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Leucaena leucocephala leaves and prickly pear (*Opuntia ficus-indica*) as substitutes for alfalfa hay (*Medicago sativa* L.) and their effects on the gas and methane production, prevalence of methanogens and patterns of ruminal fermentation *in vitro*

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Alfalfa hay has been shown to be rich in crude protein (CP) as well as rapidly degradable protein in the rumen and high inclusion in the diet usually reduces nitrogen (N) utilization and increases urine N excretion (Phelan *et al.* 2015). The use of extracts of various plants from their leaves, fruits or roots has recently been recognized for their antimicrobial and anti-methanogenic properties attributed to secondary metabolites (Malik and Singhal 2008a). Plants with bioactive contents (saponins and tannins) to modify fermentation and ruminal inhibition of methanogenesis, are an option and are generally safe, cheap, and easily available in a wide range of vegetables (Srinivas and Krishnamoorthy 2013). In this way, some plants such as *Leucaena* leaves and prickly pear may be used as alternative forage sources. *Leucaena leucocephala* is a legume species that is highly available and commonly used as forage for ruminants feeding (Montoya-Flores *et al.* 2020). Likewise, prickly pear has recently been introduced into ruminants feeding. Therefore, the aim of this study was to evaluate the effect of replacing alfalfa hay with *Leucaena* leaves and prickly pear as an alternative forage, on gas and methane production, ruminal fermentation patterns and amount total of methanogens during *in vitro* fermentation.

Four treatments were evaluated: (T1) alfalfa hay + concentrate, (50 : 50%, DM); (T2) alfalfa hay + *Leucaena* leaves + concentrate, (30 : 20 : 50%, DM); (T3) alfalfa hay + prickly pear + concentrate, (30 : 20 : 50%, DM); (T4) alfalfa hay + *Leucaena* leaves + prickly pear + concentrate (30 : 10 : 10 : 50%, DM). The gas accumulative production was recorded after 2, 4, 6, 8, 12, 16, 24, 36, 48, 72 and 96 h of incubation and the data were adjusted to the model proposed by France *et al*. (2002). The methane production measurement was carried out with a GEMTM5000 equipment. The analysis of NH_3-N and volatile fatty acids was performed by ultraviolet spectrophotometry and gas chromatography, respectively (Galyean 2010). The deoxyribonucleic acid extraction was carried out using the method described by Rojas *et al.* (2008). The primers used for the detection of bacteria and total methanogens were 16S rRNA and mcrA, respectively (Denman *et al*. 2007), while the absolute quantification was obtained with the equation proposed by Angarita *et al*. (2015). Obtained data were analyzed according to a completely randomized design using GLM procedures of SAS (2012).

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With respect to *in vitro* gas parameters and methane production, the higher A and Kd values were found in T1 and the lower in T2 ($p<0.05$) (Table 1). The A and Kd values obtained with T1, are consistent with those reported by Torres *et al*. (2020), who fermented under *in vitro* conditions, alfalfa hay plus concentrate in a range 50:50, respectively. The methane production was affected significantly by treatments ($p< 0.05$). The highest methane productions were recorded in T3 and the lowest in T2 ($p\leq0.05$). In the present study, the reduction in the methane production registered in T1 could be attributed to the increase in propionate concentration, because propionate formation consumes reducing equivalents that promote the methanogenesis (Moos *et al*. 2000), whereas the reduction in the methane production registered in T2 and T3 could be explained by the CT content in diet.

Results reported here are in agreement with that of Rira *et al*. (2015) who found under *in vitro* conditions, a reduction of 63% in methane production when they included in the diets various levels of *L. leucocephala.*

According to the data of the ruminal fermentation patterns, the highest ammonia-N concentration and propionate concentration were recorded in T1 and the lowest in T2 ($p<0.05$). The concentration of butyrate increased ($p<0.05$) in T4 compared to the other treatments ($p<0.05$). The observed ammonia-N values in this study are within

Table 1. Gas production parameters and methane production of experimental treatments.

Parameter	Treatment				
	T1	T2	T3	T4	p<
A ml/g DM	116.2±1.28[a]	102.1±1.12[d]	114.4±1.11[b]	106.7±1.32[c]	0.001
Kd/ml h	0.18±0.03[a]	0.11±0.08[d]	0.15±0.02[b]	0.13±0.05[c]	0.004
Lag h	3.0±0.12	3.3±0.16	3.1±0.22	3.1±0.39	0.412
CH_4 ml/g DM	13.7±0.19[b]	12.2±0.17[c]	15.8±0.12[a]	12.8±0.11[c]	0.050
Ammonia-N, mg d/L	12.6±0.81[a]	9.7±0.77[c]	12.3±0.54[a]	10.5±0.31[b]	0.024
Total, VFA, mM	106.8±1.11[a]	68.5±1.42[d]	79.3±2.11[b]	75.6±1.16[c]	0.048
Propionate, mol 100/mol	24.4±0.35[a]	18.2±0.47[d]	22.3±0.93[b]	19.3±0.75[c]	0.033
Butyrate mol 100/mol	5.7±0.22[c]	9.1±0.91[b]	9.3±0.15[b]	10.1±0.48[a]	0.027

Row bearing different superscripts differ significantly (p<0.05).

the optimum range to maximize the dry matter intake and organic matter utilization in the rumen which is reported between 5 and 10 mg/dl (Chandrasekharaiah *et al.* 2011). In this study, changes in propionate and butyrate concentrations could probably be explained by NFC content supplied particularly for the alfalfa hay (Wang *et al*. 2020). The results are consistent with the findings in previous studies in which alfalfa hay, *Leucaena leucocephala* and *Opuntia ficus-indica* were evaluated as forage sources in ruminant diets (Yuan *et al*. 2020).

The ruminal microorganism populations are summarized in Table 2. The total bacteria and total methanogens were not affected by treatments (p>0.05). Results obtained in total bacteria and total methanogens are higher to those reported by Angarita *et al*. (2015) and Montoya-Flores *et al.* (2020) who evaluated star grass hay, Guinea grass hay and different levels of *L. leucocephala* under *in vivo* conditions.

McSweeney *et al.* (2001) suggested that the size of the population is not the sole factor associated with methane production, and that methane emissions may be more influenced by metabolic activity and community composition of methanogens.

The results of this research showed that the replacement in the diet of alfalfa hay by *L. leucocephala* and *O. ficus-indica* did not improve *in vitro* gas production and ruminal fermentation patterns. Nevertheless, the inclusion of *L. leucocephala* in the diet decreased the methane production and did not affect the total of methanogen microorganisms.

Table 2. Ruminal microbial population of the experimental treatments after *in vitro* incubation with rumen fluid

Poblation	Treatment				
	T1	T2	T3	T4	p<
Total, bacteria	14.7±	14.9±	15.2±	15.4±	0.22
Log [ngDNA/g RC]	1.23[a]	1.45[a]	1.10[a]	2.13[a]	
Total methanogens	14.1±	13.7±	13.9±	13.6±	0.35
Log [ngDNA/gRC]	2.22[a]	1.25[a]	1.14[a]	1.17[a]	
Methanogen:	0.95±	0.91±	0.91±	0.88±	0.88
bacteria	0.09[a]	0.01[a]	0.04[a]	0.05[a]	

Row bearing different superscripts differ significantly (p<0.05).

SUMMARY

The aim of the study was to evaluate the replacement of alfalfa hay with *Leucaena leucocephala* leaves and prickly pear (*Opuntia ficus-indica*) as an alternative forage, on gas and methane production, ruminal fermentation patterns and total amount of methanogens during *in vitro* fermentation. Four treatments were evaluated: (T1) alfalfa hay + concentrate (50 : 50%, DM); (T2) alfalfa hay + *Leucaena* leaves + concentrate (30 : 20 : 50%, DM); (T3) alfalfa hay + prickly pear + concentrate (30 : 20 : 50%, DM) and (T4) alfalfa hay + *Leucaena* leaves + prickly pear + concentrate (30 : 10 : 10 : 50%, DM). The higher gas production from of soluble fraction (A) and rate gas production (Kd) values, ammonia-N (NH3-N) concentrations and propionate were found in T1 and lower in T2 (p<0.05). Nevertheless, the inclusion of *Leucaena leucocephala* in the diet decreased the methane production and did not affect the total of methanogens. Results showed that the replacement of alfalfa hay by *Leucaena leucocephala* and *Opuntia ficus-indica* in a diet do not affect the main variables of *in vitro* gas production, ruminal fermentation patterns and methanogenic microorganisms populations.

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