ABSTRACT

Preweaning diet is known to affect rumen tissue appearance at the gross level. The objectives of this experiment were to investigate effects of different preweaning diets on the growth and development of the rumen epithelium and on putative rumen epithelial stem and progenitor cell measurements at the gene and cell levels. Neonatal Holstein bull calves (n = 11) 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). Water was available ad libitum and feed and water intake were recorded daily. Putative stem and progenitor cells were labeled by administering a thymidine analog (5-bromo-2′-deoxyuridine, BrdU; 5 mg/kg of body weight in sterile saline) for 5 consecutive days and allowed a 25-d washout period. Calves were killed at 43 ± 1 d after a 6-h exposure to a defined concentration of volatile fatty acids. We obtained rumen tissue from the ventral sac and used it for immunohistochemical analyses of BrdU (putative stem and progenitor cells) and Ki67 (cell proliferation), gene expression analysis, and morphological measurements via hematoxylin and eosin staining. Epithelial stem and progenitor cell gene markers of interest, analyzed by real-time quantitative PCR, were β1-integrin, keratin-14, notch-1, tumor protein p63, and leucine-rich repeat-containing G protein-coupled receptor 5. Body growth did not differ by diet, but empty reticulorumens were heavier in MRS calves (MRS: 0.67 ± 0.04 kg; MRO: 0.39 ± 0.04 kg). The percentage of label-retaining BrdU basale cells was higher in MRS calves than in MRO calves (18.4 ± 2.6% vs. 10.8 ± 2.8%, respectively). Rumen epithelial gene expression was not affected by diet, but the submucosa was thicker in MRO calves and the epithelium and corneum/keratin layers were thicker in MRS calves. Presumptive stem and progenitor cells in the rumen epithelium were identifiable by their ability to retain labeled DNA in the long term, changed proliferative status in response to diet, and likely contributed to observed treatment differences in rumen tissue thickness.

Key words: dairy, calf nutrition, ruminant physiology, stem cell

INTRODUCTION

In mature ruminants, 63 to 80% of ME is derived from VFA (Siciliano-Jones and Murphy, 1989; Bergman, 1990). Certain VFA (mainly butyrate) are metabolized in the rumen epithelium and presumably contribute to its growth (Sakata and Tamate, 1978; Gorka et al., 2009; Malhi et al., 2013). Clearly, the rumen epithelium plays a crucial role in VFA absorption. What remains to be discovered is how the rumen epithelium grows and replenishes its absorptive surface, and how diet affects these processes as the calf consumes increasing amounts of dry feed.

The integumentary system (skin) shares similarities in architecture and organization with the rumen. For instance, the proliferation of cells in the stratified epidermis is initiated in the stratum basale layer, and cellular differentiation occurs as cells physically move upward through the spinosum, granulosum, and corneum (Zouboulis et al., 2008). In skin, the functions of stem and progenitor cells are intrinsically regulated and also responsive to external cues from the environment (Rando, 2006). Evidence of the rumen epithelium responding to external dietary stimuli was demonstrated by Goodlad (1981), who showed increased turnover rates of rumen epithelial cells in sheep fed a diet transitioning from forage to concentrate, compared with forage-based and concentrate-based diets. It can be speculated from the results of Goodlad (1981) that diet influences
ruminal stem and progenitor cell populations, and thus alters the epithelium to be equipped for the absorption and metabolism of available nutrients.

Yohe et al. (2016) recently demonstrated rumen epithelial presence of genes known to be markers of stem and progenitor cells in other organs: β1-integrin (ITGB1), keratin-14 (KRT14), notch-1 (NOTCH1), and tumor protein p63 (TP63), all of which are known for their role in epithelial stem and progenitor cell homeostasis (Janes and Watt, 2006; Senoo et al., 2007; Zhang et al., 2013, 2016), and leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), known for its role in intestinal stem and progenitor cell homeostasis (Tan and Barker, 2014; 2015).

Although we know that genes for potential stem and progenitor cells are present in rumen epithelial homogenates (Yohe et al., 2016), successful identification and quantification of rumen epithelial stem and progenitor cells has not been achieved, to our knowledge. In other organs and tissues, putative stem cells have been identified by their ability to retain labeled DNA for extended periods of time (Gunduz, 1985; Langer et al., 1985). The thymidine analog 5-bromo-2′-deoxyuridine (BrdU; Bickenbach, 1981; Capuco et al., 2009; Daniels et al., 2009), which is incorporated into cells during the S-phase of the cell cycle (Potten et al., 2002), is often used for this purpose. In the current research, our objectives were to investigate the effects of different preweaning diets on the growth (i.e., increase in cell number or size) and development (i.e., change in function) of the rumen epithelium and on putative rumen epithelial stem and progenitor cell measurements at the gene and cell level.

**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. A total of 12 healthy Holstein bull calves were purchased from a single farm and transported approximately 240 km to Virginia Tech. Within 12 h of birth at the source farm each calf was fed 2 470 g bags of colostrum replacer (≥200 g IgG; Bovine IgG Land O Lakes Colostrum Replacement; Land O Lakes Animal Milk Products Co., Shoreview, MN) mixed in 3.79 L of hot water (43 to 49°C) and the navel of each calf was dipped in an iodine tincture. Where applicable, subsequent feedings were of milk replacer (MR; 22% CP as-fed; 20% fat as-fed; Ledger, Southern States Cooperative Inc., Richmond, VA). 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 ± SEM) and initial calf age averaged 2.5 ± 0.14 d (mean ± SEM).

Upon arrival at Virginia Tech, a single jugular blood sample was collected from each calf into 10 mL glass tubes (cat# 367921; BD Vacutainer, Franklin Lakes, NJ) and used for serum IgG determination (to assess passive transfer of immunity); calf BW, hip height, and withers height were also measured. Serum IgG concentration was measured using an IgG radial immunodiffusion test (Bovine IgG Test Kit; Kent Laboratories, Triple J Farms, Bellingham, WA). After initial measurements had been obtained, each calf was moved into an individual pen (122 × 183 cm) in a temperature-controlled room (21 to 22°C) where they remained for the duration of the experiment. Each pen was placed on top of a solid rubber mat (122 × 183 cm) topped with a perforated rubber mat (91.44 × 152.4 cm) for traction and hygiene purposes. Cotton towels were used as bedding and laundered daily. Calves had visual and auditory contact with the other calves, but no physical contact.

At 10 d of age, all calves underwent rumen cannulation surgery, performed similarly to that described by Kristensen et al. (2010). Each surgically placed rumen cannula (2.75 cm 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.

Calves were assigned to 1 of 2 dietary treatments within a day of their arrival: milk replacer only (MRO; n = 5) or milk replacer and calf starter (MRS; n = 6). Treatments were balanced by calf age (2.33 ± 0.15 vs. 2.66 ± 0.15 d), BW (43.17 ± 1.70 vs. 43.74 ± 1.70 kg), serum IgG (12.48 ± 0.94 vs. 13.52 ± 0.94 mg/mL), and dam lactation number (2.83 ± 0.32 vs. 2.33 ± 0.32) for MRO and MRS calves, respectively.

The trial started with 6 calves allocated to the MRO treatment, but 1 calf died at 2 wk of age from septicemia and was not replaced; the death was deemed unrelated to dietary treatment. Initially, a power analysis was performed using preliminary data for rate of absorption of acetate, propionate, and butyrate in the rumen of steers (L. B. Harthan and R. R. White, Virginia Tech, Blacksburg; personal communication) and rumen surface area measurements from 4-wk-old preweaned calves fed either milk replacer and calf starter or milk replacer only (T. T. Yohe and K. M. Daniels, unpublished data). The resulting power (α = 0.05) was 0.91 when n = 5 calves per treatment and 0.96 when n = 6 calves per treatment. Treatment diets were formulated...
based on NRC (2001) equations and were designed to be isocaloric (4.0 Mcal of ME per day) and isonitrogenous (0.198 kg of apparent digestible protein (ADPr) per day) throughout the trial. Both groups of calves were fed MR (22% CP as-fed; Ledger, Southern States Cooperative Inc.; Richmond, VA) and included milk protein and animal fat sources.

Textured 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.

Both milk replacer and starter ME were calculated using equations from NRC (2001). The milk replacer ME = (0.057 × CP DM % + 0.092 × fat DM % + 0.0395 × lactose DM %) × 0.93; starter ME = (1.01 × digestible energy − 0.45) + 0.0046 × (ether extract – 3).

We assessed the calves for intestinal and respiratory health throughout the trial. A 4-point scale was used.
for twice-daily fecal scoring (Diaz et al., 2001). In this system, scores were as follows: 1 = firm, well-formed (not hard); 2 = soft, pudding-like; 3 = runny, pancake batter; and 4 = liquid, splatters. Calves were scored before each feeding. Any calf with a fecal score of 3 or higher was monitored closely and if necessary was offered 100 g of electrolyte powder (Diaque; Boehringer Ingelheim, Copenhagen, Denmark) dissolved into hot water for a total of 1.89 kg solution. The oral electrolyte solution was offered at least 2 h after each feeding until the scour subsided, and any signs of dehydration (e.g., eyelid depression score and skin tent score) were normal. As for respiratory health, a 4-point scale was also used for twice-daily respiratory scoring. In this system, scores were as follows: 1 = calf breathing at a normal rate; 2 = calf not showing signs of distress, but an observable increase in breathing compared with score 1; 3 = labored calf breathing with other signs of respiratory infection (e.g., lethargy, nasal discharge); 4 = score of 3 plus rectal temperature greater than or equal to 39.4°C. Calves were scored before each feeding. Any calf with a respiratory score of 3 or higher was flagged for examination after feeding. This examination included a rectal temperature measurement, examination of nose and ears for signs of infection, further observation of breathing patterns, and general behavior.

**Putative Rumen Epithelial Stem and Progenitor Cell Labeling**

A BrdU label retaining technique was used to label potential rumen stem and progenitor cells (Bickenbach, 1981; Capuco et al., 2009; Daniels et al., 2009). For this, a sterile BrdU solution [20 mg of BrdU powder per mL of sterile 0.9% NaCl (pH 8.5); BrdU powder: cat no. B5002; Sigma-Aldrich, St. Louis, MO] was administered intravenously in the jugular vein (5 mg of BrdU powder per kg of BW) once daily for 5 consecutive days (Daniels et al., 2009). For each calf, the final injection was administered 25 d before slaughter; this allowed for a 25-d washout period. The dose and washout period were determined in a pilot experiment conducted in 2015 (Virginia Tech IACUC protocol #15–165; data not shown). Right and left jugular veins were alternated daily for injections. The average injection volume was 12.5 mL/d per calf and each injection took approximately 1 min to administer.

**Analytical Procedures**

**Tissue Collection and Gross Rumen Measurements.** Calves were killed on d 43 ± 1, approximately 7 to 9 h after their last feeding and immediately after a separate VFA absorption experiment that consisted of exposing empty rumens to buffer containing concentrations of VFA typically found in 6-wk-old calves (Yohe et al., 2019). 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 stored in 10% neutral buffered formalin overnight and then switched to 70% ethanol before being processed and readied for hematoxylin and eosin and immunohistochemical staining. Rumen samples were also collected for gross examination using the punch biopsy tool, stored in saline overnight, manually dried with a paper towel the following day, and then weighed. Measurement of 2-dimensional papillae area was completed using rumen tissue samples that had been fixed in 10% neutral buffered formalin overnight and stored in 70% ethanol. Tissue sections were viewed under a dissecting scope (Olympus SZ40; Olympus Corp., Tokyo, Japan) and pictures were taken using an Olympus SZ-CTV adaptor (Olympus Corp.). Images were opened with and analyzed in Image-Pro Plus version 7.0 (Media Cybernetics Inc., Rockville, MD) where the area of 50 representative papillae was measured per calf.

**Real-Time Quantitative PCR.** Samples from the ventral sac region sample of the rumen were used to assess the relative gene abundance of potential rumen stem and progenitor cell markers via real-time quantitative PCR (qPCR). Forcrops were used to peel underlying muscle layers away from the epithelium; the muscle-containing portion was discarded, and the epithelial portion of each sample was flash-frozen in liquid nitrogen and stored on dry ice until permanent storage in a −80°C freezer later that same day. Total RNA was extracted from approximately 30 mg of tissue using a Qiagen RNeasy Pus Mini Kit (Qiagen, Valencia, CA) and our previously reported protocol (Yohe et al., 2016). For all 11 calves, the 260 nm/280 nm ratio averaged 2.11 ± 0.00, and the 260 nm/230 nm ratio averaged 1.89 ± 0.13. Single-stranded cDNA was synthesized from each RNA sample according to the method of Yohe et al. (2016). For each sample, 2.0 μg of RNA was denatured in DNase/RNase–free water for 12 min at 70°C before samples were placed on wet ice. Reverse transcription was then performed, which consisted of a master mix (8.7 μL) containing 4 μL of M-MLV RT 5X buffer (Promega, Madison, WI), 2 μL of...
0.1 M dithiothreitol, 1 μL of 10 mM dNTP (Promega), 1 μL of oligo(dT)20 primer, 0.5 μL of M-MLV reverse transcriptase (Promega), and 0.2 μL of RNase inhibitor (Promega). The 8.7 μL of master mix was added to each well of denatured RNA (11.3 μL) in a 0.2 mL thin walled PCR 8-strip well tube (World Wide Medical Services Inc., Tampa, FL) for a final reaction volume of 20 μL. Reverse transcription was carried out in an Arktik Thermal Cycler (Thermo Scientific, Waltham, MA) with cycle conditions at 40°C for 1 h, followed by 95°C for 10 min.

The resultant cDNA was then diluted 1:1 with the addition of 20 μL of DNase/RNase–free water. Each qPCR assay was performed on all samples in triplicate, with each reaction mixture (10 μL) containing 0.25 μL of each forward and reverse primer (both used at 20 μM; Eurofins Genomics, Louisville, KY), 4.75 μL of PowerUp SYBR Green Master Mix (Thermo Fisher Scientific), 3.75 μL of DNase/RNase–free water, and 1 μL of cDNA (1:1 stock). The qPCR assays were performed using a QuantStudio 6 Flex Real-Time PCR system (Thermo Fisher Scientific) with the following cycling conditions: 95°C for 3 min; 45 repeating cycles of 94°C for 15 s (denaturation), respective temperature (Table 3) for 30 s (annealing), and 72°C for 30 s (extension); 95.0°C for 2 min, 55.0°C for 30 s; and then a temperature increase at 1.0°C increments to 95.0°C (melting curve). Each assay included a no-template control and a no-reverse-transcriptase control, with the no-template control receiving 1 μL of RNase/DNase–free water instead of cDNA, and the no-reverse-transcriptase control received a 1-μL aliquot of a reverse transcription product to which no reverse transcriptase was added. The qPCR assays were repeated if the coefficient of variation for sample cycles to threshold (Ct) among triplicate reactions was greater than 10%.

Primers were designed with Primer3 (v. 0.4.0; http://bioinfo.ut.ee/primer3-0.40/; Rozen and Skaletsky, 2000). Validation of primers consisted of melting curve analyses performed after each qPCR assay to determine whether primer dimers or genomic DNA contamination were present during the assay. Target genes of interest were normalized to the geometric mean of RPS9, RPS15, and RPS26 (Yohe et al., 2016): target gene Ct – reference genes Ct = ΔCt (Vandesompele et al., 2002).

Hematoxylin and Eosin Staining. Full-thickness rumen biopsy samples from the ventral sac region of each calf that had been formalin-fixed and switched over to 70% ethanol for long-term holding underwent routine processing (Leica TP 1020; Leica Microsystems Inc., Buffalo Grove, IL), were embedded in paraffin blocks, cut to 5-μm thickness on a microtome (model HM 340 E; Micron International GmbH, Germany), and mounted onto positively charged glass microscope slides (Yohe et al., 2015). We completed hematoxylin and eosin staining using the procedure described by Tucker et al. (2016). Eight digital images were captured from each microscope slide using an Olympus BX43 microscope (Olympus Corporation of the Americas, Center Valley, PA) fitted with a Retiga R6 camera (QImaging Corporation, Surrey, BC, Canada); 4 images were taken at 40× and 4 images at 4×. Rumen tissue images were subsequently opened in Image-Pro Plus version 7.0 (Media Cybernetics, Inc.) for measurements. We used the 40× images to measure the thickness of the

<table>
<thead>
<tr>
<th>Gene symbol1</th>
<th>Primer2</th>
<th>Primer (5′–3′)</th>
<th>Annealing temperature (°C)</th>
<th>Amplicon size (bp)</th>
<th>GenBank accession no.</th>
</tr>
</thead>
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<td>LGR5</td>
<td>F: 813</td>
<td>GATACCCGAGAAGGCCCTTGTA</td>
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<td>131</td>
<td>NM_001192520.1</td>
</tr>
<tr>
<td></td>
<td>R: 943</td>
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<td>64.5</td>
<td>145</td>
<td>NM_001165751.5</td>
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<tr>
<td>ITGB1</td>
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<td>NM_174368.3</td>
</tr>
<tr>
<td></td>
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<td>62.3</td>
<td>140</td>
<td>NM_001191337.2</td>
</tr>
<tr>
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<tr>
<td></td>
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<td>62.3</td>
<td>129</td>
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<td>62.4</td>
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</tr>
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<td>108</td>
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<td></td>
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<tr>
<td></td>
<td>R: 329</td>
<td>TCAGGTCTGTTTCTTCACTGG</td>
<td>62.4</td>
<td>129</td>
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</tr>
</tbody>
</table>

1ITGB1 = β1-integrin; KRT14 = keratin-14; LGR5 = leucine-rich repeat-containing G protein-coupled receptor 5; NOTCH1 = notch-1; TP63 = tumor protein p63; RPS = ribosomal protein.

2Primer direction and hybridization position on the sequence; F = forward; R = reverse.
muscularis and submucosa layers and the 4× images to measure thickness of the epithelium and cornuem/keratin layers. In both instances, we used the line tool of the software. Distances were recorded in micrometers (µm). Within each image, 50 linear measurements of each item were recorded by a single observer who was blinded to treatment.

**Immunohistochemistry.** To prepare microscope slides for the beginning steps of immunohistochemistry protocols, we used the same protocol as that used to process and prepare rumen tissue for hematoxylin and eosin staining (Tucker et al., 2016). We used immunohistochemistry to visualize and quantify putative stem and progenitor cells (BrdU-labeled cells) and actively proliferating cells (Ki67-labeled cells). The nuclear antigen Ki67 labels cells in the active phases (G1, S, G2, M) of the cell cycle. We performed immunohistochemical procedures as previously reported (Daniels et al., 2009), with the following modifications. Microscope slides were deparaffinized (3 × 3 min) and hydrated through a descending graded series of ethanol washes (100%, 2 × 3 min; 95%, 2 × 3 min; 70%, 1 × 3 min), ending in deionized water (2 × 3 min). Antigens were retrieved by boiling slides in 10 mM citrate buffer, pH 6.0, for 30 min. Slides were allowed to cool in citrate buffer (~30 min) and then washed in PBS (3 × 2 min). Serial tissue sections were separated with a hydrophobic barrier pen (Ted Pella Inc., Redding, CA) to prevent commingling of antibodies. All tissue sections were then blocked with CAS Block (Thermo Fisher Scientific) for 30 min. The CAS Block was aspirated, and 100 µL of the primary antibody solution was added per section. Primary antibodies included anti-BrdU antibody used at 1:100 dilution (mouse monoclonal; catalog no. MAB3424; EMD Millipore, Burlington, MA) and anti-Ki67 antibody used at 1:200 dilution (rabbit monoclonal; catalog no. RM-9106; Thermo Fisher Scientific). Primary and secondary antibodies were diluted in CAS Block. For co-localization, BrdU and Ki67 primary antibodies were administered to tissue sections at the same time (Daniels et al., 2009). Slides were then incubated overnight at 4°C in a humidified chamber with designated primary antibodies. One section per slide served as a negative control, which consisted of CAS Block instead of the primary antibody solution. On d 2 of the protocol, primary antibody solution was removed by aspiration and slides were rinsed in PBS (3 × 5 min). All tissue sections then received 100 µL of the fluorescent secondary antibody solution—which contained goat anti-mouse IgG at 1:200 (catalog no. A11001; Thermo Fisher Scientific) for BrdU and goat anti-rabbit IgG at 1:200 (catalog no. A11037; Thermo Fisher Scientific) for Ki67—and incubated in the dark for 60 min at room temperature. Excess liquid was removed by aspiration, and coverslips were mounted using Prolong Gold antifade reagent containing the nuclear stain 4′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) and allowed to cure overnight in the dark.

Seven digital images per slide were acquired at 40× magnification using a Nikon Eclipse E600 epi-fluorescence microscope fitted with a Nuance FX Multispectral Imaging System (Perkin-Elmer, Waltham, MA) where excitation light was generated using a mercury lamp light source and standard filter cubes fitted with long pass emission filters. Resultant images were opened with and analyzed in Image-Pro Plus version 7.0 (Media Cybernetics). A sample image is provided in Figure 1, which shows how the basale layer of the epithelium was underlined, and then total basale cells and basale cells stained with only BrdU, only Ki67, and dual stained (BrdU + Ki67) were manually counted by a single observer blinded to treatment and reported as the percent of positive cells in the basale layer of the rumen epithelium using the method reported in Daniels et al. (2009).

**Statistical Analysis**

Intake, growth, gross rumen measurements, qPCR, and histology data were analyzed using the MIXED procedure of SAS 9.4 (SAS Institute, Cary, NC). Models included the fixed effects of treatment, with repeated measures data (intake and growth) including week and the interaction of treatment × 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 discarded. All analyses included the best-fit covariance structure when appropriate; denominator degrees of freedom were not specified. Best-fit covariance structures were as follows: MR DMI, total DMI, ME intake, ADPr intake, and hip height measurements used autoregressive; voluntary water intake and total water intake used unstructured; BW and withers height used Toeplitz; and ADG used heterogeneous autoregressive. Orthogonal polynomial contrasts were performed when significance was found for the interaction of treatment and week or the main effect of week. Raw qPCR data were initially not normally distributed (significant Shapiro–Wilk test); to remedy this, raw data were log₁₀ transformed before ANOVA; untransformed data are presented. Least squares means ± standard errors of the mean are reported for all variables. Significance was declared when P ≤ 0.05 and a tendency when 0.10 ≥ P ≥ 0.05. Health data were analyzed using the FREQ procedure of SAS 9.4 to compare the frequency of fecal and respiratory events between treatments.
RESULTS AND DISCUSSION

Intake, Growth, and Health

The dietary treatments were formulated to be both isocaloric and isonitrogenous (NRC, 2001) to prevent any confounding factors influencing rumen growth, development, and function. Intake data for MRO and MRS calves in Table 4 show differences in DMI for MR ($P < 0.01$), starter grain (MRO calves were not given starter grain), and total DMI ($P = 0.01$), but ME and ADPr did not differ for the interaction of treatment $\times$ week ($P = 0.78$ and $P = 0.52$, respectively) or for treatment alone ($P = 0.94$ and $P = 0.30$, respectively). This lack of difference in ME and ADP intake shows that the diets were successful in maintaining energy and nitrogen balance between treatments, so dietary goals were achieved. The diets provided daily ME intakes between 3.51 and 4.05 Mcal/d, which would not be considered an elevated/higher plane of nutrition (Geiger et al., 2016; Steele et al., 2017), but is within the range of ME requirements for dairy calves gaining between 0.4 and 0.6 kg/d fed either MR only or MR and starter (~2.76 to 4.31 Mcal/d; NRC, 2001). Intake of ADPr provided 0.15 to 0.18 kg ADPr/d, also within the ADPr requirements for dairy calves gaining between 0.4 and 0.6 kg/d fed either MR only or MR and starter (~0.13 to 0.19 kg of ADPr/d; NRC, 2001). We observed a notable lack of increase in ME and ADPr intake during week 2, most likely due to the rumen cannulation surgery during this time period.

Voluntary water intake increased as calves aged and was higher in MRS (Table 4; treatment $\times$ week interaction, $P = 0.01$; week, $P = 0.05$; treatment, $P = 0.01$). Total water intake (water in MR + voluntary drinking water) also increased as calves aged, but total water intake was higher in MRO calves (Table 4; treatment $\times$ week interaction, $P = 0.01$; week, $P < 0.01$; treatment, $P < 0.01$). The MRO calves were fed MR at 10.5% solids, whereas MRS calves were fed MR at 14% solids. The difference was planned, and the higher solids in MRS were intended to promote both voluntary water and starter feed intake. This approach worked (Table 4) and followed previous research that correlated dry feed intake to water intake (Meale et al., 2017). Throughout the experiment, MRO calves likely...
Table 4. Intake data for MR, starter, ME, and ADPr from MR and starter, and water for calves fed 2 differing forms of diet during the 6-wk trial\(^1\)\(^2\)

<table>
<thead>
<tr>
<th>Item</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Week 6</th>
<th>SEM</th>
<th>Treatment (MRO or MRS)</th>
<th>Week</th>
<th>Treatment × week</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR intake (kg of DM/wk)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01^5,6,7</td>
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<td>MRO</td>
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<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>6.63</td>
<td>0.20</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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<tr>
<td>MRS</td>
<td>5.38</td>
<td>5.34</td>
<td>5.29</td>
<td>4.64</td>
<td>3.80</td>
<td>3.21</td>
<td>0.18</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01^5,6,7</td>
</tr>
<tr>
<td>Starter intake (kg of DM/wk)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRO</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRS</td>
<td>0.11</td>
<td>0.19</td>
<td>0.96</td>
<td>1.74</td>
<td>3.08</td>
<td>5.23</td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total intake (kg of DM/wk)</td>
<td>5.44</td>
<td>5.18</td>
<td>5.95</td>
<td>5.95</td>
<td>5.95</td>
<td>6.63</td>
<td>0.20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRS</td>
<td>5.49</td>
<td>5.53</td>
<td>6.25</td>
<td>6.38</td>
<td>6.88</td>
<td>8.44</td>
<td>0.21</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ME intake(^8) (Mcal/wk)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRO</td>
<td>25.80</td>
<td>24.56</td>
<td>28.22</td>
<td>28.22</td>
<td>28.22</td>
<td>31.44</td>
<td>1.03</td>
<td>0.94</td>
<td>&lt;0.01^9</td>
<td>0.78</td>
</tr>
<tr>
<td>MRS</td>
<td>25.82</td>
<td>25.89</td>
<td>28.03</td>
<td>27.38</td>
<td>27.51</td>
<td>31.36</td>
<td>0.94</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADPr intake(^8) (kg/wk)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRO</td>
<td>1.13</td>
<td>1.08</td>
<td>1.24</td>
<td>1.24</td>
<td>1.24</td>
<td>1.38</td>
<td>0.05</td>
<td>0.30</td>
<td>&lt;0.01^10</td>
<td>0.52</td>
</tr>
<tr>
<td>MRS</td>
<td>1.14</td>
<td>1.14</td>
<td>1.26</td>
<td>1.25</td>
<td>1.29</td>
<td>1.51</td>
<td>0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Voluntary water intake (kg/wk)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRO</td>
<td>5.44</td>
<td>5.80</td>
<td>4.17</td>
<td>4.11</td>
<td>3.77</td>
<td>4.52</td>
<td>3.54</td>
<td>0.01</td>
<td>0.05</td>
<td>0.01^5,7</td>
</tr>
<tr>
<td>MRS</td>
<td>7.37</td>
<td>8.89</td>
<td>7.90</td>
<td>8.93</td>
<td>12.26</td>
<td>23.05</td>
<td>3.23</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total water intake(^11) (kg/wk)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRO</td>
<td>53.69</td>
<td>52.56</td>
<td>56.94</td>
<td>56.88</td>
<td>56.53</td>
<td>63.31</td>
<td>4.65</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.01^5</td>
</tr>
<tr>
<td>MRS</td>
<td>41.69</td>
<td>43.00</td>
<td>41.67</td>
<td>38.58</td>
<td>36.52</td>
<td>43.35</td>
<td>4.25</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)ADPr = apparent digestible protein; MR = milk replacer, MRO = milk replacer only; MRS = milk replacer with starter.

\(^2\)MRO group, n = 5; MRS group, n = 6. Diets were formulated to be isocaloric and isonitrogenous.

\(^3\)Significance declared at \(P \leq 0.05\).

\(^4\)Wk 6 includes from d 35 up to d 44 of the trial because calves were killed on d 42, 43, and 44.

\(^5\)Linear effect of MRO treatment.

\(^6\)Linear effect of MRS treatment.

\(^7\)Quadratic effect of MRS treatment.

\(^8\)Calculated based on equations from the NRC (2001).

\(^9\)Linear effect of week.

\(^10\)Quadratic effect of week.

\(^11\)Includes voluntary water intake and any water ingested when consuming MR.
had less need to consume water than MRS calves due to the increased amount of water fed with MR—hence the lower voluntary water consumption. As the experimental weeks progressed, water delivery to MRS calves through MR decreased, whereas water delivery through MR remained the same for MRO calves. This explains why total water intake was higher in MRO calves. When calves drink MR, 97% of the water associated with it goes to the abomasum instead of entering the rumen (Toullec and Guilloteau, 1989), whereas when calves voluntarily drink water, it presumably goes to the rumen. Ruminal water is required for supporting microbial population growth; treatment differences in water consumption and effects on rumen microbes were not studied in this research.

From a growth standpoint (Figure 2), the isocaloric and isonitrogenous nature of the diets was corroborated by the fact that BW did not differ for the interaction of treatment × week or treatment alone ($P = 0.26$ and $P = 0.41$, respectively) or ADG throughout the trial for the interaction of treatment × week or treatment alone ($P = 0.90$ and $P = 0.94$, respectively). Based on dairy NRC (2001) data, both diets provided the ME and ADPr necessary to gain between 0.4 and 0.6 kg/d throughout the trial, reflected in the ADG achieved between the treatments for the duration of the 6-wk trial (Figure 2A). As mentioned for the intake, we observed a noticeable dip in ADG around 2 wk, most likely due to the rumen cannulation surgery that had occurred a few days before weighing the calves. Compared with the ADG from the current 6-wk trial (MRO 0.46 ± 0.03 kg/d, MRS 0.46 ± 0.03 kg/d), the recommendation by Gelsinger et al. (2016) to maintain a minimum ADG of 0.5 kg/d to achieve elevated first lactation milk production shows that these diets providing 3.87 ± 0.06 Mcal of ME/d and 0.17 ± 0.003 kg/d of ADPr were not meeting the nutrient requirements to sustain somatic growth, especially after wk 4 (Figure 2A).

Diet affected fecal scores (data not shown); MRO calves had a higher frequency of scores ≥3 (i.e., runny consistency, similar to pancake batter) than MRS calves for the entire trial (27.6 vs. 16.5% of all observations, respectively; $P < 0.0001$). However, MRS calves were offered oral electrolytes more often (29 occasions) than MRO calves (18 occasions) throughout the experiment. This seems to indicate that the higher fecal scores in MRO calves were more likely caused by an absence of fecal bulk, potentially because of lower milk solids than because of bacterial or viral pathogens. In support of that, the administration of oral electrolytes commenced after only the calf had been visually inspected and checked for signs of dehydration; MRO calves were not typically dehydrated. Taken together, these observations suggest that the MRO calves may have experienced “nutritional scours,” reflecting their exclusively liquid diet instead of being a sign of illness (Roy, 1964; Leaver and Yarrow, 1972). No calf had a respiratory score ≥3, and no calves were treated for respiratory disease.

Rumen Morphometric Measurements

Results for rumen morphometric measurements are presented in Table 5. Calves on the MRS treatment had heavier empty rumens than the MRO calves (0.68 ± 0.04 vs. 0.39 ± 0.04 kg, respectively; $P < 0.01$), which aligned with previous reports that calves fed calf starter experience more rumen growth than calves fed a liquid-only diet (Harrison et al., 1960; Sutton et al., 1963; Stobo et al., 1966). We observed no difference between treatments in empty omasal plus abomasal...
weights (MRS 0.63 ± 0.07 kg; MRO 0.49 ± 0.07 kg; \(P = 0.20\)), demonstrating that even when calves are fed diets differing in the main site of digestion, the liquid diet that bypasses the rumen does not stimulate growth of the abomasum (with omasum).

Although we observed no differences in the total portion of the biopsy core samples (MRO 1.35 ± 0.10 kg; MRS 1.46 ± 0.10 kg; \(P = 0.42\)) or in the muscularis portion (MRO 0.78 ± 0.05 g, MRS 0.70 ± 0.05 g; \(P = 0.31\)), we did find a trend for an increase in the weight of the epithelial portion in MRS calves compared with MRO calves (0.77 ± 0.07 vs. 0.57 ± 0.07 g, respectively, \(P = 0.07\)). This was generally supportive of enhanced rumen epithelial growth (i.e., proliferation) when the tissue is exposed to a solid concentrate diet (Tamate et al., 1962; Sutton et al., 1963; Stobo et al., 1966). However, even without an obvious difference in the total or epithelial biopsy weights, we observed an increase in the 2-dimensional area of the rumen papillae of MRS calves compared with MRO calves (0.76 ± 0.08 vs. 0.15 ± 0.08 mm², respectively, \(P < 0.01\)), which supports many previous findings that solid concentrate diets fed to calves enhances rumen growth by affecting proliferation of the rumen tissue, resulting in larger papillae (Harrison et al., 1960; Stobo et al., 1966; Heinrichs, 2005).

### Real-Time qPCR

The primer efficiency of \(LGR5\) was not desirable (2.97), most likely due to the low expression of the gene (median cycle threshold 33.72). Thus, we chose to present \(LGR5\) data as undetectable (Table 6) to take a conservative approach in interpreting these data. In the intestine, \(LGR5\) has been shown to be important for maintaining epithelial homeostasis via its role as a co-receptor in the Wnt signaling pathway (Tan and Barker, 2015). Despite the low cycle threshold value for \(LGR5\), it is still worth noting that any expression might indicate the presence of stem cells in the epithelial tissue sample.

The other genes we included in our analysis were all relevant to skin epidermis. The following genes were not affected by treatment: \(ITGB1\), \(KRT14\), \(NOTCH1\), and \(TP63\).

### Table 5. Rumen morphometric measurements of calves fed 2 differing diets during the 6-wk trial

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>MRO</th>
<th>MRS</th>
<th>SEM</th>
<th>Test of fixed effect, (P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty reticulorumen weight (kg)</td>
<td></td>
<td>0.39</td>
<td>0.68</td>
<td>0.04</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Empty omasal and abomasal weight (kg)</td>
<td></td>
<td>0.49</td>
<td>0.63</td>
<td>0.07</td>
<td>0.20</td>
</tr>
<tr>
<td>Rumen biopsy samples (g)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscularis</td>
<td></td>
<td>0.78</td>
<td>0.70</td>
<td>0.05</td>
<td>0.31</td>
</tr>
<tr>
<td>Epithelium</td>
<td></td>
<td>0.57</td>
<td>0.77</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1.35</td>
<td>1.46</td>
<td>0.10</td>
<td>0.42</td>
</tr>
<tr>
<td>Papillae 2-dimensional area (mm²)</td>
<td></td>
<td>0.15</td>
<td>0.76</td>
<td>0.08</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

1MRO = milk replacer only; MRS = milk replacer with starter.
2MRO group, n = 5; MRS group, n = 6. Diets were formulated to be isocaloric and isonitrogenous.
3Significance declared at \(P \leq 0.05\).

### Table 6. Relative mRNA abundance of selected genes in rumen epithelial tissue relevant to potential stem cell function in the rumen epithelium of calves fed 2 differing diets at 6 wk of age

<table>
<thead>
<tr>
<th>Gene</th>
<th>Treatment</th>
<th>MRO</th>
<th>MRS</th>
<th>SEM</th>
<th>Test of fixed effect, (P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>(LGR5)</td>
<td></td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>(ITGB1)</td>
<td></td>
<td>0.021</td>
<td>0.017</td>
<td>0.0047</td>
<td>0.45</td>
</tr>
<tr>
<td>(KRT14)</td>
<td></td>
<td>0.15</td>
<td>0.22</td>
<td>0.05</td>
<td>0.33</td>
</tr>
<tr>
<td>(NOTCH1)</td>
<td></td>
<td>1.5 × 10⁻⁵</td>
<td>2.8 × 10⁻⁵</td>
<td>1.2 × 10⁻⁵</td>
<td>0.93</td>
</tr>
<tr>
<td>(TP63)</td>
<td></td>
<td>0.0028</td>
<td>0.0029</td>
<td>0.0014</td>
<td>0.93</td>
</tr>
</tbody>
</table>

1\(ITGB1\) = \(\beta_1\)-integrin; \(KRT14\) = keratin-14, \(LGR5\) = leucine-rich repeat-containing G protein-coupled receptor 5; MRO = milk replacer only; MRS = milk replacer with starter; ND = nondetectable; \(NOTCH1\) = notch-1; \(TP63\) = tumor protein p63.
2Relative mRNA abundance expressed as \(2^{-ΔCt}\) values; higher values equate to more mRNA.
3Primer efficiencies: \(LGR5 = 2.97\); \(ITGB1 = 2.11\); \(KRT14 = 1.97\); \(NOTCH1 = 2.27\); \(TP63 = 2.09\).
4Diets were formulated to be isocaloric and isonitrogenous.
5Significance declared at \(P \leq 0.05\).
TP63. Despite this, in terms of importance for skin epidermis stem cell characteristics, ITGB1 and NOTCH1 have been shown to be important for adhesion to either the basement membrane (Jones and Watt, 1993; Janes and Watt, 2006) or to neighboring cells in the basale cell layer (Watt et al., 2008; Zhang et al., 2016). A lack of difference between treatments suggests no effect of nutrients in the rumen altering the adhesive nature of the rumen epithelium when examined at the gene level on a tissue weight basis.

Both NOTCH1 and TP63 have been implicated for their roles in regulating proliferation (Watt et al., 2008; Zhang et al., 2016) and differentiation of progenitor cells (Yang et al., 2006; Senoo et al., 2007), which indicates neither treatment-exhibited differences in proliferation or differentiation events in the putative stem and progenitor cell populations in the rumen epithelium when examined at the gene level on a tissue wt/wt basis.

The results for gene expression were not isolated to potential stem and progenitor cells, but involved the entire rumen epithelium that was separated from the muscle. If only the stem cells were isolated, we might have seen a difference in gene expression; one such method could to use cryopreserved tissue sections (Choudhary et al., 2010) and subsequent laser capture microdissection that would allow for identification of putative stem cells (via BrdU labeling) and then subsequent qPCR analysis to look for gene expression of suspected stem cell markers (Steele et al., 2013). From a non–stem cell standpoint, both ITGB1 and KRT14 are the most highly expressed genes examined, which makes sense, considering that ITGB1 is an important protein for adhesion of basale cells to the basement membrane/extracellular matrix (Jones and Watt, 1993; Shen et al., 2017) and KRT14 is known to be one of the major keratin proteins found in the basale layer of skin epidermis important for cytoskeletal structure (Moll et al., 2008; Zhang et al., 2016).

Hematoxylin and Eosin Staining

Rumen muscle thickness did not differ between treatments ($P = 0.45$; Figure 3). Two potential explanations for this observation are offered. First, although others have noted that large feed particle sizes stimulate rumen muscle development (Harrison et al., 1960; Harding and Leek, 1972), the small average particle size of the starter feed we used may not have achieved this effect in MRS compared with MRO calves. The second and more likely potential explanation for the lack of rumen muscle thickness differences between MRS and MRO is that, although they were not offered starter feed, MRO calves all had at least one matted hairball (bezoar) in their rumen at slaughter (data not shown).

These hairballs likely formed through grooming and accumulated mass over time, preventing passage to the omasum, effectively trapping them in the rumen. The presence of bezoars likely contributed to at least some muscle development in the MRO calves. In our experience, rumen bezoars are common in calves fed all-milk diets and bedded with inorganic bedding.

The submucosa was thicker in MRO calves ($P = 0.05$), but the epithelium and corneum/keratin layers were thicker in MRS calves ($P = 0.02$ and $P = 0.05$, respectively). The submucosal layer consists of connective tissue that includes extracellular components as well as vasculature for nutrient transport (Baldwin, 1998). It is unclear why this layer was thicker in MRO calves, which had less nutrients in the rumen, but a potential explanation might involve invagination of the epithelium into the underlying submucosa during growth. In the ruminant forestomach, this growth of the epithelial tissue “into” the submucosal tissue has been shown in the growing fetal omasum (Lubis and O’Shea, 1978).
which might explain why the undeveloped epithelium in MRO calves had not yet invaded the submucosal layer, whereas the more developed epithelium in MRS calves had already done so.

An increase in the epithelial layer thickness of the MRS calves was expected due to the stimulating nature of feed in the rumen (Tamate et al., 1962; Sutton et al., 1963; Stobo et al., 1966). As opposed to bulk fill items such as hairballs/bezoars, feed that enters the rumen can be fermented to VFA, which can then be absorbed and metabolized by rumen epithelia. Hair is composed primarily of keratin, and to our knowledge, native rumen bacteria and fungi with specific keratinase activity are in low abundance (see Gopinath et al., 2015 and Sun et al., 2016). Also, this increase in epithelial thickness in MRS calves translated to increased corneum/keratin thickness in the rumen epithelium. Steele et al. (2011) noted no difference in thickness of the rumen corneum/keratin layer when mature cows were switched from a high-forage to a high-grain diet. Previous work has shown that a decrease in pH due to decreased forage in the diet of calves led to increased corneum thickness, supporting the protective role of the corneum/keratin layer of the rumen (Mirzaei et al., 2015). In this experiment, MRO calves had a higher rumen pH at content evacuation than MRS calves (7.52 ± 0.11 vs. 5.78 ± 0.36, respectively; $P < 0.01$), supporting the corneum/keratin thickness relationship with rumen pH. Another possibility is that the rumen contents of MRO calves (i.e., bezoars) added an abrasive element to the luminal cell layers, decreasing keratin thickness, that was absent in the starter fed to MRS calves.

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Representative immunohistochemical images of 5-bromo-2′-deoxyuridine (BrdU) and Ki67 staining in the rumen of an MRO (milk replacer only) calf during the 6-wk trial. (A) Green staining indicates a BrdU-positive cell; (B) red staining indicates a Ki67-positive cell; (C) blue staining indicates nuclei (4′,6-diamidino-2-phenylindole; DAPI); (D) composite image of BrdU, Ki67, and DAPI showing location of staining in the rumen tissue. Images were taken at 40×; the scale bars represent 50 μm.
Representative BrdU and Ki67 images for both MRO and MRS treatments are shown in Figures 4 and 5, respectively. The MRO calves had an increased proportion of BrdU-labeled stratum basale cells per unit area than MRS calves ($P < 0.01$; Figure 6A). This increase in proportion of basale cells that retained the BrdU label indicates that, per unit area, more putative stem or progenitor cells might have been present in the rumens of calves with undeveloped rumens. To our knowledge, we have no other data to compare our findings to in the rumen. However, similar studies examining BrdU-label-retaining cells in murine and bovine models will be discussed. When measuring stratified squamous epithelium (i.e., skin epidermis and oral mucosa) or a simple columnar epithelium (i.e., colon) in murine models, the percentage of label-retaining cells were 0.9 to 4.2% and 7.0%, respectively, when BrdU was allowed to wash out around 28 to 30 d (Bickenbach and Chism, 1998; Kim et al., 2004). In calves allowed 37 d for BrdU to wash out, a range of 0.24 to 5% of label-retaining cells was measured in different regions of the mammary epithelium (Capuco, 2007). Also, previous results in calves showed no difference in the proportion of BrdU-label-retaining cells (30 d washout) in mammary glands at 65 d of age when heifer calves were fed differing levels of energy and protein (Daniels et al., 2009). Soberon and Van Amburgh (2017) also compared diets differing in energy and protein fed to heifer calves and observed no difference in BrdU-labeled cells in the mammary gland. Another explanation for this observation, similar to that posited by Soberon and Van Amburgh (2017), might be that there is no difference in the percentage

**Figure 5.** Representative immunohistochemical images of 5-bromo-2′-deoxyuridine (BrdU) and Ki67 staining in the rumen of a milk replacer and starter (MRS) calf during the 6-wk trial. (A) Green staining indicates a BrdU-positive cell; (B) red staining indicates a Ki67-positive cell; (C) blue staining indicates nuclei (4′,6-diamidino-2-phenylindole; DAPI); (D) composite image of BrdU, Ki67, and DAPI showing location of staining in the rumen tissue. Images were taken at 40×; the scale bars represent 50 μm.
of BrdU-labeled cells counted per unit area, but that the larger MRS rumens (empty reticulorumen weight, MRO 0.39 ± 0.04 kg and MRS 0.68 ± 0.04 kg; \( P < 0.01 \)) would have essentially diluted the labeled cells in the MRS calves, so that the total BrdU labeled was potentially the same between treatments. Alternatively, even though the washout period should have allowed for removal of these cells, BrdU-label-retaining cells could have been growth-arrested cells and not true stem or progenitor cells (Daniels et al., 2009).

The proliferation status of the rumen epithelium, measured by Ki67-positive basale cells, indicated that MRS calves tended to have a greater proportion of proliferating basale cells than MRO calves (\( P = 0.08; \) Figure 6B). These Ki67 data were reflective of the tissue’s proliferative status at slaughter, whereas the BrdU data reflected the 25-d washout effects. Goodlad (1981) demonstrated an increased turnover rate in the rumen epithelium of sheep fed a diet transitioning from forage to concentrate (4.3 ± 2.8 d turnover time) compared with forage-based (16.5 ± 0.7 d) and concentrate-based (10.9 ± 2.0 d) diets. Ohwada et al. (1984) used a metaphase-arresting agent in adult ewes fed a diet of orchard grass hay and concentrate to determine rumen epithelial time spent in mitosis, which was found to be 2.8 h. The data from Ohwada et al. (1984) did not allow for calculation of proliferation rate, but assuming a 24-h cell cycle, they provided a growth fraction (i.e., proliferating basale cells) of approximately 9%. This growth fraction agrees with the values obtained by Goodlad (1981) for forage-fed ewes (8.95 ± 0.46%) but was drastically lower than that for ewes transitioned from a forage to a concentrate diet (32.65 ± 9.79%), and about half of that when ewes were fed only concentrate (17.25 ± 1.31%). These results, paired with those of the present study, show that calves fed a diet containing starter (MRS) had a similar proliferative rate (18.37 ± 2.57%) to ewes fed only concentrate (Goodlad, 1981). Also, calves fed MRO had a similar proliferative rate (10.84 ± 2.81%) to that of ewes fed only forage in the studies by Goodlad (1981) and Ohwada et al. (1984).

**CONCLUSIONS**

The results from this study indicate that the form of preweaning diet might play a role in the potential stem and progenitor cell populations in the rumen epithelium. An increased proportion of BrdU-labeled basale cells per unit area in MRO calves compared with MRS calves indicates a lack of dietary response in the stem and progenitor cell populations when calves are fed starter along with MR. As well, MRS calves tended to have more rumen epithelial cell proliferation than MRO calves; this trend appeared to be reflected in the increased thickness and weight of the epithelial tissue layer.

**ACKNOWLEDGMENTS**

Carrie A. Ceh, a graduate student at Virginia Tech (Blacksburg, VA), helped with calf feeding and care. We thank R. Michael Akers, Rebecca R. Cockrum, Mark D. Hanigan, Robin R. White (Virginia Tech), and Erin E. Connor (USDA, Beltsville, MD) for serving on Yohe’s PhD guidance committee and for providing editorial input on this manuscript. This work was supported by the Virginia Agricultural Experiment Station (Blacksburg, VA) and the USDA National Institute of Food and Agriculture, U.S. Department of Agriculture (Washington, DC). Additionally, N. R. Hardy was the recipient of a John Lee Pratt Summer Research Intern-
ship grant (Virginia Tech); funds supported the gene expression work reported herein.

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