ABSTRACT: It has been known for decades that microbial fermentation within the rumen is critical to postnatal rumen epithelial growth and maturation in ruminants, but the underlying mechanism is largely unknown. In this study, we determined the effect of rumen fluid, which should contain all products from rumen fermentation, on growth of rumen epithelial cells in vitro. Addition of 10% rumen fluid from cows to the culture medium inhibited (P < 0.05), whereas addition of 6.5 mM acetate, 2.5 mM propionate, or 1 mM butyrate had no effect (P > 0.1) on the proliferation of rumen epithelial cells isolated from newborn calves. Flow cytometric assays showed that 10% rumen fluid inhibited (P < 0.05) the transition of rumen epithelial cells from the G1 phase to the S phase during the cell cycle. Real-time RT-PCR analyses of mRNA for key cell cycle regulators indicated that 10% rumen fluid did not change (P > 0.1) the expression of cyclin D1, D2, D3, E1, or E2 mRNA or that of cyclin-dependent kinase inhibitor 1B or 2B mRNA, but increased (P < 0.05) the expression of cyclin-dependent kinase inhibitors 1A and 2A mRNA in rumen epithelial cells. These mRNA data support the possibility that rumen fluid inhibits proliferation of rumen epithelial cells in vitro by increasing the expression of cyclin-dependent kinase inhibitors 1A and 2A. The result that rumen fluid inhibits proliferation of bovine rumen epithelial cells in culture indicates that rumen fermentation does not stimulate the postnatal rumen epithelial growth in cattle by directly stimulating proliferation of rumen epithelial cells.

Key words: cattle, cell cycle, rumen epithelial cell, rumen fluid, volatile fatty acid

INTRODUCTION

The rumen is the primary site where high-fiber feed is digested through microbial fermentation into short-chain fatty acids (SCFA), or VFA, in ruminants. The VFA are major source of energy for ruminants, and approximately 95% of them are acetic, propionic, and butyric acids, or acetate, propionate, and butyrate, respectively (Siciliano-Jones and Murphy, 1989; Bergman, 1990; Kristensen et al., 1998). The rumen of a calf undergoes significant developmental changes from birth to 4 wk of age (Lyford, 1988). At birth, it has thin walls and short papillae, accounting for less than 30% of the total stomach mass, and is metabolically non-functional (Lyford, 1988). By 4 wk of age, the rumen represents 80% of the entire stomach weight, has long and wide dark-colored papillae, and is fully capable of absorbing and metabolizing VFA (Lyford, 1988). These physical and functional changes in rumen epithelium are believed to be caused by VFA, in particular, butyrate (Baldwin and Jesse, 1992; Lane and Jesse, 1997; Lane et al., 2000).

The mechanism by which VFA stimulate rumen epithelial growth is unclear. Added to the medium, butyrate inhibited the proliferation of cultured rumen epithelial cells (Gálfy et al., 1981) and other types of cells (Sakata et al., 1980; Sakata and Yajima, 1984; Marsman and McBurney, 1996; Fu et al., 2004; Comalada et al., 2006). These inhibitory effects of VFA on cell growth in vitro have led some to hypothesize that the stimulatory effect of VFA on rumen epithelial growth in vivo is indirect (Sakata and Yajima, 1984; Harmon, 1992; Davie, 2003). In this study, we explored the possibility that rumen fermentation directly stimulates rumen epithelial growth through products other than VFA. We tested this possibility by determining the ef-
fects of whole rumen fluid from adult cattle and the individual effects of acetate, propionate, and butyrate on proliferation of rumen epithelial cells from newborn calves in vitro.

**MATERIALS AND METHODS**

Procedures involving animals were performed according to the protocols approved by the Virginia Tech Institutional Animal Care and Use Committee.

**Rumen Fluid Preparation**

Rumen fluid samples were obtained from lactating Holstein cows via fistula used in an unrelated study (M. Hanigan, Virginia Tech, Blacksburg, unpublished data). The cows were fed corn silage, alfalfa hay, and grain. After collection, the rumen content was filtered through 4 layers of cheese cloth and then centrifuged at 12,000 × g for 1 h at 4°C. The supernatant was collected and filtered through 0.2-µm filters.

**Isolation and Culturing of Rumen Epithelial Cells**

Rumen epithelial cells were isolated from newborn calves using the serial tryptic digestion procedure as described previously (Klotz et al., 2001). The Holstein calves were killed with an overdose of pentobarbital for rumen tissue collection. The rumen tissue samples were rinsed with cold running water and transported to the laboratory in PBS on ice. In the laboratory, the epithelial layer of the rumen was separated and minced into small pieces. The minced tissue (~20 g) was digested in 50 mL of digestion solution composed of 5% trypsin (1:250, MP Biomedicals, Solon, OH), 1.08 mM CaCl₂, and 25 mM HEPES in Krebs-Ringer buffer for 15 min at 37°C in a slow-shaking incubator. After this digestion, the tissue was filtered through a 300-µm nylon mesh. The filtrate was collected on ice. The tissue remaining on the mesh was rinsed again as described above. The tissue usually underwent 7 cycles of digestion. The rumen epithelial cells were recovered by centrifuging the combined filtrates at 70 × g for 6 min at 4°C. The cells were cultured at 37°C under 5% CO₂ in minimum essential medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 µg/mL of streptomycin, and 0.25 µg/mL of amphotericin B. All reagents used in cell culture were purchased from Sigma-Aldrich (St. Louis, MO) unless indicated otherwise.

**Cell Proliferation Assay**

After 3 d of initial culturing, the cells were harvested by trypsinization and reseeded into 96-well plates at 4,000 cells per well. The culture medium was the same as described above except with addition of 1 or 10% (vol/vol) rumen fluid, or 6 mM acetate, 2.5 mM propionate, or 1 mM butyrate, or PBS. These concentrations of acetate, propionate, and butyrate approximated their concentrations in 10% rumen fluid (Bergman, 1990; Sutton et al., 2003). Each treatment consisted of 6 wells. The cells were then cultured for 0, 24, 48, or 72 h. The number of viable cells was determined using the Nonradioactive CellTiter 96 Assay kit (Promega, Madison, WI), essentially according to the manufacturer’s instructions. Briefly, 15 µL of dye solution was added to each well. After 4 h, 100 µL of solubilization/stop solution was added, and the plate was incubated overnight at 37°C before the absorbance at 570 nm was recorded using a 96-well plate reader. This cell proliferation experiment was repeated 4 times, each time using cells from a different calf.

**DNA Fragmentation Assay**

The rumen epithelial cells were cultured in the presence of 10% rumen fluid or PBS for 0, 24, 48, or 72 h before DNA fragmentation assay. This assay was performed as described previously (Kotamraju et al., 2000). Briefly, the cells were lysed with a hypotonic lysis buffer composed of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100. The lysates were digested with 0.1 mg/mL of RNase A at 37°C for 1 h and then with 100 mg/mL of proteinase K for 2 h at 50°C. The DNA was extracted with phenol, chloroform, and isoamyl alcohol mixture (Fisher Scientific, Pittsburgh, PA) and precipitated by isopropyl alcohol. The DNA was electrophoretically separated on 2% agarose gels containing ethidium bromide.

**Flow Cytometric Assay**

The rumen epithelial cells were cultured in the presence of 10% rumen fluid or PBS for 24 h as described above before being collected for flow cytometric assay. The cells were collected by trypsinization, washed with the culture medium twice, and then resuspended in PBS. Aliquots of 0.5-mL cell suspension containing 1 × 10⁶ cells were fixed in 4.5 mL of 70% ethanol on ice for 2 h. After this fixation, the cells were centrifuged at 200 × g at 4°C for 5 min and washed with PBS once. The cells were resuspended in 1 mL of propidium iodide staining solution consisting of 0.1% Triton X-100, 0.20 mg/mL of DNase-free RNase A, and 0.02 mg/mL of propidium iodide in PBS and incubated at 37°C for 15 min. The DNA content was analyzed using flow cytometry (FACS Aria, BD Biosciences, San Jose, CA). The flow cytometric data were analyzed using FlowJo (Tree Star Inc., Ashland, OR) and ModFit (Verity Software House, Topsham, ME).

**Quantitative Real-Time PCR**

The rumen epithelial cells were cultured in the presence of 10% rumen fluid or PBS for 24 h. Total RNA
was extracted with TRI Reagent, essentially according to the manufacturer’s instructions (Molecular Research Center, Cincinnati OH). Two micrograms of total RNA were reverse-transcribed in a total volume of 20 µL using ImProm-II reverse transcriptase and random primers, under conditions recommended by the manufacturer (Promega). One hundred nanograms of cDNA were amplified in a total volume of 25 µL containing 12.5 µL of SyberGreen PCR Master Mix (Applied Biosystems, Foster City, CA) and 0.2 µM of gene-specific forward and reverse primers (Table 1) under 40 cycles of 95°C for 15 s and 60°C for 1 min. The real-time PCR data were analyzed by the 2−∆∆Ct method (Livak and Schmittgen, 2001), using 18S rRNA as the internal control. Based on the Ct values, expression of 18S rRNA was not different (P > 0.1) between the rumen fluid-treated cells and the control cells.

Statistical Analyses

All data were analyzed using GLM (SAS Inst. Inc., Cary, NC). Multiple comparisons were done using the Tukey test. A difference was considered statistically significant when P < 0.05 and not significant when P > 0.1. All data were expressed as means ± SEM.

RESULTS

Rumen Fluid but Not Acetate, Propionate, or Butyrate Inhibited Proliferation of Rumen Epithelial Cells

The rumen epithelial cells from newborn calves maintained the ability to proliferate in culture (Figures 1A and 1B). Addition of 10% rumen fluid to the culture medium inhibited the proliferation of these cells (P < 0.05, Figure 1A). This inhibition was more prominent when the cells were cultured for 72 h than for 48 h or 24 h (Figure 1A). Addition of 1% rumen fluid to the culture medium did not inhibit the proliferation of these cells (P = 0.07, Figure 1A). Addition of 6 mM acetate, 2.5 mM propionate, or 1 mM butyrate to the medium did not inhibit the proliferation of these cells either, compared with the PBS control (P > 0.1, Figure 1B).

Rumen Fluid Did Not Induce Detectable Apoptosis in Rumen Epithelial Cells

Apoptosis, or programmed cell death, is characterized by the activation of endogenous endonucleases, which in turn results in the cleavage of chromatin DNA, or DNA fragmentation. As shown in Figure 2, the cells cultured in the presence of 10% rumen fluid for 24, 48, or 72 h did not show more DNA fragmentation or any detectable DNA fragmentation by DNA fragmentation assays, compared with control cells.

Rumen Fluid Induced Cell Cycle Arrest at the G1/G0 Phase

To understand the mechanism by which the proliferation of rumen epithelial cells was inhibited by rumen fluid, we used flow cytometry to identify the specific phases of cell cycle at which the cells were affected by rumen fluid. As shown in Figure 3, the cells treated with 10% rumen fluid for 24 h contained 20% more cells at the G1 or G0 (G1/G0) phase (P < 0.05) and 10% less at the G2 or M (G2/M) phase (P < 0.05) compared with the control cells. These data indicated that rumen fluid inhibited the proliferation of rumen

Table 1. The PCR primers used in this study

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Gene name</th>
<th>GenBank accession No.</th>
<th>Amplicon size, bp</th>
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<td>204</td>
</tr>
<tr>
<td>GTCAGGGCCTGATAGGAGAG</td>
<td>bCCND2</td>
<td>NM_001076372</td>
<td>163</td>
</tr>
<tr>
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<td>bCCND3</td>
<td>NM_001034709</td>
<td>178</td>
</tr>
<tr>
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<td>bCCNE1</td>
<td>XM_612960</td>
<td>187</td>
</tr>
<tr>
<td>TTCAGATCGGACTTGCGA</td>
<td>bCCNE2</td>
<td>NM_001015665</td>
<td>229</td>
</tr>
<tr>
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<td>bCDKN2B</td>
<td>NM_001075894</td>
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<td>208</td>
</tr>
<tr>
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<td>bCDKN1A</td>
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</tr>
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<td>bCDKN1B</td>
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<td>249</td>
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<tr>
<td>CCATCCTGGTTCACTCGG</td>
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</table>

1All sequences are written from 5′ to 3′. The top sequence of a pair of primers is the forward primer and the bottom sequence the reverse primer.
epithelial cells by blocking their progression from the G1/G0 phase to the S phase during the cell cycle.

Rumen Fluid Increased mRNA Expression of Cyclin-Dependent Kinase Inhibitors 1A and 2A

The transition from the G1/G0 phase to the S phase during the eukaryotic cell cycle is controlled by positive regulators such as cyclins D1, D2, D3, E1, and E2, and negative regulators including cyclin-dependent kinase inhibitors 1A, 2A, 1B, and 2B or p21, p27, p16, and p15, respectively (King and Cidlowski, 1998). As shown in Figure 4, addition of 10% rumen fluid to the culture medium did not affect the expression of cyclins D1, D2, D3, E1, E2 mRNA or cyclin-dependent kinase inhibitors 1B, 2B mRNA \( (P > 0.1) \), but caused a nearly 3-fold increase in the expression of cyclin-dependent kinase inhibitors 1A and 2A mRNA \( (P < 0.05) \).

**DISCUSSION**

Microbial fermentation within the rumen is critical to rumen epithelial growth in young ruminants, but the underlying mechanism is unclear (Zitnan et al., 1999; Baldwin et al., 2004). Previous studies in this area have focused on the role of acetate, butyrate, and propionate in this process because they are the major products of rumen fermentation. Intraruminal administration of acetate, propionate, and butyrate stimulated the growth and functional maturation of the rumen epithelium in young ruminants, with the effect of butyrate being most prominent, followed by propionate (Sakata and Tamate, 1978, 1979; Lane and Jesse, 1997). Limiting rumen production of VFA through feeding only milk inhibited rumen epithelial growth (Warner et al., 1956; Harrison et al., 1960; Tamate et al., 1962). However, VFA, including butyrate, inhibited the growth of many types of cells in vitro (Sakata and Yajima, 1984; Fu et al., 2004; Comalada et al., 2006). These seemingly conflicting effects of VFA between in vivo and in vitro led some investigators to postulate that rumen VFA stimulate rumen development through indirect mecha-

![Figure 1](image1.png)

**Figure 1.** Effects of rumen fluid and VFA on proliferation of bovine rumen epithelial cells. Rumen epithelial cells from newborn calves were treated with PBS (as a control), 1%, or 10% rumen fluid (RF) from lactating cows (panel A), or 6 mM acetate, 2.5 mM propionate, or 1 mM butyrate (panel B) for 24, 48, and 72 h, followed by cell proliferation assay. The absorbance at 570 nm on the y-axis represents the number of viable cells. The treatments not labeled with the same letter (a, b) had different effects on proliferation of the cells \( (P < 0.05, n = 4) \).

![Figure 2](image2.png)

**Figure 2.** The DNA fragmentation assay of bovine rumen epithelial cells. The rumen epithelial cells from newborn calves were treated with PBS or 10% rumen fluid (RF) for indicated times. At the end of these treatments, DNA was extracted and electrophoresed on 2% agarose gels containing ethidium bromide. The assay was repeated 4 times with similar results. A representative photograph of the gels is shown. The M denotes a DNA ladder.
nisms, perhaps through insulin, because VFA stimulate insulin secretion in ruminants (Sakata and Yajima, 1984; Bergman, 1990; Harmon, 1992). In this study, we hypothesized that rumen fermentation generates products that directly stimulate rumen epithelial cell growth and that overtake the growth inhibitory effects of VFA, and we tested this hypothesis by determining the effect of whole rumen fluid on proliferation of bovine rumen epithelial cells in culture. The study showed that 10% rumen fluid from cattle inhibited the proliferation of bovine rumen epithelial cells in culture. This result does not seem to support our original hypothesis and suggests that rumen fermentation does not stimulate rumen epithelial growth in cattle by directly increasing proliferation of rumen epithelial cells.

This study has further shown that rumen fluid inhibits the proliferation of rumen epithelial cells by inhibiting their transition from the G1 phase to the S phase during the cell cycle. The progression of the cell cycle from the G1 phase to the S phase is controlled by cyclin-dependent kinases. The activities of these kinases are regulated by cyclins D1, D2, D3, D4, E1, and E2 that bind and activate the cyclin-dependent kinases, and cyclin-dependent kinase inhibitors 1A, 1B, 2A, and 2B that bind and inactivate the cyclin-dependent kinases (King and Cidlowski, 1998). This study has shown that rumen fluid increases mRNA expression of cyclin-dependent kinase inhibitors 1A and 2A in rumen epithelial cells. This result may indicate that rumen fluid inhibits the progression of rumen epithelial cells from the G1 phase to the S phase during the cell cycle by upregulating the expression of cyclin-dependent kinase inhibitors 1A and 2A.

What components of rumen fluid are responsible for the inhibitory effect of rumen fluid on proliferation of bovine rumen epithelial cells in vitro? Because VFA are the major components of rumen fluid (except for water), and because VFA, such as butyrate, inhibit proliferation of various types of cells in vitro (Sakata and Yajima, 1984; Fu et al., 2004; Comalada et al., 2006), it is tempting to think that the inhibitory effect of rumen fluid on proliferation of bovine rumen epithelial cells is mediated by VFA. In this study, at its concentration in 10% rumen fluid, neither acetate, nor propionate, nor butyrate inhibited proliferation of bovine rumen epithelial cells. This may indicate that the inhibitory effect of rumen fluid on proliferation of bovine rumen epithelial cells is unlikely due to the effect of a single VFA if it is mediated by VFA. It remains to be determined if acetate, propionate, and butyrate combined have as strong an inhibitory effect on proliferation of rumen epithelial cells as rumen fluid. We cannot exclude the possibility that some of the growth inhibitory effect of rumen fluid on rumen epithelial cells is mediated by non-VFA components of rumen fluid, such as endotoxins and bioactive peptides processed from microbial or diet-derived protein.

Butyrate has been shown to arrest cell cycle at the G1/G0 phase (Sakata and Yajima, 1984; Li and Elsasser, 2005; Li and Li, 2006; Hatayama et al., 2007; Wang et al., 2008) and to induce cyclin-dependent kinase inhibitors 1A and 2A expression in various types of cells (Mahyar-Roemer and Roemer, 2001; Hinnebusch et al., 2002; Davie, 2003; Orchel et al., 2003; Shi et al., 2006). This study has shown that rumen fluid also halts the cell cycle of bovine rumen epithelial cells at the G1/G0 phase.
phase and increases mRNA expression of cyclin-dependent kinase inhibitors 1A and 2A in these cells. Based on these similar effects of rumen fluid and butyrate on cell cycle and gene expression and the data that butyrate alone does not inhibit proliferation of bovine rumen epithelial cells as much as whole rumen fluid, we speculate that the effects of rumen fluid on cell cycle progression and gene expression in rumen epithelial cells may be mediated in part by butyrate.

In summary, this study shows that rumen fluid inhibits proliferation of the bovine rumen epithelial cells in vitro by inhibiting their progression from the G1 phase to the S phase during the cell cycle. This growth inhibitory effect of whole rumen fluid in vitro may indicate that products of rumen fermentation do not stimulate rumen epithelial growth by directly stimulating proliferation of rumen epithelial cells. This study also shows that rumen fluid increases the expression of cyclin-dependent kinase inhibitors 1A and 2A in bovine rumen epithelial cells. This result may indicate that rumen fluid might inhibit proliferation of bovine rumen epithelial cells by increasing the expression of these cell cycle inhibitors.

**LITERATURE CITED**


Kotanraju, S., E. A. Konorev, J. Joseph, and B. Kalyanaraman. 2000. Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and eb-

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**Figure 4.** Effect of rumen fluid (RF) on gene expression of cell cycle regulators in bovine rumen epithelial cells. The rumen epithelial cells from newborn calves were treated with 10% RF or PBS for 24 h before total RNA extraction. The mRNA expression of 9 cell cycle regulators and 18S rRNA (internal control) were measured by reverse transcription coupled with real-time PCR. *Indicates \( P < 0.05 \), compared with the PBS control (\( n = 3 \)). bCCND1, bovine cyclin D1; bCCND2, bovine cyclin D2; bCCND3, bovine cyclin D3; bCCNE1, bovine cyclin E1; bCCNE2, bovine cyclin E2; bCDKN2B, bovine cyclin-dependent kinase inhibitor 2B; bCDKN2A, bovine cyclin-dependent kinase inhibitor 2A; bCDKN1A, bovine cyclin-dependent kinase inhibitor 1A; bCDKN1B, bovine cyclin-dependent kinase inhibitor 1B.