Concentrate diet modulation of ruminal genes involved in cell proliferation and apoptosis is related to combined effects of short-chain fatty acid and pH in rumen of goats

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ABSTRACT

Short-chain fatty acids (SCFA) regulate cell proliferation and cell apoptosis in gastrointestinal tissue in vitro and in vivo. We have tested the hypothesis that a medium-concentrate intake induces mRNA abundance alterations of genes involved in cell proliferation and cell apoptosis in the rumen epithelium of goats, and that these changes in mRNA abundance are related to ruminal SCFA concentration and ruminal pH. Goats ($n = 16$) were randomly allocated to 2 groups and fed either a low-concentrate (LC) diet (10% concentrate; $n = 8$) or a medium-concentrate (MC) diet (35% concentrate; $n = 8$) in 2 equal portions daily. The individually housed goats were fed separately with their respective diet for 3 wk and were slaughtered 6 h after the morning feed on d 22. In vivo, goats receiving the MC treatment exhibited a greater ruminal SCFA concentration (73.7 mM) compared with those receiving the LC treatment (53.2 mM), and the pH decreased from 6.9 to 6.5. The expression of proliferative genes of cyclin A, cyclin B1, cyclin D1, cyclin E1, *CDK1*, *CDK2*, *CDK4*, and *CDK6* mRNA in the MC group was enhanced. The gene expression of apoptosis genes (caspase 3, caspase 8, caspase 9, *p53*, and *Bax*) was significantly higher, and the ratio of *Bcl-2* to *Bax* (*Bcl-2/Bax*) expression was lower in the MC group than in the LC group. The same trend was observed in the population of apoptotic cells analyzed by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. The cell density in the stratum germinativum of the MC group was significantly increased compared with that in the LC group. During primary culture of rumen epithelial cells, SCFA or pH treatment alone of the culture medium had significant effects on the expression of most of the genes tested in the present study. Furthermore, SCFA

and pH exerted combined effects on the expression of cyclin A, cyclin B1, cyclin E1, *CDK6*, *p53*, *Bcl-2*, and *Bcl-2/Bax*. Thus, the MC diet induces alteration of gene expression of the genes that regulate both cell proliferation and apoptosis. These genes are regulated by combined effect of ruminal SCFA and ruminal pH.

Key words: rumen epithelium, gene expression, cell proliferation, cell apoptosis, short-chain fatty acid and pH

INTRODUCTION

Short-chain fatty acids (SCFA) are produced in the rumen through the microbial fermentation of food and are absorbed by the rumen papillae (Rémond et al., 1996). The rumen absorbs the energy substances [i.e., SCFA, which make approximately 70% of the energy requirement for growth, maintenance, and production in ruminants (Bergman, 1990)]. The diet influences the rumen papillae surface area and thus the rumen absorptive capacity (Gäbel et al., 1987; Bannink et al., 2008) and animal health. As ruminal fermentation products, SCFA induce morpho-functional alterations in ruminal papillae (Gäbel et al., 1991a; Krehbiel et al., 1992; Nozière et al., 2000); conversely, an increase of SCFA absorption in ruminants fed high-concentrate diets has been linked to a surface area enlargement of ruminal papillae (Dirksen et al., 1984; Gäbel et al., 1991a,b). The latter might be attributable to an increased abundance of gene transcripts related to epithelial proliferation (Penner et al., 2011).

The rumen epithelium is continually exposed to luminal SCFA. The concentration of ruminal SCFA is affected by feed intake. The changes of luminal SCFA are negatively related to changes in acidity. Accumulating study has proved that SCFA in the gut affect mucosal homeostasis (Neish, 2009; Plöger et al., 2012), epithelial absorption (Malhi et al., 2013), and gene expression (Macfarlane and Macfarlane, 2011; Yan et al., 2014). The SCFA and acidic pH also affect the growth and function of rumen epithelial cells (Steele et al., 2011; Plöger et al., 2012; Yang et al., 2012).

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Cell proliferation and cell apoptosis are 2 important processes of cell life. Mucous membrane cells are constantly in a dynamic balance between proliferation and apoptosis. Cell proliferation and cell apoptosis must be synchronously coordinated to maintain mucosal integrity and its normal function. The majority of previous studies have been conducted on animals or tissues, such as rat and human intestinal and colonic epithelium (Ichikawa and Sakata, 1998; Ichikawa et al., 2002; Blotière et al., 2003), with only a few studies using goat rumen epithelium (Lu et al., 2013; Zhao et al., 2014). To date, little is known about the molecular basis of epithelial proliferation and apoptosis in the rumen epithelial cells of the goat. The combined effect of SCFA and pH on the cell cycle in these rumen epithelial cells are not yet understood.

We have hypothesized that the feeding of a medium-concentrate diet would affect the expression of genes involved in cell growth, including those related to cell proliferation and cell apoptosis, and that dietary effects on the expression of genes are associated with ruminal SCFA and pH. Our current experiments have therefore focused on the effects of diet [low-concentrate (LC; 10% concentrate) vs. medium-concentrate (MC; 35% concentrate)] on cell proliferation and apoptosis in the ruminal epithelium of the goat and have investigated the combined effect of SCFA concentration and pH acidity on expression of genes related to cell proliferation and cell apoptosis in goat rumen epithelial cells in primary culture.

MATERIALS AND METHODS

The experimental design and procedures were approved by the Animal Care and Use Committee of Nanjing Agricultural University, following the requirements of the Regulations for the Administration of Affairs Concerning Experimental Animals (The State Science and Technology Commission of P. R. China, 1988).

Experimental Design and Goat Management

Sixteen goats (14.5 ± 1.1 kg of BW, Boer $\times$ Yangtze River Delta White), 4 mo of age at the commencement of the experiment, were used for the feeding trial. The treatments contained concentrate at 10% (low-concentrate, **LC**) and 35% (medium-concentrate, **MC**) of dietary DM. The composition of the MC and LC diets is presented in Table 1. Replacing part (almost 30%) of guinea grass with corn meal increased the dietary NFC concentration from 14.7 to 31.3% and decreased the dietary NDF concentration by almost 17%. Goats were placed in individual pens (1.2×1.0 m) with free access to water and were fed in 2 equal portions at 0800

Table 1. Composition of diets and DMI for low-concentrate (LC) and medium-concentrate (MC) treatments

Item	Treatment	
	LC	MC
Ingredient (% of DM)		
Guinea grass	90	65
Corn meal	0	25
Soybean meal	8	8
Mineral and vitamin supplement ¹	1	1
Salt	0.25	0.25
CaHPO ₄	0.75	0.75
Chemical composition		
DM (%)	91.4	89.9
CP (% of DM)	10.3	10.7
Crude fat (% of DM)	3.5	3.4
Ash (% of DM)	4.7	3.9
NDF (% of DM)	66.8	50.7
NFC ² (% of DM)	14.7	31.3
ME (MJ/kg of DM)	8.9	10.1
Intake		
Guinea grass (g/d)	421.7 $\pm$ 10.8	321.2 $\pm$ 7.0
Corn meal (g/d)	0	168
Soybean meal (g/d)	53.6	53.6
N [mg/(kg ^{0.75} ·d)]	916.0 $\pm$ 20.2	961.8 $\pm$ 13.0
ME [kJ/(kg ^{0.75} ·d)]	543.8 $\pm$ 12.4	630.1 $\pm$ 8.3*
BW of the goats (kg)		
Beginning of trial	14.4 $\pm$ 1.2	14.6 $\pm$ 1.5
End of trial	14.9 $\pm$ 1.1	15.4 $\pm$ 1.4

¹Contained 16% calcium carbonate, 102 g/kg of Zn, 47 g/kg of Mn, 26 g/kg of Cu, 1,140 mg/kg of I, 500 mg/kg of Se, 340 mg/kg of Co, 17,167,380 IU/kg of vitamin A, 858,370 IU/kg of vitamin D, and 23,605 IU/kg of vitamin E.

²NFC = 100 – (NDF + CP + crude fat + ash).

*Different from LC ($P < 0.05$).

and 1700 h daily. The amounts of feed offered and refused by each goat were recorded daily throughout the experiment. The amount of feed supplied was adjusted to ensure approximately 25% refusals. Adaptation to the diets was carried out over 10 d, and goats received their respective treatment for at least 21 d before slaughter. All goats were slaughtered, 6 h after the morning feed, at a local slaughterhouse. The goats for experiments were stunned by captive bolt and killed by exsanguination.

Feed was sampled at d 7 and 21 for chemical analysis. The samples of the feed refusals were collected for DM determination on the same day. The DM, ash, CP, and crude fat contents were determined as in our previous work (Malhi et al., 2013). The concentration of NDF was determined by using amylase and sodium sulfite (Van Soest et al., 1991).

Sample Collection

Immediately after slaughter, subsamples of ruminal fluid were collected in equal portions from the atrium ruminis, ventral rumen, caudal dorsal, and caudal ven-

tral regions separately and then combined to form 1 sample. The ruminal fluid (30 mL) was strained through 3 layers of cheesecloth and immediately subjected to pH measurement. Thereafter, 5% HgCl_2 solution (1.5 mL) was added, and the sample was stored at -20°C . Rumen tissue from the ventral blind sac was quickly excised, cleaned of loosely adherent feed particles, placed in ice-cold PBS (pH 7.4), and repeatedly rinsed until the PBS remained clear. Ruminal tissues (about 2 cm^2) for the morphometric analyses and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay were fixed in 4% paraformaldehyde solution. Epithelium for the cell cycle analysis and cell culture was transported in ice-cold PBS to the laboratory. Epithelium for extraction of RNA was separated from the muscle layers and transferred into liquid N_2 within 5 min and stored at -80°C .

Morphometric and Histomorphometric Analyses

Tissue samples from the ventral sac ruminis of 8 goats in each group were used to evaluate the histomorphology. Samples were fixed in 4% paraformaldehyde overnight, dehydrated, cleared, and embedded in paraffin. Sections of 5 to $7\text{ }\mu\text{m}$ in thickness were cut and stained by the standard hematoxylin and eosin procedure. The ruminal tissue selected for histomorphological analysis contained papillae with almost identical shape and size. For each tissue, 25 to 30 papillae were embedded for paraffin-sectioning and microscopic observation. From these, the 4 paraffin sections with the best orientation of papillae in the median sagittal plane were used to evaluate the morphological characteristics of the ruminal papillae by using Image-Pro Plus 6.0 (Media Cybernetics Inc., Bethesda, MD).

The number of cell layers forming the stratum corneum (SC) and stratum germinativum (SGv) was counted. Epithelial cell density (number/ mm^2) was measured in SGv and in the stratum basale (SB).

Cell Culture

Rumen epithelial cells were isolated from the rumen epithelium of 8 healthy goats (Boer $\times$ Yangtze River Delta White, aged 6–12 mo; both sexes) with 0.25% trypsin (Sunshine Chemical Co. Ltd., Nanjing, China) as described previously (Lu et al., 2015). Immediately after death, the epithelium from the ventral blind sac of each goat was quickly excised, and these tissues were placed in ice-cold PBS (pH 7.4). The tissues were repeatedly rinsed until the PBS remained clear. The muscle layers were then removed, and the epithelium was repeatedly digested with 0.25% trypsin in D-Hanks' buffered salt solution with antibiotics un-

til individual epithelial cells appeared in the digestion solution. Cells were washed with culture medium. For each cell culture, we prepared several cell culture flasks. The viability of the cells was confirmed by staining with trypan blue, and the cell density was adjusted to 1,000,000 cells/mL in high-glucose (4,500 mg/L) Dulbecco's modified Eagle's medium (Life Technologies Inc., Gibco/Brl Division, Grand Island, NY) with 10% fetal bovine serum (Zhejiang Tianhang Biological Technology Co. Ltd., Hangzhou, China), 2 mM L-glutamine (Sunshine Chemical Co. Ltd., Nanjing, China), 100 U/mL of penicillin, and 100 mg/mL of streptomycin. The cells were seeded in 25-cm^2 plastic tissue culture flasks (Corning Inc., Corning, NY) and incubated at 37°C in an incubator (5% CO_2) for 24 h. Thereafter, the cells were allocated across treatments in the following manner: at pH 7.4 (control; $n = 8$) or 6.8 ($n = 8$) for 24 h or in 20 mM SCFA at pH 7.4 ($n = 8$) or 6.8 ($n = 8$) for 24 h. Subsequently, the cells were scraped off the flasks and transferred to -80°C until analysis for gene expression. The mixture of SCFA contained 12 mM sodium acetate, 5 mM sodium propionate, and 3 mM sodium butyrate (Merck KGaA, Darmstadt, Germany). In the pH 6.8 group, the medium was acidified with 1 N HCl.

pH and SCFA Determination

The pH of rumen fluid was measured with a Mettler Toledo Delta 320 pH meter (Mettler-Toledo Group, Halstead, UK). An Agilent gas chromatograph HP6890N (GC HP6890N, Agilent Technologies Inc., New Castle, DE) was used to measure SCFA concentrations, as described before (Lu et al., 2015). The GC was equipped with a flame ionization detector and a $30\text{ m} \times 0.32\text{ mm}$ internal diameter $\times 0.25\text{ m}$ film thickness HP-FFAP capillary column (Hewlett-Packard, Palo Alto, CA). The carrier gas was N_2 (99.99% purity) and had a constant flow rate of 2.8 mL/min and a split ratio of 1:30. The temperature of the capillary column was set to 140°C for 4 min and then was raised by $25^\circ\text{C}/\text{min}$ to 240°C . The temperatures of the injection port and the flame ionization detector were 180 and 250°C , respectively.

Cell Isolation and Flow Cytometry Assay

Cells were isolated from rumen epithelial tissue (10 g of wet weight, sampled from the ventral blind sac ruminis) by using 0.25% trypsin. This method was described previously (Lu et al., 2015). Immediately after death, the epithelium from the ventral blind sac of each goat was quickly excised, and these tissues were placed in ice-cold PBS (pH 7.4). The tissues were repeatedly rinsed until the PBS remained clear. The muscle layers

were then removed, and the epithelium was repeatedly digested with 0.25% trypsin in D-Hanks' buffered salt solution with antibiotics until individual epithelial cells appeared in the digestion solution. Cells were washed with culture medium. Isolated cells were fixed in ethanol (75%), washed, and treated with RNase solution (100 U/mL in PBS, 37°C, 30 min), followed by incubation with propidium iodide (70 mM in PBS) for 30 min and analysis on a BD FACScan Flow Cytometer (BD Biosciences, San Jose, CA). The cell cycle was analyzed by a computer-aided Multicycle Program (Phoenix, San Diego, CA).

TUNEL Assay

The TUNEL detection of DNA fragmentation was carried out by using a TUNEL BrightRed Apoptosis Detection Kit (Vazyme, Apoptosis Detection Kit) according to the manufacturer's instructions. The cells were then observed and photographed by a Laser Scanning Confocal Microscope (Zeiss LSM700, Carl Zeiss, Jena, Germany) at 460 and 620 nm. The apoptotic index was obtained from the ratio between apoptotic and normal nuclei multiplied by 100 (Bozzo et al., 2014).

RNA Extraction and Real-Time Quantitative PCR

Total RNA extraction was carried out as previously described (Yang et al., 2012). The RNA concentration was then quantified by measuring the absorbance at 260 nm with a Biophotometer (Eppendorf, Hamburg, Germany). The absorption ratio (260/280 nm) of all samples was between 1.8 and 2.0. Aliquots of RNA samples were subjected to electrophoresis through a 1.4% agarose-formaldehyde gel to verify integrity. Complementary DNA was synthesized by using a random hexamer primer (Invitrogen, Shanghai, China) and M-MLV-reverse transcriptase according to the manufacturer's instructions (MBI Fermentas Inc., Burlington, ON, Canada). The primers for cyclin D1, *CDK4*, caspase 3, caspase 8, *Bcl-2*, and *Bax* were used as described (Deng et al., 2005; Shimizu et al., 2010; Dessauge et al., 2011; Malhi et al., 2013). The primers for the other genes were designed by Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA). The PCR products of the newly designed primers were validated by DNA sequencing. The target genes of interest and their respective sources and primer sequences are listed in Table 2. The primers were all synthesized by Shanghai Invitrogen Biological Co. (Shanghai, China).

Determination of relative gene expression was performed with real-time quantitative PCR by using the MyiQ2 2-color real-time quantitative PCR detection

system (Bio-Rad Laboratories Inc., Hercules, CA). Real-time PCR was carried out in a total volume of 20 μ L containing 1 $\times$ iQ SYBR Green Supermix (Bio-Rad Laboratories Inc.), a mixture of forward and reverse primers (500 nM each), cDNA template (1 ng), and sterile water for volume adjustment. An initial cycle for 30 s at 95°C was used to denature the cDNA. This was followed with 45 PCR cycles consisting of denaturation at 95°C for 5 s and primer annealing and extension at 60°C for 30 s. After all PCR analyses, a melting curve analysis was carried out. All investigated PCR products exhibited only a single melting peak. Before the performance of PCR for experimental samples, the amplification efficiencies of all primers were calculated by using a standard dilution series. The efficiencies of all the primers used were between 97 and 101%. All samples were analyzed in triplicate. These experiments were repeated twice with 80 to 90% similarity. Gene expression was normalized to that of *GAPDH* (Δ Ct = Ct_{target} - Ct_{GAPDH}, where Ct = cycle threshold). The average Δ CT value of the control group (LC group in vivo experiment and pH 7.4 group in vitro experiment) was then used as a reference value to calculate $\Delta\Delta$ Ct. The relative expression values were calculated by using the following formula: relative expression = $2^{-\Delta\Delta$ Ct (Livak and Schmittgen, 2001).

Statistical Analyses

Data are expressed as the means $\pm$ standard error. Differences with a *P*-value of <0.05 were considered significant. Data involving more than 2 groups were assessed by a one-way ANOVA and Student-Newman-Keuls' post hoc analysis after testing for homogeneity of variance. An independent *t*-test (2-tailed test) was used to compare data between 2 groups. The general linear model with relevant interactions was used to determine the significance of the effects of SCFA and pH in vitro. All statistical analyses were performed by using SPSS software (version 13.0.1 for Windows, SPSS, Chicago, IL).

RESULTS

Ruminal Fermentation

The goats in the MC group had a lower ruminal pH than goats in the LC group (6.5 vs. 6.9, *P* < 0.05, Table 3). In agreement with the ruminal pH results, the total ruminal SCFA was higher in the MC group compared with the LC group. Of all 6 SCFA analyzed, feeding with MC increased (*P* < 0.05) the concentration of propionate by 75%, butyrate by 85%, isobutyrate 40%, valerate by 85%, and isovalerate by 67%.

Table 2. Primers for real-time PCR

Gene ¹	Primer sequence (5' to 3'; forward, reverse) ²	Source ³	Size (bp)
<i>GAPDH</i>	TTGTCTCCTGCGACTTCA CCACCACCCTGTTACTGTT	HM043737.1	135
<i>CCNA</i>	TGGACCTTCACCAGACCTACCT GTGGGTTGAGGAGAGAAACACC	X68321.1	105
<i>CCNB1</i>	AGCGGATCCAAACCTTTGTAGTG CAATGAGGATGGCTCTCATGTTTC	NM_001045872.1	137
<i>CCND1</i>	GGTCCTGGTGAACAACTC TTGCGGATGATCTGCTT	Malhi et al., 2013	114
<i>CCNE1</i>	GGGACAAGCACCTTATGCAAC GTGTTGCCATATACCGATCAAAGA	NM_001192776.1	153
<i>CDK1</i>	CCAATAATGAAGTGTGGCCAGAAG AGAAATTCGTTTGGCAGGATCATAG	NM_174016.2	164
<i>CDK2</i>	CTGCACCGAGACCTTAAACCTCA GCTCGGTACCACAGAGTCACCA	BT020790.1	140
<i>CDK4</i>	TGAGCATCCCAATGTTGT CCTTGTCAGATACGTCCT	Malhi et al., 2013	122
<i>CDK6</i>	AGAGTGATTGCAGCTTTATGTCCA TGCCCAGGTTGCTCACTTC	GAAI01006376.1	158
Caspase 3	AGATGAGGTAAAGCCCGTCA AGCCATGGTGAAGAAGGAATCA	Dessauge et al., 2011	156
Caspase 8	ACCACAGTCCAGTTCTGTGCCT TGCCTACAGGATTCTGCTTT	Deng et al., 2005	104
Caspase 9	CCAGCTTACATTTGGCAATC TCCTTTGTTTCATCTCCTGCTTG	XM_004013798.1	115
<i>p53</i>	TTTTCTTGGCTTGGCTTTG CCCATCCTCACCATCATCAC	X81704.1	80
<i>Bcl-2</i>	GCACAAACACGCACCTCAA GATGACCGAGTATCTGAACCG	Shimizu et al., 2010	120
<i>Bax</i>	GACAGCCAGGAGAAATCAAACA TCTGACGGCAACTTCAACTG	Shimizu et al., 2010	205
	TGGGTGTCCCAAAGTAGGAG		

¹*CCNA* = cyclin A; *CCNB1* = cyclin B1; *CCND1* = cyclin D1; *CCNE1* = cyclin E1; *CDK1* = cyclin-dependent kinase 1; *CDK2* = cyclin-dependent kinase 2; *CDK4* = cyclin-dependent kinase 4; *CDK6* = cyclin-dependent kinase 6; *Bcl-2* = B-cell lymphoma 2; *Bax* = Bcl-2-associated X protein.

²The first primer listed for each gene is the forward primer, and the second primer is the reverse primer.

³The reference sequence number is given for primers whose source is the National Center for Biotechnology Information (NCBI) GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/>).

Papillae Morphology and Rumen Epithelial Growth

The cell densities in SGv ($P < 0.05$) in the ruminal ventral sac were higher in the MC group than in the LC

group, and the number of cell layers tended to increase ($P = 0.08$). However, the numbers of cell layers in SC and the cell densities in SB were not different between the LC and MC groups (Table 4).

Table 3. Effect of feeding low concentrate (LC) or medium concentrate (MC) diets on ruminal short-chain fatty acid (SCFA) and pH in goats 6 h after feeding¹

Item	Treatment		<i>P</i> -value
	LC	MC	
pH	6.9 ± 0.07	6.5 ± 0.12	0.03
Acetate (mM)	33.0 ± 1.31	42.0 ± 2.24	0.08
Propionate (mM)	11.4 ± 0.40	20.0 ± 0.93	0.01
Butyrate (mM)	4.8 ± 0.81	8.9 ± 0.69	0.04
Isobutyrate (mM)	1.0 ± 0.04	1.4 ± 0.14	0.04
Valerate (mM)	1.0 ± 0.01	1.4 ± 0.05	<0.01
Isovalerate (mM)	1.2 ± 0.04	2.0 ± 0.24	0.02
Total SCFA (mM)	53.2 ± 2.10	73.7 ± 0.84	0.01

¹Goats ($n = 8$) received the LC or MC diet at least 21 d before slaughter. Values are means ± standard error.

Analyses of Cell Cycle

Dietary MC affected the cell cycle progression (Table 5). The cell number percentage was lower in the G₀/G₁ phase (79.6 vs. 86.4%, $P < 0.05$) but higher in the S phase (5.5 vs. 4.2%, $P < 0.05$) and the G₂/M phase (15.0 vs. 9.5%, $P < 0.05$) in the MC group compared with those in the LC group.

Analyses of TUNEL Assay

The apoptotic index (Figure 1) was significantly ($P < 0.001$) different between the MC and LC groups. Supplemental Figure S1 (<http://dx.doi.org/10.3168/jds.2015-10446>) shows that the TUNEL-positive cells

Table 4. Effect of feeding low concentrate (LC) or medium concentrate (MC) diets on ruminal histometric characteristics in ruminal epithelium of goats 6 h after feeding¹

Item	Treatment		P-value
	LC	MC	
Thickness of epithelial strata (number of cell layers)			
Stratum germinativum	3.4 ± 0.14	3.7 ± 0.11	0.08
Stratum corneum	2.4 ± 0.14	2.5 ± 0.12	0.27
Cell density in epithelial strata			
Stratum germinativum (no./mm ²)	6,850.3 ± 135.13	7,268.3 ± 73.23	0.02
Stratum basale (no./mm)	266.3 ± 9.33	275.2 ± 8.43	0.42

¹Goats (n = 8) received the LC or MC diet at least 21 d before slaughter. Rumen papillae samples for histomorphometry were collected from the ruminal ventral sac of 8 goats of each group. Values are means ± standard error.

are mostly found at the superficial aspects of the ruminal epithelium, including the SC and stratum granulosum (SG), by using the TUNEL assay. The goats in the MC group had higher apoptotic indices than goats in the LC group (6.3 vs. 2.0%; $P < 0.001$).

Gene Expression In Vivo in Goats Fed by MC or LC Diet

The expression of genes related to cell-cycle-regulating proteins (Figure 2; e.g., cyclin A, cyclin B1, cyclin D1, cyclin E1, *CDK1*, *CDK2*, *CDK4*, and *CDK6*) was upregulated in the MC group compared with that in the LC group. The expression of the genes that regulate cell apoptosis (Figure 3), namely caspase 3, caspase 8, caspase 9, *p53*, *Bcl-2*, and *Bax*, was also evaluated by real-time quantitative PCR. Their relative expression in the MC group was enhanced compared with that in the LC group, with the exception of *Bcl-2*, which was decreased. The ratio of *Bcl-2* to *Bax* (*Bcl-2/Bax*) was also reduced ($P < 0.01$) in the MC group.

Table 5. Effect of feeding low concentrate (LC) or medium concentrate (MC) diets on cell cycle progression in ruminal epithelium of goats 6 h after feeding¹

Item ²	Treatment		P-value
	LC	MC	
G ₀ /G ₁ (%)	86.4 ± 1.64	79.6 ± 0.44	0.02
S (%)	4.2 ± 0.32	5.5 ± 0.16	0.02
G ₂ /M (%)	9.5 ± 1.33	15.0 ± 0.30	0.02

¹Goats (n = 8) received the LC or MC diet at least 21 d before slaughter. Samples from 8 goats of each group were used for flow-cytometric analysis. Values are means ± standard error.

²G₀/G₁ = the proportion of cells in the gap G₀ phase and G₁ phase (the percentage is based on 10,000 cells); S = the proportion of cells in the synthesis (S) phase, based on the fact that the single-cell DNA level is higher than the DNA content of resting diploid cells (the percentage is based on 10,000 cells measured); G₂/M = the proportion of mitotic cells (the percentage is based on 10,000 cells).

Gene Expression in Primary Culture of Rumen Epithelial Cells

A decrease of pH from 7.4 to 6.8 in the culture medium increased ($P < 0.05$) cyclin D1, *CDK2*, and *CDK6* gene expression by 80, 113, and 189% after 24 h of primary cell culture (Figure 4). At pH 7.4, the presence of SCFA (20 mM) in the culture medium led to an increase of ($P < 0.05$) cyclin A, cyclin D1, cyclin E1, *CDK2*, *CDK4*, and *CDK6* gene expression by 51, 107, 201, 68, 123, and 59%, respectively, as compared with the absence of SCFA in the medium. Moreover, the combined application of both low pH and SCFA (pH 6.8 + SCFA 20 mM) to the culture medium resulted in upregulated ($P < 0.05$) gene expression of cyclin B1, cyclin E1, *CDK1*, and *CDK4* by 119, 790, 53, and 79%, respectively, compared with the culture medium at pH 6.8, but no added SCFA. In the culture medium

Figure 1. Effect of feeding low concentrate (LC) or medium concentrate (MC) diets on the cell apoptotic index in the ruminal epithelium of goats. Goats received the LC or MC diet for 21 d. Values are mean ± SEM, n = 8.

Figure 2. Effect of feeding low concentrate (LC) or medium concentrate (MC) diets on the expression of genes related to cell cycle regulators in the ruminal epithelium of goats. Goats received the LC or MC diet for 21 d. Values are mean $\pm$ SEM, $n = 8$. * indicates different from LC ($0.01 < P < 0.05$).

with SCFA (20 mM), a decrease of pH from 7.4 to 6.8 resulted in the upregulation ($P < 0.05$) of gene expression of cyclin B1, cyclin E1, *CDK1*, *CDK2*, and *CDK6* by 94, 153, 47, 45, and 23%, respectively. However, it led to a reduction of the gene expression of cyclin A ($P < 0.05$).

A combination of SCFA and pH exerted synergistic effects on the expression of cyclin A, cyclin B1, cyclin E1, and *CDK6* (interaction: $P < 0.05$). The SCFA or pH treatment alone of the culture medium exhibited similar effects as they did in the combined treatment in the present study.

The expression of genes that regulate cell apoptosis; for example, caspase 3, caspase 8, caspase 9, *p53*, *Bcl-2*, and *Bax*, was also evaluated by real-time quantitative PCR in primary cell culture after 24 h of culture (Figure 5). In the culture medium at pH 6.8, the gene expression of caspase 3, caspase 8, caspase 9, and *p53* increased significantly ($P < 0.05$) by 221, 95, 93, and

61% compared with that at pH 7.4. Conversely, the ratio of *Bcl-2* to *Bax* (*Bcl-2/Bax*) was downregulated ($P < 0.05$). At pH 7.4, the presence of SCFA (20 mM) in the medium increased ($P < 0.05$) caspase 3, caspase 9, and *Bax* gene expression by 84, 101, and 126%, respectively, as compared with the medium with no SCFA, whereas the gene expression of caspase 8, *p53*, and the ratio of *Bcl-2* to *Bax* (*Bcl-2/Bax*) was decreased ($P < 0.05$) under this culture condition. Moreover, a combined application of both low pH and SCFA (pH 6.8 + SCFA 20 mM) in the medium resulted in an increase ($P < 0.05$) in the gene expression of caspase 3, *Bcl-2*, and *Bax* by 30, 126, and 35% compared with that in medium at pH 6.8 but no SCFA. However, the gene expression of caspase 8 and *p53* was reduced ($P < 0.05$). A further comparison between the effects of pH 7.4 + SCFA (20 mM) and pH 6.8 + SCFA (20 mM) showed that medium at pH 6.8 + SCFA (20 mM) upregulated ($P < 0.05$) the gene expression of caspase 3 by 126%.

Figure 3. Effect of feeding low concentrate (LC) or medium concentrate (MC) diets on the expression of genes related to cell apoptosis in ruminal epithelium of goats. Goats received the LC or MC diet for 21 d. Values are mean $\pm$ SEM, $n = 8$. * indicates different from LC ($0.01 < P < 0.05$). ** indicates different from LC ($P < 0.01$).

Figure 4. In vitro effects of acid (pH 6.8), short-chain fatty acids (SCFA; 20 mM), or acid (pH 6.8) + SCFA (20 mM) on gene expression of cell cycle regulators in rumen epithelial cells of goats after cells were incubated for 24 h. In groups with pH 6.8 treatment, the medium was acidified by 1 N HCl. The 20 mM SCFA contained (in mM) 12 sodium acetate, 5 sodium propionate, and 3 sodium butyrate. The levels of gene expression were quantified with real-time PCR in comparison with GAPDH. Values are means ± SEM, n = 8. Values within a group without a common letter (a–c) are significantly different ($P < 0.05$).

Figure 5. In vitro effects of acid (pH 6.8), short-chain fatty acids (SCFA; 20 mM), or acid (pH 6.8) + SCFA (20 mM) on expression of genes associated with cell apoptosis in rumen epithelial cells of goats after cells were incubated for 24 h. In groups with the pH 6.8 treatment, the medium was acidified by 1 N HCl. The 20 mM SCFA contained (in mM) 12 sodium acetate, 5 sodium propionate, and 3 sodium butyrate. The levels of gene expression were quantified with real-time PCR in comparison with GAPDH. Values are means ± SEM, n = 8. Values within a group without a common letter (a–d) are significantly different ($P < 0.05$).

The SCFA or pH treatment alone of the culture medium exhibited significant effects on the expression of most of the genes tested in the present study. Furthermore, we found that SCFA and pH exerted synergistic effects on the expression of *p53*, *Bcl-2*, and *Bcl-2/Bax* (interaction: $P < 0.05$).

DISCUSSION

Ruminal Fermentation

In the present study, diets varying in percentage of concentrate induce changes in ruminal fermentation. By increasing the proportion of the diet comprised of concentrate from 10 to 35%, we increased SCFA and decreased pH. This result is in agreement with previous reports that have suggested that ruminal pH decreases and total SCFA concentrations increase as the dietary concentrate to forage ratio (Yan et al., 2014), high-grain diet (Liu et al., 2013; Metzler-Zebeli et al., 2013; Mao et al., 2016), or the NFC level (Lu et al., 2015) increases. In present study, the 2 diets tested had equally energy-providing and nitrogen contents. They were in the maintenance level. However, the NFC in the MC group was higher than that in the LC group to cause a decrease in ruminal pH and an increase in total SCFA concentration. Our aim was to investigate the effect of SCFA and pH on ruminal cell proliferation and apoptosis.

Effects of Diet on Ruminal Cell Cycle and Expression of Related Genes

Cellular proliferation is controlled primarily by the regulation of the cell cycle, which consists of 4 distinct but sequential phases (G_1 , S, G_2 , and M phase). The G_1 phase is the phase of cell growth. The S phase is the phase of DNA replication. The G_2 phase distributes to the duplication of chromosomes, and the M_2 is the cell division phase. In addition to G_1 , S, G_2 , and M phases, the term G_0 is used to describe cells that have exited the cell cycle and become quiescent (Norbury and Nurse, 1992). In this study, the proportion of cells in the G_0/G_1 phase was lower, whereas that in the S-phase and G_2/M phases was higher in the MC group, indicating that the time duration of the G_1 phase was shortened and the cell cycle progression was accelerated under the MC treatment.

The cell cycle control system is based on 2 protein families: the cyclin-dependent protein kinases (CDK) and the cyclins (Vermeulen et al., 2003). The 2 G_1 cyclins are cyclin D1 and cyclin E. The binding of cyclin D1 to CDK4 and to CDK6 to form complexes is essential for entry in G_1 phase (Mathew et al., 2010).

The cyclin E, which binds to CDK2, promotes progression from G_1 into S phase (Ohtsubo et al., 1995). The complex of cyclin A and CDK2 is required in S phase (Girard et al., 1991; Walker and Maller, 1991). The cyclin A and CDK1 complex promotes cells entry into M phase. This progression is further regulated by cyclin B in complex with CDK1 (King et al., 1994; Arellano and Moreno, 1997). Any change in the duration of one or more phases of the cell cycle will affect the rate of cell production (Shen et al., 2004). In present experiments, the gene expression of the cell cycle regulators (cyclin A, cyclin B1, cyclin D1, cyclin E1, *CDK1*, *CDK2*, *CDK4*, and *CDK6*) was enhanced in goats in the MC group compared with the LC group, indicating these cell cycle regulators promoting cell cycle progression in the MC group. These data lead to the speculation that the cell cycle of the rumen was promoted when goats were fed the MC diet, a finding that is consistent with the result of the cell cycle. In the present study, the ruminal SCFA increases and pH decreases in growing goats by increasing the proportion of the dietary concentrate from 10 to 35%. This is in agreement with previous reports (Yang et al., 2012; Yan et al., 2014). It has been shown that concentrate feeding caused an increase of rumen papillae number and enlargement of papillae size. This is associated with accelerated epithelial proliferation (Shen et al., 2004; Lu et al., 2013). The effect of concentrate feeding on rumen papillae growth is partly related to luminal factors such as rumen-generated SCFA (mainly butyric acid) and pH (Gäbel et al., 1987; Uppal et al., 2003). The higher concentration of SCFA was considered as the promoter of the rumen epithelial cell proliferation (Ørskov, 1976). McLeod et al. (2007) further reported that luminal SCFA are the primary stimuli for proliferation.

Effects of Diet on Ruminal Cell Apoptosis and Expression of Related Genes

In the present experiments, we successfully used the staining and detection of TUNEL-positive cells in ruminal epithelium, with TUNEL-positive cells being most numerous at the lumen side of the ruminal epithelium. The mucosal surface of goat rumen is covered by stratified squamous epithelium, which is composed of 4 cell layers the SC, SG, stratum spinosum, and SB. The apoptotic cells usually occur in SC and SG. This result is in agreement with previous reports that the TUNEL-positive cells are most prevalent toward the base of the gastric glands in the stomach epithelium (Hall et al., 1994). The apoptotic index of rumen epithelial cells is higher in the MC group than in the LC group. This was supported by the gene expression study.

In the present experiments, the gene expression of the cell apoptosis regulators (caspase 3, caspase 8, caspase 9, *p53*, and *Bax*) was all enhanced in goats in the MC group, compared with the LC group, and the ratio of *Bcl-2* to *Bax* (*Bcl-2/Bax*) was reduced. These data lead to the speculation that cell apoptosis in the rumen is promoted when goats are fed the MC diet, as is consistent with the result of the TUNEL experiments. In brief, the intake of a 35% concentrate diet leads to both an accelerated ruminal cell cycle and the promotion of ruminal cell apoptosis. At the maintenance level, the nutritive material arising from apoptotic cell can be used for cell proliferation, which might be conducive to epithelial homeostasis.

Papillae Morphology and Rumen Epithelial Growth

We have shown that the cell densities in SGv in the ruminal ventral sac are higher in the MC group than in the LC group. In accordance with our results, previous studies have shown that an increase of SCFA absorption in ruminants fed high-concentrate diets is linked to the surface area enlargement of ruminal papillae (Dirksen et al., 1984; Gäbel et al., 1991a). The latter might be attributable to an increased abundance of gene transcripts related to epithelial proliferation (Penner et al., 2011). However, the cell densities in SGv of the MC group is only increased 6.1% compared with the LC group, and the number of cell layers in SC and the cell densities in SB were not different between the LC and MC groups. We speculate that, in this experiment, not only an increase of cell proliferation, but also an increase in cell death occurs in the ruminal epithelium. In conclusion, the 35% concentrate diet (with a normal grain level) can promote cell proliferation of the ruminal papillae.

In Vitro Combined Effects of SCFA and pH

Multicellular organisms have many potent physiological mechanisms that govern cell proliferation and tissue homeostasis. Most of the growth-control mechanisms are connected to apoptosis. Apoptosis can be induced in the case of inappropriate growth, for example, excess proliferation or growth in these cells (Schmitz et al., 2000). During the last century and more recently, a surge of studies was carried out to determine the effect of SCFA on ruminal epithelium cell proliferation (Sakata and Yajima, 1984; Neogrády et al., 1989; Malhi et al., 2013); however, most of those studies examined cell proliferation and not cell apoptosis and did not further investigate the influencing factors. Nevertheless, previous studies have shown that luminal factors, especially

SCFA and pH, can act as modulators of gene transcription (Li and Li, 2006; Lu et al., 2015; Yan et al., 2014). Furthermore, we have observed marked differences in ruminal fermentation measurements, including SCFA concentration and ruminal pH in the *vivo* experiment. Based on the above information, we have assumed that SCFA and pH affect the transcription of genes related to cell cycle regulators and cell apoptosis. To test this assumption, we have conducted *in vitro* trials.

Our primary cell culture experiments have shown that SCFA and an acidic pH both upregulate proliferation and increase senescence, sloughing, or both. These results are in accord with previous studies that have shown that SCFA, such as acetic, propionic, and n-butyric acids, stimulate the epithelial cell proliferation of the large intestine in rats (Sakata, 1987; Kripke et al., 1989) and human colonic mucosa (Scheppach et al., 1992). The SCFA, especially butyrate, stimulate cell apoptosis (Kurita-Ochiai et al., 1997; McMillan et al., 2003) in certain types of cells (murine thymocytes, splenic T cells, human Jurkat T cells, and human colon adenoma cells). The present study has indicated that epithelial gene and protein expression in the rumen is mainly affected by luminal factors that act via the apical cell membrane (Sanderson and Naik, 2000). The SCFA, which generated from microbial fermentation, can regulate host gene expression via the cell cycle and apoptosis. The study of the interactions between gut microbiota and host represents an active field of research (den Besten et al., 2013). By using the general linear model with relevant interactions, we have found that the genes cyclin A, cyclin B1, cyclin E1, *CDK6*, *p53*, *Bcl-2*, and *Bcl-2/Bax* are regulated by the combined effects of SCFA and pH.

For tissue cell homeostasis, the balance between cell gain via mitosis and cell loss by differentiation and apoptosis is critical. In consequence, enhanced proliferation might be a compensatory response to replace the cells lost by necrosis or apoptosis. This is partly related to luminal factors such as rumen-generated SCFA (mainly butyric acid) and pH in the rumen epithelium. Thus, SCFA and pH are physiological modulators of cell number in the rumen epithelium and can regulate rumen epithelium homeostasis not only by proliferation, but also by apoptosis. The above results have shown that an MC diet has a crucial role in maintaining rumen epithelial homeostasis and integrity, which is beneficial to animal health. The SCFA and proton interact with their receptors to adapt the change of energy density of the diet. The interaction of SCFA and proton with their receptors affect ruminal epithelial growth and physical structure. The further research has the potential to increase our understanding of absorptive area plasticity under changing dietary conditions.

CONCLUSIONS

An increase of dietary concentrate from 10 to 35% causes both an accelerated ruminal cell cycle and promotes ruminal cell apoptosis. In parallel, the expression of genes related to regulators of the cell cycle (cyclin A, cyclin B1, cyclin D1, cyclin E1, *CDK1*, *CDK2*, *CDK4*, and *CDK6*) and of cell apoptosis (caspase 3, caspase 8, caspase 9, *p53*, and *Bax*) is enhanced depending upon the percentage of the dietary concentrate. In primary cell culture, SCFA and acidic pH modulate ruminal genes involved in cell proliferation and apoptosis. Taken together, our data demonstrate that increase of SCFA concentration and decrease of pH are putative regulators of gene expression in rumen epithelium. This study, therefore, provides new insights into the epithelial homeostasis and molecular adaptation of rumen epithelium to adequate concentrate intake.

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