Output 1: Grass and legume genotypes with high forage quality attributes are developed

1.1 Selection of *Brachiaria* genotypes for high quality

Highlight

- In vitro dry matter digestibility (IVDMD) values for individual *Brachiaria* hybrids ranged from 48 to 63% over three sampling dates, while crude protein (CP) values ranged from 12 to 25%. Hence, we reconfirm the ample variation for these two important forage quality parameters for future genetic improvement

1.1.1 Screening of sexual and apomictic *Brachiaria* hybrids for digestibility and protein

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Rationale

One of the outstanding attributes of the first two hybrid *Brachiaria* cultivars (Mulato and Mulato II) is their very high nutritive quality, which leads to outstanding animal productivity. Hoping to maintain or improve forage quality in future commercial hybrids, special attention is required to monitor quality attributes (crude protein and dry matter digestibility), particularly in our synthetic, tetraploid breeding population where genetic gain can be achieved by selection.

Materials and Methods

Vegetative propagules of 209 BR05 and RZ05 hybrids were delivered to the Forage Quality laboratory in April 2006. Individual plants were propagated vegetatively and transplanted to single-plant experimental units in a 3-replicate, space planted (1.00 x 0.75 m) field experiment on 07 June 2006. Plants were cut to a uniform height on 27 July. Individual plants were sampled three times, at 6-wk intervals, on 07 September, 19 October, and 30 November 2006. Urea was applied at a rate of 1 gm per plant following each sampling harvest. Approximately 23-25% of plants failed to establish or died and could not be sampled. On each of the three sampling dates, 300-gm samples of leaf tissue were taken, dried at 45^{o}C for 48 hours, and ground to pass through a 1 mm screen. Dried, ground leaf tissue samples were analyzed in a NIRS System 6500 using software ISISCAN (IS-2250) version 2.71, Win ISI-III IS-1485.

Results and Discussion

Sampling date was by far the major source of variation. Genotypes differed at each sampling date and over sampling dates. Differences among genotypes were on the order of 15 percentage points for IVDMD and 12 percentage points for CP. Ten hybrids had IVDMD $\geq$ 59% with CP in excess of 14%.

Mean squares for genotypes were 2.9 times or 4.7 times larger than the mean squares for the genotype-sampling date interaction for IVDMD or CP, respectively. Correlations of genotype values between sampling dates were positive, but of only moderate magnitude: on the order of 0.3 to 0.4 for IVDMD, and on the order of 0.3 to 0.6 for CP. These results suggest that there is ample variability for genetic improvement in forage quality parameters in the *Brachiaria* breeding populations. While the digestibility and protein data reported were determined on sexual-by-apomictic hybrids, breeding progress will (or will not) be made in our synthetic tetraploid sexual breeding population. The logistics of obtaining reliable forage quality data on candidate sexual clones in an opportune manner needs to be worked out.

1.2 Forage quality of promising grasses and legumes

Highlights

- Feeding *Canavalia brasiliensis* to sheep as a supplement to a low quality grass resulted in a linear increase in dry matter intake as level of the legume offered increased

- Rumen degradability of tropical legume fibers is highly variable and at least as important to plant quality as secondary plant metabolite (tannin, saponin) content.

1.2.1 Feed value of *Canavalia brasiliensis* fed to sheep

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Rationale

The evaluation of herbaceous legumes with farmer participation in hillsides of Central America resulted in the selection of *Canavalia brasiliensis* (Canavalia) given its high biomass production and dry season tolerance. On farm evaluation of Canavalia demonstrated that it was an excellent cover legume and that when sown at the end of the dry season with maize resulted in higher grain yield in the subsequent rainy season. Anecdotal evidence suggested that Canavalia was well accepted by grazing cows in the dry season. However, given the presence of a toxic amino acid (canavalin) in Canavalia species, known to affect monogastric animals, there was concern that cows consuming the legume could exhibit some form of toxicity leading to mortality.

To investigate the feed value of Canavalia as a supplement to low quality grasses and to define if animals consuming the legume showed toxicity problems a feeding trial with sheep was carried out. Results of the feeding trial are reported in this section.

Materials and Methods

In the Quilichao research station 10 African hair sheep grouped as light (5) and heavy (5) were assigned to 5 treatments in a replicated 5 X 5 Latin Square design.

The feeding trial had duration of 70 days, divided in 5 experimental periods (14 days/ period). Each period in turn was divided in 7 days for adjustment and 7 days for measurements. Animals were housed in metabolism crates and offered sun dried *Canavalia brasiliensis* and a low quality grass (*Brachiaria humidicola*) as a basal diet in the following arrangement of treatments:

T1: Canavalia 100%
T2: 25% Canavalia + 75% Grass
T3: 50% Canavalia + 50% Grass
T4: 75% Canavalia + 25% Grass
T5: 100% Grass

Each animal received a total of 80 g of DM /kg of $BW^{0.75}$ daily in two rations AM and PM. The legume was offered alone from 8 to 10 AM and from 1 to 3PM. After the end of the two hour period the legume not consumed was measured and animals were offered the grass basal diet. In addition all animals received water and a mineral mix ad lib. Forage consumed by each animal daily was calculated by difference of grass and legume offered and grass and legume refused.

Animals were fitted with fecal collection bags on day 6 of the adjustment period. Feces from each animal were weighed from day 7 to day 14 of the measurement period. A 10% aliquot was taken from each animal and stored in a freezer for subsequent freeze drying and chemical analysis.

Laboratory measurements in freeze dried feed offered and refused and in feces included DM, OM, NDF, ADF, IVDMD, CP (N x 6.25) and minerals (P, Ca, M, K and S).

Daily intake of total DM and of grass and legume were calculated by difference of forage offered and forage refused. Digestibility of DM was calculated as follows: DM Digestibility = DM consumed (g) – DM excreted in feces (g)/ DM consumed (g).

The data was subject to an analysis of variance with animal group (heavy and light), period, animal (group), Treatment (group) and Group x Treatment as sources of variation. Given that one animal died (not related to the diet received) the SAS GLM procedure was used.

Results and Discussion

As expected the CP content of Canavalia (17%) offered was higher than in the grass (7 %). The levels of P and Ca were also higher in Canavalia (0.26 and 1.9%) than in the grass (0.09 and 0.22%). The fiber content (NDF) was lower in Canavalia (59.7%) than in the grass (83%), which did not result in higher IVDMD in the legume (61 %) as compared to the grass offered (59%).

Total intake of DM was higher for the light (31.6 g/ $BW^{0.75}$) than for the heavy sheep group (31.6 g / $BW^{0.75}$). However more interesting was that DM intake increased linearly regardless of weight of the animals as the proportion of Canavalia increased in the forage offered (Table 1).

However, results showed that DM digestibility did not differ among treatments as shown in Table 1. These results are consistent with the in vitro digestibility values recorded for Canavalia and for the grass.

One important reason for running this feeding experiment was to define if the forage of *Canavalia brasiliensis* produced toxicity symptoms when feed to sheep alone or in mixture with a low quality grass. To define if Canavalia had any effect on liver function we measured two enzymes (GGT- Gamma Glutamil Transferase and ASAT- Aspartate amino transferase). Our results showed that GGT ranged from 13 to 22.0 with an average of 17 U/ L (reference values 22 – 44 U/l).

In the case of ASAT the values recorded ranged from 38 to 97 with an average of 65 U/L (reference values 49 -123 U/L). Thus it would seem that short term intake of *Canavalia brasiliensis* did not result in any apparent liver damage. Through out the trial light or heavy sheep did not exhibit any strange behavior or toxicity symptom.

From the results of the feeding experiment we conclude that the *Canavalia brasiliensis* fed to sheep as a protein supplement resulted in a 5 to 19% increase in dry matter intake as the legume supplement increased from 25 to 75% of the forage offered. In addition, it would seem that forage of Canavalia does not produce toxic effects on sheep at least in short periods of time (70 + days).

Table 1. Intake and digestibility of *Canavalia brasiliensis* alone and as a supplement to a low quality grass.

Treatments	Intake (g DM/kg $BW^{0.75}$)	Digestibility (%)
Grass 100%	29.7 c	56.3
25% Canavalia + 75% Grass	31.1 c	57.5
50% Canavalia + 50% Grass	33.5 b	55.6
75% Canavalia + 25% Grass	35.4 a	56.3
Canavalia 100%	36.6 a	57.5

1.2.2 In vitro degradability and methane production with purified fiber from legumes with and without tannins using the Rumen Simulation Technique (Rusitec)

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Rationale

Fiber content and degradability are key factors in determining forage quality. Ruminants in tropical regions derive a major portion of their energy from the fiber fraction of forages. The low rumen digestibility of tanniniferous legumes has been mostly associated with their high tannin contents. The present experiment was conducted to better define the role of fiber quality of two tanniniferous shrub legumes (*Calliandra calothyrsus* and *Flemingia macrophylla*) on ruminal fermentation of legumes with tannins.

Material and Methods

Plant material of *Calliandra calothyrsus* (CIAT 22310) and *Flemingia macrophylla* (CIAT 21083) was harvested manually eight weeks after a uniformization cut. The material was immediately stored at 20°C and subsequently freeze dried. For *Vigna unguiculata* (cowpea; CIAT 391) eight week old herbage (the whole plant, before flowering) was harvested and sun dried for three days. The tropical low quality grass *Brachiaria humidicola* (CIAT 6133; formerly *B. dictyoneura*) was cut after a growing period of 12 weeks and sun dried for two days. The dried plant material of all species was ground in a laboratory mill using a 5 mm screen.

The material harvested was analyzed for dry- and organic matter, nitrogen, fiber, lignin and ruminal degradability. The N associated to fiber (NDF-N) was measured. Fiber content of *B. humidicola, C. calothyrsus, F. macrophylla* and *V. unguiculata* was determined with the detergent method. In the case of fiber determination in Calliandra and Flemingia sodium sulfite was added to the detergent solution.

The fiber used in the in vitro experiment was obtained applying a slightly modified detergent method. The amount of plant material used to extract NDF was 5 g placed in 250 ml of detergent solution instead of 0.5 g in 100 ml solution. The detergent was removed by intensive washings with water and ethanol 70-95% in 20 to 25 washing steps, till the detergent could not be perceived not by smell nor flavor and the fiber did feel fluffy and not sticky anymore. The isolated fiber fraction was analyzed for the same parameters as described above for the entire plant material.

Employing the in vitro rumen simulation technique (RUSITEC) fiber extracted from the three legumes was evaluated for its rumen degradability during 2×10 days periods with two replications of each treatment per period (n=4). The first four days served for adaptation of microbes to the fermentation substrates and the following 6 days for data and sample collection. As a protein source for the fermentation media, 3 g of casein were added per day.

The daily dry matter supply to the fermenters was maintained constant at 10 g fiber + 3 g casein. Gas production and methane concentration were determined daily. Substrates and solid fermentation residues were analyzed for organic matter, N, neutral detergent fiber (NDF), and acid detergent fiber (ADF). Data were statistically analyzed with an ANOVA using the GLM procedure in SAS.

Results and Discussion

Chemical composition of the tested plants. The chemical analysis of the test plants showed large differences in fiber composition and N bound to fiber (Table 2). The grass Brachiaria had by far the highest NDF value of all tested

plants. Of the legumes Flemingia had the highest NDF content (590 mg/g) followed by Vigna (530 mg/g) and Calliandra (358 mg/g). Lignin content was highest in Flemingia (154mg/g) followed by Calliandra (99 mg/g), Vigna (82 mg/g) and Brachiaria (56 mg/g).

Indigestible fiber was highest in Flemingia with 508 mg/g that corresponds to 86% of the total fiber content. In Calliandra the indigestible fiber content was 273 mg/g, corresponding to 76% of the total fiber content of the plant (Table 2). In Vigna and Brachiaria the indigestible fiber was only 170 and 192 mg/g (32% and 25% of total fiber), respectively. The hemicelluloses fraction was highest in Brachiaria followed by Vigna, Flemingia and Calliandra. Relatively to the total fiber content hemicellulose proportion in Flemingia was lower than in Calliandra, Brachiaria and Vigna (Table 2). With the fiber purification procedure used only 90-95% purity of NDF could be achieved. As we could not identify the impurities, the purified fiber was treated as homogeneous fraction.

The N bound to fiber in the plants was 59% in Brachiaria, 56% in Flemingia and 37 and 36% for Vigna and Calliandra (Table 2). Previous Rusitec trials had shown that the availability of this NDF-N for the rumen microorganisms was not sufficient to cover their needs. Thus casein was added to the medium since it is considered a good source of N for rumen microbes.

Table 2. Chemical composition of grass and legume species and their purified fibers (mg/g).

	Brachiaria		Vigna		Calliandra		Flemingia	
	plant	fiber	plant	fiber	plant	fiber	plant	Fiber
Nitrogen content	7.03	5.81	24.76	19.02	28.65	26.41	28.65	28.74
NDF-N in the plant	4.14	-	9.09	-	16.06	-	16.06	-
Not NDF-N in the plant	2.89	-	15.67	-	12.59	-	12.59	-
Neutral Detergent Fiber (NDF)	754	944	530	901	590	909	590	947
Acid Detergent Fiber (ADF)	386	468	309	507	406	547	406	636
Acid Detergent Lignin (ADL)	56	56	82	103	154	167	154	243
Hemicelluloses	368	476	221	394	184	362	184	311
Celluloses	330	412	227	404	252	380	252	393
Indigestible ADF (IADF)	192	203	170	327	508	409	508	584
Extractable condensed tannins	nd	nd	nd	nd	95	0	95	0
Bound condensed tannins	nd	nd	nd	nd	10	0	10	0.3
IVDMD	659	620	767	595	388	462	388	349

Fiber degradation and methane production. The in vitro dry matter degradation (DMD) and the degradation of organic matter (OMD) and neutral detergent fiber (NDF) differed ($P<0.05$) between all purified fibers tested. Vigna showed always the highest degradability followed by Brachiaria, Calliandra and Flemingia. Acid detergent fiber degradation was highest in Calliandra and Brachiaria and by far lowest ($P<0.05$) in Flemingia. Hemicelluloses degradation was highest ($P<0.05$) in Vigna, followed by Brachiaria, Flemingia and Calliandra. Celluloses degradation was lowest ($P<0.05$) in Flemingia and highest in Calliandra and Brachiaria.

In general, results in fiber degradation in vitro showed clear differences among legumes in fiber quality. The tanniniferous species had overall less digestible fibers than Vigna or Brachiaria. Legumes with tannins also showed particularly much lower digestibility of the hemicelluloses fraction.

Methane emission expressed as ml/d as well as methane emitted per unit of degraded organic matter differed ($P<0.05$) among species and was highest with Vigna, followed by Brachiaria, Calliandra and Flemingia (Table 3). Methane produced per unit of NDF did not differ between Brachiaria and Calliandra, and was highest with Vigna and lowest with Flemingia. These results indicate that the reduction in methane observed in other in vitro experiments and in an in vivo experiment reported in a different section when legumes with tannins were used to supplement low quality grasses was due not to the tannins per se affecting rumen methanogenic bacteria but rather to the very low degradability of the fiber of such legumes.

Table 3. Methane emission from the fermentation of purified fiber of different plant materials in an in vitro Rumen simulation system (RUSITEC) [ml/g].

	Brachiaria	Vigna	Calliandra	Flemingia
Methane [ml/d]	68b	120a	45c	13d
Methane/degraded OM [ml/g]	29.7b	38.2a	23.4c	11.8d
Methane/degraded NDF [ml/g]	25.2b	38.0a	20.1b	7.9c
Methane/degraded ADF [ml/g]	65b	119a	36c	19c
Methane/degraded Hemicelluloses [ml/g]	24.4b	81.1a	18.7b	6.5c
Methane/degraded Celluloses [ml/g]	74b	149a	51bc	30c

1.3 Utility of mixtures of legumes with and without tannins for ruminants

Highlights

- The cellulolytic bacteria *Ruminococcus flavefaciens* was not affected by tannins extracted from legumes which was not the case for *Fibrobacter succinogenes*.
- Mixing of legumes with and without tannins for silage production did not decrease nitrogen losses during storage but decreased ruminal degradability of protein which in turn could result in a more efficient utilization of nitrogen.
- Feeding tanniferous legumes to sheep as a supplement to a low quality grass decreased methane production as a result of reduced ruminal fiber degradation and not by adverse action on methanogenic microbes.
- Confirmed the high value of *V. unguiculata* (cowpea) as supplement for dual purpose cows grazing low quality pastures during the dry season as milk production increased with increasing proportion of the legume in the supplement.
- The feed value of a legume supplement based on *C. calothyrsus* high in tannins can be significantly improved by including a small proportion (e.g. 1/3) of *V. unguiculata* (cowpea) in the mixture.

1.3.1 Effect of tanniniferous forages Leucaena and Desmodium on rumen microbial populations

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Rationale

The composition of the rumen microflora can be affected by many factors one of them being the nature of the diet. A diet with high proportion of fiber tends to favor the growth of cellulolitic bacteria such as *Fibrobacter succinogenes* and *Ruminococus flavefaciens*. Thus it is possible that the inclusion in the diet of ruminants of tropical shrub legumes could favor the growth of cellulotic bacteria given their relative high fiber content. However, it is also possible that condensed tannins (CT) present in some shrub legumes could limit the availability of protein and energy needed for bacterial growth.

The present experiment was conducted to assess the effect of adding tannins extracted from *Leucaena leucocephala* and *Desmodium heterocarpum* (ovalifolium) on microbial ecology and in vitro fermentation of alfalfa.

Materials and Methods

A series of *in vitro* fermentations were carried out with rumen fluid and the addition of tannins (CT) extracted from *Leucaena leucocephala* **(**Leucaena) and *Desmodium heterocarpum* (Desmodium). Volatile fatty acid (VFA) production (mM), gas production (ml), and the relative abundance of the cellulolytic species *Fibrobacter succinogenes* and *Ruminococcus flavefaciens* by qRT-PCR were measured.

The effects of purified CT were evaluated after 24 hours of in vitro fermentation. The fermentation took place in 50 ml bottles following standard protocols. Each bottle contained 100 mg of dried and ground alfalfa, 10 mg of CT from Leucaena or Desmodium and 10 ml of a buffer solution (20% of rumen liquor and 80% of growth medium).

Results and Discussion

The CT of Leucaena or Desmodium did not cause negative effects on the abundance of *Ruminococcus flavefaciens*. In contrast numbers of *Fibrobacter succinogenes* decreased ($P < 0.05$) after treatment with CT from Leucaena and Desmodium (Table 4).

Table 4. Effect of CT from tanniniferous forages on the abundance of cellulolytic rumen microbes *F. succinogenes* and *R. Flavefaciens* ($2^{-\Delta\Delta Ct}$).

Time (h)	Treatment	*Fibrobacter*	*Ruminococcus*
0	Control	1	1
	Leucaena	0.519 a	2.64 a
	Desmodium	0.298 b	2.61 a
12	Control	1	1
	Leucaena	0.595 a	4.67 a
	Desmodium	0.221 b	4.53 a
24	Control	1	1
	Leucaena	0.266 a	3.08 b
	Desmodium	0.054 b	32.76 a

Different letters show significant differences P< 0.05

It is possible that the presence of glycocalix that is associated to cell-wall topology and chemical composition of Gram + celluloytic rumen microbes (i.e. *Ruminococcus flavefaciens*) protect them from the negative effects of CT. The mechanisms by which CT of the two tanniniferous forages increased ($P < 0.05$) numbers of *Ruminococcus flavefaciens* remain unknown. Until now there is no evidence that rumen bacteria use tannins as a carbon source. Gas production was significantly ($P<0.05$) higher when CT from Leucaena were added as compared to CT from Desmodium.

Degradability of the substrate was correlated to gas production in presence of CT of both tanniniferous legumes. Other researchers also found a negative effect of CT from *D. ovalifolium* on IVDMD degradability. Total VFA production decreased in presence of tannins extracted from Desmodium, although a positive effect was found on propionate accumulation (Table 5).

Favoring propionate production by using tannins of some tropical forage would offer an optimal scenario in the energy balance of rumen function. As found by other researchers, the CT of Leucaena increased ($P < 0.05$) VFA production as compared to the control or to the treatment with Desmodium. In summary, our results indicate that the cellulolytic bacteria *Ruminococcus flavefaciens* was not affect by tannins extracted from legumes which was not the case for *Fibrobacter succinogenes*. These results are interesting since they suggest that different species from the rumen cellulolytic population would show different tolerance to tannins present in tropical forages.

Table 5. Effect of CT of *D. ovalifolium* and *L. leucocephala* on volatile fatty acids (VFA) production after 24h of fermentation.

		Volatile Fatty Acids (nM)					
Time (h)	Treatments	Acetate	Propionate	Butyrate	Isobutyrate	Total	Ratio
1	Control	16.08 c	6.91 a	3.38 a	0.33 a	26.70 b	2.33 c
	Leucaena	20.37 a	6.63 b	2.47 b	0.17 b	29.64 a	3.07 b
	Desmodium	17.29 b	4.61 c	1.98 c	0.21 b	24.09 c	3.76 a
12	Control	23.50 c	9.74 b	4.24 b	0.89 a	38.36 b	2.42 c
	Leucaena	26.06 b	6.84 c	4.38 b	0.34 c	37.62 c	3.81 a
	Desmodium	30.76 a	10.57 a	6.93 a	0.52 b	48.78 a	2.91 b
24	Control	39.57 a	13.11 a	6.41 b	1.07	60.17 a	3.02 c
	Leucaena	39.67 a	10.29 b	5.65 c	1.08	56.70 b	3.85 a
	Desmodium	33.88 b	10.4 b	7.40 a	1.09	52.78 c	3.26 b

Different letters show significant differences $P< 0.05$

1.3.2 Nutritional value of dried or ensiled mixtures of legumes with contrasting tannin contents

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Rationale

Forage conservation represents an interesting alternative for dry season supplementation of ruminant livestock in the tropics. The Tropical Grass and Legume Project of CIAT has developed alternative technologies such as the "bag silage" that are appropriate for livestock

systems operated by smallholders. However, one important constraint of silage preparation is the loss of nitrogen during the fermentation process, particularly when legumes with high concentrations of soluble nitrogen are conserved.

Since tannins which are present in many tropical forage legumes protect proteins form microbial degradation, mixing legumes with and without tannins could contribute to reduce N losses during silage preparation and storage. The objective of the present investigation was to evaluate the ruminal fermentation characteristics and nutrient degradation of hay and silage of tropical forage legumes with (*Calliandra calothyrsus*, *Flemingia macrophylla*) or without (*Cratylia argentea*, *Vigna unguiculata*) tannins incubated either alone or in mixtures.

Materials and Methods

In Experiment 1, the shrub legume species *Calliandra calothyrsus* (CIAT 22310), *Flemingia macrophylla* (CIAT 17403) and *Cratylia argentea* (CIAT 18516 and 18668), and the herbaceous legume *Vigna unguiculata* (CIAT 1088/4, 288, 391, 9611 and 715) were cultivated at CIAT's research station Quilichao (Cauca, Colombia). All legumes were tested individually and the shrub legumes were additionally incubated in combination with *V. unguiculata* in a proportion of 1:2. Individual legumes and legume mixtures were evaluated as hay and as silage and all diets were incubated with or without the addition of polyethylene glycol (PEG) to inactivate tannins. This resulted in a total of 28 treatments (7 diets x 2 conservation methods x 2). The experiment was conducted using the gas-transducer technique.

In Experiment 2, the tanniniferous shrub legume *Calliandra calothyrsus* (CIAT 22310) and the herbaceous legume *V. unguiculata* (CIAT 1088/4, 288, 391, 9611 and 715) were tested either alone or in combination with each other in proportions of 1:2 and 2:1. Individual legumes and mixtures were evaluated as hay and as silage. This resulted in a total of 8 treatments (4 diets x 2 conservation methods). Hay was prepared by sun-drying for 3 days and silage was stored for 56 days. The experiment was conducted using a rumen simulation technique (RUSITEC).

Results and Discussion

In Experiment 1, accumulated gas production and rate of gas production were higher ($P<0.05$) with silage than with hay and were not affected ($P>0.05$) by the addition of PEG. Fermentation dynamics were clearly affected be the botanical composition of the diets. The highest ($P<0.05$) accumulated gas production and gas production rate were observed with *V. unguiculata* alone and with the mixture consisting *V. unguiculata* and *C. argentea*. The lowest values ($P<0.05$) were observed in the treatments with tanniniferous legumes alone. The treatments with *C. argentea* alone and the mixtures with *V. unguiculata* and tanniniferous legumes showed intermediate values.

In Experiment 2, chemical composition of hays and silages varied depending on the botanical composition and the conservation method. Compared to hay preparation, ensiling decreased the crude protein (CP) content in legumes without tannins (*V. unguiculata, C. argentea* and their mixtures with legumes with tannins). On the other hand, the fiber content increased with ensiling in the legumes with no tannins (*V. unguiculata* and *C. argentea*) and corresponding mixtures with legumes that had tannins. Apparent organic matter (OM) degradation was also affected by the botanical composition and the conservation method. The highest ($P<0.05$) OM degradability was observed in the treatments with *V. unguiculata* alone and in the mixtures with high proportion of this legume. The mixtures with low proportion of *V. unguiculata* showed intermediate values and the lowest OM degradability was found in the treatments with *C. calothyrsus* alone. Compared to sun drying, ensiling increased ($P<0.05$) the OM degradability in all forages except with *C. calothyrsus* alone. Apparent CP degradability was highest ($P<0.05$) with *V. unguiculata* alone and with the mixtures containing a high proportion

of this legume. Ensiling increased ($P<0.05$) apparent CP degradability in *V. unguiculata* alone but had no effect ($P>0.05$) on protein degradation in the legume mixtures.

These results confirm that ensiling may result in losses of nitrogen in legumes with no tannins. However, these losses could not be minimized by mixing legumes with and without tannins. On the other hand these results indicate that ensiling compared to sun drying increases the apparent ruminal CP degradability in tannin-free legumes, which was not the case in tannin-rich legumes. It can be concluded that mixing of legumes with and without tannins for silage production does not decrease nitrogen losses during ensiling and storage of the forage but decreases the ruminal degradability of CP which in turn could result in a more efficient utilization of nitrogen.

1.3.3 Ruminal fermentation and duodenal protein flow in sheep supplemented with legume mixtures with contrasting tannin contents

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Rationale

Nutrition of ruminant livestock in the tropics is mainly based on grasses. In general tropical grasses are of low to moderate digestibility, deficient in one or more essential nutrients (e.g. crude protein) and contain high amounts of fiber and low concentrations of soluble carbohydrates and starch. This in turn, results in low microbial activity in the rumen and may cause imbalances in digestive products and result in an inefficient use of metabolizable energy. Therefore alternative feeding strategies are needed which contribute to improve fermentation efficiency in animals fed diets based on grasses of low quality by assuring adequate ruminal ammonia levels, microbial protein synthesis and duodenal flow of undegraded feed protein. This may be achieved by supplementation with legumes which generally contain higher amounts of both rumen degradable and undegradable protein than tropical grasses. If the deficiency of fermentable nitrogen in the diet is eliminated, this may increase the activity of fibrolytic microorganisms resulting in an improved degradation of fibrous feeds.

Many of the legumes which could be used to supplement ruminant livestock in the tropics contain condensed tannins (CT). In higher concentrations, CT may have detrimental effects on animal production (e.g. suppressed intake and digestibility of nutrients). However, in lower concentration CT could have beneficial effects, such as reducing nitrogen losses during ruminal fermentation and increasing the flow of protein to the duodenum and the absorption of amino acids in the lower gut. The objective of the present investigation was to evaluate the effects of supplementing legume mixtures with different types and concentrations of CT on ruminal fermentation and the utilization of nitrogen in sheep fed a basal grass diet of low quality.

Materials and Methods

A feeding trial with sheep was conducted at CIAT's research station Quilichao (Cauca, Colombia). Then adult, castrated male sheep, fitted with rumial and duodenal canulae were divided into two groups with an average bodyweight of 26.1 and 34.6 kg, respectively. Animal were kept in metabolic crates and assigned to five treatments according to a repeated 5x5 Latin square design.

Diets consisted of *Brachiaria humidicola* (55% of dietary dry matter) and *Vigna unguiculata* either alone or in mixtures with the tanniniferous shrub legumes *Calliandra calothyrsus* and *Flemingia macrophylla*. The proportion of

tanniniferous shrub legumes in the legume mixtures was either 1/3 or 2/3. This resulted in a total of 5 treatments. Animals were offered daily 45 g of forage dry matter per kg of metabolic bodyweight ($BW^{0.75}$). Animals were offered fresh water three times per day and had free access to a mineralized salt mixture. The experimental periods consisted of 18 days each, 10 days adaptation to the experimental diets and 8 days of data and sample collection. Bodyweight was determined at the beginning and the end of each experimental period after a fasting period of 17 hours.

During days 1 to 6 of each collection period, feed refusals, and urine and feces excreted were recorded daily and samples of feeds offered and refused and of urine and feces were taken. Duodenal digesta was sampled on days 6 and 7 and on day 8 ruminal fluid was sampled every 6 hours. Indigestible acid detergent fiber (IADF) was used as internal marker to estimate duodenal dry matter and nutrient flow. Forage offered, refusals, feces and the solid phase of duodenal digesta were analyzed for organic matter (OM), neutral detergent fiber (NDF), acid detergent fiber (ADF), FAID and total nitrogen (crude protein (CP) = N x 6.25). Microbial nitrogen in duodenal digesta was estimated using purines as marker. Ruminal fluid and duodenal digesta were further analyzed for ammonia concentration and feeds offered for total condensed tannin concentration.

Results and Discussion

The grass used in this investigation presented a low CP content (64 g/kg) and high contents of NDF and ADF (810 and 425 g/kg, respectively). The tanniniferous shrub legumes presented a high CP content which was similar to that found in *V. unguiculata*. Condensed tannin content was almost three times higher in *C. calothyrsus* than in *F. macrophylla*.

One factor which probably affected the animal response variables was the fact that the proportions of tanniniferous legumes consumed were clearly below the proportions in the diets offered. As a result of this, the proportions of tanniniferous legumes in the diets consumed were 10 and 15%, respectively and not 15 and 30% as intended. Furthermore there was a relatively high variability among individual animals.

In general, OM intake tended to decrease with increasing proportion of tanniniferous legumes in the diet, and was clearly lower ($P<0.001$) with diets containing *F. macrophylla* or *C. calothyrsus* than with the diet free of tannins. No differences in OM intake were observed between the two tanniniferous legumes. Apparent OM and ADF digestibilities were lower ($P<0.05$) with diets containing high proportions of tanniniferous legumes than in the diet free of tannins. Nitrogen intake and rumen ammonia concentrations and duodenal flow of total nitrogen were also decreased ($P<0.05$) by the inclusion of tanniniferous legumes. The proportion of undegraded feed protein reaching the duodenum was not increased by the inclusion of tanniniferous legumes in the diet.

The reasons for these unexpected results are unknown but could be related to the relatively high variability in legume consumption among individual animals.

The intake of small proportions of tanniniferous legumes in mixtures with a legume free of tannins decreased OM and CP intake and suppressed fiber digestibility in sheep fed a basal grass diet of low quality. On the other hand, consumption of tanniniferous legumes did not affect N utilization in the way as it was expected. Although the inclusion of higher proportions of tanniniferous legumes decreased ruminal ammonia concentration, duodenal flow of total nitrogen was also decreased when compared to the diet free of tannins.

It can be concluded that the inclusion of small proportions of tanniniferous legumes in mixtures with legumes free of tannins did no have any positive effects on digestion and utilization of nitrogen.

1.3.4 Effects of feeding legume mixtures on energy and nitrogen utilization by sheep

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Rationale

Tropical fodder legumes are often characterized by high contents of condensed tannins (CT). The effect of CT on protein digestion is well-known but few data are available on their effects on energy utilization and methane production. To investigate effects of CT in legumes on energy and protein utilization and on methane production, an in vivo feeding trial in metabolic cages and respiratory chambers was conducted. This study formed part of a larger project on the use of tanniniferous shrub legumes as potential forage supplements in smallholder systems.

Material and Methods

The study carried out in the ETH animal facilities in Zurich, Switzerland focused on two promising tanniniferous tropical shrub legumes: *Calliandra calothyrsus* and *Flemingia macrophylla*. Six diets were tested for their effect on energy turnover and methane release in a respiratory chamber experiment in a 6 x 6 Latin square design with six lambs of the Swiss White Mountain breed (n=6) (Table 6). Dry matter offered daily was 60 g/kg of metabolic bodyweight ($BW^{0.75}$). Five of the diets consisted of mixtures of grass (*Brachiaria brizantha*) and legume (55% grass and 45% legume foliage), with a grass-only diet serving as control. The legume supplements consisted either of sun-dried CT-free Vigna unguiculata alone or of mixtures of *V. unguiculata* with air-dried leaves of *C. calothyrsus* or *F. macrophylla* in ratios of 2:1 and 1:2.

Animals were adapted to the experimental diets for 2 weeks. In the third week we measured intake, and fecal and urine excretion. In addition, samples of rumen fluid and blood were taken. For 2 consecutive days, the gaseous exchange of the animals was measured using dual open-circuit respiratory chambers.

Subsequently diets and samples of feces and urine were analyzed for nitrogen, carbon and energy content, as well as fiber composition. Blood was analyzed for nitrogen (BUN) and in the rumen fluid bacteria, protozoa, ammonia and volatile fatty acids (by HPLC) were measured. Data were subjected to analysis of variance with SAS considering diet, animal and experimental periods as sources of variation.

Results and Discussion

It is often stated that legumes with high tannin content are not palatable and therefore avoided by herbivores. However in this trial the intake of the tanniniferous legumes was high (>85% of the amounts offered) despite relatively high total CT content in Calliandra (182 g kg^{-1}) and Flemingia (219 g kg^{-1}). The differences in intake due to plant species were much smaller than differences due to animals. Also some animals became used

Table 6. Diets utilized in the feeding trial with sheep.

Diet	Grass	High quality legume	Tanniniferous legume
1	*Brachiaria brizantha* 100%		
2	*Brachiaria brizantha* 55%	*Vigna unguiculata* 45%	
3	*Brachiaria brizantha* a 55%	*Vigna unguiculata* 30%	*Calliandra calothyrsus* 15%
4	*Brachiaria brizantha* 55%	*Vigna unguiculata* 15%	*Calliandra calothyrsus* 30%
5	*Brachiaria brizantha* 55%	*Vigna unguiculata* 30%	*Flemingia macrophylla* 15%
6	*Brachiaria brizantha* 55%	*Vigna unguiculata* 15%	*Flemingia macrophylla* 30%

to the tanniniferous legumes and showed intake rates of 100% while others did not. The intake of the basal grass diet exceeded 90% of the forage offered throughout the experiment.

Rumen ammonia concentration was low in the treatment with Brachiaria only (3.9 mmol/l) and with the high proportion of Calliandra (3.8 mmol/l), which in both cases is below the minimum requirement of ruminants. The ammonia level with the Brachiaria-Vigna only diet was high (6.5 mmol/l) compared to the other treatments. Plasma urea nitrogen (PUN) was also lowest for the grass alone and high Calliandra diets, followed by the two Flemingia treatments and the diet with the low Calliandra level.

The apparent digestibility of OM was low in diets with high proportions of tanniniferous legumes and increased when their proportion was reduced. The apparently digested N in relation to the $BW^{0.75}$ was for all diets (except for the high Calliandra diet) higher than for the pure Brachiaria diet. Apparent N digestibility was lowest for the high Calliandra and for the diets with high Flemingia and low Calliandra. However, the amount of protein retained did not differ ($P > 0.05$) between diets.

Fecal N relative to N intake was elevated as the proportion of tanniniferous species increased in the diets. On the other hand N loss through urine in relation to total N loss was higher when tanniniferous legumes were include at low levels. These results suggest that N of tanniniferous legumes is mainly lost via feces as tannins protect protein from degradation in the rumen. Results also showed that the expected increase in CP absorption due to tannins in the legumes fed did not take place in a significant degree. Total N loss and N retention did not differ between diets.

Apparent digestibility of NDF showed a tendency ($P<0.1$) to be lower with higher proportions of tanniniferous legumes. Apparent digestibility of ADF was reduced in all diets containing Calliandra or Flemingia.

Apparent energy digestibility and energy metabolizability were lower in the diets with tanniniferous legumes as compared to the diet with the high quality legume and the pure Brachiaria diet. The heat energy/kg $BW^{0.75}$ was lower ($P<0.01$) for the high tannin diets than for the high quality diet. The metabolizable energy was utilized mainly for fat tissue deposition, whereas the high tannin diets also showed lower fat gain than the high Vigna diet.

Volatile fatty acids did not differ between treatments. In contrast, methane production/ $BW^{0.75}$ was reduced by the inclusion of tanniniferous legumes. This reduction was 8.4% and 25.7% for the diets containing low and high proportions of Calliandra, respectively and 9.1% and 21.8% for the diets containing low and high Flemingia, respectively. When related to intake of gross energy this effect was significant only for the diets with high proportions of legumes with tannins. There was no effect of treatments on methane production when expressed per unit of digested energy, digested NDF or digested OM.

In general, our results demonstrate that a high level of Calliandra calothyrsus in the diet resulted in lower metabolic protein and energy supply as compared to the high-quality legume diet. It was also evident that *Flemingia macrophylla* supplementation was less detrimental than that of *Calliandra calothyrsus* in metabolic protein supply, but not in energy supply. However, a high proportion of *Flemingia macrophylla* in the diet had a similar adverse effect on protein supply as the low *Calliandra calothyrsus* diet. Regarding energy supply both legume species had similar effects.

The suppressing effect of tanniniferous legumes on methane seems to have been mainly mediated by reductions in ruminal fiber and organic matter degradation and less by a direct adverse action against the methanogenic microbes. Finally, our results suggest that the CT-free Vigna could be partially replaced by *Flemingia macrophylla* for improving low-quality tropical grass-only diets in the tropics if no other option is available.

1.3.5 Milk production of dual purpose cows supplemented with legume mixtures with and without tannins

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Rationale

Milk production of dual purpose cows in livestock systems operated by smallholders in the tropics is heavily affected by the low availability and quality of the traditional forage resources during the dry season. One alternative to minimize the decrease in milk production during the dry season is to supplement livestock with legume foliage. These strategies allow smallholders to maintain livestock productivity throughout the year and reduce the dependence on purchased supplements during the dry season.

Previous studies had shown that supplementation with hay of *Calliandra calothyrsus* (Calliandra) did not increase milk production of dual purpose cows grazing low quality pastures during the dry season. This lack of response to supplementation with Calliandra has been associated to its high level of tannins, which results in low levels of ammonia production in the rumen and as a result bacterial protein synthesis is reduced. It has been hypothesized that mixing legumes with and without tannins could contribute to maximize the effects of legume supplementation on milk production due to increased production of rumen ammonia and flow of total nitrogen to the lower digestive tract.

A feeding trial with lactating dual purpose cows was conducted to test the effect of mixtures of legumes with and without tannins as supplements for milking cows grazing a low quality pasture.

Materials and Methods

The feeding trial was performed at CIAT's research station Quilichao (Cauca, Colombia). Eight Holstein x Cebu crossbred cow were assigned to 4 supplementation treatments using a repeated 4 x 4 Latin square design. The 4 supplements consisted either of the tanniniferous *Calliandra calothyrsus* (CIAT 22310) or the tannin free *Vigna unguiculata* (CIAT 1088/4, 288, 391, 9611 and 715) alone or of mixtures of these 2 legumes in proportions of 1:2 or 2:1. Legume foliage was harvested and sun dried for 3 days prior to the experiment.

The supplements were offered at a level of 1% of the cows bodyweight in two meals per day (at 05:00 and 13:00) during milking. To improve palatability of the legume foliages, the individual portions of the supplements were mixed with 100 g of molasses and 50 g of a mineralized salt for dairy cattle. The remaining time of the day, cows were grazing a pasture based on *Paspalum notatum* with an average dry matter availability of 983 kg/ha. The pasture area of 3 ha was divided into 2 paddocks of 1.5 ha each which were grazed alternately with 7 days of grazing and 7 days of rest period. The average stocking rate during the experiment was 2.6 animal units per hectare.

The experimental periods were of 14 days each, with 7 days of adaptation to the respective supplements and 7 days of measurement of daily milk production. Additionally samples of milk were taken for the determination of the contents of fat, total solids and urea nitrogen.

Results and Discussion

Despite the addition of molasses and salt to the legume foliage, consumption of the supplement was affected by its composition (Table 7). The highest ($P<0.05$) intake was observed when the supplement consisted of *V. unguiculata* (cowpea) alone or of the mixture containing 2/3 of this legume. With the supplement containing

only 1/3 of cowpea, intake was intermediate and the lowest (P<0.05) supplement intake was observed with Calliandra alone. These differences in the consumption of the supplements were also reflected in daily milk production.

The lowest (P<0.05) amount of fat corrected milk was produced in the treatment with Calliandra alone. When legume mixtures were supplemented, milk production was intermediate and when cowpea was supplemented alone, milk production was higher (P<0.05) than in any other treatment. Milk contents of fat and total solids were not affected (P>0.05) by supplementation. However, milk urea nitrogen was higher (P<0.05) with cowpea than with Calliandra, which is probably due to the higher content of readily fermentable crude protein in *V. unguiculata*.

These results confirm the high potential of cowpea as supplement for dual purpose cows grazing low quality pastures during the dry season and they indicate that milk production increases with increasing proportion of cowpea in the legume supplement.

It can be concluded that a legume supplement based on Calliandra can be significantly improved by including a small proportion (e.g. 1/3) of cowpea in the mixture. It is worth mentioning that milk urea nitrogen levels were very low throughout the experiment, indicating that rumen degradable crude protein was a limiting nutrient even in the treatment with Cowpea alone. Therefore it is not surprising that the mixtures of Calliandra and cowpea did not result in a higher milk production than cowpea alone, because a positive effect of such legume mixtures can only be expected when degradable crude protein is not limiting ruminal fermentation.

Table 7. Amount of legume foliage consumed, milk production and milk urea nitrogen concentration of cows supplemented with contrasting legume mixtures.

	Botanical composition of supplement (% of dry matter)			
C. calothyrsus	100	67	33	0
V. unguiculata	0	33	67	100
DM offered (kg/d)	4.2	4.4	4.3	4.3
DM consumed				
kg/d	1.2c	2.6b	3.5a	3.8a
g/kg BW	2.8c	6.0b	8.2a	8.7a
Milk production (kg/d)	3.6c	4.4b	4.7b	5.3a
Milk urea N (mg/dL)	3.7b	3.1b	4.5a	6.3a