

Article

Gas Endeavour Device for the Real-Time in Vitro Measurement of Carbon Dioxide and Methane Emissions Associated with Sheep Diets with Prickly Pear By-Products

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Abstract

Prickly pear by-products contain dietary fibre and bioactive components like polyphenols and flavonols, which can reduce total gas and methane emissions. To this end, an in vitro trial was carried out in duplicate utilizing three diets containing hay, concentrate, and two prickly pear by-products obtained after grinding the fruit peel and pastazzo (pulp + peel + seeds), which were ensiled with the addition of 12% wheat bran (raw weight). Based on the ingredient intake recorded in the in vivo study for 12 lactating ewes fed the three diets, an in vitro rumen fermentation study with the innovative Gas Endeavour system (GES) was performed, and the Gage R&R statistical method was used to evaluate the accuracy of the total gas and methane production detected by the GES device. Fermented liquor samples for each diet were used to calculate the disappearance of organic matter and neutral detergent fibre. Shotgun metagenome sequencing analysis was used to evaluate the effect of diet on the rumen fluid microbiota, and it was found that the parameters of repeatability and reproducibility of the total gas and the methane produced after 24 h were satisfactory. Prickly pear by-products display high fermentability for the peel and low fermentability for pastazzo silage, which generates lower total gas and methane emissions. This diminished methane gas production is not correlated with the relative abundance of methanogens. The different chemical and nutritional composition of the three diets altered the rumen bacteria, albeit only slightly, with particular reference to the *Succinivibrio* and *Selenomonas* genera. In conclusion, prickly pear peel silage displayed acceptable fermentation traits, which could support its utilization in sheep diets.

Keywords: prickly pear by-products; silages; in vitro gas production; methane emission; gas endeavour

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1. Introduction

Opuntia ficus indica (L.) Miller, commonly known as prickly pear, is a succulent shrub from the Cactaceae family, with the genus *Opuntia* being the most prominent. Native to Mexico, where it is referred to as “Nopal”, it is also found throughout the Mediterranean region, including Italy, where evidently, it has found more than favourable pedo-climatic conditions. Today, Sicily has the highest concentration of prickly pear in the Mediterranean area, making it not only a common element of the natural landscape but also a prominent symbol in literary and artistic representations of the island [1].

Undoubtedly, from an economic perspective, the primary use of prickly pear lies in the fresh consumption of its fruits, which represent the plant's most valuable food resource due to their impressive nutritional profile, being rich in vitamin C, minerals, especially calcium and phosphorus, and important antioxidant compounds [2]. Furthermore, these fruits can be processed to make juices, liqueurs, jellies, jams, and sweeteners, retaining their numerous beneficial properties while making them easier to consume. In order to produce these products, particularly juice, the processing industry often grinds the entire fruit, including the skin, resulting in a residue known as "pastazzo", which consists of the skins, seeds, and residual pulp. To enhance juice quality, the use of automatic peeling machines is becoming more common, allowing for the effective separation of the peels, seeds (which are utilized to extract valuable oil), and juice/pulp. This process creates a substantial volume of by-products, such as peels and pastazzo, which, if appropriately stabilized, can provide a valuable nutritional resource for small ruminants [3–7].

Prickly pear peel, an agro-industrial by-product, contains dietary fibre [5] and bioactive components like polyphenols and flavonols [8]. There is a growing body of data suggesting flavonoids can reduce methane emissions [9], while some studies indicate that plant secondary metabolites can adjust the rumen microbiome, changing its activity. Certain rumen microbes also improve protein and fibre digestion and decrease feed energy loss, such as through methane, in ruminants consuming tropical plants [10,11].

Tannins are the main phenolic compounds found in cactus pear seeds [12]. These substances, when introduced in ruminant diets, can decrease methane production, possibly because they hinder the breakdown of fibre [13]. In addition, research also indicates that hydrolyzable tannins might lessen methane emissions by directly impacting the rumen's microbiota, even without interfering with fibre digestion [14].

Exploring sustainable feeding methods that can efficiently decrease methane output and at the same time satisfy the nutritional needs of the growing ruminant population is therefore crucial. Utilizing agro-industrial by-products as alternative feed sources offers a possible approach for tackling the problems mentioned, thanks to their favourable nutrient content and the existence of bioactive substances [15].

Numerous investigations have evaluated the anti-methanogenic abilities of feed and additives, both inside living organisms (in vivo) and in artificial settings (in vitro). However, only a handful of studies have compared the two approaches [16].

The Gas Endeavour (GES), an automatic gas flow measurement system developed by BPC Instruments (Lund, Sweden), is a volumetric gas measurement apparatus capable of detecting extremely low gas volumes which operates on the principles of liquid displacement and buoyancy to measure GP. This technique finds multiple uses in batch fermentation experiments, and the GES has been employed across multiple fields to measure GP, including in the assessment of biomethane produced from a variety of substrate types [17]. Additionally, the GES can be used to evaluate the fermentation characteristics of various feedstuffs, including gas production kinetics and methane emissions [18–20]. The advantages of the GES in animal nutrition lie in its ability to gauge GP and MGP as well as their kinetics throughout in vitro fermentation. This is accomplished by employing sensors and software which constantly monitor and records data on a computer. An additional opportunity offered by the GES is the presentation of its results: whereas other in vitro systems frequently report gas generation in terms of pressure (psi or kPa), requiring conversion to volume by employing specific equations, the GES provides its results in terms of the volume (mL) of gas and methane generated, thus simplifying data interpretation and lessening errors [18].

Enteric methane is the largest contributor to the emissions of greenhouse gases originating from animal agriculture. The opportunity provided by Gas Endeavour to measure methane production and its production kinetics in real time allows for the development

of a better understanding of the anti-methanogenic action that agro-industrial by-products can exert on rumen fermentations. In fact, the addition of these by-products to a diet composed of hay and concentrate promotes a reduction in methane emissions during the first 24 h of fermentation [9]. From recent literature data, it is clear that the plant secondary metabolites present in by-products may be rationally utilized to modulate the rumen microbiome to achieve a reduction in methane emissions [11].

The hypothesis of this study is that the presence of polyphenols and tannins contained in prickly pear by-products may alter enteric methane production due to the modification of the ruminal microbiota. Therefore, this study evaluated whether the inclusion of prickly pear by-product silages in the diet of dairy sheep could reduce enteric methane emissions and kinetics and modify the ruminal fluid microbiota.

2. Materials and Methods

The experimental *in vivo* trial was conducted on a commercial farm in west Sicily, Italy. This farm houses 500 Valle del Belice breed ewes raised in a semi-extensive system. The trial followed the ethical principles of animal experimentation adopted by the Animal Welfare Commission of the University of Palermo (protocol number: UNPA-CLE 201954-12/12/2023).

2.1. *In Vivo* Trial

In September, 600 kg of fresh prickly pear peel (PPP) was obtained after peeling and processing prickly pear fruits using an automatic peeling machine (AGRIMAT s.r.l., Tortona, Italy), which separated the peels, seeds, pulp and juice. The PPP was immediately transported to the sheep farm and ensiled with 12% wheat bran (based on raw weight) for 50 d in hermetically sealed plastic drums equipped with a degassing valve. In addition, a local juice extraction company (Agres,, Palermo, Italy) supplied 600 kg of a mix of prickly pear peel + pulp + seeds (PPS) obtained after juice extraction (earthworm press) from whole fruits. These by-products were loaded into trucks and, after 24 h, transported to a sheep farm and ensiled with 12% wheat bran (based on raw weight) for 50 d in hermetically sealed plastic containers under the same conditions as above. Information on the production process and silage quality was reported by Gannuscio et al. [5].

Twelve Valle del Belice dairy ewes were selected from a farm group at 90 days in milk and randomly divided into three experimental groups that were homogeneous for parity (3rd-6th lambing), live weight (53.66 ± 6.57 kg) and milk yield (1.038 ± 0.144 kg/d). The ewes were housed in a farm building containing individual straw-bedded pens, each equipped with a feeder and a drinker (Piro Impianti Zootecnici Srl, Cutro, Italy). Initially, the ewe groups underwent a 2-week adaptation period to their new housing conditions and diet. After the adaptation period, each ewe group was randomly assigned to one of three experimental diets using a Latin square design (3×3) with three phases, each of which lasted for 14 d—9 d for adaptation to diets and 5 d for sampling [7]. The diets were formulated to ensure that the same fibre and crude protein contents were presents. The offered diet and each pen's refusal diet (PPP and PPS silages, hay, and concentrate) were weighed daily and sampled three times, on the 2nd, 3rd and 4th day of the sampling period, to determine the dietary intake and its composition (Table 1).

Table 1. Diets ingredients offered and intaked to lactating ewes.

Ingredients	Diets		
	CTR	PPP	PPS
Offered (g/d/head)			
Hay	3000	2700	2300

Silage	-	1500	1000
Concentrate	900	500	500
Feed Intake (g of DM/d/head)			
Hay	1499	1269	1403
Silage	-	307	410
Concentrate	795	442	442
Nutrients Intake (g/d/head)			
DM	2410	2285	2471
CP	328	274	283
EE	57.70	61.10	59.10
aNDFom	1477	1393	1647
ADFom	856	821	1030
ADL	128	126	236
NFC	398	406	324
NEL (MJ intake/d/head)	11.46	11.04	10.39

CTR: control; PPP: prickly pear peel; PPS: prickly pear peel + pulp + seeds. DM: dry matter; CP: crude protein; EE: ether extract; aNDFom: neutral detergent fibre; ADFom: acid detergent fibre; ADL: Acid Detergent Lignin; SEM: standard error of mean. NFC: non-fibrous carbohydrates = 100 - (CP + ether extract + ash + aNDFom). NEL: net energy for lactation; MJ: megajoule.

2.2. In Vitro Study

2.2.1. Preliminary Preparation of the Test

Based on feed intake recorded in the *in vivo* trial (Table 1), diets were formulated with equivalent feed ratios and employed for *in vitro* ruminal fermentation, where the gas production and its kinetics features were appraised utilizing the GES (BPC Instruments, Lund, Sweden) for the real-time observation of GP and MGP in rumen fermentation batch procedures. The GES was equipped with a water bath incubator to maintain the rumen's optimal temperature (39 °C) and ensure constant agitation throughout the fermentation process. This water bath incubator housed 18 reactors (glass bottles), each with a capacity of 250 mL, featuring a special hermetically sealed lid that allowed for the connection of two tubes, namely a tube for transport of fermentation gases and a tube connected to a tap for the introduction of the solutions. The tubes carrying fermentation gases, emerging from the top of each bottle, followed one of two routes: they either led directly to the measurement cells (working principle: liquid displacement and buoyancy; measuring resolution, 2 mL), in which case the measured gas corresponded to the entirety of gas produced, or to a CO₂ trap, comprising a glass bottle containing sodium hydroxide (concentration, 120 g/L). In this latter scenario, the fermentation gas flow was forced to pass through the CO₂ trap before reaching the measurement unit. In this case, assuming that the total volume of gases produced during fermentation was the sum of CO₂ and methane, the gas flow measurement units only measured the methane gas production (MGP), because CO₂ was captured in the traps.

The day before the experiment began, the buffer solution was prepared according to Liu et al. [18]. For each litre of buffer solution, 8.5 g of NaHCO₃, 5.8 g of K₂HPO₄, 0.5 g of (NH₄)₂HPO₄, 1 g of NaCl, 0.5 g of MgSO₄, 0.01 g of FeSO₄, and 0.10 g of CaCl₂ were dissolved in distilled water. Each freeze-dried diet (substrate) was weighed (3.000 ± 0.002 g) and placed into glass bottle anaerobic digesters.

On the day of the experiment, before it began, the buffer solution was heated to 39 °C in a water bath, while simultaneously being fully saturated with CO₂ via sparging for one hour. After this time, the first 200 mL of buffer solution was added to each bottle containing the substrate using a laboratory peristaltic pump (Innofluid, Shanghai, China). The reactors were then sealed and placed in the thermostatic bath at 39 °C. Once this step

was completed, the CO₂ cylinder was connected through an 18-outlet flow divider and to each reactor via taps, which allowed the gas to maintain saturation in the solutions and saturate the entire circuit of the instrument. Pure CO₂ gas was used to displace O₂ from the reactors to ensure an anaerobic environment before the inoculation of ruminal fluid. During this phase, the traps containing soda (sodium hydroxide) were bypassed through a tap system in order to directly connect the fermenters to the measurement cells. This also ensured that the liquid (distilled water) inside the measurement units of the instrument was saturated. The CO₂ insufflation of the entire system was carried out for 45 min.

2.2.2. Rumen Liquor Sampling

The rumen liquor (RL) was collected within 60 min after the beginning of the *in vitro* trial from two donor animals with an oro-oesophageal tube, known as stomach tubing [21]. The RL was collected from two female Valle del Belice breed sheep, 1 one-year-old lamb and 1 multiparous ewe, and then was pooled in equal amounts and transferred inside a thermos to the SAAF Department for the trial. Stomach tubing was performed by well-trained persons to minimize salivary dilution.

RL sampling was performed before sheep were fed, as recommended by Yanez et al. [22] to minimize diet–animal interactions. The diet fed to donor animals was similar in composition to the substrate incubated *in vitro*, with a similar forage/concentrate ratio (75:25).

In the laboratory, the RL was maintained under anaerobic conditions with continuous insufflation of CO₂ through a bubble tube and, maintaining a temperature of 39 °C, was filtered through four layers of cheesecloth. Finally, the RL pH was detected using a portable pH meter.

2.2.3. Gas Endeavour Trial

Once filtration was complete, 7.5 mL of RL [19] was quickly inoculated into each bottle through the tap in the lid using a 10 mL syringe, directly and without opening the bottle to maintain the anaerobic conditions established earlier. Immediately afterward, to clean the inlet tube and ensure that all ruminal fluid came into contact with the solution, 17.5 mL of buffer solution at 39 °C was added through the same tap. In this way, the total volume of buffer solution added to each bottle was 217.5 mL. Finally, the headspace of the tubes and bottles was saturated by insufflating Nitrogen 5.0 for 1 min to remove all of the CO₂. The nitrogen cylinder was directly connected to each bottle through the taps in the lid, ensuring that all the bottles remained sealed at all times. Stirring motors were used to agitate the contents of the 250 mL glass bottles.

After a 10 min waiting period to allow the system to stabilize, the experiment was started directly using the software provided with the Gas Endeavour (Bioprocess Control), and it lasted for 24 h.

The biogas volume measuring device used the principle of liquid displacement and buoyancy to measure the volumetric biogas flowrate. The CO₂-absorption unit used a concentrated solution of sodium hydroxide (3M) to remove acid gas fractions including CO₂ [23]. The remaining gas, which was assumed to be mainly methane, exited the CO₂-absorption unit to be measured by the methane volume measuring device, which also used the principle of water liquid displacement and buoyancy to measure the volumetric methane flowrate. The volumetric gas and methane flowrates were automatically normalized to one atmosphere (atm), 0 °C, and zero moisture content [23].

Each diet (CTR, PPP and PPS) was incubated in four bottles, including two for the measurement of the total GP and two for MGP, and four bottles were utilized as blank controls (RL + buffer solution).

The same in vitro procedure was repeated after 48 h (April 26th and 28th) to obtain data for another run.

2.2.4. Repeatability and Reproducibility of Gas Endeavour Measurements

To assess the repeatability and reproducibility of GP and MGP acquired in vitro tests using the Gas Endeavour system, Gage R&R was employed. Gage R&R is a method used to determine the variability present in the measurement system which would contribute to the overall variability in the result. The variability observed when the same appraiser measures a characteristic multiple times using the same gage is termed the repeatability. The variability observed when multiple appraisers or different trials measure the same components using the same gage is termed the reproducibility. The purpose of Gage R&R is to measure accuracy and precision, and a good measurement system should be responsive to small changes that are present in part-to-part variation [24].

For each bottle (two for each diet) and for the two Gas Endeavour runs, the GP and MGP detected every hour (time) were analyzed with the Gage R&R procedure with R software (4.3.3.). The various metrics evaluated to obtain the ANOVA table included the degree-of-freedom (DF), sum of squares (SS), mean square (MS), F-value (F) and *p*-value (P). The variance component table divides the total variability (TV) into part-to-part variability (PV) due to differences in the 24 readings (hours), repeatability (EV), and reproducibility (AV) of the combined gage. The value of repeatability and reproducibility (GRR) is calculated according to Equation (1):

$$GRR = \sqrt{EV} + AV^2 \quad (1)$$

The penultimate step was the calculation of measurement system suitability indicators, namely the % GRR (gage repeatability), % AV (reproducibility), and % PV (product variability). The % GRR—the value of repeatability and reproducibility—is calculated according to AIAG [25] as the proportion of combined repeatability and reproducibility divided by total variability and multiplied by 100 and is interpreted as the percentage of total variation that redounds to the combined repeatability (EV) and reproducibility (AV), representing the accuracy of the estimate. We chose to use the automotive practices [25] stating if % GRR < 10%, the measurement system is generally considered optimal; if 10% < % GRR < 30%, then the measurement system is generally considered acceptable; and if % GRR > 30%, then the measurement system is generally considered unacceptable.

As the last step in this method, the *ndc* parameter (number of district categories) was calculated, defined as the number of district categories that can be distinguished by the measurement system. AIAG [25] calculates the *ndc* parameter according to Equation (2):

$$ndc = 1.41 \times \frac{PV}{GRR} \quad (2)$$

If the *ndc* value > 5, then the measurement system is acceptable and provides reliable information about the process changes; if the *ndc* < 5, the measurement system does not provide reliable information about the process changes and thus it is not acceptable [25].

The analysis was performed in R software using the *ss.r()* function from the SixSigma package [26]. Measurements were collected across multiple parts and operators under repeat conditions. The output of the analysis included the percentage contribution of each source of variation relative to the total variability.

2.2.5. Nutrient Degradability

During the in vitro trial, the fermented liquor (FL) of two bottles for each diet was used to calculate the disappearance of organic matter (OMd) and neutral detergent fibre (NDFd). Blank bottles (RL plus buffer solution) were utilized to cancel the fermentation due to the inoculum [27]. To this end, the fermented liquor was vacuum-filtered through

pre-weighed glass crucibles (pore size #1, 100–160 µm) with a sintered filter, washed with hot distilled water, and dried to obtain unfermented residues. OM and NDF were determined according to the official procedures [28, 29], while the in vitro degradability of OM and NDF was determined as reported by Formulas (3) and (4):

$$OMd = \frac{OM(\text{feed}) - OM(\text{FL})}{OM(\text{feed})} \times 100 \quad (3)$$

$$NDFd = \frac{NDF(\text{feed}) - NDF(\text{FL})}{NDF(\text{feed})} \times 100 \quad (4)$$

2.2.6. Kinetics Study of Total Gas and Methane Emissions

For each batch (2 batch × 3 diets × 2 runs), the 24 h GP was fitted with a non-linear model. We used a sigmoid model (Equation (5)), as described by Groot et al. [30], which is a most frequent model used to fit the total GP:

$$GP = \frac{A}{1 + \left(\frac{B}{t}\right)^c} \quad (5)$$

where GP represents the total gas produced (mL/g) at a specific time t (h), A is the asymptotic gas production (mL/g), B (h) denotes the time required to reach half of the asymptote, and C is the switching characteristic of the curve.

Furthermore, since the Groot model is often used for cumulative gas production over more than 48 h [9], beyond which the curve tends to asymptote, we also used the equation of the first-degree line (Equation (6)):

$$GP = A + B \times T \quad (6)$$

where GP represents the total gas produced (mL/g) at a specific time T (h), A is the intercept, and B is the angular coefficient of the line.

For each batch, the parameters A , B and C , with an NLIN procedure of the SAS 9.2 software (2010), were estimated.

2.2.7. DNA Extraction, Amplification and 16S rRNA Sequencing

Fermented liquor was collected at the end of the trial, filtered through two layers of cheesecloth, and collected in sterile 15 mL centrifuge tubes, and then was used for DNA extraction. DNA was isolated according to the column filtration steps of the QIAamp DNA Stool Mini Kit, Hilden, Germany [31], with some modifications. The QIAamp® DNA Stool Mini Kit was purchased from QIAGEN (Canada) and used according to the manufacturer's instructions. Briefly, FL samples were centrifuged at 3000 rpm for two minutes at room temperature (15–25 °C). The clarified supernatant (250 µL) was promptly transferred to a fresh micro centrifuge tube (2 mL) and centrifuged once more to further purify the lysate. Proteinase K (25 µL) was then added to a new tube. After mixing with 600 µL of Buffer AL by means of vortexing, the mixture was incubated at 70 °C for 10 min to ensure efficient lysis and protein digestion. Subsequently, 600 µL of ethanol (96–100%) was added to facilitate DNA binding, and the lysate was loaded onto a QIAamp spin column followed by centrifugation. To wash away contaminants, the column was treated sequentially with Buffer AW1 and Buffer AW2, followed by an optional additional centrifugation step to minimize carryover of wash buffer. Finally, DNA was eluted from the column by applying 200 µL of Buffer AE directly to the membrane, allowing it to incubate for one minute at room temperature before a final centrifugation step.

Extracted DNA from ruminal fluid samples was sent to Novogene Co., Ltd. (Beijing, China) for 16S rRNA gene sequencing. The genomic DNA was randomly sheared into shorter fragments. The obtained fragments were then end-repaired, A-tailed, and further

ligated with Illumina adapters. The resulting fragments with adapters were selected based on size and PCR-amplified unless otherwise specified as PCR-free, before proceeding to purification.

The library was quantified through Qubit and qPCR, and size distribution was detected with a fragment analyzer (Agilent, Santa Clara, United States). Quantified libraries were pooled and sequenced on Illumina platforms (Illumina, San Diego, United States) according to the effective library concentration and required data amount.

The raw data obtained through sequencing contains a certain proportion of low-quality data. Quality control and host filtration were performed on the raw data to obtain clean data, which ensured that the results from subsequent analysis were accurate and reliable.

Species annotation was performed using the Kraken2 software (vs 2.0.8.), where quality-controlled sequences from each metagenomic sample were aligned to the Kraken2 database to determine the species composition of the microbiota. The Bracken (Bayesian Reestimation of Abundance with Kraken) software (vs 2.0.8.) was then used to calculate the relative abundance of each sample.

Based on the species or functional data at the phylum and gene levels, alpha diversity analysis was performed within samples, including the calculation of the alpha index (R vegan package), and significant box plots of alpha indexes were reported (R ggplot package). Beta diversity is a measure of biodiversity used to describe the extent of variation between biological communities in different groups. It quantifies the differences in species composition between different groups. Beta diversity analysis including clustering trees was carried out (Kraken cluster analysis).

Based on the table of abundance at the taxonomic level, ANOSIM (R vegan package, vs 4.3.3.) was used to test the differences between groups.

2.3. Statistical Analyses

Data on curve parameters, cumulative gas production (GP) and methane gas production (MGP) at 24 h, in vitro digestibility parameters, and taxon relative abundance were analyzed using a one-way analysis of variance (ANOVA) model (SAS 9.2 software, 2010). The following statistical model was applied:

$$Y_{ik} = \mu + D_i + \epsilon_{ik} \quad (7)$$

where Y_{ik} is the dependent variable, μ is the general average, D_i denotes the fixed effect of the i diet (i = CTR, PPP, and PPS diets), and ϵ_{ik} is the residual error. The least-squares means were compared using p -values adjusted according to the Tukey–Kramer multiple comparison test.

3. Results

3.1. Gage Repeatability and Reproducibility

The ANOVA tables (Table 2 and 3) indicate the significance of factors in the study. We first checked the significance of the interaction term: if the interaction is not significant, R software removes that term from the model and repeats the calculation again, as degrees of freedom (DF) and the sums of squares of deviations (SS) change. In our study, the interaction term was removed from the GP model (Table 2), while it remained for the MGP model (Table 3).

Table 2. Gas production: ANOVA tabulated results without interactions ($p = 0,05$ to remove interactions).

Source	DF	SS	MS	F	<i>p</i>
Time	71	1,676,726	23,616	460.4	<0.001
Run	1	1896	1896	36.9	<0.001
Repeatability	215	11,029	51		
Total	287	1,689,651			

Table 3. Methane gas production: ANOVA tabulated results.

Source	DF	SS	MS	F	<i>p</i>
Time	71	146,939	2070	50.66	<0.001
Run	1	495	495	12.12	<0.001
Time $\times$ Run	71	2900	41	6.24	<0.001
Repeatability	144	943	6.6		
Total	287	121,577			

Looking at the significance of the main effects, all samples are identical at 95% confidence level, i.e., the measurement system is capable of differentiating between gas production levels within the examined range. In addition to the statistical analysis, the acceptability needs to be evaluated, i.e., the goodness of the measurement system. Evaluation of the GRR percentages indicates that the total gage variation represented by the $\pm 6\sigma$ range is for 10.38% for GP (Table 4) and 22.42% for MGP (Table 5).

Table 4. Gas production: Gage R&R results.

Source	Standard deviation (σ)	Study Variation (SV = 6σ)	% Study Variation (%SV)
Total Gage R&R	8.01	48.04	10.38
Repeatability	7.16	42.97	9.28
Reproducibility	3.58	21.47	4.64
Run	3.58	21.47	4.64
Part-To-Part	76.75	160.52	99.46
Total variation	77.17	463.02	100.00
ndc value	13.51		

Table 5. Methane production: Gage R&R results.

Source	Standard deviation (σ)	Study variation (6σ)	% Study variation
Total Gage R&R	5.18	31.09	22.42
Repeatability	2.56	15.36	11.08
Reproducibility	4.51	27.04	19.50
Run	1.78	10.66	7.69
Time $\times$ Run	4.14	24.85	17.92
Part-To-Part	22.52	135.12	97.45
Total variation	23.11	138.65	100.00
ndc value	6.13		

Furthermore, the main focus of this analysis is not only the percentage of contribution to the total variation in the Gage R&R but also the percentage component of reproducibility, leading to the following question: is the Gas Endeavour instrument, with the same operating conditions, able to return similar information? The results indicate that the percentage component of reproducibility is 4.64% for GP, while it is 19.50% for MGP.

3.2. Gas Production and Their Kinetics

The different trends of GP and MGP were interpolated by means of two different mathematical models, namely a linear model for GP (Figure 1A) and a quadratic one for MGP (Figure 1B). The estimated means of the parameters of these curves as a function of the three diets tested are reported in Table 6.

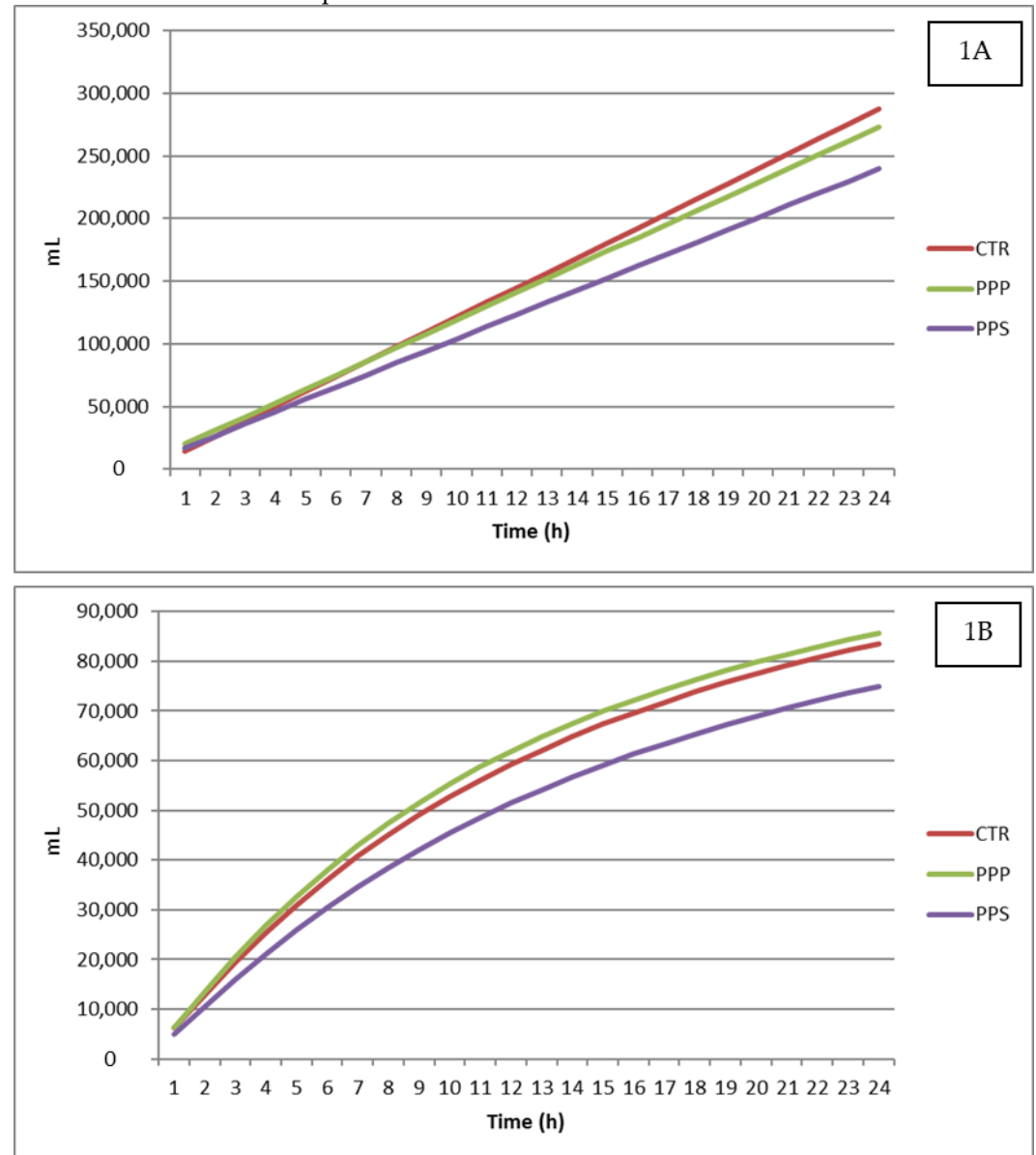


Figure 1. Trend of gas (1A) and methane (1B) production (mL).

The curves of both the GP and MGP for the PPS diet are at lower levels than those for the other diets. The slopes of the three lines that describe the GP are statistically different, with the angular coefficient for the PPS diet being significantly lower ($p < 0.01$) than those for the other two diets, while the angular coefficient for the PPP diet line is statistically lower ($p < 0.05$) than that for the CTR diet.

Table 6. Gas and methane production: function parameters (LSM).

Parameters	Diet			SEM	<i>p</i>
	CTR	PPP	PPS		
Gas production					
A	2.53	8.89	7.12	3.87	0.514
B	11.86 ^{Aa}	11.00 ^{Ab}	9.69 ^B	0.23	0.001
Methane production					
A	127	123	121	3	0.366
B	13.50 ^B	11.88 ^C	15.63 ^A	0.57	0.004
C	1.14	1.18	1.14	0.09	0.939

LSM: Least Squares Mean; Models utilized: gas production = $A+B \times \text{hour}$; methane production = $A/(1 + (B/\text{hour})^C)$. A is the asymptotic gas production (mL/g), B (h) denotes the time required to reach half of the asymptote and C is the switching characteristic of the curve. CTR: control diet; PPP silage: prickly pears peels + 12% wheat bran; PPS silage: prickly pears peels, pulp, seeds + 12% wheat bran. SEM: standard error of mean. In the row, values with different superscript letters are significant: a, b, c = $p < 0.05$; A, B, C = $p < 0.01$.

The ANOVA performed on the parameters of the MGP curves highlights a substantially ($p < 0.01$) greater value of B in the curves relating to the PPS diet. Marked differences ($p < 0.01$) were also highlighted between the other two diets; the value of parameter B for the PPP diet was significantly lower than those for the other two diets. No important differences were found for parameters A and C among the diets.

The varying progression of the fermentation processes of the three diets led to differences in the GP and MGP (Table 7). The PPS diet had a significantly ($p < 0.01$) lower GP than the other diets, while the PPP diet had a lower GP ($p < 0.05$) than the CTR diet. As regards MGP, differences were observed only for the PPS diet, highlighting lower methane production ($p < 0.01$). Overall, the percentage of MGP in relation to the total GP was between 30 and 33%, but no significant differences were found between diets.

Table 7 presents the in vitro fermentation parameters after 24 h of incubation. The pH values observed in the rumen fluid after 24 h of fermentation were similar between the CTR and PPP diets, while the PPS diet had a higher pH value ($p < 0.01$).

Regarding the organic matter digestibility (OMD), statistical differences ($p < 0.01$) were found between all diets; the CTR diet had the highest value, the PPS diet had the lowest value, and the PPP diet had an intermediate value. The neutral detergent fibre degradability (NDFD) after 24 h was similar between the diets, with no significant differences being found.

Table 7. In vitro fermentation parameters (LSM).

Items	Diet			SEM	<i>p</i>
	CTR	PPP	PPS		
pH	6.86 ^B	6.85 ^B	6.90 ^A	0.01	0.006
Organic matter degradability (%)	42.89 ^A	37.33 ^B	36.11 ^C	0.16	0.001
NDF degradability (%)	40.05	42.30	39.62	0.94	0.160
Gas production 24 h (ml/3g feed)	272 ^{Aa}	257 ^{Ab}	227 ^B	4.65	0.001
Methane production 24 h (ml/3g feed)	83.70 ^A	85.70 ^A	75.00 ^B	1.89	0.009
Methane/gas ratio (%)	30.80	33.30	33.00	1.01	0.220
Gas production 24 h (ml/g OMi)	118 ^{Aa}	110 ^{Ab}	88 ^B	2.66	0.001
Methane production 24 h (ml/g OMi)	36.20 ^A	36.60 ^A	29.10 ^B	0.68	0.001

Gas production 24 h (ml/g OMd)	223 ^B	294 ^A	291 ^A	15.07	0.013
Methane production 24 h (ml/g OMd)	73.30 ^B	98.00 ^A	94.10 ^A	4.55	0.010

LSM: Least Squares Mean; CTR: control diet; PPP silage: prickly pears peels + 12% wheat bran; PPS silage: prickly pears peels, pulp, seeds + 12% wheat bran. SEM: standard error of mean. NDF: neutral detergent fibre; OMi: Organic Matter incubated; OMd: Organic Matter disappeared. In the row, values with different superscript letters are significant: a, b, c = $p < 0.05$; A, B, C = $p < 0.01$.

The total GP detected in both in vitro studies was 273, 260, and 228 mL/3 g feed for the CTR, PPP, and PPS diets, respectively, for the first study, while the GP detected in the second study was 272, 254, and 227 mL/3 g feed for the CTR, PPP, and PPS diets, respectively. Regarding the effect of the diet on GP and MGP detected after 24 h of fermentation (mL/3 g feed), the PPS diet showed significantly ($p < 0.01$) lower values than the other diets. The highest value of GP was recorded for the CTR diet ($p < 0.05$), while no significant differences were found for MGP between the CTR and PPP diets. The reduced GP was also linked with a lower MGP, and thus the ratios between MGP and GP were not statistically distinct across the three diets.

Analyzing the GP and MGP obtained for 1 g of incubated OM, we see that the pattern between the diets mirrors what was noted above, considering the cumulative emissions at 24 h. Conversely, accounting for the disappeared of OM, the three diets generated distinct GP and MGP. The CTR diet had lower GP and MGP values per 1 g of disappeared OM ($p < 0.01$) compared to the prickly pear-based diets, while these values did not vary between the PPP and PPS diets.

3.3. Microbiota Analyses of Rumen Fluid

A total of 119.78 Gb of data was obtained from the 12 rumen liquid samples after 24 h of fermentation, with an average of 9.98 Gb per sample. After quality control and removal, 119.35 Gb of clean data, with 9.95 Gb per sample, was retained. The Q20 and Q30 of each sample were above 97.37% and 91.87%.

Shotgun metagenome sequencing analysis showed that the rumen fluid contains approximately 99% bacteria, while archaea, eukaryotes, and viruses represent less than 1% (Figure 2).

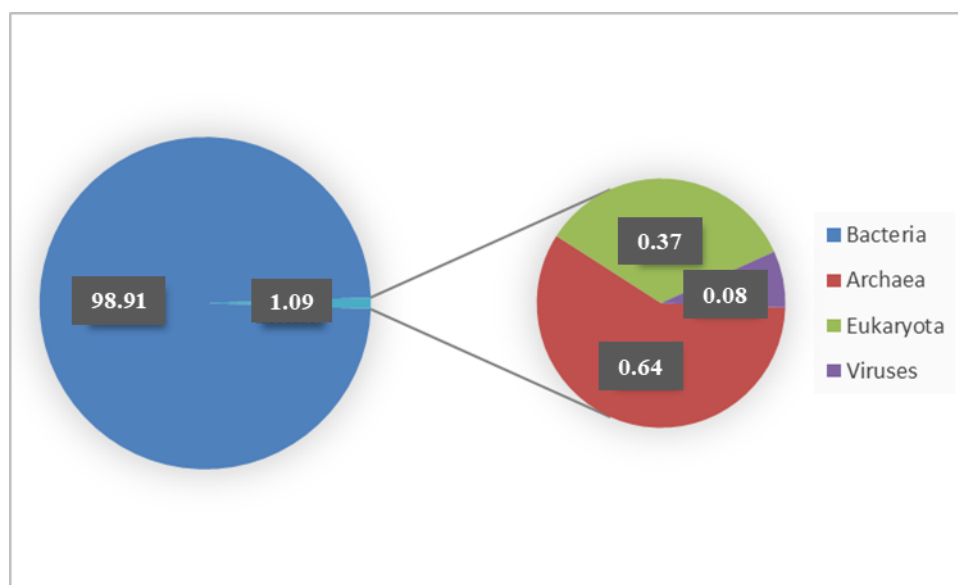
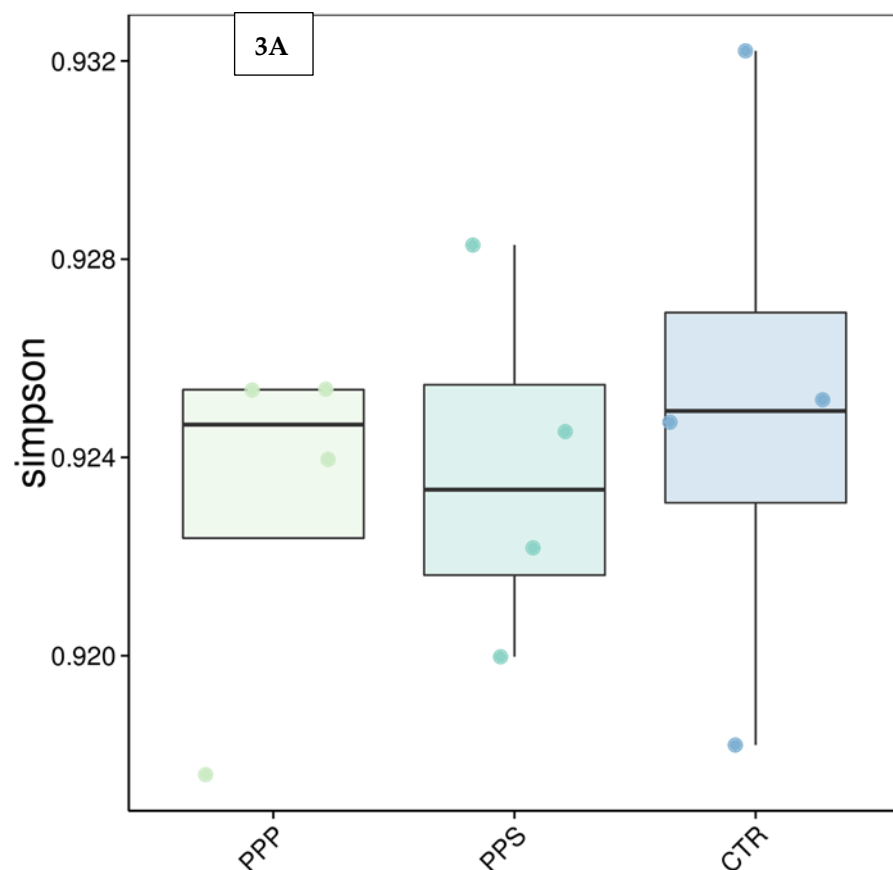


Figure 2. Rumen fluid shotgun metagenome sequencing analysis (relative abundances %).

Alpha diversity is mainly used to study the diversity of communities within a specific habitat (sample), which can be assessed by evaluating a series of Alpha diversity indices to obtain information on the richness. In Figure 3A, the Simpson index at the genus level is reported as a box plot.

This is a graphical representation of the data distribution that displays the median, quartiles, and outliers, providing a visual understanding of the data spread and extreme values. No significant differences were found between diets (sample size $n = 12$; statistical test used: Kruskal–Wallis; p value $p = 0.2457$). Beta diversity is a measure of biodiversity used to describe the extent of variation between biological communities in different groups (diets). It quantifies the differences in species composition between different groups. Beta diversity analysis at the genus level is reported as a clustering tree (Figure 3B). The results showed that microbial communities clustered within each test conducted on April 26th and 28th (*.26 and *.28), while within each trial, the differences between the relative abundances of different rumen fluid samples are smaller.



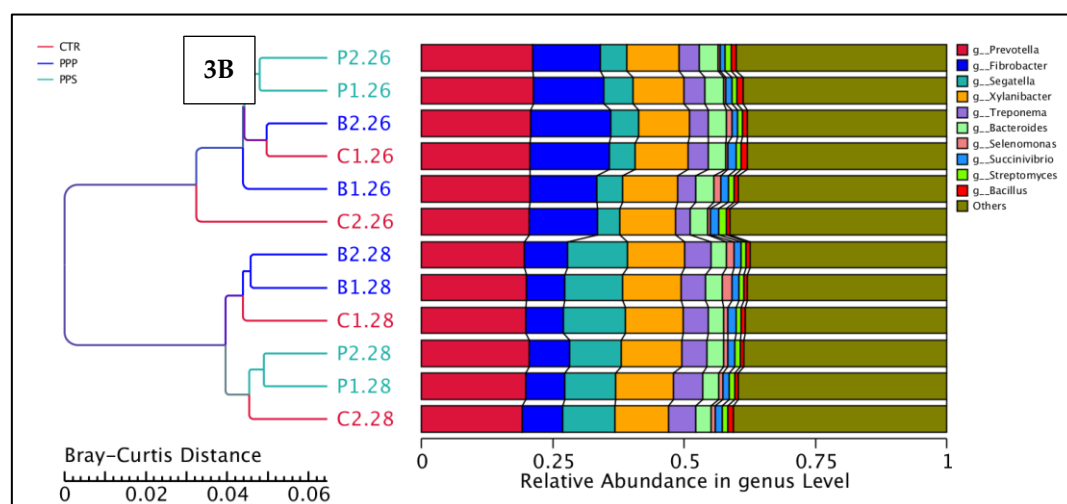


Figure 3. Effect of different diets on the α -diversity (3A) and β -diversity (3B) of sheep rumen microbiota at the genus levels.

The structure of the rumen bacterial communities is shown in Figure 4A, B. The taxonomic annotation of feature sequences was performed using a plain Bayesian classifier, resulting in the identification of 19 bacterial phyla with a relative abundance $> 0.1\%$; the first 10 phyla are reported in Figure 4A. Bacteroidota, Pseudomonadota (ex Proteobacteria), Bacillota (ex Firmicutes), and Fibrobacterota were the dominant phyla in sheep RL [32,33], with relative abundances of 50.85%, 13.08%, 12.02% and 10.65%, respectively, and these four phyla accounted for nearly 87% of all sequences.

Diet showed a significant effect only on the Bacillota phylum ($p < 0.05$). The CTR diet RL showed a higher relative abundance than the PPP diet RL (13.73% vs. 12.57%).

At the genus level, *Prevotella*, *Fibrobacter*, *Xylanibacter*, and the *Segatella* were the dominant genera, with relative abundances of 20.34%, 10.65%, 10.53%, and 7.80%, respectively (Figure 3B). Compared with the diet group, statistical differences ($p < 0.01$) were found only for two minor genera, *Selenomonas* and *Succinivibrio*. The relative abundance of *Succinivibrio* genus was higher in CTR RL than that of PPS (1.50% vs. 1.18%), while no statistical differences were found with PPP RL (1.29%). Moreover, PPP diet RL presented a higher relative abundance of *Selenomonas* genus than CTR (1.42% vs. 0.65%; $p < 0.01$) and PPS diet RL (1.42% vs. 0.56%; $p < 0.01$).

As mentioned above, RL contains 0.64% archaea, to which belong the genera considered to be the main methane producers in the rumen. Among the archaea phyla, Euryarchaeota was the most represented phylum (65% of all archaea phyla). Among the diets, significant differences were found only at the trend level ($p < 0.10$); the CTR diet RL showed a higher relative abundance than the PPP diet and the PPS diet RLs. At the genus level, *Methanomethylophilus*, *Methanobrevibacter*, *Candidatus Methanoplasma*, and *Methanosarcina* were the dominant archaea genera, with relative abundances of 0.138%, 0.036%, 0.030%, and 0.023%, respectively. No significant differences between archaea genera were found between different diets' RLs.

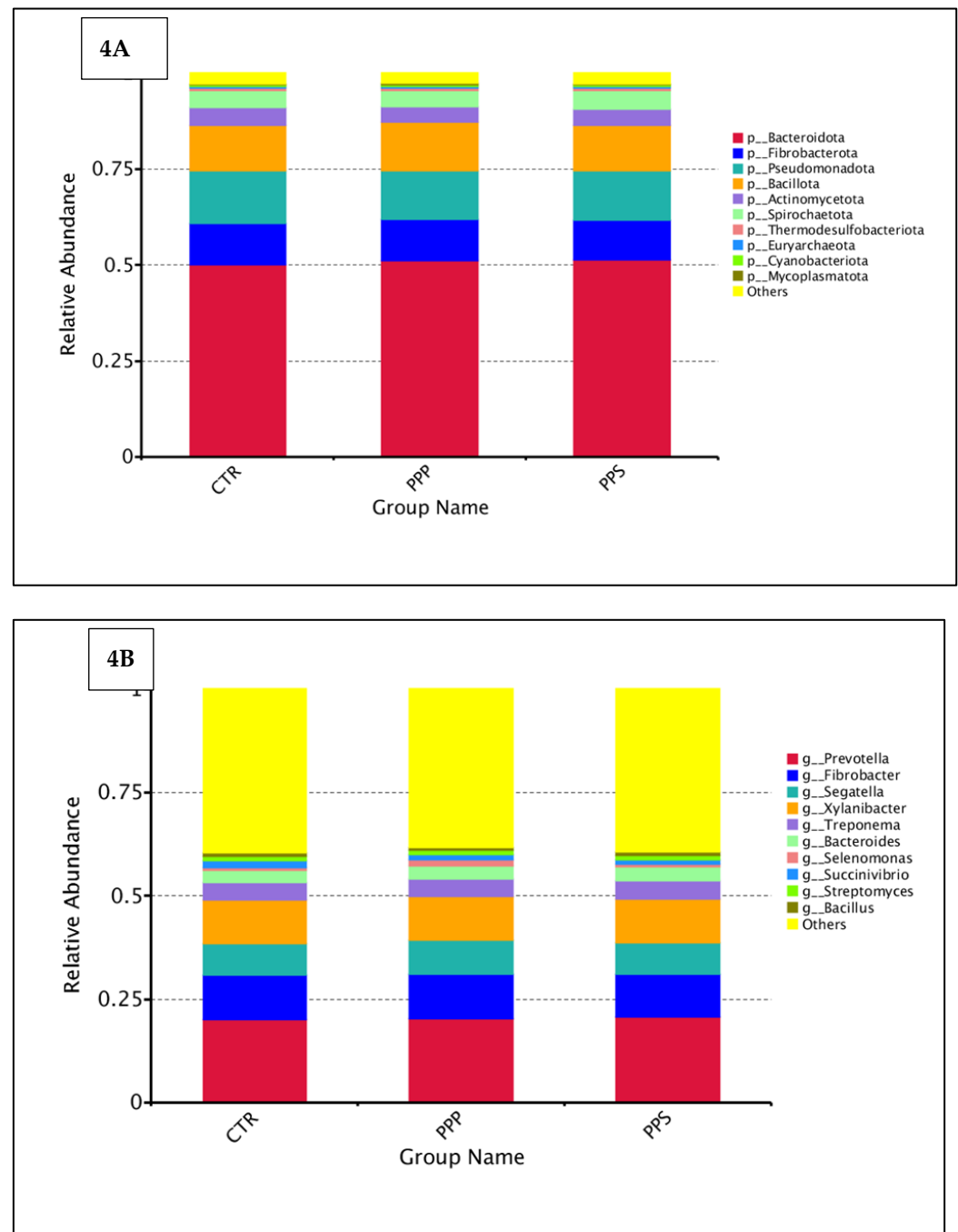


Figure 4. Distribution of bacterial taxa averaged under the phylum (4A) and genera (4B) levels of rumen fluids at the end of diets' fermentation (as a percentage of the total sequence).

However, considering the entire microbiota, no significant difference emerged between the rumen fluid of the three diets, either at the phylum or genus level (Table 8).

Table 8. Based on the abundance table of taxonomic level, the Anosim analysis was displayed at the phylum and genus levels.

Group	Phylum		Genera	
	R-value	P-value	R-value	P-value
CTR-PPP	−0.08333	0.541	−0.10417	0.587
CTR-PPS	−0.14583	0.708	−0.07292	0.503
PPP-PPS	−0.10417	0.672	0.04167	0.437

4. Discussion

In order to evaluate the repeatability and reproducibility of the GP and MGP, the Gage R&R procedure was used. The results showed that the experimental conditions adopted for the measurement of gases after 24 h of incubation fall within the acceptability range, presenting values of variation related to the Gage R&R below 30% and a ndc value > 5 [25]. With reference to the reproducibility data obtained, that related to GP was better than that for MGP. This could be explained by the fact that methanogenic bacteria are more susceptible to stress, and any stress experienced by the inoculum leads to a disproportionately greater reduction in methane emissions compared to the overall reduction in GP [34]. Therefore, in order to improve the determination of methane emission kinetics during fermentation tests with Gas Endeavour instrumentation, the number of fermenters per test or the number of runs should be increased.

A benefit of the GES is that fermentation kinetics, as well as those of gas and methane, can be analyzed for the same sample. This allows the calculation of alterations in methane production rates over time. To describe the trend of GP, a sigmoid model is frequently used [30], which fits the *in vitro* GP obtained after 48 h and until 120 h well [9]. Parameter A of the Groot model represents asymptotic gas production, which is reached after 48 h of incubation of the feed. Under our experimental conditions, the trials ended after 24 h, and the GP had not yet reached the asymptote. Therefore, the Groot model did not show good forecast ability for the GP, unlike the regression line. A different situation was observed for MGP, whose curves, after 24 h of fermentation, reached approximately 75% of the maximum gas production [18], and then the asymptote. Under these conditions, the Groot model provided good estimation capabilities in fitting the MGP over 24 h.

The slope of the GP line was statistically different between diets, with the PPS line increasing at a slower rate than that for other diets, indicating that it will consequently produce less gas than the other diets. This fact is probably due to the higher presence of an indigestible fibre fraction due to the seeds in the patazzo by-product [3].

Significant differences were observed among diets concerning parameter B in the Groot model. A lower B value in the PPP diet suggests quicker methane production. Using the same feed, Gannuscio et al. [5] and Hassan et al. [7] also reported a similar effect on GP kinetics, attributed to the higher water-soluble carbohydrate (WSC) content of this by-product. These observations appear connected to the forage fibre integrated with non-fibre carbohydrate (NFC) substrates in the PPP diet. This leads to more fermentable substances within the *in vitro* rumen, and in turn, increases MGP [35,36]. On the contrary, the MGP kinetics of the PPS diet showed a slower fermentability, which had a significantly lower MGP after 24 h than the other diets. Moreover, the presence of PPS in the diet led to a lower GP, in agreement with the results presented by Hassan et al. [7] using a different gas production method that used a pressure transducer to determine the fermentation kinetics of feeds [37]. The lower GP and MGP observed for the PPS diet is probably due to the abundant presence of seeds in the prickly pear pastazzo that reduce the digestibility; similar results were found by Albores-Moreno et al. [38], who reported that the lower methane production is due to the lower *in vitro* digestibility. This could also be explained by the presence of condensed tannins in PPS [12]. It is well known that tannins affect the degradability of proteins and carbohydrates, particularly hemicellulose, cellulose, starch, and pectins [39]. Tannins have long been known to have a secondary anti-nutritional effect on fibre degradation [40,41]. This hypothesis is confirmed by the significant lower levels of disappeared organic matter (OMD) in the PPS diet, which coincides with the higher pH of the fermented RL, probably due to the lower concentration of volatile fatty acids. In fact, the pH value at the beginning of the *in vitro* experiment was 7.70, while after 24 h of fermentation, the pH of the PPS dropped to 6.90, while that of the other diets dropped to 6.85.

When we considered the GP expressed for 1 g of incubated OM (OMi), the values detected with the Gas Endeavour system were between 88 and 118 mL, being lower than those reported by Hassan et al. [7], which ranged from 196 to 229 mL/g OMi, with the latter being detected after 120 h of fermentation. In both trials, the PPS diet was associated with a significantly lower GP than the others, while the PPP diet had a lower GP after 24 h than the CTR diet, while after 120 h, the PPP diet had a higher GP. Despite the faster fermentability of prickly pear peels reported by Vastolo et al. [4], the lower GP after 24 h recorded in the PPP diet compared to the CTR diet could be due to the forage/concentrated ratio, as the amount of concentrate in the CTR diet is double than PPP and PPS diets.

The importance of measuring substrate degradability over the incubation period has been highlighted by Navarro-Villa et al. [42], who report that GP and MGP are better expressed per unit of substrate degraded rather than per unit of substrate incubated. If we consider the GP expressed as 1 g of disappeared OM, the diets are ordered as follows: PPP > PPS > CTR. In this case, diets containing prickly pear by-products appear to have a higher GP than the control diet. This reversal of the results is certainly linked to the higher OMD found in the control diet.

A similar trend was observed for MGP: the PPS diet had a significant lower MGP than other two diets, while if we express the MGP as 1 g of OMD, prickly pear diets had a significant higher MGP than the CTR diet. In our opinion, both expressions of the results are correct depending on which question we are trying to answer. If we want to determine the methane emissions in 24 h, we must consider the MGP for 1 g of OMi, while if instead we want to compare the MGP emissions from different substrates, then we must consider the MGP for 1 g of OMD. In any case, when we considered the GP/MGP ratio, no significant differences were found between diets, with these results ranging between 30.8% and 33.3%.

Metataxonomic analysis of RL inoculated with the three diets showed the same phyla with different relative abundances, but we only observed significant differences for Bacillota. The higher relative abundance found in the CTR diet RL with respect to the PPP diet RL is probably due to the higher percentage of concentrate in this diet. These findings align with those of Wang et al. [43], who demonstrated that concentrate-dominant feed increases the abundance of Bacillota while reducing the diversity of rumen bacteria and the abundance of Bacteroidota.

A healthy rumen microbiota is characterized by the dominance of obligate anaerobic members of the Bacteroidetes and Bacillota that express relatively large numbers of genes encoding carbohydrate-active enzymes and therefore promote the breakdown of structural polysaccharides in the rumen, while also fermenting amino acids into acetate. Bacillota represent the core bacterial component that is predominant within the rumen, mainly comprising diverse fibrolytic and cellulolytic bacterial genera [33].

In all RL samples, the genera *Prevotella*, *Fibrobacter*, *Segatella*, and *Xylanibacter* were present with higher relative abundance. These genera are members of the Bacteroidetes and Fibrobacteres genera. Significant differences between diets were found only for the *Succinivibrio* and *Selenomonas* genera. The *Succinivibrio* genus comprises amylolytic bacteria [44,45], and therefore its relative abundance increases through a concentrate-rich diet, potentially contributing to the fermentation of a variety of unstructured carbohydrates [46,47]. This fact explains the significantly higher abundance in the CTR-diet RL than in that of the prickly pear diets (PPP and PPS), with the concentrate/forage ratio being clearly more shifted towards the concentrate. Significant differences were found only among CTR and PPS, while no significant differences were observed between the CTR and PPP diets, despite both prickly pear silage diets having the same amount of concentrate. The lack of difference in the relative abundance of the genus *Succinivibrio* between these last two diets is probably explained by the equal presence of non-fibrous carbohydrates due to the

greater amount of concentrate in the CTR diet and the greater amount of sugars in the PPP diet.

Moreover, in the PPP diet RL, we found a significant higher *Selenomonas* genus relative abundance than in other two diets' RLs. *Selenomonas* genus is known as a propionate producer [48] and it is the main lactate-decomposing bacteria in ruminants; in fact, it can produce propionate from lactate and maintains a stable pH value [49]. The highest and most significant relative abundance of this genus in the PPP diet RL is probably due to the higher availability of lactic acid due to the prickly pear peel silage. In fact, the lactate level detected in the PPP silage was 15 times higher than that in the PPS silage [6].

Methane emissions from ruminants are intricately tied to the rumen microbiome, particularly as methanogenic archaea, or methanogens, use H_2 and CO_2 as substrates to synthesize methane, with certain species also capable of metabolizing small organic compounds such as formate, methanol, methylamines, or acetate [50].

Archaea are widely present in the rumen and can utilize H_2 to maintain the fermentation environment of rumen microorganisms and the production of CH_4 . In our trial, the Euryarchaeota phylum, which is recognized as a classic methanogen in the rumen, was the most dominant, aligning with the findings reported in recent studies [51,52]. The higher relative abundance of the Euryarchaeota phylum in the CTR-diet RL could explain the higher methane production after 24 h of fermentation (expressed as ml/g OMi), although this does not explain why a similar MGP was also measured in the PPP diet. Even at the genus level, no significant differences were found among the archaea responsible for methane production in the rumen liquor incubated with the three different diets. Thus, the lower amount of MGP found for the PPS diet does not appear to be associated with any archaea genus in particular.

Overall, this trend indicates that the abundance of members of the Euryarchaeota phylum was reduced with the inclusion of prickly pear by-products. It therefore seems logical that the abundance of archaea would show the strongest positive correlation with methane emissions. However, some studies have refuted this assumption, indicating that CH_4 production is not based solely on methanogen abundance but also on the interplay between the community dynamics and abundance. For instance, it has been shown that there is no noteworthy relationship between the abundance of methanogens and CH_4 emissions in dairy cows [53]. One implication could be that the metabolic potential of individual methanogens has more credibility in explaining CH_4 output than abundance. A possible limitation of this study is that the ruminal microbiome and its variations were studied based on fluid obtained after the in vitro fermentation test and not on the ruminal fluid collected from sheep subjected to different feeding regimes.

5. Conclusions

The newly developed Gas Endeavour system offers additional possibilities to study gas kinetics in real time with a more accurate measurement of the low flow of highly water-soluble gases. The parameters of repeatability and reproducibility obtained in these fermentation studies are useful, and the reproducibility data for GP was better than that for MGP.

The findings derived from in vitro fermentation analyses of prickly pear by-products, PPP and PPS, thus demonstrate a considerable fermentability of the peel silage, which generates higher GP per 1 g of OMi. When PPS silages were incorporated into diets for lactating ewes, lower GP and MGP were observed, probably due to the high seed content, which makes them less digestible. Sequencing of the rumen microbiota, particularly of archaea, does not show a direct correlation between the abundance of methanogens and MGP, so the lower amount of methane produced by the fermentation of the PPS diet does not appear to be associated with the relative abundance of Euryarchaeota.

The different chemical and nutritional composition of the three diets modified the rumen bacteria, albeit only slightly, with particular reference to the genera *Succinivibrio* and *Selenomonas*, confirming that different dietary nutritional levels had significant effects on the ruminal microbial communities and metabolic functions. However, these results should be confirmed by the microbiological evaluation of the rumen fluid collected from sheep subjected to the three different diets.

Finally, the PPP silage showed better fermentation characteristics, which could justify its use in the diet of dairy sheep. Further investigation would be desirable to evaluate the most suitable amount of PPP silage to incorporate into the diet, without showing adverse effects that reduce the voluntary dry matter intake of sheep.

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Informed Consent Statement: Not applicable

Data Availability Statement: Data is unavailable due to privacy or ethical restrictions

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Abbreviations

The following abbreviations are used in this manuscript:

PPP	Prickly pear peels
PPS	Prickly pear peels + pulp + seeds
RL	Rumen liquor
FL	Fermented rumen liquor
OMd	Disappearance of organic matter
NDFd	Disappearance of neutral detergent fibre
GES	Gas Endeavour System
GRR	Combined gage the repeatability and reproducibility
GP	Gas production
MGP	Methane gas production

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