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Effect of the addition of *Enterolobium cyclocarpum* pods and *Gliricidia sepium* forage to *Brachiaria brizantha* on dry matter degradation, volatile fatty acid concentration, and *in vitro* methane production

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Abstract

The purpose of this study was to determine the in vitro fermentation, and methane (CH_4) production in the grass *Brachiaria brizantha* (B) alone or when mixed with *Gliricidia sepium* forage (G) and/or *Enterolobium cyclocarpum* pods (E). Theses substrates were incubated in the following proportions: B100 (B100%), B85E15 (B85% + E15%), B85G15 (B85% + G15%), B85GE15 (B85% + G7.5% + E7.5%) and B70GE30 (B70% + G15% + E15%). Dry matter degradation (DMD), volatile fatty acids (VFA) concentration, and CH_4 production were measured at 12, 24, and 48 hours of incubation. Experimental design was a randomized complete block. At 48 h incubation DMD ranged between 46.5 and 51.2% (*P=0.0015*). The lowest cumulative gas production (CGP) was observed in B85E15 and B85G15 (160 mL CGP/g organic matter, on average). At 48 h, B85G15 and B100 produced 28.8 and 30.2 mg CH4/g DMD, respectively, while B85E15 or the mixtures, 33.5 mg CH_4/g DMD, on average (*P≤0.05*). B85E15 and B70G30 had the highest concentration of total VFA (*P≤0.05*). Results showed that B85E15 and B70GE30 favor DMD and increased total production of VFA and CH_4 at 48 h. Supplementing livestock feed with legume forages and pods allows improves the nutritional quality of the diet and the fermentation patterns.

Keywords: *In vitro* fermentation; tropical pods; legumes; gas production.

Introduction

Livestock production must undertake a series of changes to reduce its dependence on external inputs, in addition to increasing economic benefits and reducing its carbon footprint (Peters et al. 2013; Lerner et al. 2017). Nonetheless, solutions to reduce environmental pollution due to extensive grazing have economic, technical, and practical constraints (Clark et al. 2013). Yet there are cost-effective approaches, such as supplementing diets with pods of native legumes and leguminous forages, which provide cattle and sheep with rumen fermentable nitrogen and metabolizable energy (ME) (Saminathan et al. 2016). Emission of greenhouse gases and emission intensities from cattle can be reduced by correspondingly increasing the efficiency of feed utilization and having lower emissions per MJ of gross energy consumed. As a result of high ME and crude protein content and low neutral detergent fiber content (Barahona et al. 2014), CH_4 emissions per unit of dry matter intake and per unit of product are reduced (Chará et al. 2017).

A promising option for dietary inclusion is the legume species *Gliricidia sepium* (Jacq.) Steud. (quickstick or *matarratón in Spanish*), a deciduous shrub that can reach 2–15 m in height and is widely distributed in the tropical regions (Cuervo-Jiménez et al. 2017). This species has different uses in livestock production, agroforestry, reforestation, and ecological restoration (Calle y Murgueitio 2011a). Many studies have also show *G. sepium* to have high potential as animal feed (García et al. 2008; Asaolu et al. 2014; Molina et al. 2019 a).

Enterolobium cyclocarpum (Jacq.) Griseb. is another promising species, this tree is ideal to re-forest lands, while also producing wood; its pods can be used to feed humans and animals, due to its good crude protein content, and it also reduces ruminal CH_4 emissions (Calle and Murgueitio 2011b; Molina et al. 2019 a, 2019 b). To further characterize the effect of these strategies on animal nutrition and enteric CH_4 emissions, this study aimed at determining the nutritional quality, *in vitro* fermentation, and CH_4 production in the *in vitro* incubation of *Brachiaria brizantha* grass with *E. cyclocarpum* pods and/or the foliage of leguminous species *G. sepium.*

Materials and Methods

Forage and Pods

Pods from *E. cyclocarpum* and forage from *G. sepium* and *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf. were collected in the municipalities of Mérida and Tizimín, in the state of Yucatán, México. The region is located at 10 masl, with temperature ranges between 25 and 35°C, and an average annual rainfall of 984.4 mm (INEGI, 2017). Re-growth ages for the grass and leguminous species were 60 and 90 days, respectively. The pods used for the current study were collected in the summer of 2016.

Nutritional Content and Dry Matter Quantification

After collecting the legume, pod and grass samples, they were dried in a forced-air oven at 55°C for 72 h, to determine moisture content, according to method 6496 of the International Organization for Standardization (ISO, 1999). Samples were ground and sifted through a 1-mm sieve using a Wiley Laboratory Mill (Thomas®, USA) for further analysis in the Forage Quality and Animal Nutrition Laboratory at the International Center for Tropical

Agriculture (CIAT), located in Palmira (Valle del Cauca, Colombia) and certified by the FAO-IAG proficiency test of feed constituents 2017. Crude protein (CP) content was determined using the Kjeldahl method (AOAC 984.13; 1990), as well as the neutral detergent fiber (NDF) and acid detergent fiber (ADF), following the sequential method described by Van Soest et al. (1991) in ISO standard 13906 (2008) and AOAC 973.18 (2005), respectively. Ash content was determined by incineration in a muffle furnace (AOAC, 2005) and the gross energy (GE) content, by the bomb calorimeter method, ISO 9831 (1998). Total phenol and phenolic tannin content were determined according to the method described by Makkar (2003), while the condensed tannin (CT) content was assessed following the proanthocyanidins method (Porter et al. 1985), and the saponin content, using the hemolysis test (Oleszek 1990) in the Nutrition Laboratories at UADY and CINVESTAV-IPN, Mexico.

Gas Production Kinetics

The *in vitro* gas production technique was carried out in the Forage Quality and Animal Nutrition Laboratory of CIAT- Colombia, according to the methodology suggested by Theodorou et al. (1994). After determining dry matter (DM) content, a series of treatments were formulated using *B. brizantha* (B), *G. sepium* (G) and *E. cyclocarpum* (E): B100 (*B. brizantha 100%)*, B85E15 (*B. brizantha 85% + E. cyclocarpum 15%)*, B85G15 (*B. brizantha 85% + G. sepium 15%)*, B85GE15 (*B. brizantha* 85% + *G. sepium 7.5% + E. cyclocarpum 7.5%*), and B70GE30 (*B. brizantha* 70% + *G. sepium 15% + E. cyclocarpum 15%)*. Samples of 1 g of each treatment were incubated at 39°C in hermetically sealed glass bottles containing 85 mL of the mineral mix and buffer solution, plus 4 mL of a reducing solution, and 10 mL of ruminal fluid. The latter was obtained from the solid phase of the rumen from three rumen cannulated *Brahman* calves (400 kg of live weight, on average), which consumed forages (mainly *Cynodon plectostachyus*), mineralized salt, and had *ad libitum* access to water. Thermos flasks were used to transport the ruminal fluid to the laboratory, where it was filtered through three layers of sterile gauze. During incubation, ruminal fluid was kept under anaerobic conditions, with a constant flow of CO_2 and at an optimal temperature (39°C).

At five times post inoculation (6, 12, 24, 36, and 48 h), pressure (psi) measurements were taken of the gas present in the headspace, using a pressure transducer (Sper Scientific®, USA) connected to a digital readout (Lutron Electronic Enterprise Co®. Ltd., Taiwan), a hypodermic needle, and a plastic syringe to measure and obtain the volume until the transducer reading reached zero. The total gas volume collected at each time of reading was corrected, according to the equation obtained by Gaviria et al. (*in press*), for tropical forages: $Y= 0.00052\ x^2 + 0.43175\ x - 0.23656$ ($R^2 = 0.985$)*;* where *Y* is the gas volume produced by each *x* pressure unit. Gas production data were fitted to the Gompertz non-linear mathematical model (Curve Expert 1.3® Software. Hyams 2013) proposed by Lavrenčič et al. (1998) to find values in non-measured hourly values, in addition to conversion to biological parameters, as described by Naranjo et al. (2016).

Methane Production

A sub-sample (10 mL) of total gas production at 12, 24, and 48 h post-inoculation was taken to quantify CH_4 concentration using a gas chromatographer (Shimadzu GC-2014, Shimadzu®, Japan), following Molina et al. (2013). This analysis was done in the Greenhouse Gases Laboratory of the CIAT.

Dry Matter Disappearance and Volatile Fatty Acids Contect

Dry matter degradation (DMD) at different incubation times (*i.e.* 12, 24, and 48 h) was quantified by filtering the content of each flask with a vacuum pump and glass-fiber filter. Then, filters were dried in a forced-air oven stove (Memmert® UF 750, Schwabach, Germany) at 105°C for 24 h, and then weighed on an analytical balance (Mettler Toledo®, USA). The value thus obtained was corrected for the blank flask weight (*i.e.* flask without forage sample, but containing buffer solution, reducing agent, and ruminal fluid) and DMD calculated as the ratio of the amount of incubated sample. Subsequently, 1 mL of filtered ruminal fluid was taken and added to 4 mL of 25% metaphosphoric acid to be stored at 4°C for further determination of volatile fatty acids (VFA). VFA concentration was assessed by high-performance liquid chromatography (HPLC, Shimadzu® 20A Series), using an Aminex HPX-87H column (300 mm × 7.8 mm) (Bio-Rad Laboratories®, CA, USA), with an ultra violet/visible detector (UV/Vis, SPD-20AV) at a 50°C temperature and a wavelength of 210 nm, plus, the mobile phase (H_2SO_4 0.005 M) was used with a volume flow rate of 0.7 mL/min. The injection volume for each sample was 20 µL, and retention time was 150 min/sample.

Experimental Design and Statistical Analysis

The five response variables (DMD, gas kinetics, Gompertz parameters, VFA concentration and CH_4 production) were distributed as a randomized complete block design, where each treatment had three replicates at each time

the readings were taken (three times after incubation: 12, 24, and 48 h) and 3 inoculums, the latter being the blocking factor. Means were compared by the Tukey test ($P<0.05$). The statistical model was:

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

Where: Y_{ij} are the observations of the response variables for treatment i and block j; μ is the overall mean; τ_i is the effect of the i-th treatment; β_j is the effect of the j-th block; and ε_{ij} is the random error of treatment i in block j. All analyses were done with the SAS® version 9.4 software (SAS Institute Inc®, 2012).

Results

Nutritional Content

Substitution of 15% of the grass *B. brizantha* with a combination of *G. sepium* and *E. cyclocarpum* (B85GE15) increased CP content by approximately 16 g/kg DM; this difference was doubled when comparing the treatment B70GE30 to the forage only treatment (87.2 *vs.* 55.1 g CP/kg DM for B70GE30 and B100, respectively, Table 1). A similar a difference was observed between NDF and ADF contents of the grass only and mixtures treatments, particularly when only *E. cyclocarpum* was included (B85E15). Addition of *G. sepium* contributed substantially to cellulose/lignin content (452 g ADF/kg DM), gross energy (18 MJ/kg DM), and ash content (99.7 g/kg DM). Leguminous species and the pods being evaluated in this study contained condensed tannins (CT; 46 and 41 mg CT/g DM, respectively), besides contributing with 17 and 27 mg saponins/g DM.

Table 1. Gross energy and proximate composition of *E. cyclocarpum* pods, *G. sepium* foliage, *B. brizantha* grass, and the evaluated treatments

	Enterolobium cyclocarpum	*Gliricidia sepium*	Treatments				
			B100	B85E15	B85G15	B85GE15	B70GE30
Crude Protein (g/kg DM)	154.2	170.3	55.1	70.0	72.4	71.2	87.2
Neutral Detergent Fiber (g/kg DM)	289.5	575.4	757	687	730	709	660
Acid Detergent Fiber (g/kg DM)	205.6	451.8	466	427	464	446	425
Gross Energy (MJ/kg DM)	17.7	18.3	16.4	16.6	16.7	16.6	16.9
Ash (g/kg DM)	37.8	99.7	88.8	81.2	90.4	85.8	82.8
Total Phenols (mg/g DM)	16.77	6.03	6.11	7.71	6.1	6.9	7.7
Phenolic Tannins (mg/g DM)	10.81	0.80	1.76	3.12	1.62	2.37	2.97
Condensed Tannins (mg/g DM)	41.3	45.9	0	6.20	6.89	6.54	13.08
Saponins (mg/g DM)	27	17	0	4.05	2.55	3.3	6.6

Abbreviations: B100 (*B. brizantha* 100%), B85E15 (*B. brizantha* 85% + *E. cyclocarpum* 15%), B85G15 (*B. brizantha* 85% + *G. sepium* 15%), B85GE15 (*B. brizantha* 85% + *G. sepium* 7.5% + *E. cyclocarpum* 7.5%), and B70GE30 (*B. brizantha* 70% + *G. sepium* 15% + *E. cyclocarpum* 15%).

Dry Matter Degradation

During the first 12 hours, DMD of the grass species and the mixtures with *E. cyclocarpum* and/or *G. sepium* ranged between 21 and 29.5% ($P=0.0004$. Table 2). At 24 h it had increased in all the treatments but was noticeably highest ($P=0.0001$) in B85GE15 (44.3%) compared to B100 (36%). However, by 48 h DMD in B85GE15 had only increased 5% (total = 49.3%), while in B100 it had increased by 10% to reach 48.4, and in B85E15 it had increased 12% to 51.2%. The largest differences in DMD at 48 h were observed between B85G15 (46.48%) and B85E15 (51.24%; $P=0.0015$).

Table 2. Dry matter degradability (%) of *Brachiaria brizantha* alone or mixed with *Gliricidia sepium* forage and/or *Enterolobium cyclocarpum* pods.

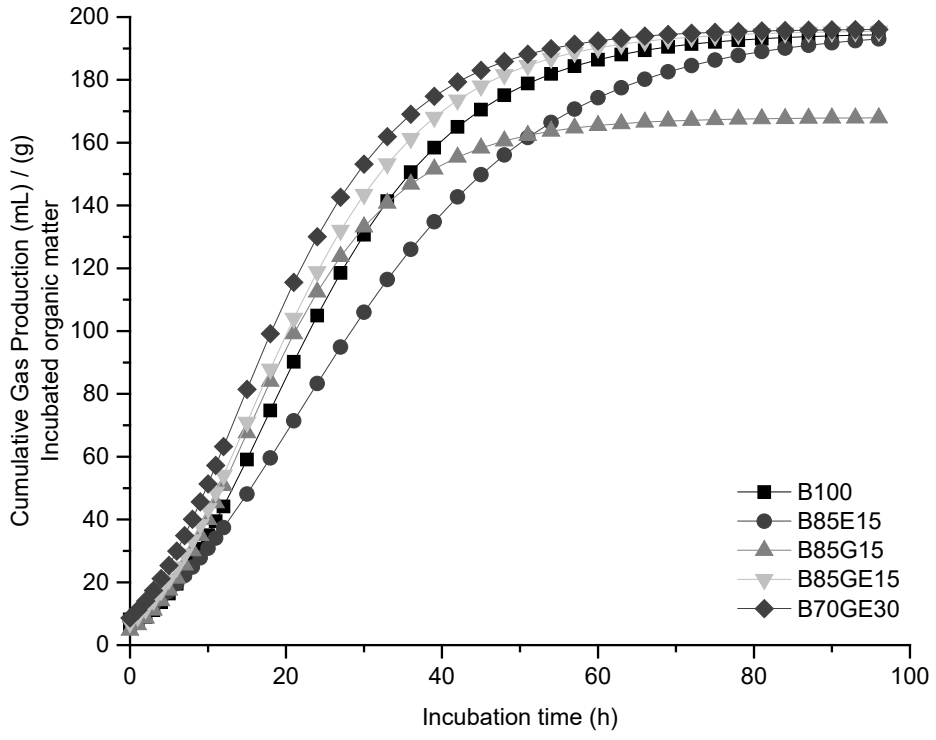
	Treatments						
Time	B100	B85E15	B85G15	B85GE15	B70GE30	MSE	p-value
12 h	21.98 [b]	27.54 [a]	21.70 [b]	29.48 [a]	29.29 [a]	1.54	0.0004
24 h	36.10 [d]	41.01 [b]	38.32 [cd]	44.29 [a]	40.11 [bc]	0.75	0.0001
48 h	48.37 [bc]	51.24 [a]	46.48 [c]	49.33 [ab]	50.01 [ab]	0.87	0.0015

[a,b,c d] Different letter superscripts in the same row indicate statistical difference ($P < 0.05$). *Abbreviations:* MSE = mean squared error B100 (*Brachiaria brizantha* 100%), B85E15 (*B. brizantha* 85% + *E. cyclocarpum* 15%), B85G15 (*B. brizantha* 85% + *G. sepium* 15%), B85GE15 (*B. brizantha* 85% + *G. sepium* 7.5% + *E. cyclocarpum* 7.5%), and B70GE30 (*B. brizantha* 70% + *G. sepium* 15% + *E. cyclocarpum* 15%).

Gas Production

The gas kinetics results indicate that after 48 h treatments B85E15 and B85G15 exhibited the lowest cumulative gas production (CGP) rate (average = 160 mL CGP/g OM; Fig 1), followed by treatment B100 (175 mL CGP/g OM) and the mixtures with *G. sepium* plus *E. cyclocarpum* (184 mL CGP/g OM). In the subsequent hours (49 to 96 h), in treatment B85G15 CGP increased 7 mL/g OM, in contrast to the single grass species and G85E15, which increased their production in 19 and 37 mL CGP/g OM, respectively.

Fig 1. Cumulative Gas Production (CGP, mL/ g Incubated organic matter) in *Brachiaria brizantha* alone or mixed in different proportions with *Gliricidia sepium* forage and/or *Enterolobium cyclocarpum* pods.



According to the Gompertz model, maximum CPG rates were similar among treatments ($P=0.2430$; Table 3). Parameters *a, b, c*, HPI, GPI and TMP did not differ among treatments. At the inflexion point, 72 mL of gas had been produced during the first 15.5 h ($P \geq 0.05$) with a maximum gas production rate per hour of approximately 5.4 mL. Lag phase of B100 was 12 times greater than that of B70GE30 (3.86 and 0.32 h, respectively. $P=0.03$)

Table 3. Parameters of the Gompertz model for the gas production observed during the incubation of the forages evaluated (*Brachiaria brizantha*, *Gliricidia sepium* and *Enterolobium cyclocarpum*).

	Treatments						
Parameters	B100	B85E15	B85G15	B85GE15	B70GE30	MSE	p-value
a	195.3	208.8	174.8	200.9	203.4	17.48	0.2430
b	1.29	1.05	1.25	1.12	1.04	0.108	0.0618
c	0.07	0.07	0.09	0.07	0.07	0.016	0.7639
TIP	17.53	15.3	15.1	15.4	14.1	1.859	0.3260
GIP	71.85	76.8	64.3	73.9	74.8	6.430	0.2436
MRGP	5.26	5.25	5.41	5.35	5.51	0.649	0.9853
LP	3.86[a]	0.65[ab]	2.78[ab]	1.57[ab]	0.32 [b]	1.835	0.0270

[a,b] Different letter superscripts in the same row indicate statistical difference ($P < 0.05$). *Abbreviations:* MSE = mean squared error, B100 (*B. brizantha* 100%), B85E15 (*B. brizantha* 85%+ *E. cyclocarpum* 15%), B85G15 (*B. brizantha* 85% + *G. sepium* 15%), B85GE15 (*B. brizantha* 85% + *G. sepium* 7.5% + *E. cyclocarpum* 7.5%), and B70GE30 (*B. brizantha* 70% + *G. sepium* 15% + *E. cyclocarpum* 15%). *a*=maximum gas production (mL); *b*= difference between initial gas and final gas at an x time; *c*= specific gas accumulation rate; TIP = time to the inflection point, h; GIP = gas at the inflection point, mL; MRGP = maximum rate of gas production, mL/h. LP= lag phase, h

Methane

Net CH_4 production differed among the treatments under evaluation (Table 4). The lowest CH_4 net values were observed when incubating *G. sepium* (15%) plus *B. brizantha*, or the single grass (B100), while the highest CH_4 production was reported for B85E15 or the mixtures of pods and the leguminous species. The relationship between CH_4 production and DMD at 12 and 48 h showed that treatment B85GE15 produced between 1.1 to 4.7 times more CH_4 than B85G15 ($P \leq 0.05$).

Table 4. Methane produced during the *in vitro* incubation of the *Brachiaria brizantha*, *Gliricidia sepium* and *Enterolobium cyclocarpum* treatments evaluated.

	Treatments						
	B100	B85E15	B85G15	B85GE15	B70GE30	MSE	p-value
Methane (mg)							
12	2.11 [b]	2.26 [b]	0.59 [c]	3.25 [a]	2.43 [b]	0.191	0.0001
24	5.84 [c]	7.74 [a]	6.76 [b]	6.68 [bc]	6.79 [bc]	0.488	0.0173
48	14.6 [c]	16.8 [ab]	13.1 [d]	16.0 [b]	17.7 [a]	0.644	0.0020
Methane (mg CH_4/ g DMD)							
12	7.50 [b]	8.34 [b]	2.31 [c]	10.75 [a]	9.11 [ab]	0.815	0.0001
24	16.2	18.9	17.7	15.1	16.9	1.033	0.0541
48	30.2 [c]	33.0 [b]	28.8 [d]	32.4 [b]	35.2 [a]	1.342	0.0295

[a,b,c d] Different letter superscripts in the same row indicate statistical difference ($P < 0.05$). Abbreviations: MSE = mean squared error, B100 (*B. brizantha* 100%), B85E15 (*B. brizantha* 85% + *E. cyclocarpum* 15%), B85G15 (*B. brizantha* 85% + *G. sepium* 15%), B85GE15 (*B. brizantha* 85% + *G. sepium* 7.5% + *E. cyclocarpum* 7.5%), and B70GE30 (*B. brizantha* 70% + *G. sepium* 15% + *E. cyclocarpum* 15%).

Volatile Fatty Acid Concentration

Regarding total VFA concentration, treatments B85E15 and B70GE30 showed the highest values, followed by B85GE15 and B85G15, while the incubation of the single grass showed the lowest concentrations ($P \leq 0.05$. Table

5). The proportion of acetic acid did not differ among treatments after 12 h of incubation. However, at 24 and 48 h, treatment B85G15 showed the highest proportions (64.4 mol/100 mol on average), while the lowest values were reported for B70GE30 and B85E15 (63 mol/100 mol on average, $P \leq 0.05$). Proportions of propionic acid ranged between 26.9 and 30.6 mol/100 mol and only showed differences between treatments at 48 h. Similar to propionic acid, butyric acid concentrations only differed after 48 h of incubation, and the maximum values were observed in B70GE30, followed by B85E15. These two treatments were nearly two units above the single grass species ($P \leq 0.05$)

Table 5. VFA concentration of treatments incubated with different proportions of *Brachiaria brizantha*, *Gliricidia sepium* and *Enterolobium cyclocarpum* for 12, 24, and 48 hours.

	Treatments						
	B100	B85E15	B85G15	B85GE15	B70GE30	MSE	p-value
Total Volatile Fatty Acids (mmol/mL)							
12 h	22.04 ^{c}	26.21 ^{a}	23.79 ^{b}	24.38 ^{b}	27.33 ^{a}	0.77	0.0014
24 h	33.34 ^{c}	37.51 ^{a}	34.18 ^{bc}	35.08 ^{bc}	36.24 ^{ab}	1.28	0.0443
48 h	43.67 ^{b}	46.01 ^{a}	42.77 ^{b}	43.21 ^{b}	45.45 ^{a}	0.53	0.0003
Acetic Acid (mol/100 mol)							
12 h	66.60	64.28	65.35	66.49	64.91	0.76	0.0665
24 h	64.52 ^{a}	63.69 ^{b}	65.02 ^{a}	63.86 ^{b}	63.77 ^{b}	0.34	0.0128
48 h	62.70 ^{b}	62.35 ^{b}	63.89 a	64.18 ^{a}	62.76 ^{b}	0.25	0.0001
Propionic Acid (mol/100 mol)							
12 h	27.17	27.53	26.90	27.24	27.14	0.24	0.2775
24 h	28.62	27.84	27.89	28.73	28.13	0.50	0.3230
48 h	30.58 ^{a}	29.41 ^{b}	28.80 ^{bc}	27.88 ^{c}	28.49 ^{bc}	0.38	0.0003
Butyric Acid (mol/100 mol)							
12 h	5.71	7.76	7.27	5.81	7.54	0.77	0.0654
24 h	6.53	8.17	6.76	7.09	7.79	0.75	0.2205
48 h	6.46 ^{d}	8.00 ^{ab}	7.04 ^{cd}	7.68 ^{bc}	8.50 ^{a}	0.42	0.0056
Acetic : Propionic Acid Ratio							
12 h	2.45	2.33	2.43	2.44	2.39	0.04	0.1069
24 h	2.25	2.29	2.33	2.22	2.27	0.03	0.0775
48 h	2.05 ^{d}	2.12 ^{cd}	2.22 ^{ab}	2.30 ^{a}	2.20 ^{bc}	0.03	0.0001

^{a,b,c,d} Different letter superscripts in the same row indicate statistical difference ($P < 0.05$). *Abbreviations*: MSE = mean squared error; B100 (*B. brizantha* 100%), B85E15 (*B. brizantha* 85% + *E. cyclocarpum* 15%), B85G15 (*B. brizantha* 85% + *G. sepium* 15%), B85GE15 (*B. brizantha* 85% + *G. sepium* 7.5% + *E. cyclocarpum* 7.5%), and B70GE30 (*B. brizantha* 70% + *G. sepium* 15% + *E. cyclocarpum* 15%).

Discussion

Addition of *G. sepium* and/or *E. cyclocarpum* to *B. brizantha* improved the quality of this forage grass. This coincides with previous studies in which inclusion of *E. cyclocarpum* pods collected from the same region as in the present study raised CP to 146±21 g/kg DM, NDF to 273±27 g/kg DM, and ADF to 193±19 g/kg DM, providing additional nutritional input in animal feed (Piñeiro-Vázquez et al. 2013; Albores-Moreno et al. 2017). On the other hand, Balogun et al. (1998), Anele et al. (2009), Molina et al. (2013), and Asaolu et al. (2014) concluded that *G. sepium* contributed 218±33 g CP/kg DM to diets or treatments. Additionally, *G. sepium* is characterized by low NDF and ADF contents (407±50 and 261±37 g/kg DM respectively; Monforte-Briceño et al. 2005; Anele et al. 2009; Molina et al. 2013). Nonetheless, in the current study, the age of this leguminous species adversely affected the content of structural carbohydrates.

Authors such as Balogun et al. (1998), Kaitho et al. (1998), Chaverri and Cicció (2015), and Rira et al. (2015) described *G. sepium* as a plant rich in anti-nutritional compounds, such as CT (2 and 121 CT g/kg DM) and to a lesser extent, saponins (17 g saponins/kg DM), flavonoids (0.45 mg/mL), alkaloids, mimosine, or coumarins. Regarding *E. cyclocarpum*, Hess et al. (2003), Albores-Moreno et al. (2017), and Lazos-Balbuena et al. (*in press*) found that the pods had a high content of saponins (36.6±10 mg/g DM). However, Pizzani et al. (2006) lists other anti-nutritional compounds in these pods (total phenols: 40 mg/g DM, CT: 11.8 mg/g DM, and steroids: 83 mg/g DM).

The grass alone and in combination with 15% of the leguminous species had the lowest DMD values, contrary to the results obtained by substituting the same amount with pods. This is closely related to the quality of forage (Getachew et al. 2004; Narváez and Lascano 2004), *i.e.*, the high soluble carbohydrate content in the pods favored DM degradation (Torres-Salado et al. 2018), in contrast to the contribution of structural carbohydrates, which were mainly from the grass and leguminous species. Indeed, Babayemi (2006), Piñeiro-Vázquez et al. (2013), and Barrientos-Ramírez et al. (2015), reported *E. cyclocarpum* ruminal *brizantha* degradability values between 60 and 87%, and Molina et al. (2019 a, 2019 b), in *in situ* experiments, observed that *E. cyclocarpum* pods contained the largest rapidly degrading fraction, while *B. brizantha, Sorghum halepense, Pennisetum maximum*, and *G. sepium* showed the lowest values, just like the potentially degradable fraction and the effective degradability.

The B100 and B85G15 treatments did not differ in terms of DMD. This contrasts with findings in which replacement of 10, 20, and 30% *Dichanthium aristatum* with *G. sepium* produced an increase from 35.1% DMD in the control to 53.3% DMD at the highest inclusion level (Molina et al. 2013). Likewise, Alayon et al. (1998), Mpairwe et al. (1998), Ondiek et al. (1999), and Asaolu et al. (2014) agree in pointing out the potential of these leguminous species when associated to tropical grasses (*Cynodon nlemfuensis, Chloris gayana, Megathyrsus maximus*, and *Pennisetum purpureum*). In this study, the presence of anti-nutritional compounds, such as CT or saponins, when substituting up to a 30% of the grass species with *E. cyclocarpum* and/or *G. sepium* did not adversely affect DMD. This contrasts with reports stating that tannins and saponins can directly and/or indirectly decrease DMD by affecting ruminal microorganisms or by encapsulating nutrients such as CP or fiber, thus preventing their degradation in the rumen (Hess et al. 2003; Archimède et al. 2015). However, the extent of degradation depends on anti-nutritional compound type and concentration, diet follow-up, and microbial community structure and adaptation to these compounds (Patra and Saxena 2009).

Cumulative gas production (CGP) and DMD values were highest in the treatments containing a mixture of *B. brizantha* with *E. cyclocarpum* and *G. sepium*, suggesting a synergistic effect. Other studies have found the opposite in the form of an additive negative effect *in vitro* when tannic acid and tannins (*Shinopsis* ssp.) were mixed with saponins (*Quillaja* ssp.) (Makkar et al. 1995), although this effect may have differed due to the amount and type of secondary compounds present. The positive results observed in the present study with the mixtures may be due to their having had a better balance between protein-fiber and energy, which would have helped the microorganism community to more efficiently degrade DM and thus produce more gas. Protein and fiber contents are reported to have positive and negative correlations ($R^2 \geq 0.50$) with gas production at 48 h incubation (Seresinhe et al. 2012; Molina et al. *submitted*); the results for the B85G15 treatment apparently support this claim. However, these results differ from those of Molina et al. (2013), who observed that including 205 mL of *G. sepium* at 96 h increased gas production by 26% (148 *vs.* 187 CGP mL/g DM). Meanwhile, Asaolu et al. (2014) compared tropical leguminous species and showed that incubating only *G. sepium* and in a proportion of 40%, plus 60% of *Megathyrsus maximus*, produced 80 and 125 mL/g DM, respectively.

In vivo experiments in cattle have shown a trend towards the reduction of CH_4 when *E. cyclocarpum* pods and/or the leguminous species *G. sepium* are included in the diets of cattle fed low-quality tropical grasses (Molina et al. 2019 a; 2019 b). However, the lowest *in vitro* net CH_4 values were reported in the B100 and B85G15, contrary to the findings for the mixtures containing pods and the leguminous species or the substitution of a 15% with *E. cyclocarpum*. This discrepancy may be explained by failure of the bacterial community to adapt to the substrate (Macome et al. 2017), or overall forage nutrient content (Kennedy and Charmley, 2012). This differs with the findings of Bhatta et al. (2007) and Danielsson et al. (2017), who claimed that *in vitro* technique is a good option to perform a first assessment of additives or diets.

Methane production is highly related to the nutrient content of the forage (Kennedy and Charmley, 2012), perhaps the low CH_4 values reported with B85G15 may be due to the low DMD, as reported by Purcell et al. (2011), who observed that CH_4 emissions/g DM incubated decreased with an increase in forage age, and fiber content. Furthermore, the presence of secondary compounds from the leguminous species could explain the

differences between B85G15 and B100. Secondary metabolites in legume species are known to eliminate protozoan microorganisms associated with methanogens (Monforte-Briceño et al. 2005; Delgado et al. 2010; Patra et al. 2017). In addition, according to type and molecular weight of the secondary compounds is the effect on digestive enzymes and microbes in the rumen (Belete and Abubeker 2018; Rira et al. 2019). In the present results, both B85GE15 and B70GE30 had higher secondary metabolite content than B100, perhaps providing them with a better nutrient balance and thus higher DMD and greater methane production. This would coincide with a report that net CH_4 production directly correlates to forage degradability and CGP ($R^2 \geq 0.86$) (Molina et al. *submitted*). Moreover, the saponin content in B85E15 may have lowered CH_4 production since *E. cyclocarpum* is characterized by high saponin content. A number of studies have also reported lower CH_4 production (10 and 28 mg CH_4/g DMD) in treatments including *G. sepium* (Babayemi 2007; Meale et al. 2012; Seresinhe et al. 2012; Asaolu et al. 2014). Although, in contrast to the present findings, when *G. sepium* was mixed with a grass species in two of these studies CH_4 production did not decline compared to the control (100% grass species) (Molina et al. 2013; Asaolu et al. 2014).

Production of VFA is directly related to DMD (Meale et al. 2012). According to Navarro-Villa et al. (2011), the greater the digestibility, the greater the VFA production, and consequently, the lower the quality of the forage the lower is the production of VFA (Rira et al. 2015). This was observed in the current experiment, in which B85E15 and B70GE30 treatments had the highest VFA production values, while incubating the single grass produced the lower VFA concentrations. The VFA concentrations in the treatments containing *G. sepium* were similar to previous reports for this species (acetic acid: 66.2± 4; propionic acid: 21±0.8; butyrate: 6.9±0.1) (Soliva et al. 2008; Meale et al. 2012; Rira et al. 2015). In addition, Judd and Kohn (2018) demonstrated that factors such as inoculum, substrate, addition or not of acetate to the incubation bottle affect gas production and VFA Profile.

Studies of the relationship between VFA concentrations and CH_4 production found that CH_4 synthesis, like that of propionate and butyrate, captures H_2 as a result of the oxidation reactions in pyruvate formation (Moss et al. 2000; Martin et al. 2010; Albores-Moreno et al. 2019). Hence, an inverse relationship exists between the amount of propionate or butyrate and CH_4 emissions. This was not clearly observed in all the treatments evaluated in the present study. Inconsistencies like these were also reported in a previous study in which a low propionate to CH_4 ratio (26%) was probably due to multiple factors such as accumulation of alternative final products or refermentation of VFA, among others (Robinson et al. 2010). Of note is that the B100 treatment exhibited the highest propionic acid concentration, which diminished hydrogen availability to methanogens and thus lowered CH_4 synthesis, apparently confirming the inverse relationship between VFA and CH_4 production.

From a broader perspective, cattle production represents a key source of income and subsistence for humans, but is simultaneously responsible for about 12% of all anthropogenic GHG emissions worldwide (Westhoek et al. 2011) and 80% of all agricultural non-CO_2 emissions (Tubiello et al. 2013). In conjunction with previous research (Navas- Camacho et al. 1993; Moscoso et al. 1995; Molina et al. 2019 a; 2019 b;), the present findings suggest that native legume pods are a viable alternative feed that may increase milk and meat production while lowering methane emissions and improving feed nutritional quality. They provide scientific support for implementation of a sustainable intensification process in which more milk and meat can be produced more quickly in less area (considering open grazing systems) utilizing forage mixtures of grass and tree-legumes which can also deliver ecosystem services. Examples of these services include carbon sequestration in the form of biomass in trees and soils, as well as atmospheric nitrogen fixation by the forage legumes. The former can aid in reducing atmospheric carbon levels and the latter in decreasing dependence on external nutrient inputs, simultaneously nurturing system forage species and important microorganisms that contribute to improving soil health parameters. The issues discussed above are graphically illustrated in Appendix A, which summarizes the *in vitro* results obtained in the present investigation and how these results relate to the *in vivo* findings reported by Molina et al. (2019 a; 2019 b), who provided equal proportions of this fruit and legume in the diet of heifers.

Conclusions

E. cyclocarpum and *G. sepium* contributed with crude protein, condensed tannins, and saponins to the diet. On top of this, the grass and leguminous species contributed structural carbohydrates. *In vitro* results showed that including 15% of *E. cyclocarpum* and the mixture of 30% leguminous species plus pods to diets based on the grass species *B. brizantha* favors *in vitro* dry matter digestibility and increased total VFA and CH_4 production after 48 h incubation.

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Compliance with ethical standards: 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 following protocol approved by the Ethics Committee of the International Center for Tropical Agriculture, assuring the welfare of animals used in the experiment.

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Appendix A: Nutritional quality (crude protein, neutral and acid detergent fiber and anti-nutritional compounds), ruminal fermentation (degradability, gas production and total volatile fatty acids), methane production, weight gain and emissions intensity *in vitro* and *in vivo* Molina et al.(2019b), experiments.