Microbial Evaluation of Fodder Tree Leaves as Ruminant Feed

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ABSTRACT : Fermentation of legume fodder tree leaves by rumen microorganisms was evaluated. The substrates were sun-dried, ground leaves. Gas and volatile fatty acid (VFAs) production were estimated. Using gas production as an index of fermentation at 12 h, the leaves tested ranked as follows; *Chamaecytisus palmensis*>*Gliricidia sepium*>*Sesbania sesban*> *Tephrosia bracteolata*>*Leucaena pallida*>*Vernonia amygdalina*>*Acacia sieberiana*>*Sesbania goetzei*>*Acacia angustissima*. Using VFA production, the ranking was as follows; *G. sepium*>*S. sesban*>*S. goetzei*>*L. pallida*>*C. palmensis*/*V. amygdalina*>*T. bracteolata*> *A. sieberiana*>*A. angustissima*. Absolute gas or VFA production rates, were also used to rank the leaves. Extracts (70% acetone) of *A. angustissima* inhibited the growth of *Ruminococcus albus* 8, *R. flavefaciens* FD-1, *Prevotella ruminicola* D3ID and *Streptococcus bovis* JBI while the growth of *Selenomonas ruminantium* D was depressed when 0.6 ml extracts were added. *C. palmensis* water extracts enhanced cellulose hydrolysis by *R. flavefaciens* FD-1. All extracts reduced celluloysis by *R. albus* 8. *R. flavefaciens* FD-1 hydrolyzed more ($p<0.001$) cellulose than *R. albus* 8. (*Asian-Aus. J. Anim. Sci. 1999. Vol. 12, No. 5 : 708-714*)

Key Words : Fodder Trees, Rumen Microorganisms, Gas Production, Volatile Fatty Acids, Acetone Extracts, Cellulose Hydrolysis

INTRODUCTION

In most developing countries ruminants subsist on native pastures and crop residues that are low in nutrients. To maintain high productivity, the nutritive value of these fibrous feedstuffs must be improved. Leguminous fodder tree leaves have been found to be potentially suitable for supplementing basal roughages. However, a number of studies (Nortorn, 1994; Kumar, 1992; Smith, 1992; Woodward and Reed, 1989) showed that some of these leaves contain anti-nutritional factors (ANFs) such as non-protein amino acids, glycosides, polyphenolics, alkaloids, saponins, oxalates etc. that limit their use as animal feed.

Ruminants can feed on plants that are toxic to monogastric animals (Cheeke and Shull, 1985). This ability is attributed to the microorganisms in the rumen that have evolved the capability to detoxify some of the ANFs, and to the natural rumen environment that provides many reductive and hydrolytic reactions which may decrease the toxicity (Kumar, 1992). Adaptation of the animal to diets containing ANFs may result in it developing resistance to some ANFs (Odenyo et al., 1997). However, in several instances, ruminants still die from intoxication by ANFs. It is therefore important to evaluate feedstuffs for ANFs and effects of these ANFs on both the rumen microbes and the host animal itself. Evaluation of feedstuffs with intact animals is extremely costly, therefore there is need for rapid, simple and less costly *in vitro* methods. In this study *in vitro* total gas and volatile fatty acids productions by mixed rumen microorganisms were used to assess various fodder tree leaves for ANFs and degradability. The hypothesis was that any leaves containing ANFs toxic or inhibitory to rumen microbes will result in less or no gas or VFAs production. Effects of acetone (70%) and water extracts of the same leaves on pure cultures of rumen bacteria and on cellulose hydrolysis were also evaluated.

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MATERIALS AND METHODS

Legume fodder leaves and their chemical analysis

Nine plants, *A. angustissima*, *A. sieberiana*, *C. palmensis*, *G. sepium*, *L. pallida*, *S. goetzei*, *S. sesban*, *T. bracteolata*, and *V. amygdalina* were evaluated. These plants were grown at the International Livestock Research Institute at the Debre Zeit station in the highlands of Ethiopia (8°47.28N; 38°59.17E; altitude 1850 M). With the exception of *V. amygdalina*, all the plants were about one year old. Only plant leaves were evaluated in this study. Both fresh and sun dried leaves were used. For the sun dried leaves, the plants were cut, dried and the leaves were shaken off the stems. Dry matter (DM), orgnic matter (OM), and nitrogen (N) were estimated following the procedures of the Association of Official Analytical Chemists (AOAC, 1990). Neutral detergent fibre (NDF) was analyzed according to Van Soest and Robertson (1985). Soluble tannins were analysed according to the methods of Reed et al. (1985). The chemical composition of the tree leaves used is shown in table 1.

Gas production

The method of Theodorou et al. (1994) was used

Table 1. Chemical composition of fodder tree leaves

Fodder trees	g/kg DM	g/kg DM					
		OM	NDF	N	Soluble tannin	S	P
Acacia angustissima	874	913	313	33	243	1	1
A. sieberiana	887	887	411	26	119	NA	NA
Chamaecytisus palmensis	866	939	336	33	129	2	1
Gliricidia sepium	849	925	414	44	70	NA	2
Leucaena pallida	882	886	273	48	213	NA	3
Sesbania goetzei	907	903	369	14	120	NA	3
S. sesban	563	870	167	38	109	2	3
Tephrosia bracteolata	951	911	420	32	97	NA	2
Vernonia amygdalina	926	884	312	24	138	2	1

NA=not available; S=Sulfur; P=Phosphorus.

to measure gas production as an index of fermentation and growth. Approximately 0.5 g of each leaf material, ground through a 0.5 mm sieve, was weighed into 125 ml anaerobic bottles (Phase Separations Ltd. Clwyd, UK). Lucerne (*Medicavo sativa*) was used as a standard feed. Defined medium (Odenyo et al. 1991) was prepared anaerobically according to Bryant (1972) without phenylpropanic acid (PPA) and phenylacetic acid (PAA). About 95 ml of medium was placed into the bottles, stoppered with butyl rubber stoppers, and sealed with aluminium crimp seals (Bellco Glass Inc., Vineland, NJ, USA). The medium was autoclaved at 121℃ for 15 minutes, cooled and stored. Rumen fluid from a rumen cannulated crossbred (*Bos taurus*×*Bos iudicus*) steer fed grass hay and cotton seed cake was collected before the morning feed into a CO_2 pre-gassed flask. The medium bottles were then inoculated with 5 ml rumen fluid using a 5 ml syringe dispenser (Astell Scientifics, Kent, UK). The rumen fluid in the flask was continuously flushed with CO_2 during inoculation. The bottles were then incubated at 39℃ for 120 h. The head space gas readings were recorded as Lkg^{-1} DM at 0, 3, 6, 9, 12, 18, 24, 36, 48, 72, 96 and 120 h using a detachable pressure transducer and LED digital readout voltmeter (Bailey and Mackey Ltd., Birmingham, UK).

Volatile fatty acid (VFA) production

During incubations with mixed rumen samples, three bottles were removed at various time points. The bottles were stored at -10℃ until the end of incubation, when the bottles were removed from the freezer, thawed, opened, the pH of the samples recorded and the samples were then centrifuged and acidified with 25% (w/v) phosphoric acid. VFAs (acetic, propionic, butyric, isobutyric, valeric, isovaleric and isocaproic acid) of chromatographic grade were used as standards according to Supelco specifications (Supelco. Inc., Bellefonte, Crans, Switzerland). A gas liquid chromatogaph (Pye Unicam 304, Cambridge, UK) was used to estimate VFAs in the samples.

Pure culture studies

Ruminococcus albus 8, *R. flaverfaciens* FD-1, *Prevotella ruminicola* D31D, *Selenomonas ruminantium* D and *Streptococcus bovis* JBI were generously donated by Prof. R. I. Mackie, Department of Animal Sciences, University of Illinois, Urbana. Acetone extracts were prepared according to Makkar (1995) from ground dried leaves passed through a 0.5 mm sieve.

Approximately 5 g of leaves from each plant source were weighed into 400 ml beakers and 100 ml 70% aqueous acetone was added. The samples were incubated in a shaking water bath (130 cycles/min) at 30℃ for 2 h, and then centrifuged at 433.4 g at 4℃ for 20 minutes. The supernatant was collected in clean beakers and evaporated in an oven at 35℃. The dried samples were re-suspended in 50 ml sterile distilled water, then filter-sterilized using filter membrane (0.45 μm pore size), and stored at 4℃ until use.

Water extracts were prepared from fresh leaves minced using a blender (Waring Products, New Hartford, Conn. USA) for a total of 10 minutes. Approximately 20 g of each plant leaf sample was weighed into a beaker containing 100 ml sterile H_2O incubated in a water bath at 30℃ for 2 h. All the liquid was squeezed through cheesecloth, centrifuged, and then filter-sterilzed.

Cellobiose medium (Odenyo, 1992) was anaerobically prepared and dispensed (9.5 ml) into Balch tubes, stoppered and sealed. The medium was autoclaved at 121℃ for 15 min. After cooling, 0, 0.1, 0.3, 0.6 ml of extracts from individual plant and 4-6 drops of B-vitamins (Lowe et al., 1981) were added. The tubes were then inoculated individually with 0.5 ml each of *R. albus* 8, *R. flavefaciens* FD-1, *P. ruminicola* D31D, *S. ruminantum* D, and *S. bovis* JBl. The tubes were incubated at 39℃ for 24 h. Optical density (OD) readings at 600 nm were recorded at 0,

Table 2. Cumulative gas production (L kg^{-1} DM) from various fodder tree leaves incubated with mixed rumen microorgainsms

Fodder trees	Incubation time (hours)						
	6	12	24	48	72	96	120
Alfalfa	106	180	234	266	278	282	284
Acacia angustissima	16	62	90	124	142	154	158
A. sieberiana	42	118	170	196	214	222	224
Chamaecytisus palmensis	84	204	268	302	334	358	374
Gliricidia sepium	72	170	224	248	272	264	278
Leucaena pallida	48	154	206	232	250	264	278
Sesbania goetzei	28	96	138	154	172	190	204
S. sesban	70	164	206	236	264	286	300
Tephrosia bracteolata	68	156	204	228	252	270	284
Vernonia amygdalina	46	136	196	224	248	266	280
SEM*	7.3	14.2	17.0	17.4	18.9	20.2	21.1
Statistical significance	***	***	***	***	***	***	***

*** $p<0.001$; * SEM=Standard error of the mean.

2, 4, 6, 8, 10, 12 and 24 h to monitor the bacterial growth using a Spectronic 20 (Beckman Instruments, Geneva, Switzerland) spectrophotometer.

Effect of leaf extracts on cellulose hydrolysis

Extracts of plant leaves that had no adverse effect on pure culture of rumen bacteria were used in this study. Cellulose medium (Odenyo, 1992) containing 0.12% acid swollen cellulose (ASC) was used as substrate. Extracts (0.3, 0.6 ml) and vitamin B complex (4-6 drops) were added to the medium. The tubes were then inoculated with 0.5 ml cultures ($OD_{600\ nm}$=0.8) of *R. albus* 8 and *R. flavefaciens* FD-1. The samples were then incubated at 39℃ for 14 d. Cellulose (relative dry matter) disappearance was determined according to Odenyo et al. (1991). At the end of incubation, the supernatant was aspirated, the cellulose residue washed five times with distilled water. The tubes with the residue were then dried at 100℃ overnight cooled in a desiccator and weighed. The weight of the residue was subtracted from the original weight of the cellulose to obtain the amount of cellulose hydrolyzed. Non- inoculated samples were treated the same way. The amount of cellulose hydrolyzed by the bacteria was recorded as g kg^{-1}.

Statistical design and analysis

A completely randomized design was used. The data were subjected to an analysis of variance procedure using a repeated measures analysis of variance available in SAS (SAS, 1987). There were three replicates for each time point. The experiment was replicated twice.

RESULTS

Non-cumulative gas production from leaf material was highest at 12 h, and was significantly ($p<0.01$) different among plants. Gas production from Lucerne was 106, 180, 234, 266, 278, 282, and 284 L kg^{-1} for 6, 12, 24, 48, 72, 96, and 120 h, respectively. The highest gas production at 12 h was from *C. palmensis* sample, while the lowest was from *A. angustissima* (table 2).

There were significant ($p<0.01$) differences in VFA concentrations among leaves tested at all incubation periods. The highest total VFA concentration at 12 h was from samples containing *G. sepium* and *S. sesban* leaves, while the lowest was from samples containing *A. angustissima*. *Leucaena pallida* samples had the highest concentration of VFA at 48 h while the lowest was from *A. angustissima* (table 3).

Table 3. Acetate, propionate, and total volatile fatty acids production ($Mm1^{-1}$) after 48 h of incubation of various fodder tree leaves with mixed rumen microorganisms

Fodder trees	Acetate	Propionate	Total VFA
Acacia angustissima	6	6	14
A. sieberiana	14	3	20
Chamaecytisus palmensis	28	11	44
Gliricidia sepium	32	19	61
Leucaena pallida	39	22	69
Sesbania goetzei	24	14	45
S. sesban	28	19	56
Tephrosia bracteolata	31	12	50
Vernonia amygdalina	31	10	48
Statistical significance	***	***	***

*** $p<0.001$.

Gas or VFA production at 12 h was used to rank plant leaves. Ranking by gas production was as follows; *C. palmensis*>*G. sepium*>*S. sesban*>*T. bracteolata*/*L. pallida*>*V. amygdalina*>*A. sieberiana*>*S.*

goetzei>A. angustissima. Using VFA production, the ranking was as follows; *G. sepium>S. sesban>S. goetzei>L. pallida>C. palmensis/V. amygdalina >T. bracteolata>A. sieberiana>A. angustissima.*

The plant leaves were also ranked using cumulative or absolute gas (Bcg) (Siaw et al., 1993) or total VFA production (BC-VFA) rates and an index of the product of the two (Bcg*BC-VFA) (table 4).

Table 4. Rankings of 9 fodder tree leaves (1=lowest, 9=highest) on the basis of absolute gas production rate (Bcg), absolute volatile fatty acid produciton rate (BC-VFA) an index of both (Bcg*BC-VFA), neutral detergent soluble (NDS), and soluble tannin contents

Fodder trees	Rankings				
	Bcg	BC-VFA	Bcg* BC-VFA	NDS	Soluble tannin
Acacia angustissima	1	2	1	6	9
A. sieberiana	3	1	2	3	4
Chamaecytisus palmensis	9	4	6	5	6
Gliricidia sepium	8	9	9	2	1
Leucaena pallida	5	5	7	8	8
Sesbania goetzei	2	7	3	4	5
S. sesban	7	8	8	9	3
Tephrosia bracteolata	6	3	4	1	2
Vernonia amygdalina	4	6	5	7	7

*** p<0.001; * SEM=Standard error of the mean.

Effects of acetone extracts from fodder tree leaves on the growth of pure cultures of rumen bacteria are depicted in table 5. The results indicated that at a level of 0.3 ml, extracts of *A. angustissima* leaves inhibited the growth of *R. flavefaciens* FD-1 and *P. ruminicola* D3ID.

Ruminococcus albus 8 and *S. bovis* were inhibited when 0.6 ml of Angustissima was added while *S. ruminantium* D was not inhibited at any of the two levels tested. At the level of 0.6 ml acetone extracts, *L. pallida* slowed the growth of *R. flavefaciens* FD-1, while the other bacteria were not significantly affected at any level. Extracts of *S. goetzei* leaves at the level of 0.6 ml suppressed the growth of *R. flavefaciens* FD-1. The rest of the extracts either did not have any effect or enhanced the growth of the bacteria tested. All water extracts had no effect on the growth of any bacteria. Cellulose (with or without added leaf extracts) hydrolysis by *R. albus* 8 was considerably lower ($p<0.001$) than by R. flavefaciens FD-1 (table 6). Acetone extract of L. pallida (0.6 ml) significantly ($p<0.01$) depressed cellulose hydrolysis by *R. flavefaciens* FD-1, while other extracts either depressed or enhanced the hydrolysis. Water extracts from *C. palmensis* leaves, enhanced cellulose hydrolysis by *R. flavefaciens* FD-1. However, addition of any of the extracts reduced cellulose hydrolysis by *R. albus* 8 (table 6).

Table 5. Effect of addition of crude acetone extracts of fodder tree leaves to cellobiose medium on the growth of rumen bacteria

MPT extracts (ML)		*Ruminococcus albus* 8	*Ruminococcus flavefaciens* FD-1	*Prevotella ruminicola* D3ID	*Selenomonas ruminantium* D	*Streptococcus bovis* JBI
Acacia angustissima	0.3	(+)	-	-	+	+
	0.6	-	-	-	(+)	-
Acacia saligna	0.3	+	+	+	+	+
	0.6	+	+	+	+	+
A. sieberiana	0.3	+	+	+	+	+
	0.6	+	+	+	+	+
Chamaecytisus palmensis	0.3	+	+	+	+	+
	0.6	+	+	+	+	+
Gliricidia sepium	0.3	+	+	+	+	+
	0.6	+	+	+	+	+
Leucaena pallida	0.3	+	+	+	+	+
	0.6	+	(+)	+	+	+
S. goetzei	0.3	+	(+)	+	+	+
	0.6	+	(+)	+	+	+
S. sesban	0.3	+	+	+	+	+
	0.6	+	+	+	+	+
Tephrosia bracteolata	0.3	+	+	+	+	+
	0.6	+	+	+	+	+
Vernonia amygdalina	0.3	+	+	+	+	+
	0.6	+	+	+	+	+

The growth was measured trubidimetrically at 600 nm wavelength.
- No growth, (+) Growth stimulation weak but significant, + Good growth.

Table 6. Cellulose disappearance (g kg^{-1}) when water extracts or acetone extracts from various plants were added and incubated with *Ruminococcus albus* 8 or *R. flavefaciens* FD-1 for 14 d

Treatment		Disappearance ($g.kg^{-1}$)	
Plant	Level (ml)	*R. albus* 8	*R. favefaciens* FD-1
Water extracts			
None		485±70	950±8
C. plamensis	0.3	194±17	970±9
	0.6	284±225	993±1
G. sepium	0.3	178±143	887±99
	0.6	399±82	934±9
L. pallida	0.3	287±135	978±15
	0.6	255±185	955±43
S. sesban	0.3	118±6	955±2
	0.6	309±15	943±2
T. bracteolata	0.3	35±15	904±130
	0.6	249±98	864±127
Acetone extracts			
C. palmensis	0.3	235±74	942±5
	0.6	241±26	877±72
G. sepium	0.3	279±84	885±62
	0.6	188±9	940±22
L. pallida	0.3	243±50	938±12
	0.6	268±52	629±75
S. sesban	0.3	335±126	900±29
	0.6	249±17	900±23
T. bracteolata	0.3	217±20	908±35
	0.6	317±5	884±58

± = Standard deviation.

DISCUSSION

Ruminants are dependent on the miciobial population in the rumen to degrade complex feedstuffs into characteristic end products, which in turn provide nutrients for the host animal. Gas and VFA are major products of fermentation in the rumen (Leng, 1970).

The concentrations of these products may therefore serve as indices of fermentation of feed substrates and growth of rumen microorgmisms. Rumen micro-organisms can only grow on those substrates, which they can degrade, transport and ferment (Russell,1985). Increases in gas and VFA production are indications of the feed material being free of ANFs and capable of supplying sufficient nutrients for the microbial ecosystem in the rumen. Both gas and VFA production can therefore be used to rank feedstuff in terms of their degradability. In this study, gas and VFA production at 12 h were used to rank plant leaves since this would be more appropriate for quick assessment in a farm situation. In the assessment, using gas or VFA production did not give similar rankings, however, the rankings were very close especially with regard to the poorly degraded leaves. Increase in gas production from plant materials indicates absence of ANFs and availability of higher concentrations of soluble carbohydrates, protein and minerals. Using gas production, *C. palmensis* was ranked first even when compared to more readily degraded materials such as Lucerne. This was not surprising. Analysis of the chemical composition of the plant leaves tested showed that *C. palmensis* contains less inhibitory substances such as tannins and high concentrations of Neutral Detergent Solubles (NDS). Recent studies using goat rumen fluid showed that *C. palmensis* had in vitro digestibility of 81% (Osuji and Odenyo, unpublished data).

Ranking using an index of the product of absolute gas and VFA production rates did not give similar rankings as either gas or VFA production except for *A. angustissima*, *G. sepium*, and *S. sesban*. Ranking based on soluble tannins showed that *A. angustissima* and *L. pallida* ranked highest. However, both gas and VFA production were markedly different between the two fodder tree leaves. This may suggest that the higher gas and VFA production by *L. pallida* may be due to higher NDS content and possibly lower hydrolyzable tannin proportion in the soluble tannin fraction. Conversely the relatively low gas and VFA production from *A. angustissima* may suggest a greater proportion of hydrolyzable tannins in the soluble tannin fraction. Several reports (McSweeney et al., 1988; Kumar, 1992; Giner-Chavez, 1996) indicate that the hydrolyzable tannin fractions are responsible for varied toxic manifestations in the rumen.

This study demonstrated that a single criterion cannot be used to effectively rank fodder trees and also that many factors must be considered. However, gas and VFA production are still very useful and quick methods and would be most beneficial when used together. It was noted that more accuracy might have been achieved by using lower concentrations of the substrate than was used in this study. When higher substrate concentrations are used, results may not be accurate due to higher concentrations of possible soluble carbohydrates. These carbohydrates may be fermented much faster resulting in accumulation of inhibitory end products and subsequently stoppage of fermentation (Hungate, 1966).

It was, however, clear that some plant loaves, particularly the acacias, contained ANFs that interfered with their fermentation by both mixed and pure cultures of numen micmorguusms. *A. angustissima* was particularly toxic to cellutolylic species. Acacias have been shown to contain many ANFs such as tannins, cyanogens, oxalates and alkaloids (Norton, 1994; Kumar, 1992). Water extracts from all leaves had no effect on microbial growth, implying that the toxic

compounds were either not water soluble or were too dilute in water extracts to have detectable toxic effect.

This study also suggested that *S. ruminantium* was more tolerant to toxic principle(s) in *A. angustissima* than other bacteria tested. This implies that *S. ruminantium* is either tolerant to the toxic principles or may have some enzymes capable of detoxifying these toxic principles, and therefore may play an important role in feeding systems involving the use of this plant. Simpson et al. (1969) had shown that various strains of *Selenomonas* and *Butyrivibrio* were able to hydrolyze the glycoside bond and ferment sugars from phenolic compounds but were not able to degrade the hetero- cyclic ring.

Results from this study suggested that *G. sepium, S. sesban, L. pallida. C, palmensis,* and *V. amygdalina* were not toxic to rumen bacteria and therefore readily degraded. Extracts from these plant leaves were also not inhibitory to most pure cultures of rumen bacteria. The effect of extracts from these plants on cellulolysis was therefore evaluated to determine their possible contribution to hydrolysis of cellulosic feeds. The results of the cellulose studies showed that water extracts of *C. palmensis* enhanced cellulose hydrolysis by *R. flavefaciens* FD-1 . This study showed that *C. palmensis* is easily fermented and contains low levels of inhibitory substances such as tannin compared with the other plant leaves evaluated. These results therefore suggested that *C. palmensis* may have a potential contribution in fibre degradation in the rumen, especially when the supplement is fed fresh. Acetone extracts of *L. pallida* at 0.6 ml suppressed cellulolysis by *R. flavefaciens* FD-1, one of the highly cellulolytic rumen bacteria. Calculations using a sheep of an average body weight of 25 kg and a daily DM intake of 750 g d^{-1} suggested that when as little as 4.5 g d^{-1} of *L. pallida* are consumed cellulolysis may be inhibited. The suppression of cellulolysis by *L. pallida* acetone extract would have important significance in feeding systems based on low quality forages. The use of *L. pallida* as a supplement aims at supplying additional N and minerals to enhance bacterial growth and therefore cellulolysis. If *L. pallida* inhibits cellulolysis at such low levels of intake, the potential gain from using it as an additional N source will not be realized.

CONCLUSION

It Is concluded that in vitro gas production is appropriate for quick assessment of the presence of ANFs and feed degradability. However, the index (absolute gas * VFA production rates) may be a better indicator for ranking. Using all the rankings, with the exception of absolute VFA producton rate, *A. angustissima* appeared to be the most inhibitory to rumen microorganisms. *G. sepium* and *S. sesban* appeared to be least toxic while *C. palmensis* was the only MPT that enhanced cellulose hydrolysis.

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