Article

In vitro methane mitigation potential of foliage of fodder trees mixed at two levels with a tropical grass

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Abstract: Enteric methane (CH_4) emitted by ruminant species is known as one of the main greenhouse gases produced by the agricultural sector. The objective of this study was to evaluate the chemical composition, *in vitro* gas production, dry matter degradation (DMD), digestibility, CO_2 production and CH_4 mitigation potential of five tropical tree species with novel forage potential including: *Spondias mombin*, *Acacia pennatula*, *Parmentiera aculeata*, *Brosimum alicastrum* and *Bursera simaruba* mixed at two levels of inclusion (15 and 30%) with a tropical grass (*Pennisetum purpureum*). Crude protein content was similar across treatments (135 g kg^{-1} DM), while *P. purpureum* was characterized by a high content of acid detergent fiber (335.9 g kg^{-1} DM) and *B. simaruba* by a high concentration of condensed tannins (20 g kg^{-1} DM). Likewise, *A. pennatula* and *P. aculeata* were characterized by a high content of cyanogenic glycosides and alkaloids respectively. Treatments SM30-PP70 (30% *S. mombin* + 70% *P. purpureum*) and BA30-PP70 (30% *B. alicastrum* + 70% *P. purpureum*) resulted in superior digestibility than *P. purpureum*, while in the AP30-PP70 (30% *A. pennatula* + 70% *P. purpureum*) was lower than the control treatment ($P \le 0.05$). At 24 and 48 h, treatments that contained *P. aculeata* and *B. alicastrum* produced higher CH_4 ml g^{-1} DOM than *P. purpureum* ($P \le 0.05$). The inclusion of *B. simaruba* at 30% reduced CH_4 at 25% compared to *P. purpureum*. Tropical tree species can improve the nutritional quality of ruminant rations and reduce CH_4 emissions to consequently contribute to the development of sustainable ruminant production systems that generate diverse ecosystem services.

Keywords: Grass; Greenhouse Gases; Ruminal Degradation; Secondary Metabolites; Tropical Livestock Systems

1. Introduction

Methanogenesis in the gastrointestinal tract of ruminant species is the main sink for hydrogen, thus assuring the appropriate fermentation of fiber in the rumen [1]. Due to CH_4 synthesis, ruminants can make use of high-fiber diets (not edible for humans) growing abundantly on enormous land areas, marginal to crop agriculture and convert it into high quality food (*e.g.*, milk, meat) for humans as well as other products derived from livestock [2]. Eructated CH_4 is part of natural cycles and can be transformed by methanotrophic bacteria and reactions with hydroxyl radicals (OH) in the air, to carbon dioxide (CO_2) that will be used in photosynthesis [3]. However, high emission rates of CH_4 in cattle constitute an energy loss and production inefficiency, especially in tropical

production systems where poorly managed grasses are used as feed [4, 5]. Additionally, due to methane´s global warming potential (GWP) and its contribution to greenhouse gas (GHG) emissions in the agricultural sector, it has gained attention in the last 20 years for research on mitigation alternatives in livestock production [1]. Some of these alternatives are focused on interfering with CH_4 synthesis in the rumen.

In the rumen there is a whole consortium of microorganisms that establish syntrophic relationships between them and of mutualism with their host [6]. The diversity and structure of microbial populations in the rumen will depend on the characteristics of the feed consumed by the ruminant [7]. These microbial populations use the compounds in ruminant diets for their growth and produce volatile fatty acids (VFA), metabolic hydrogen (H_2) and CO_2 as by-products [8]. Rumen bacteria are the most abundant and diverse group of microorganisms in the rumen ecosystem and carry out enzymatic activities for the fermentation of starch, cellulose, hemicellulose, proteins, and lipids [9]. Protozoa comprise a large proportion (approximately 20%) of the microbial mass of the rumen due to their size and can be up to 50% in some cases depending on the diet [9]. They can degrade fiber and have been directly related to the synthesis of CH_4 due to their hydrogensomes that produce H_2 that serves as a substrate for methanogenic archaea [10]. Archaea are chemolythophic organisms that use acetate, methyl groups, CO_2 and H_2 as a substrate for the synthesis of CH_4 [6]. Methanogenic archaea of the rumen produce CH_4 mainly through the hydrogenotrophic pathway and to a lesser extent through the methylotrophic and acetoclastic pathways [11]. These biochemical pathways have in common the presence of methyl-coenzyme M reductase (Mcr), responsible for the last step in the reduction of the methyl group to CH_4 [12]. Each of the steps of hydrogenotrophic methanogenesis are shown on Figure 1.
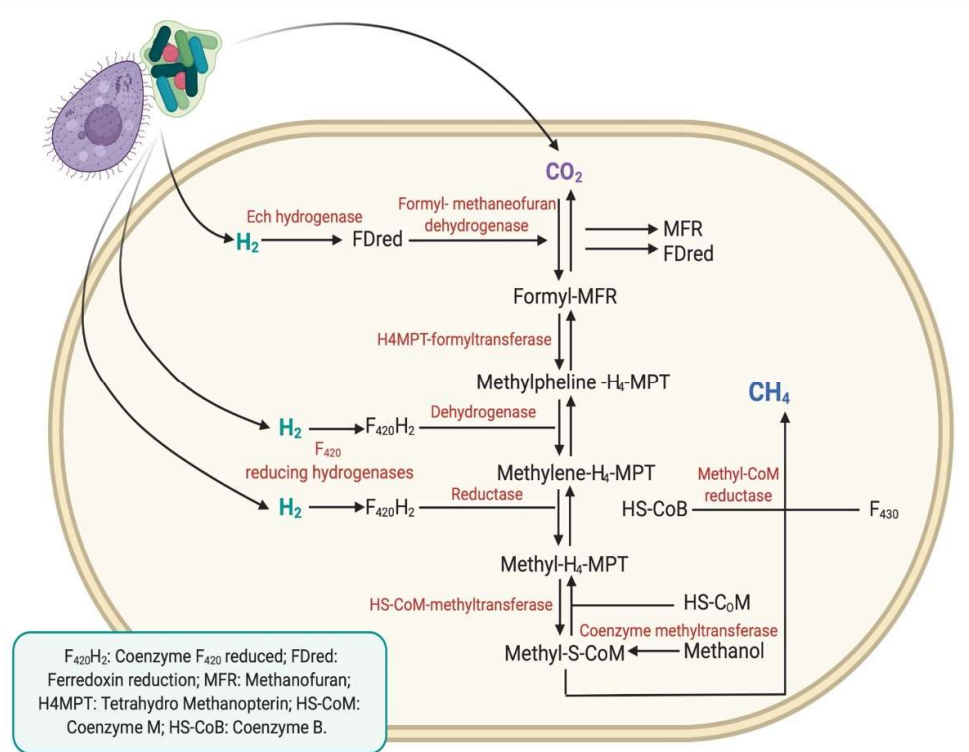

Figure 1. Hydrogenotrophic methanogenesis

Nutritional strategies for the reduction of enteric CH_4 production in ruminants have been widely studied [13]. Commercial CH_4 mitigation sources in tropical regions can have a high cost for medium and small producers and be difficult to implement in extensive grazing systems [14]. However, biomass diversity in the tropics can offer a vast diversity of low-cost alternatives for CH_4 reduction and the improvement of cattle production to more sustainable systems. Many native tree and shrub species have a high forage potential due to their nutritional quality and secondary metabolite content that have shown to be beneficial to ruminant metabolism [15]. These metabolites from plants have the capacity to modulate the rumen microbiome and reduce CH_4 synthesis [16]. In addition, the use of forestry species due to their role in biogeochemical cycles can benefit the whole production system when used in combination with well managed forage-grass species [17]. Therefore, livestock production systems have a great GHG mitigation potential within the agricultural sector due to its close relation to natural biological cycles and its capacity of transformation to efficient and sustainable systems. Furthermore, the use of these resources is directed towards using less human-edible food in ruminant production.

Livestock production in Latin America and the Caribbean is based on extensive systems that for years have promoted the deforestation of large forest areas for the sowing of pastures. Under these conditions, most of the dry matter consumed by cattle comes from medium to low quality pastures [18] and the dry seasons contribute to a scarce supply of forage and a decrease in consumption causing the animals to only cover their maintenance requirements. In this sense, five novel tropical tree species with forage potential evaluated in this study were collected at the Lacandon rainforest, one of the most important ecosystems in Mexico, however one of the main economic activities is extensive cattle production that has generated significant deforestation in the region [19]. By using the Global Forest Watch Pro platform (https://www.globalforestwatch.org), the loss of tree cover was monitored between 2011 to 2019. A loss of 145.000 ha was recorded, equivalent to a 12% decrease in tree cover since the year 2000, registering the greatest loss in 2019. Figure 2 shows the deforestation hot spots in the Lacandon rainforest region. The advance of deforestation is evident both from Guatemala and in Mexico in the Ocosingo Valley. Many Mayan indigenous and *mestizo* livestock producers use secondary vegetation in critical periods of the year to improve cattle diets due to the low nutritional quality of the pasture in those periods. However, most of the land used for cattle production is directed towards extensive grazing as most of the livestock systems in tropical regions.
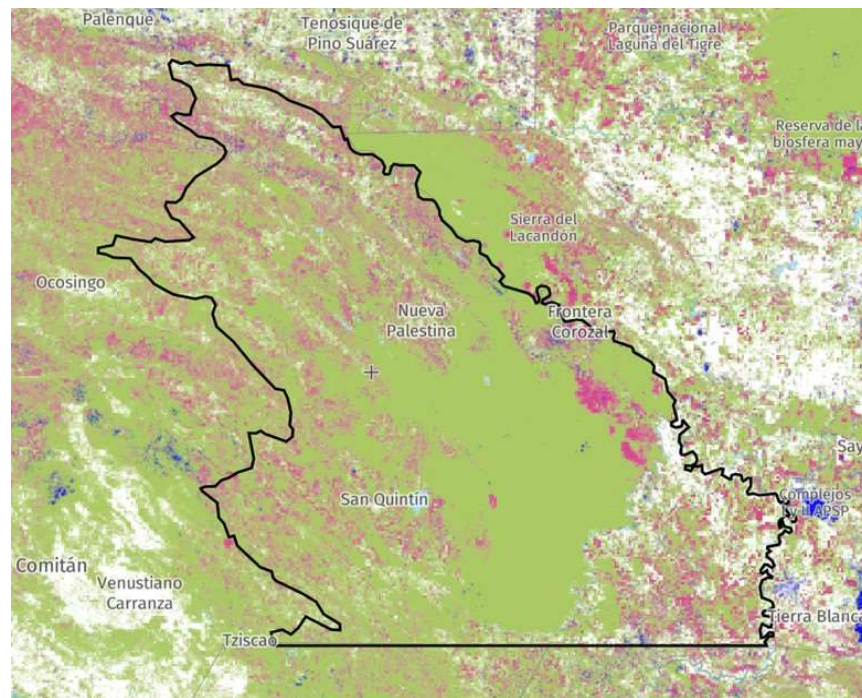
Figure 2. Tree cover loss of Lacandon Rainforest region from Global Forest Watch

Additionally, there is not much knowledge on the biodiversity that exists at the regional level that can be used for cattle production and information on the use of native resources for the design of pastoral systems and management of these species is needed. Introduced species and silvopastoral systems have been promoted and the use of native species has been minimized despite its potential for small and medium producers in tropical countries to improve the cattle´s diets and its capacity to regenerate soils and deforested regions. In this respect, the objective of this study was to quantify *in vitro* CH_4 mitigation potential, nutritional quality, and digestibility of ten experimental diets composed of *S. mombin*, *A. pennatula*, *P. aculeata*, *B. alicastrum* and *B. simaruba* replacing 15 and 30% (of DM) of grass-based rations designed to be fed to tropical cattle.

2. Materials and Methods

2.1. Description of the study area

Forage species were sampled in the Ocosingo Valley of the Lacandon Rainforest, in the East and Northeast of Chiapas, Mexico. The prevailing climate is warm-humid (23-27 °C) with an altitude that varies from 10 to 900 MASL [20]. The municipality of Ocosingo, covers the largest region of the rainforest and one of the major activities is extensive livestock production.

2.2. Sampling

Species were selected from a previous screening of fifteen species [19] for their nutritional quality and their potential to reduce *in vitro* CH_4 emissions when incubated alone. The selected species were: *S. mombin*, *A. pennatula*, *P. aculeata*, *B. alicastrum* and *B. simaruba*. Species were harvested with the help of cattle producers from the area. Leaves were collected from 5 to 9 individuals per species. Botanical samples were taken to verify the species in the herbarium of the Southern Border College (ECOSUR). Samples of *P. purpureum* grass at 60 days regrowth were taken to use as a control treatment and basal ration.

Samples were dried in a forced air oven at 55 °C or until constant weight to determine dry matter (DM) content [21]. Dried samples were ground in a Wiley Laboratory Mill (Thomas®, USA) at a particle size of 1mm and stored for transportation and chemical analysis.

2.3. Treatments

Experimental diets were the inclusion of the different tree species at two levels (15 and 30%) mixed with *Pennisetum purpureum sp.* grass (85 and 70%, respectively) simulating feeding practices commonly used by some farmers in the region. Treatments were designated as following: SM15-PP85 (15% *S. mombin* + 85% *P. purpureum*), SM30-PP70 (30% *S. mombin* + 70% *P. purpureum*), AP15-PP85 (15% *A. pennatula* + 85% *P. purpureum*), AP30-PP70 (30% *A. pennatula* + 70% *P. purpureum*), PA15-PP85 (15% *P. aculeata* + 85% *P. purpureum*), PA30-PP70 (30% *P. aculeata* + 70% *P. purpureum*), BA15-PP85 (15% *B. alicastrum* + 85% *P. purpureum*), BA30-PP70 (30% *B. alicastrum* + 70% *P. purpureum*), BS15-PP85 (15% *B. simaruba* + 85% *P. purpureum*), BS30-PP70 (30% *B. simaruba* + 70% *P. purpureum*), and control treatment 100PP (100% *P. purpureum*).

2.4. Chemical Analysis

Forage chemical analysis and the *in vitro* gas production technique were carried out at the Forage Quality and Animal Nutrition Laboratory at the International Center for Tropical Agriculture (CIAT), Palmira (Valle del Cauca, Colombia), certified by the FAO-IAG proficiency test of feed constituents 2017. Ash content was determined by combustion in a muffle furnace at 500°C for 4 h (method 942.05) [22], from which the organic matter (OM) content was determined; crude protein (CP=N×6.25) was determined by Kjeldahl (AN 3001 FOSS; method 984.14) [23]; neutral and acid detergent fiber (NDF and ADF, respectively) content were determined using the methodology proposed by Van Soest [24], adapted to an Ankom Fiber Analyzer AN 3805 (Ankom® Technology Corp. USA). An adiabatic calorimetry bomb was used to determine gross energy (GE) following the procedure described in ISO 9831 [25]. To determine digestibility, the two-stage *in vitro* technique was used [26]. Condensed tannins (TC) content of the species was determined by the vanillin extract assay [27] at the bromatology laboratory at ECOSUR, Chiapas, Mexico. Qualitative quantification of alkaloids, cyanogenic glycosides and saponins were carried out by the methodologies proposed by Domínguez [28]. Ether extract (EE) content was determined by the Soxhlet immersion method (NTC 668) [29]. Metabolizable energy (ME) was estimated according to the equation proposed by Menke et al. [30] 1979): ME (MJ kg^{-1} DM) = 2.20 + 0.136xGP + 0.057xCP + 0.0029xCP^2, where GP is the net gas production in 24 h (ml/200 mg). Likewise, net energy (NE) was calculated from the equation proposed by Menke and Steingass [31]: NE (Mcal lb^{-1}) = (2.2 + (0.0272xGP) + (0.057xCP) + (0.149xEE)) /14.64, where: GP is the net gas production in 24 h (ml g^{-1} DM), CP is crude protein (% DM), EE is the ether extract (% DM); results were transformed to MJ/kg^{-1} DM. Short chain fatty acids (SCFA) were calculated (SCFA = 0.0239 GP-0.0601 ml / 200 mg DM) [32] and transformed to mmol per liter.

2.5. In vitro gas production technique

Rumen cannulated *Brahman* bulls were treated in accordance with the Colombian normative num. 84 from 1989 following the protocol approved by the ethics committee of the International Center of Tropical Agriculture (CIAT). *In vitro* gas production was quantified using the technique proposed by Menke and Steingass [31] as modified by Theodorou et al. [33]. Rumen liquor was obtained at 08:00 am from three rumen cannulated *Brahman* bulls of 550 kg live weight, fed *Cynodon plectostachyus* and minerals. Rumen liquid and solid material were obtained from different locations in the rumen to assure a

representative sample [34]. Rumen liquor was filtered through 10 layers of gauze and mixed in a 1:9 ratio with a reduced mineral solution [31]. Ruminal content from the solid phase was liquefied and mixed with ruminal liquor and filtered again, to ensure the presence of microorganisms of both the liquid and solid phase in the inoculum. A 1 g of each diet was incubated in independent bottles of 160 ml by triplicate including blanks. Bottles were kept under constant flow of CO_2, sealed with a rubber stopper and aluminum ring and placed in a water bath at 39°C for 48 h. Gas pressure and volume in the headspace of the bottles were measured with an 840065 wide-range pressure gauge (Sper Scientific, USA) connected a PS100 2-bar pressure transducer (Lutron Electronic Enterprise Co. Ltd., Taiwan) and a three-way valve connected to a hypodermic needle that was inserted into the bottles and a 60 ml syringe was used to measure the gas volume. Gas pressure and volume were measured at 0, 4, 8, 12, 24, 30, 36 and 48 hours. The content of the bottles was withdrawn from fermentation at 24 and 48 h for the degradation of DM (DDM) and OM (DOM) as described by Valencia-Salazar et al. [19]. The pH was measured using a pH meter (AB15 Plus, Accumet®) at 24 and 48 h. The content of the bottles was filtered in crucibles with fiberglass filter and a vacuum pump. The crucibles with the fiberglass filter were dried in a forced air oven at 65°C for 48 hours and weighed with a precision balance. Data from the pressure and volume of the bottles was used to generate the following polynomial equation for the correction of the volume of gas produced:

$$y = 0.0209\,x^2 + 5.9023x - 2.984$$

$$R^2 = 0.9729$$

Gas production data was adjusted to the modified Gompertz model [35] with the following equation:

$$y = ae^{-e^{b-cx}}$$

Where, y is equal to the cumulative gas production at a time x, $a > 0$ is the maximum gas production, parameter $b > 0$ is the difference between the initial gas and the final gas at a time x and the parameter $c > 0$ describes the specific rate of gas accumulation. The application of this model requires the conversion of parameters a, b, c into parameters with biological significance. The parameters were: time at the inflection point (TIP, hours), gas at the inflection point (GIP, ml), maximum gas production rate (MGPR ml h^{-1}) and Lag phase (LP or the microbial establishment, h). For its estimation the following equations were used: TIP = b / c; GIP = a / e; MGPR = (a * c) / e; LP = ((b / c) - (1 / c)); where "e" is Euler's number, equivalent to ≈ 2,718281828459.

2.6. Methane quantification

Gas volume was stored in amber bottles with a capacity of 125 ml from samples collected from the accumulated gas at 24 and 48 h of incubation. Methane and CO_2 concentrations were quantified in the Laboratory of GHG (CIAT) using a gas chromatograph GC-2014 Shimadzu with 1/8" packed stainless-steel columns (1.0 m HayeSep T 80/100 mesh, 4m HayeSep D 80/100, 1.5 P-N, 0.7m Shimalite Q 100/180), 80°C temperature, column flow of 30.83 ml min^{-1}, injection volume handled by a loop with capacity of 2 ml and nitrogen was used as carrier gas.

2.7. Statistical analysis

For the statistical analysis a randomized block design with 10 treatments (mixed feed substrates) and a control (100% *P. purpureum*) was used with three biological replicates per

hour (24 and 48 h) and three different inoculums as blocking factor. The statistical model used was:

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

where Y_{ij} are the observations of the response variables for treatment i and block j; μ is the overall mean; i is the effect of the ith treatment; β_j is the effect of the jth block; and ε_{ij} is the random error of treatment i in block j. To test treatment effects, the PROC GLM procedure of SAS® software, version 9.4 was used [36]. The means of the treatments were compared by the Dunnett test with an Alpha of 0.05.

3. Results

3.1. Chemical composition, in vitro digestibility, and phytochemical screening

Chemical composition of forage samples and treatments is shown on Table 1. Organic matter content ranged from 821.28 to 900.82 g kg^{-1} DM between species and from 832.80 to 863.87 g kg^{-1} DM among treatments. *B. simaruba* had the lowest crude protein (CP) content (99.07 g kg^{-1} DM) and *A. pennatula* the highest (192.69 g kg^{-1} DM) among plant species. The species with the lowest acid detergent fiber (ADF) content were *S. mombin* and *A. pennatula*, with 171.18 and 210.34 g kg^{-1} DM respectively and *A. pennatula* had the lowest *in vitro* digestibility (447.44 g kg^{-1} DM). *P. purpureum* grass provides between 1.2 and 2 times more NDF than the other species. Mixed rations with the highest protein content were AP30-PP70, PA30-PP70 and AP15-PP85 with 151.56, 148.70 and 142.74 g kg^{-1} DM respectively. Gross energy content of mixed rations ranged from 15.86 to 17.44 MJ kg^{-1} DM. The highest *in vitro* digestibility was obtained when *P. aculeata* was present in the rations 588.45 and 593.02 g kg^{-1} DM for 15 and 30% inclusion, respectively. The highest metabolizable energy and net energy concentrations were obtained in the treatment BA30-PP70 with 7.82 and 4.82 MJ kg^{-1} DM, respectively. Regarding secondary metabolites, the highest content of CT was obtained with *B. simaruba* (20% CT) at inclusion of 15 and 30% in the ration with *P. purpureum*. In the phytochemical screening, presence of alkaloids was found in all species except for *B. simaruba*, and cyanogenic glycosides were found highly abundant only in *A. pennatula* as shown on Table 2. Also, saponin content was found in low abundance only in *A. pennatula*.

Table 1. Chemical composition, condensed tannin content and *in vitro* digestibility of forage species and treatments

	g kg⁻¹ DM									MJ kg⁻¹ DM		
Species	**DM**	**OM**	**NDF**	**ADF**	**CP**	**EE**	**IVDDM**	**CT**	**GE**	**ME**	**NE**
Spondias mombin (SM)	259.75	852.29	307.77	171.18	126.95	44.78	638.59	9.90	16.25		
Acacia pennatula (AP)	505.42	924.85	492.56	210.34	192.69	39.25	447.44	31.1	20.92		
Parmentiera aculeata (PA)	308.95	874.47	614.35	268.81	183.17	13.85	548.37	0.00	18.04		
Brosimum alicastrum (BA)	489.18	821.28	298.17	269.22	116.21	29.92	686.38	0.00	15.65		
Bursera simaruba (BS)	356.71	900.82	354.37	249.23	99.07	25.05	471.37	200.1	18.92		
Treatments											
SM15-PP85	213.82	839.92	542.46	311.22	132.88	23.42	542.46	1.50	15.99	7.40	4.54
SM30-PP70	221.93	842.10	501.04	286.50	131.83	27.19	501.04	3.00	16.04	7.75	4.80
AP15-PP85	250.66	850.80	570.18	317.09	142.74	22.59	570.18	4.70	16.69	7.56	4.58
AP30-PP70	295.61	863.87	556.48	298.25	151.56	25.53	556.48	9.30	17.44	7.46	4.51
PA15-PP85	221.18	843.24	588.45	325.86	141.31	18.78	588.45	0.00	16.26	7.17	4.31
PA30-PP70	236.66	848.75	593.02	315.79	148.70	17.91	593.02	0.00	16.58	7.18	4.27
BA15-PP85	248.23	835.26	541.02	325.92	131.27	21.19	541.02	0.00	15.90	7.60	4.65
BA30-PP70	290.75	832.80	498.17	315.92	128.61	22.73	498.17	0.00	15.86	7.82	4.82
BS15-PP85	228.35	847.20	549.45	322.92	128.70	20.46	549.45	30.0	16.39	6.92	4.23
BS30-PP70	251.00	856.66	515.03	309.92	123.47	21.27	515.03	60.0	16.84	6.05	3.72
100PP	205.70	837.73	583.88	335.93	133.93	19.65	583.88	0.00	15.95	7.08	4.30

DM: Dry matter; OM: Organic matter; NDF: Neutral detergent fiber; ADF: Acid detergent fiber; CP: Crude protein; EE: Ether extract; IVDDM: *In vitro* digestibility of dry matter; CT: Condensed tannins; GE: Gross energy: ME: Metabolizable energy; NE: net energy; SM15-PP85 (15% *S. mombin* + 85% *P. purpureum*); SM30-PP70 (30% *S. mombin* + 70% *P. purpureum*); AP15-PP85 (15% *A. pennatula* + 75% *P. purpureum*); AP30-PP70 (30% *A. pennatula* + 70% *P. purpureum*); PA15-PP85 (15% *P. aculeata*+ 85% *P. purpureum*); PA30-PP70 (30% *P. aculeata*+ 70% *P. purpureum*); BA15-PP85 (15% *B. alicastrum* + 85% *P. purpureum*); BA30-PP70 (30% *B. alicastrum* + 70% *P. purpureum*); BS15-PP85 (15% *B. simaruba* + 85% *P. purpureum*); BS30-PP70 (30% *B. simaruba* + 70% *P. purpureum*); 100PP: 100% *P. purpureum.*

Table 2. Secondary metabolite content of tree species from southern Mexico

Species	Alkaloids			Cyanogenic glycosides	Saponins
	Mayer	Draggendorff	Wagner		
Spondias mombin (SM)	-	+	++	-	-
Acacia pennatula (AP)	-	++	++	++++	+
Parmentiera aculeata (PA)	++++	++++	++++	-	-
Brosimum alicastrum (BA)	+++	+	-	-	-
Bursera simaruba (BS)	-	-	-	-	-

- (No presence); + (low abundance); ++ (abundant); +++ (moderately abundant); ++++ (highly abundant)

3.2. In vitro gas production parameters

Gompertz model parameters for *in vitro* gas production is shown on Table 3. Maximum gas production (*a*), time at the inflection point (TIP), gas inflection point (GIP), maximum gas production rate and Lag phase (LP) differed significantly ($P < 0.05$) between evaluated treatments and control ration (100PP). The highest maximum gas production (*a*) value was obtained in SM30-PP70 with 287.369 ml and differed from control treatment (261.304 ml) and had the highest maximum gas production rate (MGPR) value with 8.730 ml h^{-1} and 7.580 ml h^{-1} for control treatment. The lowest values for maximum gas production (*a*) and MGPR was obtained when *B. simaruba* was included in the treatment with 235.527 ml (at 15%), 214.480 ml (at 30%) and 6.61 ml h^{-1} (at 15%), 4.76 ml h^{-1} (at 30%) and were below control treatment; 261.304 ml and 7.58 ml h^{-1}, respectively for 100PP. Figure 3 shows the gas production per hour per gram of organic matter.

Table 3. Gompertz model parameters for *in vitro* gas production of forage species incorporated at two levels mixed with *P. purpureum*

	Parameters						
				TIP	GIP	MGPR	
Rations	*a*	*b*	*c*	(h)	(ml)	(ml h^{-1})	LP
SM15-PP85	266.533	1.101*	0.084	13.170	98.033	8.200*	1.206*
SM30-PP70	287.369*	1.048	0.083	12.690	105.697*	8.730*	0.583
AP15-PP85	263.756	1.017	0.079	12.866	97.010	7.670	0.216
AP30-PP70	240.909*	0.953*	0.078	12.160	88.607*	6.947*	-0.593*
PA15-PP85	220.971*	1.003	0.086*	11.616*	81.270*	6.613*	0.030
PA30-PP70	219.015*	0.925*	0.081	11.380*	80.553*	5.546*	-0.926*
BA15-PP85	277.702	0.998	0.081	12.396	102.140	8.220*	-0.020
BA30-PP70	283.543*	0.944*	0.083	11.370*	104.287*	8.676*	-0.676*
BS15-PP85	235.527*	0.979	0.076	12.826	86.62*	6.61*	-0.286
BS30-PP70	214.480*	0.917*	0.060*	15.173*	78.887*	4.766*	-1.386*
100PP	261.304	1.009	0.079	12.786	96.110	7.580	0.120
MSE	8.089	0.0201	0.003	0.456	2.975	0.177	0.267
P-Value	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001	<.0001

a: Maximum gas production (ml); *b*: difference between initial gas and final gas at an *x* time; *c*: specific gas accumulation rate;

TIP: time at the inflection point (hours); GIP: Gas inflection point (ml); MGPR: Maximum gas production rate (ml h^{-1}); LP: Lag phase;

SM15-PP85 (15% *S. mombin* + 85% *P. purpureum*);   SM30-PP70 (30% *S. mombin* + 70% *P. purpureum*); AP15-PP85 (15% *A. pennatula* + 75% *P. purpureum*); AP30-PP70 (30% *A. pennatula* + 70% *P. purpureum*); PA15-PP85 (15% *P. aculeata* + 85% *P. purpureum*);   PA30-PP70 (30% *P. aculeata*+ 70% *P. purpureum*);   BA15-PP85 (15% *B. alicastrum* + 85% *P. purpureum*);   BA30-PP70 (30% *B. alicastrum* + 70% *P. purpureum*); BS15-PP85 (15% *B. simaruba* + 85% *P. purpureum*);   BS30-PP70 (30% *B. simaruba* + 70% *P. purpureum*); 100PP: 100% *P. purpureum.*
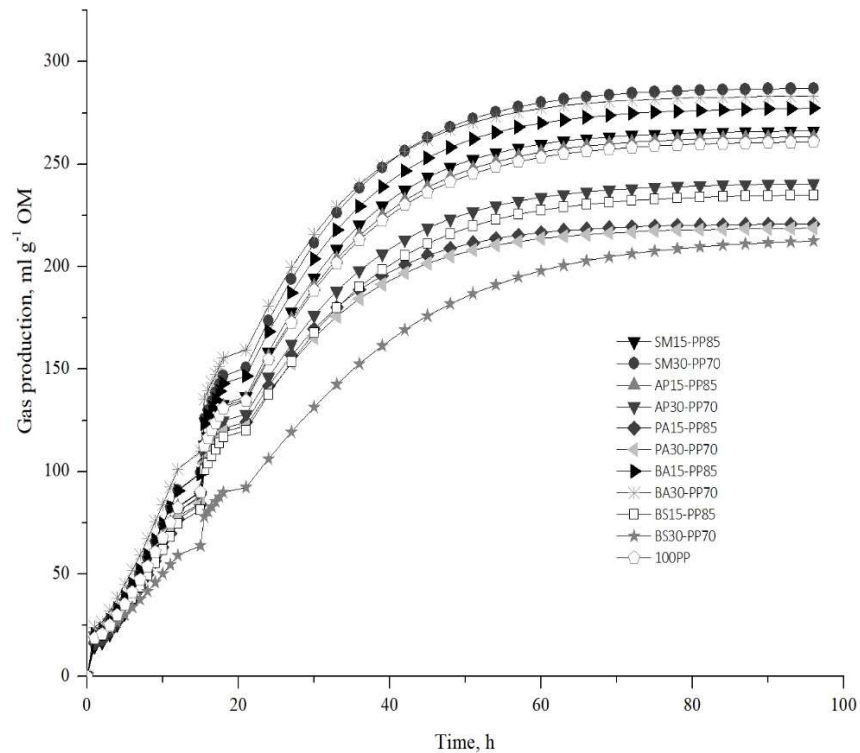
Figure 3. Gas production in ml g^{-1} Organic Matter per hour. Abbreviatures: *SM15-PP85 (15% S. mombin + 85% P. purpureum);   SM30-PP70 (30% S. mombin + 70% P. purpureum); AP15-PP85 (15% A. pennatula + 75% P. purpureum); AP30-PP70 (30% A. pennatula + 70% P. purpureum); PA15-PP85 (15% P. aculeata + 85% P. purpureum);   PA30-PP70 (30% P. aculeata+ 70% P. purpureum);   BA15-PP85 (15% B. alicastrum + 85% P. purpureum);   BA30-PP70 (30% B. alicastrum + 70% P. purpureum);   BS15-PP85 (15% B. simaruba + 85% P. purpureum);   BS30-PP70 (30% B. simaruba + 70% P. purpureum); 100PP: 100% P. purpureum*

3.3. Dry matter degradability, pH, short chain fatty acids and CH4 production

The highest degradability at 48 h was observed in SM30-PP70 (63.20%), BA30-PP70 (62.24%) and SM15-PP85 (60.69%) treatments. The lowest degradabilities at 48 h were obtained in PA30-PP70, AP30-PP70 and PA15-PP85 with 8.77, 7.91 and 7.34% lesser than the control (PP100). The pH was not affected by the inclusion of the forage tree species at two levels and the highest concentration of SCFA was observed in BA30-PP70. Methane was expressed in mg g^{-1} of incubated and DOM as suggested by Yáñez-Ruiz et al. [34] and is presented in Table 4. Methane production in mg g^{-1} DOM and mg g^{-1} IOM (incubated OM) was different between the treatments and *P. purpureum* (*P <.0001*). It was observed that PA30-PP70 had the major CH$_4$ mg g^{-1} IOM produced at 24 h and at 48 h AP15-PP85 had the lowest CH$_4$ mg g^{-1} IOM production. Methane emissions were higher and significantly different in PA30-PP70, BA15-PP85 and BA30-PP70 when compared to control (100PP). Methane reductions were observed when *A. pennatula* was included in 15 and 30% (AP15-PP85, AP30-PP70) in -10.90 and -12.58% respectively when compared to control expressed in CH$_4$ mg g^{-1} IOM at 24 hours. PA15-PP85 showed a reduction of -19% CH$_4$ mg g^{-1} IOM and -25.31% CH$_4$ mg g^{-1} DOM at 24 hours of incubation. BS30-PP70 showed reductions of -21.42 CH$_4$ mg g^{-1} IOM and -25.15% CH$_4$ mg g^{-1} DOM compared to control at 48 hours of incubation.

Table 4. DM degradability, pH, SCFA and CH$_4$ production at 24 and 48 hours of incubation of forage species mixed with *P. purpureum* at two levels

	DM DEG		pH		SCFA mMol l^{-1}	CO$_2$ ml g^{-1} IOM		CO$_2$ ml g^{-1} DOM		CH$_4$ ml g^{-1} IOM		CH$_4$ ml g^{-1} DOM	
	24	48	24	48		24	48	24	48	24	48	24	48
SM15-PP85	47.13	60.69*	6.78*	6.79	84.86*	176.37	202.58	153.78	158.15	13.58	26.20	8.22	20.47
SM30-PP70	56.65*	63.21*	6.67	6.63	92.80*	221.38	268.74*	185.08*	219.06*	16.75	37.51*	12.44	30.97*
AP15-PP85	46.29	55.02	6.72	6.68	85.29*	148.72	166.34	87.30	116.35	1256	25.81	7.38	18.04
AP30-PP70	46.04	51.43*	6.69	6.74	80.50	156.73	225.55	109.89	139.02	12.32	29.47	9.74	18.91
PA15-PP85	43.77	51.73*	6.72	6.83	77.25	142.47	244.72*	79.88	149.69	11.37	29.87	6.39	19.86
PA30-PP70	41.92*	50.95*	6.70	6.94	75.13	321.66*	267.98*	171.45	174.07*	26.16*	35.68*	13.95*	23.17*
BA15-PP85	51.37	60.11*	6.68	6.76	89.62*	259.01	272.44*	172.51	211.89*	23.61*	37.76*	14.14*	29.39*
BA30-PP70	55.98*	62.24*	6.68	6.87	95.42*	269.39	285.77*	195.74*	230.51*	22.95*	38.06*	16.68*	30.69*
BS15-PP85	45.01	54.52	6.70	6.77	75.49	305.59*	265.79*	175.28*	184.96*	20.68	31.81	12.95	22.16
BS30-PP70	39.17*	54.47	6.70	6.72	57.88*	240.56*	208.22	118.20	143.09	16.35	21.02	6.65	14.43
100PP	47.01	55.84	6.68	6.69	77.45	168.07	155.89	102.03	112.27	14.08	26.74	8.55	19.27
MSE	2.06	1.48	0.02	0.11	1.67	38.30	30.51	29.03	24.74	2.30	2.98	1.94	2.11
P-value	<.0001	<.0001	0.0028	0.1467	<.0001	<.0001	0.0003	0.0003	<.0001	<.0001	<.0001	<.0001	<.0001

DEG: degradation, IOM: incubated organic matter, DOM: Degraded organic matter; SCFA: Short chain fatty acids; SM15-PP85 (15% *S. mombin* + 85% *P. purpureum*);   SM30-PP70 (30% *S. mombin* + 70% *P. purpureum*); AP15-PP85 (15% *A. pennatula* + 75% *P. purpureum*); AP30-PP70 (30% *A. pennatula* + 70% *P. purpureum*); PA15-PP85 (15% *P. aculeata*+ 85% *P. purpureum*);   PA30-PP70 (30% *P. aculeata*+ 70% *P. purpureum*);   BA15-PP85 (15% *B. alicastrum* + 85% *P. purpureum*);   BA30-PP70 (30% *B. alicastrum* + 70% *P. purpureum*);   BS15-PP85 (15% *B. simaruba* + 85% *P. purpureum*);   BS30-PP70 (30% *B. simaruba* + 70% *P. purpureum*); 100PP: 100% *P. purpureum*; Means in the same column with same superscript are significantly different (P<0.05) when compared to control (100PP).

4. Discussion

4.1. Chemical composition and in vitro digestibility

In the present study the incorporation of the five forage tree species, independently at two different levels of inclusion (15 and 30%), into a diet based on forage grass *P. purpureum* improved nutritional composition, thus these tree forages are suitable for ruminant feeding. Crude protein content of the treatments was always above 7%, the minimum necessary for the correct function of rumen environment [24]. The mixture of grass with the foliage of native tree species at different levels can be a viable and a low-cost strategy to minimize the scarcity of nutrients in tropical cattle production systems. Gaviria-Uribe et al. [37] stated that including tree forages on ruminant's diet that are based on low quality grass, increases CP and decreases total carbohydrate intake. However, *P. purpureum* used in the present trial showed higher CP and lower values of structural carbohydrate content compared to other studies [18, 38], this is directly related to the age of regrowth of the pasture [39], as well as a higher digestibility and gas production as observed in this trial due to the high quality of the diets evaluated for tropical regions. The CP and EE values of *S. mombin* are similar to those obtained by Yusuf et al. [40] however, NDF and ADF contents were lower in the present study. The inclusion of *A. pennatula* at 30% had the highest CP content among the evaluated treatments, however the *in vitro* digestibility was reduced compared to the inclusion of 15% and this can be explained by its content of CT (31.1%) and cyanogenic (+++) glycosides that can alter the capacity of the microbiome to ferment nutrient components [15].

Metabolizable and net energy concentrations varied among treatments evaluated and was particularly high in BA30-PP70 (7.82 and 4.82 MJ kg^{-1} DM, respectively), consistent with a higher gas production observed in this treatment. A higher net energy content promotes a higher digestibility of the total nutrients as a result of increased nutritive values. Metabolizable energy ranged between 6 and 7.8 MJ kg^{-1} DM in the present investigation, this variations between species may be due to the differences in CP content and to a lesser extent to systematic errors made in the sampling of the gas produced as suggested by Tagliapietra et al. [41]. According to Krizsan et al. [42], microbial protein synthesis is favored by a higher ME input. Although, BA30-PP70 showed a higher net CH$_4$ production, its chemical composition and ME can improve animal performance and reduce CH$_4$ per kg of product.

4.2. Dry matter degradability, In vitro gas production and pH

Dry matter degradation is inversely related to structural carbohydrate content [43, 44], this is corroborated in treatments with species such as *S. mombin* or *B. alicastrum* which have higher digestibility and low NDF and/or FDA content or with mixtures constituted with PA, in which the opposite effect of carbohydrates was observed. Rumen degradation of DM at 48 h was improved in SM15-PP85, SM30-PP70, BA15-PP85, BA30-PP70 compared to control (*P* = <.0001). The degradation of *A. pennatula* at 24 and 48 h was affected due to its content of secondary metabolites (CT: 31.1 g kg^{-1} DM, alkaloids: ++, cyanogenic glycosides: ++++ and saponins: +) that can interfere in the degradation of carbohydrates and proteins. These findings are similar to those obtained by Albores-Moreno et al. [38] who supplemented *N. emargiata, L. leucocephala, P. piscipula* and *H. albicans* at 30% and increased CT content of a diet based on *P. purpureum*. Additionally, saponins can modify rumen fermentation by suppressing protozoa and selectively inhibiting some bacteria [45]. Secondary metabolites can make the diet less susceptible to the degradation of microorganism in the rumen due to their capacity to form complexes with the components of the diet, alter the mechanisms of degradation of the microorganism or reduce their population [46]. In addition, there is a direct correlation between DMD and gas

production, *i.e.,* the higher the digestibility, the higher the gas production rate expected. This is explained by the nutritional quality of the forages, especially by the structural carbohydrate content, *e.g.,* the diets that included *P. aculeata* presented a higher NDF content that will trigger a lower gas production. This observation agrees with authors such as Sánchez et al. [47] and Molina-Botero et al. [48].

The effect of plant secondary metabolites on maximum gas production can be observed when the fermentation parameters of *P. purpureum* are compared. Treatments with *A. pennatula, P. aculeata* and *B. simaruba* had the lowest gas productions and the highest content of secondary metabolites compared to *P. purpureum*. Regarding the CT content found in *A. pennatula* and *B. simaruba*, these have the capacity to inhibit enzymatic and microbial activity and consequently reduce fermentation [49]. Alkaloids present in *P. aculeata* can have negative effect on gas production as reported by Aguiar and Wink [50] due to a possible effect of these alkaloids on ruminal microorganisms. Aguiar and Wink [50] established that any outcome on the total gas production in the rumen can be interpreted as an effect on the microbial community. Fermentation kinetics of *A. pennatula, P. aculeata, S. mombin, B. alicastrum,* and *B. simaruba* incubated alone as single treatments were presented by Valencia-Salazar et al. [19]. The lowest maximum gas production observed by Valencia-Salazar et al. [19] was in *B. simaruba* and *A. pennatula* with 118.03 and 148.83 ml, respectively and *B. alicastrum* presented the highest maximum gas production (256.72 ml). These results were also observed in this study with *B. simaruba* and *B. alicastrum*, however, *A. pennatula* presented higher total gas production than *P. aculeata* in this study.

The observed pH values of the evaluated treatments are like those reported for tropical diets. Likewise, in the present investigation there is no correlation between pH and SCFA variables, as described by Li et al. [51]. This observation is perhaps because pH values were not below 6.0, which is a critical value for the activity of the fibrolytic microbial population and, therefore, for fiber degradation. As the degradation of the fiber is the main precursor of SCFA in the rumen, the observation described above (lack of correlation between pH and SCFA) can also be correlated with methanogenesis [52]. On the other hand, and according to Meale et al. [53] SCFA had consistent concentrations on leguminous, non-leguminous and grass species (68.7-105.5 mMol). However, the methodology used for the quantification of SCFA must be considered since secondary metabolites can affect its production [52]. According to Li et al. [51], VFA production in the rumen is directly related to the ME consumed, and this study corroborated this postulate as the BS30-PP70 treatment had a low ME contribution (6.05 MJ kg^{-1} DM) and resulted in a low content of SCFA (57.88 mMol l^{-1}), while with the BA30-PP70 treatment, the opposite occurred (7.2 MJ kg^{-1} DM and 95.42 mMol l^{-1}).

4.3. Methane production

Methane production at 24 h (mg g^{-1} DOM) was reduced 25% with the inclusion of 15% of *P. aculeata*, this can be explained due to the higher content of NDF, and the content of alkaloids as described before. Cyanogenic glycosides present in *A. pennatula* are either toxic to methanogens or impair their growth potential by reducing the availability of sulphur [54]. Several studies have shown the reduction effect on CH_4 synthesis of cyanogenic glycosides from cassava [54] however, further work is needed to understand the mode of action of these components on the rumen microbiome and on animal performance. On the other hand, diet components that contain alkaloids have showed in other studies to reduce CH_4 formation [55, 56]. Through the rumen simulation technique (RUSITEC), Khiaosa-ard et al. [56] showed that alkaloid supplementation at a low dose shifted the fermentation pathway to more propionate and less acetate and at a high dose an effect is observed on specific methanogenic archaea without affecting their abundance.

Results in CH$_4$ reduction were also observed in the screening carried out by Valencia-Salazar et al. [19] with *S. mombin*, *P. aculeata* and *B. simaruba* and high CH$_4$ production with B. *alicastrum*.). The high content of CT found *B. simaruba* can explain the lower CH$_4$ production at 24 and 48h compared to control treatment. Forages that contain CT have demonstrated to reduce CH$_4$ production both *in vivo* and *in vitro* trials [57]. Condensed tannins can reduce protozoal [58] and bacterial activity of archaea [59] by inhibiting enzyme activity, decreased degradation of substrates and direct action on the cell membrane [60]. The secondary effect of CT on CH$_4$ is the reduction in fiber digestion and fermentation, the decrease in the inter-specific transfer of hydrogens between protozoa and methanogenic archaea and the consequent increase in the concentration of propionic acid in the rumen [61]. At 48 h after incubation the inclusion of *B. simaruba* at 30% reduced CH$_4$ production at 21.39 and 25.15% for IOM and DOM, respectively. This result is similar to that obtained by Piñeiro-Vázquez et al. [62] when they included 30% of *B. simaruba* in sheep diets and concluded that the effect of CT from this species reduced CH$_4$ emissions. However, different animal species may vary in their response to the same mitigation strategy [34] and the results obtained in the present study must be verified in a *in vivo* trial using cattle. Bhatta et al. [63] found a positive correlation (R^2=0.98) in CH$_4$ production measured using the SF$_6$ tracer technique and the *in vitro* gas production technique proposed by Menke and Steingass [31] as used in this trial. Among CH$_4$ quantification techniques, the *in vitro* fermentation technique involving incubation of substrates in rumen fluid has been extensively used for the evaluation of ruminant feeds, complement standard laboratory analysis, and constitute a cheaper alternative to evaluate large number of samples [34].

In developing countries, livestock is predominantly kept on high-roughage diets with no concentrate supplementation which increases ruminal methanogenesis, converting forage tree species that contain secondary metabolites in an alternative in many parts of the tropics for the improvement of animal performance and the reduction of enteric CH$_4$ emissions [18, 45]. The extent of methane mitigation when feeding secondary metabolites, can be variable between *in vivo* and *in vitro* studies, so further research is necessary on the specific effect on rumen microorganisms and the chemical structure of these compounds.

5. Conclusions

Foliage of tree of the five species evaluated provided energy and protein ($\geq$123 g kg^{-1} DM) to the diet that may promote a better utilization of nutrients by ruminants and consequently reduce CH$_4$ emission intensity. Treatments that included *Bursera simaruba*, *Acacia pennatula* and *Parmentiera aculeata* contained considerable amounts of secondary metabolites such as condensed tannins, cyanogenic glycosides, and alkaloids. There was an inverse relation between NDF content and DM degradation, net gas and methane production and the content of secondary metabolites and methane production. However, the inclusion of these forage species had no statistical effect on the reduction of CH$_4$ emissions per unit of DM incubated or degraded at 24 and 48 h with respect to *P. purpureum*. The use of fodders locally available is an economic and viable strategy for mitigation of the environmental impact generated from tropical livestock systems.

Ethics approval: The work described here was conducted using rumen fluid obtained from fistulated cattle maintained in accordance with the requirements of Colombian law No 84/1989 and

Conflicts of Interest*: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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