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Article

Quantifying ruminal fermentation and methane production using the *in vitro* gas technique in the forages of a sheep silvopastoral system in Chiapas, Mexico

Ángel Jiménez-Santiago[a]

Guillermo Jiménez-Ferrer[a]*

Armando Alayón-Gamboa[b]

Esaú de Jesús Pérez-Luna[c]

Ángel Trinidad Piñeiro-Vázquez[d]

Samuel Albores-Moreno[b]

Ma. Guadalupe Pérez-Escobar[a]

Ricardo Castro-Chan[d]

[a] ECOSUR (El Colegio de la Frontera Sur, Unidad SCLC), Departamento de Agricultura, Sociedad y Ambiente. Carr. Panamericana s/n, San Cristóbal de las Casas, Chiapas, México.

[b] ECOSUR (Unidad Campeche). Campeche, México.

[c] UNACH (Universidad Autónoma de Chiapas), Facultad de Agronomía. Chiapas, México.

[d] Tecnológico Nacional de México. IT Conkal. Yucatán, México.

[e] ECOSUR (Unidad Tapachula). Chiapas, México.

* Corresponding author: gjimenez@ecosur.mx

Abstract:

Ruminal fermentation and methane production in a sheep silvopastoral system were quantified with the *in vitro* gas production technique. Evaluations were done of local energy sources (molasses, *Zea mays* L. and *Musa paradisiaca* L.), of the base forage (*Panicum maximum* cv. Tanzania), of forage tree foliage (*Gliricidia sepium* (Jacq.) and *Leucaena leucocephala* cv. Cunningham), and diets combining these elements. Ruminal fluid was collected from five sheep (Pelibuey x Katahdin; 40 ± 3 kg). Five treatments (diets) containing different mixtures of forage tree foliage, energy sources and the base forage were analyzed in a completely random experimental design. Maximum gas volume production (V) was observed in *M. paradisiaca* (544 ml/g^{-1} DM) and Z. *mays* (467 ml/g^{-1} DM) ($P \leq 0.05$). The lowest V values were for the foliage of *G. sepium* (253 ml/g^{-1} DM) and *L. leucocephala* (180 ml/g^{-1} DM) ($P \leq 0.05$). Of the diets, D4GMP (48% *P. maximum*, 30% G. *sepium*, 7% *Z. mays*, 15% *M. paradisiaca)* had the highest V value. Methane production ranged from 6.31 to 9.60 L/Kg digested DM, and did not differ between treatments ($P > 0.05$). Data were used to generate a potential fermentable gases emission index, which suggested that the diets containing slow fermenting carbohydrates resulted in higher gas emission rates. Inclusion of forage trees and local energy sources in sheep silvopastoral management systems can improve diet quality and contribute to reducing CH_4 emissions.

Key words: Mitigation, Climate Change, Energy, Agroforestry.

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Introduction

Livestock are key to the survival of more than 800 million of the world's poor[1]. However, animal production also contributes to natural resource degradation, environmental pollution and climate change[2], mainly through greenhouse gas (GHG) emissions[3]. Tropical livestock farming in Latin America is primarily based on grazing native and introduced grasses in extensive systems[4], with little or no supplementation, minimal infrastructure and low capital investment[5]. In this context, silvopastoral systems and use of local forage trees and shrubs have been shown to improve livestock production systems, reduce their environmental impact and contribute to GHG mitigation[6-9].

In silvopastoral systems, the protein in the foliage of multiple-use trees (e.g. the genera *Leucaena*, *Gliricidia* and *Erythrina*, among others) degrades rapidly in the rumen. Addition of ingredients providing energy to the diet are therefore required to improve

rumen fermentation efficiency, synchrony and nutrient balance[10,11]. High-quality commercial energy byproducts for use in livestock meat and dairy systems can be costly[12], highlighting the need to search for energy supplements among local resources that are both easily accessible and provide adequate nutritional value[13]. The foliage of many forage trees contains secondary metabolites[14], and many of these can mitigate enteric methane emissions in ruminants[15,16]. Indeed, the foliage from some forage trees is known to reduce rumen populations of protozoa and methanogenic archaea[17,18,19], leading to lower enteric CH_4 synthesis and production[20].

The present study objective was to evaluate the effect of addition of local energy sources on ruminal fermentation and methane emissions parameters when combined with forages in a sheep silvopastoral system involving *P. maximum* supplemented with *Gliricidia sepium* and *Leucaena leucocephala* foliage.

Materials and methods

Study area

Materials were obtained from a sheep ranch managed with silvopastoral techniques and located in the municipality of Chiapa de Corzo, in the state of Chiapas, Mexico (16°42' N; 93°00' W). Altitude at the ranch ranges from 400 to 450 m asl, average annual precipitation in the region is 900 mm, and average annual temperature is 26.0 °C. Soils in the area are mainly clay loam, with 2.4 % organic matter content, 7.0 pH, and slightly poor nitrogen content (0.15 %)[21]. Ranch surface area is 12 ha and average herd size is 55 Pelibuey x Katahadin sheep. Of the total area 10 ha is covered with Tanzania grass (*P. maximum*) with living fences consisting of the trees *L. leucocephala*, *G. sepium* and *Cordia dentata* (Vahl). Several paddocks (3 ha) contain *L. leucocephala* in alleys, and trees such as *Enterolobium cyclocarpum* (Jacq) and *Ceiba pentandra* L. are scattered across 7 ha of grazing areas. A nature reserve of dry tropical forest covers 2 ha. No chemical fertilization of pastures is done. Paddocks are managed in a rotation controlled by electric fences, and pastures are irrigated in the dry season. Animal production is focused on lamb meat for sale in regional and national markets.

Feed chemical analysis

Dry matter (DM) content of the forages and supplements was determined by drying in a forced air stove at 55 °C for 48 h (constant weight) and processing following the

regulation NOM-116-SSA1-1994. Crude protein content was measured by an internal method (ECOSUR-ET-BR04) based on the standard NMX-F-608-NORMEX-2002. Organic matter (OM) content was measured by incineration in a muffle oven at 550 °C for 3 h according to the standard NMX-F-607-NORMEX-2002. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were quantified following Van Soest[22], using the sequential procedure, with alpha-amylase and no ash correction in all samples (AOAC)[23]. Condensed tannins were measured with the acidified vanillin method (1% w/v vanillin in methanol)[24].

In vitro gas production

An *in vitro* gas assay was done following the cumulative gas technique suggested by Theodorou[25] and Williams[26]. Five diets (treatments) were designed using six raw materials, (Table 1): *P. maximum* as base forage (control); *G. sepium* and *L. leucocephala* foliage as protein sources; and molasses, *Zea mays* and *M. paradisiaca* as energy sources. Diets were isoenergetic and isoproteic, and formulated to meet the demands of adult sheep in the evaluated silvopastoral unit: 2,200 kcal/kg, 14% crude protein (CP).

Table 1: Treatments and percent ingredients used in *in vitro* gas experiment

Feed	*P. maximum*	*G. sepium*	*L. leucocephala*	*M. paradisiaca*	*Z. mays*	Molasses
P100 (control)	100	0	0	0	0	0
G100	0	100	0	0	0	0
L100	0	0	100	0	0	0
MP100	0	0	0	100	0	0
Z100	0	0	0	0	100	0
M100	0	0	0	0	0	100
Treatments						
D1LM	47	0	30	0	8	15
D2LMP	47	0	30	15	8	0
D3GM	47	30	0	0	8	15
D4GMP	48	30	0	15	7	0
D5GLMPM	47	16	17	5	5	10

P100 (control) = *P. maximum*; G100= *G. sepium*; L100= *L. leucocephala*; MP100 = *M. paradisiaca*; Z100= *Z. mays*; M100 = molasses; D1LM= 47% *P. maximum*, 30% *L. leucocephala*, 8% *Z. mays*, 15% molasses; D2LMP= 47% *P. maximum*, 30% *L. leucocephala* 8% *Z. mays*, 15% *M. paradisiaca*; D3GM= 47% *P. maximum*, 30% *G. sepium*, 8% *Z. mays*, 15% molasses ; D4GMP= 48% *P. maximum*, 30% *G. sepium*, 7% *Zea mays*, 15% *M. paradisiaca*; D5GLMPM= 47% *P. maximum*, 16% *G. sepium* 17% *L. leucocephala*, 5% *M. paradisiaca*, 5% *Z. mays*, 10% molasses.

Sheep were managed and ruminal fluid extracted from them according to Alexander and McGowan[27] and Blummel and Orskov[28], and following the animal welfare norms of the ECOSUR Sustainable Livestock Production Research Group. Ruminal fluid was extracted from five ewes in the experimental area; all had a live weight of 40.0 ± 3.0 kg,

were of similar ages and good body condition. An esophageal probe was used to extract 300 ml ruminal fluid from each animal, for a total of 1.5 L ruminal fluid. All ruminal fluid samples were stored at 39 °C and protected from sunlight.

In vitro fermentation of each treatment was done by introducing 0.5 ± 0.001 g substrate in 90 ml amber glass vials and evaluating fermentation as represented by gas production at different times (0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, 36, 48, 60, 72 h). Three replicates were done per treatment. The pressure generated in each vial was monitored with an analog manometer (Metron Mod. 63100, Range: 0^{-1} kg/cm^2), and the resulting data used to calculate six response variables: maximum gas volume (V); gas production rate (S); lag phase (L); rapid fermentation fractional gas volume generated in first eight hours (V8); intermediate fermentation volume generated from eight to 24 h (V24); and slow fermentation volume generated from 24 to 72 h (V72). Two batches were incubated simultaneously, each comprised of three replicates (vials) per feed and treatment. In the first batch total accumulated gas production at 72 h was evaluated in each fermentable fraction: rapid, intermediate and slow. For each fraction three groups of fermentable carbohydrates were estimated (monosaccharides, starch and cellulose) based on the gas volumes recorded in three time intervals: 0 to 8 h incubation (Vf0-8); 8 to 24 h (Vf8-24); and 24 to 72 h (Vf24-72). These volumes were used to estimate the rapid (FR), intermediate (FI) and slow (FS) fermentable fractions using the linear regression equations proposed by Miranda *et al*[29]: FR = Vf0-8/0.4266, FI = Vf8-24/0.6152, and FL = Vf24-72/0.3453). Values for accumulated gas production were fit to the model of Menke and Steingas[30]:

$$\mathbf{Y= v/ (1+exp (2-4*s*(t-L)))},$$

Where:

Y = Total volume of gas produced;

v = Maximum production volume;

s = Constant gas production rate;

t = Time;

L = Lag or delay phase.

In vitro dry matter digestibility (IVDMD) was measured by gravimetric analysis, considering initial dry matter weight, and final weights at 24 and 72 h fermentation. Dry matter (DM) weight was measured by recovering the matter with a 200 μm filter and drying at 65 °C to constant weight. Calculation of IVDMD was done with the formula:

$$\%\, \boldsymbol{IVDMD} = \frac{\boldsymbol{IW-FW}}{\boldsymbol{IW}} * \mathbf{100},$$

Where:

% IVDMD = percentage *in vitro* dry matter digestibility;

IW = initial weight incubated dry matter in grams;

FW = final weight incubated dry matter in grams.

Using the data for $IVDMD_{24/72}$ and emitted gas volumes a potential fermentable gas emission index (PFGEI) was generated. This refers to the amount of gas that can be produced by a substrate per gram of fermented DM or OM in the rumen[31].

Methane and carbon dioxide production

Production of CO_2, CH_4 and minor gases was analyzed during the first 24 hours of fermentation in samples from the second incubation batch. Following Bartha and Pramer[32] as modified by Miranda[29], CO_2 separation was done using a trap (hermetically sealed glass jar with rubber stopper and aluminum ring) containing 90 ml 1 M potassium hydroxide (KOH) and a dilution of 56.10 g KOH in 1 L deionized water. Samples were taken and placed in sterile vials under a vacuum for later analysis with gas chromatography and quantification of CH_4 for each substrate. Analysis of CH_4 production was done in a gas chromatographer (Clarus 500, Perkin Elmer; Software version 6.3.2.0646; 0.530 mm column diameter; 50 m length; 35 °C injection temperature). Analysis was done of a total of 36 samples collected during the 24 h *in vitro* fermentation, in the second incubation run; 20 µl of sample were used in each assay. Correction of CH_4 concentrations was done for each treatment by subtracting average methane production from the three blanks. For the purposes of calculating CH_4 concentration and the effect of the treatments on CH_4 production, it was expressed as L CH_4/kg DMDIG.

Statistical analysis

Gas production parameters, IVDMD and methane production were analyzed with an ANOVA in a completely random design. The mathematical model was:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij}$$

Where:

$\boldsymbol{Y_{ij}}$= Response variable in *j*-th replicate (flasks) of *i*-th treatment;

$\boldsymbol{\mu}$= overall mean of all experimental data;

T_i = Effect of treatment *I*;

ε_{ij} = experimental error associated with *j* under treatment *i*.

Data from all the response variables were processed with an ANOVA[33], and differences between treatment means compared with a Tukey test ($P \leq 0.05$) using the PROC GLM procedure in the SAS statistics package[34].

Results and discussion

Analysis of forage, energy source and treatment (diet) chemical composition showed crude protein (CP) content to be high in the *G. sepium* and *L. leucocephala* foliage (Table 2); indeed, it was higher than reported elsewhere[32,33]. As expected, the energy sources had low CP and NDF contents. The grass *P. maximum* (control) had a CP higher than the 7 to 9 % average in many tropical grasses. This relatively high grass CP may be linked to natural fertilization via sheep feces in the studied controlled grazing management system. The *P. maximum* also had high NDF and ADF contents. Compared with previous reports[35,36], the *L. leucocephala* leaves analyzed in the present study contained very little tannins (CT). This discrepancy could be due to variability in the nutritional value of foliage from the same fodder tree species in response to site conditions, management, phenological stage and specific characteristics of the study area[37]. Lignin content in *L. leucocephala* was high but within the range suggested by the FAO. This lignin content very probably affected the digestibility of *L. leucocephala*, and ration components, reducing energy use[38,39].

Table 2: Chemical composition (g/Kg DM) of forages, energy sources, and treatments used in *in vitro* gas experiment

	DM	OM	CP	Lignin	NDF	ADF	CT	CHO
P. maximum (control)	933	853	124	103	712	490	NA	231
G. sepium	930	889	367	133	353	250	0	269
L. leucocephala	932	883	261	207	462	308	56	352
M. paradisiaca	925	953	52	NA	137	37	NA	763
Z. mays	866	984	59	6	86	16	NA	795
Molasses	788	866	53	3*	8*	5*	NA	600
D1LM	906	874	149	111	481	324	16	368
D2LMP	926	887	149	111	501	329	16	392
D3GM	905	876	181	89	448	307	NA	343
D4GMP	926	888	182	90	474	317	NA	361
D5GLMPM	914	877	172	105	482	326	9	349

DM= dry matter; OM = organic matter; CP= crude protein; NDF= neutral detergent fiber; ADF = acid detergent fiber; CT= condensed tannins; CHO= carbohydrates; NA = not analyzed. * https://www.feedipedia.org/01/05/2018. ; D1LM= 47% *P. maximum*, 30% *L. leucocephala*, 8% *Z. mays*, 15% molasses; D2LMP= 47% *P. maximum*, 30% *L. leucocephala*, 8% *Z. mays*, 15% *M. paradisiaca*, D3GM= 47% *P. maximum*, 30% *G. sepium*, 8% *Z. mays*, 15% molasses; D4GMP= 48% *P. maximum*, 30% *G. sepium*, 7% *Z. mays*, 15% *M. paradisiaca*; D5GLMPM= 47% *P. maximum*, 16% *G. sepium*, 17% *L. leucocephala*. 5% *M. paradisiaca*, 5%, *Z. mays*, 10% molasses.

Gas production data at 8, 24 and 72 h fermentation showed the highest gas volumes (V) to be 544.0 ml/g^{-1} DM in MP100 (*M. paradisiaca*), 467.3 ml/g^{-1} DM in Z100 (Z. *mays*) and 325.7 ml/g^{-1} DM in M100 (molasses)(Table 3). These levels differed (P<0.05) between each other and from the diets. This behavior is typical of foods containing carbohydrates such as monosaccharides and starches[40]. Both *G. sepium* (G100) and *L. leucocephala* (L100) had relatively low gas production volumes (V), which differed from each other (P<0.05) (Figure 1). These low production levels may be due to the presence of secondary metabolites (tannins) in *L. leucocephala*[40], and/or the high lignin and fiber contents in both species' leaves (111 g/kg DM), all of which can result in lower gas production compared to higher carbohydrate content diets[41]. Treatments with energy-protein mixtures had higher gas production (V) (P<0.05) due to the additive effect of the carbohydrates to *L. leucocephala* and *G. sepium* leaves (Figure 2). Overall, gas production rate (S) was similar among the treatments although slight differences were present (P<0.05).

Table 3: Total gas production parameters and fractional volumes in feed ingredients and treatments in *in vitro* gas production experiment

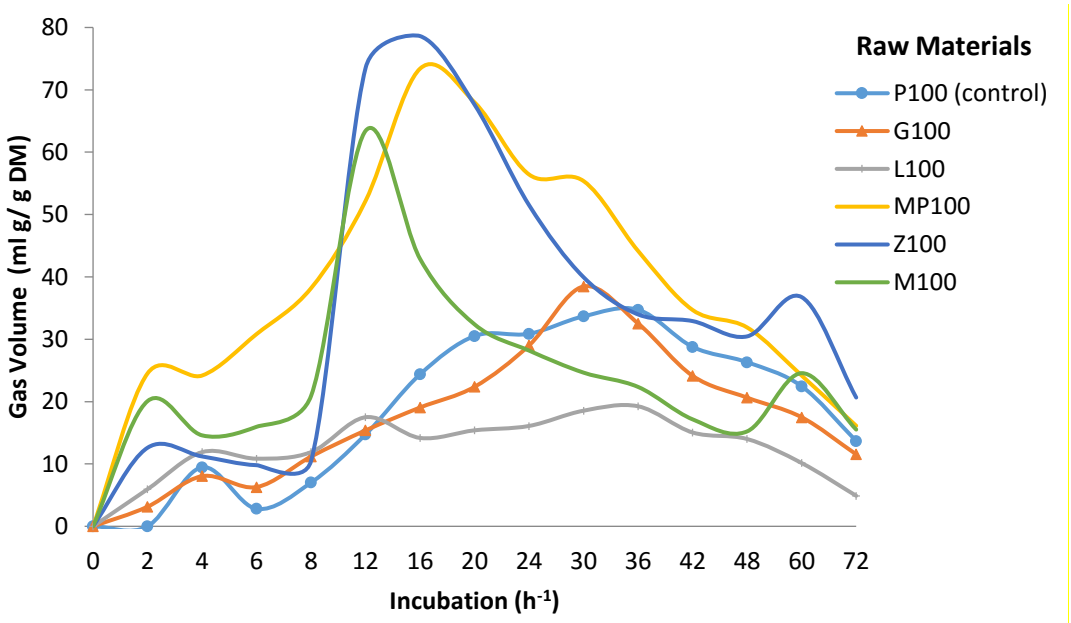
Feed ingredients	Parameters			Fractional volumes (ml g^{-1} DM)		
	V (ml / g^{-1} DM)	S (ml h^{-1})	L (h)	V_8	V_{24}	V_{72}
P100 (control)	266.3^{de}	0.03^{ab}	11.2^{a}	15.1^{e}	100.5^{d}	159.7^{b}
G100	253.0^{e}	0.03^{ab}	9.0^{b}	28.7^{ed}	85.9^{de}	144.8^{bcd}
L100	180.8^{f}	0.03^{ab}	2.7^{f}	40.6^{cd}	63.2^{e}	81.9^{e}
MP100	544.9^{a}	0.03^{ab}	3.7^{ef}	117.7^{a}	250.0^{a}	206.4^{a}
Z100	467.3^{b}	0.04^{a}	6.2^{c}	44.1^{cd}	271.2^{a}	194.7^{a}
M100	325.7^{c}	0.04^{a}	2.6^{f}	71.6^{b}	166.9^{b}	119.5^{d}
Treatments						
D1LM	299.8^{cd}	0.03^{b}	4.7^{cde}	51.7^{c}	105.0^{c}	149.9^{bc}
D2LMP	308.9^{cd}	0.03^{ab}	5.7^{cd}	46.8^{c}	119.7^{cd}	152.4^{bc}
D3GM	293.6^{cde}	0.03^{ab}	4.5^{de}	54.0^{c}	115.6^{cd}	134.3^{bcd}
D4GMP	337.4^{c}	0.03^{ab}	5.6^{cd}	52.6^{c}	147.1^{bc}	151.5^{cb}
D5GLMPM	292.3^{cde}	0.03^{ab}	3.6^{fe}	57.5^{cb}	122.2^{cd}	128.7^{cd}

V = maximum gas production volume; S = constant gas production rate; L = Lag phase (h); V_8 = fractional volume generated in rapid fermentation fraction (0-8 h); V_{24} = fractional volume generated in intermediate fermentation fraction (8-24 h); V_{72} = fractional volume generated in slow fermentation fraction (24-72 h).

P100 (control)= *P. maximum*; G100= *G. sepium*; L100= *L. leucocephala*; MP100= *M. paradisiaca*; Z100= *Z. mays*; M100 = molasses; D1LM= 47% *P. maximum*, 30% *L. leucocephala*, 8% *Z. mays*, 15% molasses; D2LMP= 47% *P. maximum*, 30% *L. leucocephala*, 8% *Z. mays*, 15% *M. paradisiaca*; D3GM= 47% *P. maximum*, 30% *G. sepium*, 8% *Z. mays*, 15% molasses; D4GMP= 48% *P. maximum*, 30% *G. sepium*, 7% *Z. mays*, 15% *M. paradisiaca*; D5GLMPM= 47% *P. maximum*, 16% *G. sepium*, 17% *L. leucocephala*, 5% *M. paradisiaca*, 5% *Z. mays*, 10% molasses.

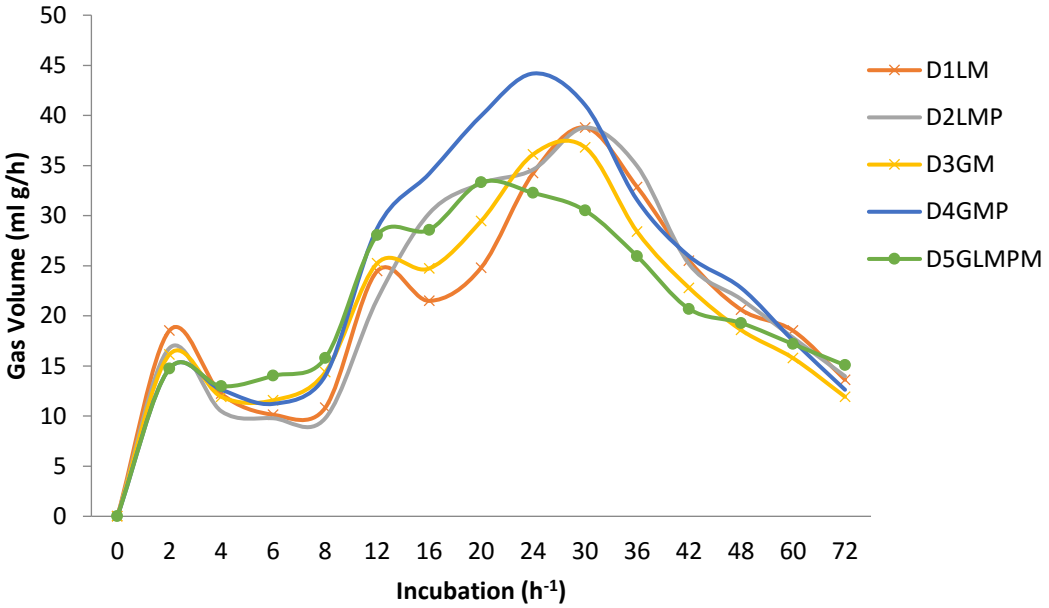
^{abcdef} Different letter superscripts in the same column indicate significant differences between treatments (α= 0.05).

Figure 1: Gas volume over time in control treatment and raw material ingredients in *in vitro* gas production trial



P100 (control)= *Panicum maximum*; G100= *Gliricidia sepium*; L100= *Leucaena leucocephala*; MP100= *Musa paradisiaca*; Z100= *Zea mays*; M100= molasses.

Figure 2: *In vitro* gas production (ml gas/h) of five diets used in sheep in a silvopastoral system in Chiapas, Mexico.



P100 (control)= *P. maximum*; D1LM= 47% *P. maximum*, 30% *L. leucocephala*, 8% *Z. mays*, 15% molasses; D2LM= 47% *P. maximum*, 30% *L. leucocephala*, 8% *Z. mays*, 15% *M. paradisiaca*; D3GM= 47% *P. maximum*, 30% *G. sepium*, 8% *Z. mays*, 15% molasses; D4GMP= 48% *P. maximum*, 30% *G. sepium*, 7% *Zea mays*, 15% *M. paradisiaca*; D5GLMPM= 47% *P. maximum*, 16% *G. sepium*, 17% *L. leucocephala*, 5% *M. paradisiaca*, 5% *Z. mays*, 10% molasses.

The fermentation profiles clearly varied between the energy sources, forages and treatments. Energy sources such as bananas (MP100) and molasses (M100) began to ferment quickly, increased gas production during the intermediate incubation phase and then declined rapidly. In the treatments containing mixtures of forages with energy sources gas production and fermentation rate were initially slow but increased notably in the intermediate phase and remained higher for longer (Figure 2). During fermentation the substrate is hydrated and colonized by ruminal microorganisms. The quantity and type of carbohydrates present in the substrate influence gas volume and its effect on DM digestibility[42,43].

In vitro dry matter digestibility (IVDMD) was lowest at 72 h with *P. maximum* (50.9 %) and *L. leucocephala* (29.9 %), which differed from *G. sepium* and the diets ($P \leq 0.05$) (Table 4). The IVDMD values for *L. leucocephala* were lower than reported in other *in vitro* and *in vivo* studies[34,42,43], probably due to the maturity of the forage tree foliage used in the present study and its consequently high lignin content. At both 24 and 72 h IVDMD was highest ($P \leq 0.05$) in M100 (*Z. mays*), Z100 (molasses) and MP100 (*M. paradisiaca*). The treatments (D1LM, D2LMP, D3GM, D4GMP and D5GLMPM) exhibited a range of values between these highs and lows ($P < 0.05$). The linear increases observed in the treatments resulted from the contributions of *G. sepium* and *L. leucocephala* foliage to fermentation and digestibility (Figure 1). Inclusion of energy sources (D3GM and D4GMP) improved digestibility ($P \leq 0.05$) compared to D2LMP, and

P100 and L100 ($P \leq 0.05$). The digestibility observed for *G. sepium* was similar to that reported elsewhere[43]. The energy sources' (MP100, Z100 and M100) high digestibility was due to their high soluble sugars contents. When diets are balanced with high *G. sepium* and molasses contents, digestibility and utilization are higher due to the synchrony between protein and energy contents[44].

Table 4: CH_4, CO_2, IVDMD, PFGEI and Total CH_4 produced by fermentation of treatments in *in vitro* gas production experiment

Treatments	CH_4 (%)	CO_2 (%)	IVDMD 24 h (%)	IVDMD 72 h (%)	PFGEI/DM 24 h	PFGEI/DM 72 h	CH_4 (L CH_4/kg DMDIG)
P100 (control)	22.5^{bcd}	77.5^{abc}	33.7^{f}	50.9^{e}	791.0^{a}	523.5^{cd}	1.55^{d}
G100	23.2^{bcd}	76.8^{abc}	51.0^{cd}	60.1^{cd}	496.8^{e}	420.9^{e}	1.68^{d}
L100	30.8^{a,b}	69.2^{cd}	28.8^{f}	29.9^{f}	628.1^{bcd}	606.6^{ab}	1.94^{d}
MP100	18.1^{d}	81.9^{a}	77.0^{b}	83.6^{b}	708.1^{ab}	652.1^{a}	15.75^{b}
Z100	16.4^{d}	83.6^{a}	80.1^{b}	87.0^{b}	583.6^{cde}	537.2^{cd}	28.59^{a}
M100	17.9^{d}	82.1^{a}	92.7^{a}	92.4^{a}	351.4^{f}	352.5^{f}	9.03^{c}
D1LM	31.9^{a}	68.1^{d}	44.4^{e}	56.6^{d}	678.6^{bc}	529.7^{cd}	8.82^{c}
D2LMP	27.0^{abc}	73.0^{bcd}	44.9^{de}	50.9^{e}	690.0^{b}	606.5^{ab}	8.83^{c}
D3GM	24.2^{abcd}	75.8^{abcd}	55.1^{c}	61.9^{c}	533.0^{de}	474.2^{de}	6.32^{cd}
D4GMP	21.9^{cd}	78.1^{ab}	54.5^{c}	61.1^{cd}	619.4^{bcd}	552.1^{bc}	9.60^{c}
D5GLMPM	22.3^{bcd}	77.7^{abc}	51.7^{c}	56.6^{d}	565.5^{de}	516.9^{cd}	6.31^{cd}

CH_4= *in vitro* methane + minor gases; CO_2= *in vitro* carbon dioxide; IVDMD 24 h= *in vitro* dry matter digestibility at 24 h; IVDMD 72 h= *in vitro* dry matter digestibility at 72 h; PFGEI/DM 24 h= potential fermentable gas emission index at 24 h; PFGEI/DM 72 h= potential fermentable gas emission index at 72 h; CH_4= methane concentration at 24 h.

P100 (control)= *P. maximum*; G100= *G. sepium*; L100= *L. leucocephala*; MP100= *M. paradisiaca*; Z100= *Z. mays*; M100= molasses; D1LM= 47% *P. maximum*, 30% *L. leucocephala*, 8% *Z. mays*, 15% molasses; D2LMP= 47% *P. maximum*, 30% *L. leucocephala*, 8% *Z. mays*, 15% *M. paradisiaca*; D3GM= 47% *P. maximum*, 30% *G. sepium*, 8% *Z. mays*, 15% molasses; D4GMP= 48% *P. maximum*, 30% *G. sepium*, 7% *Z. mays*, 15% *M. paradisiaca*; D5GLMPM= 47% *P. maximum*, 16% *G. sepium*, 17% *L. leucocephala*, 5% *M. paradisiaca*, 5% *Z. mays*, 10% molasses.

^{abcdef} Different letter superscripts in the same column indicate significant differences between treatments (α= 0.05).

Total CH_4 production (L/Kg DMDG) was highest in the *Z. mays* (Z100) and *M. paradisiaca* (MP100) energy sources ($P \leq 0.05$) (Table 4). The lowest production values were in the control (P100), *G. sepium* (G100) and *L. leucocephala* (L100), which did not differ ($P > 0.05$). The diet treatments (D5GLMPM, D3GM, D1LM, D2LMP and D4GMP) exhibited intermediate values ($P > 0.05$). Of the treatments containing mixed energy source and protein, D5GLMPM had the lowest CH_4 production, highlighting the importance of associating forages with carbohydrates[45,46]. These authors emphasize that carbohydrate type determines transit time, thus affecting CH_4 production per gram of digested substrate. Carbohydrate type appears to be a determining factor in CH_4 production[47], since it can be mediated by lower availability of digestible

carbohydrates[48]. Concentrations of 550 g kg^{-1} DM surpass the concentration which negatively affects voluntary consumption of feed and nutrient digestibility in animals[49]. In addition, tree and shrub foliage contains low concentrations of structural fractions[44], making them more susceptible to degradation and bacterial action, resulting in increased transit time, which decreases total gas production and therefore results in lower enteric CH_4 production[36,50].

Both research and development agencies have been focusing on quantification of GHG from ruminal fermentation, creation of indices to evaluate the potential for environmental pollution from ruminal GHG, and design of sustainable animal management strategies[51,52]. In the present results wide variation (P<0.001) was apparent in the PFGEI/DM, both at 24 and 72 h, and in the evaluated energy sources and treatments (Table 4). Of note is that the lowest PFGEI rates at 24 and 72 h correspond to M100 (496.8 $ml.g^{-1}$/IVDMD) and G100 (420.9 $ml.g^{-1}$/IVDMD), whereas the highest rates occurred with MP100 at 24 h (708.1 $ml.g^{-1}$/IVDMD) and 72 h (652.1 $ml.g^{-1}$/IVDMD). Of the treatments including tree foliage and energy sources, the lowest index corresponded to the D3GM mixture. The present data suggest that the type of foliage from forage trees, in association with carbohydrate type, can affect ruminal GHG production, especially if the carbohydrate exhibits slow fermentation, as is the case with starches[53].

Conclusions and implications

The present results suggest that in silvopastoral systems the combination of foliage from forage trees with local energy sources, especially molasses and bananas, can improve diet nutritional value and animal production parameters while mitigating generation of greenhouse gases such as methane. The combination of 30% DM foliage from trees such as *G. sepium* and *L. leucocephala* with local energy sources such as molasses and bananas contributed to lowering CH_4 emission in sheep. Management of forage trees (e.g. *G. sepium* and *L. leucocephala*) is recommended in silvopastoral systems because they improve diet quality, particularly when combined with local energy sources, and contribute to lowering CH_4 emissions. Future research will need to address animal response (e.g. weight gain) and bio-economic balance in these systems to understand how to make them economically and socially viable, and to develop adaptation strategies that will improve animal production, contribute to producers' social welfare and mitigate greenhouse gas emission.

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