Effects of Centrifugation Speed and Freezing on Composition of Ruminal Bacterial Samples Collected from Defaunated Sheep

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ABSTRACT

Triplicate ruminal samples from four rumen-cannulated defaunated sheep (50 kg BW) were used in a completely randomized design experiment with a $2 \times 3$ factorial arrangement of treatments to test effects of two pretreatments (fresh vs. freezing) and three centrifugation speeds ($4640 \times g$ for 30 min; $9820 \times g$ for 20 min; $25,900 \times g$ for 20 min) on quantity of ruminal bacteria harvested and composition of ruminal bacterial samples. There were no differences among centrifugation speeds on quantity of ruminal bacteria harvested, organic matter, total N, RNA, and diaminopimelic acid concentrations (% of OM), or RNA:N, RNA:organic matter, N:organic matter, and diaminopimelic acid:organic matter ratios. However, there were differences between fresh and frozen samples for all measurements except diaminopimelic acid concentration (% of DM), or RNA:N, RNA:organic matter, N:organic matter, and diaminopimelic acid:organic matter ratios. However, there were differences between fresh and frozen samples for all measurements except diaminopimelic acid concentration, and the diaminopimelic acid:organic matter ratio, suggesting that freezing ruminal fluid is not appropriate for obtaining truly representative bacterial samples. (Key words: centrifugation speed, freezing, ruminal bacteria)

INTRODUCTION

Although many experiments involving the measurement of ruminal bacterial yield use centrifugation to collect the bacterial samples, there is no standard method of centrifugation. Very wide ranges in centrifugation speeds have been used by different groups ($22,000 \times g$ for 30 min (11); $25,000 \times g$ for 15 min (19); $27,000 \times g$ for 15 min (3); $27,750 \times g$ for 20 min (17); $43,000 \times g$ for 30 min (6); $49,000 \times g$ for 15 min (26)). No specific reason for centrifugation speed used has been reported.

Most ruminal bacteria are within the range of .4 to 1 μm in diameter and 1 to 3 μm long (9). Some extremely large ruminal bacteria also have been identified. *Selenomonas ruminantium* is 2.5 to 3 μm in diameter and 4 to 9 μm long (9). Quin's oval is 2.1 to 3.5 μm in diameter and 3.5 to 6.5 μm long (25). Ruminal bacterial sizes approximate the eucaryotic mitochondrial size range of .2 to 5 μm (12). The centrifugation speeds used for pelleting mitochondria ($7,000 \times g$ for 10 min (13); $10,000 \times g$ for 15 min (12); $20,000 \times g$ for 15 min (22)) are within a more narrow range than those used for ruminal bacteria.

During the process of harvesting bacteria from ruminal contents, lysis and death of some bacteria due to exposure to oxygen or change in temperature cannot be prevented. This will result in contaminating cell debris in intact bacterial cell samples. Without use of proper centrifugation speeds, unrepresentative samples (e.g., intact bacterial samples contaminated by bacterial membranes or cell wall debris with loss of nucleic acids and cytoplasmic proteins of broken cells into the supernatant) may be obtained, which could introduce error in use of an RNA: N or a diaminopimelic acid:N ratio to determine bacterial protein outflows.

Ruminal bacteria have different cell wall characteristics (5). Different types or thicknesses of cell walls may have different physical strengths to protect bacteria from bursting under freezing or thawing stresses. Freezing, with or without prior formaldehyde addition, is a method often used to preserve ruminal contents or fluid for subsequent collection of ruminal bacteria. The effects of freezing on the quantity of ruminal bacteria harvested or compositional changes in bacterial samples collected have not...
been studied. The present experiment was designed to determine the effects of ruminal fluid pretreatment (fresh vs. freezing) and centrifugation speed on these criteria.

**MATERIALS AND METHODS**

Four defaunated Suffolk × Hampshire cross-bred wethers (BW 50 kg) with ruminal cannulas and fed twice daily were used for collecting ruminal contents. Defaunation was accomplished with doses of 25 ml of alkanate 3SL3 (ICI Australia Petroleum Chemical Ltd., Melbourne, D309.129/sheep per d for 3 d, with no feed provided during the first 2 d, and a single supplemental dose of 70 ml of alkanate 3SL3 8 d later (8). Animals were housed in a 3.6 × 3.6 m elevated, wire-mesh pen in a controlled-environment room with constant fluorescent lighting. No faunated animals were present in the room. Water was available ad libitum throughout the trial. Diet composition was 45% bromegrass hay, 18% soybean hulls, 10% com, 8% molasses, 10% soybean meal, 5.7% cornstarch, .5% vitamins A, D, and E mix, .5% trace-mineral mix, 1% dicalcium phosphate, and 1.3% NaCl. The diet had DM, organic matter (OM), CP, NDF, and ADF concentrations of 89.9, 90.3, 14.8, 49.6, and 31.1% on a DM basis, respectively. Feed intake was 1076 g DM/sheep per d.

Sheep were allowed to adjust to feed for 7 d. Four hours after the morning feeding on d 8, 10, and 12, 1.5 L of ruminal contents were collected from each sheep, diluted with 200 ml of .9% saline, blended in a Waring blender (T. K. Heller Corp., Floral Park, NY) for 1 min, squeezed through eight layers of cheesecloth and composited. Pooled ruminal fluid was divided into two aliquots of equal volume. One of the aliquots was immediately frozen and kept frozen for 1 mo. During the process of harvesting bacterial samples, each aliquot was centrifuged at 150 × g for 10 min to remove small feed particles, and then further divided into three aliquots of equal volume. One of the aliquots was immediately frozen and kept frozen for 1 mo. The weight of the dried bacterial samples was measured. Dry matter, OM, and total N concentrations of bacterial samples were determined by 105°C oven drying, 600°C muffle furnace ashing, and Kjeldahl analysis, respectively, according to AOAC (1). Bacterial RNA was determined by the procedure of Zinn and Owens (27). Bacterial diaminopimelic acid was determined by the procedure of Rahnema and Theurer (18) using 94 min to change buffer pH from 3.25 to 3.81.

Data were analyzed by the method of least squares ANOVA using the General Linear Model procedures of SAS (21). Data were analyzed by ANOVA for a completely randomized design with a 2 × 3 factorial arrangement of treatments. Treatment means were compared by the method of least significant difference protected by a significant F-value (4).

**RESULTS AND DISCUSSION**

The purpose of using defaunated sheep in this study was to prevent possible contamination with protozoal debris and organelles of bacterial samples harvested. The centrifugation speed of 9820 × g for 20 min was chosen because it approximated the middle range of centrifugation speed used for pelleting mitochondria (12, 13, 22). A lower speed with longer centrifugation time (4640 × g for 30 min) was chosen to test the possibility of obtaining cleaner bacterial samples without contamination with cell debris. The highest centrifugation speed (25,900 × g for 20 min) approximated the upper limit of the centrifuge rotor used. This speed was chosen to test the possibility of obtaining more contamination with cell debris.

Statistical analysis indicated no interaction (P>.3) of pretreatment and centrifugation speed. Therefore, data are presented separately as a pretreatment comparison (fresh vs. frozen) and a centrifugation speed comparison (Table 1). Statistical comparisons were made on DM, OM, total N, RNA, and DAPA concentrations, and RNA:N, RNA:OM, N:OM, and DAPA:OM ratios. The RNA:N ratio often is used to calculate bacterial protein outflow; therefore, we thought it important to test whether it could be altered by different bacterial harvesting pro-

Journal of Dairy Science Vol. 73, No. 1, 1990
PROcedures for Harvesting Rumenal Bacteria

Table 1. Effects of pretreatment and centrifugation speed on chemical composition of ruminal bacteria.

<table>
<thead>
<tr>
<th>Item</th>
<th>Pretreatment</th>
<th>Centrifugation force and time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fresh</td>
<td>Frozen</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM, Bacteria in ruminal</td>
<td>8.0^a</td>
<td>6.8^b</td>
</tr>
<tr>
<td>contents, g/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>69.3^a</td>
<td>63.9^b</td>
</tr>
<tr>
<td>Total N</td>
<td>6.5^a</td>
<td>6.2^b</td>
</tr>
<tr>
<td>RNA</td>
<td>7.6^a</td>
<td>6.0^b</td>
</tr>
<tr>
<td>RNA:N</td>
<td>1.16^a</td>
<td>.98^b</td>
</tr>
<tr>
<td>RNA:OM</td>
<td>11^a</td>
<td>.09^b</td>
</tr>
<tr>
<td>N:OM</td>
<td>.09^a</td>
<td>.10^b</td>
</tr>
<tr>
<td>DAPA:OM</td>
<td>28</td>
<td>30</td>
</tr>
</tbody>
</table>

^a,bMeans in the same row within pretreatment with different superscripts differ (P<.05).

cesses. After three saline washings, the ash content of bacterial samples is increased artificially. Therefore, to prevent the variation caused by the increase in ash content, the RNA:OM, N:OM, and DAPA:OM ratios were used to allow for a more precise estimation of bacterial composition changes.

There were no differences (P>.05) among centrifugation speeds on quantity of ruminal bacteria harvested, OM, total N, RNA, and DAPA concentrations or on RNA:N RNA:OM, N:OM, and DAPA:OM ratios. However, there were differences (P<.05) between fresh and frozen samples for all measurements except DAPA concentration and the DAPA:OM ratio.

Although no significant effects of centrifugation speed were detected, we observed microscopically (Zeiss Standard LAB16 microscope, Zeiss, Thornwood, NY; 1000 x magnification) more contamination with cell debris in fresh bacterial samples subjected to the higher centrifugation speeds. Therefore, to avoid this contamination, we suggest using a centrifugation speed of 4640 x g for 30 min to harvest ruminal bacterial samples.

Mackie and Therion (15) indicated that gram-negative bacteria are more sensitive to an osmolality change than gram-positive bacteria. Stainer et al. (23) noted that the cross-linkage of bacterial cell wall peptidoglycan is the primary factor for countering turgor pressure and preventing osmotic lysis. They also indicated that gram-positive bacteria have much thicker and more crosslinked cell walls than gram-negative bacteria. Freezing can change the physical structure of membrane lipid components and result in a widening of the membrane pores (24). The swelling of the water volume upon freezing also can induce a tremendous turgor force. Therefore, the freezing procedure used for preserving ruminal fluid samples may result in relatively more destruction of gram-negative than gram-positive bacteria. Arambel et al. (2) reported that gram-positive bacteria have a lower total N content and a lower RNA:N ratio than gram-negative bacteria. Therefore, the osmotic lysis of gram-negative bacteria could be one of the major causes of a lower total N content and a lower RNA:N ratio in the frozen samples in the present study. Exponentially growing bacteria have a higher RNA content (14), a lower cell wall content (20), and show greater susceptibility to cold shock (24) than those in the stationary growth phase, suggesting that lysis of exponentially growing bacteria in the frozen samples also may be important in altering the components measured in the present study. The tendency for a higher DAPA:OM ratio in frozen samples indicates the possible higher cell wall content of frozen ruminal samples, which, in turn, might be due to the destruction of gram-negative bacteria, or exponentially growing bacteria, or both.

Freezing of harvested and washed bacterial samples has been found to cause some degradation of bacterial RNA (10, 16). However, Ha and Kennelly (7) reported that using the purine
assay of Zinn and Owens (27), instead of directly measuring RNA, could overcome this problem. Therefore, we think that harvesting bacteria from fresh ruminal contents followed by freeze-drying is an important criterion to obtain truly representative bacterial samples.

REFERENCES