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Leucaena leucocephala and *Opuntia ficus-indica* reduce the ruminal methane production *in vitro*

Leucaena leucocephala y *Opuntia ficus-indica* reducen la producción de metano *in vitro*

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Abstract

This study aimed to evaluate the inclusion of *Leucaena leucocephala* (LL) and *Opuntia ficus-indica* (OFI) fresh and fermented as alfalfa hay substitute in the forage fraction on the ruminal fermentative parameters, gas production kinetics and methane production *in vitro*. Four treatments were formulated as beef cattle diets (T1: 50% HA; T2: 30% LL y 20% HA; T3: 30% OFI y 20% HA; T4: 30% OFI fermentado y 20% HA). Total phenolics compounds and condensed tannins (CT) increased over 400% when LL was included (P<0.05). Additionally, CT increased 45% when fermented OFI was included in the ration in T4 (P<0.05). Ammonia-nitrogen, volatile fatty acids, gas production and acetate:propionate ratio were different among treatments. The maximum gas production was observed when LL was included in T2 (P<0.05). However, no changes were observed among T1, T2 and T3 (P>0.05). In addition, lag phase decreased in T2 (P<0.05). Furthermore, methane production was different among treatments (P<0.05); reductions of 26 and 14% were observed when including LL and OFI, respectively. Thus, according to the obtained results, LL and OFI are ingredients, which may be included in the bovine diets and thereby contribute to the greenhouse gases mitigation.

Keywords: methanogenesis, *Saccharomyces cerevisiae*, ruminal fermentative, prickly pear.

Resumen

El objetivo fue evaluar la inclusión de *Leucaena leucocephala* (LL) y *Opuntia ficus-indica* (OFI) fresco y fermentado como sustituto del heno de alfalfa (HA) en la fracción forrajera sobre los patrones de fermentación, cinética de producción de gas y metano *in vitro*. Cuatro tratamientos (T1: 50% HA; T2: 30% LL y 20% HA; T3: 30% OFI y 20% HA; T4: 30% OFI fermentado y 20% HA) fueron formulados como dietas para bovinos. El contenido de compuestos fenólicos totales y taninos condensados se incrementaron más de 400% con T2 (P<0.05); los taninos condensados aumentaron 45% cuando se incluyó OFI fermentado en T4. La concentración de nitrógeno amoniacal, ácidos grasos volátiles, producción de gas y la relación acetato:propionato fueron diferentes entre tratamientos (P<0.05). La máxima producción de gas se observó cuando se añadió LL a la ración (P<0.05); no se observaron cambios entre T1, T2 y T3 (P>0.05). Además, la fase lag disminuyó con T2 (P<0.05). Adicionalmente, la producción de metano fue diferente entre tratamientos (P<0.05); se observaron reducciones del 26% cuando se incluyó LL, y del 14% con OFI. De acuerdo con los resultados obtenidos, LL y el OFI son ingredientes que pudiesen incluirse en la dieta de bovinos y así, contribuir a la mitigación de gases de efecto invernadero.

Palabras clave: metanogénesis, *Saccharomyces cerevisiae*, fermentación ruminal, nopal forrajero.

INTRODUCTION

The livestock sector has undergone substantial transformations in recent decades. The growing demand resulting from the demographic explosion requires increases in livestock production (INECC, 2018). In Mexico, the production of bovines has had a constant growth of 1.6% in the last decade alone, which currently boasts a production of 1.88 million tons of carcass meat (INECC, 2018).

However, an increase in livestock production leads to an increase in greenhouse gas (GHG) emissions from ruminal enteric fermentation, mainly methane. Furthermore, ruminal methane synthesis represents an energy loss of up to 12% to the animal (Johnson and Johnson, 1995). Methane is a gas that has up to 28 times more calorific value than CO_2, so it seeks to reduce its emissions. Anthropogenic methane reaches up to 40% of the total methane emitted into the atmosphere, of which 18% is attributable to livestock through ruminal enteric fermentation (IPCC, 2015). Only in Mexico, in 2015 a total of emissions of 510,043 Gg of CO_2e (CO_2 equivalents) was registered, of which 13% is related to livestock activity; approximately 40% of these emissions is methane (INECC, 2018).

Due to this, infinity of investigations around the world have tried to create diverse strategies that decrease the production of rumen methane, through: diet modifications, the use of various additives, vaccines, use of natural extracts, administration of chemical compounds and, lately, the supplementation of bio-active compounds extracted from plants (Martin *et al*., 2010; Pámanes-Carrasco *et al.*, 2019).

Currently, efforts have been focused on the use of unconventional forage sources, which present a food alternative in animal production. In this way, some plants whose use is not common in ruminant feed can be used as an alternative to reduce GHG emissions. In fact, due to the limited availability of forage in arid and semi-arid areas, some trees, shrubs and cacti could be used as a source of forage. In this regard, *Leucaena leucocephala* (LL) is known for having a high nutritional quality, mainly protein 22-27% DM; Aye and Adegun, 2013).

It usually adapts to defoliation and regrowth in the dry season; in arid zones it can have a production of up to 112 t ha^{-1} in areas of up to 430 mm of annual precipitation and an average temperature of 32 ºC (Singh and Toky, 1995). Furthermore, in previous investigations, no changes in dry matter consumption and daily weight gain have been reported when 40% of LL was added to the ration (Piñeiro-Vázquez *et al*., 2017). Similarly, certain spineless cacti, such as the *Opuntia ficus*-indica (OFI) variety, are considered fresh and palatable forages that are produced in the dry season, and represent an important source of water for animals (González-Arreola *et al*., 2019).

However, the protein content in cacti is low, so researchers have developed biotechnological procedures that improve crude protein content through solid state fermentations, using different yeast cultures successfully (Flores-Ortiz and Reveles-Hernández, 2010; Herrera *et al*., 2014, 2017). Additionally, the addition of live cells, such as yeasts, show a reduction in methanogenesis in *in vitro* experiments (Hristov *et al*., 2013). Furthermore, both species (LL and OFI) contain secondary metabolites, such as condensed tannins, saponins and/or flavonoids, which can act as inhibitors of methane synthesis (Aye and Adegun, 2013; Alves *et al.,* 2017; Pámanes-Carrasco *et al.*, 2019;). Consequently, the use of these species as forage sources in ruminant feed can be an alternative in reducing GHG emissions, mainly methane without affecting productive development.

Therefore, the objective of the present work was to evaluate the inclusion of fresh and fermented *Leucaena leucocephala* and *Opuntia ficus-indica*, as a substitute for alfalfa hay in the forage fraction, on the fermentation patterns, kinetics of gas and methane production *in vitro.*

MATERIAL AND METHODS

Study area and materials

This research was carried out in the Animal Nutrition Laboratory of the Juárez University of the State of Durango, Mexico. *L. leucocephala* plants randomly collected during the fall of 2017, from a cultivar located at the same University. The collected plants were 1 m long and the foliage was manually after harvesting removed. The leaves of *O. ficus-indica* (variety AV6) were collected from a plantation located on land adjacent to the University. *Saccharomyces cerevisiae* yeast cultures were purchased from a local store. Table 1 shows the proximal chemical analysis of the main ingredients (alfalfa, LL, OFI and OFI fermented).

Fermentation of *O. ficus-indica*

OFI samples were fermented with *Saccharomyces cerevisiae* at 32 °C, for 48h according to the procedures reported by Herrera *et al.* (2014). Yeast cultures were added to 1% (DM). After fermentation, the samples were dried and reduced to a particle size of 1 mm for later analysis.

Formulation of experimental treatments and chemical analysis

Four experimental treatments were formulated (Table 2), such as diets for cattle with the inclusion of *L. leucocephala* (T2), *O. ficus-indica* (AV6), fresh (T3) and *O. ficus-indica* fermented (T4), as partial substitution of the alfalfa hay fraction in the control treatment (T1).

Table 1. Chemical and nutritional characterization of the ingredients of the forage fraction in experimental treatments

	Ingredients (% DM)			
	Alfalfa	Leucaena	Prickly pear	Fermented prickly pear
DM Partial	-	44.9	9.3	8.3
DM Total	89.7	89.5	90.0	88.1
Organic matter	87.1	91.5	72.0	79.6
Crude protein	16.7	21.3	5.3	17.4
Ethereal extract	1.4	3.1	1.7	3.1
FDN	45.0	42.9	53.9	42.2
FDA	27.6	13.8	13.5	21.2
Hemicellulose	17.3	29.1	40.3	21.0
Cellulose	18.6	7.5	8.3	9.1
Lignin	6.5	8.1	4.8	5.3
DIVDM	55.7	45.7	51.5	65.8
DIVOM	50.0	42.3	40.0	59.8
CFT (mgEAG/gDM)	47.8	252.2	71.4	70.5
TC (mgEC/gDM)	5.3	69.3	3.1	2.0

DM: dry matter; FDN: neutral detergent fiber; FDA: acid detergent fiber; DIVDM: *In vitro* digestibility of dry matter at 48h; DIVOM: *In vitro* digestibility of organic matter at 48h; CFT: total phenolic compounds; TC: condensed tannins; mgEAG: equivalent milligrams of gallic acid; mgEC: milligrams of catechin equivalent.

10 kg of each experimental treatment were prepared and mixed in a 150 L capacity rotary mixer (Gladiator PRO, model H8155/16); then, a representative sample of 1 kg was taken and by the quartering method, sub-samples were taken for each analysis. All experimental treatments were subjected to chemical composition (Table 3) analysis according to the standardized procedures by AOAC (2010). Furthermore, neutral detergent fiber (FDN) and acid detergent fiber (FDA), as well as cellulose, hemicellulose and the lignin fraction, were determined in a Fiber Analyzer 200 kit (ANKOM Technology, USA), as proposed by the manufacturer (ANKOM, 2020). The *in vitro* digestibility of dry material (DIVDM) and *in vitro* digestibility of organic matter (DIVOM) were according to the procedures suggested by ANKOM (2018) analyzed.

Table 2. Ingredients of the experimental treatments

	Treatments			
Ingredients (% DM)	T1	T2	T3	T4
Alfalfa hay	50	20	20	20
L. leucocephala	0	30	0	0
O. ficus-indica	0	0	30	0
O. ficus-indica fermentado	0	0	0	30
Ground corn	30	30	30	30
Harinoline	19	19	19	19
Mineral mix	1	1	1	1

Total phenolic compounds and condensed tannins

Total phenolic compounds (CFT) were determined according to the methods proposed by Heimler *et al*. (2005). Furthermore, condensed tannins (TC) were analyzed as reported by Porter *et al.* (1986).

***In vitro* fermentation patterns**

For the *in vitro* fermentation tests, ruminal fluid was obtained from two Brangus calves, equipped with a ruminal cannula, weighing approximately 450 kg, and fed with corn silage and concentrate in a 50:50 ratio. Approximately 1 g of sample from each experimental treatment was incubated with 120 mL of ruminal buffer-inoculum solution in a 2: 1 ratio at 39 °C in ANKOM glass modules (ANKOM Technology, USA), with hermetic rubber and plastic caps. , as proposed by the manufacturer in triplicate (ANKOM 2018). After 24h of incubation, the modules were opened and the pH was measured. Immediately, 10 mL aliquots were taken to be placed in glass jars, for subsequent analysis of volatile fatty acids (AGV) and Galyean (2010) proposed ammoniacal nitrogen, according to what.

***In vitro* gas production**

Approximately 1 g of each experimental treatment was placed in ANKOM glass modules (ANKOM Technologies, USA), equipped with a triplicate wireless pressure transducer. The fermentations were carried out according to what was proposed by the manufacturer (ANKOM, 2018), incubating the sample with a mixture of ruminal buffer-inoculum solution in a 2: 1 ratio. Incubations were carried out until 96h and pressure changes were recorded every hour during the process. The kinetics of *in vitro* gas production was estimated by fitting the data obtained to the Gompertz model, according to the following equation (Murillo-Ortiz *et al*., 2018):

$$GP = Ae^{-Le^{-(k_d t)}}$$

Where GP = gas production at time t (mL); A = maximum gas production (mL); k_d=gas production constant (h^{-1}); and L=latency time before gas production begins (h). Additionally, for the measurements of the proportions of methane and CO_2, the pressure relief valve of the modules was opened for 2 s, and the released gas was led through a tube to a portable gas analyzer, according to the procedure proposed by the manufacturer (GEM™5000, LANDTEC, USA) and adapted by González-Arreola *et al*. (2019).

Metabolizable energy (ME) was estimated according to the equation proposed by Menke *et al.* (1979), which is presented below:

$$EM = (1.1456 * GP_{24}) + (0.07675 * PC) + (0.1642 * EE) + 1.198$$

Where ME=metabolizable energy (MJ/kg DM); GP24=gas production after 24 h of incubation (mL); PC=crude protein (% DM); EE = ethereal extract (% DM).

Statistical analysis

The experimental data obtained was analyzed according to a completely randomized design, using the GLM procedure of the SAS statistical package (2011). The comparison of means was carried out using the Tukey test, declaring significant differences with a P <0.05.

Table 3. Chemical analysis of experimental treatments

	Treatments			
Nutrients (% DM)	T1	T2	T3	T4
OM	90.3	91.3	87.6	87.3
PC	17.4	18.1	14.3	17.5
EE	1.6	2.0	1.5	1.3
FDN	53.3	52.8	52.6	46.5
FDA	23.8	23.4	17.7	15.6
Hemicellulose	29.5	29.4	34.9	30.9
Cellulose	16.3	14.5	11.9	11.6
Lignin	7.4	8.8	5.8	3.9
DIVOM	53.7	47.6	59.8	59.4
ME (Mcal/kgDM)	4.7	4.4	4.7	4.6

OM: organic matter; PC: crude protein; EE: ethereal extract; FDN: neutral detergent fiber; FDA: acid detergent fiber; DIVOM: *In vitro* digestibility of organic matter at 48h; ME: metabolizable energy; T1: 50% alfalfa + 50% concentrate; T2: 20% alfalfa + 30% LL + 50% concentrate; T3: 20% alfalfa + 30% OFI + 50% concentrate; T4: 20% alfalfa + 30% OFI fermented + 50% concentrated.

RESULTS AND DISCUSSION

The fermentative patterns of the experimental treatments are presented in table 4. The ammoniacal nitrogen concentration was different between the treatments (P <0.05); the highest value was obtained with the control treatment (T1). Thus, when adding LL and OFI to the ration, reductions of 26.5, 28.7, and 18.4% were observed in T2, T3, and T4, respectively, compared to T1. Furthermore, the values obtained for N-NH_3 in this study are within the optimal range (5 to 10 mg/dL), which maximizes the consumption of dry matter and the use of organic matter in the rumen (Chandrasekharaiah *et al*., 2011). Likewise, the concentration of volatile fatty acids (AGV) and total volatile fatty acids (AGVT) presented differences between treatments (P <0.05). According to what was exposed by Sutton *et al*. (2003), the normal concentrations of acetic, propionic and butyric acid in a ruminal fermentation are approximately 60, 20 and 15%, respectively; however, the amounts reported in this study differ from those previously reported.

The highest concentrations of total volatile fatty acids (AGVT), as well as acetic acid, occurred in T3. In contrast, butyric and propionic acid concentrations were lower at T3. However, when calculating the molar concentration of each volatile fatty acid, the amounts of acetate, propionate and butyrate are higher in T3 than in the other treatments. These changes in the concentration of AGV may be related to the increase

in the carbohydrate content, such as hemicellulose; due to the addition of fresh OFI to the ration. In this regard, Johnson and Johnson (1995) comment that a decrease in the acetate: propionate ratio represents a more efficient fermentation process and a reduction in energy losses through the formation of its final products. This can be observed by decreasing the production of propionate, when fresh OFI is included at T3.

Table 4. Patterns of ruminal fermentation *in vitro* and concentration of phenolic compounds from experimental treatments

	Treatments				
	T1	T2	T3	T4	SEM
pH	6.9 ± 0.01	7.0 ± 0.04	6.9 ± 0.01	6.9 ± 0.01	0.05
N-NH_3 (mg/dL)	13.6 ± 0.62^a	10.0 ± 0.17^b	9.7 ± 0.04^b	11.1 ± 0.22^b	0.34
AGVT (mM)	78.7 ± 1.32^b	78.7 ± 2.04^b	132.9 ± 6.39^a	76.3 ± 1.88^b	1.03
Acetic (% AGVT)	46.8 ± 0.39^b	46.4 ± 0.77^b	57.0 ± 1.28^a	45.1 ± 0.65^b	0.84
Propionic (% AGVT)	32.7± 0.32^a	33.2 ± 0.51^a	25.1 ± 1.05^b	34.0 ± 0.46^a	0.64
Butyric (% AGVT)	15.0± 0.02^a	14.9 ± 0.15^a	12.4 ± 0.15^b	15.4 ± 0.07^a	0.11
Acetate:Propionate	1.4 ± 0.02^b	1.4 ± 0.4^b	2.2 ± 0.14^a	1.3 ± 0.03^b	0.08
CFT (mgEAG/gDM)	14.1± 0.43^c	77.1±1.04^a	20.9 ± 0.25^b	20.1 ± 0.43^b	0.61
TC (mgEC/gDM)	1.55 ± 0.03^b	20.4 ± 0.17^a	0.90 ± 0.07^c	0.50 ± 0.08^c	0.10

^{ab} Different letters in the same row indicate significant difference (p <0.05). SEM: Standard Error of the difference between means; N-NH_3 = Ammoniacal nitrogen concentration after 24 hours of *in vitro* fermentation; AGVT = Total Volatile Fatty Acids; CFT = Total phenolic compounds; TC = condensed tannins; mgEAG: equivalent milligrams of gallic acid; mgEC: milligrams of catechin equivalent.

As can be in Table 1 seen, there is a marked difference in the concentrations of total phenolic compounds (CFT), and condensed tannins (TC) between the different ingredients of the forage fraction. Because of this, Table 4 shows differences between treatments in CFT and TC (P <0.05). CFT contents increased more than four-fold when LL was included in the T2 ration. Similarly, CFTs increased about 45% when OFI was included at T3. Karimi *et al*. (2013) observed three times lower CFT contents when they compared alfalfa with LL. Furthermore, the CFT contents in OFI cladodes depend on the maturity of the plant; same that were reported in values close to 33 mg/g of DM (Figueroa-Pérez *et al*. 2016). Similarly, the CT contents are different between the experimental treatments (P <0.05); when LL was included to T2; TC content was increased by about 20 mgEC/g DM. These results agree with those reported by Berard *et al*. (2011). Because LL is considered a taniferous plant, the CFT and TC contents must be higher in T2 compared to the others. Furthermore, when OFI was added to T3 and T4, a reduction in TC concentration was observed. Márquez and Suárez (2008) reported a CT content in alfalfa close to 0.5 mg/g DM while in OFI cladodes concentrations were lower.

This study found the same effect. In this regard, attribute the high CFT contents with the inclusion of OFI to other phenolic compounds other than TC, as stated by Cardador-Martínez *et al.* (2011). Furthermore, Koenig *et al.* (2018) stated that the

addition of pure TC extracts greater than 3.5%, does not affect the consumption of dry matter in fattening calves.

The parameters of the kinetics of gas production are presented in Table 5. The maximum gas production “A” was different between treatments (P <0.05). The value of "A" recorded in T4 was different from that obtained in T2, presenting an average of 138.4 (mL/g DM) (P <0.05); while the values in the lag phase (L) decreased when LL was included in T2, when compared with T3 and T4 (P <0.05). According to what Van Soest (1994) says, cellulolytic microorganisms take less time to cross the forage cell wall with a lower lignin content; however, this effect is not observed in this study. Apparently, the increases in the cellulose fraction in T3 and T4 lead to an increase in the lag phase (L). Grilli *et al*. (2015), also found this effect, when they measured the degradability of hemicellulose in various forages; the hemicellulose contained in alfalfa degraded in less time than that contained in other forage species.

Table 5. Parameters of the kinetics of gas, methane and carbon dioxide production from ruminal fermentations *in vitro*

	Treatments				
	T1	T2	T3	T4	SEM
A (mL/g DM)	155.4 ± 1.78^{ab}	162.9 ± 9.06^{a}	142.6 ± 0.14^{ab}	138.4 ± 3.14^{b}	1.65
k_d (h^{-1})	0.1 ± 0.02	0.1 ± 0.01	0.1 ± 0.01	0.1 ± 0.01	0.05
L (h)	2.0 ± 0.15^{ab}	1.6 ± 0.14^{b}	2.3 ± 0.01^{a}	2.4 ± 0.04^{a}	0.10
PG_{24h} (mL/gDM)	123.7 ± 6.30	111.2 ± 0.22	122.0 ± 0.56	120.0 ± 1.01	1.31
CH_4 (mL/gDM)	15.0 ± 0.65^{a}	11.1 ± 0.08^{c}	13.1 ± 0.27^{b}	12.9 ± 0.07^{b}	0.35
CO_2 (mL/gDM)	102.8 ± 3.85^{a}	90.5 ± 0.39^{b}	98.9 ± 2.32^{ab}	93.7 ± 0.24^{ab}	1.22
CO_2:CH_4	6.8 ± 0.04^{c}	8.1 ± 0.09^{a}	7.5 ± 0.02^{b}	7.2 ± 0.06^{b}	0.05

^{ab} Different letters in the same row indicate significant difference (p <0.05). SEM: Standard Error of the difference between means; A=maximum gas production (mL/g DM); k_d = Constant rate of gas production (%/h); L=Latency phase (h). T1: 50% alfalfa + 50% concentrate; T2: 20% alfalfa + 30% leucaena + 50% concentrate; T3: 20% alfalfa + 30% prickly pear + 50% concentrate; T4: 20% alfalfa + 30% fermented prickly pear + 50% concentrate.

On the other hand, gas production at 24h did not show changes between treatments (P> 0.05). However, reductions of 26 and 14% in methane production were observed with the inclusion of LL and OFI in T2 and T3, respectively; of which T2 registered the lowest methane production (P <0.05). Reductions in methane production can be attributed to the presence of secondary metabolites in the ration. In this sense, the highest concentrations of CFT and TC occurred in T2. These results agree with those reported by Tavendale *et al*. (2005), who establish that the highest TC contents tend to form complexes with proteins, which limits its degradation due to the action of microorganisms present during fermentation. Additionally, methane production decreases by reducing the digestion of the fibrous fraction. The latter was observed in T2, which presented lower digestibility. In the same way, Tan *et al.* (2011) reported that the CT contained in LL could reduce the population of methanogenic microorganisms, due to the greater presence of protozoa, and thus affect the transfer of hydrogen ions. Thus, the production of methane as an electron scavenger is limited. Furthermore, the reduction in methane production in T3 and T4 is directly related to the presence of CFT. This coincides with previous research carried out by Tavendale *et al*. (2005) y Murillo-Ortiz *et al*. (2018). These authors affirm that the presence of

secondary metabolites, such as CFTs, are soluble and non-fermentable molecules that are negatively related to methane synthesis.

On the other hand, the lowest CO_2 production was registered in T2, compared to T1 ($P < 0.05$). In contrast, the highest CO_2: CH_4 ratio was found in T2 compared to T1. These results are consistent with the presence of condensed tannins; plant CT scans reduce rumen methanogenesis by inhibiting methanogens (Tavendale *et al.*, 2005). Likewise, the high values in the CO_2: CH_4 ratio in T1 indicate that at some point in the metabolic pathway, metanogenesis is being inhibited, since there is more volume of CO_2 present, which is not synthesized to methane.

The ruminal synthesis of methane involves the successive reduction of CO_2 to methane, through different levels of formyl, methylene and methyl, and its reaction with the coenzymes, among them M Coenzyme in the last step of the synthesis (Liu and Whitman, 2008). In this regard, Patra and Saxena (2010) mention that the anti-methanogenic activity of tannins could lie in the activity on certain functional proteins (enzymes), located in certain accessible sites of the methanogens. This action could interrupt ruminal methanogenesis, showing CH_4 inhibition at a point where CO_2 is as a substrate consumed; Murillo-Ortiz *et al*. (2018) observed the same effect.

CONCLUSIONS

These results indicate that the substitution of alfalfa by *Leucaena leucocephala*, maintains the nutritional quality; as well as similar values in the gas production and the amount of AGV *in vitro*. Additionally, the inclusion of 30% of *Leucaena leucocephala* and *Opuntia ficus-indica* reduce the concentration of methane *in vitro* by 26 and 14%, respectively. Therefore, due to its nutritional quality and its fermentative characteristics, they could be included in the bovine diet, and thus contribute to the mitigation of greenhouse gases.

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