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Comparative *in vitro* evaluation of forage legumes (prosopis, acacia, atriplex, and leucaena) on ruminal fermentation and methanogenesis*

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ABSTRACT

Two experiments *in vitro* were conducted to evaluate four Egyptian forage legume browses, i.e., leaves of prosopis (*Prosopis juliflora),* acacia *(Acacia saligna),* atriplex *(Atriplex halimus),* and leucaena *(Leucaena leucocephala*), in comparison with Tifton (*Cynodon* sp.) grass hay for their gas production, methanogenic potential, and ruminal fermentation using a semi-automatic system for gas production (first experiment) and for ruminal and post ruminal protein degradability (second experiment). Acacia and leucaena showed pronounced methane inhibition compared with Tifton, while prosopis and leucaena decreased the acetate:propionate ratio ($P<0.01$). Acacia and leucaena presented a lower ($P<0.01$) ruminal NH_3-N concentration associated with the decreasing ($P<0.01$) ruminal protein degradability. Leucaena, however, showed higher ($P<0.01$) intestinal protein digestibility than acacia. This study suggests that the potential methanogenic properties of leguminous browses may be related not only to tannin content, but also to other factors.

KEY WORDS: *in vitro*, methane, rumen fermentation, tannins, ruminants

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INTRODUCTION

The productivity of ruminant livestock in the tropical and subtropical areas of developing countries is limited by poor nutritional conditions that are characterized by highly lignified, low digestible feed from poor and nitrogen (N)-limited native grass pastures and crop residues, or suffer from a general lack of feed during drought, or even both (Goel and Makkar, 2012).

This substandard productivity not only results in high absolute methane (CH_4) emissions that make these countries responsible for almost three-quarters of enteric CH_4 emissions, but also in a very high cost in terms of CH_4 emissions per unit of product (Aluwong et al., 2011).This is especially the case when grass-based forages are the main ingredient in ruminants' diets. Ruminants fed grass-based forages may produce more CH_4 than ruminants fed legume forages (Eckard et al., 2010); this also represents a significant loss of dietary energy that could potentially be redirected towards the production of milk and meat (Eckard et al., 2010). In this respect, forage legume browses seem promising in overcoming the limitations of feed sources in many parts of the tropics as they can be grown by small-scale farmers and contain higher amounts of both degradable and undegradable protein than grasses (Goel and Makkar, 2012). Additionally, they contain natural bioactive phytofactors like tannins which, depending on their type, concentration, and activity, form natural tannin-protein complexes in these plants and have an antiprotozoal activity. This is related to the protection of dietary protein against rumen degradation by ruminal microorganisms, decreasing ammonia production, increasing the flux of dietary protein for absorption in the intestines, and may result in improving the amount of amino acids absorbed from intestinal digestion (Szumacher-Strabel and Cieślak, 2010; Goel and Makkar, 2012).

Even though these forage legumes are useful as feed alternatives to typical low-quality diets (Sallam et al., 2010), their potential in terms of CH_4 mitigation in their native country is still limited. Apparently these legumes are promising for CH_4 reduction partly because of their lower fibre content, faster rate of passage, and in some cases, the presence of condensed tannins (CTs) (Bhatta et al., 2012). Various studies have reported that legume CTs are able to suppress ruminal methanogenesis directly through their antimethanogenic activity and in directly through their antiprotozoal activity (Bhatta et al., 2012; Goel and Makkar, 2012). In most of those studies, however, the reduction in CH_4 was confounded by concomitant changes in tannin content and forage quality (legumes vs grass), such as lower NDF content and nitrogen content. Thus, there is still considerable uncertainty about the effectiveness of forage legume tannin content to reduce enteric CH_4 emissions from ruminants (Rodríguez et al., 2010).

Thus it is hypothesized that comparative evaluations of different, less-well

researched forage legumes will yield promising candidates to reduce ruminal methanogenesis while improve the nitrogen supply compared with grasses. This hypothesis was investigated in two *in vitro* experiments, testing several common Egyptian forage legumes [prosopis (*Prosopis juliflora),* acacia *(Acasia saligna)*, atriplex *(Atriplex halimus)*, and leucaena *(Leucaena leucoce-phala)*] for ruminal degradability, fermentation, methanogenesis, and post ruminal protein digestibility evaluations compared with Tifton hay as the standard grass hay.

MATERIAL AND METHODS

The chemical analysis and *in vitro* assays were carried out at the Center for Nuclear Energy in Agriculture, University of Sao Paulo (Piracicaba-Brazil); all treatments and techniques used were in accordance to the Internal Commission for Environmental Ethics in Experimentation and Internal Commission of Ethics in Experimentation with Animals of CENA/USP. Two experiments were performed independently. The first experiment was designed to study the influence of forage legume plants on rumen methane production, degradation, and fermentation characteristics using an *in vitro* gas production technique. The second experiment was performed to evaluate the potential of these plants for ruminal protein degradability and post ruminal protein digestibility *in vitro*.

Browse samples

Four Egyptian legume browses were used: fodder leaves of prosopis (*Prosopis juliflora),* acacia *(Acasia saligna)*, atriplex *(Atriplex halimus)*, and leucaena *(Leucaena leucocephala)*. Each plant sample (branches with a diameter of 5 mm or less) had been harvested between 08.00 and 10.00 h during the wet season (February and March, 2008) while they were still green and growing in the flowering stage. About 20 kg of fresh leaves of each browse were collected on the north western coastal region of Borg El Arab, Alexandria (Egypt). In general, the climate of this region is arid Mediterranean with a scarcity of rain and high radiation. The atmospheric relative humidity ranges from 50% to 75% and the average annual rainfall is about 100-150 mm, distributed over a period of 15-25 rainy days during the wet season. The leaves were collected from 20 trees and pooled individually by browse. After oven drying at 40°C for 72 h and milling through 1 mm screen, this pool was used for the chemical analysis and the *in vitro* assays. Tifton *(Tifton-85 Cynodon* sp.*)* hay was used as the control grass plant and was obtained from the local Brazilian market.

Chemical analysis

Plant samples were analysed for dry matter (DM) basis according to AOAC (1995) as for organic matter (OM), crude protein (CP, as 6.25×N), and ether extract (EE). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were measured sequentially using the same sample in filter bags and expressed exclusive of residual ash according to Van Soest (1991) and adapted to Mertens (2002), using an Ankom Fiber Analyzer. The NDF was assayed with a heat-stable amylase. Acid detergent lignin (ADL) was determined by solubilization of cellulose with sulphuric acid (72%) according to Van Soest et al. (1991). Total phenols (TP) were determined with the Folin-Ciocalteau reagent according to Makkar (2003). Total tannins (TT) were determined as the difference in TP before and after treatment with insoluble polyvinyl polypyrrolidone (PVPP) (Makkar et al., 1993), and condensed tannins (CT) were measured by the HCl-butanol method according to Makkar (2003). TP and TT were expressed as tannic acid equivalents and condensed tannins were expressed as a leucocyanid in equivalent (Makkar, 2003).

Experiment 1

Inoculum donors and preparations

Six adult rumen-cannulated Santa Inês sheep (60±2.5 kg body weight) grazing a tropical grass pasture and supplemented with ground maize and soyabean meal (0.7 kg/100 kg liveweight, 20% crude protein) with free access to a mineral premix and fresh water were used as inoculum donors. Ruminal liquid and solid fractions were collected separately from each animal before the morning feeding and kept in pre-warmed thermo containers (39ºC) under anaerobic conditions. A liquid fraction was obtained by using a stainless steel probe (2.5 mm screen) attached to a large capacity syringe. Similar volumes (500:500 v/v) of both fractions were blended for 10 s, squeezed through three layers of cheesecloth, and maintained in a water bath (39ºC) under CO_2 until inoculation took place. Each forage sample was incubated in three inocula, each prepared using two animals per inoculum. The different inocula were used to avoid the individual animal effect. In each inoculum, six flasks were prepared for each plant sample: three for determining truly degraded organic matter (TDOM) and the other three for sample collections for fermentation parameters and protozoa count. The same system was used for the blank (flasks without substrate containing inoculum + medium) to correct the GP from the inoculum; an internal standard was included for each inoculum to enable adjustments among inocula. Biased incubation inocula (variations above 10%) were rejected. If inocula were within this acceptance range, standard results were used to correct all results.

In vitro gas and methane production

The *in vitro* gas production (GP) assay (Theodorou et al., 1994) was adapted to a semi-automatic system (Bueno et al., 2005) using a pressure transducer and a data logger (Pressure Press Data 800, LANA, CENA/USP, Piracicaba, Brazil).

A half gram of the air-dried ground samples was incubated in serum glass flasks of a total volume of 160 ml and head space 85 ml with 50 ml of incubation medium (Menke's buffered medium) and 25 ml of inoculum. Flasks were sealed immediately with 20 mm butyl septum stoppers (Bellco Glass Inc., Vineland, NY, USA), manually mixed, and incubated at 39°C in a forced air oven (Marconi MA35, Piracicaba, SP, Brazil) for 24 h. Head space gas pressure was measured at 3, 6, 9, 12, and 24 h. Gas production was calculated by the following equation:

$$V = 7.365 \times p \qquad (n = 500;\ r^2 = 0.99;\ \text{data not reported})$$

where: V - gas volume (ml); p - measured pressure (psi).

For CH_4 determination, 2 ml of gas were sampled at each measuring time using a 5 ml syringe (Becton Dickson Indústria Cirúrgica LTDA, Curitiba, Brazil) and stored in a 10 ml vacuum tube. After each gas sampling, flasks were vented, mixed, and returned to the oven. The methane concentration was determined using a gas chromatograph (Shimadzu 2014, Tokyo, Japan) equipped with a Shincarbon ST 100/120 micro packed column (1.5875 mm OD, 1.0 mm ID, 1 m length; Ref. no 19809; Restek, Bellefonte, PA, USA) using an external calibration analytical curve (0, 30, 60, 90 and 120; ml/l) prepared with pure CH_4 (White Martins PRAXAIR Gases industrial Inc., Osasco, Brazil; 995 ml/l purity). The temperatures of the column, injector, and flame ionization detector were 60ºC, 200ºC, and 240ºC, respectively. Helium at 10 ml/min was used as the carrier gas. The methane concentration was determined according to Longo et al. (2006) as follows:

$$CH_4, \text{ml} = (\text{total gas, ml} + \text{headspace, 85 ml}) \times CH_4 \text{ concentration, ml/ml}$$

Both GP and CH_4 were expressed as ml/g IOM and ml/g TDOM and calculated by correcting the values of total gas production and incubated or truly degraded organic matter for the corresponding blank.

Rumen degradability and fermentation characteristics

After termination of the incubation (24 h), truly degraded organic matter (TDOM) was determined in three flasks for each inoculum by inclusion of

70 ml of neutral detergent solution (Van Soest et al., 1991) without heat stable α-amylase in each flask and incubated at 105ºC for 3 h. The flasks' contents were filtered in pre-weighed crucibles, washed with hot water followed by acetone, and the residual DM and ash were determined. The partitioning factor (PF) was calculated as the ratio between mg of TDOM and gas volume (ml) at 24 h incubation (Blümmel et al., 1997).

The contents of the other three flasks were used for determining fermentation characteristics. The net release values of NH_3-N were measured according to Preston (1995) using the micro-Kjeldahl method by steam distillation with sodium tetraborate solution (5%), collected in boric acid solution (20%) and determined by titration with a solution of H_2SO_4 (0.05N).

Short-chain fatty acids (SCFA) were determined according to Palmquist and Conrad (1971). Rumen fluid was centrifuged (15.000 *g* for 10 min at 4°C) to remove large feed particles; 1.6 ml of the supernatant was centrifuged (15.000 *g* for 15 min at 4°C) after adding 0.4 ml solution of metaphosphoric acid (3:1) 25% and formic acid 98%-100% plus 0.2 ml of 2-ethyl-butyric acid 100 mM (internal standard, MW=116.16; Sigma Chemie Gmbh, Steinheim, Germany). After centrifugation, approximately 1.2 ml was transferred to the chromatographic vial. One µl was injected onto the gas chromatograph (GC HP 5890 Series II/ integrator HP 3396 Series II/automatic injector HP 6890 Series, Agilent Technologies, Palo Alto, CA, USA). A mixture of SCFA of known concentrations was used as the external standard for integrator calibration. Two ml of rumen fluid were mixed with 2 ml of methyl green-formalin saline solution for determination of ruminal protozoa count microscopically following the procedure described by Dehority et al. (1983).

Experiment 2

Ruminal protein degradability and post ruminal protein digestibility

A three-step *in vitro* procedure was used to estimate the rumen protein degradability and intestinal digestion of proteins according to Calsamiglia and Stern (1995) with some modifications. Four clean and dry dacron bags (18×9 cm; 520 mm pore size) were used for each experimental sample. The bags containing 3 g of substrate were incubated in one run using an *in vitro* incubator (Tecinial incubador *in vitro*, model TE-150) for 16 h with 3 different inoculums (12 replicates/plant) obtained from the same six cannulated animals of the first GP experiment. Inoculum collection and preparation of incubation medium were done following the same procedure as in the GP experiment. The *in vitro* incubator had 3 units, each unit acted as a separate inoculum and contained 1300 ml medium and 650 ml rumen inoculum. At the end of the incubation time all of the bags were washed for 1 h using cold tap water and 2 h in a washing

machine with the water being changed every 15 min, then kept in the freezer for 48 h and rewashed again in the washing machine for 2 h and dried at 50ºC for 48 h. The residue in the bags was used to analyse the N that was not degraded in the rumen (rumen undegradable protein, RUP).

The residue after ruminal exposure (0.1 g) was then incubated for 1 h with a pepsin-HCl (Sigma, Chemie Gmbh, Steinheim, Germany) (pH=1.9) solution followed by a pancreatin solution (Sigma, Chemie Gmbh, Steinheim, Germany) (pH=7.8) for 24 h at 38°C. After incubation, 3 ml of trichloroactetic acid (TCA, 100%) were added to each tube, then vortexed. The tubes were left for 15 min and centrifuged at 10.000 *g* for 15 min. Five ml of the supernatant were taken for determination of N (intestinal digested protein - IPD) by the Kjeldahl method.

Statistical analysis

The experimental plants were statistically compared using a random regression design and means were compared using the Duncan test. Analysis of variance was carried out using the general linear model procedure (PROC GLM) of SAS (2002). The experimental unit in the first experiment was the composite sample of each plant species replicated in three bottles (5 plants x 3 rumen inocula x 3 bottles) and in the second experiment, the composite sample of each plant species replicated in four dacron bags (5 plants x 3 rumen inocula x 4 bags) in the second experiment.

RESULTS

Chemical analysis of browses

The nutrient composition and tannin content of experimental plants are shown in Table 1. The lowest OM content was observed in atriplex leaves (838 g/kg DM). In terms of CP content, all forage legumes had a higher CP content compared with the Tifton grass hay, with leucaena leaves having the highest value (268 g/kg DM). The highest contents of NDF and ADF were found for Tifton hay (803 and 461 g/kg DM, respectively), whereas acacia leaves presented the highest lignin content (163 g/kg DM). There were wide variations in the tannin content of the investigated browses, in particular TP and TT, which ranged between 6 to 103 eq-g tannic acid/kg DM and 3 to 88 eq-g tannic acid/kg DM, respectively. Leucaena and acacia leaves showed the highest content of TP and TT (103, 91, and 88, 71 eq-g tannic acid/kg DM, respectively). Acacia and leucaena leaves were rich in CT content (63 and 46 eq-g leucocynidin/kg DM, respectively), while the other investigated plants had negligible contents of TP, TT, and CT.

Table 1. Nutrient composition (g/kg DM) and tannin content of the experimental plants

Item	Experimental plant				
	Tifton hay	acacia	atriplex	prosopis	leucaena
Organic matter	898	905	838	921	931
Crude protein	74	138	162	179	268
Neutral detergent fibre	803	465	372	494	336
Acid detergent fibre	461	428	223	384	228
Lignin	65	163	144	144	60
Total phenols[1]	6	91	8	29	103
Total tannins[1]	0	71	4	18	88
Condensed tannins[2]	0	63	0.2	0.4	46

[1] expressed as eq-g tannic acid/kg DM; [2] expressd as eq-g leucocyanidin/kg DM

Experiment 1

Gas and methane production

Ruminal GP and CH_4 production at 24 h are shown in Table 2. The GP calculated for the incubated organic matter (ml/g IOM) was highest (P<0.0001) for atriplex and lowest for acacia, while the tanniniferous browses showed a similar decrease (P<0.0001) in GP expressed for the truly degraded organic matter (ml/g DOM) compared with the Tifton hay.

Table 2. *In vitro* ruminal gas production (GP) and methane production (CH_4) of the experimental plants

Indices	Experimental plants					SEM	P
	Tifton hay	acacia	atriplex	prosopis	leucaena		
GP, ml/g IOM	150^{bc}	125^{c}	191^{a}	152^{b}	131^{bc}	18.00	<0.0001
GP, ml/g TDOM	376^{a}	297^{b}	250^{b}	236^{b}	266^{b}	39.70	<0.0001
CH_4, ml/g IOM	13.3^{a}	8.2^{b}	14.7^{a}	11.7^{ab}	8.5^{b}	2.68	<0.0001
CH_4, ml/g TDOM	26.9^{a}	16.7^{b}	19.8^{ab}	19.9^{ab}	17.4^{b}	5.77	0.01

[a,b,c] means within a row without a common superscript letter differ significantly; IOM - incubated organic matter; TDOM - truly degraded organic matter; SEM - standard error of means

When ranking the plants according to their methanogenic properties, acacia and leucaena showed high inhibition (by 37.9% and 35.31% based on TDOM, respectively), whereas atriplex and prosopis were similar in CH_4 reduction (26% based on TDOM, respectively) compared with Tifton as the control.

Rumen degradability and fermentation characteristics

Tifton hay and prosopis leaves decreased (P=0.002) the PF compared with the other browses (Table 3). Acacia and Tifton hay had the lowest (P<0.0001) TDOM compared with the other plants. In the current study, the experimental plants had no significant (P>0.05) effect on pH, but ruminal NH_3-N concentrations were

higher (P=0.0002) for atriplex and prosopis compared with the other plants. All browses had lower (P<0.0001) total counts of protozoa compared with the control, whereas prosopis showed the least number of protozoa followed by leucaena (2.21 and 2.56 10^5/ml, respectively).

Table 3. Partitioning factor (PF), truly degraded organic matter (TDOM), protozoa count and rumen microbial fermentation parameters of the experimental plants

Indices	Experimental plants					SEM	P
	Tifton hay	acacia	atriplex	prosopis	leucaena		
PF	2.42^{b}	3.78^{a}	3.69^{a}	3.08^{ab}	4.12^{a}	0.84	0.002
TDOM, g/kg OM	403^{d}	424^{d}	767^{a}	641^{b}	504^{c}	54.2	<0.0001
pH	6.93	6.95	6.94	6.98	6.87	0.131	0.46
NH_3-N, mg/100 ml	24.7^{b}	24.5^{b}	27.4^{a}	30.9^{a}	26.4^{b}	2.580	0.0002
Protozoa, 10^5/ml	3.33^{a}	3.04^{b}	3.00^{b}	2.21^{d}	2.56^{c}	0.134	<0.0001
SCFA, mmol/l							
C2	45.24	45.57	44.22	46.38	41.98	4.200	0.17
C3	11.28^{b}	10.80^{b}	10.73^{b}	12.22^{a}	11.43^{ab}	0.645	0.002
C4	8.54^{a}	6.87^{ab}	7.09^{ab}	6.65^{ab}	6.50^{b}	1.360	0.03
C5	1.13^{a}	0.86^{b}	0.91^{ab}	0.88^{ab}	0.89^{ab}	0.180	0.03
IC4	1.011^{a}	0.75^{b}	0.85^{ab}	0.84^{ab}	0.79^{b}	0.130	0.01
IC5	1.76^{a}	1.32^{b}	1.64^{ab}	1.31^{b}	1.38^{ab}	0.301	0.01
Total SCFA	68.97	65.96	65.72	68.37	62.93	5.072	0.11
C2:C3	4.01^{abc}	4.21^{ab}	4.30^{a}	3.79^{bc}	3.70^{c}	0.301	0.004

^{a,b,c} means within a row without a common superscript letter differ significantly; SCFA - short chain-fatty acids; C2 - acetate; C3 - propionate; C4 - butyrate; C5 - valerate; IC4 - isobutyrate; IC5 - isovalerate; C2:C3 - acetate:propionate ratio; TDOM - truly degraded organic matter; SEM - standard error of means

The molar proportion of acetate was not significantly different among the experimental plants, while propionate was higher (P=0.002), with a corresponding decrease in the acetate:propionate ratio (P=0.004) with prosopis and leucaena. Among the experimental browses, leucaena decreased (P=0.03) the proportion of butyrate, whereas there were no significant differences among the plants in total SCFA.

Experiment 2

Ruminal protein degradability and post ruminal protein digestibility

There was a similar decrease (P<0.0001) of rumen degradable protein (RDP) between acacia and leucaena leaves, while acacia and Tifton hay presented the lowest (P<0.0001) mean values of intestinal protein digestibility (IPD) in comparison with other browses (Table 4). The ranking order of the browse species on the basis of their potential IPD was atriplex ≥ prosopis>leucaena ≥ control ≥ acacia.

Table 4. Ruminal protein degradability and post ruminal protein digestibility of the investigated plants, g/kg DM

Indices	Experimental plants					SEM	P
	Tifton hay	acacia	atriplex	prosopis	leucaena		
RDP	614[a]	81[c]	631[a]	519[b]	117[c]	5.18	<0.0001
RUP	386[c]	919[a]	369[c]	481[b]	882[a]	5.18	<0.0001
IPD	416[cd]	339[d]	628[a]	535[ba]	464[bc]	6.78	<0.0001

[a,b,c,d] means within a row without a common superscript letter differ significantly; RDP - rumen degradable protein; RUP - rumen undegradable protein; IPD - intestinal protein digestibility; SEM - standard error of means

DISCUSSION

There were wide variations in the chemical composition of the investigated browses. Generally, the high CP contents of all of the forage legume browses studied compared with Tifton grass hay suggested that these legumes would be a good source of protein to improve productivity of ruminant livestock in tropical regions. In terms of CT content, that of prosopis was negligible, while Bhatta et al. (2002) reported that prosopis leaves contain 7%-11% tannins. Variations in the analytical method can lead to large variations in the final tannin results (Makkar, 2003). Acacia and leucaena leaves can be considered tanniniferous plants because of their high CT compared with the other browses.

There was a wide range within the *in vitro* GP values per unit of incubated OM and degraded OM. Atriplex showed the highest value for GP per unit of incubated OM compared with the other browses, but when expressed per unit for degraded OM, Tifton hay had the highest value, which may be due to differences in rumen degradability. García-González et al. (2008) observed that GP and CH_4 emissions are closely related to the amount of ruminally fermented OM or the amount of digestible OM. Thus GP and CH_4 are better expressed per unit degraded OM than per incubated OM. The high CH_4 reduction for acacia and leucaena was associated with the highest CT content, where prosopis and atriplex had negligible CT contents and still reduced the amount of CH_4 by 26% (based on TDOM) compared with Tifton hay. These results suggest that the antimethanogenic properties of theses experimental legumes browses may not be related only to their tannin content, but also to other ingredients since Tifton had the highest NDF content and highest CH_4 production compared with all of the experimental browses. Jayanegara et al. (2009) studied the relationships between chemical constituents, including TP, TT, and CT for 17 different plant materials and found that NDF had a high correlation (r=0.86) with CH_4 concentration, while a very weak relationship (r=0.09) was found between CT and CH_4 production.

These results confirm that improving forage quality, either through feeding

forage with a lower fibre and/or higher protein content, can reduce CH_4 production (Rodríguez et al., 2010). In addition, the better synchronization of nitrogen and energy available for microbial utilization, the less CH_4 is produced by forage legumes compared with forage grass (Rodríguez et al., 2010). This hypothesis is supported by the increasing PF values associated with the CH_4 reduction of the experimental forage legumes compared with Tifton grass hay. Therefore, these results suggest that the forage legumes improved the efficiency of microbial protein synthesis by increasing the incorporation of ruminally degraded organic matter into microbial cells, while the percentage partitioned into CH_4 was decreased. The increase in GP with Tifton could have been associated with a lower partitioning of nutrients to microbial protein synthesis (Blümmel et al., 1997).

Although acacia and Tifton were different in their tannin content and CH_4 production, they showed similar decreases in the rumen TDOM values compared with the other browses (Table 3). Generally, the high CT content of acacia might have affected the ruminal OM degradability through interfering with microbial attachment to feed particles and, consequently, also inhibiting CH_4 production (Barry and McNabb, 1999). On other hand, the higher NDF content of Tifton could be the reason for decreasing the ruminal TDOM and increasing CH_4. McAllister et al. (1996) found that cellulose and hemicellulose ferment at slower rates than do non-structural carbohydrates, thus yielding more CH_4 per unit substrate digested. The results of the current study confirmed that not only tannin content affected rumen degradability but a higher fibre content did, too.

Even though atriplex and prosopis showed similar inhibition of CH_4 per TDOM, this was not associated with similar protozoa counts or TDOM. In this sense, it seems that there were other anti-protozoal factors than tannin responsible for the reduction of the protozoa count by prosopis.

Bhatta et al. (2012) confirmed that the effects of tannin on protozoal numbers are varied, probably because some of the tannins have a direct effect on those methanogenic archaea, which are not associated with the protozoa. Accordingly, Goel and Makkar (2012) suggested that upon inhibition of protozoa, the species belonging to the *Methanobacteriaceae* (living in association with protozoa) declined with an increase in the number of free-living *Methanobacteriales*. A reduced rate of association of protozoa and methanogens could result in higher interspecies hydrogen transfer between an increased population of both hydrogen-producing bacteria (*R. flavefaciens* and *F. succinogenes*) and free-living *Methanobacteriales* and thus might remain without effect on methane production. Therefore, our results and those of Bhatta et al. (2012), as well as Goel and Makkar (2012), confirm that the unidirectional relationship between protozoal numbers and methanogenesis, as affected by tannins, is not obligatory.

The decreases in the acetate:propionate ratio, together with the increasing propionate found with prosopis and leucaena without decreasing total SCFA or pH, indicates that their CH_4 reduction was mediated by directing hydrogen from CH_4 to propionate and not as general fermentation inhibition (Szumacher-Strabel and Cieślak, 2010). These results suggest that the fermentation response patterns with both prosopis and leucaena are similar to the CH_4 reduction obtained by ionophores (Goodrich et al., 1984). In the last few years the interest of nutritionists in bioactive plant factors that reduce CH_4 emission without adversely affecting ruminal degradability or fermentation has been increasing (Szumacher-Strabel and Cieślak, 2010). Apparently, among the experimental tanniniferous browses, leucaena tannins were more favourable in this respect compared with acacia, which indicates differences in their CT types as well as activity.

The low concentrations of NH_3-N in rumen fluid found with acacia and leucaena could be attributed to the inhibition of rumen protein degradation and deamination processes by CT (Szumacher-Strabel and Cieślak, 2010; Goel and Makkar, 2012).

The association of the decrease in the ruminal NH_3-H concentration and RDP for acacia and leucaena shows that the high concentration of CT decreases ruminal CP degradation mainly through formation of tannin-protein complexes that are hardly degraded by ruminal microbes (McSweeney et al., 1999). Leucaena showed a higher IPD than acacia. This may be related to differences in their CP content and in the affinity for re-binding of proteins to CT in the jejunum and ileum as the pH increases (Min et al., 2003). The reactivity between CT and proteins depends partly on the molecular weight, type of tertiary structure, and amino acid profile of the proteins (Min et al., 2003). In a review, Min et al. (2003) noted that at equivalent concentrations, different CT sources had variable effects on degradation of CP due to differences in molecular weight and chemical structure influencing the biological activity of CT.

The use of prosopis decreased RDP and increased IPD compared with atriplex, which can be associated with the decreasing TDOM. These results confirmed the antiprotozoal activity of prosopis. The described antiprotozoal activity of some of these plants is also favourable in this sense, as it may improve the efficiency of microbial protein synthesis due to suppression of the bacteriolytic activity of the ruminal protozoa (Goel and Makkar, 2012). This could increase protein flow to the duodenum by protein by-passing the rumen (Min et al., 2003). A decrease in ruminal protein degradability is beneficial for ruminants as they increase the supply of dietary nitrogen to the lower intestine for production; however, the limitation in ruminally degradable protein may result in an inadequate ruminal ammonia concentration and suboptimal microbial growth as well as fibre fermentation. Thus, a combination of such high-tannin content with highly fermentable diet ingredients

will improve microbial protein synthesis and protect the dietary protein without adversely affecting microbial growth efficiency and overall animal productivity.

CONCLUSIONS

The results of the present study confirmed the higher protein and lower fibre content of selected forage legume browses compared with Tifton grass hay. Their use also reduced CH_4 emission without adversely affecting ruminal fermentation and nutrient degradation. The presence of higher condensed tannins levels in acacia may affect ruminal degradability, however. Our interpretation of the obtained results is that in developing countries, leucaena and prosopis could be used as supplements to well-digestible diets in order to protect the dietary protein from microbial activity as well as to reduce CH_4 production from ruminants. More studies, however, are necessary to evaluate possible synergistic effects in complete diets when combinations of these plants are used, and this must be demonstrated *in vivo*.

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