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Effects of milk replacer and whole milk feeding on rumen development, expression of genes related to volatile fatty acid absorption, and rumen bacteria in calves

Ahmet CEYLAN[1], Ali ÇALIK[2], Burcu EKİM[3], Alev Gürol BAYRAKTAROĞLU[1], Oğuz ÇALIŞICI[4], Doğukan ÖZEN[5], Pınar SAÇAKLI[2,*]

[1]Department of Histology Embryology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey
[2]Department of Animal Nutrition & Nutritional Diseases, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey
[3]Life Sciences Research and Application Centre, Gazi University, Ankara, Turkey
[4]Clinic for Cattle, University of Veterinary Medicine Hannover, Hannover, Germany
[5]Department of Statistics, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey

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Abstract: This research examined the effects of liquid feeds on calf performance, rumen development, rumen cellulolytic bacteria count, and total bacteria diversity. Twenty Holstein bull calves were allocated to 2 groups either fed with milk replacer (MR) or whole milk. Calves fed milk tended to have higher (P = 0.058) average daily weight gain as compared to calves fed with MR during the entire experimental period. Starter mixture intake was not influenced by either milk or milk replacer feeding. Calves fed with milk had higher (P < 0.001) proliferative cells in the rumen papilla. The expressions of MCT1, MCT4, NHE1, NHE3, ACSS1, AceCS2, and ACSM1 genes, which are related to volatile fatty acid absorption, in the dorsal sac of the rumen were similar for calves fed either MR or whole milk. *Fibrobacter succinogenes* (P = 0.017) and *Ruminococcus flavefaciens* (P = 0.037) were significantly higher in the rumen of the milk-fed calves. Moreover, denaturing gradient gel electrophoresis (DGGE)-derived dendrograms showed noticeable differences in ruminal microbial population between MR-fed and milk-fed calves. In conclusion, feeding calves with whole milk, which contains better quality protein and nutrients, instead of milk replacers, positively affects rumen development, cellulolytic bacteria numbers and total bacteria diversity.

Key words: Calf, denaturing gradient gel electrophoresis (DGGE), gene expression, milk, milk replacer

1. Introduction

Unlike adult ruminants, newborn calves have a physically and metabolically undeveloped rumen [1]. However, with the initiation of solid feed consumption, the rumen epithelium undergoes physical and metabolic development due to the growth of rumen bacteria and a subsequent increase in volatile fatty acid (VFA) concentrations [2]. Serving a limited amount of liquid feed and the early introduction of solid feeds are common practice in dairy farms and are highly important for functional development of the rumen and for early weaning [1–3]. Besides, liquid feed is the main nutrient and energy source for calves until they achieve enough of a solid feed intake amount for weaning. Therefore, liquid feed type and its nutritional quality is undoubtedly important for growth, health, and gastrointestinal tract (GIT) development [4,5].

Whole milk is the best liquid feed source for young ruminants due to its nutrient composition and bioactive peptide content. However, utilizing saleable whole milk for calf nutrition, which is originally intended for human consumption, is not an economic feeding strategy in modern dairy cattle farming [6]. From an economic point of view, a variety of milk substitutes have been developed as a blend of less expensive alternatives to provide nutritional components for newborn ruminants [6]. However, even if the milk replacers are formulated to mimic whole milk's nutrient composition, significant differences can be expected between calves fed whole milk versus milk replacer in terms of growth performance and GIT development [5,7,8]. Previous studies showed that stimulation of milk replacers on abomasum and small intestinal development is less than whole milk, mostly due to its protein source and quality [3,9]. Moreover, bioactive peptides, like hormones (insulin, somatostatin, prolactin etc.) and growth factors (epidermal growth factor I–II, insulin like growth factor etc.), which are present in whole milk, also play important roles in GIT development of neonatal calves [10]. Therefore, due to the lower protein

* Correspondence: psacakli@ankara.edu.tr

quality, presence of antinutritional factors, and lack of bioactive components, milk replacer feeding may also slow down rumen papilla development by influencing lower GIT development, in contrast to whole milk feeding, where the liquid feed is digested and absorbed [5, 11].

Earlier studies showed that liquid feed type and composition influenced calf performance [3,5,9], rumen papilla development [5,11], and rumen MCT1 mRNA expression [8]. While the liquid feed bypasses the reticulorumen b ythe reticular groove and is digested and absorbed in lower GIT, it nevertheless indirectly influences the development of the rumen by providing ample nutrients [8,11,12]. Based on the previous findings that suggest a beneficial effect of whole milk feeding on calf performance and rumen development, the current study aimed to compare milk replacer or whole milk feeding on calf performance, gene expression level of important genes related to VFA transport and metabolism, rumen epithelial cell proliferation, rumen cellulolytic bacteria counts and structure of the bacterial population applying PCR-DGGE.

2. Materials and methods

2.1. Animals and management

This study was approved and conducted under the guidelines of Animal Ethics Committee of Ankara University (2014-17-119). Twenty Holstein bull calves were obtained from a commercial farm between 06-18 March 2016, with 42.43 ± 0.75 kg (mean ± SE) of body weight (BW). The calves were separated from their dams following birth. All calves were received postpartum colostrum before involving the study. Animals were allocated into 2 groups, each group comprised of 10 calves, according to their BW and date of birth, to balance the average body weight and age between the groups. Calves were housed in individual hutches with straw bedding. Bedding material was observed daily and changed with new straw at least weekly. Groups were fed with either milk replacer, which contains skimmed milk powder, whey protein concentrate, whey, wheat gluten, palm oil powder, lysine, methionine, and vitamin-mineral premix (80% of the crude protein was milk based) or whole milk. Nutritional composition of the whole milk was analysed on a regular basis. Amount of the milk replacer (powder-to-water ratio) was adjusted according to the whole milk nutrient composition to ensure approximate energy consumption between the treatments. Milk replacer was prepared in a mixer with warm water and served to calves around 40–42 °C. Milk was obtained from main milk cooling tanks at the farm, warmed to 40–42 °C and served without pasteurization. Milk replacer or milk were offered via nipple buckets twice daily in two equal portions between 07:30–08:30 and 17:00–18:00 from d 0 to d 49 and once daily from d 50 to d5 6. Refusals of liquid feeds were recorded daily. Nipple buckets were cleaned and disinfected after each use. All calves had free individual access to fresh water and calf starter (22.91% CP). Nutrient composition of the milk replacer and starter mixture were analysed according to AOAC [13]. Calf starter was offered between d 10 and d 56 of the study and concentrate intake was recorded. Calves were individually weighed on d 0 (day of birth), 15, 25, 40, and 56 of the study. Feed intake (FI) was recorded for three consecutive days and mean values for individual calves were calculated on d 25, 40, and 56 of the study. On d 56 of the study, four calves from each group were slaughtered for sampling. After slaughter, the abdominal cavity of each calf excised to collect rumen tissue samples (from dorsal sac of the rumen) and rumen fluid for further analysis. Samples were snap frozen in liquid nitrogen and then placed to –86 °C.

2.2. Immunohistochemical analysis

Rumen samples from each calf were fixed for 7 h in Bouin's solution. Following paraffin embedding, the specimens were cut into 4 μm sections. IHC staining of the tissue samples were performed collectively on serial sections with the standard streptavidin–biotin complex method. Briefly, after deparaffinization and rehydration, slides were pretreated with 0.01 M citric acid buffer (pH6) according to a heat-mediated antigen retrieval method. Slides were then placed in a humidity chamber and incubated for 1 h with the Ki67 antibody (clone MIB-1, Dako, Glostrup, Denmark) at dilutions of 1:100 at room temperature. Tissue staining was visualized with a 3,3′-diaminobenzidine tetrahydrochloride (DAB, Invitrogen, Carlsbad, CA, USA) substrate chromogen solution. Slides were counterstained with Gill's hematoxylin, dehydrated, and mounted with Entellan (Merck, Darmstadt, Germany). Images were captured with a Leica DM2500 light microscope connected to a Leica DFC450 digital camera (Leica Microsystems, Wetzlar, Germany). Cell counting was processed using software (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA) with a manual tag function in selected 10 high power fields. Each stained nucleus was considered positive, regardless of intensity.

2.3. Short chain fatty acid analysis

Rumen fluid samples were thawed and 5 mL fluid was transferred to screw-cap tubes. Samples were centrifuged at 4.000 rpm for 15 min at 4 °C. One millilitre of supernatant was transferred to a 1.5 mL microcentrifuge tube containing 0.2 mL ice-cold metaphosphoric acid solution (25%). Tubes were placed on ice bath for 30 min to precipitate proteins. Subsequently tubes were centrifuged at 11.000 rpm for 15 min at 4 °C. Samples were analysed using gas chromatography (GC) coupled with a 30m × 0.25mm × 0.25μm column (Nukol, Product Number: 24107) and flame ionization detector (FID) to

determine the concentrations of various volatile fatty acids (Shimadzu GC, Shimadzu Co., Kyoto, Japan). The injector-port and FID temperatures were fixed at 240 °C. The volume of injection was set at 1 µL. The initial GC oven heat was started at 120 °C for 1 min and raised by 4.5 °C/min to 185 °C. Helium was used as the carrier gas. Concentration of these compounds were determined using a volatile free acid mix standard (Supelco, 46975-U; Sigma-Aldrich, St. Louis, MO, USA).

2.4. Total RNA isolation and reverse transcriptase (RT) reaction

Total RNA was extracted from each rumen tissue using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA) following the treatment with RNase-free DNase I recombinant (DNaseI; Roche Diagnostics, Mannheim, Germany). Total RNA was measured at 260 nm using a NanoDrop ND-2000 spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized from 1 µg total RNA from each sample using cDNA synthesis kit (NG dART RT kit, Eurx Molecular Biology Products, Gdansk, Poland) according to the manufacturer's protocol. All cDNA products were stored at –20 °C for further analysis.

2.5. Quantitative real-time PCR

Real-time polymerase chain reaction was performed for the specified genes [Monocarboxylic acid transporter type 1 (MCT1), monocarboxylic acid transporter type 4 (MCT4), Na+/H+ exchange protein 1 (NHE1), Na+/H+ exchange protein 3 (NHE3), Acyl-CoA synthetase short-chain family member 1 (acyl-CoA synthetase), Acetyl-CoA synthetase 2 (acetyl-CoA synthetase 2), Acyl-CoA synthetase medium-chain family member 1 (butyrl-CoA synthetase), and β-actin] using Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA, USA) according to the manufacturer's protocols. The qRT-PCR was done using a Power SYBR Green PCR Master Mix (Roche Applied Science, Mannheim, Germany) to analyse gene expression. Primer details are listed in Table 1. During the PCR reaction, samples were subjected to an initial denaturation phase at 95 °C for 15 min, followed by 40 cycles at 95 °C for 15 s, annealing at 60 °C for 20 s and an extension period at 72 °C for 20 s. Each sample was run in triplicate. The β-actin gene was as an endogenous control. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative expression levels of the genes of interest [14]. The calibrator for each gene was the average ΔCt value from the milk fed group.

2.6. Rumen cellulolytic bacteria

Total genomic DNA was isolated from frozen rumen content samples using a bead beating method (GeneMATRIX Stool DNA Purification Kit, EurX Molecular Biology Products). Approximately 0.2 mg of rumen content was homogenized and total DNA was isolated according to the manufacturer's protocol. DNA was quantified by measuring the absorbance at 260 nm using a NanoDrop ND-2000 spectrophotometer (NanoDrop 2000, Thermo Fisher Scientific).

Table 1. Primer sequences of the genes analysed by quantitative RT-PCR.

Gene name	Primer (5' to 3')	
Monocarboxylate transporter type 1 (MCT1)	Forward	AACCTCTTCTTCCAGGCAAGC
	Reverse	CAGCAGGCTTCTCCCCTAA
Monocarboxylate transporter type 4 (MCT4)	Forward	GGTCAGCGTCTTCTTCAAGG
	Reverse	TGAAGAACATGGCAAAGCTG
Na^+/H^+ exchange protein 1 (NHE1)	Forward	ATCGCCCTCTGGATCCTACT
	Reverse	ACCACGAAGAAGCTCAGGAA
Na^+/H^+ exchange protein 3 (NHE3)	Forward	GCAAGTCCGTGGACTCGTTC
	Reverse	CGTCGGAGCCGGTGTC
Acyl-CoA synthetase short-chain family member 1 (ACSS1)	Forward	GGATCAACGATGCCCAATGC
	Reverse	GGAAACATCCAGATGCCCCA
Acetyl-CoA synthetase 2 (AceCS2)	Forward	TGCAGGGTAAGCCGAAAGAG
	Reverse	GGGTCTTCAGCATCACACCA
Acyl-CoA synthetase medium-chain family member 1 (ACSM1)	Forward	ACCAAGCGTTTCCTAGACAGT
	Reverse	TGATAAAGATCGGCAGCGCA
β-actin	Forward	CGTGAGAAGATGACCCAGATCA
	Reverse	TCACCGGAGTCCATCACGAT

2.7. Preparation of external standards and qPCR analysis
Modified chopped meat medium was used for *Ruminococcus flavefaciens* (ATCC 19208) strain and incubated at 37 °C for 2–3 days under anaerobic conditions. For *Fibrobacter succinogenes* (ATCC 19169) culture, 1943 Fibrobacter medium was used in anaerobic conditions at 37 °C for 3–4 days. Bacterial colonies were collected in potassium phosphate buffer solution (PBS). Pure bacterial DNA was isolated using the GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit (EurX Molecular Biology Products). DNA isolates were stored at –20 °C. For each bacterial standard, dilutions of 1/10, 1/100, 1/1,000, 1/10,000, 1/100,000 were prepared and species-specific standardization curves were generated using each bacterial specific primer sequences (Table 2). The amplification reaction for quantitation was performed using SYBR Green (Promega, Madison, WI, USA) in Applied Biosystems 7500 Fast Real-Time PCR System. For each qRT-PCR reaction, 18µL of qPCR master mix containing 10 µL of GoTaq qPCR master mix (Promega), 1 µL forward and reverse primers each, and 6 µL of sterile nuclease-free water per reaction were added to each well. Subsequently, 2 µL of the diluted DNA was added each well to a final volume of 20 µl. PCR condition was follows: initial denaturation for 15 min. and then 45 cycles of denaturation at 95 °C for 15 s, followed by annealing at 60 °C for 20 s and elongation at 72 °C for 20 s.

2.8. PCR-DGGE analysis
Total bacterial 16S rDNA obtained from rumen samples were amplified by touchdown PCR using primers V3-GCF and V3-R (Table 3). Denaturing gradient gel electrophoresis (DGGE) analysis was performed on DCode Mutation Detection System (Bio-Rad Laboratories GmbH, Feldkirchen, Germany) on an 8% polyacrylamide gel with 35%–60% denaturing gradient [100% denaturant 7M urea (Merck), and 40% (v/v) formamide (Merck) in 1×TAE-buffer]. Samples were run at 60 °C and 50 V for 10 min followed by 130 V for six and a half hours. Gel was stained for 10 min in 1×TAE buffer with ethidium bromide stain (1%) and photographed under UV transillumination. DGGE bands were detected using the band-search algorithm. Similarity matrices were formed between the samples by evaluating the bands of the DGGE; the presence of a band at a certain level coded as "1" and the absence as"0". The similarity score and cluster analysis of the DGGE gel profiles of samples were performed using the unweighted pair group method (UPGMA) and the similarity matrix arithmetic mean based on the dice equation and the microflora dendrograms of the samples were drawn. All analyses were carried out using the NTSYS 2.1 (numerical taxonomy and multivariate analysis system) program.

2.9. Statistical analysis
Before applying any significance test procedures, the assumption of normality was investigated using the Shapiro–Wilk test whereas the assumption of homogeneity of variances was examined using the Levene's test. Descriptive statistics for each variable in the study were calculated and expressed as "mean ± standard error of the mean". To examine the significance of statistical difference between treatment groups, independent sample t test was used. $P < 0.05$ criteria was used to evaluate statistical significance. All computational works were done with the help of SPSS 14.01 software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Diet composition, calf performance and health
Analysed and calculated compositions of milk, milk

Table 2. Primer sequences for detection of rumen cellulolytic bacteria.

Target Bacteria	Strain	Primer(5' to 3')		Annealing temperature
Ruminococcus flavefaciens	ATCC 19208	Forward	CGAACGGAGATAATTTGAGTTTACTTAGG	60 °C
		Reverse	CGGTCTCTGTATGTTATGAGGTATTACC	
Fibrobacter succinogenes	ATCC 19169	Forward	GTTCGGAATTACTGGGCGTAAA	60 °C
		Reverse	CGCCTGCCCCTGAACTATC	

Table 3. Universal primers and sequences of bacterial V3 16S rRNA regions.

Name of the primer	Sequence (5' to 3')
338F 16S rDNA V3 F+GC tailed	CGC CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GAC TCC TAC GGG AGG CAG CAG
534R 16S rDNA V3-R	ATT ACC GCG GCT GCT GG

replacer and starter feed are presented in Table 4. Milk replacer was formulated to contain 24% CP and 19% fat. Analysed results showed that MR contained 24.05% CP (N × 6.38) and 18.77% fat. Milk samples were analysed on a regular basis with results expressed as the average of the analysed data. As expected, milk had more CP, fat and ME as compared with MR. However, lactose content higher in MR in comparison to that of whole milk. Average daily gain (ADG), BW and calf starter intake are presented in Table 5. Body weight was not affected by liquid feed regimen throughout the entire experiment period (Table 5). However, ADG tended to be higher in calves fed with milk as compared to calves fed with milk replacer (P = 0.058). Starter mixture intake were not influenced by either milk or milk replacer feeding on d 25, 40, and 56 of the study.

3.2. Rumen immunohistochemistry

Numbers of Ki67 positive cells in the rumen papilla of the calves fed either MR or milk are shown in Figure 1. Additionally, an image of the positive cells is given in Figure 2. Calves fed with milk had significantly higher (P < 0.001) proliferative cells in the rumen papilla in comparison to those fed MR.

3.3. Rumen volatile fatty acid concentration

The effect of liquid feed treatments on VFA profiles in the rumen are presented in Table 6. Molar concentrations of acetic, propionic, and butyric acids were not influenced by liquid feed treatments. Similarly, no differences were observed between milk and MR feeding in terms of total VFA concentration (mmol/L).

3.4. Rumen epithelium gene expression

The mRNA abundances of MCT1, MCT4, NHE1, NHE3, ACSS1, AceCS2, and ACSM1 in the dorsal sac of the rumen were similar for calves fed either MR or whole milk (Figure 3).

3.5. Rumen cellulolytic bacteria

Populations of rumen cellulolytic bacteria are presented in Figure 4. Milk-fed calves had significantly higher number of *Fibrobacter succinogenes* (P = 0.017) and *Ruminococcus flavefaciens* (P = 0.037) as compared with MR-fed calves.

3.6. PCR-DGGE profile of bacterial community

Total number of bands from rumen samples of milk and MR groups ranged from 12 to 19 and 11 to 17, for each group respectively. Bands were very consistent, with minimal animal-to-animal variations. According to the clustering analysis of rumen fluids, the DGGE dendrogram revealed that samples from MR- and milk-fed calves were distinctly grouped (Figure 5).

4. Discussion

Liquid feed is the most important energy and protein source from birth to weaning. In a modern calf rearing system, a certain amount of liquid feed is provided to increase solid feed intake for early weaning and maximum rumen development. However, previous studies revealed that liquid feed type and composition also had a significant indirect effect on rumen development by improving abomasum and small intestine morphology [5,8,11]. Based on the previous findings, this research focused to determine the effects of either MR or whole milk feeding on calf performance, expression level of important genes related to VFA transport and metabolism, rumen epithelial cell proliferation, rumen cellulolytic bacterial counts and bacterial diversity in the rumen fluid using PCR-DGGE.

In this study, no differences were determined in body weight of calves fed either MR or milk during the entire experimental period. However, ADG was tended to be higher (529.6 vs. 441 g/day) in milk fed calves in comparison to those fed MR (P = 0.058). Gorka et al. [5] revealed that calves fed MR containing 24% soy protein concentrate had lower weight gain than those fed with whole milk. Authors concluded that positive effects of whole milk feeding on calves' performance might be related to their effect on abomasum, small intestine, and rumen papilla development [5]. Similarly, Niwińska and Strzetelski [4] noted that milk feeding significantly improved daily weight gain as compared with milk replacer feeding. Reduction in growth performance is an expected

Table 4. Analysed and calculated composition of liquid feeds and starter mixture[1].

	Liquid feed		Solid feed
Item	Milk replacer[2]	Milk	Calf starter
Analysed composition			
Dry matter, %	96.31	12.25	88.78
Crude protein, %	24.97	25.55	22.91
Crude fat, %	19.50	28.16	3.58
Crude fiber, %	0	0	5.85
Ash, %	7.68	7.85	5.90
Calculated composition			
Lactose[3], %	47.85	38.44	0
ME[4] (Mcal/kg)	4.60	5.02	3.09

[1]Results are given on a dry matter basis.
[2]Contains skimmed milk powder, whey protein concentrate, whey, wheat gluten, palm oil powder, lysine, methionine, and vitamin-mineral premix (80% of the crude protein was milk based).
[3] Lactose (%) = 100–crude protein–crude fat–ash (calculations are made on a 100% dry matter basis).
[4]Metabolizable energy.

Table 5. Body weight and average daily gain of calves fed milk replacer or milk[1].

	Treatment[2]		Statistics
Item[3]	MR	Milk	P
BW, day of birth	43.07 ± 1.23	41.79 ± 0.89	0.411
BW, d 15	49.57 ± 0.95	44.50 ± 1.21	0.170
BW, d 25	49.57 ± 1.15	50.80 ± 1.35	0.497
BW, d 40	60.02 ± 1.06	60.60 ± 1.47	0.752
BW, d 56	68.17 ± 1.26	72.10 ± 2.22	0.140
Calf Starter Intake, d 25	160.1 ± 28.63	122.8 ± 26.44	0.351
Calf Starter Intake, d 40	336.9 ± 78.52	401.0 ± 73.09	0.558
Calf Starter Intake, d 56	562.9 ± 52.65	621.8 ± 99.64	0.608
ADG, g/d	441.0 ± 21.76	529.6 ± 35.2	0.058

[1]Data represent mean values of 10 replicates per treatment.
[2]MR: milk replacer, Milk: milk, nontreated.
[3]BW: body weight, ADG: average daily gain.

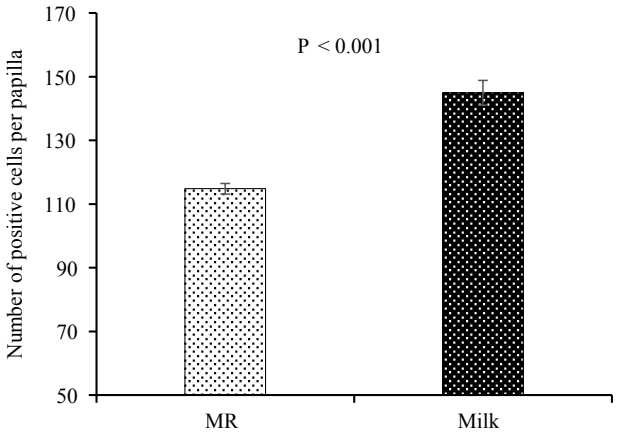

Figure 1. Number of proliferating cells in the rumen papilla of calves fed MR (milk replacer) or milk (P < 0.001). Data represent mean ± SE values of 4 replicates per treatment.

outcome for newborn calves when they are fed with milk replacers compared with whole milk [15]. The possible reasons underlying the growth-reduction in MR-fed calves might be attributed to protein quality of the MR, presence of antinutritional factors, especially in the plant-based MR and their direct effect on lower GIT development. Ragionieri et al. [11] suggested that improvements in lower GIT not only influenced growth performance, but may also increase solid feed consumption. In newborn calves, solid feed consumption and thereby an increase in VFA concentration are considered a main factor influencing proliferative activity of rumen epithelium cells [2]. However, our results show that liquid feed type (MR vs. milk) had no impact on starter mixture intake during the entire study. In agreement with our results, previous studies did not observe differences between milk-fed calves and MR-fed calves in terms of solid feed intake [4,5]. The reason behind the unchanged feed intake in this study might be related to the quality of the milk replacer which contains plenty of milk protein (approximately 80% of the protein was milk derived).

A number of anatomical and functional changes take place in newborn calves, especially in their forestomach, while they are developing towards an adult rumen [16]. Observed changes in the rumen, like an increase in rumen volume and development of rumen papillae, mainly need solid feed and ruminal fermentation. Sakata and Tamate [17] suggested that an increase in the rate of intraruminal concentration of butyrate promotes the proliferation of epithelial cells. Propionate and acetate also have proliferative effects on rumen epithelium, however, their effects were observed to be lower than butyrate [18]. Moreover, Mentschel et al. [19] revealed that butyrate induced ruminal papillary growth not only by increasing mitotic index, but also by lowering apoptosis. According to our results molar proportions or concentrations of acetate, propionate, and butyrate were not influenced by either MR or whole milk feeding, most likely due to the unchanged solid feed consumption. However, we observed an increase in proliferative activity of rumen epithelial cells in calves fed with whole milk. Gorka et al. [5] revealed that calves fed with whole milk had significantly lower acetate and propionate in comparison to calves fed with MR. On the other hand, they did not observe any differences between whole milk and MR-fed calves in terms of

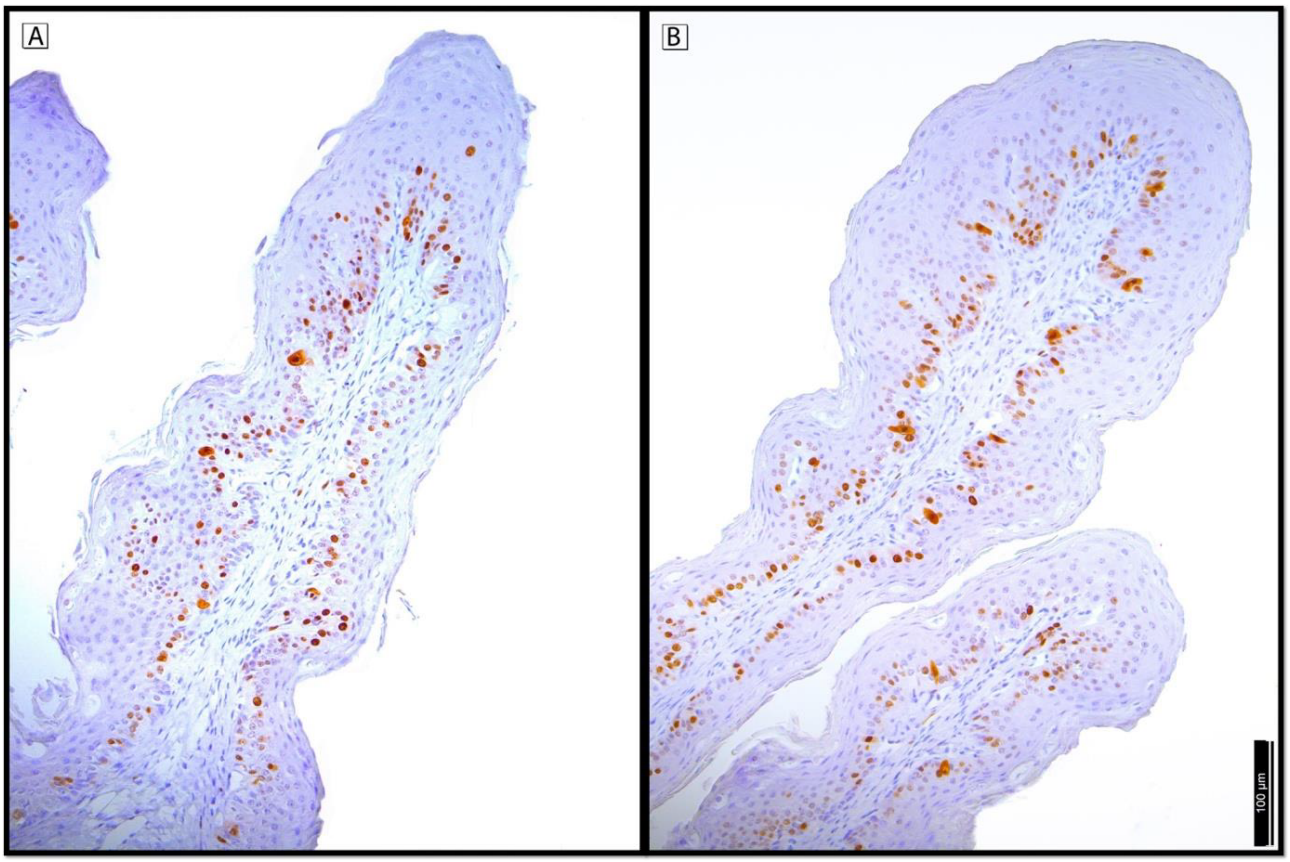

Figure 2. Quantitative immunohistochemical analysis of rumen proliferating cells with Ki67 proliferation marker. Bar 100 µm. A: milk replacer, B: whole milk.

Table 6. Rumen VFA profile in calves fed MR or milk on d 56[1].

	Treatments		Statistics
Item[2]	MR	Milk	P
Acetic acid, %	67.20 ± 2.14	63.05 ± 0.37	0.105
Butyric acid, %	7.94 ± 0.89	8.49 ± 1.49	0.763
Acetate/propionate ratio	2.75 ± 0.46	2.23 ± 0.21	0.085
Total VFA mmol/L	57.79 ± 8.60	62.55 ± 2.33	0.613

[1]Data represent mean values of 4 replicates per treatment.
[2]VFA: volatile fatty acid.

butyrate concentration. In agreement with our results, authors noticed significant improvement in rumen papilla development [5]. It can be assumed that the improvements in rumen epithelial mitotic index are related to beneficial effects of whole milk on abomasum and small intestinal development. Another possible explanation for increased rumen epithelial cell proliferation might be related to the higher digestibility and absorption rate of milk nutrients as compared with milk replacer nutrients. Both energy and protein intake markedly increased plasma insulin-like growth factor-1 concentration, which plays a crucial role in the proliferation of many cell types, including rumen epithelial cells [20–22].

In addition to their effects on functional development of rumen epithelium, VFA's are also important substrates in ruminant energy metabolism [23,24] and they need to be effectively absorbed and transferred across the rumen epithelium prior to use by the ruminant [25].

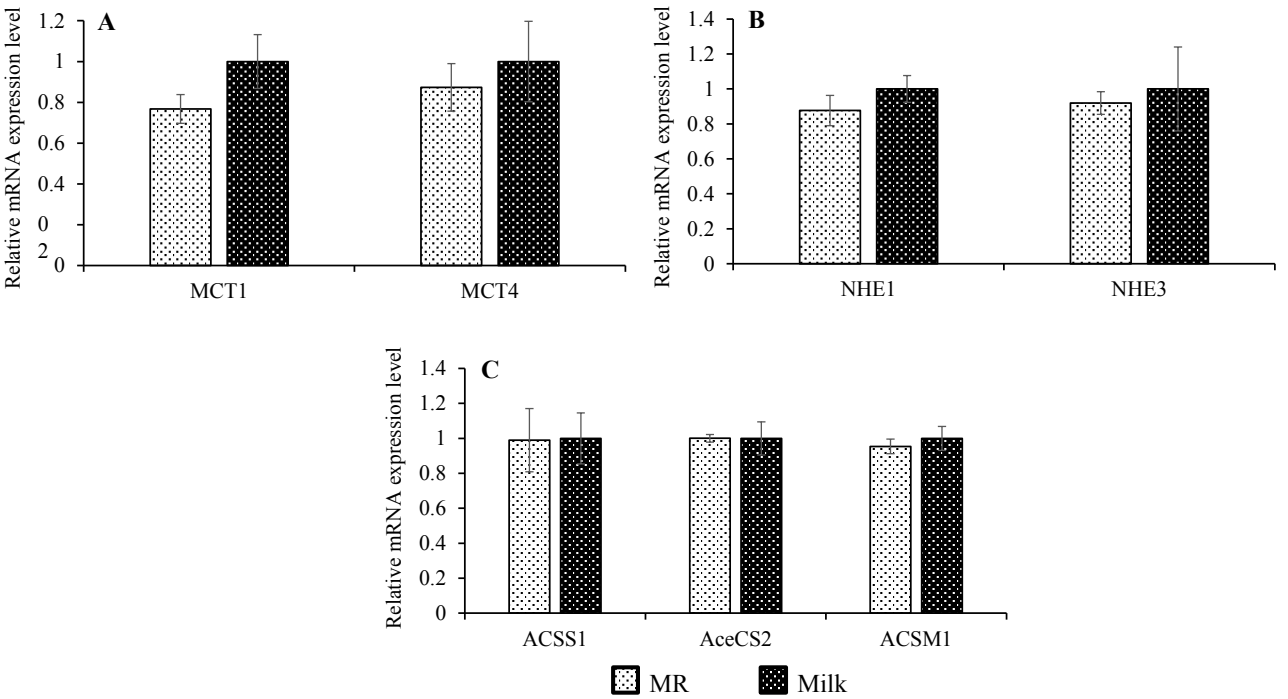

Figure 3. Effects of MR or milk feeding on MCT1 (monocarboxylate transporter type 1) and MCT4 (monocarboxylate transporter type 1) (A), NHE1 (Na^+/H^+ exchange protein 1)and NHE3 (Na^+/H^+ exchange protein 3) (B), ACSS1 (Acyl-CoA synthetase short-chain family member 1), AceCS2 (Acetyl-CoA synthetase 2), and ACSM1 (Acyl-CoA synthetase medium-chain family member 1) (C) mRNA abundances in the rumen epithelium on d 56. Data represent mean ± SE values of 4 replicates per treatment.The calibrator for each gene was the average ΔCt value from the milk fed group.

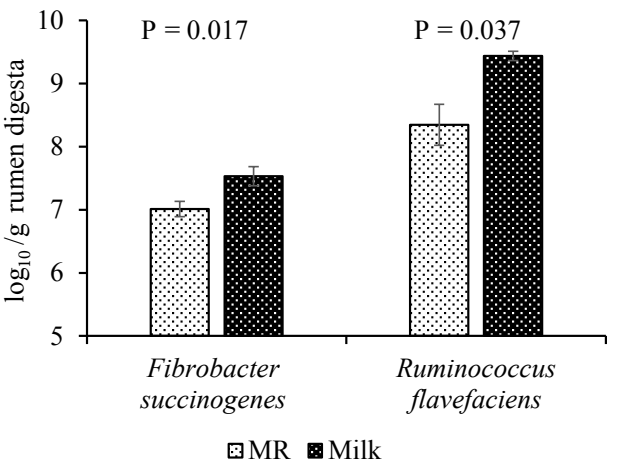

Figure 4. Effects of MR or milk feeding on rumen *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*. Data represent mean ± SE values of 4 replicates per treatment.

After being taken up via passive diffusion across the apical surface of rumen epithelium, these compounds are degraded intracellularly to monocarboxylates and transported into the bloodstream by specific proteins, known as monocarboxylene transport (MCT) [26–28]. Among the previously described fourteen members of MCT proteins, only the first four (MCT1 through MCT4) have been shown to catalyse proton linked transport of monocarboxylates [24,29,30]. According to our results, no differences were observed between calves fed either MR or milk in terms of MCT1 and MCT4 mRNA abundance in the dorsal sac of the rumen. The main reason for the unaffected MCT1 and MCT4 gene expression is most likely related to the unchanged ruminal VFA concentration and solid feed intake, because previous studies revealed a positive relationship between butyrate concentration and MCT1 and/or MCT4 activity [31,32]. However, the effect of liquid feed on MCT1 gene expression was addressed by Flaga et al. [8] who found a significant increase in mRNA abundance of MCT1 in the rumen epithelium of calves fed milk in comparison to those fed MR.The lack of consistency between our findings and those of Flaga et al. [8] might be related to protein quality of the milk replacers.

Na^+/H^+ exchange proteins play an essential role in pH regulation in rumen [33]. Graham et al. [34] suggested that expression of NHE1 reduces the extracellular pH and promotes the non-ionic diffusional uptake of VFA to cells. Previous studies showed a significant relationship between NHE activities and rumen pH

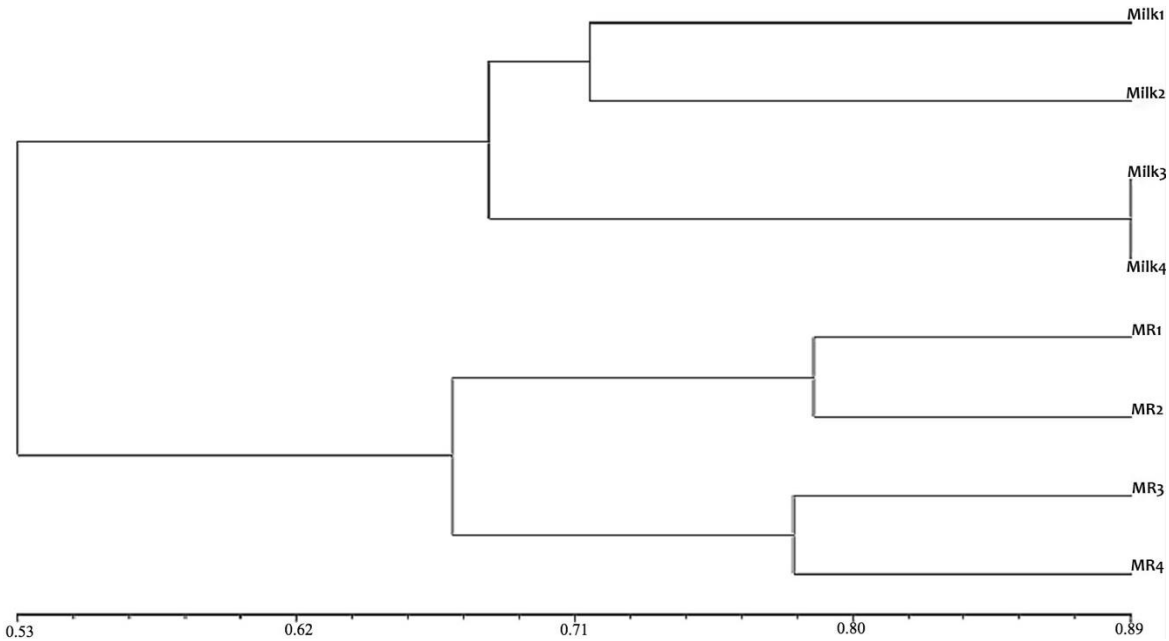

Figure 5. Dendrogram of PCR-DGGE analysis of MR and milk group. Results were clustered with the Dice similarity coefficient (optimization, 1.0%; tolerance, 1.0%) and UPGMA.

and VFA concentration [35]. NHE1 and NHE3 are the predominant isoforms among the previously described NHE proteins in rumen epithelium [36], and they import Na^+ to the cell and export H^+ to the rumen [37]. Similar to MCT gene expressions, we did not observe differences in NHE1 and NHE3 mRNA abundances in rumen epithelium. On the other hand, lower NHE3 expression was reported in calves fed with milk replacer and starter mixture by Laarman et al. [31] in despite of a higher butyrate concentration in the rumen. Along with the genes related to membrane transport, we also did not observe any differences in the expression of ACSS1, AceCS2, and ACSM1, which are associated with VFA metabolism in rumen epithelium. A previous study carried out with cows fed either high or low concentrate diets concluded no differences between the groups in terms of AceCS2, ACSS1, and ACSM1 expression in rumen epithelium [38]. More recently, Ma et al. [39] suggested that the expressions of ACSS proteins were not influenced by the NDF and starch ratio. However, as far as we know, no available data exists about the effects of liquid feed type on the expression of the genes affiliated with the VFA transport (except MCT1) and metabolism. In consideration of the results of starter feed intake and rumen VFA concentration, it can be assumed that liquid feed type had no contribution to gene expressions related to VFA transport and metabolism either directly or indirectly. However, it should be noted thatt hese results might be changed with the use of low-quality milk replacers that contain plenty of plant-based proteins.

Physical and functional development of the rumen is anoutcome of intimate interaction among rumen microorganisms, their end products, and diet [40,41]. In newborn calves, the initial rumen microbial community is acquired from the cow and surrounding environment [12]. Moreover, colostrum and milk may also contribute to rumen microbiota establishment even though they directly enter the abomasum via the reticular groove [42,43]. All major rumen microorganisms, such as cellulolytic bacteria, proteolytic bacteria, and sulphate reducing bacteria can be found in the rumen within the first week after birth [40,43]. More recently, Guzman et al. [44] suggested that rumen fibrolytic bacteria might initially use milk as a nutrient source to grow throughout the early monogastric phase. Previous studies revealed that two major cellulolytic bacteria, *R. flavefaciens* and *R. albus* were found in the rumen as early as first week [41,45], but *F. succinogenes* was detected in 2 month-old and older ruminants [41]. However, most of the studies focused on the effects of age and solid feed type on rumen microbial population, and based on our knowledge, there is very little evidence about the effects of milk or MR feeding on cellulolytic bacteria counts. We observed a significant increase in *F. succinogenes* and *R. flavefaciens* numbers in rumen fluid of calves fed with milk as compared to MR. Rey et al. [43] suggested that cellulolytic bacteria were settled within the first week after birth and their relative abundances have ceased to increase. In this context, results of the current trial might be important for the calves' future rumen microbiota. In addition to cellulolytic

bacteria numbers, DGGE-derived dendrograms showed noticeable differences in ruminal microbial populations between MR-fed and milk fed-calves. According to our results, it might be assumed that milk feeding influenced rumen cellulolytic bacteria numbers and bacterial diversity. However, to date, this study is the first report of the effect of liquid feed type on rumen bacterial diversity. For future studies, investigation of the origin of these bacteria and how they are transferred to a host's rumen will help us understand the establishment pathways and interactions between host and microbiota. In addition, high-throughput sequence analysis may help to define the functions and interactions of the ruminal microbiome between milk fed or MR fed calves.

Our results showed that feeding whole milk instead of high-quality milk replacer, which contained plentiful amounts of milk protein, significantly improved rumen development and colonization of cellulolytic bacteria without affecting the solid feed intake and rumen VFA concentration. We also noticed distinct rumen bacterial diversity between the experimental groups. Findings from the current trial might be important since there is increasing evidence that early and effective bacterial colonization has a significant effect on rumen development and animal performance.

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References

1. Khan MA, Weary DM, Von Keyserlingk MA. Invited review: effects of milk ration on solid feed intake, weaning, and performance in dairy heifers. Journal of Dairy Science 2011; 94: 1071-1081.
2. Baldwin R, McLeod K, Klotz J, Heitmann R. Rumen development, intestinal growth and hepatic metabolism in the pre-and postweaning ruminant. Journal of Dairy Science 2004; 87: E55-E65.
3. Gorka P, Kowalski ZM, Pietrzak P, Kotunia A, Kiljanczyk R et al. Effect of sodium butyrate supplementation in milk replacer and starter diet on rumen development in calves. Journal of Physiology and Pharmacology 2009; 60 (Suppl. 3): 47-53.
4. Niwińska B, Strzetelski J. Effects of type of liquid feed and feeding frequency on calf performance. Journal of Animal and Feed Sciences 2004; 13: 167-170.
5. Gorka P, Kowalski Z, Pietrzak P, Kotunia A, Jagusiak W et. al. Is rumen development in newborn calves affected by different liquid feeds and small intestine development? Journal of Dairy Science 2011; 94: 3002-3013.
6. Moore D, Taylor J, Hartman M, Sischo W. Quality assessments of waste milk at a calf ranch. Journal of Dairy Science 2009; 92: 3503-3509.
7. Lee HJ, Khan MA, Lee WS, Yang SH, Kim SB et al. Influence of equalizing the gross composition of milk replacer to that of whole milk on the performance of Holstein calves. Journal of Animal Science 2009; 87: 1129-1137.
8. Flaga J, Gorka P, Zabielski R, Kowalski ZM. Differences in monocarboxylic acid transporter type 1 expression in rumen epithelium of newborn calves due to age and milk or milk replacer feeding. Journal of Animal Physiology and Animal Nutrition 2015; 99: 521-530.
9. Drackley JK, Blome RM, Bartlett KS, Bailey KL. Supplementation of 1% l-glutamine to milk replacer does not overcome the growth depression in calves caused by soy protein concentrate. Journal of Dairy Science 2006; 89: 1688-1693.
10. Zabielski R. Bioactive peptides in young animal nutrition. Journal of Animal and Feed Sciences 2001; 10: 169-180.
11. Ragionieri L, Cacchioli A, Ravanetti F, Botti M, Ivanovska A et al. Effect of the supplementation with a blend containing short and medium chain fatty acid monoglycerides in milk replacer on rumen papillae development in weaning calves. Annals of Anatomy 2016; 207: 97-108.
12. Abecia L, Ramos-Morales E, Martinez-Fernandez G, Arco A, Martin-Garcia A et al. Feeding management in early life influences microbial colonisation and fermentation in the rumen of newborn goat kids. Animal Production Science 2014; 54: 1449-1454.
13. AOAC. Official Methods of Analysis of AOAC International. Gaithersburg, MD, USA: AOAC; 2000.
14. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Research 2002; 30: e36.
15. Seegraber FJ, Morrill JL. Effect of protein source in calf milk replacers on morphology and absorptive ability of small intestine. Journal of Dairy Science 1986; 69: 460-469.
16. Beharka AA, Nagaraja TG, Morrill JL, Kennedy GA, Klemm RD. Effects of form of the diet on anatomical, microbial, and fermentative development of the rumen of neonatal calves. Journal of Dairy Science 1998; 81: 1946-1955.
17. Sakata T, Tamate H. Rumen epithelial cell proliferation accelerated by rapid increase in intraruminal butyrate. Journal of Dairy Science 1978; 61: 1109-1113.

18. Sakata T, Tamate H. Rumen epithelium cell proliferation accelerated by propionate and acetate. Journal of Dairy Science 1979; 62: 49-52.

19. Mentschel J, Leiser R, Mülling C, Pfarrer C, Claus R. Butyric acid stimulates rumen mucosa development in the calf mainly by a reduction of apoptosis. Archiv für Tierernaehrung 2001; 55: 85-102.

20. Baldwin RL. The proliferative actions of insulin, insulin-like growth factor-I, epidermal growth factor, butyrate and propionate on ruminal epithelial cells in vitro. Small Ruminant Research 1999; 32: 261-268.

21. Shen Z, Seyfert H-M, Löhrke B, Schneider F, Zitnan R et al. An energy-rich diet causes rumen papillae proliferation associated with more IGF type 1 receptors and increased plasma IGF-1 concentrations in young goats. The Journal of Nutrition 2004; 134: 11-17.

22. Zitnan R, Kuhla S, Sanftleben P, Bilska A, Schneider F et al. Diet induced ruminal papillae development in neonatal calves not correlating with rumen butyrate. Veterinarni Medicina 2005; 50: 472-479.

23. Bergman EN. Energy contributions of volatile fatty acids from the gastrointestinal tract in various species. Physiological Reviews 1990; 70: 567-590.

24. Kirat D, Inoue H, Iwano H, Hirayama K, Yokota H et al. Expression and distribution of monocarboxylate transporter 1 (MCT1) in the gastrointestinal tract of calves. Research in Veterinary Science 2005; 79: 45-50.

25. Kirat D, Masuoka J, Hayashi H, Iwano H, Yokota H et al. Monocarboxylate transporter 1 (MCT1) plays a direct role in short-chain fatty acids absorption in caprine rumen. The Journal of Physiology 2006; 576: 635-647.

26. Aschenbach J, Penner G, Stumpff F, Gäbel G. Ruminant nutrition symposium: role of fermentation acid absorption in the regulation of ruminal pH. Journal of Animal Science 2011; 89: 1092-1107.

27. Pfannkuche H, Taifour F, Steinhoff-Wagner J, Hammon HM, Gabel G. Post-natal changes in MCT1 expression in the forestomach of calves. Journal of Animal Physiology and Animal Nutrition 2014; 98: 140-148.

28. Sehested J, Diernæs L, Møller PD, Skadhauge E. Ruminal transport and metabolism of short-chain fatty acids (SCFA) in vitro: effect of SCFA chain length and pH. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 1999; 123: 359-368.

29. Halestrap AP, Meredith D. The SLC16 gene family—from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. European Journal of Physiology 2004; 447: 619-628.

30. Halestrap AP, Price NT. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochemical Journal 1999; 343: 281-299.

31. Laarman A, Ruiz-Sanchez A, Sugino T, Guan L, Oba M. Effects of feeding a calf starter on molecular adaptations in the ruminal epithelium and liver of Holstein dairy calves. Journal of Dairy Science 2012; 95: 2585-2594.

32. Dengler F, Rackwitz R, Benesch F, Pfannkuche H, Gäbel G. Both butyrate incubation and hypoxia upregulate genes involved in the ruminal transport of SCFA and their metabolites. Journal of Animal Physiology and Animal Nutrition 2015; 99: 379-390.

33. Müller F, Aschenbach J, Gäbel G. Role of Na+/H+ exchange and HCO3− transport in pHi recovery from intracellular acid load in cultured epithelial cells of sheep rumen. Journal of Comparative Physiology B 2000; 170: 337-343.

34. Graham C, Gatherar I, Haslam I, Glanville M, Simmons NL. Expression and localization of monocarboxylate transporters and sodium/proton exchangers in bovine rumen epithelium. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 2007; 292: R997-R1007.

35. Yang W, Shen Z, Martens H. An energy-rich diet enhances expression of Na/H exchanger isoform 1 and 3 messenger RNA in rumen epithelium of goat. Journal of Animal Science 2012; 90: 307-317.

36. Etschmann B, Heipertz KS, Von der Schulenburg A, Schweigel M. A vH+-ATPase is present in cultured sheep ruminal epithelial cells. American Journal of Physiology-Gastrointestinal and Liver Physiology 2006; 291: G1171-G1179.

37. Connor EE, Li RW, Baldwin RL, Li C. Gene expression in the digestive tissues of ruminants and their relationships with feeding and digestive processes. Animal 2010; 4: 993-1007.

38. Penner GB, Taniguchi M, Guan LL, Beauchemin KA, Oba M. Effect of dietary forage to concentrate ratio on volatile fatty acid absorption and the expression of genes related to volatile fatty acid absorption and metabolism in ruminal tissue. Journal of Dairy Science 2009; 92: 2767-2781.

39. Ma L, Zhao M, Zhao LS, Xu JC, Loor JJ et al. Effects of dietary neutral detergent fiber and starch ratio on rumen epithelial cell morphological structure and gene expression in dairy cows. Journal of Dairy Science 2017; 100: 3705-3712.

40. Li RW, Connor EE, Li C, Baldwin V, Ransom L et al. Characterization of the rumen microbiota of pre-ruminant calves using metagenomic tools. Environmental Microbiology 2012; 14: 129-139.

41. Jami E, Israel A, Kotser A, Mizrahi I. Exploring the bovine rumen bacterial community from birth to adulthood. ISME Journal 2013; 7: 1069-1079.

42. Curtis TP, Sloan WT. Prokaryotic diversity and its limits: microbial community structure in nature and implications for microbial ecology. Current Opinion in Microbiology 2004; 7: 221-226.

43. Rey M, Enjalbert F, Combes S, Cauquil L, Bouchez O et al. Establishment of ruminal bacterial community in dairy calves from birth to weaning is sequential. Journal of Applied Microbiology 2014; 116: 245-257.

44. Guzman CE, Bereza-Malcolm LT, De Groef B, Franks AE. Uptake of milk with and without solid feed during the monogastric phase: effect on fibrolytic and methanogenic microorganisms in the gastrointestinal tract of calves. Animal Science Journal 2016; 87: 378-388.

45. Flint HJ, Bayer EA. Plant cell wall breakdown by anaerobic microorganisms from the Mammalian digestive tract. Annals of the New York Academy of Sciences 2008; 1125: 280-288.