Chemical characterization and *in vitro* biological activity of four tropical legumes, *Styzolobium aterrimum* L., *Styzolobium deeringianum*, *Leucaena leucocephala*, and *Mimosa caesalpiniaefolia*, as compared with a tropical grass, *Cynodon* spp. for the use in ruminant diets

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ABSTRACT: *Leucaena leucocephala* (LEU) and three under-utilized tanniferous legumes, *Styzolobium aterrimum* L. (STA), *Styzolobium deeringianum* (STD), and *Mimosa caesalpiniaefolia* Benth (MIC) were chemically characterized and the biological activity of tannins was evaluated using *in vitro* simulated ruminal fermentation through tannin-binding polyethylene glycol (PEG) and compared with a non-tanniferous tropical grass hay, *Cynodon* spp. (CYN). The Hohenheim gas test was used and gas production (GP) was recorded at 4, 8, 12, 24, 32, 48, 56, 72, 80, and 96 h incubation with and without PEG. Kinetic parameters were estimated by an exponential model. STA, STD, and LEU contained higher ($P < 0.05$) crude protein than MIC, which had greater neutral detergent fibre and acid detergent fibre. Total phenols, total tannins, and condensed tannins (CT) were consistently the highest in MIC. Gas production was the lowest from MIC ($P < 0.05$) and the highest in LEU and STA. MIC + PEG largely reduced ($P < 0.05$) the lag phase and the fractional rate of fermentation and increased potential GP. Kinetic parameters of STA + PEG and LEU + PEG were not affected. LEU + PEG produced greater gas increment ($P < 0.05$) than STD + PEG, although both legumes had the same CT. All legumes except MIC were more extensively degraded than CYN. However, fermentation of the legumes was differently affected by the presence and proportions of CT, indigestible fibre or both.

Keywords: tannins; tropical forages; rumen fermentation; gas production; kinetics; PEG

Utilization of protein supplements in ruminant diets can have nutritional, economic, and ecological advantages. Many tropical plants have so far not been fully utilized due to the presence of secondary metabolites, such as condensed tannins (CT). Condensed tannins are phenolic compounds classified as polymers of flavan 3-ol with different molecular weights that can bind protein and other macromolecules (Mueller-Harvey, 2006). The binding is reversible according to pH and molecular type (McAllister et al., 2005) and is sometimes considered beneficial as it can reduce nematode damages, bloat, and ruminal protein degradation (Mueller-Harvey, 2006). Some of the negative ef-

fects are low palatability, digestibility reduction, and reduced performance (Makkar, 2003a). Level of tannins cannot be used alone to indicate suitability of a fodder species as a protein supplement. Factors such as reactivity, structure, molecular weight, and interactions of different secondary compounds in the plant are important. Loss of nitrogen (N) as ammonia should be related to tannins (level and quality) but also to rate of degradation and synchronization pattern (Barry et al., 1986; Waghorn et al., 1994; Kaitho et al., 1998). Some studies have shown that enteric methane was reduced in the presence of CT (Waghorn et al., 2002; Hess et al., 2003; Puchala et al., 2005). However, inconsistent results have also been found. Beauchemin et al. (2007) applying 20 g/kg DM of quebracho (*Schinopis* sp.) tannin extract failed to reduce methane but protein-binding effect was observed. Therefore, beneficial effects of tannins rely upon their level and the source (Jayanegara et al., 2012).

The *in vitro* technique as a batch culture is the simplest *in vitro* method, which monitors the accumulation of gases being limited to the rumen environment. It is recognized to improve information of nutritional values of feedstuffs and browses rich on secondary compound not as a simulation of rumen fermentation but the estimation of potential rate and extent of feed fermentation (Krishnamoorthy et al., 2005). Evaluation of CT effects on rumen fermentation can be done using *in vitro* gas production (GP) techniques with the addition of polyethylene glycol (PEG) as a CT-complexing agent (Makkar et al., 1995).

Typical vegetation in the northwest of Brazil is composed of leguminous shrubs and trees with high crude protein (CP) and tannin contents, the latter reducing their utilization by domestic animals (Vitti et al., 2005). Around 70% of the woody species from some range sites may contribute significantly to bovine, ovine, and goat diets (Araújo Filho et al., 1991). *Styzolobium aterrimum* L. (STA), *Styzolobium deeringianum* (STD), *Leucaena leucocephala* (LEU), and *Mimosa caesalpiniaefolia* (MIC) belong to these legumes. Leucaena species occur naturally from the southern United States through middle South America (Argel and Pérez, 1997) and have been intensively studied (Kaitho et al., 1998; McNeill et al., 1998; Karda and Dryden, 2001; Vitti et al., 2005). *Mimosa caesalpiniaefolia* is a tanniferous legume mainly used as ornamental plant that has recently gained increased interest as protein supplement due to its high CP concentration and high acceptability by ruminants. Some studies with MIC from the northeast of Brazil were carried out to characterize the CT at different phenological stages (Guimarães-Beelen et al., 2006) and to evaluate *in vitro* fermentation kinetics (Nozella, 2006).

The objective of this study was to chemically characterize LEU and three underutilized tanniferous legumes, namely STA, STD and MIC, and to evaluate the biological activity of tannins on *in vitro* ruminal fermentation through the use of tannin-biding polyethylene glycol (PEG) in comparison to a non-tanniferous tropical grass hay, *Cynodon* spp. (CYN). Degradabilities and other ruminal variables of these plants were evaluated in other assays.

MATERIAL AND METHODS

Experimental plants and site

Four high-CT legumes, STA, STD, LEU, and MIC, commonly called mucuna preta, mucuna rajada, leucaena, and mimosa, respectively, were obtained from an experimental area of the Polo Regional Centro-Sul – Apta, Sao Paulo, Brazil (latitude 22°43'31''S, longitude 47°38'57''W, 554 m height, humid subtropical climate (Koeppen *Cwa*) with 1300 mm average annual precipitation, 28°C maximum and 15°C minimum average temperature) that received no lime and no other fertilization.

Sample collection

The whole aerial biomass was harvested from three different sub-areas of 1 m^2 each between November 7th 2004 and January 5th 2005 when STA and STD were in late vegetative stage. Plants carrying stems with diameter larger than 0.5 cm were discarded. The LEU and MIC materials were cut from stem tips of re-growth branches of ten randomly chosen plants of each species. The collected materials were bulked to give a composite sample of each species, wilted under shade for 24 h before being dried to constant weight in a forced-air oven at 40°C. *Cynodon* spp. (CYN), 'Tifton 85' grass hay, was obtained from the local market and used as a CT-free (CT < 0.1 g/kg dry matter (DM)) experimental control. All materials were ground to pass a 1-mm screen in a Wiley mill and stored at 4°C before analyses.

Chemical analyses

All forage materials were analysed for DM (AOAC, 2005: method 934.01), ash (AOAC, 2005: method 942.05), CP (AOAC, 2005: method 954.01), acid detergent fibre (ADFom) (AOAC, 2005: method 973.18) and neutral detergent fibre (aNDFom) (AOAC, 2005: method 2002.04 adapted to Ankom Fibre Analyzer) with heat stable amylase and expressed without residual ash. Phenolic compounds were extracted in an ultra-sound bath with 10 ml of aqueous acetone solution (700 ml/l) (Makkar, 2003b). Total phenols (TP) and total tannins (TT) were determined by adding 0.25 ml Folin-Ciocalteau reagent (2 N) and 1.25 ml sodium carbonate solution (200 g Na_2CO_3/l) to an aliquot of the supernatant and absorbance readings at 725 nm. To determine TT, a binding tannin agent, insoluble polyvinyl pyrrolidone (PVPP) was added to the extract. A calibration curve was prepared from aliquots of the acid tannic solution (0.1 mg/ml; Merck GmbH, Darmstadt, Germany). The difference of TP and the PVPP extract readings was an estimate of TT. The concentrations of TP and TT were calculated as tannic acid equivalents (eq) and expressed as g /kg DM.

Condensed tannins were expressed as leucocyanidin equivalent (% of DM) and were determined using Butanol-HCl and concentrations calculated by the formula:

$$\frac{\text{Absorbance at 550 nm} \times 78.26 \times \text{Dilution factor}}{\%\ \text{DM}}$$

The dilution factor was equal to 1 if no 70% acetone was added or 0.5 ml per volume of the extract was taken (Porter et al., 1986; Makkar, 2003b).

In vitro gas production measurement

The Hohenheim gas test was carried out at the Animal Nutrition Laboratory, University of Bonn, Germany according to Menke and Steingass (1988) except that the concentration of $NaHCO_3$ was reduced to 33 g/l and that of $(NH_4)HCO_3$ increased to 6 g/l to prevent shortage in N during prolonged incubation times (Liu et al., 2002). Two castrated male adult sheep, Blackface breed, fed on a diet of 600 g hay and 600 g mixed concentrate per day that covered maintenance energy requirements, served as rumen liquid donor animals. The animals had never received tanniferous and/or tropical feeds before. Rumen fluid was collected immediately before morning feeding and strained through two layers of cheesecloth into a pre-warmed and insulated bottle. All laboratory handling of rumen fluid was carried out under a continuous flow of CO_2. Samples (200 ± 10 mg) with and without PEG (200 ± 10 mg) of the air-dry substrate were accurately weighed into 100 ml glass syringes and the syringe pistons were lubricated with vaseline and inserted into the syringes. *In vitro* incubation of the samples was conducted in triplicate. As a routine, triplicates of bottles without substrate (blanks) and standard hay and concentrate obtained from the Institute of Animal Nutrition, Hohenheim University, Stuttgart, Germany, were included as a laboratory controls with a known chemical composition and expected GP (Menke and Steingass, 1988). Incubations were repeated when gas volumes of the standards deviated by more than 10% from the reference values. The syringes were placed in a rotor inside an incubator (39°C). The GP (ml) was measured at 4, 8, 12, 24, 32, 48, 56, 72, 80, and 96 h of incubation.

The dynamics of GP over time was estimated from an exponential model (France et al., 1993):

$$Vt = A\{1 - e^{[-b(t-L) - c(\sqrt{t} - \sqrt{L})]}\};\ \mu = b + \left\{\frac{c}{2\sqrt{t}}\right\}$$

where:
Vt = final gas volume accumulated (ml)
L = lag phase (h)
A = potential GP (ml)
μ = fractional fermentation rate (%/h)
b, c = constants in the model
t = temperature

The parameters A, b, c, and L were estimated using nonlinear regression analysis (procedure NLIN of SAS, 2001), with minimum values defined as 80, 0.05, −0.15, and 5, respectively.

The time $t_{½}$, which represents the time when half of the potential GP was produced, was estimated as $t_{½} = \{\ln 2/c\} + L$ (Grings et al., 2005). Estimating the mean retention time in the rumen to be 48 h, it would be desired that all materials consumed by the animal were completely fermented during this period. Bueno et al. (2005) suggested that the ratio of GP at 48 h to that at 96 h (REL1) indicates how much of the total fermentation was reached until 48 h, and that the ratio of GP at 96 h to A (REL2) represents how much of the potential GP was reached during the

observed fermentation period. The gas increment due to PEG addition was calculated at 8, 24, and 48 h and hereafter denoted Inc8, Inc24, and Inc48, respectively.

Statistical analyses

Data were subjected to analysis of variance using the GLM procedure of SAS (SAS, 2001) in a 5 (plants) × 2 (tannin-binding agent) factorial arrangement, where the experimental unit was the composite sample of each species replicated in three bottles. The model used was:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + E_{(ij)k}$$

where:

Y_{ijk} = dependent variable (gas production (GP8, GP24, G48, and GP96), gas increment (Inc8, Inc24, and Inc48) and kinetic parameters (*A*, *L*, μ8, μ24, μ48, REL1, and REL2))

μ = population mean

α_i = effect caused by a plant

β_j = effect of PEG

$E_{(ij)k}$ = residual error

Treatment means were compared by linear contrasts. The following contrasts were evaluated: (1) influence of legumes (Grass vs. Legumes), (2) influence of legumes excluding MIC due to its high CT concentration (no MIC: Grass vs. (STA, STD, LEU)), (3) species of the genus *Styzolobium* compared with the other legumes ((STA + STD) vs. (LEU + MIC)), and (4) influence of assumed nutritional quality, i.e. high quality vs. low quality (HQ vs. LQ: (STA + LEU) vs. (STD + MIC)), plants with low tannin (STA) or low fibre (LEU) concentrations vs. plants with high tannin and high fibre contents (MIC and STD). Correlations between gas production and phenolic compounds (TP, TT, and CT) were tested with and without PEG using CORR procedure of SAS (SAS, 2001).

Chemical composition data were analysed by one-way analysis of variance using the GLM procedure and the means were compared by Tukey's test. Differences among means with $P < 0.05$ were accepted as representing statistically significant differences. Probability values lower than 0.001 were expressed as $P < 0.001$ rather than the actual value.

RESULTS

Based on chemical composition (Table 1), CYN and MIC had lower CP contents compared with STA, STD, and LEU. *Cynodon* spp. had the lowest CP and the greatest aNDFom values ($P < 0.05$), however it contained as much hemicellulose as LEU ($P > 0.05$), intermediate ADFom, and had the lowest concentrations of phenolic compounds. On the other hand, MIC had the greatest values ($P < 0.05$) for phenolic compounds and ADFom and intermediate CP and hemicellulose concentrations. Greater CP concentrations were observed in STA, STD, and LEU ($P < 0.05$) while STD and LEU had intermediate CT contents (64 and 56 g of leucocyanidin equivalents/kg DM, respectively).

Table 1. Chemical composition (in g/kg dry matter) of *Cynodon* spp. 'Tifton 85' (CYN), *Styzolobium aterrimum* L. (STA), *Styzolobium deeringianum* (STD), *Leucaena leucocephala* (LEU), and *Mimosa caesalpiniaefolia* (MIC)

	CYN	STA	STD	LEU	MIC	SEM
Dry matter	930^{c}	923^{d}	944^{a}	922^{e}	932^{b}	0.7
Organic matter	904^{d}	937^{c}	942^{bc}	971^{a}	960^{ab}	5.1
Crude protein	78^{c}	241^{a}	236^{a}	246^{a}	191^{b}	8.0
aNDFom	774^{a}	637^{bc}	642^{bc}	620^{c}	706^{ab}	20.1
ADFom	393^{b}	412^{ab}	405^{ab}	275^{c}	494^{a}	23.4
Hemicellulose	381^{a}	225^{b}	237^{b}	346^{a}	212^{b}	20.3
Total phenols (tannic acid equivalent)	6^{e}	55^{d}	72^{c}	114^{b}	151^{a}	4.3
Total tannins (tannic acid equivalent)	3^{c}	38^{b}	49^{b}	98^{a}	110^{a}	4.4
Condensed tannins (leucocyanidin equivalent)	0.2^{d}	20^{c}	64^{b}	56^{b}	105^{a}	3.0

^{a,b,c,d,e}means ($n = 3$) with different letters within row differ ($P < 0.05$)

SEM = standard error of means, aNDFom = neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash, ADFom = acid detergent fibre expressed exclusive of residual ash. Hemicelluloses calculated as NDF-ADF

Table 2. Gas production observed *in vitro* (ml/200 mg DM) with and without polyethylene glycol (PEG, tannin-binding agent) after 8 (GP8), 24 (GP24), 48 (GP48), and 96 h (GP96) and gas increment (values of unsupplemented forage equal to 100) due to PEG addition after 8, 24, and 48 h (Inc8, Inc24, and Inc48)

	CYN		STA		STD		LEU		MIC		SEM	Main factors		Interaction plant × PEG
	no PEG	PEG	no PEG	PEG	no PEG	PEG	no PEG	PEG	no PEG	PEG		plants	PEG	
GP8	10.2	10.4	25.4	26.2	24.2	26.8	25.7	31.5	8.0	20.1	0.42	< 0.001	< 0.001	< 0.001
GP24	24.7	24.5	36.8	38.2	34.8	36.9	37.6	41.3	10.8	30.4	0.40	< 0.001	< 0.001	< 0.001
GP48	34.5	34.7	41.7	42.9	39.2	41.1	43.4	46.7	16.2	36.0	0.81	< 0.001	< 0.001	< 0.001
GP96	39.3	41.0	45.2	45.7	44.1	45.1	47.2	50.9	22.8	40.3	1.07	< 0.001	< 0.001	< 0.001
Inc8	0	1.5	0	3.3	0	10.5	0	22.8	0	152.1	5.36	< 0.001	< 0.001	< 0.001
Inc24	0	0	0	3.8	0	6.3	0	9.8	0	183.4	5.60	< 0.001	< 0.001	< 0.001
Inc48	0	0.7	0	2.9	0	4.9	0	7.6	0	123.7	5.70	< 0.001	< 0.001	< 0.001

Linear contrasts[1]	*P*										
	No PEG				PEG						
	G8	G24	G48	G96	G8	G24	G48	G96	Inc8	Inc24	Inc48
Grass vs. legumes	< 0.001	< 0.001	0.599	0.751	< 0.001	< 0.001	< 0.001	0.002	0.003	0.002	0.013
No MIC	< 0.001	< 0.001	0.002	0.011	< 0.001	< 0.001	< 0.001	< 0.001	0.272	0.449	0.651
Styz vs. other legumes	< 0.001	< 0.001	< 0.001	< 0.001	0.140	0.003	0.327	0.833	< 0.001	< 0.001	< 0.001
HQ vs. LQ	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

CYN = *Cynodon spp.* – 'Tifton 85', STA = *Styzolobium aterrimum* L., STD = *Styzolobium deeringianum*, LEU = *Leucaena leucocephala*, MIC = *Mimosa caesalpiniaefolia,* SEM = standard error of means (n = 6)
[1]grass vs. legumes = CYN vs. (STA, STD, LEU, MIC), No MIC = CYN vs. (STA, STD, LEU), Styz. vs. other legumes = STA + STD vs. LEU + MIC, HQ vs. LQ = STA + LEU vs. STD + MIC

The two *Styzolobium* species did not differ ($P > 0.05$) with respect to CP, aNDFom, ADFom, and hemicellulose contents but STA contained less ($P < 0.05$) TP and CT than STD and the difference was particularly pronounced for CT with STA having about one-third of the CT content of STD. The two other legumes, MIC and LEU, although both were stem tips of re-grown plants, had different ($P < 0.05$) fibre and phenolic compound concentrations, with MIC being higher than LEU in aNDFom, ADFom, TP, and CT.

Gas production after 8, 24, 48, and 96 h and gas increment due to PEG addition are presented in Table 2. There were significant interactions between PEG and plants for GP and gas increment at all measured time intervals ($P < 0.05$). Legumes without PEG had higher GP than the grass but only up to 24 h of incubation ($P < 0.05$). *M. caesalpiniaefolia* had the least GP, ranging only from 8 to 23 ml/200 mg DM. Consequently, when MIC was not included, the positive ($P < 0.05$) influence of legumes on GP was seen in all incubation periods. Among the legumes, the highest ($P < 0.05$) GP without PEG was recorded for LEU and STA. The same trend was not observed in STD, which had similar CT concentrations as LEU and similar chemical characteristics as STA, but produced less ($P < 0.05$) gas than the two other legumes after 24 and 48 h. The GP of *Styzolobium* spp. (STA and STD) with no PEG was different from the other legumes during the whole incubation period ($P < 0.05$).

With PEG addition, GP was significantly affected by legumes with or without MIC inclusion at every measured time ($P < 0.05$). Despite this, increment of gas produced by PEG addition (Inc8, Inc24, and Inc48) did not show any difference ($P > 0.05$) when MIC was not included. *Styzolobium* spp. legumes (STA and STD) were different from the other legumes ($P < 0.05$) regarding GP after 24 h. There was no correlation ($P > 0.05$) between GP in any time and the TP, TT or CT.

Addition of PEG did not improve the kinetic parameters of STA. Moreover, Inc24 and Inc48 (3.8 and 2.9%, respectively) in STA was lower ($P < 0.05$) than

Table 3. Potential gas production (*A*; ml), lag phase (*L*; h), and fractional fermentation rate (μ; %/h) after 8, 24, and 48 h in the *in vitro* incubation of *Cynodon* spp. 'Tifton 85' (CYN), *Styzolobium aterrimum* L. (STA), *Styzolobium deeringianum* (STD), *Leucaena leucocephala* (LEU), and *Mimosa caesalpiniaefolia* (MIC) using an exponential model[1] and the gas production ratios of GP48/GP96 (REL1) and GP96/*A* (REL2)

	CYN		STA		STD		LEU		MIC		SEM	Main effects		Interaction plant × PEG
	no PEG	PEG	no PEG	PEG	no PEG	PEG	no PEG	PEG	no PEG	PEG		plants	PEG	
A	40.2	41.9	69.9	70.2	48.7	66.3	67.7	76.3	36.3	47.4	1.25	< 0.001	< 0.001	< 0.001
L	0.5	0.7	3.0	2.7	2.0	2.7	2.5	2.4	10.8	1.9	0.44	< 0.001	< 0.001	< 0.001
μ8	3.7	3.4	1.8	1.9	2.3	1.9	2.0	2.2	–0.6	2.3	0.14	< 0.001	< 0.001	< 0.001
μ24	4.3	3.9[b]	3.0	3.1	3.2	3.0	3.2	3.4[c]	1.1	3.2	0.10	< 0.001	< 0.001	< 0.001
μ48	4.5	4.2[b]	3.5	3.6	3.6	3.5	3.6	3.8	1.8	3.6	0.08	< 0.001	< 0.001	< 0.001
REL1	0.88	0.85	0.92	0.94	0.89	0.91	0.92	0.92	0.71	0.89	0.01	< 0.001	< 0.001	< 0.001
REL2	0.98	0.98	0.65	0.65	0.91	0.69	0.70	0.67	0.63	0.85	0.01	< 0.001	0.375	< 0.001

Linear contrasts[2]	*P*													
	No PEG							PEG						
	A	μ8	μ24	μ48	*L*	REL1	REL2	*A*	μ8	μ24	μ48	*L*	REL1	REL2
Grass vs. legumes	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.002	0.163	< 0.001
No MIC	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	0.039	0.064	< 0.001
Styz vs. other legumes	0.002	0.004	0.004	0.008	0.002	< 0.001	< 0.001	0.003	0.001	< 0.001	< 0.001	0.001	< 0.001	< 0.001
HQ vs. LQ	< 0.001	0.886	0.085	0.017	0.085	< 0.001	< 0.001	0.003	0.001	< 0.001		0.002	< 0.001	< 0.001

[1] $V = A\{1 - e^{[-b(t-L) - c(\sqrt{t} - \sqrt{L})]}\}$

[2] grass vs. legumes = CYN vs. (STA, STD, LEU, MIC), No MIC = CYN vs. (STA, STD, LEU), Styz. vs. other legumes = STA + STD vs. LEU + MIC, HQ vs. LQ = STA + LEU vs. STD + MIC

SEM = standard error of means (n = 6)

in STD (6.3 and 4.9%, respectively). At the beginning of incubation, Inc8 was as high as 10% for STD and 23% for LEU being Inc48 still observed for these legumes but at lower values (5 and 8%, respectively).

Kinetic parameters of GP are presented in Table 3. The $t_{½}$ values were 17, 6, 6, 7, and 47 h for CYN, STA, STD, LEU, and MIC, respectively. Interactions were observed ($P < 0.05$) between plants and PEG for all studied kinetic parameters. The greatest potential GP had STA and LEU ($P < 0.05$). These two plants differed in all kinetic aspects from the other two legumes, except for lag phase and fractional rate at 8 and 24 h. Lag phase of MIC was approximately three times longer than that of the other legumes. *Cynodon* spp. had the shortest ($P < 0.05$) lag phase, differing significantly from the legumes with or without MIC inclusion. *Cynodon* spp. also had the greatest fermentation rate, however, it had lower ($P < 0.05$) GP and potential GP (A) compared with legumes with or without MIC. A remarkable discrepancy was observed between 96 h gas production (Table 2) and the potential gas production (A; Table 3), e.g., around 25 ml difference for STA. For us this clearly indicates a poor fit of the exponential model to the data. From a look at the data plot it appears that a simple mono-exponential model would fit better and thus it was tested the mono-exponential model of Blummel and Orsko (1993)

$$p = a + b\,(1 - e^{-ct})$$

where:

p	= dry matter loss at time t (ml)
a	= immediately soluble material
b	= insoluble but fermentable material
$a + b$	= potential gas production
c	= rate constant
t	= temperature
e	= mathematical constant

However, the mono-exponential model presented a lower potential gas than the final 96 h, e.g., $a + b$ should at least be on the size of 96 h. It seems there is no single perfect equation for all plants/treat-

ments (± PEG) and that perhaps no single model fits the different curves perfectly. Therefore, we kept analysing the results of the exponential of France et al. (1993). Fractional fermentation rates at 8, 24, and 48 h differed ($P < 0.05$) between grass and the legumes with or without MIC inclusion. The legumes responded differently to the addition of PEG. When MIC was incubated with PEG, the lag phase was drastically reduced and the potential GP increased by 31%. Moreover, gas increment and fermentation rates had values greater than 100% at all incubation times in this plant with PEG. Addition of PEG to STD increased ($P < 0.05$) lag phase and potential GP values but did not ($P > 0.05$) affect fermentation rate at all measured incubation times.

The values for REL1 and REL2 largely differed among forages (Table 3). REL1 increased ($P < 0.05$) with legumes regardless of PEG addition. Although, MIC had the lowest REL1 value ($P < 0.05$), contrast between grass and legumes with no MIC had higher REL1 for the legumes. STA and LEU had the greatest values (92% on average). Inclusion of PEG resulted in higher REL1 with legumes (0.91 on average) than with grass (0.85) ($P < 0.05$) and also when MIC was not considered. The REL2 (GP96/*A*) values ranged from 63 to 98% and were in the following order: MIC ≤ STA ≤ LEU < STD < CYN. Expectedly, there was no change in REL1 and REL 2 with the addition of PEG for CYN. However, REL2 increased by 35% for MIC + PEG and reduced by 25% for STD + PEG, the other forages did not respond to PEG addition.

DISCUSSION

The nutritive value of forages depends on how efficiently they are solubilised and degraded in the gut and thus, how much energy and nutrients can be delivered to rumen microorganisms and ruminant animals. Based on the chemical composition of the plants, CYN fed as sole forage would not meet the nutrient requirements of growing lambs (NRC, 1985) without a form of supplementation. Contrary to lower fibre values expected from MIC and LEU because they were cut from stem tips of re-grown branches, MIC had aNDFom and ADFom contents higher than expected. Higher fibre and phenolic concentrations of MIC seemed not to be related to plant maturity or phenological stage or these factors were more influenced by soil fertility (Barry and Forss, 1983; Lees et al., 1994), water deficiency, and excessive heat (Reed et al., 1990; Vitti et al., 2005) which may be directly associated with increased concentrations of secondary compounds in plants.

Guimarães-Beelen et al. (2006) investigated the leaves of MIC from a semi-arid site in the northeast of Brazil and observed greater CT (154 vs. 105 g/kg DM) and much lower aNDFom (476 vs. 706 g/kg) concentrations compared with MIC utilized in this study. Different geographic, weather conditions and individual characteristics of the plants and species have their own bearing on the nutritive quality of forages.

Crude protein concentrations in LEU were similar to values reported by Bonsi et al. (1997) and Kaitho et al. (1998) but CT and aNDFom values were greater. A good performance of LEU was observed also by Bonsi et al. (1997) in an *in situ* study with four treatments: LEU + crushed maize, *Sesbania sesban* + crushed maize, cotton meal + crushed maize, and *Eragrotis tef* grass as control. The greatest DM disappearance after 6 and 48 h was obtained with the LEU treatment. The result is in agreement with the high REL1 value of LEU in our study. Also, the lag phase of LEU reported by Bonsi et al. (1997) was similar to the one in our study (2.2 and 2.5 h, respectively).

In vitro studies using tanniferous plants with and without PEG supplementation showed the effect of tannin in reducing the potential GP (Makkar et al., 1995; Getachew et al., 2000). Addition of PEG in STA led to low gas increment at 24 and 48 h with no effect on other parameters. This indicated a weak effect of CT and other phenolic compounds as other factors such as the high ADFom concentration of STA may have restricted GP more than CT. In LEU, tannin-binding by PEG did not affect the kinetic parameters, however it increased total GP.

Of the two *Styzolobium* species, STD contained more CT and TP than STA and its incubation with and without PEG resulted in lower GP after 24 and 48 h. However, the gas increment due to PEG addition was higher for STD than STA which indicated a direct influence of CT in STD. However, when STD was compared with LEU, which had similar CT concentration, lower gas increment for STD suggested that other factors such as greater aNDFom and ADFom contents or their low digestibility may have limited fermentation. Moreover, several authors emphasized that tannin effects should be considered not only by the amount present but also by the size, weight of molecules, and the number

of linkage sites (Kaitho et al., 1998; McNeill et al., 1998; Min et al., 2003; Mueller-Harvey, 2006). Therefore, it is also possible that the types of tannins in STD and LEU were different and thus exerted a differential influence on fermentation, however this requires further studies.

According to France et al. (1993), the fractional fermentation rate (μ) cannot be negative and must satisfy the condition $t \geq$ lag. As MIC had longer lag (10.8 h) than observed time t = 8 h, this condition was not satisfied, which shows the absence of fermentation at 8 h for this legume and thus a negative value for μ. When PEG was supplemented, MIC produced at least two times more gas than CYN at 8, 24, and 48 h. However, even when PEG attached to tannins, MIC had less GP than the other legumes, suggesting a combined action of fibre and phenolic compounds, which did not allow MIC to be well fermented. Vitti et al. (2005) also observed gas increments higher than 100% when they incubated *Anadenanthera macrocarpa* (Angico) and *Astronium urundeuva*, Engl. (Aroeira), plants from northeastern Brazil. Mlambo et al. (2007) incubated tanniferous plants from Zimbabwe using also rumen fluid from unadapted animals and observed 80% gas increment with *Dichrostachys cinerea*.

The lowest REL1 value was observed for MIC when it was incubated without PEG and this may be related to the long lag phase and the low fractional rate during the whole incubation period. The low REL2 values of MIC, STA, and LEU demonstrate that the potential GP plateau was not reached within the 96-h incubation period. These legumes had longer lag phases and lower fractional fermentation rates than the control (CYN) and as such would require a longer turnover time to achieve a larger proportion of their potential. When PEG was added to MIC, REL1 increased as a consequence of the increase of GP at 48 h. The enhancement of REL2 for MIC + PEG reflected increase in the fractional fermentation rates, the potential GP, and the drastic reduction (> 80%) of the lag phase. These changes enabled MIC to increase GP during the 96 h of *in vitro* fermentation. Similar *in situ* results with PEG-supplemented MIC were reported by Guimarães-Beelen et al. (2006), who observed increases of 112% for the degradation rate, 25% for potential degradability, and 32% for effective degradability.

On the other hand, STD + PEG reduced the REL2 due to an increased lag phase, whereas fractional rates did not change and thus the accumulated gas at 96 h was low, not allowing STD to reach the fermentation plateau.

CONCLUSION

With the exception of MIC, all the legumes had better fermentative performance than the tropical grass. However, fermentation was still limited by the presence of CT or fibre fractions or by both. The fermentation of MIC and STD was most likely affected by CT and fibre fractions, i.e., aNDFom and ADFom. On the other hand, LEU had CT as the main limiting factor, whereas performance of STA was low due to the high amount of fibre and its presumably low degradability.

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