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 (CH₄) 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 acid (VFA) concentration, and CH₄ production were measured at 12, 24, and 48 h 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 CH₄/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 CH₄ 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 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), CH4 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 and Murgueito 2011a). Many studies have also shown G. sepium to have high potential as animal feed (Garcia et al. 2008; Asaolu et al. 2014; Molina et al. 2019a).

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 CH4 emissions (Calle and Murgueito 2011b; Molina et al. 2019a, b). To further characterize the effect of these strategies on animal nutrition and enteric CH4 emissions, this study aimed at determining the nutritional quality, in vitro fermentation, and CH4 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 (2005b), respectively. Ash content was determined by incineration in a muffle furnace (AOAC 2005a) 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 sapogenin 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 plectostachys), 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 CO2 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: \[ Y = 0.00052x^2 + 0.43175x - 0.23656 \] (\( R^2 = 0.985 \)), 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 CH₄ 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 CIAT.

Dry matter disappearance and volatile fatty acid Content

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 (VFAs). 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 (H₂SO₄ 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 CH₄ 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:

\[ Y_{ij} = \mu + \tau_i + \beta_j + \epsilon_{ij} \]

where \( Y_{ij} \) are the observations of the response variables for treatment \( i \) and block \( j \); \( \mu \) is the overall mean; \( \tau_i \) is the effect of the \( i \)th treatment; \( \beta_j \) is the effect of the \( j \)th block; and \( \epsilon_{ij} \) 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 mixture 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.

Dry matter degradation

During the first 12 h, 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%; \( P = 0.0015 \)).

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.
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 inflection 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 ($P = 0.03$).

**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 B85G15 produced between 1.1 to 4.7 times more CH$_4$ than B85G15 ($P \leq 0.05$).

**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 ($P \leq 0.05$).
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), Kaithe 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. (in press) found that the pods had a high

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*)

| Parameters | Treatments          | 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        |                     | 15.3 | 15.1   | 15.1   | 15.4    | 14.1    | 1.859  | 0.3260  |
| GIP        |                     | 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         |                     | 3.86a| 0.65ab | 2.78ab | 1.57ab  | 0.32 b  | 1.835  | 0.0270  |

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)
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).

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

| Treatments | MSE | P value |
|------------|-----|---------|
| B100 | B85E15 | B85G15 | B85GE15 | B70GE30 |
| Total volatile fatty acids (mmol/mL) | | | | | |
| 12 h | 22.04 c | 26.21 a | 23.79 b | 24.38 b | 27.33 a |
| 24 h | 33.34 c | 37.51 a | 34.18 bc | 35.08 bc | 36.24 ab |
| 48 h | 43.67 b | 46.01 a | 42.77 b | 43.21 b | 45.45 a |
| Acetic acid (mol/100 mol) | | | | | |
| 12 h | 66.60 | 64.28 | 65.35 | 66.49 | 64.91 |
| 24 h | 64.52 a | 63.69 b | 65.02 a | 63.86 b | 63.77 b |
| 48 h | 62.70 b | 62.35 b | 63.89 a | 64.18 a | 62.76 b |
| Propionic acid (mol/100 mol) | | | | | |
| 12 h | 27.17 | 27.53 | 26.90 | 27.24 | 27.14 |
| 24 h | 28.62 | 27.84 | 27.89 | 28.73 | 28.13 |
| 48 h | 30.58 a | 29.41 b | 28.80 bc | 27.88 c | 28.49 bc |
| Butyric acid (mol/100 mol) | | | | | |
| 12 h | 5.71 | 7.76 | 7.27 | 5.81 | 7.54 |
| 24 h | 6.53 | 8.17 | 6.76 | 7.09 | 7.79 |
| 48 h | 6.46 d | 8.00 ab | 7.04 cd | 7.68 bc | 8.50 a |
| Acetic:propionic acid ratio | | | | | |
| 12 h | 2.45 | 2.33 | 2.43 | 2.44 | 2.39 |
| 24 h | 2.25 | 2.29 | 2.33 | 2.22 | 2.27 |
| 48 h | 2.05 d | 2.12 cd | 2.22 ab | 2.30 a | 2.20 bc |

Different letters 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%)
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. (2019a, 2019b), 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), Mpaware 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 tannin 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. 2019a, 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 B85GE15 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. 2019a, 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 Fig. 2, 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. (2019a, 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.

**Conflict of interest**  The authors declare that they have no conflict of interest.
Appendix

Fig. 2  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.
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