# Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats

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**ABSTRACT**

Rumen cannulation is the reference method for collection of representative samples of rumen digesta. However, it is not always viable, which obliges to depend on less invasive techniques, such as stomach tubing. The aim of this work was to study if the differences in fermentation parameters and rumen microbial populations observed between species (sheep and goats), diets (forage and forage plus concentrate) and sampling times (pre- and post-feeding) are consistent when collecting the samples through stomach tube or rumen cannula, in an attempt to validate the use of the former as an alternative to the latter. Four sheep and four goats, fitted with ruminal cannula, were fed either forage (F diet; alfalfa hay) or forage plus concentrate (1:1; FC diet), in two 15-d periods. At the end of each period (d 14 and 15), samples of rumen digesta were taken by stomach tube and rumen cannula, before and 4 hours after morning feeding, for determination of ruminal fermentation parameters (pH, and lactate, ammonia and total VFA concentrations). The three main rumen microbial groups (bacteria, protozoa and methanogenic archaea) and two fibrolytic bacteria (*Ruminococcus flavefaciens* and *Fibrobacter succinogenes*) were quantified by real time PCR and, additionally, PCR-DGGE analysis of the bacterial community on the rumen digesta samples collected post-feeding was carried out. Overall, sampling through ruminal cannula and stomach tube gave similar results regarding fermentation parameters when comparing species, diets and sampling times. Despite samples for microbiology assays contained liquid plus solid fractions when collected through rumen cannula and mostly liquid when collected through stomach tube, both techniques showed certain consistency in the effects of treatments on the rumen microbiota (e.g., both revealed no differences between species in total bacteria, archaea and *R. flavefaciens* concentrations, and higher protozoa numbers in goats than

in sheep). However, there was also some discrepancy regarding microorganism concentrations, particularly concerning sampling times (e.g., differences between pre- and post-feeding samplings were only observed in rumen cannula samples for total bacteria and methanogenic archaea, and in stomach tube samples for *R. flavefaciens* concentrations). Therefore, this study supports that non-invasive stomach tubing is a feasible alternative to surgical rumen cannulation in sheep and goats to examine ruminal fermentation. Nonetheless, caution should be taken when using this technique to assess the structure and composition of the rumen microbial community.

*Keywords*: rumen cannula, stomach tube, small ruminants, fermentation, microorganisms

*Abbreviations*: ADF, acid detergent fibre; DMI, dry matter intake; CP, crude protein; D, diet; DGGE, denaturing gradient gel electrophoresis; DM, dry matter; F, forage; FC, forage plus concentrate; FM, fresh matter; G, goat; LW, live weight; MEI, metabolizable energy intake; N, nitrogen; NDF, neutral detergent fibre; OM, organic matter; PCA, principal components analysis; PCR, polymerase chain reaction; qPCR, real-time quantitative PCR; S, sheep; Sp, species; VFA, volatile fatty acids; T, sampling time.

# 1. Introduction

Rumen cannulation is considered the reference method for collection of representative samples of rumen digesta and is therefore widely used in ruminant nutrition research (Komarek, 1981; Kristensen et al., 2010). However, rumen cannulation is not feasible in lactating ewes or goats, because of potential adverse effects on animal performance, which obliges to depend on less invasive alternatives, such as oral stomach probing.

Rumen cannulation and stomach tubing have been mainly used to assess ruminal fermentation (Geishauser and Gitzel, 1996; Duffield et al., 2004) and, more recently, to analyse the structure of the rumen microbial community (Hook et al., 2009; Lodge-Ivey et al., 2009; Terré et al., 2013). In the few studies in which the two techniques were used together, comparisons of fermentation profile and microbiota resulted in either significant differences (e.g., Geishauser and Gitzel, 1996; Duffield et al., 2004) or similar results (e.g., Lodge-Ivey et al., 2009; Shen et al., 2012; Terré et al., 2013) and the reasons for this discrepancy are probably related to the probing procedure to avoid saliva contamination, the type of sample obtained and the rumen sampling site.

While negligible amounts of solid material can be collected with stomach probe, rumen cannula allows collection of both solid and liquid fractions of the rumen digesta. This may be relevant when the treatments to be studied are not expected to have the same effect on microbial populations attached to solids or inhabiting the liquid phase (Martínez et al., 2010).

Regarding the rumen sampling site, Shen et al. (2012) obtained significant variations in ruminal fermentation parameters (pH, VFA, ammonia N and ion concentrations) when sampling at different locations through ruminal cannula.

Differences between samples collected via cannula or stomach tube were also observed and attributed to the sampling site when the probe was not inserted to a depth enough to reach the central sac. Otherwise, no significant differences were detected between methods (Shen et al., 2012). Unfortunately, probe insertion in an accurate location of the rumen is very complicated in small ruminants.

To our knowledge, reports analysing methods of rumen sampling are very scant in sheep and practically non-existent in goats. Therefore, this experiment was conducted with ruminally-cannulated sheep and goats to validate the use of the stomach probing as an alternative to rumen cannulation in small ruminants. The main aim of this work was to assess the ability of both approaches to detect differences between treatments (i.e., species, diets or sampling times) in ruminal fermentation and microbial community, rather than a direct comparison of methods.

## 2. Materials and methods

*2.1. Animals, diets and experimental design*

Four Segureña sheep (S; mean live weight 56.4 ± 2.66 kg) and four Murciano-Granadina goats (G; 37.8 ± 1.65 kg), fitted with a ruminal cannula (35 mm internal diameter), were individually penned and fed alfalfa hay for 2 weeks. After that adaptation, animals were fed two different diets in two consecutive 15-d periods (for each period, 2 animals/species and diet): forage (F diet; alfalfa hay) or forage plus concentrate (1:1; FC diet). Concentrate (Pacsa Sanders, Seville, Spain) was provided as pellets. Chemical composition of the diets (g/kg DM) and dry matter intake (DMI; g/kg) and metabolizable energy intake (MEI; MJ/d) is shown in Table 1. Experimental diets were offered in two meals (60% at 9:00 h and 40% at 18:00 h) at estimated energy requirements for maintenance for sheep (Aguilera et al., 1986) and

goats (Prieto et al., 1990). Clean water and mineral supplement were always available.

All experimental procedures were approved and completed in accordance with the Spanish Royal Decree 53/2013 for the protection of animals used for experimental purposes.

*2.2. Measurements and sampling procedures*

On days 14 and 15 of each period, samples of rumen digesta were obtained, via stomach tube and rumen cannula, from each animal.

For stomach tube sampling, a flexible PVC tube (2 mm of wall thickness and 6 mm of internal diameter; Cristallo Extra, FITT S.p.A., Sandrigo, Italy) with about 20 holes of 3 mm diameter in the 12 cm-probe head was warmed-up using hot water and inserted to a depth of approx. 120-150 cm via the esophagus. Rumen samples (ca. 50 ml) were obtained using an electric vacuum pump (down to 7 mbar; Vacuubrand MZ 2C, Wertheim, Germany). Before being strained through a nylon membrane (400 µm; Fisher Scientific S.L., Madrid, Spain), these samples were subjected to visual and tactile examination to ensure that they were not contaminated with saliva. A 20 cm long handle sampling scoop was used to collect rumen contents samples through the cannula from different parts of the dorsal sac in the rumen. An average of 5 samples were taken, composited, aliquoted (ca. 20 mL) and strained through the nylon membrane. For each animal, samples were first collected via stomach tube and immediately afterwards via rumen cannula, both before morning feeding and 4 h post-feeding.

The pH was measured using a pH-meter (Crison GLP 21, Barcelona, Spain) and a 4 mL subsample was acidified with 4 mL of 0.2 M HCl for ammonia

determinations. Further 4 and 0.8 mL aliquots of strained ruminal fluid were taken, respectively, for the analysis of lactic acid and VFA (deproteinized with 0.5 mL of 20 g/L metaphosphoric and 4 g/L crotonic acids in 0.5 M HCl). All these samples were stored at –30ºC until analysis. Additionally, on day 15 non-strained subsamples (ca. 30 g) of rumen digesta were collected, before the morning feeding and 4 hours after feeding, first via stomach tube and subsequently via rumen cannula, immediately frozen at –80ºC, freeze-dried, and stored again at –80ºC until subsequent molecular analyses.

*2.3. Chemical analysis*

Feed samples (i.e., alfalfa hay and concentrate) were prepared (ISO 6498:2012) and analysed for DM (ISO 6496:1999), ash (ISO 5984:2002), and CP (ISO 5983-2:2009). The aNDF and ADF were determined as described by Mertens (2002) and the AOAC (2006; Official Method 973.18), respectively, using an Ankom$^{2000}$ fibre analyser (Ankom Technology Corp., Macedon, NY, USA). Neutral detergent fibre was assayed with sodium sulphite and α-amylase and expressed with residual ash (the latter also for ADF). The content of ether extract in the diets was determined by the Ankom Filter Bag Technology (AOCS, 2008; Procedure Am 5-04). Starch content was analysed by a total starch assay kit obtained from Megazyme (K-TSTA; Megazyme Intl. Ireland Ltd., Wicklow, Ireland).

Ammonia and lactic acid concentrations were determined by colorimetric methods (Weatherburn, 1967, and Taylor, 1996; respectively) and VFA by gas chromatography, with crotonic acid as an internal standard (Ottenstein and Bartley, 1971), in centrifuged samples.

*2.4. DNA extraction, quantitative PCR and Denaturing Gradient Gel Electrophoresis analyses*

Freeze-dried samples of rumen digesta were mixed by physical disruption using a bead beater (Mini-bead Beater 8, BioSpec Products, Bartlesville, OK, USA). The DNA extraction was performed from 50 mg samples following the QIAamp DNA Stool Mini Kit (Qiagen Ltd, West Sussex, UK) manufacturer's instructions but with higher temperature (95ºC) for lysis incubation. The DNA samples were used as templates to quantify the copy numbers of *16S rRNA* (for bacteria), methyl coenzyme M reductase A (*mcrA*) gene (for methanogenic archaea), and *18S rRNA* (for protozoa) by real-time quantitative PCR (qPCR) as described by Abecia et al. (2012b). Primer set used for *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* were described by Denman and McSweeney (2006).

The PCR-DGGE analysis of bacterial community on the rumen digesta samples collected post-feeding was carried out as previously described (Abecia et al., 2012a). The DGGE banding profiles were compared using the similarity matrix obtained by using the Bray-Curtis algorithm. The Shannon index and richness (number of bands obtained in the DGGE gel for each sample) were used to estimate the bacterial diversity in each sample.

*2.5. Statistical analysis*

For each sampling method, all data (rumen fermentation characteristics and microbiological results) were analysed by 3-way ANOVA, using the MIXED procedure of SAS (2012, version 9.3). The statistical model included the fixed effects of species (Sp), diet (D), sampling time (T) and their interactions. In all cases, the period (mean values of days 14 and 15) was considered as a blocking term and the

animal as a random effect. Since microbiology results did not satisfy the assumptions of normality, data were $\log_{10}$ transformed before the statistical analysis. The model for diversity indices included the fixed effects of species (Sp), diet (D) and their interaction. Differences were declared significant at $P<0.05$ and considered as tendencies towards significance at $P<0.10$. Least squares means are reported throughout. Principal components analysis (PCA) plots were obtained using R-statistical software (R Core team, 2013) and Vegan package.

## 3. Results

### *3.1. Fermentation parameters*

The amount of FC and F diets provided daily was consumed by all animals with the exception of two sheep that left refusals of the F diet. This was reflected in a lower MEI intake of the F diet by sheep than it was expected (Table 1).

Differences in pH values and ammonia concentrations due to the animal species were only observed when sampling through rumen cannula. Whereas pH values were lower ($P=0.04$) in sheep than in goats, ammonia concentrations tended to be higher ($P=0.09$) in the former species. For the rest of fermentation parameters studied, both sampling techniques gave similar results: either no differences (for lactate concentration, molar proportions of propionate and butyrate, and acetate/propionate ratio) or higher concentration of total VFA ($P<0.05$) and molar proportion of acetate ($P<0.10$), for sheep in comparison to goats (Table 2).

Both rumen cannula and stomach tube sampling showed significant differences for most of the fermentation parameters measured when feeding F or FC diets. The pH values as well as the molar proportions of acetate and propionate were higher ($P<0.01$) for the F diet, whereas the concentration of ammonia and lactate, the molar

proportion of butyrate, and the ratio acetate/propionate resulted in higher values ($P<0.05$) with the FC diet. For total VFA and the sum of isobutyrate, isovalerate, valerate and caproate, no differences were detected by any of the two methods (Table 2).

Collecting the samples before the morning feeding or 4 h afterwards, either through rumen cannula or stomach tube, resulted in significant differences ($P<0.05$) for all the parameters considered but the ammonia concentration (Table 2). Both methods showed that sampling 4 h after feeding resulted in lower pH and higher concentrations of lactate and total VFA ($P<0.05$). Molar proportions of acetate and propionate were higher whereas that of butyrate was lower post-feeding ($P<0.01$). The ratio acetate/propionate was, however, lower ($P<0.001$) when sampling post-feeding.

*3.2. Microbial abundances*

The concentrations of protozoa in the rumen of goats were higher ($P<0.10$) than in sheep for samples obtained using both sampling methods. However, only sampling through stomach tube revealed differences between animal species ($P=0.09$) in the gene copy numbers of *F. succinogenes* (Table 3). No significant differences between sheep and goats were detected in the concentration of total bacteria, archaea and *R. flavefaciens*, regardless the sampling technique.

With respect to the diets, only sampling through stomach tube was able to detect differences in the concentration of bacteria, which was higher ($P=0.09$) when animals were fed the F diet. However, both sampling methods showed differences in the numbers of protozoa, methanogenic archaea and *R. flavevaciens*: the concentrations of protozoa and *R. flavefaciens* were greater whereas that of archaea was lower for FC

diet in comparison to F diet (P<0.05). Besides, none of the techniques resulted in changes in *F. succinogenes* numbers in relation to the diet (Table 3).

With respect to sample collection times (pre- or post- feeding), both techniques revealed differences in concentrations of protozoa and *F. succinogenes* (P<0.05), values being higher before feeding. Nevertheless, differences due to the sampling time were only observed when samples were taken through rumen cannula for bacteria (P<0.001) and methanogenic archaea (P=0.07), and through stomach tube for *R. flavefaciens* concentrations (P=0.01). Although the abundances of protozoa and R. flavefaciens were lower than those normally published in the literature (Patra and Yu, 2013 and 2014), the relevance of such underestimation for the purpose of our work is negligible.

*3.3. Analysis of the bacterial community structure and diversity*

The PCA plot of bacterial community (Figures 1a and 1b) segregated samples by component 2 in two groups, for both rumen cannula and stomach tube techniques, corresponding to sampling period regardless of diet or species. Within each period, samples tended to be grouped by animal species, although the pattern was more evident for rumen cannula samples than for stomach tube ones. Percentages of variance explained by the principal components were 54.7% and 46.5%.

Diversity indexes (Table 4) were higher in goats than in sheep when sampling through rumen cannula (P=0.02). However, these differences between species were not observed when sampling through stomach tube. Both techniques were unable to detect variations due to feeding F or FC diets.

## 4. Discussion

Given the great potential of stomach tubing as non-invasive technique in small ruminant nutrition research and the very few studies that have evaluated its suitability in comparison to rumen cannulation in sheep and goats (Geishauser and Gitzel, 1996; Duffield et al., 2004), the aim of this work was to study if the differences in fermentation parameters and rumen microbial populations observed between species (sheep and goats), diets (F and FC) and sampling times (pre- and post- feeding) were consistent when samples were collected by both approaches.

Rumen samples were obtained first via stomach tube and immediately afterwards via rumen cannula. Terré et al., (2013) collected samples through rumen cannula first to avoid a possible contamination of rumen digesta with saliva, due to stimulation of its flow by the stomach probing. Nevertheless, Geishauser and Gitzel (1996) reported that differences in fermentation parameters observed when sampling through oro-ruminal probe and rumen cannula did not depend on the sampling order (probe prior to cannula or vice versa). Despite a widespread perception that samples obtained by stomach tube may be considerably contaminated by saliva, it has been demonstrated that salivary contamination is rarely a problem if the person collecting the sample is experienced, the tube is not frequently relocated, the probed animal does not move, and the collection is completed in a short time (Geishauser and Gitzel, 1996; Lodge-Ivey et al., 2009). After visual and tactile examination of our samples, it seems very unlikely that they were contaminated with saliva to a considerable extent. However, the fact that water and saliva are delivered to the reticulo-rumen through the esophageal orifice, may imply an unavoidable minor dilution of rumen contents when the sample is collected from that point via stomach tube.

*Ruminal fermentation parameters*

The collection of samples of rumen digesta through stomach tube or rumen cannula revealed similar differences between animal species, diets and sampling times, with the exception of pH values in sheep and goats, which were significantly different only when samples were obtained via rumen cannula. The latter may be related to a possible saliva contamination. On the other hand, a greater VFA concentration in rumen cannula than in stomach tube samples has been reported previously (Geishauser and Gitzel, 1996; Terré et al., 2013) and attributed to saliva contamination.

Results obtained using any of the two techniques are consistent with those reported by other authors when comparing species (Yañez Ruiz et al., 2004) and sampling times (Salles et al., 2003). Ruminal $NH_3$-N and VFA concentrations have been found to be lower in goats than in sheep, and post-feeding sampling is known to decrease pH and increase ammonia, lactate and VFA concentrations. Li et al. (2009) attributed post-feeding differences in fermentation parameters to changes in cells numbers for particular bacteria species in response to the availability of the substrate over time. Regarding diets differing in the amount of concentrate, it has been reported that starch-rich diets may yield greater lactic acid concentration and hence lower ruminal pH (Cantalapiedra-Hijar et al., 2009), in agreement with the results obtained in this study for the FC in comparison to the F diet. Increases in $NH_3$-N concentrations in response to increasing levels of concentrate in the diet have also been observed previously (Cantalapiedra-Hijar et al., 2009). With respect to total VFA, we did not detect differences when feeding F or FC diets, which may probably be due to their similar chemical composition and, especially, the high content of NDF in the FC diet. Overall, sampling through ruminal cannula and stomach tube gave similar results regarding fermentation parameters when comparing species, diets and sampling times,

which was also supported by the similar average coefficients of variation of fermentation parameters between techniques (10.24 and 10.98, respectively for rumen cannula and stomach tube).

*Quantification of microorganisms and structure and diversity of rumen bacteria*

Recent studies have reported that although there is an overall resemblance in microbial community structure between samples collected through rumen cannula and stomach tube, the relative abundance of certain microbial groups differs depending on the sampling method (Lodge-Ivey et al., 2009; Henderson et al., 2013). This has been related to the different composition of the samples in terms of liquid and solid fractions and is in agreement with known variations in the relative abundances of some microbial groups between liquid (present in both stomach tube and rumen cannula samples) and solid phases (present only in negligible amounts in stomach tube samples) (Henderson et al., 2013).

In spite of the different physical composition of the samples, certain consistency was also evidenced by both techniques in the effects of treatments on the rumen microbiota. Nevertheless, there was also some discrepancy regarding concentration of microorganisms, particularly when sampling times were considered. Thus, lower post-feeding concentrations of total bacteria and methanogenic archaea were only revealed when samples were obtained via rumen cannula. The decrease in total bacteria is in line with the pattern observed by Leedle et al. (1982): a decrease in post-feeding numbers after which these increase steadily, reaching the highest value at 16 h. On the contrary, numbers of *R. flavefaciens* were lower post-feeding when animals were sampled with stomach tube. This could be due to the stomach tube not allowing the collection of small pieces of fibre and therefore underestimating the numbers of

microorganisms associated to plant material. However, that was not the case for *F. succinogenes* results, which could be explained by differences in preference of plant tissues as growth substrate by these two fibrolytic bacteria (Shinkai and Kobayashi, 2007).

The higher protozoa numbers detected by both sampling methods in goats than in sheep agrees with previous reports (Santra et al., 1998; Yáñez Ruiz et al., 2004). Differences between animal species were also accompanied by a decrease in protozoa after feeding that has been attributed to the dilution effect of saliva influx as well as to the sequestration of entodiniomorphs over time (Dehority, 2003). A greater concentration of protozoa with the FC diet was also detected by both techniques and is in agreement with other studies (Cantalapiedra-Hijar et al., 2009) on the effect of increasing proportions of concentrate in the diet on microbial growth.

The PCA plots derived from the DGGE banding profiles suggested that, within period, animal species was the factor driving the grouping pattern, although this was more evident in samples collected through rumen cannula. Kong et al. (2010) reported that richness in bacterial species of the solid fraction is 3.5 times higher than the in the liquid fraction, which may explain the less evident segregation of samples obtained by stomach tubing. Furthermore, discrepancy between techniques was observed for the diversity indexes in goats and sheep, values being higher for goats only when sampling via rumen cannula. As mentioned above, the fact that samples obtained through cannula include both liquid and solid fractions would allow the detection of certain microorganisms associated to the solid phase that would not be included in samples collected by stomach tube. Terré et al. (2013) reported that, in calves, the comparison of specific rumen bacteria or fingerprintings of bacteria communities can be acceptable regardless of the sampling technique (stomach tube or rumen cannula),

although these authors did not consider different species, diets or sampling times as in the present study. However, Lodge-Ivey et al. (2009) compared sampling through rumen cannula or stomach tubing in sheep and cattle, obtaining similar Shannon index with both sampling methods (2.1 and 2.2) and therefore supporting the use of stomach tube for bacterial community studies. It is probably worth mentioning that in this study only post-feeding samples were analysed so different results could have been observed if pre-feeding samples had also been considered. Although it has been reported that sampling time has little impact on the assessment of bacterial diversity in the rumen (Li et al., 2009), changes in numbers of particular species in response to the availability of substrate over time may occur.

With respect to diets, none of the techniques detected significant variations in the bacterial diversity when F and F:C were compared, this result being not expected. Differences in favour of the rumen cannulation were anticipated as it has been reported that the diet has a greater effect on solid associated bacteria, assumed to be much less abundant in samples obtained through stomach tube, than on liquid associated bacteria (Larue et al., 2005; Martínez et al., 2010). In addition, it cannot be ruled out that the lack of variations due to the consumption of F or FC diet was due, as mentioned previously, to the lack of substantial differences in diet composition. Larue et al., (2005) and Martínez et al. (2010) found higher diversity in solid associated bacteria when sheep were fed a high forage diet than when it was high in concentrate.

Nevertheless, the overall effect of different diets on rumen microbiota might not depend on whether it is assessed in rumen digesta or liquid samples, even if the microbial composition was significantly changed by the treatments. This have been observed by Castro-Carrera et al. (2014) who reported that the effect of diet

supplementation with marine lipids, evaluated by 454 pyrosequencing, was rather consistent in rumen content or fluid samples despite inherent differences between these fractions in their bacterial composition. However, caution should be taken when the composition of the diets do not differ to a great extent, as occurs in the present study.

## 5. Conclusions

This study supports that stomach tubing is a feasible alternative to surgical rumen cannulation for sampling rumen digesta from sheep and goats to examine ruminal fermentation. Stomach tubing allows the collection of a highly diverse bacterial community and is able to detect most of the effects observed when sampling through cannula. However, further studies including other microbial groups and using high-throughput sequencing tools, are recommended to explore differences in the abundances of some microbial taxa.

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**Table 1.** Chemical composition of the diets (g/kg DM) and dry matter intake and metabolizable energy intake of sheep and goats.

| | Diet | |
|---|---|---|
| | F[1] | FC[2] |
| Organic matter | 891 | 883 |
| Crude protein | 192 | 185 |
| Neutral detergent fibre | 397 | 355 |
| Acid detergent fibre | 306 | 224 |
| Starch | 11 | 107 |
| Ether extract | 35 | 43 |
| Metabolizable energy (MJ/kg DM) | 8.1 | 9.25 |
| Dry matter intake (g/d) | | |
| Sheep | 1040±97 | 1050±49 |
| Goats | 906±30 | 804±29 |
| Metabolizable energy intake (MJ/d) | | |
| Sheep | 8.42±0.8 | 9.71±0.45 |
| Goats | 7.34±0.25 | 7.43±0.27 |

[1]Forage diet (alfalfa)

[2]Forage plus concentrate diet (1:1). The concentrate (Pacsa Sanders, Seville, Spain) contained wheat flour (35%), sunflower meal (20%), malt sprouts (8%), canola meal (13%), soybean hulls (20%), calcium carbonate (1%), rumen-inert fat (2.5%), sodium chloride (0.50%).

**Table 2.** Ruminal fermentation characteristics determined in samples obtained via rumen cannula (RC) or stomach tube (ST).

| | | Sp | | | D | | | T | | | P[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Method | S | G | SED | F | FC | SED | Pre | Post | SED | Sp | D | T | Sp×D | Sp×T | D×T | Sp×D×T |
| pH | RC | 6.53 | 6.77 | 0.095 | 6.76 | 6.54 | 0.063 | 7.02 | 6.28 | 0.063 | 0.04 | 0.01 | <0.001 | 0.78 | 0.49 | 0.63 | 0.48 |
| | ST | 7.16 | 7.12 | 0.118 | 7.25 | 7.03 | 0.075 | 7.44 | 6.85 | 0.075 | 0.78 | 0.01 | <0.001 | 0.84 | 0.47 | 0.79 | 0.84 |
| Ammonia (mg/L) | RC | 249 | 170 | 39.0 | 169 | 250 | 21.9 | 195 | 223 | 21.9 | 0.09 | 0.01 | 0.21 | 0.66 | 0.82 | 0.07 | 0.75 |
| | ST | 225 | 160 | 34.1 | 154 | 231 | 18.4 | 178 | 207 | 18.4 | 0.11 | <0.001 | 0.13 | 0.94 | 0.81 | 0.09 | 0.59 |
| Lactate (mg/L) | RC | 185 | 182 | 17.8 | 160 | 207 | 8.3 | 171 | 196 | 8.3 | 0.86 | <0.001 | 0.01 | 0.27 | 0.09 | 0.04 | 0.16 |
| | ST | 111 | 129 | 16.7 | 100 | 140 | 9.2 | 110 | 130 | 9.2 | 0.34 | <0.001 | 0.04 | 0.97 | 0.21 | 0.01 | 0.07 |
| Total VFA (mmol/L) | RC | 83.6 | 56.6 | 8.98 | 70.1 | 70.1 | 4.71 | 42.2 | 98.0 | 4.71 | 0.02 | 0.99 | <0.001 | 0.99 | 0.38 | 0.42 | 0.70 |
| | ST | 69.7 | 51.0 | 7.28 | 61.9 | 58.9 | 3.72 | 38.5 | 82.3 | 3.72 | 0.04 | 0.43 | <0.001 | 0.85 | 0.09 | 0.95 | 0.99 |
| Molar proportion (mol/100 mol) | | | | | | | | | | | | | | | | | |
| Acetate | RC | 70.5 | 68.5 | 0.79 | 70.9 | 68.9 | 0.40 | 68.8 | 70.2 | 0.40 | 0.04 | <0.001 | 0.01 | 0.90 | <0.001 | 0.35 | 0.32 |
| | ST | 71.1 | 69.2 | 0.91 | 71.6 | 68.8 | 0.47 | 69.4 | 70.9 | 0.47 | 0.08 | <0.001 | 0.01 | 0.71 | 0.01 | 0.28 | 0.42 |
| Propionate | RC | 14.4 | 14.8 | 0.41 | 15.1 | 14.1 | 0.27 | 12.6 | 16.6 | 0.27 | 0.35 | 0.01 | <0.001 | 0.76 | 0.04 | 0.14 | 0.31 |
| | ST | 14.1 | 14.5 | 0.47 | 14.8 | 13.8 | 0.25 | 12.2 | 16.4 | 0.25 | 0.39 | <0.001 | <0.001 | 0.83 | 0.03 | 0.12 | 0.31 |
| Butyrate | RC | 9.9 | 10.1 | 0.45 | 8.3 | 11.7 | 0.26 | 10.8 | 9.2 | 0.26 | 0.68 | <0.001 | <0.001 | 0.73 | 0.06 | 0.88 | 0.18 |
| | ST | 9.7 | 9.6 | 0.44 | 7.9 | 11.4 | 0.30 | 10.5 | 8.9 | 0.30 | 0.90 | <0.001 | <0.001 | 0.66 | 0.25 | 0.85 | 0.36 |
| Others[b] | RC | 5.2 | 6.5 | 0.45 | 5.6 | 6.1 | 0.30 | 7.8 | 4.0 | 0.30 | 0.03 | 0.13 | <0.001 | 0.68 | <0.001 | 0.99 | 0.41 |
| | ST | 5.1 | 6.6 | 0.51 | 5.7 | 6.0 | 0.30 | 7.9 | 3.8 | 0.30 | 0.03 | 0.39 | <0.001 | 0.98 | <0.001 | 0.57 | 0.63 |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A/P ratio | RC | 5.0 | 4.8 | 0.19 | 4.8 | 5.0 | 0.10 | 5.5 | 4.2 | 0.10 | 0.24 | 0.04 | <0.001 | 0.61 | 0.76 | 0.02 | 0.22 |
| | ST | 5.2 | 4.9 | 0.23 | 4.9 | 5.1 | 0.10 | 5.7 | 4.3 | 0.10 | 0.31 | 0.04 | <0.001 | 0.63 | 0.78 | 0.02 | 0.26 |

[a] Probability of significant effect due to species (Sp; S: sheep and G: goat), diet (D; F:forage and FC:forage plus concentrate), sampling time (T) and their interactions.

[b] Calculated as the sum of isobutyrate, isovalerate, valerate and caproate.

**Table 3.**

Ruminal concentration ($log_{10}$ gene copies/g fresh matter) of bacteria (*16S rRNA*), protozoa (*18S rRNA*), methanogenic archaea (*mcrA*), *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* determined in samples obtained via rumen cannula (RC) or stomach tube (ST).

| | | Sp | | | D | | | T | | | P[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Method | S | G | SED | F | FC | SED | Pre | Post | SED | Sp | D | T | Sp×D | Sp×T | D×T | Sp×D×T |
| Bacteria | RC | 10.43 | 10.48 | 0.088 | 10.49 | 10.42 | 0.039 | 10.54 | 10.37 | 0.039 | 0.65 | 0.13 | <0.001 | 0.43 | 0.22 | 0.44 | 0.11 |
| | ST | 9.76 | 9.89 | 0.155 | 9.91 | 9.74 | 0.093 | 9.89 | 9.77 | 0.093 | 0.44 | 0.09 | 0.22 | 0.87 | 0.42 | 0.85 | 0.37 |
| Protozoa | RC | 4.20 | 4.33 | 0.066 | 4.03 | 4.50 | 0.054 | 4.32 | 4.20 | 0.054 | 0.09 | <0.001 | 0.04 | 0.26 | 0.63 | 0.37 | 0.93 |
| | ST | 3.35 | 3.64 | 0.082 | 3.17 | 3.82 | 0.076 | 3.62 | 3.37 | 0.076 | 0.02 | <0.001 | 0.01 | 0.04 | 0.21 | 0.95 | 0.02 |
| Methanogenic archaea | RC | 8.30 | 8.22 | 0.106 | 8.38 | 8.14 | 0.086 | 8.35 | 8.18 | 0.086 | 0.47 | 0.01 | 0.07 | 0.92 | 0.86 | 0.89 | 0.82 |
| | ST | 7.58 | 7.69 | 0.187 | 7.95 | 7.32 | 0.133 | 7.74 | 7.53 | 0.133 | 0.59 | <0.001 | 0.13 | 0.48 | 0.87 | 0.56 | 0.26 |
| *Ruminococcus flavefaciens* | RC | 3.53 | 3.44 | 0.198 | 3.21 | 3.76 | 0.184 | 3.62 | 3.35 | 0.184 | 0.68 | 0.01 | 0.16 | 0.14 | 0.57 | 0.94 | 0.89 |
| | ST | 2.19 | 2.12 | 0.269 | 1.73 | 2.58 | 0.144 | 2.41 | 1.90 | 0.144 | 0.78 | <0.001 | 0.01 | 0.09 | 0.42 | 0.14 | 0.01 |
| *Fibrobacter succinogenes* | RC | 8.71 | 8.65 | 0.083 | 8.69 | 8.67 | 0.076 | 8.85 | 8.51 | 0.076 | 0.49 | 0.76 | <0.001 | 0.61 | 0.81 | 0.27 | 0.52 |
| | ST | 9.03 | 8.47 | 0.283 | 8.80 | 8.70 | 0.232 | 9.08 | 8.42 | 0.232 | 0.09 | 0.66 | 0.01 | 0.22 | 0.19 | 0.23 | 0.09 |

[a] Probability of significant effect due to species (Sp; S: sheep and G: goat), diet (D; F:forage and FC:forage plus concentrate), sampling time (T) and their interactions.

**Table 4.**

Richness and Shannon index calculated from bacterial denaturing gradient gel electrophoresis profiles in rumen samples obtained post-feeding via rumen cannula (RC) or stomach tube (ST).

| | | Sp | | | D | | | P[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Method | S | G | SED | F | FC | SED | Sp | D | Sp×D |
| Richness | RC | 36.1 | 42.3 | 2.265 | 41.0 | 37.4 | 2.265 | 0.02 | 0.14 | 0.63 |
| | ST | 34.3 | 32.8 | 0.997 | 33.0 | 34.0 | 0.997 | 0.16 | 0.34 | <0.001 |
| Shannon index | RC | 3.58 | 3.72 | 0.056 | 3.70 | 3.60 | 0.056 | 0.02 | 0.13 | 0.45 |
| | ST | 3.53 | 3.49 | 0.031 | 3.49 | 3.52 | 0.031 | 0.22 | 0.37 | 0.01 |

[a] Probability of significant effect due to species (Sp; S: sheep and G: goat), diet (D; F:forage and FC:forage plus concentrate) and their interaction.

**Fig. 1.** PCA plots of total bacteria present in rumen samples obtained via rumen cannula (a) or stomach tube (b). Numbers 1 to 4 indicate individual animals. S: sheep; G: goat; F: forage diet; FC: forage plus concentrate diet. Open dots represent goat and filled dots represent sheep.