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Effects of long-term diet supplementation with *Gliricidia sepium* foliage mixed with *Enterolobium cyclocarpum* pods on enteric methane, apparent digestibility, and rumen microbial population in crossbred heifers[1][2]

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ABSTRACT: In the last decades, strategies have been evaluated to reduce rumen methane **(CH_4)** production by supplementing tropical forages rich in secondary compounds; however, most of these beneficial effects need to be validated in terms of their persistence over time. The aim of this study was to assess CH_4 emissions over time in heifers fed with and without *Gliricidia sepium* foliage **(G)** mixed with ground pods of *Enterolobium cyclocarpum* **(E)**. Two groups of four crossbred (*Bos taurus* x *Bos indicus*) heifers (284 ±17 kg initial weight) were fed with two diets (0 and 15% of a mixture of the pods and foliage [**E+G:0** and **E+G:15**, respectively] over 80 days, plus two weeks before the experiment, in which every animal was fed a legume and pod-free diet. Every 14 days, CH_4 production, apparent digestibility, volatile fatty acids **(VFA)**, and microbial population were quantified for each animal. The experiment was conducted with a repeated measurements design over time. Diets fed differed in terms of their crude protein **(CP)**, condensed tannins **(CT)** and saponins content supplied by *E. cyclocarpum* and *G. sepium*. For most of the experiment, dry matter intake **(DMI)** and digestible dry-matter intake **(DDMI)** were 6.3 kg DMI/d and 512 g DDMI/kg, respectively for both diets (Diet: *P>0.05*). Apparent digestible crude protein (**DCP**) was reduced by 21 g DCP/kg DM when the diet was supplemented with E+G:15 (*P*=0.*040*). Molar proportions of VFA's in the rumen did not differ between diets or in time (*P>0.05*). Daily methane production, expressed in relation to DMI was 23.95 vs 23.32 g CH_4/kg DMI for the diet E+G:0 and E+G:15 respectively (Diet: *P=0.016*; Time: *P>0.05*). Percent gross energy loss as CH_4 (***Ym***) with grass-only diets was above 8.1%, whereas when feeding heifers with the alternate supplementation, ***Ym*** values of 7.59% (*P=0.016*) were observed. The relative abundance of total bacterial, protozoa, and methanogenic archaeal replicates was not affected by time nor by the incorporation of legume and pods into the diet (*P>0.05*).

Results suggest that addition of *G. sepium* mixed with *E. cyclocarpum* pods can reduce CH_4 production in heifers and this response remains over time, without effect on microbial population and VFA concentration and a slight reduction in crude protein digestibility.

Keywords: cattle, greenhouse gas, legumes, long term feeding, microbial population

INTRODUCTION

Ruminal microorganisms have been known for decades to be of major importance to the host, as they are largely responsible for the health and the conversion efficiency of feedstuffs in domestic animals (Cammack et al., 2018). In addition, methane **(CH_4)** is produced in the rumen by microorganisms from the *Archaea* domain (Lui and Whitman, 2008). These microorganisms benefit from the end products of fermentation (ATP, N-NH_3) carried out by bacteria, fungi, and protozoa (Martin et al., 2010). Some strategies to reduce CH_4 production propose regulating ruminal fermentation by supplying secondary compounds o improve nutritional quality of diets (Eckard et al., 2010). Condensed tannins **(CT)** and saponins may directly or indirectly reduce CH_4 emissions between 10 and 35% (Hess et al., 2006; Albores-Moreno et al., 2017; Piñeiro-Vázquez et al., 2018). These secondary metabolites can negatively affect the diversity or activity of methanogens as a result of the changes in the cellular membrane of microorganisms, reducing the availability of hydrogen, changing fermentation pattern, or by forming complexes with other nutritional compounds (Jayanegara et al., 2012; Wallace et al., 2014; Patra et al., 2017).

In this respect, *Enterolobium cyclocarpum* (“parota”, “orejero” or “piñón de oreja”) pods and *Gliricidia sepium* (“matarraton” or “madero negro”) foliage have shown promising results as animal feed and in reducing CH_4 in a short period of time, due to the crude protein (**CP**), saponin, and CT contained (Pizzani et al., 2006; Narayan et al., 2013; Asaolu et al., 2014; Archimède et al., 2015; Torres-Salado et al., 2018). However, some authors, such as Newbold et al. (1997), Wina et al. (2006) and Ramos-Morales et al. (2017), reported that the actions of secondary compounds may be transient, since microorganisms are able to degrade or develop protection mechanisms against these

compounds. Therefore, the main aim of this study was to evaluate CH_4 emissions and changes in microbial population over time in crossbred heifers fed with and without *Gliricidia sepium* foliage mixed with *Enterolobium cyclocarpum* pods.

MATERIALS AND METHODS

All animals in this research were handled according to a protocol approved by the Ethics Committee of the Faculty of Veterinary Medicine and Animal Science, University of Yucatan (UADY), Mexico.

Study site, animals and experimental design

The study was conducted at the Climate Change and Livestock Production Laboratory of the Faculty of Veterinary Medicine and Animal Science (Merida, Mexico, latitude 21°15' N; longitude 83°32' W) from May to September 2017. During this period, temperatures ranged between 21 and 36°C; monthly rainfall ranged between 69 and 183 mm (INEGI, 2017).

The experiment was conducted using a repeated measures design (Littell et al., 1998; Robinson et al., 2015). For this purpose, two groups of four crossbred (*Bos taurus* x *Bos indicus*) heifers with an initial weight of 284 ±17 kg and 18 ± 3 months of age were used. These animals were fed two types of diets (with and without *E. cyclocarpum* (**E**) mixed with *G. sepium* (**G**): E+G:15 and E+G:0, respectively) for 80 days. During this period, every 14 days, and for two consecutive days, enteric CH_4 production and apparent digestibility were determined; ruminal fluid was also sampled (Figure 1). In addition, there

was a 14-day period prior to the experiment (Phase 0), where all animals were fed the same diet (without legumes and pods) to cleanse the rumen from the carryover effect of previous diets on all variables evaluated. To ensure the repeatability of the experiment, the diets fed to the animals did not change in quality and quantity throughout the experiment.

Experimental diets, feed intake, and digestibility

The control diet consisted of two tropical grass hays (*Megathyrsus maximus* [syn. *Panicum maximum*] cv. Guinea and *Sorghum halepense* [L.] Pers. (Johnson grass), respectively, plus soybean meal, wheat bran, cane molasses, and a commercial mineral mixture. In the experimental diet, grasses were substituted by 7.5% *Enterolobium cyclocarpum* (Jacq.) Griseb. ground pods mixed with 7.5% of *Gliricidia sepium* (Jacq.) Steud. Leaves. Diets were formulated according to the National Research Council (NRC, 2016) guidelines to meet the maintenance and growth requirements of heifers. Table 1 shows the nutrient composition and proportion of ingredients and experimental diets. All animals had free access to fresh water and were housed individually in pens measuring 3 m long and 3 m wide.

Pods of *E. cyclocarpum* and foliage of *G. sepium* were collected in central and east regions of Yucatan (Mexico). The legume was harvested at a regrowth age of 60 days, while the grasses hay had 120 days of regrowth. Legume leaves were air-dried and pods were dried at 55°C for 72 h in a forced air oven. All materials were ground to pass a 2-mm hammer mill (Azteca®, Nuevo Leon, Mexico) and properly stored in plastic bags to prevent moisture. Heifers were fed at 8:30 h and the next day, orts were collected and weighed to calculate dry mater intake **(DMI)** daily. Total fecal production was collected in trays and

weighed for three consecutive days (2 days of CH_4 measurements in open-circuit respiration chambers and one additional day in metabolic crates. A sub-sample (10%) was also taken and dried at 55°C for 48 h to determine dry mater digestibility (**DMD**, Schneider and Flatt, 1975) and perform further chemical analyses (described below). Heifers were weighed using a one-ton scale (Revuelta®, DF. Mexico) at the beginning of the experiment and every 17 days to quantify the daily weight gain.

In situ incubation

Rumen degradation measurements were conducted through the method proposed by Ørskov et al. (1980) for all the ingredients of the diets. The main ingredients were incubated in the rumen of three crossbred (*Bos taurus* x *Bos indicus*) mature cows, each fitted with a plastisol rumen cannula, which were fed 74.9% of *Megathyrsus maximus* hay and 24.1% of a balanced feed based on soybean meal, ground corn, urea and cane molasses (11.1% CP and 8.6 MJ ME/kg DM); heifers had free access to fresh water.

Samples (5 g DM) were weighed by triplicate in nylon bags (7 x 14 cm; 53-micron pore size). Bags were removed from the rumen at 3, 6, 12, 24, 36 and 48 h post-incubation (for soybean meal); at 72 h for *G. sepium*, *E. cyclocarpum*, and wheat bran; and at 96 h for the two grasses. Zero-hour degradation bags were soaked in water for 5 min. At the end of the incubation period, bags were dried at 55°C for 72 h and then weighed. Dry matter degradation kinetics was calculated using the equation $Y_t = a + b \times (1-e^{-c*t})$ proposed by Ørskov and McDonald (1979), where Y is the percentage of rumen degradability at time (t, h) of incubation. SAS 9.4 nonlinear regression model procedure was used (SAS Inst. Inc., 2012) in calculations. For the interpretation of 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). Effective rumen degradability (ERD) was calculated as $ERD\ (g/kg\ DM) = (a + b \times c) \div (c + K_p)$, where the parameters a, *b, and c*, had the aforementioned meaning, and *'K_p'* is the forage passage rate, which is 0.05 per hour for ruminants fed at low levels of production (NRC, 2001).

Methane production

The Laboratory of Climate Change and Livestock Production at the University of Yucatan has two open-circuit respiration chambers to accommodate one animal per chamber. Therefore, measurements of CH_4 production were planned sequentially to enter the animals into the respiration chambers. Heifers remained inside the chambers for two consecutive days, approximately 23 h each day, and one hour was used to clean up and collect the feces. Protocols detailing the construction and operation of the chambers are described elsewhere Canul-Solis et al. (2017) and Valencia-Salazar et al. (2018). Respiration chambers were built with metal sheet panels and their dimensions were: 300 cm in length, 214 cm in height, and 144 cm in width. Temperature (23 ± 1 °C) and relative humidity (55 ± 10%) within the chamber were continuously monitored. Air was extracted from the chambers at 250 L/min with the help of mass flow meters (Sable Systems International, Las Vegas, NV, USA), then a sample of chamber air is passed through an infrared CH_4 analyzer (MA-10 Sable Systems International, USA) to measure CH_4 concentration (Arceo-Castillo et al., 2019). Before each CH_4 measurement in the chambers, pure nitrogen and CH_4: (1,000 ppm; Praxair® Industrial Gases Inc., Mexico) were used for zeroing and calibrating the CH analyzer respectively. At the beginning of the experiment

high purity methane (99.997%; Praxair® Industrial Gases Inc., Mexico) was injected from a cylinder into the chambers to assess recovery rates which ranged between 97 and 102%.

Rumen fermentation parameters and DNA quantification

One day after each CH_4 measurement, approximately 1 L of ruminal fluid was aspirated from heifers four hours after feeding using an oesophageal tube, to measure pH and to quantify volatile fatty acids (VFA) and microbial populations. Ruminal fluid was immediately filtered through sterile gauze and the pH was measured (Hannah® Instruments, Woonsocket, USA). To determine VFA concentration, 4 mL of ruminal fluid were taken and 1 mL of a 25% of metaphosphoric acid solution was added. The mixture was kept at -20°C for further analysis.

Another sub-sample (100 mL) was frozen at -20°C to determine microbial population. The extraction method was based upon the deoxyribonucleic acid (**DNA**) adsorption onto silica without use of phenol, ethanol precipitation, or a cesium chloride gradient, as described by Rojas-Herrera et al. (2008). The procedure was carried out at the Biotechnology Laboratory of the Faculty of Chemical Engineering at UADY, Mexico, using 1 mL of ruminal fluid. Then, the samples were preserved at -20°C for further analysis at the Molecular and Environmental Biology Laboratory at the International Center for Tropical Agriculture- (CIAT) in Colombia. DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific®, Wilmington, DE, USA). After DNA was extracted, a quantitative real-time polymerase chain reaction (**qPCR**) was conducted to quantify the abundance of total bacteria, total methanogens (16S), and total

protozoa (18S). The absolute quantities of each microorganism were determined using standards. Standard curves for each primer set were created using serial dilutions of plasmid DNA of each microbial group. Standards were obtained cloning fragments of the plasmid using the pGEM®-T Easy Vector, System I kit (Promega®, Madison, WI, USA), followed by transformation of *Escherichia coli* competent cells. Quantification of samples was performed on a Rotor-Gene Q (QIAGEN®, MD, USA) including three replicates and a negative control (without template DNA) in every run. Each qPCR reaction mixture (20 µL of final volume) contained 10 µL of SYBR® Green (QIAGEN®, MD, USA), 0.5 µM of each primer, 20 ng of DNA samples at a concentration of 20 ng, plus 6 µL of ultrapure water. The oligonucleotide primers used and annealing temperature conditions by qPCR amplification are described in Table 2. Estimation of copy numbers for the samples was obtained from the linear relationship between the threshold amplification and the logarithm of 16S or 18S DNA copy numbers from the standard (r^2 0.998, with a primer efficiency of approximately 97.8± 2%, and a slope value of 3.4). Copy numbers for each sample were calculated using the equation developed by Faseleh et al. (2013) and the absolute abundance was expressed as the Log of copies/mL of the culture sample.

Chemical analysis

Chemical analyses were carried out on samples of ingredients, orts, and collected feces. In addition, DM of ingredients was monitored every week to make sure the variation of this parameter would not exceed 3% throughout the whole experiment. Sample DM was calculated as the difference between fresh weight and final weight after being dried at 55°C for 48 h in a forced air oven. Samples were ground to pass a 1-mm screen in a Wiley® mill. Ash and crude protein (**CP**) were obtained in accordance with methods 942.05 (AOAC,

2005) and 984.14 (AOAC, 1990. CP=N×6.25; Kjeldahl AN 3001 FOSS). Neutral detergent fiber and acid detergent fiber content (**NDF**, **ADF**, respectively) was 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 (**GE**) was determined in accordance with ISO 9831:1998 specifications. Total phenolic and tannin contents were determined using the Folin-Ciocalteu's method (Makkar, 2003); condensed tannins (**CT**) were measured using the proanthocyanidins method (Porter et al., 1986) with butanol-HCl reagent. Content of saponins was determined through the method proposed by Oleszek, (1990) (haemolytic micro-method test). VFA proportions were quantified using a high-performance liquid chromatography (**HPLC**; Shimadzu® series 20A) equipped with an ultra-violet/visible (UV/Vis) detector (SPD-20AV) and a chromatography column (BIO-RAD Aminex HPX-87H. Dimensions: 300 mm x 7.8 mm). HPLC conditions consisted of a mobile phase with H_2SO_4 0.005 M, oven temperature of 50°C, with a flow of 0.7 mL/min, detector wave length of 210 nm and a sample injection volume of 20 µL. Results were determined using a commercial standard curve for acetic, propionic, butyric, and isobutyric acids. All measurements and calculations were performed at the Forage Quality and Animal Nutrition Laboratory of CIAT, Colombia.

Statistical analysis

In order to determine the effect of treatments and time on feed intake, apparent digestibility, VFA's, pH, CH_4 production, rumen microbial population and weight gain, the PROC MIXED procedure of SAS software®, version 9.4 (SAS Institute Inc., Cary, NC, USA, 2012) was used. Mean separation was made using the Tukey test with an alpha of 0.05. The model is described below:

$$Y_{ijk} = \mu + \delta_i + \bar{\imath}_k + \beta_{ji} + (\delta * \bar{\imath})_{ik} + \epsilon_{ijk}$$

Where: Y_{ijk} is the response of subject *j* under ration *i*, during time *k;* μ is the population mean, δ_i is the effect of the *i-th* diet (*i* = E+G:0 and E+G:15); $\bar{\imath}_k$: is the effect of the *k-th* time (*k*=1…, 5); $(\delta * \bar{\imath})_{ik}$: Interaction between *i-th* diet and *k-th* time; β_{ji}: effect of the *j-th* heifer (*k*=1…, 4) into diets, and ϵ_{ijk} is the experimental error.

A completely randomized design was used to determinate the differences between groups (group 1 and 2) under the same control diet in the first phase:

$$Y_{ij} = \mu + \delta_i + \epsilon_{ij}$$

Where: Y_{ij} is the response of subject *j (j= 1...4)* under group *i,* μ is the population mean, δ_i is the effect of the *i-th* group (*i* =1 and 2), and ϵ_{ij} is the experimental error.

RESULTS

Experimental diets

Diets fed were similar in nutrient, ADF, GE, and ash contents. The main difference between diets was the content of CT and saponins supplied by *E. cyclocarpum* pods and *G. sepium* foliage. This diet had also higher CP and lower NDF content.

Intake and apparent digestibility

Average DMI per animal was 6.0 kg DMI/d in the first phase (*P=0.131*); this is 0.3 kg DMI/d less than during the following 80 days, where there was a difference in time (*P=0.01*), but not between treatments *(P=0.121*. Table 3. Figure 2(*a*)). In the first 14 days

(Phase 0), CP, NDF, ADF, and ash intake did not differ between groups (*P≥0.073*). However, CP intake in the diet containing *E. cyclocarpum* mixed with *G. sepium* was 0.3 g/kg DM greater than with the control diet in Phases 1 to 5. Moreover, CT and saponin content in the diet supplemented with the legume and the pods had 43 and 22 more g/d, respectively, than the tropical grass-based diet. All the nutrients ingested by the heifers showed differences in time (*P≤0.003*).

In this study, DMD and apparent digestibility of Organic Matter (**OMD**) averaged 512 and 534 g/kg DM, respectively. These parameters did not differ between groups in Phase 0, treatments (with and without legume mixed with pods), nor in the subsequent five phases (*P≥0.086*). CP digestibility (**DCP**) was reduced by 21 g/kg DM when heifers were supplemented with *E. cyclocarpum* mixed with G. *sepium* (*P=0.039.* Figure 2(*b*)). There was an interaction between time and treatment in NDF and ADF digestibility (**DNDF** and **DADF**, respectively), as well as in the total digestible CP, NDF, and ADF intake and gross energy intake (*P≤0.043*). Furthermore, over time, items such as digestible NDF intake and gross energy intake differed with time (*P≤0.045*).

In situ degradation

Ingredient ruminal degradations showed that *E. cyclocarpum* pods had the greatest rapidly degradable fraction values, while grasses were approximately 500g/kg DM lower (623 vs 130 g/kg DM. *P=0.001*. Table 4). When calculating the potentially degradable fraction (*a* + *b*), it was observed that soybean meal, *E. cyclocarpum*, and wheat bran showed the greatest values, contrary to those reported for the legumes and two grass species (*P=0.001*). Passage rate ranged between 0.2 and 23.7, while the rate of degradation

per hour ranged between 0.2 and 1.18 (*P=0.001*). Soybean meal had an effective rumen degradation (991 g/kg DM) greater than *B. brizantha*, *G. sepium*, and *S. halepense* (460 g/kg DM in average. *P=0.001*).

Ruminal fermentation parameters

Rumen pH ranged between 6.58 and 6.97 without difference between animals in Phase 0 (*P=0.081*), or between response variables, treatments, or in the subsequent 5 Phases (*P≥0.06*. Table 5). However, between diet and time there was an interaction (*P=0.001*. Figure 2(*c*)). Molar proportions of acetic, propionic and butyric acids averaged 61.0, 19.4, and 9.8 mmol/100 mol, respectively. These values were similar throughout the whole experiment, as well as when including *E. cyclocarpum* mixed with *G. sepium.* The same occurred in the acetic: propionic acid ratio (average = 3.2 *P≥0.374*). Total VFA, expressed in relation to DMI or DOMI averaged 12 and 24 (mmol/l)/(kg/d), respectively (*P≥0.079)*

Methane production

Methane production per heifer ranged between 140 and 149 g/d throughout the whole experiment (Table 6). Methane production, expressed in relation to DM, DDMI, and DCP intake, was lower in heifers fed with E+G:15 (*P≤0.033*) but without differences over time (*P≥0.2698*. Figure 2 (*e*)). Percent gross energy loss as methane gas (***Ym***) with grass-only diets (from Phase 0 to 5) was above 8.1%, whereas when feeding heifers with the alternate supplementation, ***Ym*** values of 7.59% (*P=0.0163*) were observed. The largest difference between diets was observed when projecting annual CH_4 production corrected by weight gain, it was reduced by 38% when animals were fed E+G:15 – 0.4 vs 0.65 kg CH_4/kg ADG, respectively (*P=0.001*). These differences remained over the time period measured (*P=0.5780*).

DNA quantification

Table 7 shows the quantification of the ruminal microbial population. The copy number of total bacterial, protozoan, and methanogenic archaeal replicates was 9.6, 7.3, and 6.6 [log_{10}], respectively. These microorganisms were not affected by the incorporation of the legume and the pods into the diet; moreover, this response remained over time (*P≥0.1954*. Figure 2 (*f*)) from the initial phase.

DISCUSSION

This study shows the differences between tropical grasses and legume or pods when used in diets for cattle. The main contrasts were observed in parameters related to CP, NDF, and secondary compounds. Condenses tannins and saponins values obtained in this study are similar to those previously reported for the *E. cyclocarpum* pods and *G. sepium* legume by other authors (Piñeiro et al., 2013; Seresinhe et al., 2012; Archimède et al., 2015; Albores-Moreno et al., 2017). In the literature, these chemical compounds (CP, CT and saponins) are directly and indirectly related to rumen fermentation, CH_4 production, and microbial population (Patra and Saxena, 2009; Hammond et al., 2015).

Dry matter intake was similar in both treatments, possibly because CT and total saponins contents in the diet did not exceed 5%, as this could negatively affect palatability of the diet (Waghorn, 2008). Pods of *E. cyclocarpum* have a high soluble carbohydrate fraction and low fiber content which could have a positive effect on feed intake (Martin et al., 2010; Kahn et al., 2015). However, this was not evidenced in the current study possibly due to the low level of inclusion of this substrate in the diet. Dry matter intake and nutrient

intake differed in time; this could be because during the first phase, heifers had to adapt to the change in diet, due to the inclusion of *E. cyclocarpum* and G. *sepium*. Authors such as Grant et al. (2015) and Machado et al. (2016) concluded that 7 to 14 days are required to stabilize voluntary intake in cattle fed tropical diets. Nonetheless, the intake behavior and feed selectivity depend on factors specific to animals, social factors and habitat conditions, as well as to feed characteristics and diet composition (energy density, crude protein and fiber contents), among other (Tarazona et al., 2012; Grant et al., 2015).

There are several factors which affect feed digestibility (Schneider and Flatt, 1975). For instance, Makkar et al. (1995) argued that when CT and saponins are available in the same diet, they may have an additive effect on the reduction in feed digestibility. This was not observed in the present study, since DM and OM digestibilities were similar between treatments. Additionally, Kahn et al. (2015) stated that DDMI is closely related to feed rumen degradability and passage rates, which in turn are associated with NDF content in the diet. This was demonstrated by Seresinhe et al. (2012), who found that including *E. cyclocarpum* increased digestibility due to the soluble carbohydrate content. It is feasible to expect such a result when pods intake is higher, as this feedstuff showed the highest values in rapidly and potentially degradable fractions in the rumen, as well as in effective degradability (*a*, *a+b*, and *ERD*, respectively).

Crude protein digestibility was reduced when heifers were fed *E. cyclocarpum* pods and the legume *G. sepium*. This could be due to the presence of tannins in the diet, which may have reduced microbial degradability of dietary CP in the rumen, thus increasing the passage of crude protein (low degradable) to the small intestine and rendering the amino acids available for absorption (Patra and Saxena, 2009). As discussed above, feed intake of

heifers differed in terms of nutritional compounds during the transition phase from a control to a supplemented diet; this had an effect on fiber digestibility (ADF and NDF). Furthermore, the low dose of anti-nutritional compounds fed to the animals did not have a negative effect on Gram positive bacteria (*Ruminococcus albus* and *R. flavefaciens*) and fungi with a fibrolytic role in carbohydrate degradation in the rumen (Wina et al., 2006; Rira et al., 2015).

Enteric CH_4 production values fall within the range reported for cattle fed in tropical production systems (20 to 160 g CH_4/d. Molina et al., 2016; Ku-Vera et al., 2018; Valencia-Salazar et al., 2018). Methane production (g/ kg DMI, g/ kg DDMI; or g/ kg DCP) showed a reduction between 2.6 and 10.5% when animals were fed *E. cyclocarpum* mixed with *G. sepium*; these differences were maintained over time. Methane production depends on factors such as level of feed intake and diet composition and digestibility (Pinares-Patiño et al. 2011; Hristov et al. 2013; Ramin and Huhtanen, 2013). Huhtanen et al. (2016) found that for every additional hour of fermentation in the rumen, production of CH_4 increases by 0.37 and 0.33 g CH_4/ kg DMI in cows and sheep, respectively. In addition, Cabezas-García et al. (2017) argued that passage rate affecting DMI and microbial nitrogen efficiency is closely related to CH_4 emission variation among animals. According to Hess et al. (2006), reduction of CH_4 per unit of fermented nutrient comes from the effect of tannins reducing nutrient degradation in the rumen.

The loss of dietary gross energy as CH_4 with the control diet was higher than for the diet with alternate supplementation (8.1 vs 7.6% GE). These values are within the range reported by authors such as Kennedy and Charmley, (2012), Richmond et al. (2015), Kaewpila and Sommart, (2016) and Molina et al. (2016) for cattle fed improved or native

tropical grasses, including or not including legumes species. Annual CH_4 projection corrected by weight gain was reduced when animals were fed *E. cyclocarpum* mixed with *G. sepium*. These findings are consistent with those of Warner et al. (2017) who stated that reducing the nutritional quality increased CH_4 emission intensity (g CH_4/kg of fat- and protein-corrected milk) by 28%. This is due to the fact that when increasing animal performance, the energy requirements for maintenance are lowered in relation to the total energy requirements, thus reducing the energy demand per additional unit of product (Clark et al., 2013). The relationship between CH_4 emission and animal productivity is important when ruminants provide high-quality food for humans (Waghorn and Hegarty, 2011).

Despite the fact that there were no differences between treatments regarding the concentration of VFA's in the rumen that could help interpret the results obtained in CH_4 reduction, it would be expected that hydrogen sinks (*i.e.* propionate) would increase, while the hydrogen providers, like that with an acetate or butyrate type of fermentation, would be reduced (Martin et al., 2010), due to a decrease in rumen pH that favors propionate producing bacteria, but inhibits other microorganisms (Spanghero et al., 2008). On this subject, Palarea-Albaladejo et al. (2017), asserted that the VFA balance ([acetate plus butyrate]/propionate) describes better their contribution to CH_4 production (g/kg DMI). However, Robinson et al. (2010) argue that VFA concentrations are poorly associated to daily CH_4 production, since these proportions reflect the balance between production and absorption rates, which depend on other factors, such as intake level, ruminal volume, and osmolarity in the rumen. All pH values fell within the normal range reported in the literature (6.8 on average) (Van Kessel and Russell, 1996; Shaani et al., 2017) and they did not change when *G. sepium* mixed with *E. cyclocarpum* were incorporated. According to

Boda et al. (2012), under low pH conditions, saponins have a more pronounced effect on microbial populations and, indirectly, on methane production.

The copy number of total bacterial and methanogenic archaeal replicates was not affected by the incorporation of legumes species and pods into the diet. In agreement with the findings of Navas-Camacho et al. (1993), Hess et al. (2003), and Soliva et al. (2008), the number of total bacteria was not affected by including *E. cyclocarpum* or *G. sepium* in the diets of sheep or lambs, nor in *in-vitro* studies. As for the discrepancy between methane reduction without affecting both populations, Wallace et al. (2014) claimed that both parameters are moderately correlated, probably because there is an intrinsic host effect that remains over time. In addition, it is possible that the reduction in CH_4 emissions observed in this study by the treatment incorporating *E. cyclocarpum* mixed with *G. sepium* was not due to the effect of tannins and saponins on the quantity of microorganisms, but probably due to the inhibition of methanogenic activity, as described by Guo et al. (2008), who argued that the expression of the methyl-coenzyme M reductase (*mcrA*) gene is reduced by the action of saponins. On the other hand, it could be that the effect of these secondary compounds could affect other microbial populations, such as anaerobic fungi, *Succinovibrionaceae* and *Prevotella*, which were not measured in this study, but which are associated with methane production (Tapio et al., 2017). Moreover, when these secondary compounds are included in the diet, there is an inverse correlation between ruminal pH and the abundance of the archaea population (r = -0.95) (Díaz-Carrasco et al., 2017) due to the reduction of available hydrogen caused by a decrease and/or lower activity of cellulolytic bacteria (Brossard et al., 2004), thus reducing rumen CH_4 emissions. However, Hünerberg et al. (2015) claimed that methanogens can adapt to a lower pH to produce methane,

therefore, both variables (pH and g CH_4/kg DMI) are slightly correlated. Additionally, it is possible that no variations in microbial populations between treatments were observed because of the technique used to obtain ruminal fluid (oesophageal tube), since according to Henderson et al. (2013) the abundance of one of the predominant CH_4 producers (*Methanobrevibacter*) varies depending on whether the sample is obtained in the liquid or solid fraction in the rumen. However, the results reported by Hess et al. (2006) and Rira et al. (2015) are in agreement with ours, since they did not find any changes in their quantities when they conducted their research with *E. cyclocarpum* pods and *G. sepium* legume.

Similarly, while there was no difference in the quantity of archaea, there was also no difference in protozoa between treatments; both microorganisms are closely related, since archaea are able to develop endosymbiosis with protozoa, because some of them may contain organelles in their membranes, which produce H_2 (Patra et al., 2017). In contrast to the findings of this study, Navas-Camacho et al. (1993) reported an increase in the number of protozoa when supplying diets with leaves of *Enterolobium cyclocarpum* (10% DMI), due to an increased availability of slowly degradable protein that these microorganisms use as a source of nitrogen for cell synthesis. Nevertheless, at high doses, they have an adverse effect, as demonstrated by Monforte-Briceño et al. (2005) and Albores-Moreno et al. (2017) who suggested that there is a reduction in the number of protozoa given the sensibility of their cell membrane to the presence of saponins in the diet. When evaluating protozoa over time, Wina et al. (2006) reported that, in the short term, protozoa adapted quickly to saponins, but in the long term, the number or protozoa was reduced, depending on the dose. Other studies state that the effect of saponins on protozoa is transient, as bacteria may degrade saponins into sapogenins, a compound that is not toxic to protozoa

(Newbold et al., 1997; Ramos-Morales et al., 2017). It has also been reported that these microorganisms are able to thicken their cell walls or produce extra-cellular polysaccharides around the membrane, thus avoiding its degradation in the rumen (Wina et al., 2006).

In this study, supplementing diets with the pods and legumes did not result in weight gains, perhaps because feed intake was restricted in order to prevent feed selection by cattle. Nonetheless, numerous studies on sheep and cattle have reported weight gains (29- 89 g/d and 355- 695 g/d, respectively) by supplying of those legume or pods (Navas-Camacho et al., 1993; Abdulrazak et al., 1997; Mpairwe et al., 1998).

CONCLUSION

It can be concluded that the incorporation of 15% of *E. cyclocarpum* mixed with *G. sepium* into the diet of crossbred heifers for 80 days reduces methane emissions (g/kg DMI, g/kg DDMI, g/kg DCP, energy loss as CH_4, kg/kg ADG/year). However, this reduction in CH_4 production cannot be ascribed to the effect of CT and saponins on DM digestibility, variations in the concentration of VFA's, or microbial populations (total bacteria, protozoa, archaea). Further research is required to assess changes in population dynamics at the level of Phylum or other microbial populations that could have had an influence on these results and on nitrogen kinetics at the whole animal level.

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Table 1. Proportion of ingredients and chemical composition of the ingredients and experimental diets.

Items	Ingredients							Diets[1]	
	Sorghum halepense	***Panicum maximum***	***Enterolobium cyclocarpum***	***Gliricidia sepium***	**Soybean meal**	**Wheat bran**	**Cane molasses**	**E+G:0**	**E+G: 15**
Chemical composition									
Dry matter	911.9	883.5	955.7	912.1	916.4	915.0	860.0		
Crude protein, (g/kg DM[2])	27.7	43.3	156.3	163.6	431.0	164.3	31.5	9.7	10.0
Neutral detergent fiber (g/kg DM)	753.2	767.5	310.1	556.7	287.8	479.8	n.d.[3]	64.4	61.8
Acid detergent fiber (g/kg DM)	502.1	513.4	212.9	429.3	81.3	139.8	n.d.	39.5	39.1
Gross energy (MJ/kg DM)	16.9	16.3	17.3	17.9	18.1	17.2	14.7	16.4	16.4
Ash (g/kg DM)	57.3	63.5	36.3	98.2	67.4	59.6	119.0	6.16	6.26
Total phenols (mg/g)	10.5	3.6	14.2	6.4	n.d.	n.d.	n.d.	0.55	0.64
Tannins phenols (mg/g)	0.6	0.5	8.2	1.1	n.d.	n.d.	n.d.	0.28	0.32
Condensed tannins (mg/g)	16.5	0.0	41.3	45.9	n.d.	n.d.	n.d.	0.68	1.24
Saponins (mg/g)	0.0	0.0	27.0	17.0	n.d.	n.d.	n.d.	0.00	3.30

	Sorghum halepense	***Panicum maximum***	***Enterolobium cyclocarpum***	***Gliricidia sepium***	**Soybean meal**	**Wheat bran**	**Cane molasses**	**Minerals**	**$CaCO_3$**
Proportion of ingredients (%)									
E+G:0	42.27	30.48	0	0	11.94	11.94	2.43	0.65	0.28
E+G: 15	37.05	26.80	7.5	7.5	8.9	8.9	2.43	0.65	0.28

[1] E+G:0= Control diet; E+G:15= Diet with *E. cyclocarpum* 7.5% + *G. sepium* 7.5%

[2] DM = Dry matter

[3] n.d. = not determined

Table 2. Specific primers used for qRT-PCR

Organisms	Sequences (5' – 3')	Annealing (°C)	Amplicon size (bp)	References
Total bacteria	Fw: ACTCCTACGGGAGGCAG	57	552	Stevenson and Weimer (2007)
	Rv: GACTACCAGGGTATCTAATCC			
Methanogenic archaea	Fw: GGATTAGATACCCSGGTAGT	60	173	Hook et al. (2009)
	Rv: GTTGARTCCAATTAAACCGCA			
Total protozoa	Fw: GCTTTCGWTGGTAGTGTATT	55	237	Sylvester et al. (2004)
	Rv: CTTGCCCTCYAATCGTWCT			

Table 3. Nutrient and energy intake and digestibility of heifers fed (long-term) with and without *Gliricidia sepium* mixed with *Enterolobium cyclocarpum.*

	Phase 0				Phases 1 to 5					
								P-Value		
Items	E+G:0	E+G:0	SEM	P-Value	E+G:0	E+G:15	SEM [3]	Diet[1]	Time	D*T[2]
ADG (g/d)	129.75	116.75	50.76	0.731	354.92	395.24	46.94	0.598	0.018	0.383
Intake										
DM (kg/d)	5.81	6.25	0.36	0.135	6.11	6.62	0.20	0.121	0.001	0.031
OM (kg/d)	5.46	5.87	0.33	0.132	5.73	5.20	0.19	0.121	0.001	0.036
CP (g/d)	586	605	37.2	0.494	595 [b]	670 [a]	19.2	0.031	0.003	0.458
NDF (kg/d)	3.68	4.02	0.22	0.091	3.92	4.07	0.13	0.484	0.002	0.023
ADF (kg/d)	2.26	2.47	0.14	0.073	2.40	2.57	0.08	0.198	0.002	0.019
Condensed Tannins (g/d)	0.00	0.00			0.00 [b]	43.22 [a]	0.15	0.0001	0.001	0.001
Saponins (g/d)	0.00	0.00			0.00 [b]	21.81 [a]	0.08	0.0001	0.001	0.001
Apparent nutrient digestibility (g/kg)										
DM	498.8	505.5	24.9	0.722	512.1	531.7	7. 25	0.086	0.198	0.412
OM	520.9	527.4	23.8	0.713	537.2	548.8	6.52	0.267	0.239	0.201
CP	619.9	625.4	18.7	0.691	627.8 [a]	606.8 [b]	5.68	0.039	0.197	0.052
NDF	494.7	504.3	25.2	0.613	517.5	504.6	6.71	0.223	0.112	0.043
ADF	448.0	451.1	29.3	0.896	472.4	461.8	7.26	0.342	0.094	0.023
Digestible intake										
OM (kg/d)	2.85	3.10	0.23	0.187	3.08	3.40	0.13	0.136	0.157	0.065
CP (g/d)	366.7	375.3	27.5	0.670	372.9	406.1	14.7	0.161	0.117	0.016
NDF (kg/d)	1.87	1.99	0.15	0.313	2.04	2.05	0.09	0.907	0.045	0.005
ADF (kg/d)	1.01	1.12	0.10	0.206	1.14	1.19	0.05	0.545	0.058	0.003
Energy intake (MJ/d)										
Gross Energy	94.89	102.21	5.76	0.123	99.84	108.49	3.21	0.107	0.001	0.007

Digestible energy	47.21	50.58	4.00	0.283	51.40	56.20	2.31	0.175	0.282	0.061

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

[1] E+G:0= Control diet; E+G:15= Diet with *E. cyclocarpum* 7.5% + *G. sepium* 7.5%

[2] D*T = Interaction of Diet and Time

[3] SE = standard error

ADG = average daily gain;

DM = dry matter;

OM = organic matter;

CP = crude protein;

NDF = neutral detergent fiber;

ADF = acid detergent fiber;

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

	Ingredients							
Items	**Soybean meal**	**Wheat bran**	***Enterolobium cyclocarpum***	***Gliricidia sepium***	***Brachiaria brizantha***	***S. halepense***	**SE**	**p-value**
a	542.0 [b]	475.6 [c]	623.3 [a]	301.9 [d]	129.8 [e]	142.5 [e]	16.4	0.001
b	449.1 [ab]	317.2 [c]	247.4 [c]	336.0 [bc]	278.9 [c]	466.9 [a]	41.7	0.001
c	1.80 [ab]	1.11 [ab]	0.63 [ab]	0.23 [bc]	0.16 [bc]	1.12 [ab]	0.42	0.001
a+b	991.1 [a]	792.9 [b]	871.2 [ab]	637.9 [c]	408.7 [d]	609.4 [c]	51.0	0.001
Kp	19.8 [b]	1.12 [d]	0.71 [d]	23.7 [a]	14.1 [c]	0.24 [d]	1.31	0.001
ERD	990.8 [a]	791.4 [b]	856.3 [ab]	479.8 [c]	369.1 [c]	528.3 [c]	52.6	0.001

[a,b,c,d,e] 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. Concentrations of volatile fatty acids (VFA) and pH in the rumen of heifers fed (long-term) with and without *Gliricidia sepium* mixed with *Enterolobium cyclocarpum.*

	Phase 0				Phases 1 to 5					
								P-Value		
Items	**E+G:0**	**E+G:0**	**SEM**	**P-Value**	**E+G:0**	**E+G:15**	**SEM [3]**	**Diet[1]**	**Time**	**D*T[2]**
pH	6.58	6.90	0.22	0.081	6.87	6.72	0.05	0.060	0.193	0.001
Total VFA (mmol/L)	72.25	71.10	4.73	0.648	73.34	70.97	6.45	0.882	0.094	0.967
Acetic acid (mmol/100 mol)	65.72	65.37	1.70	0.673	60.88	60.97	3.97	0.987	0.082	0.991
Propionic acid (mmol/100 mol)	20.58	20.35	2.72	0.588	19.28	19.51	1.25	0.898	0.109	0.964
Butyric acid (mmol/100 mol)	10.00	10.53	7.80	0.389	10.15	9.489	0.46	0.347	0.197	0.722
Iso-acids (mmol/100 mol)	3.725	3.775	8.30	0.829	4.029	4.062	0.12	0.854	0.495	0.392
Acetic:propionic acid ratio	3.200	3.175	3.79	0.779	3.184	3.289	0.09	0.982	0.374	0.591
Total VFA (mmol/L)/ DMI (kg/d)	11.56	12.49	0.87	0.221	12.05	11.95	0.45	0.874	0.505	0.409
Total VFA (mmol/L)/ DOMI (kg/d)	25.55	23.23	1.90	0.151	24.55	23.23	0.94	0.216	0.313	0.079

[1] E+G:0= Control diet; E+G:15= Diet with *E. cyclocarpum* 7.5% + *G. sepium* 7.5%

[2.] D*T = Interaction of Diet and Time

[3] SE = standard error

DMI = dry matter intake;

DOMI = digestible organic-matter intake

Table 6. Enteric CH_4 production in heifers fed (long-term) with and without *Gliricidia sepium* mixed with *Enterolobium cyclocarpum.*

Items	Phase 0				Phases 1 to 5					
								P-Value		
	E+G:0	E+G:0	SEM	P-Value	E+G:0	E+G:15	SEM [3]	Diet [1]	Time	D*T [2]
CH_4 (g)/ d	140.3	149.3	7.84	0.1550	145.99	147.78	3.02	0.6319	0.2662	0.4407
CH_4 (g)/ DMI (kg)	23.89	24.15	0.56	0.5532	23.95 [a]	23.32 [b]	0.33	0.0166	0.8858	0.6827
CH_4 (g)/ DDMI (kg)	47.36	48.52	2.93	0.5991	47.01 [a]	42.08 [b]	1.14	0.0258	0.2698	0.2323
CH_4 (g)/ DCP (kg)	383.44	396.34	15.0	0.2013	392.3 [a]	361.4 [b]	8.50	0.033	0.4143	0.2235
CH_4 (g)/ DNDF (kg)	75.17	75.51	4.77	0.9231	73.03	71.48	1.89	0.6901	0.0974	0.2019
CH_4 (g)/ DADF(kg)	134.94	139.39	10.5	0.5728	131.24	123.24	3.71	0.2457	0.0577	0.1891
Energy loss as CH_4 (% GE)	8.13	8.21	0.19	0.5621	8.15 a	7.59 b	0.11	0.0163	0.8863	0.6828
CH_4 (kg)/ (ADG (kg) · year)	0.62	0.66	0.03	0.1439	0.65	0.40	0.01	0.0001	0.5780	0.6391

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

[1] E+G:0= Control diet; E+G:15= Diet with *E. cyclocarpum* 7.5% + *G. sepium* 7.5%

[2.] D*T = Interaction of Diet and Time

[3] SE = standard error

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;

Table 7. Total population of bacteria, protozoa, and archaea (copy number/mL ruminal fluid) in crossbred heifers fed (long-term) with and without *Gliricidia sepium* mixed with *Enterolobium cyclocarpum*

Rumen microbes (copy number/mL)	Phase 0				Phases 1 to 5					
								P-Value		
	E+G:0	E+G:0	SEM	P-Value	E+G:0	E+G:15	SEM[3]	Diet [1]	Time	D*T [2]
Total bacteria [log_{10}]	9.59	9.46	0.26	0.603	9.50	9.59	0.08	0.610	0.812	0.662
Total protozoa [log_{10}]	7.48	7.43	0.24	0.195	7.25	7.21	0.06	0.730	0.901	0.692
Methanogenic archaea [log_{10}]	6.72	6.76	0.28	0.751	6.62	6.59	0.08	0.873	0.831	0.655
Relation archaea:bacteria	0.70	0.71	0.02	0.356	0.70	0.69	0.01	0.685	0.740	0.668

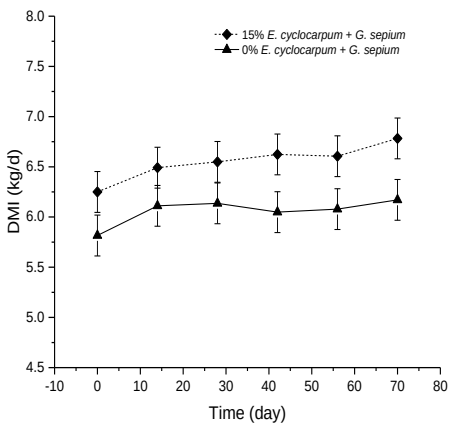
[1] E+G:0= Control diet; E+G:15= Diet with *E. cyclocarpum* 7.5% + *G. sepium* 7.5%

[2.] D*T = Interaction of Diet and Time

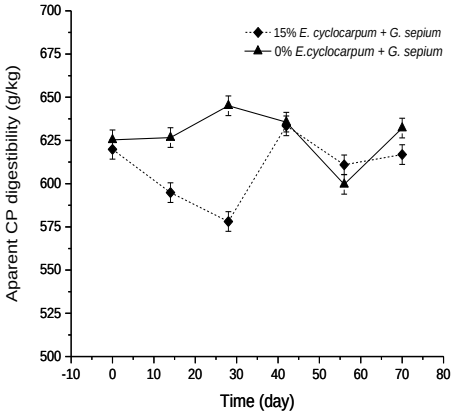
[3] SE = standard error

Figure 1. Experimental design of heifers fed (long-term) with and without *Gliricidia sepium* mixed with *Enterolobium cyclocarpum*

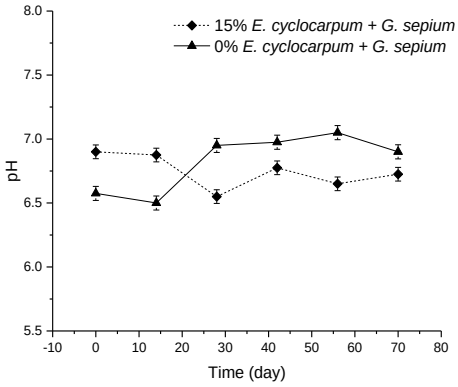
 *Control diet*					*Crossbred heifers fed with and without Gliricidia sepium (G)mixed with Enterolobium cyclocarpum (E)* *E+G:0= Control diet*  *E+G:15= Diet with E. cyclocarpum 7.5% + G. sepium 7.5%* 																				***Diet***
0					***1***					***2***					***3***					***4***					***5***					***Phase***
-14	*…*	*-2*	*-1*	*0*	*1*	*…*	*14*	*15*	*16*	*17*	*…*	*28*	*29*	*30*	*31*	*….*	*42*	*43*	*44*	*45*	*…*	*56*	*57*	*58*	*59*	*…*	*70*	*71*	*72*	***Days***
		•	•	•			•	•	•			•	•	•			•	•	•			•	•	•			•	•	•	***Measurement of response variables***



(a)



(b)



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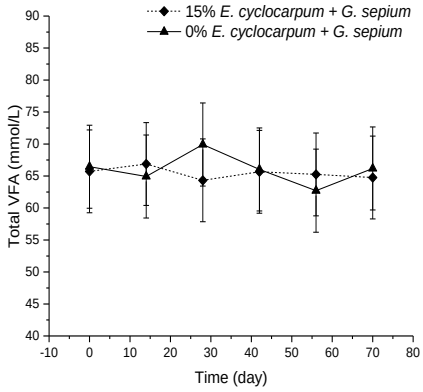

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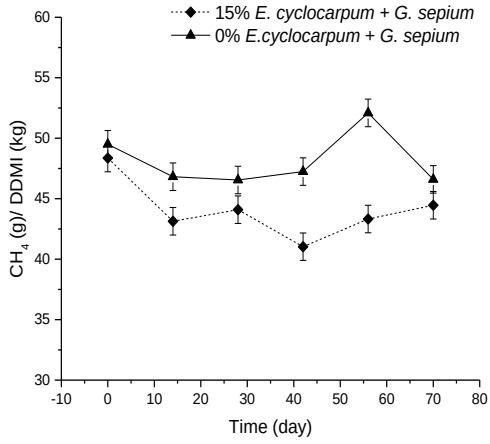
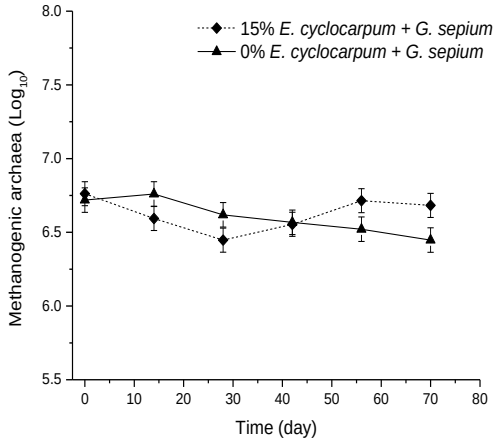


(e)



(f)

Figure 2. Mean ***(a)*** Mean DMI (kg/d), ***(b)*** apparent CP digestibility (g/kg), ***(c)*** pH, (d) total VFA (mmol/L), ***(e)*** CH_4 (g) / DDMI (kg), ***(f)*** methanogenic archaea (Log_{10}) averaged over the treatment period from crossbred heifers fed with or without 15% of *E. cyclocarpum* mixed with *G. sepium.*