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In vitro Ruminal Gas Production Kinetics of Four Fodder Trees Ensiled With or Without Molasses and Urea

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Abstract

This study investigated if the addition of urea (U), molasses (M) or their 1:1 (v/v) mixture during ensiling increases the nutritional value of forage from four fodder trees (*Prunus persica*, *Leucaena esculenta*, *Acacia farnesiana*, and *Prunus domestica*). Forage samples of fodder trees were collected in triplicate (three individual samples of each species) and subjected to an *in vitro* gas production (GP) procedure. Fermentation at 24 h (GP_{24}), short-chain volatile fatty acids (SCFA), and microbial crude protein production (MCP), *in vitro* organic matter digestibility (OMD), metabolizable energy (ME) and dry matter degradability (DMD) were estimated. Forage samples were incubated for 72 h in an incubator at 39ºC and the volume of GP was recorded at 2, 4, 6, 8, 10, 12, 24, 48, and 72 h of incubation using the reading pressure technique. The rumen fermentation profiles were highest for *P. persica*, which showed the highest (P<0.0001) DMD, ME, OMD, SCFA, GP_{24} and MCP. On the other hand *L. esculenta* had the lowest (P<0.0001) DMD, SCFA, MCP; *P. domestica* had the lowest (P<0.0001) OMD. The addition of M to silage increased (P<0.0001) ME and OMD, as well as GP. However, the addition of U and the mixture of U and M reduced (P<0.0001) DMD, ME, OMD, SCFA, GY_{24} and MCP. These results show that *P. persica* has the highest nutritive value and *L. esculenta* the lowest for ruminants. Additionally, the addition of M to forage from fodder trees increases rumen GP and fermentation, which may improve nutrient utilization in ruminants.

Key words: fodder trees, urea, molasses, gas production

INTRODUCTION

Fodder trees and shrubs constitute an important feed supplement in harsh environment with long dry periods, because they provide forage for grazing ruminants throughout the year or at specific critical periods of the year, particularly when herbage is scarce or when the quantity and quality of herbaceous species sharply declines (Devendra 1990).

Grazing studies in Mediterranean shrublands indicated that browse constitutes more than 60% of goats' diets, and it is an integral component of sheep diets (Perevolotsky *et al*. 1998). In some regions of Mexico,

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fodder trees and shrubs are heavily used by the producers to alleviate the nutritional shortage of grazing animals (Nahed *et al*. 1998). In a global context, various studies have shown the biological and economical efficiency achieved for meat, milk and wool production using fodder trees and shrub in ruminants (Shelton *et al*. 1991; Nahed *et al*. 1998).

Many fodder trees and shrubs are high in crude protein and plant secondary metabolites (Salem *et al*. 2006; Rop *et al*. 2009; Scandellari *et al*. 2010; Kaensombath and Frankow-Lindberg 2012). However, the presence of tannins and other secondary metabolites in a large number of nutritionally important shrubs and trees reduce their digestibility and acceptability by herbivores (Tolera *et al*. 1997; Rubanza *et al*. 2007). It is therefore important to enhance rumen efficiency and the delivery of nutrients to animals when tannin-rich forages are fed. One approach could be cutting the leaves of trees and ensilaging with additional nitrogen in order to enhance microbial protein synthesis and spare plant protein through altered chemical activity of protein-tannin complexing in the rumen (Kumar and Singh 1984). Therefore, the present study was conducted to evaluate the effect of adding U, M or the combination of both additives to four fodder trees (*Prunus persica* (PP), *Leucaena esculenta* (LE), *Acacia farnesiana* (AF), and *Prunus domestica* (PD)) during the ensiling process on *in vitro* gas production (GP), chemical composition and ruminal fermentation profiles.

RESULTS

The levels of plant secondary metabolites and chemical composition of fodder trees ensiled either with urea (i.e., U), molasses (i.e., M) or both supplements (i.e., UM) are presented in Table 1. Total phenols (TP) and the aqueous fraction (AF) contents of *P. persica* were higher (P<0.001) than those found in the other trees, which did not differ from each other. Saponins (SP) content of *P. domestica* was lower (P<0.001) compared to the other trees; its ash content was higher (P<0.001) than that of the other trees (Table 1). The TP, SP and AF contents in all trees ensiled with U, M or the mixture UM were lower than those of the control. The TP content decreased (P<0.001) by 22.6, 30.8 and 43.2%; the SP content decreased (P<0.001) by 13.8, 35.3 and 23.3% (P<0.001), and the AF content decreased (P<0.001) by 14.7, 21.7 and 8.8%, respectively, with the ensiling process compared to the control. The ash content of all trees ensiled with M was higher (P<0.001) than the control, but ash content diminished in the ensiled samples (P<0.001) compared to the control when forage from the four trees were ensiled with U and the UM.

Crude protein (i.e., CP) content of *P. persica* was higher (P<0.001) than that of other trees. There were no differences for NDF and ADF contents among tree species. Ensiling the leaves of trees with U, M or UM resulted in a greater (P<0.001) CP content for all species and it was highest with the addition of U. The CP content increased by 28.2, 13.2 and 15.5%, respectively, when leaves from the four trees were ensiled with U, M or UM, compared to the control. On the other hand, NDF and ADF contents of the four trees were not affected by the addition of U, M or UM to the silage, compared with control.

Table 1 Levels of plant secondary metabolites and chemical composition of fodder trees ensiled with urea, molasses or the mixture of both additives

	Tree leaves						Treatments					
	P. persica	*L. esculenta*	*A. farnesiana*	*P. domestica*	SEM	*P*	Control	Urea (U)	Molasses (M)	UM	SEM	*P*
Plant secondary metabolites[1)]												
TP	13.2 a	10.4 b	9.7 b	11.1 b	0.85	0.005	14.6 a	11.3 b	10.1 bc	8.3 c	0.60	<0.0001
SP	12.9 a	7.8 b	11.9 a	5.4 c	0.96	<0.0001	11.6 a	10.0 ab	7.5 c	8.9 bc	0.68	0.005
AF	61.1 a	35.8 b	45.1 b	39.3 b	6.54	0.007	51.1	43.6	40.0	46.6	4.62	0.4042
Chemical composition[2)]												
CP	22.9 a	16.9 c	21.3 b	13.6 d	0.62	<0.0001	15.8 c	22.0 a	18.2 b	18.7 b	0.44	<0.0001
NDF	29.8	30.9 6	31.2	31.1	4.29	0.985	25.6	30.2	32.8	34.4	3.04	0.224
ADF	17.5	21.2	21.1	19.7	3.11	0.611	16.7	17.9	20.8	24.1	2.20	0.125
Ash	8.4 b	6.6 c	5.7 d	16.9 a	0.14	<0.0001	9.7 b	8.6 c	10.4 a	8.8 c	0.10	<0.0001

[1)] TP, total phenols; SP, saponins; AF, aqueous fraction.

[2)] CP, crude protein; NDF, natural detergent fiber; ADF, acid detergent fiber.

Values are expressed as g kg^{-1} DM. a, b, c, within rows means in tree leaves and treatments without a common letter differ (P<0.01). SEM, standard error of the mean. The same as below.

Table 2 presents the *in vitro* GP parameters and cumulative gas volume after different incubation time of the tree leaves ensiled with U, M or UM. There were variations in GP parameters of the different tree leaves, with *b* ranging from 57.9 to 118.4, *c* from 0.027 to 0.055 and *L* from 0.70 to 2.08. *P. persica* recorded the highest value of *b*, *c* and *L*. The GP parameters differed (P<0.001) among treatments, and all the GP parameters for U, M and UM treatments decreased compared to the control; the *b* value of the M treatment was higher (P<0.001) than that of the control.

Cumulative GP at 6, 12, 24, 48 and 72 h of incubation are shown in Table 2 and Fig. 1. Cumulative GP at 6, 12, 24, 48 and 72 h of incubation was different (P<0.001) among fodder trees. The ranked cumulative GP of the four fodder trees from the highest to the

Table 2 *In vitro* gas production parameters and cumulative gas volume at various incubation times of foliage of fodder trees ensiled with urea, molasses or the mixture of urea and molasses

	Fodder trees						Treatments					
	P. persica	*L. esculenta*	*A. farnesiana*	*P. domestica*	SEM	*P*	Control	Urea (U)	Molasses (M)	UM	SEM	*P*
In vitro gas production parameters[1)]												
b	118.4 a	57.9 c	73.6 b	70.3 b	3.94	<0.0001	94.7 a	61.0 b	99.1 a	65.5 b	2.79	<0.0001
c	0.055 a	0.036 b	0.027 c	0.038 b	0.0041	<0.0001	0.052 a	0.028 b	0.047 a	0.029 b	0.003	<0.0001
L	2.08 a	0.70 b	1.67 a	1.92 a	0.287	<0.0001	2.07 a	1.48 ab	1.74 a	1.07 b	0.203	0.0093
In vitro accumulative gas production (mL g^{-1} DM)[2)]												
GP6	35.4 a	11.7 b	11.2 b	13.9 b	3.85	<0.0001	28.5 a	9.4 b	24.8 a	9.6 b	2.72	<0.0001
GP12	57.5 a	20.90 b	20.4 b	25.1 b	4.92	<0.0001	46.1 a	17.2 b	42.9 a	17.6 b	3.48	<0.0001
GP24	82.5 a	33.9 b	34.3 b	41.0 b	4.9	<0.0001	66.5 a	29.2 b	66.1 a	29.9 b	3.431	<0.0001
GP48	103.4 a	47.5 c	50.9 bc	57.8 b	4.16	<0.0001	84.0 a	43.8 b	86.7 a	45.1 b	2.940	<0.0001
GP72	111.3 a	53.2 c	59.6 bc	64.9 b	3.96	<0.0001	90.4 a	51.3 b	94.0 a	53.3 b	2.80	<0.0001

[1)] *b*, asymptotic gas production (mL g^{-1} DM); *c*, rate of gas production (h^{-1}); *L*, initial delay before gas production begins (h).

[2)] Mean of the cumulative gas volume at time of 6, 12, 24, 48 and 72 h of incubation.

a, b, c, mean values within a row in fodder trees and treatments without a common letter differ (P<0.01). The same as in Table 3.

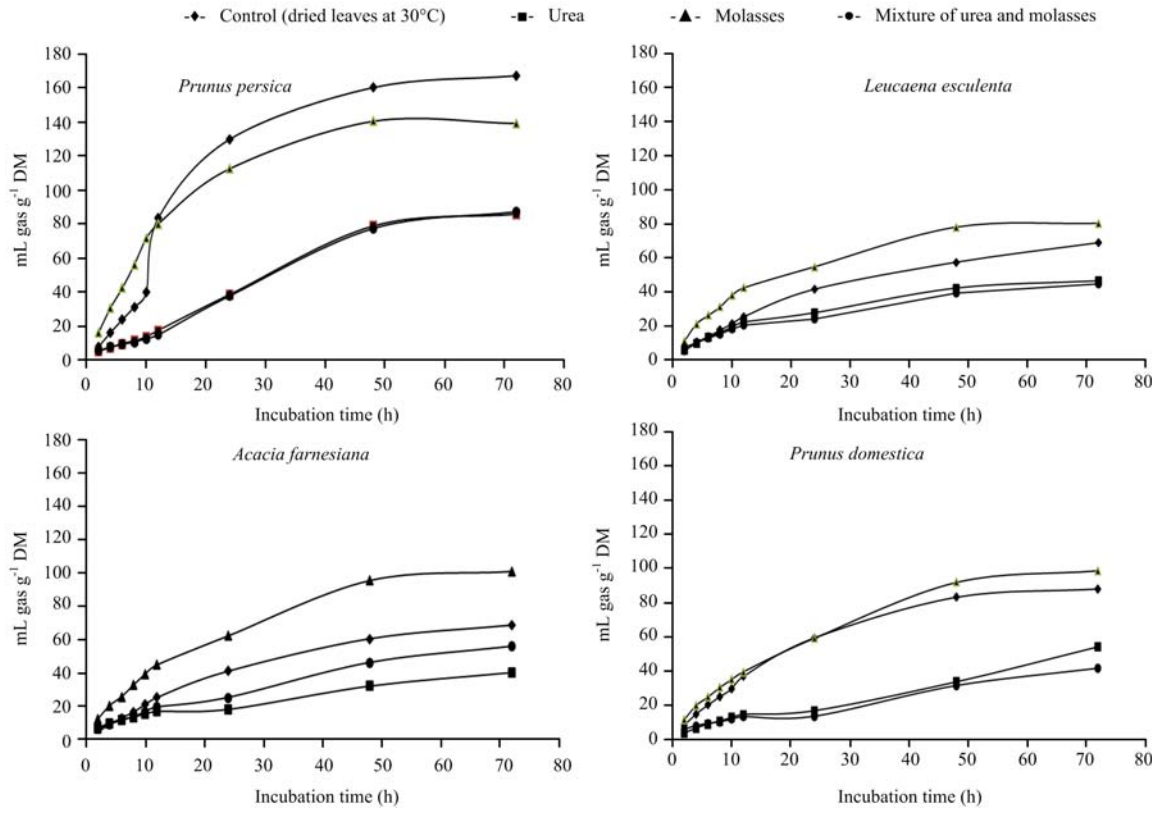

Fig. 1 Cumulative gas production profiles (mL gas g^{-1} DM) from *in vitro* fermentation of fodder trees ensiled with different treatments.

lowest was *P. persica*, *L. esculenta*, *A. farnesiana* and *P. domestica*. The cumulative GP of tree leaves ensiled with U and UM decreased $P<0.001$); but there were no differences between M and control.

The *in vitro* fermentation profiles of fodder trees ensiled with U, M or UM are presented in Table 3. As expected, *in vitro* fermentation profiles differed ($P<0.001$) among tree species. *P. persica* recorded the highest ($P<0.001$) DMD, ME, OMD, SCFA, GY_{24} and MCP and the lowest ($P<0.001$) pH and PF_{72}. *In vitro* fermentation values of tree leaves ensiled with U and UM were lower ($P<0.001$) than that of control. There were no differences between the M treatment and the control for DMD, ME, OMD, SCFA and MCP. The pH and GY_{24} of the M treatment was higher ($P<0.001$) than that of control, and inverse for PF_{72}.

DISCUSSION

Chemical composition of fodder tree leaves

Secondary metabolites of shrubs across arid and semi-arid regions represent a basic mechanism for plant competitiveness and appear to be especially important for adaptation to harsh, resource-limited environment (Freeland 1991). Thousands of plant secondary metabolites from numerous structural classes exist in nature, and their presence and concentration in a given plant are influenced by genetics, phenology, and a host of biotic and abiotic environmental factors. Thus, their concentrations vary temporally and spatially among and within species, and their proportions relative to other compounds (both primary and secondary) are in constant flux (Estell 2010), which explains why secondary metabolites were different among tree leaves in the present study.

In the current study, CP, NDF and ADF contents of tree leaves were inconsistent with previous reports (Rop *et al*. 2009; Rubio-Covarrubias *et al*. 2009), which could be a reflection of differences in plant growing conditions, plant species, soil type, plant parts and stage of maturity (Norton and Waterfall 2000). The increased CP content of ensiled treatments are in line with data of Khan *et al*. (2006) who reported that CP content increased by 18.4% when fermented for 30 d. An important increase in CP with the addition of U to the silage might be attributed to more availability of ammonia, as a result of more urea hydrolysis. Increased CP with ensiling with M may be attributed to accelerated microbial activity driven by more supply of readily fermentable energy, consequently more urease and urea hydrolysis leading to higher CP level of tree leaves. These results are in line with other (Lopez-Guisa *et al*. 1991) who have found that increased CP content of tree leaves ensiled with M might be due to the increased availability of fermentable carbohydrates and suitable growth media for microbial multiplication, leading to more ammonia fixing in tree leaves. Energy is usually the limiting factor for growth of anaerobic microbes and provision of U and M might have increased the microbial mass that leads to increased CP (Staples *et al*. 1981). Thus, provision of carbon skeleton and energy for microbial growth in tree leaves might have synchronized with ammonia released from urea hydrolysis, consequently increasing the CP content of forages ensiled.

The use of fodder trees for ruminants is limited by the presence of a wide range of secondary metabolites

Table 3 *In vitro* fermentation profiles of foliage from fodder trees ensiled with urea, molasses or the mixture of urea and molasses

	Fodder trees						Treatments					
	P. persica	*L. esculenta*	*A. farnesiana*	*P. domestica*	SEM	*P*	Control	Urea (U)	Molasses (M)	UM	SEM	*P*
Rumen fermentation profile[1)]												
pH	6.9 c	7.0 b	7.0 ab	7.1 a	0.02	<0.0001	6.7 d	7.2 b	6.9 c	7.2 a	0.02	<0.0001
DMD	611.6 a	414.0 b	415.6 b	442.9 b	19.75	<0.0001	546.7 a	395.0 b	544.9 a	397.7 b	13.96	<0.0001
ME	5.7 a	4.1 b	4.3 b	4.1 b	0.13	<0.0001	4.9 a	4.2 b	5.0 a	4.1 b	0.09	<0.0001
OMD	40.4 a	28.9 c	30.9 b	28.8 c	0.86	<0.0001	34.3 a	30.4 b	35.3 a	29.0 b	0.61	<0.0001
SCFA	1.81 a	0.73 b	0.74 b	0.89 b	0.108	<0.0001	1.46 a	0.63 b	1.44 a	0.64 b	0.076	<0.0001
PF_{72}	8.2 d	13.2 b	14.8 a	11.5 c	0.37	<0.0001	9.4 c	15.4 a	8.6 d	14.4 b	0.26	<0.0001
GY_{24}	126.9 a	79.8 c	78.1 c	90.4 b	2.15	<0.0001	112.2 b	71.5 c	118.3 a	73.2 c	1.52	<0.0001
MCP	430.2 a	339.4 b	340.1 b	352.7 b	9.07	<0.0001	400.4 a	331.0 b	399.5 a	331.9 b	6.42	<0.0001

[1)] pH, ruminal pH; DMD, dry matter degradability (mg g^{-1} DM); ME, metabolizable energy (MJ kg^{-1} DM); OMD, *in vitro* organic matter degradability (g kg^{-1} MS); SCFA, short chain fatty acids (mmol g^{-1} DM); PF_{72}, partitioning factor (mg DMD/mL gas); GY_{24}, gas yield at 24 h (mL gas g^{-1} DMD); MCP, microbial crude protein production (mg g^{-1} DM).

including tannins, saponins, alkaloids, non protein amino acids, steroids, essential oils, terpenes, oxalic acids, and resins (Aganga and Tshwenyane 2003; Makkar 2003). These compounds do not form part of the plant nutrients as they do not take part in primary metabolic processes, but instead they have diverse roles including protecting plants from pest, fungal and herbivore attack (Aganga and Tshwenyane 2003). Several methods have been used to counteract the deleterious effects of anti-nutritional factors in fodder trees. Among these are use of alkalis (Makkar and Singh 1993; Vitti *et al*. 2005), metal ions, oxidising agents (Makkar 2003), exogenous microbial enzymes (McSweeney *et al*. 2001; Makkar 2003) and tannin-binding compounds (Priolo *et al*. 2005; Mlambo *et al*. 2007). Oven, freeze and sun-air drying techniques have also been used to lessen the adverse effects of phenolics in browse legumes (Dzowela *et al*. 1995; Vitti *et al*. 2005). The present study showed that secondary metabolites contents decreased when tree leaves were ensiled with U, M and the mixture of UM probably due to the polymerization of tannins and polyphenols as noted by Makkar (2003), in mature oak.

In vitro gas production

Gas produced in *in vitro* fermentation reflects the extent of feed fermentation and digestibility (Getachew *et al*. 1998). Cumulative GP values indicate large differences in fermentation for *P. persica*, *L. esculenta*, *A. farnesiana*, and *P. domestica* (Table 2). According to Cone and van Gelder (1999), comparison of GP data of samples differing widely in CP content can lead to misinterpretations. Generally, low GP would indicate low degradability, but feedstuffs high in CP normally produce less gas during fermentation, even if their extent of degradation is high, because protein fermentation produces ammonia, which influences the carbonate buffer equilibrium by neutralizing H^+ ions from VFA without release of carbon dioxide (Cone and van Gelder 1999). In the present study, leaves of *P. domestica* contained low CP but produced more gas compared to *L. esculenta* and *A. farnesiana*, indicating that *P. domestica* has a higher fermentation potenctial than *L. esculenta* and *A. farnesiana*.

The addition of M to tree leaves increased GP after 48 h of incubation, suggesting that during the ensiling process M might have removed some chemical linkages of hemicelluloses and thus enhanced their solubility in detergent solutions (Chaudhry 1998), and also possibly due to the ability of rumen microorganism to degrade the plant secondary metabolites like alkaloids (Wachenheim *et al*. 1992), saponins (Hu *et al*. 2005; Hart *et al*. 2008) and phenolics (Varel *et al*. 1991) and utilize them as an energy source. Results with the addition of U and UM on GP to tree leaves in the present experiment are consistent with the results of Cone and van Gelder (1999).

In vitro rumen fermentation profiles

The pH values obtained in the present work were similar to those previously observed in goats and sheep (Yanez-Ruiz *et al*. 2004; García *et al*. 2006). For each fodder tree and treatment, average pH values near 7.0, might have resulted from a combined effect of slow fermentation and continuous buffer infusion, and reflected low VFA concentration. Variable responses of *in vitro* digestibility among tree leaves could be due to variable levels of secondary metabolites, or to variations in anti-nutritive activity among tree leaves. The anti-nutritive activity of phenolic compounds has been reported to vary between forage species due to the nature of tannin and chemical structure (Dalzell and Kerven 1998), degree of polymerization (Schofield *et al*. 2001), biochemical processes (Wong 1973) and to the tannin structure-biological activity relationship (Haslam 1998). Decreased GP at 24 h, OM and ME digestibility for U and UM treatments to *in vitro* fermentation systems indicated an accelerative effect of phenolic compounds on degradation of feed nutrients in the rumen and, probably, their enhanced ruminal digestibility after ensiled with U and UM (Rubanza *et al*. 2005). The stimulatory effect of protein supplementation has been reported (Carro and Miller 1999; Ranilla *et al*. 2001) and our results seem to indicate, in disagreement with Soto *et al*. (1994), that stimulatory effect of ensiling with P are dependent on the levels of available protein.

McSweeney *et al*. (2001) and Min *et al*. (2003) reported that the protein-tannin complexes reduce the availability of fermentable N for microbial activity in

the rumen. Kumar and Singh (1984) reported that tannins in tree leaves inhibited proteolysis of casein and subsequent ammonia production *in vitro*. Therefore, U addition would provide fermentable N for stimulating microbial fermentation in the rumen (Russell and Lolley 1989; Kumar and Vaithiyanathan 1990). However, Kumar and Singh (1984) suggested that a quantitative relationship between tannins and urea for improving feed quality need to be worked out. Therefore, in the present study, the lower MP yield *in vitro* expected with ensiling with U, M and UM may explain the lack of difference in N retention between treatments.

CONCLUSION

Among the fodder trees examined, *P. persica* had the highest ruminal fermentation, highest microbial protein synthesis and efficiency, as well as the highest digestibility and metabolizable energy, indicating that, of the four fodder trees tested, *P. persica* constitute a source of high quality forage. Also, these results indicate that the addition of M to foliage of fodder trees during the ensiling process results in a marked, improvement in *in vitro* ME, OMD, and GP, indicating that M could positively modify rumen GP and fermentation, which may improve nutrient utilization by ruminants.

MATERIALS AND METHODS

Tree leaves and treatments

Leaves from four fodder trees: *P. persica*, *L. esculenta*, *A. farnesiana* and *P. domestica* were collected in triplicate (i.e., three individual samples from each tree) from central Mexico. The samples were randomly and manually harvested from different parts of the trees to obtain representative samples of both young and mature leaves from each tree species. 100 g of fresh tree leaves from each species were chopped to pass a 1-3 cm sieve. The treatments included ensiled (i) dried leaves (30ºC for 72 h) without any additive (control), (ii) tree leaves mixed with 40 mL of urea solution (U, 5 g of urea per 100 mL of water), (iii) tree leaves mixed with molasses solution (M; 10 g of molasses per 100 mL of water) and (iv) tree leaves mixed with urea and molasses (UM; 40 mL of the mixture of U and M solutions, 1:1, v:v) in nylon bags (20 cm×50 cm) for 30 d, respectively. After the ensiling period, samples of each treatment were dried at 30ºC for 72 h and ground to pass through a 1-mm sieve and stored at ambient temperature (25-27ºC) in plastic bags for later determination of chemical components, secondary metabolites and *in vitro* GP.

Animals as rumen inoculum donors

Rumen liquor was obtained from eight growing Katahdin×Pelibuey lambs (live weight, (24±0.3) kg) using a stomach tube. Lambs were fed a total mixed ration (TMR) based on soybean meal, 220; alfalfa hay, 150; sorghum grain, 550; fishmeal, 35; mineral/vitamin premix, 25 and salt, 20 (g kg^{-1} dry matter (DM)) *ad libitum*. Ruminal contents from each lamb were obtained before the morning feeding, mixed and strained through four layers of cheesecloth into a flask with O_2-free headspace. The TMR was formulated to meet the lamb's nutrient requirements according to NRC (1985). Fresh water was available at all time.

In vitro incubations

Forage samples (0.5 g) from each tree was weighed into 120 mL serum bottles and 10 mL of particle-free ruminal fluid were added to each bottle followed by 40 mL of a buffer solution described by Goering and van Soest (1970), without trypticase, in a proportion of 1:4 (v/v). A total of 432 bottles (4 tree species×3 individual samples×3 runs on different weeks×3 replication (three individual samples of each species)×4 treatments) with three bottles as blanks (rumen fluid only) at each run, were incubated for 72 h. Once all bottles were filled, they were immediately closed with rubber stoppers, shaken and placed in the incubator at 39ºC. The volume of GP was recorded at 2, 4, 6, 8, 10, 12, 24, 48, and 72 h of incubation using the reading pressure technique (RPT; DELTA OHM, Italy) described by Theodorou *et al*. (1994). At the end of incubation period (72 h), bottles were uncapped; pH was immediately measured using a pH meter (GLP 22, Crison Instruments, Barcelona, Spain) and fermentation was stopped by swirling the bottles in ice. The contents of each bottle were filtered to get the residue (undegraded forage) for determination of apparently degraded substrate.

In vitro dry matter degradability

At the end of the incubation period the contents of each serum bottle were filtered under vacuum through glass crucibles with a sintered filter (coarse porosity no. 1, pore size 100-160 μm, Pyrex, Stone, UK). Fermentation residues were dried at 105ºC overnight to estimate potential DM disappearance. Loss in weight after drying indicated undegradable DM. The DM degradability at 72 h of incubation (dry matter degradability, DMD; mg g^{-1} DM) was calculated as the difference between DM content of sub-

strate and its undegradable DM.

Chemical analyses and secondary metabolites

Samples of forages were analyzed for DM (#934.01), ash (#942.05), N (#954.01) and EE (#920.39) according to AOAC (1997). The neutral detergent fiber (NDF, van Soest *et al*. 1991), acid detergent fiber (ADF) and lignin (AOAC 1997; #973.18) were determined with an ANKOM200 fiber analyzer unit (ANKOM Technology Corporation, Macedon, NY, USA). NDF was assayed without use of alpha amylase but with sodium sulfite in the NDF. Both NDF and ADF are expressed without residual ash.

Plant secondary metabolites of leaves forage were determined in all tree species. 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 by drying and quantifying the total phenolics layer in the funnel. After the separation of total phenolics, a double volume of n-butanol (99.9/100, analytical grade, Fermont®, Monterrey, Mexico), was added to fractionate saponins (Makkar *et al*. 1998). The remaining solution was considered to be the aqueous fraction which has other secondary metabolites such as lectins and polypeptides (Cowan 1999).

Calculations

To estimate kinetic parameters of GP, gas production results (mL g^{-1} DM) were fitted using the NLIN option of SAS Institute (2002) according to France *et al*. (2000) using the following model:

$A=b\times(1-e^{-c(t-L)})$

Where A is the volume of GP at time t; b is the asymptotic GP (mL g^{-1} DM); c is the rate of GP (h^{-1}) from the slowly fermentable feed fraction b, and L (h) is the discrete lag time prior to GP.

Metabolizable energy (ME, MJ kg^{-1} DM) and *in vitro* organic matter degradability (OMD, g kg^{-1} organic matter) were estimated according to Menke *et al*. (1979) as:

ME=2.20+0.136 GP (mL 0.5 g^{-1} DM)+0.057 CP (% DM)

OMD=148.8+8.89 GP+4.5 CP (g kg^{-1} DM)+0.651 ash (g kg^{-1} DM)

Where GP is net gas production in mL from 200 mg of dry sample after 24 h of incubation.

The partitioning factor at 72 h of incubation (PF_{72}; a measure of fermentation efficiency) was calculated as the ratio of *in vitro* dry matter degradability (DMD, mg) to the volume of gas (mL) produced at 24 h (DMD/total GP (GP_{24})) according to Blümmel *et al*. (1997). Gas yields (GY_{24}) were calculated as the volume of gas (mL gas g^{-1} DM) produced after 24 h of incubation divided by the amount of DMD (g) as follows:

Gas yields (GY_{24})=mL gas g^{-1} DM/g DMD

Short chain fatty acids concentration (SCFA) was calculated according to Getachew *et al*. (2002) as follows:

SCFA (mmol 200 mg^{-1} DM)=0.0222 GP-0.00425

Where GP is the 24 h net GP (mL 200 mg^{-1} DM).

Microbial crude protein production (MCP) was calculated according to Blümmel *et al*. (1997) as:

MCP (mg g^{-1} DM)=mg DMD-(mL gas×2.2 mg mL^{-1})

Where 2.2 mg mL^{-1} is a stoichiometric factor which expresses mg of C, H and O required for the SCFA gas associated with production of 1 mL of gas (Blümmel *et al*. 1997).

Statistical analyses

Data of *in vitro* ruminal GP and fermentation parameters were analyzed as a 4×4 factorial experiment (four fodder trees (random effect) and 4 treatments (fixed effect)) according to a randomized block design using the MIXED procedure of SAS Instituten (2002). Data of each of the three runs within sample were averaged and used as the mean value of each within tree species. Mean values of each individual sample within each tree species (three samples per tree) were used as the experimental unit. The full statistical model was:

$Y_{ikl}=\mu+S_i+T_k+E_{ikl}$

Where Y expressed every observation of the kth individual sample within the ith tree species, S expressed the tree species effect, T expressed the treatments and E is the experimental error.

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