Small Ruminant Research 262 (2026) 107816



Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres





Greenhouse gases, nutrition, rumen fermentation, and microbiome of lambs fed sugarcane silage versus sugarcane/*Moringa oleifera*

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ARTICLE INFO

Keywords:
Lamb
Greenhouse gases
Rumen microbiota
Sugarcane
Moringa
Feeding

ABSTRACT

Silage is widely used in tropical ruminant systems, although the low crude protein content of some conserved forages may limit nutrient supply and ruminal function. This study evaluated the effects of partially replacing sugarcane silage with *Moringa oleifera* silage on feed intake, physiological responses, rumen microbial abundance, and greenhouse gas emissions in lambs. Sixteen Santa Inês × Dorper lambs were assigned to two dietary treatments: sugarcane silage (C) or sugarcane silage partially replaced with *M. oleifera* silage (C+M; 50:50 on a dry matter basis). Feed intake dynamics, serum biochemical parameters, infrared thermography, rumen microbial abundance assessed by qPCR, and gaseous emissions (CH_4, CO_2, N_2O, and NH_3) measured in respiration chambers were evaluated. The C + M diet increased dietary crude protein without markedly changing fiber fractions and was associated with a more stable intake pattern over time. Physiological indicators remained within normal reference ranges, suggesting no evidence of systemic metabolic stress. Methane and nitrous oxide emissions were not affected by dietary treatment ($P > 0.05$), whereas carbon dioxide and ammonia emissions were higher in lambs fed the C + M diet (CO_2: 173.82 vs. 88.36 g/kg DMI, $P = 0.039$; NH_3: 1.35 vs. 0.31 g/kg DMI, $P = 0.022$). Fungal abundance was lower under the C + M diet ($P = 0.016$), while total bacterial abundance remained unchanged and protozoal populations declined over time in both treatments. Overall, partial replacement of sugarcane silage with *M. oleifera* improved the dietary protein profile and was associated with changes in intake dynamics, selected rumen microbial groups, and emission profiles, without reducing methane emissions under the conditions of the present study. These findings suggest that *M. oleifera* may be a promising forage resource for tropical sheep production systems, although further studies are needed to clarify the mechanisms involved and optimize inclusion levels.

1. Introduction

Sheep production plays a strategic role in global livestock systems due to the adaptability of small ruminants and their capacity to efficiently convert fibrous feed resources into high-quality animal protein (Rakib et al., 2022). In tropical production systems, however, improvements in productivity are increasingly constrained by the need to enhance nutrient utilization efficiency while reducing environmental impacts associated with rumen fermentation. Enteric methane (CH_4) represents the primary greenhouse gas (GHG) emitted by ruminants and accounts for energy losses ranging from 3% to 12% of gross energy intake, directly reducing feed efficiency (Huhtanen et al., 2015).

Methane formation is tightly coupled to rumen fermentation dynamics, hydrogen disposal pathways, and the structure of the microbial

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https://doi.org/10.1016/j.smallrumres.2026.107816
Received 10 March 2026; Received in revised form 27 May 2026; Accepted 27 May 2026
Available online 1 June 2026
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ecosystem. In addition to CH_4, nitrogen losses in the form of ammonia (NH_3) and nitrous oxide (N_2O) represent inefficiencies in ruminal protein metabolism and nitrogen utilization, further contributing to the environmental footprint of sheep production systems (Khanaki et al., 2024; Barbosa et al., 2024). In this context, nitrogen–energy synchrony refers to the temporal balance between rumen-available nitrogen and fermentable energy required to support microbial growth and nutrient utilization.

In Brazil and other tropical regions, sugarcane silage is widely adopted during dry seasons due to its high biomass yield and agronomic resilience. However, sugarcane silage is characterized by low crude protein concentration and a carbohydrate profile that may limit efficient synchronization between rumen-available nitrogen and fermentable substrates after ensiling. (Suong et al., 2022; Oliveira et al., 2023). These nutritional constraints underscore the need for complementary forage sources capable of improving dietary protein supply without negatively affecting intake or growth performance (Cordeiro et al., 2023).

Moringa oleifera has emerged as a promising tropical forage due to its elevated protein concentration (Dong et al., 2019), favorable digestibility, and presence of secondary metabolites such as tannins, saponins, and flavonoids. These compounds have been reported to selectively modulate rumen microbial populations, influencing protozoal abundance, hydrogen flux, and methanogenic pathways (Benhammouche et al., 2021). Nevertheless, the effects of Moringa on methane emissions remain inconsistent and appear to depend on inclusion level, basal diet composition, and overall nutrient balance (Scanes and Pierzchała-Koziec, 2024), suggesting that its primary impact may involve functional modulation of rumen fermentation rather than direct suppression of methanogenesis.

Despite increasing interest in plant-based dietary strategies, integrative studies simultaneously assessing feed intake, growth performance, rumen microbial structure, and multiple greenhouse gases (CH_4, CO_2, N_2O, and NH_3) in lambs fed sugarcane-based diets are scarce, particularly under tropical conditions. Such integrated approaches are essential, as gaseous emissions reflect not only microbial abundance but also nutrient partitioning efficiency and the animal's capacity to convert consumed feed into productive output.

Therefore, this study evaluated the effects of partially replacing sugarcane silage with *Moringa oleifera* silage on feed intake, growth performance, rumen microbial populations, and greenhouse gas emissions in lambs. The hypothesis of this work is that partial replacement of sugarcane silage with *Moringa oleifera* silage would increase dietary nitrogen supply and alter rumen microbial and emission patterns, without necessarily producing a direct reduction in methane emissions.

2. Materials and methods

2.1. Experimental design and animals

The experiment was conducted at the Animal Science Institute (APTA/SAA), Nova Odessa, São Paulo, Brazil. All animal procedures were approved by the institutional Animal Care and Use Committee (CEUA/IZ 346–2022; CEUA/CENA-USP 011/2016) and complied with national guidelines for the ethical use of animals in research. Sixteen Santa Inês × Dorper male lambs (6 months of age; 23 ± 2 kg initial body weight) were individually identified and randomly assigned to one of two dietary treatments (n = 8 per treatment) in a completely randomized design. The individual animal was considered the experimental unit. Animals were housed under confinement conditions throughout the 75-day experimental period (15 for adaptation and divided in 60 for feeding and data evaluation). The dietary treatments consisted of: (1) Control (C): sugarcane silage; (2) C + M: sugarcane silage partially replaced (50:50 on a dry matter basis) with *Moringa oleifera* silage.

Chemical composition of experimental silages and their respective ingredients is presented in Table 1. Dry matter (DM), crude protein (CP), ether extract (EE), ash, and crude fiber were determined according to (AOAC, 2016) procedures. Procedures of NDF and ADF followed (Van Soest et al., 1991).

Table 1
Chemical composition of experimental silages (C and C+M) and their raw ingredients.

Component	C	C + M	Concentrate	Sugarcane (fresh)	*M. oleifera* (fresh)
Dry matter (g/kg DMI)	252.9	205.2	900.1	266	158.4
Crude protein	26.1	75.5	160	26.0	223.8
Ether extract	14.2	19.4	25	20.0	135.0
Crude fiber	142.2	172.1	100.0	243.9	28.0
Ash	70.4	77.6	100	67.0	94.6
NFE	747.1	655.5	-	665.0	508.8
TDN	-	643.4	-	683.0	622.0
NDF	540.8	544.9	-	440.0	551.0
ADF	335.5	378.8		237	448.0
Calcium	-	-	1.30	-	-
Phosphorus	-	-	0.40	-	-
Magnesium	-	-	1.65	-	-

C = sugarcane silage (control); C + M = sugarcane silage partially replaced with *Moringa oleifera* (50:50, dry matter basis). Fresh sugarcane and fresh Moringa correspond to the raw materials used for silage preparation. NDF = neutral detergent fiber; ADF = acid detergent fiber; NFE = nitrogen-free extract and TDN = Total Digestible Nutrients.

Moringa oleifera plants were cultivated, harvested at vegetative stage (Patricio et al., 2015), manually chopped, and immediately ensiled in 25 kg plastic bags at the experimental farm of the institute. The whole Moringa plants were used for silage preparation, sugarcane forage was obtained from a local supplier, located in Nova Odessa, São Paulo, Brazil, chopped, and ensiled following the same procedure. All diets were formulated with a commercial concentrate, resulting in a forage-to-concentrate ratio of 60:40 (dry matter basis). Feed was offered at least twice daily, allowing for refusals, which were collected and weighed prior to the subsequent feeding to determine intake. Animals had free access to clean drinking water throughout the experiment. Feed intake was monitored using an electronic feeding system (Intergado®, Brazil).

2.2. Infrared thermography and blood biochemistry

Infrared thermographic images were obtained using a FLIR Ignite 5 PRO E54 camera (FLIR Systems, Estonia), with a reported accuracy of ±2 °C and thermal sensitivity < 0.05 °C. Images were captured at a fixed distance of approximately 1 m, at the same time of day, under standardized environmental conditions to minimize the influence of solar radiation, wind, and ambient temperature fluctuations.

Five anatomical regions were selected as physiological indicators of thermal status: orbital area, muzzle, thoracic flank (rumen region), hind limb, and rump. Regions of interest (ROI) were manually delineated using FLIR Tools® software (FLIR Systems Inc.), and minimum, maximum, and mean surface temperatures were extracted. For statistical analysis, only mean surface temperature values were considered, as recommended for livestock thermal assessment (Ghezzi et al., 2024).

Blood samples were collected via jugular venipuncture into vacuum tubes without anticoagulant, during the morning, between 8 and 9 AM, before feeding time. Samples were centrifuged at 10,000 × g in 10 min, and serum was stored at −20 °C until biochemical analysis. Serum metabolites were quantified using commercial colorimetric kits (LabTest Diagnóstica®, São Paulo, Brazil) in an automated analyzer (LabTest 100®), following the manufacturer's instructions.

The following parameters were determined: albumin (bromocresol green method), total protein (biuret method), glucose (hexokinase method), total cholesterol (enzymatic Trinder reaction), creatinine (Jaffé method), urea (UV enzymatic method), alkaline phosphatase, and γ-glutamyltransferase (GGT). Instrument calibration and internal

quality control procedures were conducted according to established clinical chemistry standards for ruminants (Kaneko et al., 1997; Souza et al., 2020).

2.3. In vivo gas emissions

Greenhouse gas emissions (CH_4, CO_2, N_2O, and NH_3) were quantified using semi-open circuit respiration chambers at the Animal Facility of the Center for Nuclear Energy in Agriculture (CENA/USP), Piracicaba, São Paulo, Brazil. Four chambers were available, and measurements were conducted in two consecutive experimental batches, each including eight lambs. Within each batch, animals were randomly allocated to chambers (four lambs per treatment). The individual animal remained the experimental unit.

The respiration chambers (57 × 71 × 167 cm) were developed at the Laboratory of Animal Nutrition (LANA/CENA/USP) and operated according to the methodological principles described by Abdalla et al. (2012). Animals remained inside the respiration chambers for four consecutive days following a standardized protocol. The first two days were used for acclimation and were not included in emissions calculations. The subsequent two days were used for continuous gas measurement. Daily emissions were calculated for each measurement day, and the mean of the two measurement days was used as the individual emission value for statistical analysis. During chamber confinement, lambs received the same experimental diets as in the feeding phase and had *ad libitum* access to water.

Airflow rates were continuously monitored and maintained under controlled conditions. Outgoing air was continuously extracted using a vacuum pump connected to a microcapillary sampling system (Fixed Speed Peristaltic Pump, 115 V/60 Hz). Gas concentrations were quantified in real time using cavity ring-down spectroscopy (CRDS; Picarro G2508®, Picarro Inc., Santa Clara, CA, USA), with a temporal resolution of 1 Hz.

Emission rates (g/d or mg/d) were calculated from the difference between gas concentrations measured in chamber air and ambient air, combined with airflow corrected to standard temperature and pressure (STP). Air flow at STP was calculated as:

$$\textit{Air flow at STP}\ (L) = ((H \times TCA\ )/(T + 273.15)\ )/(101.325/273.15)$$

where H is the measured air humidity (%), TCA is the total chamber airflow (L/d), T is the chamber temperature (°C), *273.15* is the factor used to convert temperature from degrees Celsius to Kelvin, and 101.325 is the standard atmospheric pressure at 1 atm. Gas volume at STP was calculated as:

$$\textit{Gas at STP}\ (L) = \frac{(GCC - GAC) \times \textit{Air flow at STP} \times (22.4/M)}{1000000}$$

where GCC is the concentration of the evaluated gas measured in chamber air, GAC is the concentration of the same gas measured in ambient air, *22.4* is the molar volume occupied by one mole of gas at STP (L/mol), M is molecular weight of the gas (g/mol), and *1000000* is the factor used to convert ppm to volumetric fraction. Daily gas production was calculated as: $Gas(L/d) = \textit{Gas at STP} \times 86,400/\textit{Sampling time}$ where *86,400* is the number of seconds in 24 h and *sampling time* is the duration of gas sampling (s). Final gas mass emission was calculated as: $Gas(\mathrm{g}/d \text{ or } \mathrm{mg}/\mathrm{d}) = Gas(L/d) \times \textit{Gas density}$ where *Gas density* corresponds to the density of each evaluated gas. The following values were adopted: CH_4, 0.7159 kg/m^3 and 16.0430 g/mol; CO_2, 1.9638 kg/m^3 and 44.0100 g/mol; N_2O, 1.9639 kg/m^3 and 44.0130 g/mol; and NH_3, 0.7599 kg/m^3 and 17.0310 g/mol, for gas density and molecular weight, respectively. Final values were expressed as absolute emissions (g/d or mg/d) and, when appropriate, standardized relative to dry matter intake (g/kg DMI or mg/kg DMI).

2.4. Rumen microbiota

Rumen fluid samples were collected at two time points: at the beginning of the experimental period (initial phase, IN) and during the gas-measurement period (final phase, FIN). Samples were obtained using an oral esophageal probe following standardized rumen sampling procedures. The first aliquot was discarded to minimize saliva contamination. Immediately after collection, samples were transferred to sterile microtubes, kept on ice, and stored at −80 °C until laboratory analysis (Zeoula et al., 2003).

Genomic DNA was extracted from rumen fluid using the QIAamp® DNA Stool Mini Kit (Qiagen, Germany). DNA concentration and purity were assessed by spectrophotometry (NanoDrop ND−1000, Thermo Fisher Scientific, USA), and samples were standardized to a uniform concentration prior to amplification.

Quantification of major rumen microbial groups was performed by quantitative PCR (qPCR), targeting total bacteria (16S rRNA gene), rumen fungi (ITS region), and protozoa (18S rRNA gene). Previously validated primer sets were used (Sylvester et al., 2004; Silva et al., 2020). Amplification reactions were conducted in triplicate using SYBR Green chemistry in a StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA), including no-template controls in all runs to detect potential contamination.

Standard curves were generated from serial dilutions of cloned target amplicons to determine amplification efficiency and linearity (R^2). Amplification efficiency ranged within acceptable limits (90–110%). Microbial abundance was expressed as $\log_{10}$ gene copy number per milliliter of rumen fluid.

2.5. Statistical analysis

The experiment followed a completely randomized design with two dietary treatments: sugarcane silage (C) and sugarcane silage partially replaced by *Moringa oleifera* silage (50:50 on a dry matter basis; C+M), using sixteen lambs (n = 8 per treatment). Statistical analyses were performed using SAS Studio (SAS Institute Inc., Cary, NC, USA). Residual normality and homogeneity of variances were assessed using the Shapiro–Wilk and Levene tests, respectively. Variables with a single observation per animal that met model assumptions were analyzed by analysis of variance (ANOVA) using the PROC GLM procedure according to the model: $Yij = \mu + Ti + eij$

Where Yij represents the observation for animal j receiving treatment i, μ is the overall mean, Ti is the fixed effect of dietary treatment, and eij is the residual error. Means were compared using Tukey–Kramer adjustments, and statistical significance was declared at $P < 0.05$. When the assumptions of normality and/or homogeneity were not met, non-parametric procedures were applied as appropriate. In the case of thermographic variables, treatment effects were evaluated using the Kruskal–Walli's test. Feed intake, recorded over time, was analyzed using linear mixed models (PROC MIXED), including treatment as a fixed effect and animal as a random effect. Degrees of freedom were estimated using the Satterthwaite approximation.

Greenhouse gas emissions were analyzed as one observation per experimental unit, corresponding to the mean daily emission calculated from the two-days chamber measurement period for each animal. Associations between gaseous emissions (CH_4, CO_2, N_2O, and NH_3) and productive variables [dry matter intake (DMI) and average daily gain (ADG)] were explored using Pearson correlation analysis. When normality assumptions were not satisfied, Spearman correlation coefficients were used. Linear trends were illustrated graphically, to visualize relationships between variables within treatments.

The temporal dynamics of feed intake were visualized using scatter plots with fitted trends generated in Python (Matplotlib library) during the experimental period. Statistical significance was declared at $P < 0.05$, whereas values between 0.05 and 0.10 were interpreted as tendencies.

3. Results

3.1. Ingestive pattern

Dry matter intake (DMI) exhibited a significant curvilinear response over the evaluation period ($P < 0.001$), reflecting the typical adaptation and subsequent decline pattern observed under confinement conditions (Fig. 1). Overall mean DMI did not differ between treatments ($P = 0.891$). However, a significant treatment × day interaction was detected ($P = 0.001$), indicating distinct temporal intake dynamics between diets.

Although both groups displayed a progressive reduction in intake toward the end of the experimental period, lambs fed the C + M diet showed a less pronounced decline compared with the control group. Thus, while total DMI was not affected by treatment, the C + M diet was associated with a more stable intake pattern over time.

3.2. Thermography

Infrared thermography revealed limited differences between dietary treatments (Table 2). Among the evaluated anatomical regions, only orbital temperature was affected by diet ($P = 0.049$), with lambs fed the C + M diet presenting lower mean values compared to the control group. No significant differences ($P > 0.05$) were observed for snout, rumen, hind limb, or scrotal surface temperatures between treatments.

3.3. Gas emissions

Greenhouse gas emissions expressed per unit of feed intake differed partially between dietary treatments (Table 3). Methane (CH_4) and nitrous oxide (N_2O) emissions did not differ between treatments ($P > 0.05$). Although CH_4 emissions were numerically greater in lambs fed C + M, this difference was not statistically significant ($P = 0.104$). In contrast, CO_2 emissions were higher in lambs receiving C + M than in the control group (173.82 vs. 88.36 g/kg DMI; $P = 0.039$). Similarly, ammonia (NH_3) emissions were greater in C + M (1.35 vs. 0.31 g/kg DMI; $P = 0.022$).

When analyses were separated by treatment, distinct association patterns were observed between intake, growth performance, and gaseous emissions (Supplementary Figure 2). In the control diet (C), CO_2 emissions were positively correlated with dry matter intake ($r = 0.59$; $P = 0.020$). In lambs fed C + M, this relationship was not observed ($r = 0.07$; $P = 0.79$). For CH_4 emissions, weak and non-significant correlations were observed in both treatments. The correlations were positive in the C group ($r = 0.33$; $P = 0.23$) and negative in the C + M group ($r = -0.16$; $P = 0.56$).

For nitrogen related gaseous emissions, distinct intake emission relationships were also observed (Supplementary Figure 2). In the control diet, N_2O emissions were positively correlated with dry matter intake ($r = 0.70$; $P = 0.003$). A similar but weaker relationship was observed in lambs fed the C + M diet ($r = 0.54$; $P = 0.037$). For NH_3, correlations with intake were weak and not statistically significant in both

Table 2
Mean ($\bar{x}$) and standard error (SE) of temperatures in the evaluated areas of animals under both treatments, C and C + M, using a thermal imaging camera (FLIR Ignite 5 PRO).® Nose, Eye, Rumen, Leg and Scrotum.

Area	Trat	$\bar{x}$	SE	H-stat	p-value
Snout	C	34.5	1.05	0.0028–0.95	
	C + M	33.64	2.68		
Eye	C	38.46[a]	1.03	3.86–0.049	
	C + M	37.56[b]	0.64		
Rumen	C	33.06	1.58	0.45–0.49	
	C + M	33.76	2.02		
Hind limb	C	33.62	0.74	1.93–0.16	
	C + M	34.57	1.38		
Scrotal region	C	33.75	0.76	0.59–0.44	
	C + M	33.57	0.96		

Differences between treatments were assessed using the Kruskal–Walli's test. C refers to sugarcane only, and C + M, sugarcane with *Moringa oleifera*

Table 3
Gas emissions from the Picarro® system, divided according to treatment. The following are CH_4 (g/kg DMI), CO_2 (g/kg DMI), N_2O (mg/kg DMI), and NH_3 (g/kg DMI).

Evaluated gas	C (LSMeans ± SE)	C + M (LSMeans ± SE)	p-value
CH4	4.63 ± 1.11	6.81 ± 1.11	0.1036
CO2	88.36 ± 23.26[a]	173.82 ± 23.26[b]	0.0392
N2O	16.37 ± 6.45	30.27 ± 6.45	0.2091
NH3	0.31 ± 0.25[a]	1.35 ± 0.25[b]	0.0220

Means followed by different letters in the same line differ according to Tukey's test ($p < 0.05$). Treatment C (control) refers to sugarcane only, and C + M refers to sugarcane treated with *Moringa oleifera*, both silages.

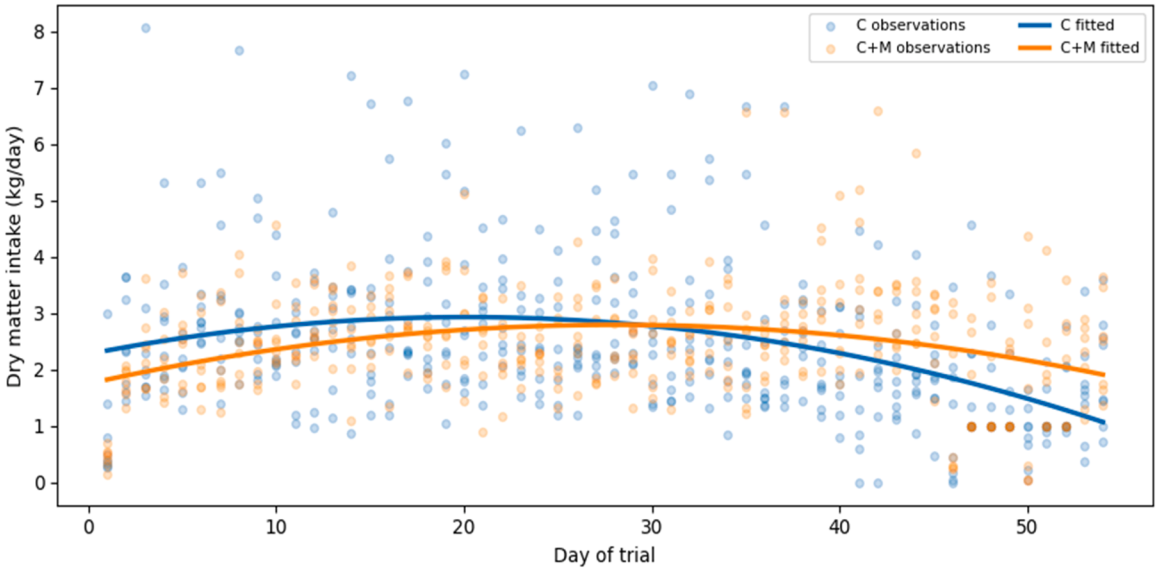


Fig. 1. Daily dry matter intake (DMI) of lambs fed sugarcane silage (C) or sugarcane silage partially replaced by *Moringa oleifera* (C+M). Points represent individual observations, and lines represent mixed-model fitted curves.

treatments. When growth performance was considered, positive associations between average daily gain (ADG) and N_2O emissions were observed in the control group ($r = 0.53$; $P = 0.035$), No significant relationships were detected in C + M.

3.4. Rumen microbiota

The abundance of rumen microbial groups revealed diet and phase-dependent responses (Table 5). When treatments were compared overall, total bacterial abundance was not affected by the inclusion of *Moringa oleifera*, indicating no detectable treatment effect on total bacterial abundance. In contrast, fungal populations were significantly lower in animals fed the C + M diet ($P = 0.0157$), showing a lower fungal abundance under the C + M diet.

Temporal analyses revealed additional shifts in the rumen microbiota during the experimental period. In the control group, bacterial abundance decreased over time ($P = 0.028$), while protozoal populations also declined significantly between the initial and final phases ($P = 0.003$). A similar reduction in protozoa was observed in lambs fed the C + M diet ($P = 0.032$), although bacterial abundance remained relatively stable.

Despite these differences in microbial abundance, methane emissions were not affected by treatment. Therefore, under the present experimental conditions, the observed shifts in qPCR-based microbial abundance were not accompanied by detectable differences in CH_4 emissions. (Santos et al., 2025).

4. Discussion

The partial replacement of sugarcane silage with *Moringa oleifera* silage improved the nutritional profile of the forage, primarily through a marked increase in crude protein content This response is consistent with the high nitrogen concentration found in *Moringa oleifera* biomass and suggests its potential to compensate for the intrinsic protein deficiency commonly observed in sugarcane-based silages. (Oliveira et al., 2023). Despite a slight reduction in dry matter resulting from the higher moisture content of Moringa (Table 1), fiber fractions remained largely unchanged, indicating that the nutritional improvement was mainly driven by an increase in nitrogenous compounds rather than alterations in the structural carbohydrate fraction. This pattern aligns with previous reports on alternative tropical forages that enhance protein supply and support ruminal nutrient supply without substantially modifying the physical structure of the diet (Benhammouche et al., 2021).

From a functional perspective, changes in chemical composition were accompanied by differences in the temporal dynamics of feed intake. Lambs fed the control diet (C) exhibited the typical curvilinear decline in intake during the confinement period (Fig. 1), a pattern frequently reported in sugarcane-based feeding systems. This behavior may be related to the lower protein supply of sugarcane silage, which can limit the balance between dietary nitrogen and fermentable substrates in the rumen. In contrast, animals receiving the C + M diet displayed a more stable intake trajectory throughout the experimental period, suggesting that the C + M diet favored more consistent intake over time.

This greater stability of intake may be related to the higher protein content of the C + M diet and, potentially, to compositional characteristics of *Moringa oleifera* that may have influenced feeding behavior during confinement. Previous studies evaluating protein-rich tropical forages containing secondary metabolites have reported similar effects on voluntary intake and feeding consistency in ruminant production systems (Piluzza et al., 2014).

These findings suggest that *Moringa oleifera* can be considered a promising forage resource for improving the nutritional quality of sugarcane-based diets while promoting greater intake stability during confinement. Such responses indicate potential use for Moringa in tropical production systems, that rely heavily on conserved forages during periods of feed scarcity.

The physiological responses observed in this study suggest that the inclusion of *Moringa oleifera* did not induce systemic metabolic stress in lambs. Serum biochemical parameters remained within the reference ranges reported for sheep (Kaneko et al., 1997; Souza et al., 2020), indicating maintenance of hepatic, renal, and energetic homeostasis. The increase in serum urea observed in lambs fed the C + M diet likely reflects greater ruminal nitrogen availability resulting from the higher protein content of Moringa, leading to increased ammonia absorption and subsequent hepatic conversion into urea. This pattern suggests intensified protein metabolism without indications of marked metabolic imbalance (Valizadeh et al., 2021).

Thermographic analysis (Table 2) was consistent with the physiological responses observed in this study. The lower ocular temperature recorded in animals receiving the C + M diet may indicate differences in peripheral thermal response between treatments. Because the orbital region is sensitive to variations in blood flow and autonomic activity, changes in this area may reflect differences in physiological regulation. Although bioactive compounds from *Moringa oleifera* have been associated with antioxidant and metabolic effects in previous studies (Diya, 2023), such mechanisms were not directly evaluated here. Therefore, the thermographic findings should be interpreted as indicative of a distinct peripheral thermal profile rather than as direct evidence of improved thermoregulatory efficiency.

Regarding gaseous emissions (Table 3), CH_4 emissions remained below the classical ranges reported for sheep (8–20 g/kg DMI; Hammond et al., 2016), a pattern frequently observed in tropical diets characterized by moderate intake levels. The absence of a significant effect of dietary treatment on methane production suggests that, under the conditions evaluated, partial replacement of sugarcane silage with *Moringa oleifera* silage did not result in a detectable reduction in methane emissions. This result is consistent with the dose-and-diet dependent nature, commonly reported for plant derived bioactive compounds (Durmic et al., 2025).

In contrast, CO_2 and NH_3 emissions were higher in animals fed the C + M diet, indicating differences in carbon, and nitrogen-related emissions between dietary treatments. Higher CO_2 emissions may be associated with increased metabolic activity and substrate oxidation, whereas the increase in NH_3 likely reflects greater ruminal deamination of soluble proteins and other nitrogenous compounds (Soltan et al., 2018). Nitrous oxide emissions, however, exhibited substantial inter-individual variability and no consistent treatment effect, which is compatible with the complex and indirect microbial pathways involved in its formation (Carvalho et al., 2022).

Together, the results indicate that the inclusion of *Moringa oleifera* did not reduce CH_4 emissions under the conditions of the present study but was associated with higher CO_2 and NH_3 outputs. These responses suggest that the C + M diet altered the pattern of carbon, and nitrogen related emissions, likely reflecting differences in dietary composition, particularly protein supply. Therefore, the present results reinforce that the relationship between dietary nitrogen availability and emission profiles should be considered when evaluating feeding strategies for tropical production systems (Redoy et al., 2021).

Exploratory correlation analyses further characterized the relationships between intake, performance, and gaseous emissions (Table 4). Absolute emissions of CH_4 and CO_2 were positively associated with dry matter intake. However, when emissions were expressed relative to animal performance, both gases tended to decline as average daily gain increased. These results suggest a possible dilution of emission intensity as animal growth increased (Capper, 2023).

Nitrogen-related gaseous emissions exhibited distinct response patterns compared with carbon-derived gases. Nitrous oxide (N_2O) showed positive associations with both feed intake and growth performance, indicating that N_2O output was more closely associated with productive variables under the conditions of this study. In contrast, ammonia (NH_3)

Table 4
Pearson's correlation coefficients (r) and associated P-values for the relationships between gaseous emissions and productive variables in lambs fed sugarcane silage (C) or sugarcane silage partially replaced with *Moringa oleifera* (C+M).

Gas	Variable	Treatment	r	P-value
CH_4	DMI	C	0.33	0.23
CH_4	DMI	C + M	−0.16	0.56
CO_2	DMI	C	0.59	0.020
CO_2	DMI	C + M	0.07	0.79
CH_4	ADG	C	−0.04	0.88
CH_4	ADG	C + M	−0.27	0.34
CO_2	ADG	C	0.46	0.07
CO_2	ADG	C + M	0.27	0.32
NH_3	DMI	C	0.02	0.93
NH_3	DMI	C + M	0.05	0.85
N_2O	DMI	C	0.70	0.003
N_2O	DMI	C + M	0.54	0.037
NH_3	ADG	C	−0.17	0.54
NH_3	ADG	C + M	0.40	0.14
N_2O	ADG	C	0.53	0.035
N_2O	ADG	C + M	0.37	0.18

DMI = dry matter intake; ADG = average daily gain; CH_4 = methane; CO_2 = carbon dioxide; NH_3 = ammonia; N_2O = nitrous oxide.

emissions were not correlated with productive performance, suggesting that NH_3 release was not directly linked to tissue accretion and may reflect differences in nitrogen metabolism (Koluman, 2023; Pardo et al., 2024). These results indicate that nitrogen-related emissions displayed different association patterns from carbon-derived gases. The corresponding graphical patterns are presented in Supplementary Figures 1 and 2.

Microbial data (Table 5) are consistent with this interpretation. Although total bacterial abundance remained stable across treatments, Moringa supplementation was associated with lower fungal populations and temporal reductions in protozoal abundance. Bioactive compounds previously reported in *Moringa oleifera*, including tannins and saponins (Gupta et al., 2012), have been associated with selective effects on protozoa and other microorganisms involved in ruminal nitrogen metabolism (Aguirre et al., 2020; Elghandour et al., 2024). Under the present experimental conditions, the reduction in protozoal abundance may help explain part of the differences observed in emission patterns, although no substantial reduction in absolute methane production was detected.

Collectively, these results indicate that the inclusion of *Moringa oleifera* was associated with changes in intake pattern, selected rumen microbial groups, and carbon- and nitrogen-related emissions. The C + M diet was characterized by stable bacterial abundance, reduced protozoal populations over time, and higher CO_2 and NH_3 emissions, while CH_4 emissions were not affected under the conditions of the present study. Thus, *Moringa oleifera* may be considered a promising forage resource for tropical sheep production systems, although the mechanisms underlying these responses require further investigation.

Table 5
Rumen microbial abundance in lambs fed sugarcane silage (C) or sugarcane silage partially replaced with *Moringa oleifera* (C+M), according to treatment and experimental phase.

Treatment/Phase	Bacteria (log_{10})	Fungi (log_{10})	Protozoa (log_{10})
C	8.04 ± 0.08	8.98 ± 0.17	7.04 ± 0.10
C + M	8.01 ± 0.09	8.84 ± 0.25	6.88 ± 0.13
p-value	0.80	0.0157	0.45
IN C	8.14 ± 0.09^a	7.48 ± 0.81	6.92 ± 0.18^a
FIN C	7.83 ± 0.19^b	8.04 ± 0.22	6.86 ± 0.20^b
p-value	0.028	0.60	0.003
IN C + M	8.03 ± 0.11^a	7.98 ± 0.24	7.08 ± 0.12^a
FIN C + M	8.04 ± 0.11^b	7.98 ± 0.24	6.88 ± 0.18^b
p-value	0.004	0.08	0.032

Within each microbial group and treatment, different superscript letters indicate significant differences between the initial and final phases according to Tukey's test ($P < 0.05$). Overall treatment comparisons are presented in the first three rows.

Some limitations should be considered when interpreting the present results. First, the relatively small number of animals per treatment may have limited the statistical power to detect differences in variables with high inter-individual variability, particularly CH_4 and N_2O emissions. Second, gas emissions were measured during a standardized chamber-evaluation period, consisting of two days of acclimation followed by two days of continuous measurement, and therefore should not be interpreted as representing cumulative emissions over the entire 75-day feeding period. In addition, chamber measurements were conducted in two consecutive batches due to chamber availability, and batch was not explicitly included in the statistical model. The dietary comparison should also be interpreted as the combined effect of partial replacement with *Moringa oleifera* silage and the associated changes in dietary crude protein and energy supply, because the experimental diets were not formulated to be isonitrogenous or isoenergetic. Therefore, treatment responses cannot be attributed exclusively to *Moringa oleifera* per se. Finally, volatile fatty acids, nitrogen balance, plasma ammonia, and quantitative concentrations of secondary metabolites in the silages were not determined, and microbial assessment was based on qPCR quantification of selected groups. Therefore, mechanistic interpretations regarding ruminal fermentation, nitrogen utilization, and methanogenesis should be considered exploratory.

5. Conclusion

Partial replacement of sugarcane silage with *Moringa oleifera* did not compromise productive performance and was associated with differences in intake stability, rumen microbial abundance, and gaseous emissions. The C + M diet increased CO_2 and NH_3 emissions, whereas CH_4 and N_2O emissions remained unchanged under the present experimental conditions. Reductions in protozoal and fungal abundance were also observed. These findings suggest that *Moringa oleifera* silage may be a promising forage resource for tropical sugarcane-based sheep production systems. Because the diets were not isonitrogenous or isoenergetic, the observed responses should be interpreted as diet-related effects, rather than isolated effects of *Moringa oleifera*. Future studies should focus on clarifying the metabolic and microbial mechanisms involved and on optimizing inclusion levels to better balance animal performance and emission profiles.

Funding Declaration

This study was carried out with financial support from the Coordination for the Improvement of Higher Education Personnel – CAPES. Process code: 88887.802359/2023–00, and São Paulo State Research Support Foundation (FAPESP), Proc Fapesp 2021/03409–3. R.L.D.C held productivity research fellowship from CNPq – Brazil (Conselho Nacional de Desenvolvimento Científico e Tecnólogico) 302089/2022–5. The Article Processing Fee for the publication of this research was funded by the Coordination for the Improvement of Higher Education Personnel - CAPES (identifier ROR: 00x0ma614). For open access purposes, the authors have assigned a Creative Commons CC BY license to any accepted version of the article.

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draft, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition. **Gustavo Delmilho:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Adibe Luiz Abdalla:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Márcia Mayumi Harada Haguiwara:** Writing – review & editing, Resources, Methodology.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Ricardo Lopes Dias da Costa reports financial support was provided by State of Sao Paulo Research Foundation. Gustavo Delmilho reports financial support was provided by Coordination for the improvement of Higher Education Personnel. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.smallrumres.2026.107816.

Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.smallrumres.2026.107816.

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