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In vitro gas production of foliage from three browse tree species treated with different dose levels of exogenous fibrolytic enzymes

D. López[1], J. F. Vázquez-Armijo[1], N. López-Villalobos[1,2], H. A. Lee-Rangel[3], A. Z. M. Salem[4], J. L. Borquez-Gastelum[4], I. A. Domínguez-Vara[4] and R. Rojo-Rubio[1]

1 Centro Universitario UAEM Temascaltepec, Universidad Autónoma del Estado de México Temascaltepec, México, Mexico
2 Institute of Veterinary, Animal and Biomedical Sciences, Massey University Palmerston North, New Zealand
3 Facultad de Agronomía, Universidad Autónoma de San Luis Potosí Soledad de Graciano Sánchez, San Luis Potosí, Mexico, and
4 Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México El Cerrillo Piedras Blancas, México, Mexico

Summary

The aim of this study was to evaluate the effect of different dose levels of exogenous fibrolytic enzymes (EFE) on *in vitro* ruminal fermentation kinetics and energy utilization of foliages from three browse trees (*Pithecellobium dulce, Heliocarpus velutinus* and *Guazuma ulmifolia*). Mixture of EFE product was added to the leaves of the three browse tree species at three dose levels: 0 (control), 3.5 and 7.0 mg/g of DM. Chemical composition of the foliages, including plant secondary metabolites such as total phenolics (TP), saponins (SAP) and aqueous fraction (AF), was determined. In addition, *in vitro* assaying of ruminal gas production kinetics was determined for the three browse three foliages treated with EFE. *P. dulce* had the highest crude protein content ($p < 0.05$), whereas *G. ulmifolia* had the highest content of neutral detergent fibre and SAP ($p < 0.05$) and *H. velutinus* had the lowest content of TP ($p < 0.05$). The interaction between tree species and dose level of EFE was significant ($p < 0.05$) for gas production (GP) at 24 h of incubation, parameters *b* and *c* of the accumulated GP curve, short-chain fatty acids (SCFA) and metabolizable energy (ME). The lowest ($p < 0.01$) extent of accumulated GP as well as the *b* and *c* values occurred in *G. ulmifolia* at 0 mg EFE/g DM. *P. dulce* had the highest ($p < 0.05$) values for ME and SCFA at the highest dose of EFE. Tree species and dose level had significant ($p < 0.05$) effects on all parameters describing *in vitro* ruminal fermentation kinetics and energy utilization. Addition of EFE improved the fermentation kinetics of the browse species considered in this study.

Keywords browse tree species, exogenous enzyme, gas production

Correspondence R. Rojo-Rubio, Centro Universitario UAEM Temascaltepec, Universidad Autónoma del Estado de México, Km. 67.5 Carretera Federal Toluca-Tejupilco, 51300, Temascaltepec, Estado de México, México. Tel: +52 7162665209; Fax: +52 7162665209;
E-mail: dr_rojo70@yahoo.com.mx

Introduction

Livestock production in tropical and subtropical regions is limited by the scarcity of high-quality feed, mainly during the long dry season (Hove et al., 2001). Some forage as such legume fodder, trees and shrubs present high protein values and are potentially useful to correct the nutrient deficiencies (Camacho et al., 2010a). Tree and shrub leaves are important components of goat and sheep diets and play an important role in small ruminant nutrition under extensive livestock production systems (Salem et al., 2006; Camacho et al., 2010a). However, they also contain some secondary metabolites such as tannins and other secondary compounds (Wina et al., 2005). Tannins are polyphenolic compounds, which occur widely in plants, with the ability to bind proteins and other nutrients. Some tannins can also produce toxic and secondary metabolites in monogastric and ruminant animals and cause reduced intake, lower nutrient digestibility and protein availability (Szumacher-Strabel and Cieślak, 2010), although other secondary metabolites, such as saponins, alkaloids, essential oils and the aqueous fraction of lectins, polypeptides and starch, may also have negative impacts on carbohydrates and protein digestion (Salem et al., 2006).

The use of enzymes as feed additive for ruminant diets has attracted considerable interest recently

(Beauchemin et al., 2003). However, there are increasing evidences indicating that the mode of action of these enzymes in ruminants is a combination of pre- and post-feeding effects (Colombatto et al., 2003). Improvements in ruminant production with supplemental fibrolytic enzymes are generally attributed to increased ruminal fibre digestion, but the exact mechanism is not yet fully understood. Numerous potential mechanisms have been proposed (Beauchemin et al., 2000, 2003; McAllister et al., 2001), including pre-ingestive and ruminal effects such as direct hydrolysis, structural changes in the fibre, increased ruminal microbial attachment, stimulation of ruminal microbial populations and synergism with ruminal microbial enzymes. A product containing cellulases, xylanases, α-amylase and proteases from an anaerobic bacterium showed a positive effect on browse leaves degradation in rabbits (El-Adawy et al., 2008), ruminant performance and nutrient utilization of low-quality forages *in vivo* (Gado, 1997) and *in vitro* (Gado et al., 2007). Therefore, the objective of this work was to evaluate the effect of different dose levels of exogenous fibrolytic enzymes treatment (i.e. EFE) on *in vitro* fermentation, gas production kinetics and energy utilization of *Pithecellobium dulce*, *Heliocarpus velutinus* and *Guazuma ulmifolia* leaves.

Materials and method

Location for sample collection and experimental treatments

Foliages for the three tree species was collected from the South of Mexico state. Geographically, this is located at 19°02′04″ north latitude and 100°02′14″ west longitude at an elevation of 1720 masl. The climate is moderately humid with an average temperature of 15–18 °C and annual rainfall of 950–1000 mm (García, 1987). Laboratory analyses and *in vitro* gas production study were undertaken in the Animal Nutrition Laboratory of the Centro Universitario UAEM Temascaltepec, located in the municipality of Temascaltepec de González, México.

Browse species

During the dry season (April/May), a mixture of young and mature leaves was sampled from three browse species – Pinzan (*Pithecellobium dulce*), Guácimo (*Heliocarpus velutinus*) and Cuahuilote (*Guazuma ulmifolia*) from several locations in the Southern part of Mexico state. Three individual replicates samples (∼2.5 kg each one pooled of at least 7 trees) were randomly collected. These samples were air-dried in the shade to minimize changes in secondary compounds activity (Makkar and Singh, 1991).

Exogenous fibrolytic enzymes

The tested exogenous fibrolytic enzyme was a preparation containing xylanase and cellulase activities. According to the manufacturers, this additive (Fibrozyme®) is a fibrolytic enzyme powder preparation containing xylanase and cellulase activities (Fibrozyme, Alltech, Nicholasville, KY, USA) from *Aspergillus niger* and *Trichoderma viride* fermentation which have cellulase and xylanase activity of 31.0 and 43.4 UI respectively. Enzymes activity was determined in the Fibrozyme® according to the methods of Ramírez et al. (2005). Doses of exogenous enzymes tested were as follows: 0 (control), 3.5 and 7.0 mg/g DM of dried leaves.

Analytical methods

Fresh samples were dried at 45 °C for 48 h for moisture determination and ground in a Willey-mill to pass through a 1-mm screen. Samples were assayed in triplicate according to the AOAC (1990) for DM, crude protein (CP, N × 6.25) and ash content by methods 930.15, 976.05 and 942.05 respectively. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) content (Van Soest et al., 1991) were analysed using the ANKOM F-57 filter bags in an Ankom^{200} fibre analyser (Ankom Technolgy, Macedon, NY, USA). For a NDF analysis, samples were treated with α-amylase (Sigma A-3403 Sigma-Aldrich®, St. Louis MO, USA), and the neutral detergent solution (NDS) contained sodium sulphite and the residues were not corrected for residual ash. Hemicellulose content was calculated from the difference between a NDF and ADF.

Plant secondary metabolites (PSM): total extractable phenolics, saponins and aqueous fraction, were determined by methodology described by Salem et al. (2006). Fresh leaves were chopped (1–2 cm) and immediately 10 g of each species were extracted with 80 ml of solvent mixture. The solvents mixture contained 10 ml methanol (99.8/100, analytical grade, Fermont®, Monterrey, Mexico), 10 ml ethanol (99/100, analytical grade, Fermont®, Monterrey, Mexico) and 80 ml distilled water. Plants materials were individually incubated with the solvent in closed flasks at 25–30 °C for 48 h. After the incubation time, all flasks were incubated in water bath at 39 °C for 1 h, and then immediately filtered and the filtrates were collected and stored at 4 °C for further use.

PSM were determined in triplicate for each plant extract (*P. dulce*, *H. velutinus* and *G. ulmifolia*). 10 ml of extract was fractionated by funnel separation with a double volume of ethyl acetate (99.7/100, analytical grade, Fermont®, Monterrey, Mexico) to determine total phenolics (TP) by drying and quantifying the TP layer in the funnel. After TP separation, 20 ml of n-butanol was added (99.9/100, analytical grade, Fermont®, Monterrey, Mexico) to fractionate the saponins (Makkar et al., 1998). The remaining solution in the funnel was considered to be the aqueous fraction (AF) that has the other secondary compounds such as lectins, polypeptides and starch (Cowan, 1999).

In vitro ruminal gas production

Donor animals

Rumen fluid used for the *in vitro* gas production of the fodder tree species was withdrawn by stomach tube from four growing goats (Boer, LW 18 ± 0.3 kg) before the morning feed which have the following forage:concentrate (70:30 DM, basis) diet. The forage contained a mixture of oat hay (677 g/kg), alfalfa hay (176 g/kg), *H. velutinus* dried leaves (44 g/kg) and *G. ulmifolia* dried leaves (103 g/kg) with a concentrate mixture (210 g CP/kg DM), containing ground sorghum (380 g/kg), ground corn (380 g/kg), soya bean meal (120 g/kg), sugarcane molasses (80 g/kg), urea (2 g/kg) and minerals (20 g/kg DM). The diet was offered twice daily, and all the animals had access to clean water *ad libitum*.

In vitro gas production

The gas production assay was carried out according to the procedure described by Theodorou et al. (1994) as modified by Mauricio et al. (1999). Approximately 1 ± 0.002 g of DM of each species was weighed in triplicate into 160 ml serum bottles. Exactly 250 mg of enzyme product was dissolved in 50 ml of distilled water, and 0, 0.7 and 1.4 ml were added to each bottle. Three hours later, 90 ml anaerobic buffer (containing micro- and macro-elements, a reducing agent and a reduction indicator of resazurin) was added to the bottles (Mauricio et al., 1999), and they were then stored at 20–22 °C for 17 h (Colombatto et al., 2007). Thus, total enzyme–feed interaction time was 20 h. Ten millilitres of ruminal fluid, obtained pre-feeding (7:00 h) from the four goats by stomach tube, was inoculated into the bottles. The ruminal fluid was obtained from multiple sites in the rumen, strained through two layers of muslin and then kept at 39 °C under a continuous CO_2 stream. Negative controls containing buffered rumen fluid with or without enzyme but no substrate, were also included in triplicate for correction of gas produced from small particles present in the ruminal fluid or sugars present in the enzyme products. Cumulative gas production (ml/g DM) was recorded at 2, 4, 6, 8, 10, 12, 15, 19, 24, 30, 36, 48, 72 and 96 h post-incubation at 39 °C. Volume of gas (ml/g DM) produced after 24 h of incubation (GP24) was used as an index of energy feed value of tree fodder samples.

In vitro dry matter degradability

At the end of incubation (96 h), the contents of each serum bottle were filtered using sintered glass crucibles (porosity 1, 100- to 160-μm pore size, Pyrex, Stone, UK) under vacuum. Fermentation residues were dried at 105 °C overnight to estimate the DM disappearance.

Calculation

The pressure generated by the gas accumulated in the upper part of the incubation serum bottles was measured through a pressure transducer connected to a digital data reader. The equation was previously obtained using the REG procedure of the SAS (2002) program:

$$Y = 0.024 + 5.34X + 0.031X^2$$

where Y is volume (ml) and X is pressure (psi). $R^2 = 0.99$.

Then, gas production data (ml/g DM) were fitted using the NLIN procedure of SAS (2002) with a non-linear model as described by France et al. (2000):

$$A_t = b \times \left[1 - e^{-c(t-L)}\right]$$

where A_t is the volume of gas production at time t; b, the asymptotic gas production (ml/g DM); c is the rate of gas production (ml/h) and (L/h) is the lag time.

Metabolizable energy (ME, MJ/kg DM) was estimated according to Menke and Steingass (1988) as:

$$\mathrm{ME} = 2.20 + 0.1357\mathrm{GP}_{24} + 0.0057\mathrm{CP} + 0.0002859\mathrm{EE2}$$

where GP_{24} was gas produced after 24 h of incubation and CP was that crude protein of tree leaves (% DM).

Short-chain fatty acids (SCFA) were estimated depending on the relationship ($R^2 = 0.94$) between gas production at 24 h and SCFA concentration of tannin-containing browses following the equation of Getachew et al. (2002):

$$\text{mmolSCFA} = -0.00425 + 0.0222 \text{ (ml gas at 24 h)}$$

Experimental design and statistical analysis

Nutrient and secondary compounds contents from tree foliage were statistically analysed using the GLM procedure of SAS (2002) with a linear model that included the effect of browse tree species. Least squares means and standard errors for each browse tree species for each dependent variable were obtained and used for multiple comparisons using the Tukey test.

Data for *in vitro* ruminal fermentation were analysed using a randomized complete design with 3 browse tree species × 3 dose levels of EFE (0, 3.5 and 7.0 mg/g of DM) in factorial arrangement with tree repetitions. The linear model was:

$$y_{ijk} = \mu + B_i + D_j + BD_{ij} + e_{ijk}$$

where y_{ijk} = the dependent variable; μ = overall mean; B_i = effect of i-browse tree species; D_j = effect of j- dose level of EFE; BD_{ij} = interaction between the i-browse tree species and j-dose level of EFE; and e_{ijk} = the residual error ~ NI (0, σ^2).

Least squares means and standard errors for each browse tree species, dose level of EFE and each combination of browse tree species and dose level were obtained and used for multiple comparisons using the Tukey test.

Results

Chemical composition and secondary compounds

The CP content of *P. dulce* (222 g/kg DM) was higher ($p < 0.05$) than the remaining tree species, while *G. ulmifolia* showed the lowest CP value (148 g/kg DM) (Table 1). *G. ulmifolia* showed higher ($p < 0.05$) contents of NDF, ADF and hemicellulose than the other two tree species.

The tree species differed in secondary compounds contents ($p < 0.05$). *P. dulce* and *G. ulmifolia* had higher ($p < 0.05$) contents of TP than *H. velutinus*. *G. ulmifolia* showed the highest ($p < 0.05$) contents of SAP, while *P. dulce* had the highest ($p < 0.05$) AF contents.

In vitro rumen fermentation

Browse tree species and dose level had a significant effect ($p < 0.02$) on all variables describing the *in vitro* ruminal fermentation kinetics and energy utilization of foliages, excepting that dose level had no significant effect on IVDMD (Table 2). *P. dulce* and *H. velutinus* showed higher values of SCFA, ME and IVDMD than *G. ulmifolia* (Table 2). Addition of EFE improved the *in vitro* fermentation kinetics of the browse tree leaves (see Table 2 and Figs 1–3). High dose levels of EFE decreased the lag time for all species ($p < 0.05$).

The interaction between browse tree species and dose level of EFE was significant ($p < 0.05$) for GP_{24}, parameters b and c of the accumulated GP curve, SCFA and ME, but this interaction was not significant

Table 1 Chemical composition and secondary compounds (g/kg DM) of browse tree species

Item	*P. dulce*	*H. velutinus*	*G. ulmifolia*	SEM	p-Value
Chemical composition					
OM	896.99[b]	921.92[a]	897.59[b]	0.87	<0.0001
CP	222.10[a]	154.30[b]	147.76[c]	1.23	<0.0001
NDF	435.23[c]	455.81[b]	478.06[a]	1.96	<0.0001
ADF	305.79[b]	323.57[b]	368.46[a]	6.73	0.0002
Hemicellulose	129.43[a]	132.23[a]	109.59[b]	5.87	0.0164
Ash	103.01[a]	78.08[b]	102.41[a]	0.87	<0.0001
Secondary compounds					
TP	51.93[a]	15.67[b]	48.93[a]	2.86	<0.0001
SAP	16.57[b]	16.87[b]	29.40[a]	0.85	<0.0001
AF	157.30[a]	133.70[b]	130.90[b]	4.49	<0.0020

Means in the same row with different superscripts differ ($p < 0.05$). OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre; TP, total phenols; SAP, saponins; AF, aqueous fraction; SEM, standard error of the mean.

Table 2 *In vitro* gas production at 24 h of incubation (ml/g DM), gas production parameters (b, c and L), energy utilization (ME and SCFA) and *in vitro* dry matter degradability of three browse tree foliages added with different dose level of exogenous fibrolytic enzymes (mg/g DM)

Browse	Enzyme level	GP_{24}	*b*	*c*	L	SCFA	ME	IVDMD
P. dulce	0	100.3^{b}	197.9^{a}	0.029^{c}	3.83^{ab}	1.00^{b}	9.6^{b}	515^{a}
	3.5	103.4^{b}	201.3^{a}	0.031^{bc}	2.83^{bc}	1.01^{b}	9.7^{b}	519^{a}
	7.0	117.7^{a}	206.0^{a}	0.035^{a}	2.48^{bc}	1.16^{a}	10.6^{a}	532^{a}
H. velutinus	0	77.53^{c}	169.8^{b}	0.026^{d}	4.50^{a}	0.78^{d}	7.9^{d}	499^{a}
	3.5	96.4^{b}	173.2^{b}	0.033^{ab}	4.29^{ab}	0.85^{cd}	8.3^{cd}	511^{a}
	7.0	98.1^{b}	175.5^{b}	0.034^{ab}	2.61^{bc}	0.90^{c}	8.6^{c}	528^{a}
G. ulmifolia	0	49.7^{e}	155.1^{c}	0.016^{e}	5.42^{a}	0.41^{f}	5.6^{f}	434^{b}
	3.5	58.3^{de}	172.2^{b}	0.017^{e}	3.48^{bc}	0.51^{e}	6.2^{e}	455^{b}
	7.0	61.5^{d}	175.3^{b}	0.018^{e}	2.93^{bc}	0.55^{e}	6.5^{e}	460^{b}
SEM		2.515	2.978	0.0012	0.509	0.026	0.161	7.923
p-Value								
Browse tree species (B)		<0.0001	<0.0001	<0.0001	0.0149	<0.0001	<0.0001	<0.0001
Dose level (D)		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0002
B × D		0.0015	0.0094	0.0002	0.1290	0.0414	0.0403	0.5304

Means within a column with different superscripts differ ($p < 0.05$). GP_{24}, cumulative gas production at 24 h; b, asymptotic gas production (ml/g DM); c, fractional rate of gas production (/h); L, lag time (h); SCFA, short-chain fatty acid concentration (mmol); ME, metabolizable energy content (MJ/kg DM); IVDMD, *in vitro* dry matter degradability (g/kg DM); SEM, standard error of the mean.

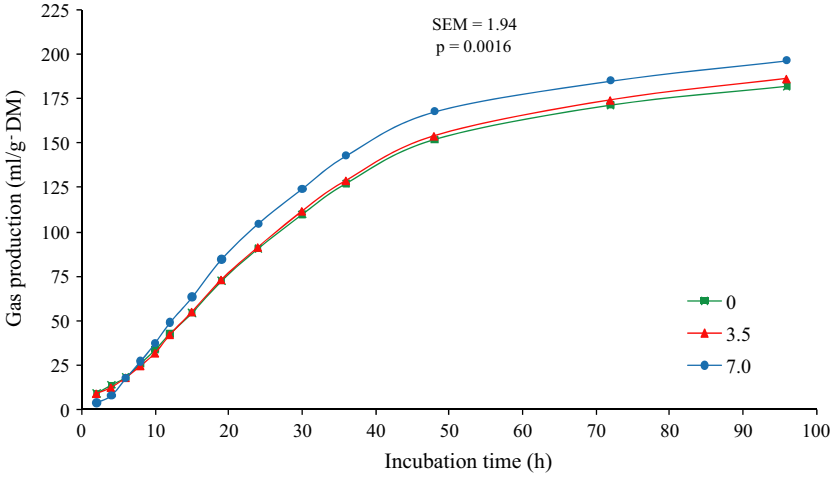

Fig 1 Cumulative gas production profiles (ml/g DM) from *in vitro* fermentation of *Pithecellobium dulce* leaves treated with different dose levels (0, 3.5 and 7.0 mg/g of DM) of an exogenous fibrolytic enzyme preparation. SEM is for the overall fit, and p-Value is for the effect of enzyme level.

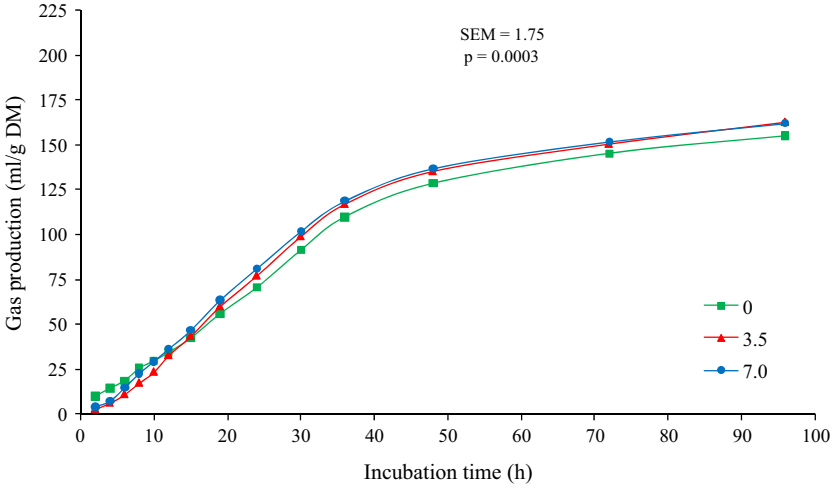

Fig 2 Cumulative gas production profiles (ml/g DM) from *in vitro* fermentation of *Heliocarpus velutinus* leaves treated with different dose levels (0, 3.5 and 7.0 mg/g of DM) of an exogenous fibrolytic enzyme preparation. SEM is for the overall fit and p-Value is for the effect of enzyme level.

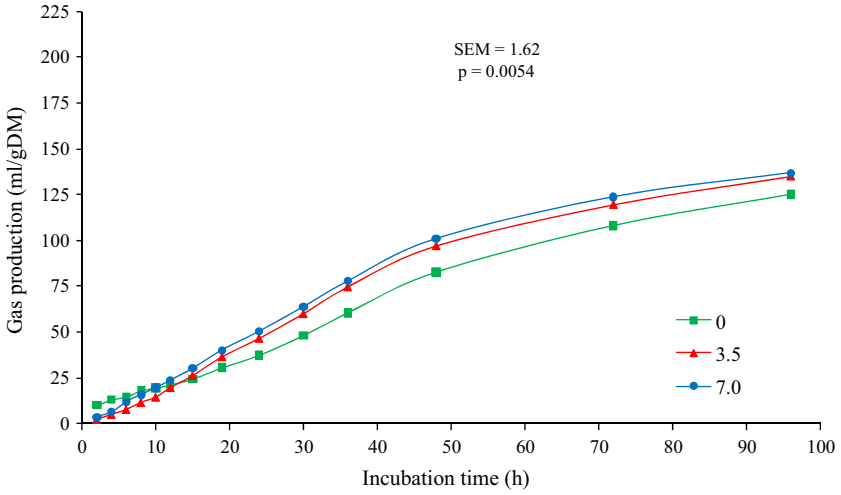

Fig 3 Cumulative gas production profiles (ml/g DM) from *in vitro* fermentation of *Guazuma ulmifolia* leaves treated with different dose levels (0, 3.5 and 7.0 mg/g of DM) of an exogenous fibrolytic enzyme preparation. SEM is for the overall fit, and p-Value is for the effect of enzyme level.

for L and IVDMD (Table 2). *P. dulce* treated with the highest dose level of EFE (i.e. 7 mg EFE/g DM) showed the highest ($p < 0.01$) value of G_{24}, SCFA and ME. The lowest ($p < 0.01$) extent of accumulated GP as well as the *b* and *c* values occurred in *G. ulmifolia* at 0 mg EFE/g DM.

Discussion

Browse tree leaves are a useful current option to feed goats during the dry season when availability of higher quality forages is low. Compared with the values reported in this study, Camacho et al. (2010b) reported similar values of CP, NDF and ADF for *P. dulce* during wet and dry seasons in a region near to the present study site. The CP content of the three browse tree species used in our study indicated that they provide ruminal degradable N when used as supplements during the dry season in semi-arid regions. Furthermore, the relatively low fibre contents in *P. dulce* and *H. velutinus* suggest that these browse three species will show high DM digestibility (Reed, 1986; Salem et al., 2006). However, content of secondary compounds such as TP and SAP in *P. dulce* and *G. ulmifolia* may cause some negative effects, for example, depressing feed intake, impairing digestibility and/or having a toxic effect on rumen microorganisms (Mangan, 1988; Salem et al., 2006). Secondary compounds in foliages are mainly a property of plant genetic factors controlling physiological synthesis and accumulation of secondary compounds (Okuda et al., 1993; Kelman et al., 1997). Other factors associated with high rates of polyphenolic synthesis include high environmental temperatures, drought stress and plant-defensive mechanisms against pests, pathogens and predators (Mangan, 1988).

In vitro GP parameters (*b* and *c*) appear related to the chemical composition of the substrate, in particular to the fibre content and its structural polysaccharides (Kamalak et al., 2005). The high value of GP_{24} in *P. dulce* at the highest dose level of EFE suggests a higher extent of fermentation in the first 24 h of fermentation *vs.* the other leaves, especially *G. ulmifolia*. Differences in GP among the leaves could be due to the proportion, and nature, of their fibre (Rubanza et al., 2003). Higher asymptotic GP (i.e. *b*) of *P. dulce* could be due to its lower fibre and SAP content, although some variation among leaves could be due to genetic characteristics relative to the type of secondary compound activity on digestibility (Salem et al., 2006). Direct-fed EFE have been shown to enhance microbial colonization of feeds by increasing the numbers of ruminal fibrolytic microbes (Morgavi et al., 2004) resulting in an increased degradation rate of ruminal fibre (Giraldo et al., 2008). Pre-treatment of forages with EFE can solubilize some fibre and improve the digestibility at shorts incubation times (Moharrery et al., 2009). However, some research has shown that efficiency of forage utilization was increased at increasing dose levels of EFE (Miller et al., 2008), whereas others suggest that EFE produced better results at a specific level, rather that showing a dose response (Jalilvand et al., 2008). In this study, the interaction between browse tree species and dose level of EFE was significant ($p < 0.05$) for *in vitro* ruminal kinetics parameters and energy utilization, confirming that efficiency of EFE depends on the forage type and dose level.

The lower GP and energy utilization (ME and SCFA) of *G. ulmifolia vs.* other browse tree species was probably due to higher fibre contents, as well as high levels of TP and SAP. This effect is probably due to

reduced microbial adherence of feed particles (McAllister et al., 1994) and inhibition of microbial growth. The antinutritive effects of phenolic compounds in tree and shrub leaves, particularly tannins, are associated with their ability to combine with dietary proteins, polymers such as cellulose, hemicellulose and pectin, and minerals thus retarding the DM digestion (McSweeney et al., 2001). Other secondary metabolites, such saponins, alkaloids, essential oils and the aqueous fraction of lectins, polypeptides and starch, may also have negative impacts on the digestibility of DM (Salem et al., 2006).

However, in our study high dose levels of EFE decreased the lag time for all species. Colombatto et al. (2003) mentioned that enzymes could degrade complex substrates to simpler forms at early stages of fermentation to allow faster ruminal microbial colonization and fermentation. Some authors have suggested that pre-treatment of feed with enzymes could create a stable enzyme–feed complex (Kung et al., 2000), but others have indicated an alteration in fibre structure, which would stimulate microbial colonization (Nsereko et al., 2000).

Increased IVDMD of *P. dulce* and *H. velutinus* reflects its higher fermentation, and lower fibre and saponins content. In contrast, lower values of IVDMD in *G. ulmifolia* represent less fermentation and higher secondary compound levels (Salem et al., 2006). Digestibility of tree leaves was adversely affected by secondary compounds *in vitro* (Peng et al., 2005; Rakhmani et al., 2005) and *in vivo* (Salem et al., 2006). Rubanza et al. (2003) reported a negative relationship between chemical composition, and phenolic compounds, with *in vitro* degradability of legumes at 24 h of *in vitro* incubation.

Conclusion

Among the examined browse tree leaves, *P. dulce* had the highest protein content, as well as higher gas production and metabolizable energy, indicating that, of the browse leaves examined, it had the best potential as a ruminant feed. In contrast, the nutritional value of *G. ulmifolia* was the lowest. This study indicated that responses to level of added enzyme differ with browse specie, so that the additive was more effective with *P. dulce*. The EFE product (Fibrozyme®), added to some browse tree leaves can improve the fermentation kinetics in diet for goats. Further *in vivo* studies on the effect of EFE of the nutritive utilization of tree leaves in goats are needed.

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