Short communication: Comparison of pH, volatile fatty acids, and microbiome of rumen samples from preweaned calves obtained via cannula or stomach tube

M. Terré,^{*1} L. Castells,* F. Fàbregas,* and A. Bach^{*†}

^{*}Department of Ruminant Production, Institut de Recerca i Tecnologia Agroalimentàries (IRTA), 08140 Caldes de Montbui, Spain

[†]Institució Catalana de Recerca i Estudis Avançats (ICREA), 08010 Barcelona, Spain

ABSTRACT

The objective of this study was to compare rumen samples from young dairy calves obtained via a stomach tube (ST) or a ruminal cannula (RC). Five male Holstein calves (46 ± 4.0 kg of body weight and 11 ± 4.9 d of age) were ruminally cannulated at 15 d of age. Calves received 4 L/d of a commercial milk replacer (25% crude protein and 19.2% fat) at 12.5% dry matter, and were provided concentrate and chopped oats hay ad libitum throughout the study (56 d). In total, 29 paired rumen samples were obtained weekly throughout the study in most of the calves by each extraction method. These samples were used to determine pH and volatile fatty acids (VFA) concentration, and to quantify *Prevotella ruminicola* and *Streptococcus bovis* by quantitative PCR. Furthermore, a denaturing gradient gel electrophoresis was performed on rumen samples harvested during wk 8 of the study to determine the degree of similarity between rumen bacteria communities. Rumen pH was 0.30 units greater in ST compared with RC samples. Furthermore, total VFA concentrations were greater in RC than in ST samples. However, when analyzing the proportion of each VFA by ANOVA, no differences were found between the sampling methods. The quantification of *S. bovis* and *P. ruminicola* was similar in both extraction methods, and values obtained using different methods were highly correlated ($R^2 = 0.89$ and 0.98 for *S. bovis* and *P. ruminicola*, respectively). Fingerprinting analysis showed similar bacteria band profiles between samples obtained from the same calves using different extraction methods. In conclusion, when comparing rumen parameters obtained using different sampling techniques, it is recommended that VFA profiles be used rather than total VFA concentrations, as total VFA concentrations are more affected by the method of collection. Furthermore, although comparisons of pH across studies should be

avoided when samples are not obtained using the same sampling method, the comparison of fingerprinting of a bacteria community or a specific rumen bacterium is valid.

Key words: rumen cannula, rumen sample, stomach tube

Short Communication

Rumen development in young dairy calves has been widely studied because of the importance of this digestive compartment in the transition from a liquid (milk or milk replacer) to a solids (forage plus concentrate)-based diet. Some studies have obtained rumen liquid samples directly from the rumen via cannula (Beharka et al., 1998; Lesmeister and Heinrichs, 2004), whereas others have used a stomach tube (Abdelgadir et al., 1996; Coverdale et al., 2004; Khan et al., 2008). Each sampling technique has some advantages and disadvantages. Rumen cannulation requires a surgical action, whereas placing the stomach tube is a noninvasive technique. However, the latter is susceptible to saliva contamination. Rumen cannulation allows monitoring rumen parameters more exhaustively using rumen probes or conducting repeated samplings within a day, in contrast to the stomach tube technique, which it is usually performed in samplings at time intervals. It is well known from cow studies (Duffield et al., 2004) that the rumen sampling technique can affect the rumen parameters measured, making comparisons among studies difficult. The objectives of this study were to (1) compare pH, VFA, and microbial populations of rumen fluid obtained via stomach tubing (ST) or via a rumen cannula (RC), and (2) assess whether comparison of rumen fluid parameters obtained using these 2 sampling techniques is feasible.

Five male Holstein calves (46 ± 4.0 kg of BW and 11 ± 4.9 d of age) were purchased from a commercial farm, and raised in the facilities of the Institute of Research and Technology of Food and Agriculture (IRTA) at Prat de Llobregat, Spain. All calf interventions were approved and supervised by the Animal Care Com-

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¹Corresponding author: marta.terre@irta.cat

mittee of IRTA (expedient number 3885). Calves were housed individually and received 500 g of DM/d of a commercial milk replacer (25% CP, 19.2% fat, and 4.32 Mcal of ME/kg of DM, Sprayfo Excellent 60, Sloten BV, Deventer, the Netherlands) in 4 L of water offered over 2 feedings at 0800 and 2000 h throughout the study. A pelleted starter concentrate (20.4% CP, 21.0% NDF, 2.7% fat, and 3.21 Mcal of ME/kg of DM), chopped oats hay (9.7% CP, 72.1% NDF, 46.2% ADF, and 1.77 Mcal of ME/kg of DM), and water were offered ad libitum in buckets throughout the study. The study ended when calves were 71 ± 5.8 d of age.

Four days after arrival, calves were ruminally cannulated following the procedure described by Kristensen et al. (2010) and allowed to recover for 1 wk after surgery. Rumen liquid was harvested 2 h postfeeding weekly. Sometimes, ST samples could not be obtained, and the corresponding RC sample was discarded from the study. In total, 29 paired rumen samples were completely analyzed. Samples (150 mL) were obtained first via the rumen cannula and then immediately after from the stomach tube, to avoid the possible contamination of rumen fluid with saliva from the ST method. Between samplings, collection flasks were washed with a 1:10 (vol:vol) dilution of sodium hypochloride solution and distilled water and then rinsed several times with distilled water before the next use. Immediately after the sample was obtained, rumen pH was measured using a pH meter (Crison pH25, Barcelona, Spain), and 40 mL of rumen liquid was frozen in dry ice and then stored at -80°C until subsequent quantitative PCR microbial determination and denaturing gradient gel electrophoresis (DGGE). Additionally, 4 mL of rumen liquid was immediately acidified with 1 mL of a solution containing 2 g/L mercuric chloride, 20 mL/L orthophosphoric acid, and 2 g/L 4-methylvaleric (internal standard) in distilled water, and stored at -20°C for subsequent VFA analyses. The VFA in ruminal contents were analyzed by gas chromatography (HP-6890 Series II Agilent, Hewlett Packard, Palo Alto, CA) using a capillary column ($10 \text{ m} \times 0.10 \text{ mm} \times 100 \text{ }\mu\text{m}$; DB5, Agilent, Hewlett Packard).

The DNA of each rumen sample was extracted following the procedure described by Yu and Morrison (2004a) using the RBB+C method, which employs bead beating in the presence of high concentrations of sodium dodecyl sulfate, NaCl, and EDTA, and the subsequent DNA purification with QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Real-time PCR was performed using specific primers assembled from the literature and tested for specificity (Table 1). A total reaction volume of 20 μL was used, containing 100 ng of DNA, 10 μL of SYBR Green (Bio-Rad Laboratories, Hercules, CA), and the optimized primer concen-

tration for each gene (Table 1). The PCR reactions for *Streptococcus bovis* (Fernando et al., 2010) consisted of an initial denaturing cycle of 10 min at 95°C , followed by 40 cycles of 30 s at 95°C and 10 s at 60°C , 30 s at 72°C , and a final extension of 5 min at 72°C . For *Prevotella ruminicola* (Stevenson and Weimer, 2007), the conditions were as follows: an initial denaturing cycle of 5 min at 95°C , followed by 40 cycles of 30 s at 95°C and 15 s at 54°C , 30 s at 72°C , and a final extension of 10 min at 72°C . Bacteria quantification was evaluated using the cycle threshold (C_t) values, defined as the number of PCR cycles over which product amplification occurs. Therefore, the greater the C_t value, the lower the concentrations of rumen bacteria. Furthermore, to assess the similarity between bacteria populations in samples obtained with the 2 methods, a DGGE analysis was performed with the rumen samples obtained in wk 8 of the study. In brief, the v3-v5 region of 16S rRNA eubacteria was amplified using the forward primer F-341-GC (5'-CCTACGGGAGGCAGCAG-3' with a 40-bp GC clamp at the 5' end), and the reverse primer R-907 (5'-CCGTCAATTCCTTTGAGTTT-3') (Yu and Morrison, 2004b), with the following conditions: an initial denaturing cycle of 5 min at 94°C , followed by 30 cycles of 60 s at 94°C , annealing at 55°C for 30 s, an elongation at 72°C for 45 s, and a final elongation of 10 min at 72°C . After testing the amplification results in 1.5% agarose gel and PicoGreen DNA quantification, the DGGE was performed with the DGGE-4001 equipment (CBS Scientific Co., Solana Beach, CA) in a polyacrylamide gel at 8% concentration with a denaturing gradient from 30 to 70% (urea formamide). An aliquot of 600 ng was applied per well, and samples were electrophoresed for 16 h at 60°C using a fixed voltage of 100V. Gels were stained by SybrGold under blue fluorescence, and analyzed with Gene Flash (Syngene Bio Imaging, Frederick, MD) and Blue Converter Plate (UV Products, Chicago, IL).

After checking for normality of the data, a paired t -test was used to compare samples from the same calf and day obtained via cannula or stomach tube. Furthermore, data were analyzed with an ANOVA with a mixed-effects model for repeated measures. The statistical model included sampling technique, week of sampling after cannulation, and the interaction between both parameters as fixed effects, and calf as random effect. Furthermore, for pH data, visual saliva contamination was included as a block. A linear regression analysis within calf and day was conducted between RC and ST samples. To determine the similarity among samples analyzed by DGGE, a dendrogram was built using the nearest neighbor method in a Pearson correlation. A principal component analysis was conducted to establish the band profile changes among samples

Table 1. Primers, annealing temperature (T_m), and optimized DNA primer concentrations of 16S ribosomal RNA for *Prevotella ruminicola* and *Streptococcus bovis*

Species	Forward (5' → 3')	Reverse (5' → 3')	T _m , °C	Concentration, μ M
<i>P. ruminicola</i>	GAAAGTCGGATTAATGCTCTATGTTG	CATCCTATAGCGGTAAACCTTTGG	54	0.25
<i>S. bovis</i>	ATTCTTAGAGATAGGGTTTCTCTT	ACCTTATGATGGCAACTAACAATA	60	0.50

considering the distribution and the intensity of the bands in the gel. A power analysis for pH and VFA parameters was performed to determine whether the sample size needed to establish significant differences in studies using RC or ST differed. The analysis was conducted for an 80% power, and the difference to detect and the standard deviation of each parameter are reported in Table 2.

Rumen pH was greater ($P = 0.05$) in ST compared with RC samples by 0.30 pH units. This pH difference can be attributed to saliva contamination in ST samples, because no differences were observed when visual saliva contamination was included in the ANOVA model as a block (Table 3). Similarly, Duffield et al. (2004) on adult cows reported differences of 0.44 and 0.35 pH units from samples obtained with an oro-ruminal probe and ruminocentesis. When rumen samples that were visually contaminated with saliva were excluded from the paired t -test analysis, the difference in pH units between ST and RC samples was reduced to 0.12, but this difference continued to be significant at $P = 0.002$. Therefore, even when discarding visually contaminated samples, a greater pH can be expected from samples obtained with ST compared with those from RC. Data from pH values from ST and RC samples were moderately correlated ($R^2 = 0.66$, $P < 0.01$), but they were highly correlated ($R^2 = 0.86$, $P < 0.01$) when 5 samples that were visually contaminated with saliva were excluded from the analysis. Because of the greater standard deviation of ST samples compared with RC samples, the power analysis indicated that more ani-

mals needed to be sampled when using the ST technique compared with RC as a method for measuring rumen pH (Table 2).

Similar to Geishauser and Gitzel (1996) with rumen-fistulated sheep, both a paired t -test and an ANOVA determined that rumen VFA concentrations were greater ($P < 0.05$) in RC than in ST samples (Table 3). Again, it is likely that saliva contamination diluted ST samples, decreasing VFA concentrations. Nevertheless, VFA concentrations from ST or RC samples were moderately or highly correlated (R^2 of 0.53, 0.57, 0.87, 0.75, 0.89, and 0.73 for acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate acids, respectively, $P < 0.01$). The ANOVA did not reveal differences in the molar proportions for each VFA between RC and ST samples (Table 3), and the paired t -test analysis revealed differences only in the molar proportions of butyrate and valerate acids between the 2 sampling techniques. However, in contrast to the VFA concentrations, VFA molar proportions from ST and RC samples were all highly correlated (R^2 of 0.97, 0.97, 0.99, 0.89, 0.99, and 0.73 for acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate acids, respectively, $P < 0.01$). Furthermore, the ANOVA revealed that the interaction between sampling technique and week of sampling was not significant for any of the parameters analyzed (Table 3), suggesting that the results found for the 2 techniques would remain consistent over time. Last, after running a power analysis considering the standard deviations of the samples obtained in the present study, we concluded that the number of animals

Table 2. Parameters used in a power analysis with a power of 0.80 and the number of animals per treatment needed to detect significant differences for each rumen parameter on samples obtained via a stomach tube (ST) or via a ruminal cannula (RC)

Item	Difference	Standard deviation		Number of animals	
		ST	RC	ST	RC
pH	0.6	0.54	0.40	14	9
Concentration, mM					
Acetic acid	20	19	16	15	12
Propionic acid	15	17	16	22	19
Butyric acid	10	13	16	28	42
Molar proportion, %					
Acetic acid	9	6.6	6.6	10	10
Propionic acid	6	7.0	7.0	23	23
Butyric acid	3	7.9	7.7	110	105

Table 3. Least squares means of rumen parameters of rumen samples obtained via stomach tube (ST) or rumen cannula (RC) from calves between 22 and 71 d of age

Item	Technique		SEM	<i>P</i> -value ¹		
	ST	RC		T	Week	T × Week
Rumen pH ²	5.9	5.6	0.24	0.05	0.12	0.55
Rumen pH ³	6.3	6.2	0.51	0.34	0.09	0.97
Total VFA, mM	129.7	157.3	10.50	<0.05	<0.05	0.79
VFA concentrations, mM						
Acetic acid	56.7	67.6	3.35	<0.01	<0.01	0.96
Propionic acid	47.1	56.2	4.23	<0.05	0.06	0.98
Isobutyric acid	0.89	1.07	0.120	0.07	0.05	0.95
Butyric acid	19.9	24.0	4.79	<0.05	0.13	0.99
Isovaleric acid	0.79	1.03	0.136	<0.01	0.18	0.90
Valeric acid	5.9	7.1	2.58	0.54	0.19	0.97
VFA proportion, %						
Acetic acid	44.4	44.1	2.57	0.86	0.79	1.00
Propionic acid	35.7	35.7	2.24	0.99	0.08	1.00
Isobutyric acid	0.67	0.69	0.146	0.87	0.42	1.00
Butyric acid	14.5	14.7	2.99	0.91	0.18	1.00
Isovaleric acid	0.56	0.66	0.158	0.27	0.26	0.97
Valeric acid	5.9	7.1	2.58	0.54	0.19	0.97
<i>Streptococcus bovis</i> , Ct ⁴	28.8	29.6	1.19	0.53	<0.05	0.97
<i>Prevotella ruminicola</i> , Ct	22.8	22.9	1.92	0.97	0.05	1.00

¹T = effect of the sampling technique; week = effect of week of sampling after cannulation; T × Week = interaction between sampling technique and week of sampling after cannulation.

²Rumen pH derived omitting the effect of visual saliva contamination as a block in the statistical analysis.

³Rumen pH derived including the effect of visual saliva contamination as a block in the statistical analysis.

⁴Cycle threshold value.

needed to detect differences in VFA concentrations or proportions was very similar whether samples were obtained using ST or RC (Table 2).

Both a gram-negative (*P. ruminicola*) and a gram-positive (*S. bovis*) species were chosen to assess if the rumen sampling method influenced the results of bacteria quantification. The ANOVA did not show differences between the 2 sampling techniques (Table 3). However, the paired *t*-test revealed that the Ct value of *P. ruminicola* was similar ($P = 0.61$) in RC and ST samples. In contrast, the paired *t*-test for *S. bovis* indicated that the quantification of *S. bovis* was greater ($P < 0.01$) in ST than in RC samples. Nevertheless, samples obtained with the 2 methods were highly correlated ($R^2 = 0.98$; $P < 0.01$; $R^2 = 0.89$; $P < 0.01$ for *P. ruminicola* and *S. bovis*, respectively). In humans, *Streptococcus* is a common oral genus detected in periodontal samples (Wade, 2011). Therefore, the increase of *S. bovis* in ST samples might be due to potential hybridization of the primers used in the present study for *S. bovis* with other bacteria of the *Streptococcus* genus from the oral cavity that could have contaminated the samples obtained with the stomach tube.

The rumen bacterial communities obtained from the DGGE analysis were similar between ST and RC samples as shown in the dendrogram depicted in Figure 1. In P2 and P5 calves, the band profile between cannula

and oral samples were practically the same (the coefficients for the Pearson correlation matrix were 1.00), and the other 3 calves showed some slight differences (the coefficients for the Pearson correlation matrix were 0.97, 0.98, and 0.92 for P1, P3, and P4, respectively). The different bands obtained from the DGGE analysis were evaluated using principal component analysis. This technique identified some bands that differed among calves but not within calves when using different techniques for collecting the rumen fluid. Therefore, these bands were not sequenced because the identification of differences between calves was beyond the scope of the current study.

In conclusion, when comparing rumen parameters from different techniques, VFA molar proportions are preferred to total VFA concentrations, as the latter are more affected by the method of collection. Furthermore, pH comparisons across studies should be avoided when samples are not obtained by the same sampling method. The comparison of specific rumen bacteria or fingerprintings of bacteria communities can be acceptable, regardless of sampling technique. In general, despite some differences between RC and ST samples, results obtained with the 2 techniques are highly correlated and thus the potential exists for using a correction factor to approximate both methods and allow comparisons across studies.

Figure 1. Similarity dendrogram from denaturing gradient gel electrophoresis analysis of rumen samples obtained using a rumen cannula or a stomach tube. Labels P1 to P5 indicate individual animals. In the second column, odd numbers refer to samples obtained via stomach tube, and even numbers to samples obtained via rumen cannula.

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