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EFFECT OF THE INCLUSION OF FOLIAGE OF TROPICAL TREES ON DIGESTIBILITY, FERMENTATION, AND GAS PRODUCTION UNDER *IN VITRO* CONDITIONS

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ABSTRACT

The objective of this study was to determine the effects of different levels of inclusion of tree species in combination with fiber (corn stover) on digestibility, fermentation and total gas production under *in vitro* conditions. Different levels of inclusion of tree foliage were evaluated: + fiber, F-100%; *Brosimum alicastrum* (Ba): *Ba-10%+F-90%*, *Ba-20%+F-80%*, and *Ba-30%+F-70%; Guazuma ulmifolia* (Gu)*: Gu-10%+F-90%*, Gu*-20%+F-80%*, and *Gu-30%+F-70%; Moringa oleifera* (Mo): *Mo-10%+F-90%*, *Mo-20%+F-80%*, and *Mo-30%+F-70; Gliricidia sepium (Gs): Gs-10%+F-90%*, *Gs-20%+F-80%*, and *Gs-30%+F-70%; Piscidia piscipula* (Pp): *Pp-10%+F-90%*, *Pp-20%+F-80%*, and *Pp-30%+F-70%;* and *Viguiera dentata* (Vd): *Vd-10%+F-90%*, *Vd*-20%+*F*-80%, and *Vd*-30%+*F*-70%. The in vitro gas technique was used for 72 h. The experiment was conducted using in a randomized complete block design. Digestibility, total gas production and protozoan population were measured. The treatments *Gu*30%+70%F and Gu20%+80%F had higher crude protein content, with 12.8 and 9.79%, respectively. Regarding gas

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volume, the lowest value was recorded by *Gu10%+90%F* with 81.43%, followed by *Pp*20%+80%F with 110.01%. The lowest protozoal population was found in *Ba*30%+70%F and *Gu*20%+80%F, recording 3.12% and 3.36% log_{10}, respectively. Overall, the most suitable treatments as nutrient supply for ruminants were *Gu*30%+70%F, Gu20%+80%F, *Ba*30%+70%F, and *Gs*30%+70%F.

Keywords: emissions, fiber, ruminants, tropical resources.

INTRODUCTION

Currently, it is necessary to increase meat and milk production worldwide due to the increase in global population. According to FAO (2017), there will be 10 billion people in the world by 2050. This challenge is complex and requires more sustainable or environmentally friendly livestock production. Various studies have indicated that livestock farming contributes to 14.5% of the global methane emissions (Haque, 2018), but livestock farming represents a primary activity in undeveloped areas.

Methane is produced through the fermentation of carbohydrates in the rumen, which is determined by the type and quality of the diet offered to ruminants. When forages are in a phenological stage of maturity, the content of structural carbohydrates increases. This reduces the amount of rapidly fermentable carbohydrates, causing a reduction in digestibility. For this reason, forages with higher lignin contents usually cause greater methane production (Hristov et al., 2015; Canul et al., 2020). In tropical regions, where there is a great diversity of trees and shrubs, alternate shrub species containing secondary metabolites have been evaluated. The effects of these species have become of interest in livestock production because of their capacity to improve fermentation patterns and increase the concentration of propionic acid, which reduces the availability of hydrogen for the synthesis of CH_4. Therefore, the objective of this study is to determine the effects of the inclusion of foliage on digestibility, fermentation, and gas production under *in vitro* conditions.

MATERIALS AND METHODS

Location

The study was carried out in the Laboratory of Digestive Physiology and Nutrition of the Conkal Technological Institute,Yucatan State, México (20 ° 05 'N and 89 ° 32' 0). The climate of the region is AW0 (according to the Köppen classification, modified by García, 1973), considered as a warm subhumid climate, with an average precipitation of 1100 mm per year and a maximum temperature of 36°C and a minimum of 16°C. There is a rainy season, from June to December followed by a dry season, from January to May. The flora is characterized by low deciduous forest with secondary vegetation where the Fabaceae family predominates (Gutiérrez-Báez and Zamora- Crescencio, 2012). The composition of the soil corresponds to the Litosol type in the northern and northwest and Rendzina regions (García, 1973).

Collection of plant material

Foliage of *Brosimum alicastrum, Guazuma ulmifolia, Moringa oleifera, Gliricidia Sepium, Piscidia piscipula, Viguiera dentata* and the fiber (corn stover) were collected in the dry season (April and May). The collected foliage was subsequently placed in paper bags and transferred to the laboratory for dehydration at 60 °C until constant weight was obtained. Dehydration was performed in a forced-air oven and then the samples were ground to a particle size of 1 mm with a Thomas-Wiley® mill. The collected foliage comes from biomass and protein banks available in the Conkal Technological Institute. In the livestock research area, these protein banks have constant maintenance (cleaning, pruning and irrigation). In this case, the plants were of an age of regrowth of 60 days. Twenty plants were used to obtain the material used in this and other subsequent experiments.

Ruminal inoculum

The ruminal fluid was taken from four sheep using an esophageal probe (Ramos-Morales et al., 2014). The donors had an average live weight of 30±1 kg. The animals were fed for a period of four weeks with a cellulose-based diet offering freely available fresh African star grass (*Cynodon nlemfuensis*) and supplemented with a concentrated feed containing 16% crude protein (CP) and 2.3 Mcal/kg dry matter (DM). The feed was formulated in a proportion of 60% grains and 40% forage in the form of fiber. The ruminal fluid was obtained before feeding. Once the ruminal fluid was extracted, it was placed in insulated thermos at 39 °C under anaerobic conditions and subsequently transported to the laboratory.

In vitro gas production

The in vitro gas production technique of Menke and Steingass (1988) modified by

Theodorou et al. (1994) was used. Amber flasks (120 mL) were used, and 1.00 g of DM was added with different levels of inclusion of tree foliage + fiber (F 100%). *Brosimum alicastrum* (Ba): *Ba-10%+F-90%*, *Ba-20%+F-80%*, and *Ba-30%+F-70%; Guazuma ulmifolia (Gu): Gu-10%+F-90%*, Gu-*20%+F-80%*, and *Gu-30%+F-70%; Moringa oleifera (Mo): Mo-10%+F-90%*, *Mo-20%+F-80%*, and *Mo-30%+F-70%; Gliricidia sepium (Gs): Gs-10%+F-90%*, *Gs-20%+F-80%*, and *Gs-30%+F-70%*; *Piscidia piscipula (Pp)*: *Pp-10%+F-90%*, *Pp-20%+F-80%*, and *Pp-30%+F-70%;* and *Viguiera dentata* (Vd): *Vd-10%+F-90%*, *Vd*-20%+*F*-80%, and *Vd* -30%+ *F*-70%. Each treatment had four replicates per block. Subsequently, the preparation of the mineral solution, resarzurin, and reducing solution was carried out. For this, 90 mL of the previously filtered ruminal inoculum was added under a constant flow of CO_2. Once filled with 90 mL of inoculum, all the vials were hermetically sealed with plastic caps and aluminum rings. Subsequently, the flasks were incubated in a water bath at 39 °C. The pressure of the gas contained in each bottle was recorded with a manometer at 0, 2, 4, 6, 8, 12, 16, 20, 24, 30, 36, 42, 48, 60 and 72 h of incubation and subsequently transformed to a volume of gas with the linear regression equation V = (P + 0.019) (0.024), where V is the volume of gas produced and P is the pressure recorded on the manometer.

Rumen protozoan population

The protozoan count was performed using the technique described by Rosales et al. (1999), which consists of mixing 1 mL of ruminal fluid and 1 mL of MSF solution (35 mL/L formaldehyde, 0.14 mM NaCl, 0.92 mM methyl green) and centrifuging at 2000 rpm for 20 min. Subsequently, an aliquot was taken and placed in a Neubauer chamber and observed using a microscope (Leica-DM500) with a 40X objective. The number of ciliated protozoa was reported as $\log_{10}$ per mL of ruminal fluid and estimated with the following formula: Number of cells per mL = [(n1 + n2 + n3 + n4 + n5) / 5] /0.001 $mm^3 \times \mathbf{10}^3 \times \mathbf{d}$; where: n1...n5= number of protozoa per large square, and d = dilution factor (Rosales et al., 1999). The classification of protozoa was carried out at the genus level according to Ogimoto and Imai (1981).

Experimental design and statistical analysis

The experimental design was randomized blocks repeated in time (Cochran and Cox, 1990). Three series of experimental runs were carried out at three different times. Each run was considered as a block and four replicates per foliage were used in each run (block). *In vitro* fermentation data (Vm, S, L, RF, MF, LF), *in vitro* dry matter digestibility (IVDMD), *in vitro* organic matter digestibility (IVOMD) were analyzed using the SAS PROC GLM procedure (SAS Inst. Inc., Cary, North Carolina, 2004). The comparison of means was performed using the Tukey test at P≤0.05.

The following mathematical model was used:

$Yij = \mu + \eta i + \alpha j + \varepsilon ij$ (Montgomery, 2013).

where:

Y ij = Score of subject i in treatment j
μ = Global average of all experimental data
η i = μ i - μ = Effect associated with block i
αj = μ j - μ = Effect of treatment j
εij = Experimental error associated with subject i under treatment j.

RESULT AND DISCUSSION

The chemical composition of the different treatments had similar results in terms of DM content. The CP content of Gu30%+70%F and Gu20%+80%F reached the highest values of 12.8 and 9.79, respectively, followed by Pp30%+70%F with 9.27. Conversely, the treatment consisting of 100% fiber recorded the lowest DM content, reaching 5.3%. These results agree with those of Ávila-serrano (2020), who evaluated different levels of tree species incorporating *Cynodon nlemfuensis* and reported similar values with *Guazuma ulmifolia* at 15 and 30%. However, the results obtained with the inclusion of the *Moringa oleifera* species in the same study were higher than those obtained in the present study.

The neutral detergent fiber (NDF) fractions of the different levels of inclusion ranged from 55.01 to 68.46% of DM. However, the 100% fiber treatment resulted in a DM content of 72%, being the highest value compared to the tree species, which agrees with the results of Avila-Serrano (2020). In this sense, a study carried out by Jiménez-Santiago (2019) reported that the species *Gliricidia sepium* had a value of 35.5% in NDF and 25% in ADF. In addition, Valencia-Salazar (2022) conducted a study on different tree species and found that *Brosimum alicastrum* at 15 and 30% resulted in NDF and FDA values ranging from 54.1 to 49.8 and from 32.5 to 31.5%, respectively. These results are lower compared to those obtained in our study since NDF values increased at different levels of inclusion of tree species. This can be explained by the fact that tropical trees have a higher content of protein and a lower proportion of cell wall compared to grasses, and thus their phenological state

and period of the year in which the species are collected are important factors that determine their chemical composition (Edwards et al., 2012).

Total gas production and its fractional values under *in vitro* conditions of the different levels of inclusion of the foliage of the tree species are shown in Figs. 1, 2, 3 and in Tables 1 and 2. There was a decrease in the maximum volume variable (MV) in Gu10%+90%F (Fig.1), Mo20%+80%F (Fig. 2), and Gs30% +70%F (Fig. 3) at 10%, 20% and 30% inclusion, respectively. However, no differences were observed between the species, fermentation rate (S) or lag phase (L), nor were any differences found after 72 h of incubation. However, there were differences between the tree species in the initial phase of the fractional volume at 8 h, with the highest volume being recorded with Mo30%+70%F with 199.04%, followed by Vd30%+70%F with 184.43%. The other species and fiber treatments behaved similarly, with Gu10%+90%F recording the lowest value of 81.43%, followed by Pp20%+80%F with 110.01%. A similar study conducted by Avila-Serrano (2020) evaluated the effects of incorporating trees in C. *nlemfuensis* diets under *in vitro* conditions using three different levels of inclusion of M. oleifera, *L. leucocephala*, *G. ulmifolia* at 15, 30 and 45%, and 100% *C. nlemfuensis*. The author found differences at 72 hours, reporting that the lowest gas production was recorded with the *L. leucocephala species* at 45%, while higher levels were obtained with the *C. nlemfuensis* diet. It should be noted that differences were found in the first 8 h of fermentation in our study. This is related to the fermentation of microorganisms as well as the chemical composition of the diet (Gaviria, 2015). Likewise, Cuartas (2015) mentions that gas production is also related to NDF degradation, and this relationship is usually linear because more gas is produced in forages with higher NDF content. In the present study, greater gas production was obtained in the treatments 100% fiber, Mo10%+90%F, Gu10%+90%F, presenting the highest NDF percentage. These data coincide with the study carried out by Rodríguez (2014), who reported that *L. leucocephala* and *M. oleifera* resulted in higher gas production after 72 hours. This may also be explained by organic matter (OM) availability and the content of carbohydrates, such as monosaccharides and starches, which can be easily degraded by rumen microorganisms (Jiménez, 2019). Conversely, a low concentration of the soluble fraction of the food reduces the amount of gas produced per unit (Candelaria-Martínez, 2022).

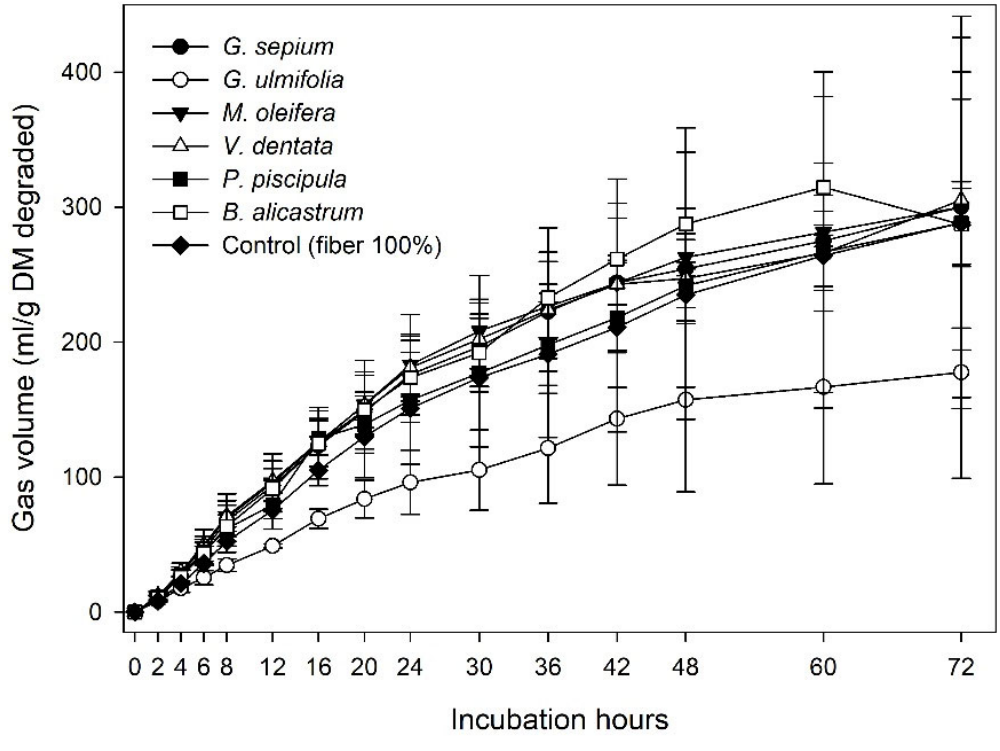

Figure 1. Total gas production at 10% inclusion of tree species foliage

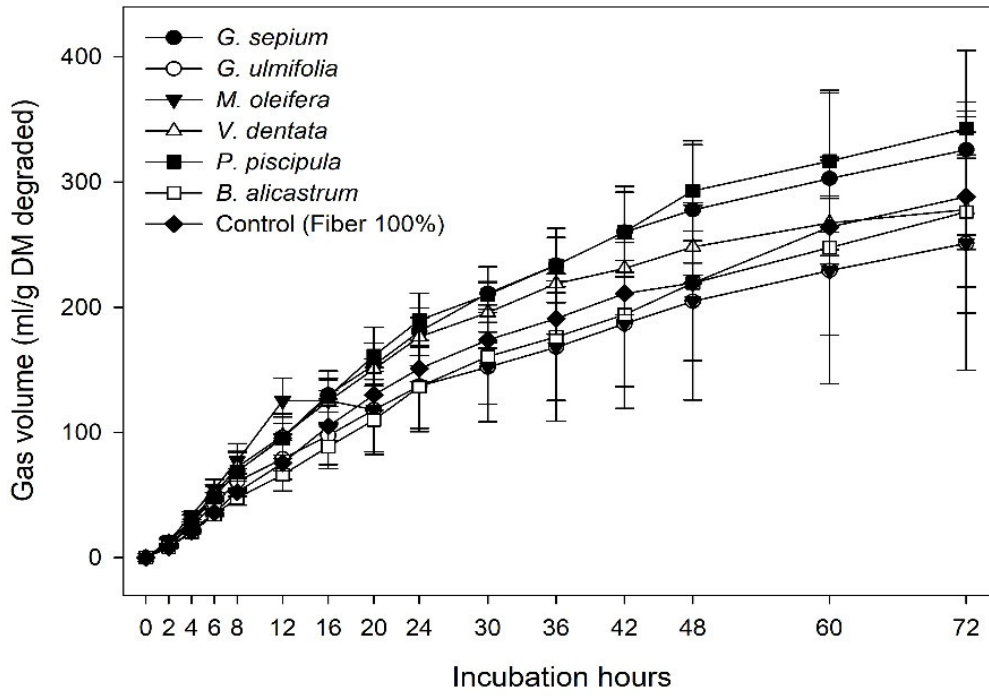

Figure 2. Total gas production at 20% inclusion of tree species foliage

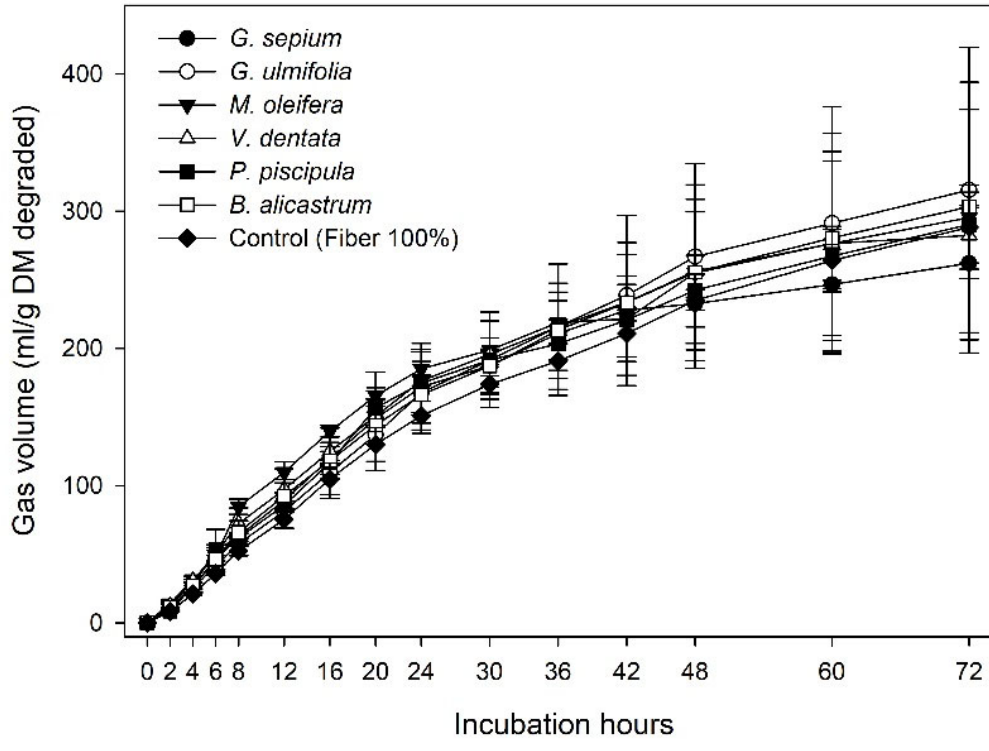

Figure 3. Total gas production at 30% inclusion of tree species foliage

Table 1. Chemical composition (% of DM) of different inclusion levels of tropical tree foliage under *in vitro* gas conditions.

Species	DM	CP	NDF	ADF
Legumes				
Gs10%+90%F	91.3	6.01	68.46	44.63
Gs20%+80%F	90.8	7.03	64.93	42.52
Gs30%+70%F	90.5	8.05	61.40	40.41
Pp10%+90%F	92.3	6.42	67.60	44.13
Pp20%+80%F	92.2	7.84	63.20	41.52
Pp30%+70%F	92.2	9.27	58.80	38.90
Not legumes				
Ba10%+90%F	90.4	6.01	68.00	44.51
Ba20%+80%F	90.2	7.02	65.65	42.28
Ba30%+70%F	90.0	8.04	62.48	40.05
Gu10%+90%F	92.0	7.39	66.33	43.55
Gu20%+80%F	90.9	9.79	60.67	40.36
Gu30%+70%F	90.7	12.18	55.01	37.17
Mo10%+90%F	90.2	5.68	68.40	44.51
Mo20%+80%F	90.1	6.36	64.80	42.27
Mo30%+70%F	89.8	7.04	61.20	40.03
Vd10%+90%F	89.5	5.81	66.46	43.51
Vd20%+80%F	89.3	6.62	60.93	40.28
Vd30%+70%F	89.1	7.43	55.40	37.05
Fiber				
F (100%)	93.87	5.30	72.10	46.75

DM = dry matter, CP = crude protein, NDF = neutral detergent fiber, ADF = acid detergent fiber

IVDMD, IVOMD, and digestibility at 72 hours of the different inclusion levels of tropical trees were all greater than 41.94% (Table 3), being in agreement with the results of Rodríguez (2014) and Valencia-Salazar (2022). The facts that the tree species under study have a low content of carbohydrates, which are easily fermented, and that there is a direct relationship between IVDMD and gas production can account for this situation. That is, the higher the digestibility, the higher the expected gas production rate (Molina-Botero, 2020). The different levels of inclusion of the tree species did not present differences in the pH at 72 hours of incubation since these values were higher than 6 pH, being suitable for a good fermentation process and, consequently, good development and activity. Rumen microorganisms, especially cellulolytic batteries, are responsible for the degradability of cell walls (Cobos et al., 2018; Araiza-Rosales et al., 2021).

The total protozoan population, including holotrichs, at the different inclusion levels was similar between the treatments. However, the highest inclusion of Gs10%+90%F and Gs20%+80%F resulted in an entodinium population of 3.86 and 3.85 (Table 4), followed by Pp20%+90%F and Gs30%+ 70%F with 3.75 $\log_{10}$. The lowest populations were found in Ba30%+70%F and Gu20%+80%F, with 3.12 and 3. 36 $\log_{10}$, respectively. On the other hand, the largest total population of protozoans was in the Control, Gs10%+90%F, and Vd10%+90%F, with 4.57, 4.50 and 4.41 $\log_{10}$, respectively. The reduction in the population of protozoans of the genus entodinium may be related to the highest inclusion levels, as well as the presence of secondary metabolites such as tannins and saponins. In this sense, Pérez-Can et al. (2020) reported that *B. aliscastrum* and *G. ulmifolia* contain condensed tannins and saponins that influence protozoan populations. Similarly, Velez et al. (2014) mentioned that saponins decrease methanogenesis indirectly by reducing the availability of H_2 and the protozoan population,

Table 2. Total gas production parameters of different inclusion levels of foliage of tropical trees under *in vitro* gas conditions.

Species	MV (mL g^{-1})	S (h^{-1})	L (h)
Legumes			
Gs10%+90%F	281.9 a	0.036 a	3.11 a
Gs 20%+80%F	312.9 a	0.031 a	2.94 a
Gs30%+70%F	243.2 a	0.043 a	2.85 to
Pp10%+90%F	272.8 a	0.029 a	5.49 a
Pp20%+80%F	162.1a _	0.049 to	5.31 a
Pp30%+70%F	265.2a _	0.034 a	1.82 to
Not legumes			
Ba10%+90%F	326.4a _	0.028 to	3.79 a
Ba 20%+80%F	87.7 a	0.018 to	- 5.72 a
Ba30%+70%F	289.3 a	0.045 to	3.15 a
Gu10%+90%F	175.9 a	0.011 to	8.81 a
Gu20%+80%F	241.3 a	0.025 to	-0.05 to
Gu30%+70%F	309.9a _	0.032 a	4.29 a
Mo10%+90%F	42.6 a	0.022 to	- 10.55 a
Mo20%+80%F	268.6 a	0.046 a	1.52 to
Mo30%+70%F	261.4a _	0.043 a	0.99 to
Vd10%+90%F	292.1a _	0.022 to	2.76 a
Vd20%+80%F	324.1a _	0.028 to	2.57 a
Vd30%+70%F	265.6 a	0.059 to	1.83 to
Fiber			
Fiber (100%)	272.7 a	0.029 a	3.28 a
HE	95,091	0.011	47.93
P- value	0.1979	0.3167	0.4723

MV = Maximum volume; S = fermentation rate; L = lag phase; SE= standard error of the mean. [a, b, c, d] Means within rows with different letters indicate differences (P<0.05).

which are found in an endosymbiosis with methanogenic archaea whose relationship can generate 9 to 37% of the total CH_4 emissions. In addition, saponins can favor an increase in the concentration of propionate and reduce the production of acetate and butyrate, which results in a lower production of hydrogen and thus a reduction in the production of methane (Min et al., 2014). A study conducted by Valencia-Salazar et al. (2022) reported the absence of secondary metabolites in the species *B. alicastrum*. It should be noted that the content of these metabolites depends on many factors. Ku-Vera et al. (2014) found that condensed tannins cause a reduction in ruminal fiber degradation, which produces a decrease in hydrogen release.

CONCLUSIONS

Overall, the combination of Ba30%+70%F, Gu20%+80%F, Gu30%+70%F, and Gs30%+70%F had the capacity to reduce gas production and the population of protozoans as well as improve diet quality. Those combinations could be recommended for ruminant feed as they could be considered environmentally friendly. Further studies under on-farm conditions are recommended.

Conflict of interest: We certify that there is no conflict of interest with any financial organization with respect to the material discussed in the manuscript.

Table 3. Fractional volume and digestibility of DM and OM of different inclusion levels of foliage of tropical trees under *in vitro* gas conditions.

Species	V0-8 (mL g $^{-1}$)	V8-24 (mL g $^{-1}$)	V24-72 (mL g ^{1})	IVDMD (%)	IVOMD (%)	pH
Legumes						
Gs10%+90%F	158.33 ab	176.38 a	359.5 a	50.72 a	44.07 a	6.2
Gs20%+80%F	161.21 ab	181.80 a	420.20 a	62.77 a	57.45 a	6.2
Gs30%+70%F	148.46 ab	176.38 a	261.2a _	53.15 a	46.52 a	6.2
Pp10%+90%F	124.20 ab	169.68 a	364.3 a	41.94 a	34.13 a	6.3
Pp20%+80%F	110.01 ab	108.94 a	160.6 a	50.32 a	44.82 a	6.3
Pp30%+70%F	145.38 ab	147.29 a	367.6 a	45.62 a	39.56 a	6.3
Not legumes						
Ba10%+90%F	148.67 ab	187.22 a	475.3 a	51.36 a	45.09 a	6.3
Ba 20%+80%F	111.86 ab	144.30 a	404.2a _	53.30 a.m.	47.42 a	6.3
Ba30%+70%F	154.84 ab	162.83 a	396.8 a	60.82 a	54.88 a	6.2
Gu10%+90%F	81.43b _	99.81 a	235.7 a	47.70 to	41.38 a	6.2
Gu20%+80%F	142.70 ab	123.91 a	330 a	48.82 a	42.13 a	6.1
Gu30%+70%F	135.30 ab	178.80 a	427.8 a	65.31 a	59.81 a	6.2
Mo10%+90%F	163.68 ab	184.36 a	338.4a _	53.27 a	47.17 a	6.2
Mo20%+80%F	181.15 ab	149.72 a	314.5 a	52.78 a	46.83 a	6.2
Mo30%+70%F	199.04 a	163.12a _	281.7 a	60.12 a	54.23 a	6.2
Vd10%+90%F	143.32 ab	154.99 a	380 to	48.20 a	42.64 a	6.3
Vd 20%+80%F	161.83 ab	196.20 a	440 a	52.35 a	45.10 a	6.2
Vd30%+70%F	183.42 ab	157.13a _	334.6 a	52.63 a	47.40 a	6.3
Fiber						
Fiber (100%)	122.76 ab	160.27 a	397.8 a	48.89 a	45.10 a	6.2
HE	18.77	26.91	83.57	4.54	4.74	
P- value	0.0010	0.1851	0.0002	0.1293	.0001	

Fractional volume of 8, 24 and 72h; SE= standard error of the mean. [a, b, c, d] Means within rows with different letters indicate differences (P<0.05).

Table 4. Protozoan population (log ^{10} /ml) at different inclusion levels of foliage of tropical trees under *in vitro* gas conditions.

Species	Holotriches (log_{10})	Entodiniomorphs (log_{10})	Total protozoa (log_{10})
Legumes			
Gs10%+90%F	1.5	3.86 ^{to}	4.50ab _
Gs20%+80%F	1.5	3.85 ^{to}	4.37 ABC
Gs30%+70%F	1.5	3.75ab _	4.20 abc
Pp10%+90%F	1.5	3.54 abcd	4.38 abc
Pp20%+80%F	1.5	3.75ab _	4.10 abc
Pp30%+70%F	1.5	3.52 abcd	4.04 acb
Not legumes			
Ba10%+90%F	1.5	3.42 bcde	4.39 ABC
Ba20%+80%F	1.5	3.64 abc	4.01 abc
Ba30%+70%F	1.5	3.12e _	3.78c _
Gu10%+90%F	1.5	3.52 abcd	4.30 abc
Gu20%+80%F	1.5	3.22 of	4.07 abc
Gu30%+70%F	1.5	3.36 cde	4.09 abc

Mo10%+90%F	1.5	3.56 abcd	4.29 ABC
Mo20%+80%F	1.5	3.52 abcd	4.09 abc
Mo30%+70%F	1.5	3.47 bcde	3.96 abc
Vd10%+90%F	1.5	3.59 abcd	4.41 abc
Vd 20%+80%F	1.5	3.56 abcd	4.04 abc
Vd30%+70%F	1.5	3.43 bcde	3.91 BC
Fiber			
Fiber (100%)	1.5	3.71 abc	4.25 abc
HE	0.500	0.065	0.113
P- value	1.0000	<.0001	0.0045

SE= standard error of the mean. [a, b, c, d] Means within rows with different letters indicate differences (P<0.05).

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