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Effects of tannins and saponins contained in foliage of *Gliricidia sepium* and pods of *Enterolobium cyclocarpum* on fermentation, methane emissions and rumen microbial population in crossbred heifers

Short title: Methane mitigation in cattle fed foliage and pods of tropical legumes.

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Abstract

Incorporation of foliage and pods of tropical legumes in ruminant rations is an alternative to mitigate enteric methane emissions. The objective of this research was to evaluate the effect of adding increasing levels of ground pods of *Enterolobium cyclocarpum* (Jacq.) Griseb. mixed with foliage of *Gliricidia sepium* (Jacq.) Steud. on emissions of ruminal methane (CH_4), volatile fatty acid proportions, rumen pH and microbial population in cattle. Four

heifers (218 ±18 kg LW) were fed (13 days) 0, 15, 30, and 45% of pods of *E. cyclocarpum* mixed with foliage of *G. sepium*, which were supplemented to a basal ration of *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf. Data were analyzed as a 4×4 Latin square. After three days of CH_4 measurements in open-circuit respiration chambers, rumen fluid was collected to determine volatile fatty acid (VFA) molar proportions and quantify the microbial population. Samples of ration ingredients, refusals and feces were collected to evaluate nutrient composition. Foliage and pods of legumes provided crude protein (CP), condensed tannins (CT) and saponins, while grass was characterized by higher concentrations of neutral detergent fiber (NDF). Dry matter intake (DMI) was 5.35 kg/day on average (P=0.272). Apparent fiber digestibility was reduced (81 g/kg) and digestible CP intake (13 g/kg) increased when *E. cyclocarpum* mixed with *G. sepium* in rations were given (P<0.05). Incorporation of legume foliage and pods had a linear effect on molar proportions of butyric acid and acetic to propionic acid ratio (P<0.05). Methane production, expressed on basis to digestible dry matter intake (DDMI), ranged between 43.22 and 49.94 g/kg DDMI (P=0.131) and when CH_4 was related to digestible CP (347 *vs*. 413 g CH_4 /kg DCP) or annual weight gains (0.30 *vs*. 0.38 kg CH_4/kg weight gain, P<0.001) there were differences between the *E. cyclocarpum* mixed with *G. sepium* rations compared to the control treatment, respectively. Rumen population of total bacteria, methanogenic archaea, and total protozoa was not affected by the increasing levels of condensed tannins and saponins in rations (P>0.05). Substitution of 15 and 30% of pods of *E. cyclocarpum* mixed with foliage of *G. sepium* in the ration, decreases annual methane emissions per unit product, without affecting dry matter intake or rumen microbial population, on the contrary, digestible CP intake and animal productivity increased due to supply of CP, CT and saponins.

Keywords: Cattle, greenhouse gas, secondary compounds, tropical feed.

Introduction

In spite of its contributions to food security and employment for rural families, agriculture has an environmental impact (Hyland et al., 2016), since it contributes between 20 and 35% of the total greenhouse gas emissions (Jayanegara et al., 2015). Livestock produces greenhouse gases such as nitrous oxide and methane; the latter has a warming potential 28 times higher than carbon dioxide and an increase of 86% has been predicted to occur over the next twenty years (Myhre et al., 2013). Production of methane during carbohydrate fermentation in the rumen represents an inefficiency in energy utilization at the whole-animal level (Millen et al., 2016; Patra & Lalhriatpuii, 2016) which negatively affects the economy of producers. Therefore, it is important to identify methods and strategies to find a balance between minimizing environmental impacts and intensifying animal productivity to meet requirements of animal protein by the world population (Roehe et al., 2016; Rojas-Downing et al., 2017; Richards et al., 2018) in the years to come.

In the search for new knowledge that highlights the importance of intensive silvopastoral systems as modalities of land use contributing to mitigation and adaptation to climate change, several authors have shown that including legume foliages and pods in the ration is an alternative to reduce greenhouse gas emissions in the tropics (Barahona et al., 2014; Ku-Vera et al., 2014). Those forages species commonly used in this systems usually contain condensed tannins and saponins that alter the numbers of rumen microbes such as archaea, protozoa, and fibrolytic bacteria affecting fermentatives processes such as to reducer fiber digestibility (Bodas et al., 2012), while increasing the supply of protein and energy available to the animal (Patra and Saxena, 2009; Saminathan et al., 2016). Thus, the inclusion of local forage resources such as foliage of *Gliricidia sepium* and pods of *Enterolobium cyclocarpum* to a basal ration of grass is a viable means to provide protein and secondary compounds (*e.g.,* CT and triterpenic saponins; Rastrelli et al., 1999; Delgado

et al., 2012) in practical ruminant rations. Hence, some other reports indicate an additional effect in the reduction of net methane emissions when *G. sepium* and *E. cyclocarpum* are independently included in the diets The emission reductions were calculated between 5 and 10% when compared to the emissions of similar diets lacking these ingredients (Rira et al., 2015; Albores-Moreno et al., 2017). The objective of this research was to evaluate the effect of increasing levels of pods of *Enterolobium cyclocarpum* mixed with foliage of *Gliricidia sepium* in a ration based on tropical grass on emissions of enteric methane, rumen fermentation, and microbial population of crossbred heifers.

Materials and methods

Location

The study was conducted at the Laboratory of Climate Change and Livestock Production of the Faculty of Veterinary Medicine and Animal Science, University of Yucatan, located at 21°15' North latitude and 83°32' West longitude, in Merida, Mexico. Average temperature and annual rainfall in the region are 26.2°C and 1014 mm, respectively (INEGI, 2017).

Animals and experimental design

This experiment was conducted in accordance with a protocol approved by the Ethics Committee of Faculty of Veterinary Medicine and Animal Science for the welfare of experimental animals.

Four crossbred (*Bos taurus* X *Bos indicus*) heifers with an initial weight of 218 ±18 kg (12 months old) were used. Experimental rations were fed to each animal in a 4 x 4 Latin-square design. Each period of the experiment consisted of a 13-day adaptation period, 3 days of CH_4 measurements, and 3 additional days to collect ruminal fluid. An additional rumen

cleaning period consisting of 10 days between feeding periods was given, the ration fed during the cleaning period consisted of 79.9% *Brachiaria brizantha* (Hochst. ex A. Rich.) hay and 20.1% of balanced mix based on soybean meal, wheat bran, cane molasses and minerals (10.7% CP and 9.2 MJ ME/kg DM).

Experimental rations and feed intake

Pods of *Enterolobium cyclocarpum* (Jacq.) Griseb. and foliage of *Gliricidia sepium* (Jacq.) Steud. were collected in Merida and Tizimin (Yucatan), respectively, in the June and July 2016. Pods were dried at 55°C in a forced air oven for three days, while the leaves of *G. sepium* were air-dried; both materials were ground to a particle size of 2 mm in a hammer mill (Azteca®, Monterrey, Nuevo León, México) to be fed to the animals at a later date. Leaves of *G. sepium* were harvested at an age of regrowth of 60 days.

Four levels of incorporation of foliage of *G. sepium* mixed with pods of *E. cyclocarpum* (both in equal proportions) were evaluated (0, 15, 30, and 45% DM basis). Basal ration was *Brachiaria brizantha* (Hochst. ex A. Rich.) Stapf. hay (90 days regrowth) supplemented with soybean meal, wheat bran, cane molasses, and 65 g/day of a commercial mineral mixture. Table 1 and 2 shows proportions and nutritional composition of ingredients and experimental rations. Feeding regimes met the maintenance and growth requirements of heifers, according to the National Research Council (NRC, 2016). Heifers were randomly assigned to four experimental rations and housed in individual pens with free access to water. Daily feed intake was calculated as the difference between the amount fed and that refused the following day. Feces were collected daily in trays which allowed for the separation of urine from feces prior to weighing. Cattle were weighed at the beginning and end of each period on a one-ton scale (Revuelta®, DF. Mexico).

[Insert Table 1 around here]

[Insert Table 2 around here]

Chemical analysis and digestibility

Samples of ration ingredients, refusals and feces were pooled by period and frozen at -4°C before being analyzed. All materials were dried at 55°C for 48 h in a forced-air oven to calculate dry matter (DM), ground to pass a 1-mm screen in a Wiley® mill and analyzed in the Forage Quality and Animal Nutrition Laboratory, CIAT (Colombia) for ash (4 h at 500°C; AOAC, 2005: method 942.05) and crude protein (CP=N×6.25; Kjeldahl AN 3001 FOSS; AOAC, 1990: method 984.14). Neutral detergent fiber and acid detergent fiber content were determined using the method proposed by Goering and Van Soest (1970), adapted to an Ankom Fibre Analyzer AN 3805 (Ankom® Technology Corp. USA). Gross energy was analyzed following the procedure described in ISO 9831.1998. Total phenol and phenols tannin contents were determined using the Folin-Ciocalteu's method (Makkar, 2003) following the equation: Y=0.0391x + 0.0246; where Y is the amount of tannins and x is the standard (R^2 = 0.9962) obtained from the standard tannic acid solution (Sigma-Aldrich® MO, USA). Condensed tannins were measured using the Proanthocyanidins method (Porter et al., 1986) with the butanol-HCl reagent. Content of saponins was determined through the method proposed by Oleszek, (1990) (haemolytic micro-method test) with the Saponin Quiallaja sp standard (Sigma-Aldrich® MO, USA). Digestibility of DM was determined from the total feces output (Schneider and Flatt, 1975) during days 17-19 of each period. Feces were weighed and a sub-sample (10%) was taken, then dried (55°C) and ground (1 mm) for further analysis.

***in situ* incubation of feed ingredients**

Three crossbred (*Bos taurus* X *Bos indicus*) mature cows each fitted with a plastisol rumen cannula were used to estimate *in situ* degradation (Ørskov et al., 1980) of feed ingredients. Cattle were fed a diet of 74.9% of *Megathyrsus maximus* hay and 24.1% of balanced feed

based on soybean meal, ground corn, urea and cane molasses (11.1% CP and 8.6 MJ/kg DM) with free access to water. In each incubation time, five grams of each forage, pods and grains were weighed by triplicate into nylon bags (7 x 14 cm; 53-micron pore size). Bags for zero time determination were soaked in tap water for 5 minutes while the other bags were removed from the rumen at 3, 6, 12, 24, 36, 48 hours post-incubation (for soybean meal), at 72 h for *G. sepium*, *E. cyclocarpum,* and wheat bran, and at 96 h for *B. brizantha* hay to be washed, dried (55°C for 72 h), and weighed. Percentage of rumen degradability (Y) at time (t, h) of incubation was obtained from the equation $Y_t = a + b \times (1-e^{-c*t})$ proposed by Ørskov and McDonald (1979) using the SAS 9.4 nonlinear regression model (SAS Inst. Inc., 2012). For interpretation of the parameters: *'a'* is the soluble and rapidly degradable fraction, *'b*' is the slowly degradable fraction, *'c'* is the constant rate of disappearance (/h), and *'t'* is the time of incubation (h) *'e'* is the base of natural logarithms (2.71828). Effective rumen degradability (ERD) was calculated as $ERD\ (g/kg\ DM) = (a + b \times c) / (c + K_p)$, where the parameters *a, b, and c*, have the aforementioned meaning and $'K_p'$ is the forage passage rate, which is 0.05 for hour for ruminants fed at low levels of production (NRC, 2001).

Methane production

Methane production was measured in two open-circuit respiration chambers, details on its description, operation, and calibration are given in Canul-Solis et al. (2017) and Valencia-Salazar et al. (2018). Respiration chambers were made of insulated fenestrated panels mounted on a profiled floor, incorporating airlocks for entry and feeding. Total volume of chambers was 9.38 m^3, and air was extracted by mass flowmeters (Sable Systems International®, NV, USA) set at 250 L/min. Temperature and humidity were controlled with air-conditioning units set at 23±1°C and 55±10% relative humidity, respectively. Chambers were operated under negative pressure (-275 Pa). Methane emission was measured with an infrared CH_4 analyser (MA-10 Sable Systems International®, USA) for three consecutive

days for approximately 23 h per day after the cows were entered into the chambers individually. Respiration chambers were previously calibrated by infusion of a known amount of pure CH_4 (Praxair® Gases Industrial Inc., Mexico; 99.997% purity), its recovery values ranged between 97–102%. The values obtained (ppm) in the ExpeData® software (Sable Systems International®, USA) were transformed to liters of methane per day, according to the equation developed by Arceo-Castillo et al. (*submitted).* CH_4 energy was calculated using the conversion factor 55.24 kJ/g CH_4 (Kriss, 1930).

DNA quantification

During the last three days of each period (days 17 to 19) ruminal fluid was collected via oesophageal tube four hours after feeding for the quantification of the rumen microbial population. DNA was extracted from 1 mL of ruminal fluid using a previously described purification method (Rojas-Herrera et al., 2008) in the Biotechnology Laboratory of the Faculty of Chemical Engineering at UADY, Mexico; then the samples were preserved at -20°C for further analysis in the MEB Laboratory at CIAT, Colombia. Concentration and purity of extracted DNA was measured with the Nanodrop 2000 spectrophotometer (Thermo Scientific®, Wilmington, DE, USA) to be diluted ten times (1:10 *i.e.* DNA: ultrapure water). Abundance of total bacteria, total methanogens (16S) and total protozoa (!8S) was quantified running specific quantitative polymerase chain reactions (qPCR; Rotor- Gene Q, QIAGEN®, MD, USA). Each qPCR reaction included 10 µL SYBR® Green (QIAGEN®, MD, USA), 1 µL of each primer, 2 µL of DNA samples plus 6 µL of ultrapure water. Primers for total bacteria used were Forward: 5´-ACTCCTACGGGAGGCAG-3´ and Reverse: 5´-GACTACCAGGGTATCTAATCC-3´ (Stevenson and Weimer, 2007). Methanogenic archaea primers were Forward: 5´- GGATTAGATACCCSGGTAGT-3´ and Reverse: 5´-GTTGARTCCAATTAAACCGCA-3´ (Hook et al., 2009). Total protozoa primers were Forward: 5´- GCTTTCGWTGGTAGTGTATT-3´ and Reverse: 5´-

CTTGCCCTCYAATCGTWCT-3´ (Sylvester et al., 2004). Temperature of annealing by qPCR amplification were 57, 60 and 55°C for total bacteria, methanogenic archaea and total protozoa, respectively. Standard curves for microbes were generated with 10^1 to 10^8 copies of recombinant plasmids per µL from the cloning and transformation of competent cells of *Escherichia coli*. Number of copies of the DNA template was calculated using the equation developed by Faseleh et al. (2013) and the absolute abundance was expressed as copies/mL of culture sample and transformed to Log_{10}. Estimation of copy numbers for the samples was made from the linear relationship between the threshold amplification (Ct) and the logarithm of 16S o 18S DNA copy numbers from the standard (r^2, 0.999, with a primer efficiency of approximately 98± 2%, and a slope value of 3.4). Each estimate had an average of three replications.

Rumen fermentation parameters

Four mL of rumen fluid were added to a tube containing 1 mL of 25% metaphosphoric acid and frozen at -20°C for subsequent analysis of volatile fatty acid (VFA). VFA proportions were determined by high-performance liquid chromatography (HPLC; Shimadzu® series 20A) equipped with an UV/VIS detector at a wavelength of 210 nm (SPD-20AV) and a chromatography column (BIO-RAD Aminex HPX-87H). The mobile phase was prepared for analysis with H_2SO_4 0.005 M with a flow of 0.7 mL/min and an injection volume of 20 µL. Molar proportions of volatile fatty acids were calculated by the retention times and the peak area of the commercial standards of acetic, butyric, and isobutyric acids (10,100, 250, 1000, 1500, 2000, and 3000 ppm) and corrected by the molar mass of each of them. All measurements and calculations were performed at the Forage Quality and Animal Nutrition Laboratory at CIAT, Colombia. pH of the rumen liquor sample was determined immediately after being taken with a portable pH meter (Hannah® Instruments, Woonsocket, USA).

Statistical analysis

In order to determine the effect of treatments on the intake, digestibility, volatile fatty acids, pH, production of methane, rumen population, and gain weight, the PROC MIXED procedure of SAS® software, version 9.4 (SAS Institute Inc., Cary, NC, USA, 2012) was used. Mean separation was made by using the Tukey test with an alpha of 0.05. The model is described below:

$$Y_{ijk} = \mu + \delta_i + P_j + \beta_k + e_{ij}$$

where: Y_{ijk} is the response of subject *k* under ration *i, during period j*; μ is the population mean, δ_i is the effect of the *i-th* diet (*i* =1…, 4), P_j is the effect of the *j-th* period (*j*=1…, 4), β_k is the effect of the *k-th* heifer (*k*=1…, 4), and e_i is the experimental error.

Results

Intake and apparent digestibility

Heifers consumed an average of 5.4 kg DM per day, this value, along with intake of OM, NDF and ADF (4.96, 3.26, and 1.99 kg/d, respectively) was not affected by the incorporation of foliage of *G. sepium* mixed with pods of *E. cyclocarpum* in the ration (P>0.05). However, intake of crude protein, tannins, and saponins increased linearly when the content of grass was reduced in the ration (Table 3). Cattle with access to legume leaves and pods showed lower apparent digestibility of NDF and ADF than when they were fed only grass. On the other hand, the apparent digestibility of CP had a quadratic trend (P<0.05). Digestible DM, OM, NDF, and ADF intakes were similar between the control (*Brachiaria brizantha*) and experimental treatments, the only difference was that digestible CP intake increased by 0.14 times on average in the treatments with incorporation of *G. sepium* and *E. cyclocarpum* (P=0.009) compared to the control. Gross and digestible energy intake by heifers (88.4 and

71.8 MJ/d, respectively) did not show a significant difference among diets (P>0.05). Average daily gain of heifers were 453 g/d (P=0.585).

[Insert Table 3 around here]

in situ degradation and rumen fermentation parameters

In situ incubation of all ration ingredients showed that the rapidly degradable fraction from DM in the rumen was highest for the pods (623.3 g/kg DM), while *Brachiaria brizantha* and soybean meal showed the highest values for the slowly degradable fraction (458.0 and 449.1 g/kg DM, respectively. P<0.001, Table 4). Degradation rate (*k*) of raw materials ranged between 0.34 and 0.18 per hour (P<0.001). Effective rate of degradation (ERD) in the grass species was 0.45 times (average) lower than the pods and soybean meal ((454.8 vs. 892.6 and 761.4 g/kg DM ERD, P<0.001, respectively).

[Insert Table 4 around here]

Inclusion of up to 45% of *E. cyclocarpum* pods mixed with *G. sepium* to a *Brachiaria brizantha* ration did not affect rumen pH, which ranged between 6.66 and 7.03 (P=0.350. Table 5). Although the proportions of acetic and propionic acid in the rumen did not show variation between treatments with and without pods and foliage (P=0.063 and P=0.079, respectively), a linear response was observed for butyric when they were incorporated in the ration (P= 0.034). Ratio of acetic to propionic acids in the rumen (A: P) had an average value of 3.1 (P=0.079) and a linear contrast (P=0.031)

[Insert Table 5 around here]

Methane production

Average enteric CH_4 emission expressed as g CH_4 per kg DMI of heifers were 26.61(P=0.165. Table 6) and this had a linear contrast when *G. sepium* mixed with *E.*

cyclocarpum were incorporated in the ration (P=0.050). There was an effect (P=0.003) of the supplementation with legume foliage mixed with pods on methane production, expressed as CH_4 g/kg of DCP, this decreased an average of 0.16 times (413 vs. 347 g CH_4/kg DCP) when rations were supplemented with *G. sepium* mixed *E. cyclocarpum*, compared to unsupplemented treatment. Addition of 15 and 30% of *G. sepium* and *E. cyclocarpum* to the grass ration showed a reduction of methane emissions of 0.10 kg CH_4/kg WG/year, compared to treatments containing 0 and 45% (P<0.001).

[Insert Table 6 around here]

DNA quantification

Effects of feeding different levels of foliage of *G. sepium* mixed with pods of *E. cyclocarpum* on the rumen microbial population of heifers are shown in Table 7. In the present study, the copy numbers transformed in logarithms of the total bacteria, archaea and protozoa were not significantly affected by the dietary treatments. Total bacteria and methanogenic archaea were 9.8 y 7.7 [log_{10}] on average, respectively. Copy numbers transformed of the 18S gene (total protozoa) had a quadratic trend when including increasing levels of pods and foliage of tropical legumes in the ration.

[Insert Table 7 around here]

Discussion

Implementation of sustainable systems of cattle production in the tropics with local forage supplements improves nutritional characteristics of the ration which may favour fermentative activity in the rumen, mitigate greenhouse gas emissions, and increase animal productivity (Rao et al. 2015; Arango et al., 2016; Gemeda and Hassen, 2018). In the present study,

pods of *E. cyclocarpum* and foliage of *G. sepium* foliage provided CP, CT and saponins. The content of CT of *G. sepium* was in the range reported in the literature 2 to 121 mg CT/g DM (Balogan et al., 1998, Schofield et al., 2001, García and Medina, 2006, Rira et al., 2015;). The ample range hereby reported could have been due to the fact that the content of CT depend on age, variety or ecotype, part of the plant, processing, environmental or agronomic factors, among others (Ojeda et al., 2015; Patra et al., 2017). Concentrations of CT and saponins in pods of *E. cyclocarpum* were similar to those reported by Hess et al. (2003) and Albores-Moreno et al. (2017).

In the present experiment dry matter intake was not affected in the heifers, despite differences in rumen degradation parameters (*A, B,* K_p, and ERD) between the grass and other components of the ration that could negatively affect retention time of digesta in the rumen (Valderrama and Anrique, 2011). Similarly, Briceño-Poot et al. (2012), Piñeiro-Vazquez et al. (2013), Phesatcha and Wanapat (2016) and Valencia-Salazar et al. (2018) found that supplementation of ruminants with pods and legume foliage rich in CT and saponins had no effect on feed intake, possibly because CT and saponin concentrations in the rations were not higher than 50 and 40 g/kg DM, respectively (Patra and Saxena, 2009 and 2011), which could negatively affect feed palatability (Laudau et al., 2000).

An quadratic effect on the apparent digestibility of dry matter (DMD) by supplementation with *G. sepium* and *E. cyclocarpum* was registered, when levels exceeded 30% in a ration based on tropical grasses this decrease; this was explained by the variation in passage rate from the rumen (Cabezas-Garcia et al., 2017). For instance, Briceño-Poot et al. (2012) and Piñeiro-Vázquez et al., (2013) reported a 9-13% reduction in DMD with the inclusion of *E. cyclocarpum* pods (up to 50%), while *G. sepium* increased DMD between 2 and 14% (Alayon et al., 1998; Mpairwe et al., 1998; Widiawati et al., 2007). These parameters did not differ between treatments. However, there was a reduction in the apparent digestibility of NDF

and ADF by including *E. cyclocarpum* and *G. sepium*; this can be explained by the multiple adverse effects of CT's on animal fermentative processes. These secondary metabolites can negatively affect the adhesion of cellulolytic bacteria to the substrate, reduce the activity of fibrolytic enzymes, inhibit the growth of the cellulolytic population or form complexes with cellulose that inhibit degradation in the rumen (Silanikove et al., 1996; Bento et al., 2005; Batta et al., 2015; Patra and Saxena, 2017). In addition, saponins can also change the site of fiber degradation in the digestive tract of ruminants or inhibit microbial growth (Patra and Saxena, 2009, Morgavi et al., 2012). In the present experiment, a quadratic response to increasing amounts of saponins on apparent CP digestibility was observed, substitution of 45% of the grass, gave lower values of CP digestibility, than when the ration incorporated only 15% of the legume mixture. Nonetheless this could be possibly explained by the concentration of CT in the ration. This can be rationalized by the concentration of CT in the ration, as it can form a CT-CP complex that prevents this nutrient from being degraded in the rumen by microorganisms, to be made available further down the lower gastrointestinal tract (Barahona et al., 1997, Patra and Saxena, 2011). This in general can increase the efficiency of microbial protein synthesis in the rumen (Gemeda and Hassen, 2018).

This study suggest that CH_4 emissions (g/d) by cattle were not affected by the inclusion of foliage of *G. sepium* mixed with pods of *E. cyclocarpum* in the ration at increasing levels. This could be due to a possible effect of time (*i.e.* an increase intake due to growth of animal) which biased a possible response. Such results differ from those obtained by Rira et al. (2015), which found that CH_4 production decreased linearly due to the inclusion of these tannin-rich legume species or those reported by Albores-Moreno et al. (2017), which showed a 0.35 times mitigation of CH_4 emissions when hair sheep consumed 300 and 450 g DM of ground pods of *E. cyclocarpum.* However, this tendency was observed when CH_4 production was corrected for intake (g/kg DMI). Level of feed intake is one of the factors that directly

affects the emissions CH_4 (Hristov et al. 2013). Values of CH_4 production on a DDMI basis (43.22 to 49.9 g/kg DDMI) were higher than those reported by Seresinhe et al. (2012), Molina et al. (2013), Aderinola and Binuomote (2014) and Asaolu et al. (2014) when incubating *G. sepium* (16.8-28.1 CH_4 mg/g DDMI) with a tropical grass.

Some of the factors that influence production of enteric methane are related to rumen pH. According to Janssen (2010), the low partial pressure of H_2 promotes synthesis of CH_4. The results hereby described agree with those from authors such as Silivong et al. (2013), Rira et al. (2015) and Albores-Moreno et al. (2017), who did not find changes in rumen pH when they supplied or incubated *G. sepium* or *E. cyclocarpum* in different proportions in the ration. In addition, pH values obtained in this study are within the optimal range (7±0.5) for the growth of microorganisms and production of volatile fatty acids (Van Kessel and Russell, 1996; Lana et al., 1998). Molar proportions of acetic and propionic acid in the rumen found in this study did not differ among treatments, perhaps because the levels of CT and saponins in the ration did not drastically affect salivation, pH, rate of passage, or VFA absorption through the rumen wall (Bhatta et al., 2009; Saxena and Patra 2009; Bodas et al., 2012; Roehe et al., 2016). In studies with legumes such as *Leucaena leucocephala* or *G. sepium*, Rira et al. (2015) concluded there were no changes in VFA proportions due to their inclusion in the rations. However, in the present research a linear trend was observed to increase butyric acid and decree acetic: propionic ratio when the amount of *E. cyclocarpum* plus *G. sepium* increased. Other studies have reported increases in the molar proportion of propionic and butyric acids because the steroidal saponins change the H_2 pathways for the synthesis of these energy products and not of CH_4 (Albores-Moreno et al., 2017; Valencia-Salazar et al., 2018).

Microorganisms play an important role in proper rumen functioning (Cammack et al., 2017). For example, protozoa use starch, cellulose, hemicellulose, pectin, and soluble sugars to

produce VFA and H_2, but the archaea attached to their surface use free H_2 to produce CH_4 and thus reduce the negative pressure in the rumen (Cersosimo et al., 2015; Millen et al., 2016). As a result, there is an association between methanogenic bacteria and rumen protozoa (Finlay et al., 1994). In their absence, methane can be decreased by around 11% (Newbold et al., 2015). However, CH_4 emissions are more related to methanogenic activity than to the total microbial population present in the rumen (Popova et al., 2011); although this relationship is more complex when dietary changes occur. In the present study, inclusion of saponins did not affect the amount of microorganisms, probably because the concentration of saponins was low and, in contrast, it is possible that permeability of the membrane was increased in a controlled way, producing higher absorption of nutrients, which could result in benefits to the host or perhaps the effect of saponins was transient (Patra and Saxena, 2009). According to Newbold et al. (1997) bacteria can degrade saponins to non-toxic compounds for protozoa. A study carried out by Martinele et al. (2014) did not report differences in 10 species of protozoa with respect to the control (274.2 vs. 248.1x10^4 protozoa/mL) when they replaced 13 to 40% of corn silage with *Gliricidia sepium* in lamb rations. Contrary to this, authors such as Monforte-Briceño et al. (2005), Degaldo et al. (2010), and Albores-Moreno et al. (2017) indicated that protozoa defaunation occurred (up to 40%) due to the presence of secondary metabolites contained in *G. sepium* or pods of *E. cyclocarpum,* because of cell lysis. According to Rira et al. (2015) or Angarita et al. (2015) there was no difference in the methanogen population among the treatments under study (species rich in tannins such as *Leucaena leucocephala*, *G. sepium* or *Manihot esculenta*).

In the rumen, synthesis of methane has an energy cost, in stoichiometric terms, in rations high in grains, the loss of gross energy would be minimal (2%); the opposite occurs when the contribution of structural carbohydrates (cellulose) is high, increasing this value up to

15% of gross energy (Johnson et al., 2007; Patra and Lalhriatpuii, 2016). In the present study, energy loss as CH_4 (% GE) ranged between 8.8 and 9.6%; these values are comparable to those reported for beef cattle fed low-quality rations in tropical regions (Y_m between 7-9%; Kaewpila and Sommart, 2016, Molina et al., 2016).

Weight gains of heifers ranged between 382 and 556 g/d. In spite of not showing differences between treatments, this values are consistent with other experiments with cattle, where their weight gain was increased by adding *G. sepium* to the ration (355 to 753 g/d. Abdulrazak et al., 1997). In addition, when calculating annual CH_4 emissions by weight gain, differences were found between treatments with or without legume or pod inclusion, this coincides with the results reported by Hyland et al. (2016), who stated that both parameters are inversely correlated and the explanation for this trend lies is the dilution of maintenance energy (Hristov et al., 2013). In addition, Ellis et al. (2012) argued that the increase in protein concentration and soluble sugars in the ration can decrease production of CH_4 per unit product down to 13%.

Conclusion

Substitution of 15 and 30% of ration dry matter as pods of *E. cyclocarpum* mixed with foliage of *G. sepium*, decreases the annual emissions of methane per unit product, without affecting daily dry matter intake or the rumen microbial population. On the contrary, digestible CP intake increased due to the supply of crude protein, condensed tannins and saponins contained in the foliage and pods of the two legume species. Studies on the long term effects of feeding those legumes to cattle and nitrogen balance are warranted as well as the study of supplying those plants at different physiological stages in cattle.

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Conflict of Interest

The authors state that there is no conflict of interest in relation to this work.

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Table 1. Chemical composition of ingredients used in the experimental diets

	Brachiaria brizantha	***Enterolobium cyclocarpum***	***Gliricidia sepium***	**Soybean meal**	**Wheat bran**	**Cane molasses**
Dry matter	925.9	962.8	924.9	926.8	927.0	860.0
Crude protein (g/kg DM)	55.1	154.2	170.3	427.6	166.7	31.5
Neutral detergent fiber (g/kg DM)	757.3	289.5	575.4	257.5	474.5	n.d.
Acid detergent fiber (g/kg DM)	466.4	205.6	451.8	73.7	134.8	n.d.
Gross energy (MJ/kg DM)	163.7	176.9	182.5	181.6	173.0	146.9
Ash (g/kg DM)	88.8	37.8	99.7	69.7	62.4	119.0
Total phenols (mg/g)	6.11	16.77	6.03	n.d.	n.d.	n.d.
Tannins phenols (mg/g)	1.76	10.81	0.80	n.d.	n.d.	n.d.
Condensed tannins (mg/g)	0	41.3	45.9	n.d.	n.d.	n.d.
Saponins (mg/g)	0	27.0	17.0	n.d.	n.d.	n.d.

DM = Dry matter

n.d. = not determined

Table 2. Proportion of ingredients and chemical composition of the experimental diets

	Level of incorporation of *E. cyclocarpum* + *G. sepium* in the ration (% DM)			
	0	**15**	**30**	**45**
Ingredients (g/kg DM)				
Brachiaria brizantha	747	653	557	461
Gliricidia sepium	0.0	75	150	225
Enterolobium cyclocarpum	0.0	75	150	225
Soybean meal	104	8.0	57	34
Wheat bran	104	8.0	57	34
Cane molasses	32	24	17	8.0
Minerals[1]	13	13	13	13
Chemical composition (g/kg DM)				
Crude protein	104.0	108.6	113.8	118.9
Neutral detergent fiber	641.8	617.9	593.3	568.6
Acid detergent fiber	370.1	370.5	370.3	370.0
Gross energy (MJ/kg DM)	16.4	16.6	16.8	17.0
Ash	83.9	81.7	79.7	77.3
Total phenols	4.6	5.7	6.8	7.9
Tannins phenols	1.3	2.0	2.7	3.4
Condensed tannins	0.0	6.5	13.1	19.6
Saponins	0.0	3.3	6.6	9.9

DM= dry matter.

[1] Mineral premix contained (minimum values per kg): 40 g P, 120 g Ca, 0.74 g Fe, 10 g Mg, 400 g NaCl, 1.50 g Mn, 1.5 g Zn, 0.15 g Cu, 0.0018 g I, 0.001 Co.

Table 3. Intake and digestibility of nutrients and energy by heifers fed increasing levels of *G. sepium* foliage mixed with pods of *E. cyclocarpum* in the ration.

	Level of incorporation of *E. cyclocarpum* and *G. sepium* in the ration (% DM)						Contrast		
Items	**0**	**15**	**30**	**45**	**SE**	**p-value**	**L**	**Q**	**C**
LW (kg)	231	234	244	242	9.72				
ADG (g/d)	382	556	465	410	183.2	0.585	0.989	0.258	0.500
Intake									
DM (kg/d)	5.18	5.23	5.57	5.57	0.33	0.272	0.085	0.873	0.423
DM (% LW)	2.23	2.24	2.27	2.30	0.07	0.561	0.197	0.815	0.770
OM (kg/d)	4.75	4.80	5.13	5.14	0.30	0.219	0.064	0.886	0.416
CP (g/d)	542^{c}	573^{bc}	635^{ab}	663^{a}	33.8	0.008	0.001	0.946	0.426
NDF (kg/d)	3.32	3.23	3.31	3.17	0.21	0.725	0.455	0.865	0.439
ADF (kg/d)	1.91	1.93	2.06	2.06	0.13	0.259	0.081	0.895	0.407
Condensed tannins (g/d)	0^{d}	34.2^{c}	72.9^{b}	109^{a}	6.22	0.001	0.001	0.672	0.580
Saponins (g/d)	0^{d}	17.3^{c}	36.8^{b}	55.2^{a}	2.63	0.001	0.001	0.688	0.575
Apparent nutrient digestibility (g/kg)									
DM	528	572	594	568	38.97	0.225	0.158	0.127	0.778
OM	651	643	649	634	18.16	0.568	0.301	0.699	0.416
CP	654^{ab}	687^{a}	661^{ab}	641^{b}	16.00	0.035	0.121	0.018	0.124
NDF	669^{a}	627^{ab}	628^{ab}	586^{b}	23.30	0.014	0.003	0.985	0.156
ADF	631^{a}	591^{ab}	607^{ab}	552^{b}	24.20	0.019	0.006	0.533	0.059
Digestible intake									
OM (kg/d)	3.05	3.07	3.30	3.23	0.25	0.317	0.118	0.648	0.282
CP (g/d)	350^{b}	391^{ab}	416^{a}	422^{a}	20.6	0.009	0.002	0.140	0.958
NDF (kg/d)	2.19	2.01	2.05	1.84	0.16	0.095	0.029	0.845	0.212
ADF (kg/d)	1.19	1.13	1.24	1.12	0.10	0.366	0.766	0.575	0.117
Energy intake (MJ/d)									
Gross energy	84.90	85.82	91.33	91.41	5.31	0.260	0.080	0.878	0.433
Digestible energy	68.36	69.24	74.81	74.88	0.30	0.214	0.008	0.889	0.426

LW = live weight;

ADG = average daily gain;

DM =dry matter;

OM =organic matter;

CP =crude protein;

NDF = neutral detergent fiber;

ADF = acid detergent fiber.

L = linear contrast;

Q = quadratic contrast;

C = cubic contrast;

SE = standard error.

Table 4. Rumen DM degradation (g/kg DM) of different feed components.

	Ingredients						
Items	**Soybean meal**	**Wheat bran**	***Enterolobium cyclocarpum***	***Gliricidia sepium***	***Brachiaria brizantha***	**SE**	**p-value**
a	542.0[b]	475.7[c]	623.3[a]	338.2[d]	286.5[e]	10.5	0.001
b	449.1[a]	317.2[c]	247.8[d]	341.6[b]	458.0[a]	.8.32	0.001
c	1181[a]	1.12[ab]	00.62[bc]	00.71[bc]	0.34[c]	4.81	0.001
a + *b*	9911[a]	792.9[c]	871.3[b]	678.8[e]	744.4[d]	0.35	0.001
K p	20.04[b]	1.03[b]	0.71[b]	0.78[b]	30.9[a]	0.35	0.001
ERD (%)	892.6[a]	695.9[b]	761.4[ab]	542[b]	454.8[c]	53.3	0.001

[a,b,c,d] Means in the same column with different letters are statistically different according to Tukey's test (*P<0.05*).

a = very rapidly degradable fraction; *b* = slowly degradable fraction;

a + *b* = degradation potential;

K_p = rate of passage;

c = constant rate of degradation (per hour);

ERD = effective degradability expected at a rate of rumen outflow of 0.05/h

Table 5. Molar proportions of volatile fatty acids (VFA) in the rumen of heifers fed *Brachiaria brizantha* and increasing levels of *E. cyclocarpum* pods mixed with foliage of *G. sepium* in the ration

Items	Level of incorporation of *E. cyclocarpum* and *G. sepium* in the ration (% DM)				SE	p-value	Contrast		
	0	15	30	45			L	Q	C
Rumen pH	6.94	6.68	6.66	7.03	0.25	0.350	0.932	0.094	0.865
Acetic acid (mmol/L)	60.7	59.6	59.0	58.4	3.10	0.774	0.337	0.861	0.961
Propionic acid (mmol/L)	17.0	19.0	19.6	19.0	1.29	0.105	0.057	0.089	0.929
Butyric acid (mmol/L)	10.1	11.1	12.5	13.2	1.78	0.155	0.034	0.892	0.817
Isobutyric acid (mmol/L)	3.51	3.82	3.50	4.38	0.90	0.513	0.293	0.545	0.399
Acetic:propionic ratio	3.58	3.16	3.01	3.07	0.27	0.079	0.031	0.116	0.919

L = linear contrast;

Q = quadratic contrast;

C = cubic contrast;

SE = standard error.

Table 6. Enteric CH_4 production in heifers fed *Brachiaria brizantha* and increasing levels of *E. cyclocarpum* pods mixed with foliage of *G. sepium* in the ration.

Items	Level of incorporation of *E. cyclocarpum* and *G. sepium* in the ration (% DM)				SE	p-value	Contrast		
	0	15	30	45			L	Q	C
CH_4 (g)/d	144.8	140.1	141.3	143.3	4.88	0.570	0.788	0.218	0.655
CH_4 (g)/DMI (kg)	28.20	26.80	25.56	25.89	1.50	0.165	0.050	0.299	0.689
CH_4 (g)/DDMI (kg)	49.93	47.28	43.22	46.08	3.33	0.131	0.081	0.149	0.307
CH_4 (g)/DCP (kg)	413.1[a]	359.5[b]	339.7[b]	341.8[b]	17.7	0.003	0.001	0.013	0.452
CH_4 (g)/DNDF (kg)	121.9	125.6	114.1	129.0	10.6	0.313	0.691	0.331	0.128
CH_4 (g)/DADF (kg)	65.9	70.4	68.8	78.8	5.93	0.091	0.031	0.392	0.233
Energy loss as CH_4 (% GE)	9.57	9.09	8.67	8.80	0.51	0.157	0.051	0.281	0.683
kg eq CO_2 /year	1094	1059	1068	1084	36.9	0.571	0.788	0.218	0.655
CH_4 (kg)/ADG (kg)/year	0.38[a]	0.25[c]	0.30[b]	0.35[a]	0.01	0.001	0.179	0.001	0.001

[a,b] Means in the same column and item with different letters are statistically different according to Tukey's test (P>0.05).

CH_4 (g/d) = grams of CH_4 per day;

DMI = dry matter intake;

DDMI = digestible dry matter intake;

DCP = digestible crude protein;

DNDF = digestible neutral detergent fiber;

DADF = digestible acid detergent fiber;

GE = gross energy;

ADG = average daily gain.

L = linear contrast;

Q = quadratic contrast;

C = cubic contrast;

SE = standard error.

kg eq CO_2 /year = [(CH_4 (g)/d *360) / 1000] * 21

Table 7. Effect of treatments on the total population of bacteria, protozoa and archaea (copy number/mL rumen fluid) in heifers fed *Brachiaria brizantha* and increasing levels of pods of *E. cyclocarpum* mixed with foliage of *G. sepium* in the ration.

Rumen microbes (copy number/mL)	Level of incorporation of *E. cyclocarpum* and *G. sepium* in the ration (% DM)				SE	p-value	Contrast		
	0	15	30	45			L	Q	C
Total bacteria [log_{10}]	9.88	9.73	9.74	9.95	0.19	0.366	0.661	0.107	0.898
Total protozoa [log_{10}]	4.91	4.72	4.77	4.94	0.13	0.149	0.639	0.035	0.708
Methanogenic archaea [log_{10}]	7.77	7.69	7.61	7.61	0.27	0.974	0.814	0.837	0.759

[a,b] Means in the same column and item with different letters are statistically different according to Tukey's test (P>0.05).

L = linear contrast;

Q = quadratic contrast;

C = cubic contrast;

SE = standard error.