regardless of dietary treatment, resulting in a lack of effect on VFA absorption rates.

Other aspects of the paper written by Sutton et al. (1963) are vague, which could explain the differences between our studies. For example, the concentration of the buffer used in the first experiment of Sutton et al. (1963) was not described, but if it was the same buffer used in Sutton et al. (1962) then the clear differences in VFA and salt concentrations would help elucidate why we had different results. Another discussion point is that in the experiment by Sutton et al. (1963) calves fed milk, grain, and hay received rumen inoculations of adult rumen contents, whereas the milk-fed calves did not. As well, Sutton et al. (1963) did not measure the absorption rates of propionate or butyrate, so we cannot directly compare the studies. These questions are not meant to disparage the work by Sutton et al. (1963): their work was thorough and carefully executed. However, considering the uncertainties, the gap in time, and the lack of corroborating data, it is worth revisiting

the question of dietary effects on preweaned calf rumen VFA absorption.

Another aspect of VFA absorption that was not explored by Sutton et al. (1963) or in the current study is epithelial blood flow. Previous work has demonstrated the importance of epithelial blood flow for VFA absorption (Storm et al., 2011, 2012). Dietary considerations that may have affected blood flow are CP intake (Storm et al., 2011) and butyrate concentration (Dobson et al., 1971), but considering that the diets were isonitrogenous and the buffers introduced into the rumen contained the same concentration of butyrate, it is fair to assume that these did not have an effect. Storm et al. (2012) demonstrated that rumen epithelial metabolism of VFA may influence blood flow, which may have been altered in the current study due to larger papillae (Table 6), a tendency for heavier epithelial biopsy weight (Table 6), and thicker epithelial cell layer (Yohe et al., 2019) leading to an increased opportunity for VFA metabolism in the rumen epithelium. In the end, we did not measure blood flow and we found no apparent differences in VFA absorption, so none of these speculations can be confirmed.

We also considered other factors when assessing rumen VFA absorption, shown in Table 6. The covariate had an effect in only a handful of instances. One of these was a tendency for total rumen biopsy weight to influence acetate and butyrate absorption rate ($P = 0.10$ and $P = 0.06$, respectively). Increased epithelial biopsy weight was positively associated with acetate, propionate, and butyrate absorption rate ($P < 0.01$, $P = 0.01$, and $P = 0.01$, respectively). Finally, a denser epithelium increased acetate and propionate absorption rate ($P < 0.01$ and $P < 0.01$, respectively) and had a trending influence on butyrate absorption rate ($P = 0.08$). All of these results support the idea that a more developed rumen (i.e., thicker and heavier epithelium) influences VFA absorption rate. This was shown by Sutton et al. (1963), but was not demonstrated in our

results. The reason for this lack of difference in our VFA absorption rates is unclear, but perhaps data used to perform the power analysis was not representative, affecting our ability to detect statistical differences. Interestingly, though, when looking at total VFA absorbed over the 6 h period, the only trending influence relevant to rumen growth was epithelial density and total butyrate absorbed ($P = 0.07$). As well, MCT4 protein abundance had an effect on total acetate absorbed ($P = 0.04$) and a trending influence on total propionate absorbed ($P = 0.10$). These data indicate that butyrate tends to undergo passive diffusion more readily than acetate and propionate, which will instead use protein-mediated transport. This supports the commentary of Bergman (1990) that rate of ruminal VFA absorption increased with increasing VFA chain length because passive diffusion is a quicker process.

CONCLUSIONS

Our hypothesis was not supported: form of calf diet, whether solely MR or MR and starter, did not alter VFA absorption when the rumen was exposed to a defined concentration of VFA at 6 wk of age. This finding was supported by a lack of difference in the abundance of protein transporters (MCT1 and MCT4) and by rates of VFA absorption and total VFA absorbed during the 6 h experimental period. The transport of VFA via passive diffusion also did not seem to be altered in preweaning calves under the experimental conditions. Total VFA absorption was most likely increased in calves fed MR and starter compared with MR only because they had larger rumens and increased rumen surface area. Regardless, these results indicate the rumen has the innate ability to absorb VFA, regardless of external stimuli.

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