



Levels of the *Enterolobium cyclocarpum* pod in feedlot diet on growth performance, ruminal fermentation and biogas production of lambs-hair

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ABSTRACT

Enterolobium cyclocarpum (Ec) is a native legume that grows from southern Mexico to South America; its seeds are traditionally used for feeding ruminants. The objective of this study was to evaluate the addition of Ec pods in diets of hair lambs and their effect on the productive variables, ruminal fermentation and *in vitro* production of methane (CH₄) and carbon dioxide. Thirty male lambs fed diets containing 0% (Ec0), 20% (Ec20) and 40% (Ec40) based on dry matter were evaluated. The addition of Ec40 in the integral diet of the lambs did not affect the growth performance. The pH of the ruminal fluid did not show differences between the treatments. The addition of Ec40 increased propionate and decreased acetate. Protozoa decreased in 47 and 59% with Ec20 and Ec40, respectively; while the population of cellulolytic bacteria decreased with Ec40. *In vitro* degradability of dry matter was lower in 8.8% with the inclusion of Ec40 in diets. The novelty of the study was that Ec40 diet decreased the ruminal methane production.

Keywords: Methane, Rumen, Ruminal microorganisms, Volatile fatty acid

In Latin American countries including Mexico, sheep production in tropical regions is based on the grazing of animals with pastures with high amount of structural carbohydrates and low protein level (Archimede *et al.* 2014). Unfortunately, the availability of forages during the year is variable and most of times lacking during dry season. Generally, animals that consume these forages are native lambs owned by small producers in poor conditions (Santacoloma 2011). One of the strategies to improve productivity of the lambs, is by elaborating complex diets, with ingredients cultivated in their own regions. Specifically, the use of tropical fruits, which can provide the necessary nutrients to formulate rations (Anantosook *et al.* 2015, Albores *et al.* 2017, Vélez *et al.* 2018). The fruits or pods of *Enterolobium cyclocarpum* (Ec) are used as a protein and energy supplement in the diet of ruminants in the tropical regions; the crude protein content of the pod varies from 19 to 30% and the metabolizable energy (ME) content is from 6 to 10 MJ/kg of dry matter (DM) (Álvarez *et al.* 2003, Barrientos *et al.* 2015). Ec pods have been included in rations for ruminants from 2.5 to 30% in the diet, obtaining response variations (Moscoso *et al.* 1995, Galindo *et al.* 2001); toxicity occurred in animals when 60% of Ec was included (Briceño-Poot *et al.* 2012). The differences may be due to the amount of anti-nutritional components in the pod or the use of ruminal inoculum

obtained from animals not adapted to diets with the pod, among other several causes. Therefore, the objective of our study was to evaluate 3 levels of Ec pods in lamb diets by evaluating the productive parameters and the rumen fermentation variables that may occur with ample feeding time in each of the assigned groups.

MATERIALS AND METHODS

The experiment was regulated with the standards of ethics and animal welfare required by the Official Mexican Standard published in accordance with the guidelines of the Mexican Council on Animal Care (NOM-062-ZOO 1999). The experiment was performed in the Biochemistry and Animal Nutrition laboratories of the Universidad del Mar, Puerto Escondido in the state of Oaxaca, Mexico.

The collection of Ec pods was carried out for 6 days in the month of April 2017, in communities of Puerto Escondido, Bajos de Chila and Pochutla, Oaxaca. These were obtained directly from the soil, indicating a good state of maturity by the natural fall of the tree. The pods had the following characteristics: mature pods of circular shape with measurements between 7 and 15 cm in diameter; flattened and curled brown. Its external and internal consistency was woody and spongy, respectively. The pods contained from 10 to 16 ovoid seeds with measures of 1.5–2.3 cm.

Thirty male lambs (Pelibuey × Katadin) of 3 months old were selected and transferred to the experimental unit of the university and housed in individual cages 1 m wide by

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1.96 m in length. During the management, the lambs were fed and gradually adapted to a diet high in grain (plus 50%) for 2 weeks. Previously the animals were dewormed with a combination of fenbendazole (Panacur® 10% suspension, Intervet), closantel (5% closantil suspension, Chioin) and ivermectin (Virbamec® L.A. Virbac). At the beginning of the experiment, the lambs had an average weight of 12.88 ± 0.48 kg. Each group (8 animals) was randomly assigned to one of the three evaluated treatments, viz. Eco (no supplementation of *Enterolobium cyclocarpum*), Ec20 (supplemented with 20% of Ec) and Ec40 (supplemental with 40% of Ec). The diets were formulated and evaluated in accordance with the nutritional requirements of the NRC (2007) for growing lambs. Corn grain and soybean meal were substituted by the amounts of Ec pods. All the ingredients used in the diets were sown and cultivated in the study region, with the exception of soybean meal (Table 1). The corn and pods were milled with a hammer mill, using 1 cm² mesh.

Table 1. Composition of experimental diets fed to lambs hair

Item (%)	Ec0	Ec20	Ec40
<i>Enterolobium cyclocarpum</i> pod	0.00	20.00	40.00
Seeds	0.00	8.00	16.10
Shell	0.00	12.00	23.90
Corn grain	27.50	14.00	0.00
Soybean meal	12.50	6.00	0.00
Peanut straw	30.00	30.00	30.00
Alfalfa hay	28.50	28.50	28.50
Limestone	1.00	1.00	1.00
Trace mineral salt ^a	0.50	0.50	0.50
<i>Nutrient composition (DM basis)</i>			
Dry matter (% ^b)	89.10	87.50	86.90
Organic matter (% ^b)	90.80	91.60	92.70
ME (MJ/kg ^c)	9.21	8.92	8.42
Protein (% ^b)	14.30	14.30	14.50
Neutral detergent fibre (% ^b)	33.00	31.30	29.50
Acid detergent fibre (% ^b)	22.40	24.80	25.40
Calcium (% ^b)	0.81	0.77	0.74
Phosphorous (% ^b)	0.22	0.18	0.19

^aTrace mineral salt contained: NaCl, 92.96%; CuSO₄, 1.04%; CoSO₄, 0.068%; FeSO₄, 3.57%; ZnO, 1.24%; MnSO₄, 1.07%; KI, 0.052%. ^bIndividual values measured in laboratory. ^cBased on tabular values for individual feed ingredients (NRC, 2007) with the exception of *Enterolobium cyclocarpum* pod, which the total energy was measured.

The animals were fed daily at 8:00 and 16:00 h. Amounts of total mixed ration fed to lambs were calculated according to previous days of animal intake, and adjustments were made when needed so that refused feed did not exceed 15% of daily intake. Dry matter intake (DMI) and average daily weight (ADG) were measured every 14 days. The feed conversion (FC) was calculated between the DMI and the ADG. The averages in each variable were only reported at the end of the experiment.

The amount of 100 mL of ruminal fluid from each animal

was extracted with an esophageal tube (ET) 3 h after giving the morning meal, during the last day of the experiment (60 d). The saliva impregnated in the tip and outer layer of the ET was cleaned, preventing contamination with ruminal fluid deposited in a beaker. Then, the duplicate samples were strained through three layers of cheese cloth and pH, total bacteria amount, number of cellulolytic bacteria and the amount of protozoa were recorded (per mL of ruminal fluid). Other ruminal fluid samples were mixed with 25% metaphosphoric acid, ratio 1 : 4 and frozen (–20°C) until the VFAs (acetic, propionic and butyric) analysis.

Two incubation runs were performed in 10 days. Then, an amount of 0.5 g DM of each experimental diet was weighed in vials of 120 mL. Subsequently, 45 mL of the buffer solution (Cobos and Yokoyama 1995) and 15 mL of fresh rumen fluid, obtained from 2 lambs per treatment. The vials were maintained in anaerobic conditions with CO₂, and they were incubated in a water bath at 39°C connecting to a Tygon® hose (inner diameter 3/32) with two needles at each end (Terumo® 20 gauge GX1). The biogas (CH₄ and CO₂) production was quantified with the water displacement technique at 24, 48 and 72 h of fermentation. Then, 500 µL samples of the biogas were taken by duplicate to quantify the amounts of CH₄ and CO₂. The *in vitro* degradability of dry matter (IVDDM) from each culture incubation were measured at 72 h and calculated using the following equation:

$$IVDDM = 1 - [(R - B)/S]$$

where R is g of the residue of the substrate, B is g of the residue of the blank, and S is the DM weight of the initial substrate.

Chemical analysis: The chemical composition of the diets as dry matter, ash, and crude protein, was determined as per AOAC (2005). The content of neutral detergent fibre (NDF) and acid detergent fibre (ADF) was determined by the method described by Van Soest *et al.* (1991). Gross energy was measured with adiabatic calorimeter bomb (Model-1271, Parr-Moline, IL).

Ruminal pH: pH was measured with a portable Orion® potentiometer, calibrated at pH 4 and 7.

Total rumen bacteria count: The total bacteria was determined by direct counting in a Petroff-Houser® camera using a 1000× Motic® optical microscope. The concentration of cellulolytic bacteria in the rumen fluid was determined using the most probable number technique (Harrigan and McCance 1979).

Ruminal cellulolytic bacteria count: Fresh ruminal fluid was inoculated into culture tubes (13×100 mm) containing liquid anaerobic culture medium for cellulolytic bacteria (Hungate 1969). Bacterial growth was confirmed with degradation of Whatman 541 paper by 10 d of incubation at 39°C.

Ruminal protozoa count: They were performed with a Neubauer-improved camera (Marienfeld, USA) on a Motic® optical microscope at a magnification of 400×.

Ruminal VFAs molar concentration: The VFAs were

measured with a Perkin Elmer® gas chromatograph with a flame ionization detector. The equipment conditions were oven temperature 130°C, the injector and capillary column (15×0.32 m) 250°C. The retention times were 1.26 min for acetate, 1.6 min propionate and 2.09 min butyrate (Erwin *et al.* 1961).

Ruminal methane and carbon dioxide: The amounts of CH₄ and CO₂ were analyzed with a gas chromatograph (Perkin Elmer®) equipped with a thermal conductivity detector and a Poro-pack packed column. The detection conditions were as follows: oven temperature 80°C, column 170°C and thermal conductivity detector 130°C. The retention times were 0.71 and 1.005 min for CH₄ and CO₂, respectively. Helium was used as a carrier at a flow of 23 mL/min. The molar concentration of CH₄ and CO₂ were calculated as described by Posada and Noguera (2005).

Data were analyzed as a complete randomized design using the GLM model procedure of the SAS Institute, Inc. (2011) with treatment as the fixed effect. For chemical composition or individual tube treatments were used as random factor. Differences among treatments were differentiated using LSMEANS with the PDIF option. The data of the bacteria and protozoa were transformed to Log 10, prior to the statistical analysis.

RESULTS AND DISCUSSION

The protein content in the seed was higher than in the shell (Table 2). Other study report 22.8 and 31.1% PC contents for the seed and pod, respectively (Álvarez *et al.*

Table 2. Characteristics of *Enterolobium cyclocarpum* pod

Weight of the seeds (g)	12.01±3.60
Cover-shell weight (g)	17.88±4.21
Total weight (g)	29.89±3.92
Dry matter (g/100 g)	92.7±4.12
Protein in the seed (g/100 g)	24.78±3.80
Protein in the pod (g/100 g)	16.67±2.16
Neutral detergent fibre (g/100 g)	34.03±4.24
Gross energy (MJ/kg)	18.38±0.93

2003). The content of FDN in Ec pods was similar with previous works (Albores *et al.* 2017). There is no data available in gross energy in Ec pods. Several studies report levels from 6.6 to 10.4 MJ EM/kg to seed and shell, respectively (Álvarez *et al.* 2003, Albores *et al.* 2017). But these values were calculated indirectly with data obtained from the proximal bromatological analysis.

The addition of Ec40 in the integral diet of the lambs did not affect the growth performance, indicating that corn grain and soybean meal can be substituted as a source of protein and energy respectively (Table 3). Other studies indicate similar results when the maximum level of Ec-30% has been used in diets for lambs, obtaining ADG from 111 to 135 g/d (Peralta *et al.* 2004, Esquivel *et al.* 2010, Briceño-Poot *et al.* 2012). In this study, the cost of the ration decreased 5 and 14%, as Ec increased by 20 and 40%, respectively.

Table 3. Influence of *Enterolobium cyclocarpum* pod level on growth-performance response of lambs-hair

Item	Ec0	Ec20	Ec40	SEM
Days on test	60	60	60	
Live weight (kg)				
Initial	12.80 ^a	12.84 ^a	12.82 ^a	0.48
Final	20.29 ^a	20.0 ^a	20.95 ^a	0.51
Weight gain (g/d)	0.124 ^a	0.111 ^a	0.135 ^a	0.08
DM intake (g/d)	0.701 ^a	0.692 ^a	0.738 ^a	0.09
DM intake/gain	5.73 ^a	6.21 ^a	5.87 ^a	0.17

Corn grain and soybean meal were substituted by *Enterolobium cyclocarpum* pod at 0% (Ec0), 20% (Ec20) and 40% (Ec40) in the diet. SEM, Standard error mean. ^{a,b}Different superscripts in the same row indicate differences (P<0.05) between treatments.

VFA and pH in rumen fluid: The pH of the ruminal fluid did not show difference (P>0.05) between the treatments (Table 4) coinciding with another study when Ec was included up to 50% of the diet (Hess *et al.* 2003). The incorporation of Ec40 increased (P<0.05) the propionate (37%) and decreased the acetate (9.3%). Although, in this study, the concentration of saponins in diets was not determined, it is reported that amounts of 8–15 g of saponins/animal/d included in the pod of Ec increased propionate and decreased acetate (Albores *et al.* 2017, Serratos *et al.* 2008, Patra and Saxena 2009). The ideal activity of methanogens is given by the range of pH 6.0–7.0 (Rea *et al.* 2007) as recorded in this study.

Number of bacteria and protozoa in ruminal fluid: The number of total bacteria in the ruminal fluid showed no difference (P>0.05) between the treatments (Table 4). While the population of cellulolytic bacteria decreased 57% (P<0.05) with Ec40 oscillated from 3.9 to 9.3 × 10⁵/ml of ruminal fluid. Possibly the presence of saponins in the pods of Ec affected the development of cellulolytic bacteria due to their antibacterial activity (Serratos *et al.* 2008, Patra and Saxena 2009). Saponins have antibacterial effects by

Table 4. Influence of *Enterolobium cyclocarpum* pod level on ruminal characteristics of lambs-hair

Item	Ec0	Ec20	Ec40	SEM
pH	6.12 ^a	6.20 ^a	6.07 ^a	0.19
<i>Ruminal microorganisms</i>				
Total bacteria, 10 ¹⁰ /ml	1.76 ^a	1.83 ^a	1.27 ^a	0.15
Cellulolytic bacteria, 10 ⁵ /ml	9.26 ^a	7.48 ^a	3.92 ^b	0.57
Protozoa, 10 ⁶ /mL	11.16 ^a	5.93 ^b	4.56 ^b	0.54
<i>Volatile fatty acids (mmol/100 moles)</i>				
Acetate	75.52 ^a	73.23 ^a	68.52 ^b	1.03
Propionate	15.04 ^a	16.67 ^a	20.71 ^b	0.69
Butyrate	9.42 ^a	10.42 ^a	10.76 ^a	0.41

Corn grain and soybean meal were substituted by *Enterolobium cyclocarpum* pod at 0% (Ec0), 20% (Ec20) and 40% (Ec40) in the diet. SEM, Standard error mean. ^{a,b}Different superscripts in the same row indicate differences (P<0.05) between treatments.

altering the permeability of the cell membrane, affecting surface tension and giving rise to cellular content, destroying gram positive and negative bacteria (Wina *et al.* 2005, Sung and Lee 2008). Protozoa decreased ($P < 0.05$) in 47 and 59% with Ec20 and Ec40, respectively (Table 3). Ivan *et al.* (2004) reported high amounts (more than 20%) of Ec in the diet of ruminants caused defaunation by the effect of saponins and consequently decreased the level of acetic acid in the rumen (Albores *et al.* 2017). Saponins are toxic to the protozoa of the rumen, an insoluble mass is formed with the sterols located in the cellular membranes of the protozoa, causing perforations in the membrane and inducing cell death and lysis (Francis *et al.* 2002).

IVDDM was lowered ($P < 0.05$) 8.8% with the inclusion of Ec40 in the diets (Table 4). This difference is due to the higher digestibility rate of corn grain and soybean meal (Álvarez *et al.* 2003) or the higher content of saponins contained in Ec (Morgavi *et al.* 2012, Albores *et al.* 2017). Substitution in diets with corn grain and soybean meal by Ec decreased the IVDDM, perhaps this response was due to the lower amount of starch, the greater amount of FDA (25.4%) contained in the pods of Ec (Patra and Saxena 2009). In addition, the decrease of IVDDM in the diet has been associated with the reduction of cellulolytic bacteria, caused by the presence of saponins contained in Ec (Wina *et al.* 2005, Yogiando *et al.* 2014).

Treatment with Ec40 at 72 h of fermentation decreased the CH_4 content by 39% and increased CO_2 by 28% ($P < 0.05$), compared to Ec0 (Table 5). As it is known to increase the content of structural carbohydrates in diets, the production of CH_4 increases (Tiemman *et al.* 2008) because acetate fermentation, CO_2 and H_2 are produced in the fermentation process of structural carbohydrates as final products (Ramírez *et al.* 2012, Saminathan *et al.* 2015), which are used by the methanogenic Archaeas to produce energy and CH_4 (Patra and Saxena 2010). The reduction in methane emissions was 28% in the evaluated diets, due to decrease in the IVDDM of the diets containing Ec pod,

Table 5. Influence of *Enterolobium cyclocarpum* pod level on *in vitro* digestibility of dry matter (IVDDM) and biogas production in diets used to fed lambs hair

Item	Ec0	Ec20	Ec40	SEM
IVDDM (%)	52.48 ^a	49.63 ^{ab}	47.86 ^b	1.11
Methane (mL)				
24 h	16.37 ^b	18.07 ^a	17.44 ^a	0.17
48 h	32.75 ^b	35.61 ^a	24.65 ^c	0.32
72 h	50.53 ^a	49.15 ^a	36.31 ^b	0.54
Carbon dioxide (mL)				
24 h	114.63 ^c	148.93 ^a	134.56 ^b	3.02
48 h	146.25 ^b	166.39 ^a	163.35 ^a	2.99
72 h	162.47 ^b	178.85 ^b	208.69 ^a	4.21

Corn grain and soybean meal were substituted by *Enterolobium cyclocarpum* pod of 0% (Ec0), 20% (Ec20) and 40% (Ec40) in the diet. SEM, Standard error mean. ^{a,b}Different superscripts in the same row indicate differences ($P < 0.05$) between treatments.

which provides a lower amount of water-soluble carbohydrates (Wina *et al.* 2005) and a greater quantity of saponins (Albores *et al.* 2017); decrease in the population of the protozoa caused less formation of CH_4 (Hess *et al.* 2003). The main microbial groups that inhabit the rumen are facultative anaerobes and contribute to the degradation of the dry matter of the diet. It is known that protozoa contribute half of the microbial biomass in the rumen, therefore defaunation affects fibre digestion. The increase in propionate was due to the decrease in cellulolytic bacteria and the increase in amylolytic microbes.

The inclusion of Ec20 and Ec40 in diets for growing hair lambs in the tropics can replace part of the corn grain and soybean meal in the diet. Although digestibility was reduced but the productive parameters were not affected. The novelty of the study was that the Ec40 diet decreased the ruminal methane production.

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