Comparison of Different DNA Sampling and Extraction Protocols for Bacterial and Archaeal Populations Analysis in Water Buffalo

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Abstract: Methane (CH_4) is a potent greenhouse gas, and ruminants are a significant source of agricultural emissions. It has been hypothesized that the host's genome controls rumen microbial communities, but robust results require numerous samples. The feasibility of a research project will depend on the ease and representativeness of the sampling method, as well as the cost-efficiency of large-scale sequencing. This study aimed to compare different protocols to investigate whether non-invasive samples could serve as a substitute for ruminal digesta. DNA recovery was tested in various matrices (whole rumen content, feces, and buccal swabs) from five cannulated buffalo cows. Three types of buccal swabs were tested, as well as feces in different forms (as-is, pelleted, or in a glycerol solution) and the rumen content. The study compared different protocols for DNA extraction, including WUR protocol, Maxwell®, and Quick Extract™, and two sampling times. Saliva was a challenging matrix to process, obtaining unsatisfactory DNA yield. Feces showed higher yields when pelleted but lower than rumen. The highest amount of DNA was obtained from whole rumen content using all three DNA extraction methods. Quick Extract was the easiest method to perform, while WUR resulted in the highest yield of DNA, swabs excluded. The Maxwell® method gave satisfactory results with all three matrices. However, further metagenomic analysis is required to verify if the species composition is comparable.

Keywords: Rumen cannula, rumen microbiome, feces, buccal swabs, methane emission.

1. INTRODUCTION

Methane (CH_4) emissions account for 40% to 45% of greenhouse gas emissions from ruminants. Over 90% of these emissions come from enteric fermentation [1]. Methane production in the rumen represents a loss of energy for animal growth and production, ranging from 2 to 12% of gross energy intake. Therefore, reducing CH_4 emissions would benefit the environment and improve livestock production efficiency.

It has been hypothesized that the host genome controls rumen microbial communities [2, 3], but robust results require a large number of samples, and it is essential to sample ruminal digesta to exploit the technology's benefits. Oral intubation is an unpleasant procedure for the animal, and samples taken in this way frequently contain a high proportion of saliva. Samples obtained from a ruminal cannula are the most reliable, although this method is impractical for sampling many animals.

The ease and representativeness of the sampling method and the cost-effectiveness of large-scale sequencing determine (through the robustness of collected data) the reliability and feasibility of a research project.

Several methodologies and kits have been developed for the extraction of DNA from a variety of biological samples. The quantity and quality of the DNA obtained by such methods are crucial for successfully completing research studies [4]. Therefore, selecting an appropriate and relevant DNA extraction protocol can result in cost savings, time efficiencies and the acceleration of experimental work [4]. Previous studies [4, 5] indicated that the choice of DNA extraction method can impact the representation of microbial communities within samples sourced from different habitats, including the rumen. The advantages and disadvantages of various fast DNA extraction methods and kits are examined related to the sample, yield, application, materials and methodology, and the expenses involved.

Additionally, the sampling technique (e.g., oral stomach tubing and collecting through a rumen fistula) and rumen sample fractionation (e.g., liquid and solid) can also influence microbial community parameters [5, 6].

The fecal community is significantly different from that of the rumen [7-9], but there may be useful indicators analogous to the presence of fecal archaeol,

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a membrane lipid of ruminal archaea, being used as a marker for ruminal methanogenesis [10-12].

The water buffalo (*Bubalus bubalis*) is a valuable domestic animal globally, providing milk, meat, leather, and draft power. Compared to cattle, buffaloes are more efficient at using low-quality feed due to differences in the anatomy and physiology of their digestive system [13]. Buffalo has been reported to have less potential impact on methane production [14].

The present experiment aimed to compare potential alternative sampling and DNA extraction methods to evaluate their suitability as proxies for direct sampling of ruminal digesta. The same hypothesis was previously explored using buccal samples in sheep [15] and dairy cows [16]. This study investigated whether this non-invasive sampling could be applied to buffalo species. To our knowledge, such information is not yet available.

2. MATERIAL AND METHODS

2.1. Animals

Five non-lactating Mediterranean buffalo (*Bubalus bubalis*) cows with rumen cannulas were used in the trial conducted at the CREA experimental field station in Monterotondo, Rome (Tor Mancina, 42° 05′ 26.0″ N 12° 36′ 44.7″ E). The animal study research protocol was approved by the National Ethics Committee (Ministry of Health Decree 26/2014, authorization n◦1103/2020-PR, Italy) by the guidelines established by the EU Council/Directives 86/609/EEC. Animals were fed 25 kg/head Total Mixed Ration (TMR) with a forage-to-concentrate ratio of 60:40 and 35% humidity. The TMR had the following characteristics on a Dry Matter basis (DM): crude protein 13.8%, Neutral Detergent Fiber (NDF) 45%, and Milk Forage Units (MFU) 0.9/kg.

2.2. Sampling Methods

The experimental plan included three consecutive sampling days on five fistulated animals: two hours after feeding during the first two days and one hour before morning feeding on the third day.

2.2.1. Rumen Samples

Whole rumen content samples were collected from four regions (anterior dorsal, anterior ventral, posterior dorsal, and posterior ventral) within the rumen-reticulum. After collection, ruminal digesta samples were mixed thoroughly and kept under CO_2 flow before bag filtration using Bag Page (280 µm, Interscience, France). A portion of the rumen content was divided into two aliquots. The first aliquot was used for the direct determination of pH. The second aliquot was homogenized for 5 minutes using a Stomacher (VWR International, USA) to detach microorganisms from feed particles. Subsequently, 1 mL aliquots of the homogenate were frozen at -20°C for microbial analysis.

2.2.2. Fecal Samples

Fecal samples (50 g) were collected by stimulating rectal activity during rumen sampling. A preliminary analysis using feces as-is or preserved with different solutions: 1) Tapio [16] protocol- 30% v/v saline-glycerol PBS, 1:2 feces/PBS-glycerol ratio; 2) feces/PBS ratio 1:2; 3) feces/molecular H_2O 1:2, tested at 0,5 and 1 mL of quantity, pelleted or liquid samples was performed. The best results were obtained with 1 mL of pelleted feces (data not shown), preserved following the Tapio *et al*. protocol [16]. These samples were frozen at -20°C before performing qPCR.

2.2.3. Buccal Swabs

After a commercially available material survey, the following three types of swabs were used for daily sampling of mouth fluids of each animal: PG-100 Nasal (PERFORMAgene, DNA Genotek Inc., Canada), OMR-110 (DNA Genotek Inc., Canada), and a commercial dry swab (STD). The first two are used in animal tests, whereas the third (STD) is used in humans.

Buccal PG-100. Buccal swabbing was performed using a Performagene kit (buccal PG-100). The sponge included in the Performagene sample collection kit which measured 3 cm in length, was inserted into the animal's mouth and swabbed several times across the inner side of the left cheek. The sample was placed in a Performagene test tube with a stabilizing solution and sealed according to the manufacturer's instructions. The tube was stored at -20°C. When the DNA was extracted, the collection sponges were heated in a water bath at 50°C for 1 hour, following the manufacturer's protocol, and then squeezed against the inner wall of the collection tube to release most of the liquid. The resulting saliva sample was transferred to a sterile 1.5-mL Eppendorf. For DNA extraction, 300 µl of the samples were used in the three protocols under testing, reported below in paragraph 2.4.

OM-110. sampling the animal's inner side of the cheeks was swabbed multiple times using the cotton roll provided with the kit. The cotton roll was then

placed in an Omnigene test tube containing 1 mL of stabilizing solution (DNA Genotek Inc., Ottawa, Canada), and the tube was sealed according to the manufacturer's instructions. The Omnigene tubes containing one swab were heated in a water bath at 50°C for 1 hour according to the manufacturer's protocol. The saliva sample was collected by removing the bottom tip (approximately 5 mm) of the cotton roll and placing it without the lid inside a sterile 15-mL tube. The sample was centrifuged at 1,000 g for 3 min to extract the saliva from the cotton roll, and the resulting liquid was used for analysis. The contents of the 15-mL tube were transferred to a sterile 1.5-mL reaction tube. The saliva samples were stored at -20°C until further analysis. For DNA extraction, 300 µL of the samples were used following the three protocols under test reported below in the 2.4 paragraph.

STD sampling. A sterile cotton roll in a polypropylene test tube Ø 12 x 150 mm (Aptaca Spa, Italy) was inserted into the animal's mouth and swabbed the inside of both cheeks several times. The cotton roll was placed in its tube. The tubes were stored at -20°C. The cotton roll was removed from the tube with sterile forceps, and approximately 1/3 of the swab was cut off and processed.

The samples were used undiluted.

2.3. Chemical Analysis

Fibre components (NDF and ADF, cellulose, hemicellulose, and lignin) were analyzed according to the Van Soest method [17], as modified by Martillotti *et al*. [18]. A pH meter (GLP 21, Crison, Spain) recorded the rumen pH. One mL of sample was added with 50% of 0.1 N sulphuric acid, centrifuged at 14.000 rpm, 4°C per 10 minutes, 3 times, and was used to determine the ammonia nitrogen (N-NH_3). In the ammonia nitrogen measurement, the supernatant was diluted with demineralized water to a final volume of 40 mL and then quantified using the APAT 4030 method [19]. The HPLC analysis of VFAs was conducted using an Aminex 85 HPX-87H column (Bio-Rad, USA) on isotherm conditions at 40°C with a 0.6 mL/min flow rate and isocratic 0.008 N H_2SO_4. The Shimadzu system (Japan) was used for the analysis, and a UV detector at 220 nm was employed.

2.4. DNA Extraction

The trial aimed to compare three DNA extraction methods to verify the yield and quality of the material extracted from the three types of matrices (rumen, feces and saliva) as described previously.

WUR (Wageningen University & Research). DNA was extracted using a protocol according to van Lingen *et al.* [20], involving an initial sample treatment step with a Bead Beater homogenizer (Biospec, USA) to improve cell disruption and facilitate DNA recovery and thus the efficiency of the associated extraction tool. The Maxwell® 16 (Promega, USA; see details below) was used with specific Kits: a buccal swab lev DNA purification kit for buccal swabs and a Tissue DNA Purification Kit for feces and rumen.

Mx (Maxwell® 16 Instrument). An automated DNA purification system for various types of samples, using the specific Kits (Buccal Swab LEV DNA Purification Kit for swabs and Tissue LEV DNA purification Kit for rumen and feces -Promega, USA) for recovering eluted DNA suitable for quantification and use for amplification analysis was obtained. The system uses paramagnetic particles (PAMPs) to purify the sample, which optimizes DNA capture, washing, and elution while avoiding common problems such as clogged tips or partial transfer of reagents.

QE (QuickExtract™). DNA Extraction Solution (Lucigen, UK) offers a simple, fast, and cost-effective method for preparing genomic DNA for PCR amplification. This method does not require the use of columns or chemicals. The extraction process involves only heat treatment to lyse the cell or tissue material, release the DNA, and degrade compounds, inhibiting amplification. This makes the DNA ready for use in automated systems.

The quantity of DNA was determined using a Quantity-one fluorimeter (Promega, USA), and the quality was checked on an agarose gel (1,5%).

2.5. Primer Identification

The microorganisms to be studied were selected based on a literature review. The primers used to identify total Bacteria [21] and Archaea [22] are reported in Table **1**.

2.6. qPCR

The samples extracted with Maxwell® 16 Instrument were chosen for absolute quantification of bacteria and Archaea, given the good results with all three matrices. SYBR green qPCR assays were performed using an iCycler IQ (Bio-Rad Laboratories, USA) following the protocols of Huws *et al*. [23] for bacteria, and van Lingen *et al*. [20] for archaea. qPCR efficiency for all assays was between 90-110, and the

Table 1: Primers Used in the Trial

Primer name	Target group	Sequence (5'-3')	Reference
EUB F	Bacteria	GTGSTGCAYGGYTGTCGTCA	
EUB R	Bacteria	ACGTCRTCCMCACCTTCCTC	Maeda *et al*. (2003)
Arch787F	Archea	ATTAGATACCCSBGTAGTCC	
Arch1059R	Archea	GCCATGCACCWCCTC	Hook *et al*. (2009)

correlations of genomic DNA standards for all qPCRs were >0.97. The bacteria qPCR analyses were performed in triplicate with a reaction volume of 25 µL, including 2 µL of 1:50 dilutions of sample DNA extracts. A bacterial standard was created by combining equal amounts of genomic DNA from eight different pure cultures of bacteria, including *Butyrivibrio sp*., *Clostridium proteoclasticum, Eubacterium ruminantium, Fibrobacter succinogenes, Megasphaera elsdenii, Prevotella brevis, Prevotella bryantii*, and *Selenomonas ruminantium*. For archaea determinations, the reaction volume was 22 µL, which contained 2 µL of 1:20 dilutions of sample DNA extracts. Standard curves were generated using serial dilutions of custom-synthesized DNA prepared from known 16S rRNA gene sequences of *Methanobrevibacter ruminantium*.

2.7. Protozoa Quantification

Like bacteria, protozoa reside in the ruminal liquid phase or are attached to the ruminal wall or food particles [24]. Due to their cellulolytic activity [25], they are involved in the degradation and fermentation process of the fiber and, consequently, in the rumen methane production. Therefore, a microscopic quantification of protozoa was carried out in the rumen using the Fuchs Rosenthal counting chamber [26].

2.8. Statistical Analysis

The microbial concentration and fermentation data were analyzed using the one-way ANOVA of the PAST software version 3.22 (2018, Øyvind Hammer, University of Oslo, Norway, https://www.nhm.uio.no/english/research/resources/past/, accessed on 29 April 2024). Homoscedasticity and normality were checked before testing. If the normality of distributions was verified, the data were analyzed using one-way ANOVA. In this case, Tukey's honestly significant difference (HSD) test was used to separate the means. When the data distribution deviated from normality, the Kruskal-Wallis non-parametric test was applied, and the differences were tested according to the Mann-Whitney pairwise test.

3. RESULTS AND DISCUSSION

This study compared different protocols to investigate whether non-invasive samples (feces, buccal swabs) could serve as a proxy for ruminal digesta. It also aimed to determine the most suitable method of sampling and extracting DNA from bacterial and archaeal populations in water buffaloes.

The chemical analysis of the administered diet is reported in the supplementary material (Table **S1**), together with the chemical composition of the rumen (Table **S2**).

Fresh rumen liquid sampled before feeding represents the inoculum most commonly used when the aim is to minimize inter-animal variation. Although, Belanche *et al*. [27] showed that sampling at 3h after feeding provides the most diverse and active rumen microbial inoculum in dairy cows. In this trial, no statistical differences in DNA yield were observed between the three sampling times: two hours after feeding and one hour before feeding. Nevertheless, the analysis showed higher data variability in the samples taken before feeding. Figure **1A** shows the boxplot of DNA yield among sampling times.

Concerning the matrices, a statistically significant difference was revealed among the three, and the rumen expressed the highest yield (Figure **1B**). The ruminal cannula has already proven to be the best sampling method for microbial analysis. Still, it requires a cannulated animal and is unsuitable when many subjects are needed [28].

Obtaining swab samples from the buccal cavity of ruminants has been identified as a promising method for sampling by various authors on other species [15, 16], given the non-invasive nature of the procedure

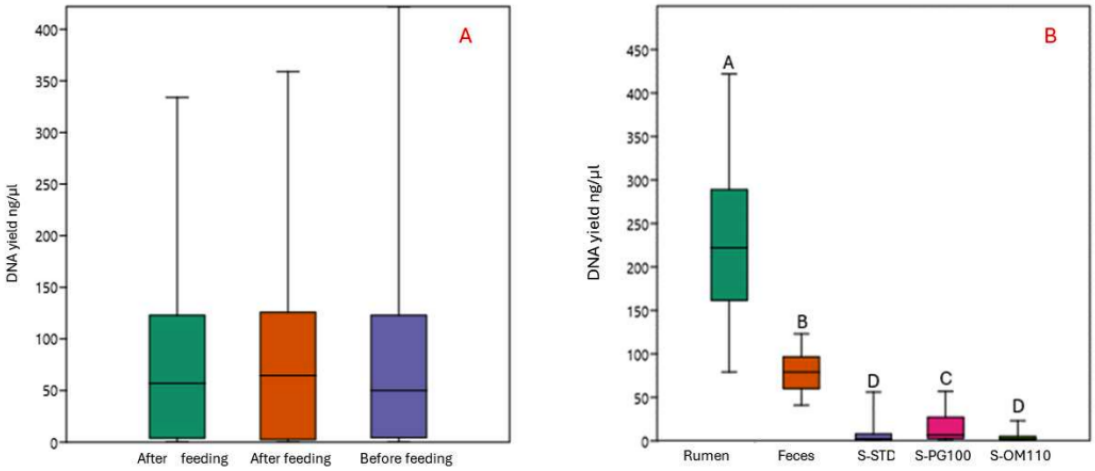

Figure 1: Boxplot of DNA quantity according to sampling time (**A**) and matrices (**B**). Different letters above each boxplot indicate a statistically significant difference for p ≤ 0.01 following the post-hoc Mann-Whitney test.

S-STD (Commercial standard dry swab); S-PG100 (PerformagenePG-100 Nasal swab); S-OMR-110 (Genotek Inc. buccal swab).

compared to the rumen cannula or stomach tube. However, concerns about the use of buccal swabs include the lower amount of genomic DNA isolated and the lower quality of the DNA [29]. The low quality of the DNA obtained could be related to enzymatic contamination and its subsequent loss of integrity [29] or perhaps to the high keratinization level of oral mucosa in buffalo that limits the presence of cell nucleus in the superficial layer, as reported by Sa *et al*. [30]. In our study, the buccal samples showed the lowest DNA yield among the three matrices. Recovery decreased from PG100 swabs to STD and OM100, respectively.

The kit utilized for the buccal PG-100 method included a sterile collection swab. Nevertheless, the stick was short, and consequently, reaching the inner part of the mouth to obtain sufficient material without contaminating or damaging the swab was challenging, confirming what was reported by [15]. Using an STD swab with the PG-100 test tube before direct extraction could be an option. In that case, the cost of the kit should be considered.

One meaningful advantage of the OM-110 or PG-100 kits is that they did not require the sample to be stored at 20°C immediately following collection. According to the manufacturer's specifications, the specimen can be stored at room temperature for up to 12 months after sampling. This would allow researchers, veterinarians, or farmers in remote locations to collect buccal swab samples without compromising the subsequent processing [15].

Contrary to the findings for sheep [15], OM swabs processed with Qiagen kits did not give acceptable results. The PG-100 300 µl of storage fluid yielded enough DNA to be processed. Nevertheless, the results of direct quantification were poor, which could be due to the initial lower concentration of target DNA [31, 32].

According to our results, samples collected by buccal swabbing turned out to be a difficult matrix to process that did not provide a satisfactory DNA amount with every used method, and it was not possible to directly quantify either archaea or bacteria by qPCR.

Although the loss of individual samples is a significant limitation, the use of Next Generation Sequencing (NGS) enabled the acquisition of substantial sequence data from a sufficiently large number of animals to assess the potential of buccal swabs as an effective alternative to stomach tubing in sheep [15] or cattle [16]. Further NGS investigations are needed to provide the same conclusion for Buffalo.

The efficiency of various DNA extraction methods is shown in Figure **2**. No statistical difference was highlighted between methods in DNA yield, even though the WUR method allowed the recovery of the highest amount, followed by QE and Mx, respectively. Nevertheless, the WUR method is more laborious and time-consuming. Moreover, it is unsuitable for DNA recovery with STD cotton heads because the step in Bead beater flakes the head and does not allow recovery of supernatant for further processing.

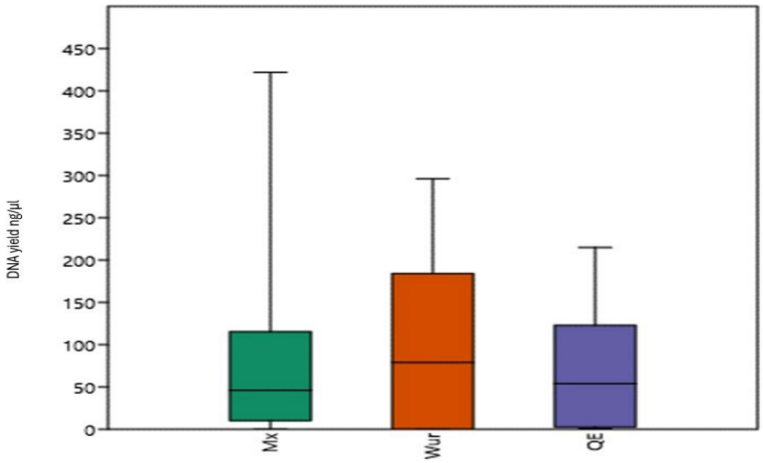

Figure 2: Boxplot of different extraction protocols. Mx: Maxwell® 16 Instrument; Wur: Wageningen University & Research; QE: QuickExtract™.

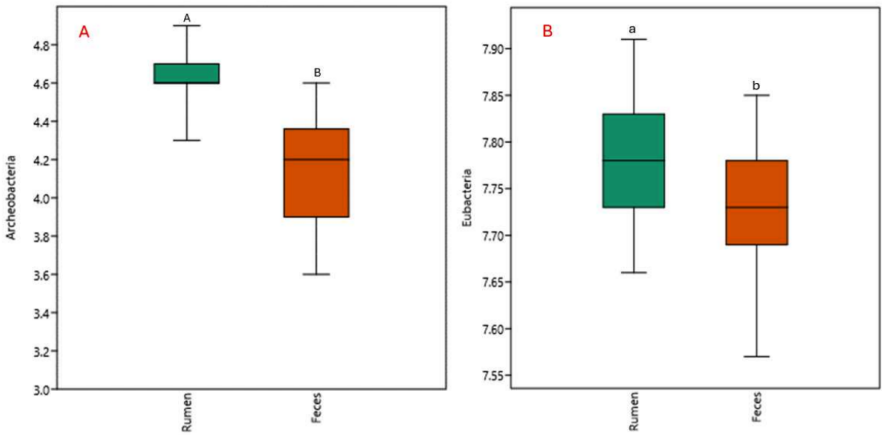

Figure 3: Boxplot of Archaeal (**A**) and Bacterial (**B**) qPCR quantification in the two matrices, rumen and feces. The different letters above each boxplot indicate a statistical difference for p ≤ 0.01 (uppercase) or for p≤ 0.05 (lowercase), according to Tuckey's HSD test.

Table 2: Ph, Protozoa Count, and Qpcr Quantification of Archaeal and Bacterial Populations (Means ± SD)

pH	After feeding	6.70±0.29
	Before feeding	6.94±0.27
Protozoan mL^{-1}	After feeding	4.22^{-5} ± 2.53^{-5}
	Before feeding	4.61^{-5} ± 2.15^{-5}
Archea	Rumen*(Log pgmL^{-1})*	4.64±0.14 A
	Feces*(Log pgmL^{-1})*	4.13±0.27 B
Total Bacteria	Rumen*(Log pgmL^{-1})*	7.78±0.06 a
	Feces*(Log pgmL^{-1})*	7.72±0.07 b

Within each group (Archea or Bacteria), the letters indicate a statistical difference for p ≤ 0.01 (uppercase) or for p≤ 0.05 (lowercase).

The QE was the easiest and fastest method to perform compared to the other two.

3.1. qPCR Microbial Analysis

As reported in Figure **3** and Table **2**, ruminal samples differed markedly from corresponding fecal samples in terms of the absolute quantification of bacterial and archeal populations.

Archaea were significantly lower in feces than rumen (4.13±0.27 and 4.64±0.14, respectively, $p \leq 0.01$) as well as total bacteria (7.78±0.06 and 7.72±0.07 rumen and feces, respectively, $p \leq 0.05$).

Several studies have highlighted the differences between the ruminal and fecal microbiota in terms of the diversity and composition of bacterial families [7,8, 9]. Nonetheless, to the best of our knowledge, no information is available on buffalo, and further in-depth metagenomic investigations are required to confirm this tendency in buffalo species.

The number of protozoa present before the meal was found to be higher than the resulting number after the meal (Table **2**), as reported by various authors [33-35]. However, the difference was not statistically significant.

Several studies suggest that protozoan abundance correlates with methane emissions [35, 36], while others do not [37, 38]. Their involvement in methanogenesis is partly due to their abundant H_2 production [36].

CONCLUSIONS

The objective of the present experiments was to compare alternative sampling and DNA extraction methods to process ruminal digesta via rumen cannula, buccal fluid, and feces to assess the feasibility of utilizing non-invasive samples as proxy indicators for ruminal digesta.

Rumen content obtained through the rumen cannula represents the best source of DNA, given the highest concentration obtained using all three DNA extraction methods. Buccal swabs resulted in a challenging matrix to sample, while feces ranked intermediate, as they showed higher yields when pelleted but lower than rumen.

No differences were revealed between sampling times.

However, additional metagenomic analysis will be necessary to ascertain the extent to which the species composition observed in the three matrices is comparable to that reported by other authors on sheep and cattle and in mitigation genetic programs where a large number of subjects are needed.

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AUTHOR CONTRIBUTIONS

AC Conceived and designed the experiments. AC, ER, and MCL performed the sampling. MCL performed the molecular analysis. ER and MC performed the chemical analyses. DMZ performed the chemical analysis of the diet. AC and ES statistically analyzed the data. AC wrote the manuscript. All authors read and approved the final manuscript.

SUPPLEMENTARY TABLES

The supplementary tables can be downloaded from the journal website along with the article.

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