Use of DNA Probes to Monitor Nutritional Effects on Ruminal Prokaryotes and *Fibrobacter succinogenes* S85\(^1,2\)

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**ABSTRACT:** We used DNA probes to study dietary effects on the prokaryotic population in the rumen. Procedures used to isolate and quantify prokaryotic 16S ribosomal RNA (rRNA) from the rumen using universal and species-specific DNA probes were evaluated. In this experiment, three ruminally fistulated steers were fed orchardgrass hay, and ruminal digesta were collected at 0, 3, and 9 h after offering hay (0800). Samples of ruminal digesta were taken from the interior portion of the digesta mat and from the fluid below the mat in the dorsal rumen. Freezing (\(-65°C\)) and blending samples both increased \((P < .07)\) the yield of 16S rRNA from ruminal digesta.

Extraction of prokaryotic rRNA was greater \((P < .04)\) when phenol buffered with sodium acetate was used than when it was buffered with hydroxymethyl-amino-methane. Prokaryotic 16S rRNA concentration of the fluid phase was similar \((P > .10)\) at 0, 3, and 9 h after offering hay. Prokaryotic 16S rRNA concentration of the mat phase increased up to the 9 h after feeding. The proportion of *Fibrobacter succinogenes* remained constant in both digesta phases at all times measured. From these data we concluded that DNA probes can be used to monitor bacterial population shifts in the rumen.

Key Words: Rumen Bacteria, DNA Probes, RNA, Oligonucleotides

**Introduction**

Establishment and maintenance of cellulolytic and hemicellulolytic ruminal bacterial populations in the rumen is a key factor affecting efficient forage utilization by ruminants. Although several methods exist to measure ruminal bacterial populations (Bryant and Burkey, 1953; Kistner, 1900), these procedures are laborious, subject to variation, and difficult to implement in a general nutrition laboratory if the necessary equipment for microbiological methods is not readily available. Recent advances in molecular biology have made possible the phylogenetic analysis of ruminal prokaryotes (Pace et al., 1985). Phylogenetic analysis of bacteria is based on ribosomal RNA for several advantageous reasons (Pace et al., 1985). Because ruminal degradative capacity is influenced primarily by the population of bacterial species in the rumen, we hypothesized that phylogenetic analysis could be used to study dietary effects on the ruminal bacterial population, which would enhance predictability of dietary supplementation on ration digestibility.

Stahl et al. (1988) synthesized oligonucleotide DNA probes, homologous to conserved regions of bacterial 16S rRNA, which were used to quantify relative bacterial populations in the ruminal ecosystem. A universal probe was synthesized to estimate total prokaryotes and a species-specific probe was used to estimate the relative proportion of total rRNA contributed by a prokaryotic species of interest.

The objectives of our study were 1) to evaluate different procedures used to harvest prokaryotic 16S rRNA from ruminal digesta and 2) to determine the effect that time after feeding and ruminal digesta phase (fluid vs mat) had on prokaryote 16S rRNA mass and relative proportion of *Fibrobacter succinogenes* S85.

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**Materials and Methods**

**Animals and Feeding.** Three ruminally cannulated crossbred steers (average weight, 550 kg) were used in this experiment as donors of ruminal digesta. Steers were cannulated under approved University of Missouri Office of Laboratory Animal Medicine guidelines. Steers were fed (1.5% BW orchardgrass hay; 7.5% CP) once daily at 0800 at a level equal to their maintenance energy requirements (NRC, 1984). Steers had ad libitum access to water and a trace mineral salt block. Steers were housed individually in a confinement facility with partial out-of-doors pens. Animals were fed for 10 d before ruminal digesta collections. Ruminal digesta were collected (100 mL) as described below on d 11 at 0, 3, and 9 h after offering hay.

**Isolation of Prokaryotic rRNA from Ruminal Digesta.** All labware was washed in sterile water treated with 0.2% (vol/vol) diethylpyrocarbonate. Ruminal contents were collected from the mat or the liquid phase of the ruminal digesta. Digesta were placed directly into sterile 100-mL specimen containers and placed on dry ice during transport to the laboratory. The digesta suspension was thoroughly mixed and evenly divided (by volume). One-half of the digesta collected was frozen (−65°C), and the other one-half of the digesta was immediately subjected to rRNA extraction procedures. Frozen samples were thawed (37°C) and 10 g of mat or fluid digesta was either placed in a jar and blended (3 min) with cold, sterile 85% saline (wt/vol) or was placed in a 50-mL centrifuge tube and vortexed for 3 min with cold, sterile saline. This allowed the merit of blending samples to be evaluated.

Mat samples were taken from the medial area of the ruminal mat. Fluid samples were taken from below the mat in the dorsal area of the ventral rumen by pushing a covered specimen container down through the mat and then opening it slightly to allow filling of the container.

Blended samples were washed thoroughly into centrifuge tubes with cold saline. All samples were centrifuged (400 × g, 4°C, 15 min) two times. The supernatant was decanted into a clean centrifuge tube and placed on ice and the pellet was washed in cold saline (400 × g, 4°C). Supernatants from each centrifugation were combined and then centrifuged for 15 min (10,000 × g, 4°C) to collect a bacterial pellet. After aspirating the supernatants with a sterile Pasteur pipette, bacterial pellets were gently resuspended in equal volumes of saline, and 750 μL of these suspensions was used for rRNA extraction.

**Extraction of RNA.** The entire extraction procedure was performed in a cold room (4°C), and samples were kept on ice between steps. All centrifuge tubes, pipette tips, glassware, and solutions were treated with 0.02% (vol/vol) diethylpyrocarbonate and autoclaved. All reagents used were molecular biology grade. Approximately 250 μL of 200-μm autoclaved glass beads (Sigma Chemical Co., St. Louis, MO) were placed in extraction tubes (2-mL, polypropylene screw cap) with 550 μL of buffered phenol (1.1% 8-hydroxyquinoline equilibrated with 50 mM sodium acetate and 10 mM EDTA [pH 5.20], 50 μL of 10% (wt/vol) SDS, 15 μL of diethylpyrocarbonate, and 750 μL of bacterial suspension. Samples were beaten on a Mini Bead-Beater (Biospec Products, Bartlesville, OK) (4°C, 3 min) to lyse bacterial cells. Samples were centrifuged at 14,000 × g (15 min, 4°C), and the aqueous layer was removed into sterile 1.5-mL polypropylene microtube tubes. A saturated solution of NaCl (250 μL) was added to each tube. Samples were then placed on ice for 10 min, centrifuged (14,000 × g, 4°C, 10 min), placed on ice for 10 min, and centrifuged. Supernatant was extracted once with 1 mL of buffered phenol: chloroform:isoamyl alcohol (100:24:1), removed, and reextracted with chloroform:isoamyl alcohol (24:1).

Phenol buffered with Tris, routinely used to extract rRNA (Sambrook et al., 1989), was compared to buffered phenol as an extraction medium for bacterial RNA. In Tris-buffered phenol extractions, samples were treated similarly to the samples extracted with buffered phenol, with the exception that bacterial cell suspensions were made using 1 mL of lysing buffer (100 mM NaCl, 30 mM Tris, 5 mM EDTA, and 0.1% SDS; pH 8.0). These samples were beaten (1.5 min), digested with proteinase K (100 μg from 20 mg/ml stock) at 37°C for 30 min, and again beaten for 1.5 min. Samples were then phenol-extracted using 500 μL of Tris-equilibrated phenol.

All samples were precipitated in ethanol in 2× final aqueous volume of absolute ethanol at −65°C overnight. Samples were centrifuged for 15 min (14,000 × g, 4°C), dried under vacuum, resuspended in 30 to 50 μL of TE buffer (10 mM Tris and 1 mM EDTA; pH 7.6), and stored at −65°C until further analysis.

**Oligonucleotide Synthesis and Endlabeling.** Oligonucleotide DNA probes were synthesized at the University of Missouri DNA core facility. The *F. succinogenes* S85 probe was constructed as published (Stahl et al., 1988). The universal probe sequence used in this experiment was constructed as follows: 5' ACG GCC GGT GTG TAC AAG GCC 3'. Oligonucleotide probes were endlabeled with 200 μCi of Iγamma-32P]ATP with T4 polynucleotide kinase (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in kinase buffer (500 mM Tris [pH 7.5], 100 mM MgCl2, and 50 mM dithiothreitol). The kinase reactions were carried out at 37°C for 45
min and stopped by placing in a waterbath at 65°C for 5 min. Labeled oligonucleotides were purified with NENSORB (Dupont NEN Products, Boston, MA) columns for removal of unincorporated 32P.

Hybridization of RNA. All RNA samples (2 μg) were denatured with 15.5 μL of denaturing solution (23.3% formalin, 66.7% deionized formamide, 13.3% 5x MOPS [19.4 g of 3-N-morpholinopropanesulfonic acid, 8.1 g of Na acetate, and 10 mL of 0.5 EDTA Na2] in 1 L) plus sterile water treated with diethylpyrocarbonate for a final volume of 20 μL at 65°C for 15 min and quickly placed on ice before electrophoresis or dotting onto a nylon membrane. Samples of RNA were subjected to electrophoresis on 1.2% denaturing agarose gels (1.2 g of agarose, 10 mL of 10x MOPS, and 17.7 mL of formaldehyde in 100 mL) in 1x MOPS (100 volts, 4 h) and transferred onto positively charged nylon membranes (Optiplot, IBI Corporation, New Haven, CT) with the aid of an electroblot (Model T, Biorad, Richmond, CA) apparatus in 0.5x BE (50 mM Tris, 50 mM borate, 1 mM EDTA). Blots were baked at 80°C for 2 h. These blots were hybridized with the universal probe to verify the presence of the 16S ribosomal band and to verify hybridization (Figure 1). Escherichia coli MRE 600 rRNA was used as a positive control for the 16S band and for the universal probe.

For each dot blot, 2 μg of RNA was denatured by adding 15.5 μL of 37% formaldehyde, formamide, and 5x MOPS (22.5:64.5:13.0, respectively) and dotted in triplicate onto a nylon membrane with the aid of a vacuum manifold (Model 3181, GIBCO BRL, Gaithersburg, MD). Both E. coli MRE 600 and F. succinogenes S85 rRNA (extracted from pure culture by blending and buffered phenol) were included on each blot as standards. After baking, membranes were prehybridized in 6x SSPE (52.6 g of 3M NaCl, 8.3 g of .2M NaH2PO4. H2O, .02M EDTA-Na2 to 1 L; pH 7.4), 10x Denhardt's solution (1 g of polyvinylpyrrolidone, 1 g of BSA, 1 g of Ficoll 400 to .5 L), .1% SDS, 50 μg/mL tRNA (Promega, Madison, WI) and 50 μg/mL of salmon sperm DNA (50 μl/blot) at 42°C for 2 h. Blots were hybridized (16 h, 52°C) with 1 x 107 counts per minute (cpm) 32P-labeled oligonucleotide per blot in 50 mL of 6x SSPE and .1% SDS. Blots were washed three times for 15 min at room temperature and once for 15 min at 52°C in 6x SSPE, .1% SDS (50 mL/blot).

Quantification of rRNA. Blots were hybridized with either universal or F. succinogenes S85 probe and scanned for 20 min on an AMBIS radioanalytic imaging system (Ambis Systems, San Diego, CA). Autoradiograms were taken of each blot for a permanent image (Figure 2). The probe was then stripped (65°C, 2 h) with probe removal solution (5 mM Tris-HCl [pH 8.0], .2 mM EDTA, .5% sodium pyrophosphate) and blots were checked for complete probe removal by autoradiography. Blots were then rehybridized with the other probe.

Blots were compared against themselves for calculation of proportions of F. succinogenes S85. A correction factor was used to compare a blot hybridized with the universal and with the species-specific probe. A standard dot of F. succinogenes should give equivalent binding with the universal probe as with the species-specific probe. Therefore, the correction factor was calculated by dividing the cpm of the F. succinogenes S85 standards by the cpm of the same dots with the

Figure 1. Autoradiogram of a Northern blot showing several 16S rRNA bands extracted from ruminal digesta that were hybridized with the universal [32P]-labeled oligonucleotide probe.

Figure 2. Autoradiogram of a dot blot, blotted with various concentrations of E. Coli 16S rRNA and hybridized with the universal [32P]-labeled oligonucleotide probe, similar to that used for calculating a standard curve to quantify the 16S rRNA concentration.
universal probe. To make comparisons among blots, a correction factor was calculated by dividing the standard cpm on each blot by the standard with the highest cpm to normalize the data. All cpm for samples were then adjusted using these correction/normalization factors before statistical analyses.

A separate experiment was undertaken to determine the relationship of growth rate to 16S rRNA concentration, as measured by oligonucleotide hybridization. A 1-mL overnight culture of E. coli XL Blues was used to inoculate (100 μL) 50 mL of Terrific broth (6 g of bactotryptone, 1.2 g of bactoyeast extract, .4 mL of 50% glycerol, 5 mL of potassium phosphate buffer, and 45 mL of water). This culture was grown in a sterile 250-mL flask at 37°C with shaking. Samples were removed over time for optical density determinations (600 nm). The optical density samples were taken such that a growth curve could be determined. In addition, five intermittent samples were taken for enumeration and RNA extraction. Samples were kept on ice until cell enumeration was complete and then frozen (−65°C). Samples of the culture were vortexed, and a 100-μL subsample was taken for cell enumeration. Cell numbers were quantified using a hemocytometer and light microscope. Three counts were taken on each sample that had rRNA quantified.

Statistical Analyses. The experiments were conducted as completely randomized designs. Data were analyzed by ANOVA (SAS, 1984) with treatment effect tested by the error mean square. When there was F-test (P < .05) for treatment, means were compared by the ls1 method (Steel and Torrie, 1980). Animal served as the replication in this experiment. Treatment was the dependent variable. Samples were analyzed in triplicate. The mean value calculated from the analysis of a sample in triplicate was used as the value for that experimental observation.

Results

Freezing ruminal digesta samples enhanced (P < .07) the yield of prokaryotic 16S rRNA from ruminal digesta (Table 1). Blending the ruminal mat fraction increased (P < .06) rRNA yield but did not increase (P > .10) the yield from the fluid fraction (Table 2). Extraction using phenol buffered with Tris had a lower (P < .04) yield of 16S rRNA than extraction with sodium acetate-buffered phenol (941 vs 1154 cpm for the Tris- and sodium acetate-buffered phenol extractions, respectively; Table 3). Therefore, rRNA extractions in the following experiments were conducted with sodium acetate-buffered phenol.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cpm a</th>
</tr>
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<tbody>
<tr>
<td>Fresh</td>
<td>359b</td>
</tr>
<tr>
<td>Frozen</td>
<td>498c</td>
</tr>
<tr>
<td>SE</td>
<td>14.2</td>
</tr>
</tbody>
</table>

aCounts per minute.
b,cMeans with unlike superscripts differ (P < .07).

<table>
<thead>
<tr>
<th>Table 1. Effect of freezing (−65°C) on hybridization of the universal oligonucleotide probe to prokaryotic 16S rRNA from ruminal digesta</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>Fresh</td>
</tr>
<tr>
<td>Frozen</td>
</tr>
<tr>
<td>SE</td>
</tr>
</tbody>
</table>

Total prokaryotic and F. succinogenes S85 rRNA concentrations in the mat and fluid phases of ruminal digesta were compared at three different times after offering feed (0, 3, and 9 h; Table 4). A sampling time × digesta phase (mat vs fluid) interaction (P < .05) occurred for total prokaryotic rRNA. At 0 h, 16S rRNA concentration of the bacterial population in the fluid phase was greater (P < .01) than that in the mat. The rRNA concentration of the fluid remained similar (P > .10) during the sampling times. The 16S rRNA concentration of bacterial population in the mat increased (P < .05) as time after feeding increased. At 9 h after feeding, the mat rRNA concentration was greater (P < .05) than the fluid-associated rRNA concentration. The dominant trend in this study was that the bacterial rRNA associated with the mat phase increased as time after feeding increased, but apparently there was little change in the bacterial rRNA concentration of the fluid. There were no differences (P > .05) in the proportion of F. succinogenes S85 rRNA to total prokaryotic 16S rRNA over time or between digesta phases (Table 4).

<table>
<thead>
<tr>
<th>Table 2. Effect of blending the fluid and mat phases of ruminal digesta on hybridization of the universal oligonucleotide probe to prokaryotic 16S rRNA</th>
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<tbody>
<tr>
<td>Blended a</td>
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<tr>
<td>-----------</td>
</tr>
<tr>
<td>Yes</td>
</tr>
<tr>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>SE</td>
</tr>
</tbody>
</table>

aRuminal digesta were blended in cold, sterile .85% saline for 3 min.

bRuminal digesta were harvested from the dorsal area of the rumen.

Counts per minute.
d,eMeans with unlike superscripts differ (P < .05).
USE OF DNA PROBES TO MONITOR RUMINAL BACTERIA

Table 3. Comparison of Tris-buffered phenol to sodium acetate-buffered phenol as an extraction media

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>941</td>
</tr>
<tr>
<td>Sodium acetate</td>
<td>1,153</td>
</tr>
<tr>
<td>SE</td>
<td>69.3</td>
</tr>
</tbody>
</table>

aCounts per minute.  
b,cMeans with unlike superscripts differ (P < .04).

and total cellular RNA concentration had similar curves because cell number was the primary factor controlling RNA concentration. Although some variation existed, the ratio of 16S rRNA to total cellular RNA seemed to be unaffected by growth stage of the culture. This ratio varied from .05 to .13, with a mean of .08.

Discussion

Figure 1 shows the 16S rRNA band isolated from prokaryotic RNA that was extracted from ruminal digesta. The tailing in each lane is degraded 16S rRNA that the probe hybridized. Analyses were conducted to ensure that tailing was not a result of probe hybridization to contaminant protein. Figure 2 shows a dot blot strip that had increasing levels of rRNA loaded onto the dot blot and then hybridized with the 32P-labeled universal probe. The gamma radioactivity was measured for each dot on the dot blot; the level of radioactivity was a function of the quantity of 16S rRNA that had been hybridized by the labeled probe. Blending and freezing were evaluated for their ability to enhance harvest yield of bacterial cells. The enhanced yield of prokaryotic rRNA due to freezing and blending was expected. Chilling and blending have previously been reported to enhance dissociation of bacteria from digesta particles (Merry and McAl- lan, 1983; Furchtenicht and Broderick, 1987). The main objective of evaluating the freezing step in this experiment was to ensure that 16S rRNA did not degrade during sample storage. Although samples must be stored at -60°C or lower to prevent substantial 16S rRNA degradation, these results showed that samples could be stored without increased 16S rRNA degradation and that freezing increased 16S rRNA yield.

Blending had no effect on 16S rRNA yield from fluid phase ruminal digesta (Table 2). Blending mat samples, which contained large digesta particles, resulted in a 1.7-fold increase in 16S rRNA extracted. This was most likely because the blending action homogenized the digesta particles, allowing more complete extraction of 16S rRNA from the bacterial cells present in the interior of digesta particles. From this research we concluded that the optimum procedure for preparing ruminal digesta for extraction of prokaryotic 16S rRNA was to freeze (-65°C) and blend digesta samples before extraction.

Ribosomal RNA extraction with phenol buffered with Tris was evaluated because it is routinely used for RNA extraction (Sambrook et al., 1989) and offered some advantage in time and

Table 4. Change in prokaryotic rRNA (counts per minute; cpm) and Fibrobacter succinogenes S85 percentage [% of total rRNA] of mat [M] and fluid [F] ruminal digesta at 0, 3, and 9 hours after feeding

<table>
<thead>
<tr>
<th>Item</th>
<th>0</th>
<th>3</th>
<th>9</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M</td>
<td>F</td>
<td>M</td>
<td>F</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Prokaryotic, cpm</td>
<td>1,588&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2,500&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2,838&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2,900&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>3,361&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F. succinogenes, %</td>
<td>5.4</td>
<td>6.6</td>
<td>5.6</td>
<td>6.1</td>
<td>5.7</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup>Means with unlike superscripts differ (P < .01).
<sup>d</sup>Percentage of total population.
likely due to an increase in bacterial mass ingestation (Orpin, 1980). Therefore, the increasing has been reported to occur rapidly after feed concentration of the mat increased. The increasing bacterial attachment and colonization of digesta particles. Attachment to ruminal digesta particles relatively constant, whereas the 16s rRNA concentration in the mat was most found that heifers had greater total bacterial numbers in the dorsal rumen than in the ventral rumen regardless of the diet they were fed. In addition, they found that numbers peaked between 2.5 and 5.5 h postfeeding. The trends found in our study based on 16S rRNA quantification agree with data of Bryant and Robinson (1988). We hypothesized that the increasing 16s rRNA concentration of mat digesta was due to microbial colonization of the digesta particles. Although steers were given ad libitum access to feed, they were offered hay at 0800 daily and consumed the largest amount of hay soon after feeding. Because their next period of eating activity was usually 10 to 12 h later, the sampling times used in this experiment should reflect changes that occurred as degradation proceeded in the rumen with minimum dilution by new feed particles entering the rumen.

At 0 h (before offering hay), the fluid phase had a greater 16S rRNA concentration than the mat (Table 4). As time progressed to 9 h post-feeding, the 16S rRNA concentration of the fluid remained relatively constant, whereas the 16S rRNA concentration of the mat increased. The increasing 16S rRNA in the mat was most likely due to bacterial attachment and colonization of digesta particles. Attachment to ruminal digesta particles has been reported to occur rapidly after feed ingestion (Orpin, 1980). Therefore, the increasing 16S rRNA concentration in the mat was most likely due to an increase in bacterial mass because cell division and colony development occurred. Perhaps more important is that as 16S rRNA concentration increased, the capacity for enzyme synthesis likewise would have increased. Based on these results, we concluded that the mat would be the most representative of fiber degrading capacity in the rumen. However, further research is needed to determine the relationship between time after feeding and degrading capacity in the rumen.

We found that total RNA concentration in E. coli XL Blues followed the culture's growth rate (Figure 3). We believe this indicates that RNA would serve as a valid marker for establishment and developmental studies of prokaryotic populations. Because there was no apparent relationship between cell numbers in the culture and the ratio of 16S rRNA to total cellular RNA, we believe that 16S rRNA can be used to study prokaryotic populations.

We concluded from this study that DNA probes can be used to monitor dietary effects on microbial population shifts in the rumen. The more important value of this procedure, however, will be its potential use to monitor enzyme concentration changes in the rumen as mediated through population shifts. This will be useful in understanding how dietary supplementation affects degradation of specific polymers (i.e., protein, starch, and structural polysaccharides) in the rumen as diet is altered because of changes in forage quality or supplementation.

Because 16S rRNA:total cellular RNA did not change over the growth curve, we concluded that 16S rRNA would be an appropriate measure of bacterial population shifts.

Implications

A sensitive measure of the relative concentration of prokaryote and prokaryotic species in the rumen is possible using DNA probes. This measure will allow more detailed studies of microbial processes in the rumen. In addition, results from these studies are expected to allow a more detailed understanding of how ruminal microfloral development is affected by dietary manipulation.

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