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

1.1 Development of *Brachiaria* hybrids with high quality

Highlights

- Using NIRS we detected differences in CP and IVDMD in a selected population of sexual *Brachiaria* hybrids planted in pots. The variability observed in CP and IVDMD in the sexual population examined indicates that there is considerable scope for genetic improvement of quality in *Brachiaria*.
- The magnitude of genetic variation for key nutritional quality parameters in a set of *Brachiaria* hybrids was greater than the interaction of genotypes with sampling dates.
- Milk yield of cows with limited milking genetic potential was similar in well managed pastures of *B. brizantha* cv Toledo as compared to pastures with Br*achiaria* hybrids
- Breeding *Brachiaria* for adaptation to acid soils has resulted in cultivars (i.e. cv Mulato II) with mineral concentrations (i.e. P) in leaf tissue similar to what is recorded in commercial cultivars with adaptation to edaphic constraints (i.e. cv Toledo).

1.1.1 Methodology development for screening *Brachiaria* hybrids for high digestibility and protein

Contributors: P. Avila, G. Ramirez, C. Lascano and J.W. Miles (CIAT)

Rationale

In 2004 we reported that with a small set of *Brachiaria* hybrids grown in the field there was a significant sampling period and genotype effect for crude protein and in vitro digestibility and that sampling x genotype interaction was not significant for both quality variable measured. This year we wanted to reconfirm results from last year, but using a larger population of *Brachiaria* hybrids transplanted in pots rather than in the field.

Materials and Methods

A total of 119 *Brachiaria* sexual hybrids (selected in 2003) were transplanted in replicated (3) pots. Initially the plants were cut at a uniform height and after 6 weeks of growth, leaves were harvested in three successive samplings. After each harvest 1 g of urea was applied per plant. All samples (leaves) were analyzed in CIAT's Forage Quality Laboratory for crude protein (CP) and in vitro digestibility (IVDMD) using NIRS. Results were subject to an analysis of variance using SAS version 9.1.3.

Results and Discussion

Results showed that average CP and IVDMD values recorded in the sexual hybrids differed between entries and sampling periods, but that the interaction hybrid x sampling period was not significant (Table 1).

Variation in quality of the hybrids across the three sampling periods was 14.4 to 22.9 % for CP and 57.9 to 68.1 % for IVDMD. The mean CP (19 %) and IVDMD (64 %) observed were high for a tropical grass probably related to genetic factors as well as management factors (i.e. N fertilization and age of leaf at harvest).

These results agree with results from last year that showed that in a population of 50 hybrids

Table 1. Analysis of Variance associated with crude protein (CP) and in vitro digestibility (IVDMD) measured in three successive samplings in a population of *Brachiaria* sexual hybrids.

Variable	Source of Variation (Significance)		
	Hybrid	Sampling	Hybrid x Sampling
CP	0.0001	0.0024	0.8364
IVDMD	0.0001	0.0008	0.1125

from the cross *B. ruziziensis* x *B. brizantha* cv. Marandu there were significant sampling ($P < 0.0001$) and genotype ($P< 0.002 –0.006$) effects for both CP and IVDMD, but a non significant sampling date x genotype interaction. Variation in CP (12.1-21.8%) and IVDMD (63.5-74.1%) measured in the hybrids last year was similar to the variation observed in the sexual population evaluated this year.

Last year we indicated that to screen for quality *Brachiaria* hybrids coming out of the improvement program we needed to standardize a protocol for plants grown in pots in the greenhouse. Results from this year confirm that it is possible to detect with NIRS differences in CP and IVDMD among entries in a population of *Brachiaria* hybrids planted in pots. The variability observed in CP and IVDMD in leaf tissue from the sexual population examined indicates that there is scope for genetic improvement of quality in *Brachiaria.* Screening for quality in the latest population of sexual hybrids selected by the breeder is currently underway.

1.1.2 Nutritional quality parameters (dry matter digestibility, crude protein content) of *Brachiaria* hybrids (series BR04), preselected at Quilichao and Matazul in 2004

Contributors: C. Lascano; J.W. Miles; P. Ávila (CIAT)

Rationale

Inherent nutritional quality (dry matter digestibility [IVDMD] and crude protein content [CP]) will be important in determining the commercial success of new hybrid *Brachiaria* cultivars. It is clear from past results that ample genetic variation for quality parameters exists in the breeding population, probably owing to the heavy contribution of high quality *B. ruziziensis* germplasm in the synthesis of the original population. Reliable selection to maintain and improve quality attributes will depend on quantification of the stability of expression of quality traits over environmental variation so as to design effective and efficient evaluation and selection protocols.

Materials and Methods

Three hundred thirty-nine preselected BR04, hybrid *Brachiaria* clones were delivered to the forage quality lab (as small, rooted cuttings, in 4-in pots) early in the year. These plants were propagated and a thrice-replicated field trial established at CIAT-Quilichao. Plants were sampled three times during the season and IVDMD and CP determined by NIRS (Near Infra-Red Spectroscopy).

Results and Discussion

Significant genetic variation over sampling dates among hybrid clones was detected for both IVDMD and CP. Large variation among sampling dates was detected (For IVDMD, mean square = 7.9 vs. 878.8 for genotypes or sampling dates, respectively; for CP, mean square = 6.9 vs. 1,716.0 for genotypes or sampling dates, respectively). However, it is encouraging that despite the large effect of sampling date on both quality parameters, the interaction of genotype with sampling, while statistically significant, was less than that for genotypes (For IVDMD, mean square = 7.9 vs. 1.8 for genotypes or genotype-sampling date interaction, respectively; for CP, mean square = 6.9 vs. 2.6 for genotypes or genotype-sampling date interaction, respectively).

Hence, it appears that sampling field-grown plants even on only a single date should be effective in identifying genotypes superior for quality traits. Given the significant genotype-sampling date interaction, prudence would suggest sampling in multiple environments (dates or locations). A strategy aimed at efficient use of laboratory resources might be to cull on the results of a single sampling and then assess the remaining genotypes more intensively to identify those with consistently superior nutritional quality.

1.1.3 Milk yield of cows grazing *Brachiaria* pastures managed with high grazing pressure

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Rationale

We had reported that milk yield of cows grazing *Brachiaria* hybrid cv Mulato and the newly released *Brachiaria* hybrid cv Mulato II was higher than in *B. brizantha* cv Toledo when no N was applied. In both hybrids MUN (Milk Urea Nitrogen) was greater as compared with *B. brizantha* cv Toledo and this was associated with higher crude protein in the leaf tissue. Thus we concluded that the potential to produce milk in the absence of N fertilization was similar in the two *Brachiaria* hybrids and superior to *B. brizantha* cv Toledo, which is an accession selected from the collection held by CIAT.

An observation made when grazing *B. brizantha* cv Toledo in several on-farm trials was that it had a very fast regrowth after grazing or cutting and that as a result if not properly managed (i.e. adequate stocking rate and grazing frequency) it tended to mature and loose quality (lower crude protein) very fast. Thus this year we were interested in comparing milk production in the two *Brachiaria* hybrids with cv Toledo in N fertilized pastures managed with similar grazing pressure in two contrasting seasons of the year.

In addition, we wanted to document that breeding *Brachiaria* for adaptation to acid soils with low fertility (i.e. low bases and low P) did not result in cultivars with lower quality and concentration of minerals as compared to commercial cultivars.

Material and Methods

Two grazing trials were carried out in the Quilichao research station in a rainy period (March 21 to May 3, 2005) and a dry period (July 4 to August 16, 2005) to measure milk production in pastures of *B. brizantha* cv Toledo (control), *Brachiaria* cv Mulato *and Brachiaria* cv Mulato II arranged in reversible 3 x 3 LSD. Each period was of 14 days of which 7 were for adjustment and 7 for measurements. Total rainfall in the wet and dry periods was 242 and 27 mm, respectively.

In the two experiments, pastures were mowed, fertilized with 50 kg of N/ha and allowed to grow for 6 weeks before being grazed by 6 cows (crossbreds and Holstein) with similar days of lactation and initial milk production (5 liters/ day). The average stocking rate used in both seasons of the year was 3 cows/ha (each cow averaged 450 kg liveweight).

Measurement in the pastures included forage on offer, quality (CP and IVDMD) in pluck samples and mineral composition in leaf tissue. Milk yield was measured AM and PM and milks samples were analyzed for fat, non-fat solids and MUN (Milk Urea Nitrogen). Results from the vegetation and from the animals were subject to analysis of variance using SAS program package version 9.1.3 and the Ryan- Einot- Gabriel-Welsch Multiple Range Test was used to separate means.

Table 2. Quality and forage availability in pastures of *Brachiaria* grazed by milking cows in two contrasting seasons of the year

Pastures	Forage on offer (kg DM/ha)	CP (%)	IVDMD (%)
Maximum rainfall			
Toledo (control)	2905 (58)*	9.1 b	66.6
Mulato	2666 (60)	9.7 b	67.2
Mulato II	3042 (58)	11.4 a	66.3
Significance	NS	P <0.05	NS
Minimum rainfall			
Toledo (control)	3082 (78)*	7.4 b	57.9
Mulato	2815 (48)	7.5 b	61.1
Mulato II	3269 (52)	8.4 a	61.0
Significance	NS	P <0.05	NS

* Values in parenthesis are the proportion of green leaf in the forage on offer

Results and Discussion

The amount of forage on offer did not vary among pasture treatments in both seasons of the year (Table 2). As expected the proportion of green leaf was higher in the wet season (58 to 60%) than in the dry season (48 to 52 %) with the exception of *B. brizantha* cv Toledo, which had a very high proportion of leaf (78%) in the dryer period (Table 2), which is an indication of the high adaptation of this grass to the dry season.

As expected, CP and IVDMD in the three grasses were lower in the dry period as compared to the wet period (Table 2). On the other hand, IVDMD did not vary among grasses in the wet or dry periods. In contrast the level of CP was higher in Mulato II as compared to the other grasses in both seasons of the year. The fact that all pastures were managed with a high grazing pressure undoubtedly controlled the growth rate of Toledo which in turn contributed to maintain a relatively high leaf proportion and high CP content on the forage on offer.

In this study we were also interested in determining variation in mineral concentration of the three grasses grown in acid soil with low fertility and only fertilized with N. Results presented in Table 3 are consistent with what is expected for a tropical grass. For example, the concentration of P, which is a key mineral in the nutrition of livestock, averaged 0.22% which is similar to the concentration recorded in grasses grown in acid-low fertility soils. It was also

Table 3. Mineral composition of leaf tissue in pastures of *Brachiaria* grazed by milking cows in two contrasting seasons of the year

Pastures	Ca (%)	P (%)	S (%)	K (%)	Mg (%)
Maximum rainfall					
Toledo (control)	0.33 b	0.22	0.14	1.68	0.34 b
Mulato	0.49 a	0.19	0.11	1.82	0.37 b
Mulato II	0.54 a	0.24	0.14	1.56	0.44 a
Significance	P <0.05	NS	NS	NS	P < 0.05
Minimum rainfall					
Toledo (control)	0.39 c	0.17 b	0.11	1.57	0.32
Mulato	0.47 b	0.20 a, b	0.10	2.24	0.35
Mulato II	0.52 a	0.25 a	0.13	1.62	0.43
Significance	P <0.05	P <0.05	NS	NS	NS

Table 4. Milk yield and composition of cows grazing *Brachiaria* pastures in two contrasting seasons of the year

Pastures	Milk yield (l/cow/d)	Fat (%)	Non fat solids (%)	MUN (Mg/dL)
Maximum rainfall				
Toledo (control)	4.7	4.4 a	9.4	6.1 a
Mulato	4.7	4.2 b	9.4	5.2 b
Mulato II	4.4	4.0 c	9.5	7.8 a
Significance	NS	P <0.05	NS	P <0.05
Minimum rainfall				
Toledo (control)	5.4	4.2	8.8	3.0
Mulato	5.5	4.2	8.8	3.0
Mulato II	5.2	3.9	8.9	3.6
Significance	NS	NS	NS	NS

interesting to observe that with the exception of Ca and Mg (higher in the two *Brachiaria* hybrids) the concentration of other minerals measured was not affected by grass cultivar. Results presented in Table 4 indicate that milk yield was similar in pastures sown with Toledo, in spite of the higher CP level in Mulato II in both seasons of the years.

The finding that milk yield in *Brachiaria* hybrids Mulato and Mulato II was similar than in Toledo could be the result of: a) using cows with limited ability to respond to improvements in quality of the forage on offer and/or b) using high grazing pressure to manage the pastures that prevented Toledo from becoming mature as result of its fast growth rate. In general, results indicate that milk yield of cows with limited ability to produce milk can be as high in pastures of *B. brizantha* cv Toledo as compared to *Brachiaria* hybrids provided they are well managed in terms of grazing pressure and fertilization with nitrogen. In addition, results suggest that breeding *Brachiaria* for adaptation to acid soils has resulted in cultivars (i.e. cv Mulato II) with concentration of minerals (i.e. P) in leaf tissues similar to commercial cultivars with adaptation to edaphic constraints (i.e. cv Toledo).

1.2 Conservation of forages for dry season feeding in smallholder systems

Highlights

- The addition of small quantities of molasses to good quality grasses ensiled in plastic bags is an insurance to obtain good quality silage for use in the farm or for sale to other farmers.

- Supplementing (1% DM of BW) medium quality grass hay (60-65% IVDMD and 9-10% CP) to cows grazing pastures with limited forage availability but adequate CP resulted in ½ liter more milk per day**.**

1.2.1 Quality of different grass silages with and without additive

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Rationale

A major constraint of small milk producers is lack of high quality feed in the dry season, which results in large economic losses. One known alternative to overcome the shortage of feed in the dry season is forage conservation in the form of silage. However, the adoption of forage conservation technologies by smallholders has

been very limited in part due to lack of information and of machinery to make good quality silage.

One alternative to the traditional "bunker" type silage is the "bag silage" technology which has several advantage for smallholders: (a) forage can be harvested and ensiled anytime during the year when labor is available either in small or large quantities (a person can make about 1 bag of 30-35 kg per hour), (b) machinery is not needed to harvest the forage material to be conserved; (c) silage produced can be used in the farm or sold to other farmers to obtain additional income; and (d) silage can be produced by either livestock or non-livestock owners. Generally two bags are used: one made of any material to put the forage biomass, and then this bag is inserted into the plastic bag that will avoid the penetration of oxygen to allow the fermentation process to take place.

As part of two special projects (CIAT led BMZ and ILRI led CFC) operating in Central America we are testing with smallholders with and without cattle the option of producing silage in plastics bags. Several farmer training courses have been carried out with practical demonstrations on principles for making good quality "bag silage" using grasses and legumes. One concern expressed by some farmers in Honduras and Nicaragua is the need for using molasses as an additive when ensiling grasses given that most of the local experience is ensiling maize or sorghum with adequate sugar content. Thus this year we evaluated the quality of grass silage with and without molasses in order to support on –farm work on forage conservation through feedback to extensionists and farmers in Central America.

Material and Methods

Material to make the silage was hand- harvested after 6 weeks regrowth from pastures sown with different *Brachiaria* cultivars (the hybrids cv Mulato and Mulato II and *B. brizantha* cv Toledo) in Quilichao and fertilized with 50 kilos of N/ha. The material was cut (3 cm) and one kilogram (fresh material with 25% DM) was placed in small plastic bags (23 x 32 cm). Molasses (5% on DM basis) was applied and mixed with the forage in bags prior to compaction.

Treatments were arranged in a randomized design with two factors (grasses and molasses) and 4 replications. The process of anaerobic fermentation was allowed to proceed for 40 days before opening the bags for chemical analysis. All silage samples were analyzed for crude protein, fiber content, and pH. In addition, we measured fermentation parameters (extent and rate of gas production) using the gas transducer

Table 5. Effect of adding molasses on quality of silage made from three *Brachiaria* grasses

Treatments	CP (%)	NDF (%)	ADF (%)	pH
Mulato + Molasses	9.7	58.4	25.6	3.8
Mulato - Molasses	9.5	64.4	33.9	5.4
Mulato II + Molasses	9.3	56.7	25.1	3.9
Mulato II - Molasses	10.6	62.4	31.5	5.6
Toledo + Molasses	8.9	59.9	30.1	3.9
Toledo - Molasses	9.4	72.9	37.5	5.5
Significance (P)				
Grass	0.002	0.0001	0.0001	NS
Molasses	0.003	0.0001	0.0001	0.001
Grass x Molasses	0.002	0.0001	0.009	NS

technique. Results were subject to analysis of variance using the SAS program package version 9.1.3.

Results and Discussion

The effect on quality of adding molasses to the three grasses ensiled is shown in Table 5. The protein content of the two *Brachiaria* hybrids was higher than in Toledo and was slightly lower when molasses was added. In contrast, both fiber components measured (NDF and ADF) were lower with the addition of molasses.

The pH of the ensiled material, a good indicator of quality in silages, was not affected by the material being conserved but varied significantly with addition of molasses. A good quality silage should have a pH of around 4 or less, which was the case with the grasses ensiled with molasses. Silage with a low pH is associated with fermentation producing high lactic acid whereas a high pH (5.5 or greater) is associated with inadequate fermentation and as a result with low quality silage.

The results on extent and rates of degradation by ruminal microorganisms of the three grass silages are presented in Table 3. Adding molasses to the material had a greater effect on fermentation parameters than cultivar of the grass. With all three grasses the extent and rate of gas production was greater when 5% (DM) molasses was added. However, we did observe that the two *Brachiaria* hybrids had a slightly higher digestibility than Toledo, which is consistent with having lower cell wall content (Table 6).

The addition of low levels of molasses when ensiling tropical grasses with low sugar content provides insurance for making good silage. Thus small farmers making silage in plastic bags for their own use or for sale should be advised on the advantages of adding molasses or other sources of fermentable sugars to the material being ensiled, particularly if they are planning to conserve grasses and legumes with low sugar contents. It is also important that farmers keep in mind that silage with high pH does not only have poor quality in terms of nutrient composition but that it also consumed in low quantities.

Table 6. Fermentation parameters of *Brachiaria* silage with and without additives.

Treatments	Maximum gas production (ml)	Rate of gas production (ml/h)
Mulato + Molasses	224	0.0602
Mulato - Molasses	200	0.0533
Mulato II + Molasses	232	0.0622
Mulato II - Molasses	210	0.0555
Toledo + Molasses	232	0.0518
Toledo - Molasses	219	0.0466
Signficance (P)		
Grass	0.0001	0.0001
Molasses	0.0001	0.0001
Grass x Molasses	0.03	NS

One final comment has to do with the process of reducing humidity of the forage before ensiling in small plastic bags through wilting. As part of the work for adjusting appropriate technologies for conservation of grasses as silage by smallholders, we investigated wilting vs. no wilting of grasses (6 weeks regrowth and 25% DM).

Results (not shown) indicated that wilting had a negative effect on the quality of the silage made with the three grasses which is contrary to what is expected. One explanation was that the forage was wilted for 24 hours under shade which favored growth of fungi. Thus if wilting of the forage is not done properly it can result in poor quality silage.

1.2.2 Milk yield of cows supplemented with grass hay

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Rationale

The Forage Program of CIAT is developing/ adjusting forage technologies to feed livestock in the dry season that are appropriate for smallholders. Farmers in dry hillsides of CA are rapidly adopting improved grasses such as *B. brizantha* cv Toledo and *Brachiaria* hybrid cv Mulato, which have shown to be drought tolerant. In addition, there is growing interest among small farmers in conserving forages as hay or silage for dry season feeding and/ or for sale. One option is to harvest excess forage from improved pastures in the wet season to make high quality silage or hay for the dry season.

There is abundant literature on production and utilization of silage and hay in different livestock systems, but in most cases the technology available is not useful to small farmers given that it relies on machinery (i.e. tractors and mechanical forage harvesters) out of the reach of these farmers. Thus we have been investigating alternative technologies such as the "bag silage" (see previous activity) and "bag hay" that could be more appropriate for livestock systems operated by smallholders.

In this section we report the results of feeding grass hay to milking cows grazing a native pasture with limited forage availability but with adequate level of protein.

Materials and Methods

Pastures of *B. brizantha* cv Toledo, *Brachiaria* hybrid cv Mulato and *Brachiaria* hybrid cv Mulato II were used to make hay for supplementing milking cows grazing a *Paspalum notatum* pasture in the Quilichao Research Station. The *Brachiaria* pastures were mowed and fertilized with 50 kg N/ha and allowed to grow for 6 weeks. The regrowth was cut with a mower, collected manually and then sun –dried for three days. After drying the hay was stored in plastic bags and subsequently utilized to feed cross bred cows in late lactation.

A total of 8 cows were assigned to one of the following treatments arranged in 4 x 4 reversible LSD: T1*: Paspalum notatum* (control), T2: *Paspalum notatum* + Toledo hay, T3: *Paspalum notatum* + Mulato hay and T4: *Paspalum notatum* + Mulato II hay. Cows milked twice a day were offered hay of the three grasses at a level of 1% DM of BW. Each experimental period was of 14 days of which 7 days was for adjustment and 7 days for measurements. Measurements in the experimental pasture included forage on offer and quality parameters. To assess quality of the three hays we measured extent and rate of gas production, in vitro digestibility and crude protein. Hay consumption and milk yield and composition were measured daily.

Table 7. Quality of three hays made from *Brachiaria* pastures of similar regrowth age*

Hay	Maximum gas production (ml)	Rate of gas production (ml/h)	IVDMD %	CP %
Toledo	228 b	0.061 a	64.0 b	9.1
Mulato	230 b	0.048 c	64.8 b	9.2
Mulato II	241 a	0.055 b	74.8 a	10.8
Significance	P <0.05	P <0.05	P <0.05	

* Six weeks regrowth fertilized with 50 kg of N/ha

Table 8. Milk production and composition of cows grazing a *Paspalum notatum* pasture and supplemented with grass hay.

Hay	Milk yield (l/cow/d)	Fat (%)	Non fat solids (%)	MUN (mg/dL)
Toledo *	5.3	4.8 b	8.4	3.8 b
Mulato **	5.2	5.3 a	8.2	3.7 b
Mulato II ***	5.5	4.8 b	8.4	3.0 b
Control	4.8	5.0 b	8.3	4.2 a
Significance	P<0.06	P<0.005	NS	P <0.001

*Intake 0.74% DM of BW
** Intake 0.63% DM of BW
*** Intake 0.79% DM of BW

Results were subject to analysis of variance using the SAS -GLM program package version 9.1.3, given that one cow was declared as missing cell due to abnormal behavior.

Results and Discussion

Forage availability in the experimental pasture was low (750 kg DM/ha), with medium digestibility (55%) but with high CP content (11%). The quality of the three hays (Table 7) was different since Mulato II hay had higher digestibility (gas production and IVDMD) and CP, and this was associated with slightly higher intake (7%) relative to the other two hays.

Supplementing hay to cows grazing a pasture with low forage availability resulted in 8 to 15% (0.4 to 0.7 liters/cow/d) more milk than unsupplemented cows (Table 8). Milk fat varied among treatments, but no consistent trend was observed with hay supplementation.

In a previous study we had evaluated the utility of supplementing legume hay (cowpea) and legume silage (Cratylia) to milking cows grazing *B. decumbens* pastures in the dry season. Results from those studies indicated that supplementation of high quality legume- based silage and hay resulted in 11 to 18% more milk yield when compared with milk production of the control cows, which is similar to the increments recorded with grass hays in the present experiment.

In summary, results indicate that supplementing relatively small quantities (1% DM of BW) of medium quality grass hay (60-65% IVDMD and 9-10% CP) to cows grazing pastures with limited forage availability but adequate CP resulted in ½ liter more milk per day. These results need to be confirmed in on -farm experiments in order to assess the economics of grass hay production for feeding dairy cows with different genetic potential in the wet and dry season.

1.3 Assessment of the potential of tanniniferous legumes to improve ruminant nutrition in the tropics

Highlights

- Biomass production of selected shrub legumes was affected by soil fertility in planting site and by fertilization, but the extent of response varied widely among species. *Leucaena leucocephala* was by far the most affected species, while *Flemingia macrophylla* was not affected.

- Tannins from *Calliandra calothyrsus* 22310 showed higher solubility in ethanol than those from *Calliandra calothyrsus* 22316. In addition, results revealed that *C. calothyrsus* 22310 had a larger proportion of tannins of low polarity than *C. calothyrsus* 22316.

- A major part of the tannin-bound protein in shrub legumes, which is protected from microbial degradation in the rumen, was digested with acid-pepsin in an in vitro system.

- The PEG: tannin ratio required to maximize ruminal fermentation varied among shrub legume species and is probably influenced by the type of tannins present in the plants.

- Tannins from *Leucaena leucocephala* and *Flemingia macrophylla* were less effective in decreasing ruminal nutrient degradation and VFA production than tannins from *Calliandra calothyrsus*. Between two accessions of *Calliandra*, tannins from the accession 22310, were more reactive than those from the accession 22316.

- Of three protocols evaluated for extraction of DNA from ruminal fluid from tanniniferous diets the best results were obtained with the protocol recommended by IAEA**.**

1.3.1 Assessment of the effects of plant nutritional status on forage yield, quality, concentration and chemical properties of condensed tannins of selected shrub legume species

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Rationale

Type and concentration of secondary metabolites in plants may be affected by environmental factors. Results from experiments carried out during 2004 indicated that forage quality of tanniniferous shrub legumes could be improved by the selection of appropriate planting sites or by improving the growing conditions (e.g. by fertilization). Therefore the present activity concentrates on defining the environmental factors (i.e. soil type and fertility) responsible for differences in forage quality and type and concentration of condensed tannins of a range of tanniniferous shrub legumes. For this purpose, experimental plots were established at two contrasting sites in Colombia. The aim of this activity is to identify growing conditions which could contribute to an improved forage quality of tanniniferous legumes.

Material and Methods

In Experiment 1, the shrub legume species *Cratylia argentea* (CIAT 18516), *Calliandra calothyrsus* (CIAT 22310), *Desmodium velutinum* (CIAT 33443), *Flemingia macrophylla* (CIAT17403) and *Leucaena leucocephala* (CIAT 17502) were raised in Jiffy™ pots and after 6-7 weeks transplanted to the plot in May 2004 at the beginning of the rainy season on a Mollisol (fine silty, mixed, isohypothermic, Aquic Hapludoll, pH: 7.5, rainfall is bimodal, average annual precipitation: 896 mm; mean annual temperature: 24.3°C) at CIAT's headquarters in Palmira and on an Oxisol in Matazul (typic isohipertermic Caolinitic Haplustox, pH 4.5, low natural fertility (in particular deficient in P, basic compounds and organic matter); rainfall is unimodal, annual total: 2251 mm (April-November); annual mean temperature: 26°C) in the Eastern Plains of Colombia, and two levels of fertilization were applied (low fertilization level [kg/ha]: 20 P, 20 K, 500 lime, 10 S; high level: 50 P, 60 K, 1000 dolomite, 40 S) in order to assess the effect of fertilization as affected by soil type.

In Experiment 2, the same legume species were established in Matazul and four fertilizer treatments were applied in order to assess the effect of S and P fertilization: (i) no fertilizer, (ii) lime, P, K, S, Ca, and Mg applied in a water soluble form in a quantity equivalent to the amount of elements that can be taken up by the plant within 18 months, (iii) the same amounts of

nutrients without S and (iv) the same amounts of nutrients without P. The five legume species were transplanted in three replicates per site and fertilizer treatment, and arranged in a randomized complete block design. One year after transplanting the first standardization cut was carried out, with cutting heights of 100 cm for *Leucaena leucocephala* and of 50 cm for the remaining species. Since then the plants have been cut every 9 weeks and one sampling has been carried out. Prior to sampling, the number of surviving plants and their growth performance were assessed visually. In order to determine the feeding value, edible forage (branches up to 1cm diameter) from actively growing shoots was collected from five randomly chosen plants from every legume species per fertilizer level and replicate.

To assess dry matter production, plants were cut as described above and the harvested material was separated into leaves and stems. Samples for quality analyses were packed in plastic bags stored on ice and transported to the laboratory. Subsequently the samples were kept frozen at –20°C until freeze-drying. Dried samples were ground in a laboratory mill to pass a 1-mm screen and stored in air-tight containers at –20°C. Plant tissues will be analyzed for C, P, N, K, S, Ca and Mg, and the concentration, astringency, monomer composition and molecular weight of condensed tannins will be determined. Furthermore, biological N fixation by the legumes will be estimated by comparing the natural ^{15}N abundance in legumes and in weedy grasses growing in the same plot. For this purpose one grass and one herbal weed species from the borders of the plots were harvested.

Results and Discussion

In Experiment 1, on the acid soil in Matazul, *L. leucocephala* showed the lowest survival rate and performance in all parameters. Given that it did not reach the minimum size for harvesting, it was discarded. The establishment of the remaining legume species in Matazul was successful and survival rates ranged between 94 and 100%, with no significant differences ($P>0.05$) between species and fertilization level.

On the more fertile and less acidic soil in Palmira, *L. leucocephala* and *D. velutinum* showed the best survival rates of 100% followed by *C. calothyrsus* with 97.5%. *F. macrophylla* showed an intermediate survival rate of 89% and *C. argentea* presented the lowest survival rate. Fertilization increased survival rate of *C. argentea* from 77% to 88% but had no effect of the survival rates of the remaining legume species.

In Matazul, fertilizer application increased ($P<0.001$) biomass production from 88 to 147 g DM per plant on average among all legume species. The highest increase was observed in *C. argentea* (from 26 to 112 g DM per plant) and the lowest in *F. macrophylla* (from 166 to 179 g DM). Independent of fertilizer application *F. macrophylla* (173 g) and *C. calothyrsus* (139 g) showed higher ($P<0.05$) DM yields than *D. velutinum* (90 g) and *C. argentea* (69 g). *L. leucocephala* did not reach the minimum height for the standardization cut and no evaluation could be conducted in Matazul.

With exception of *F. macrophylla*, which did not show any differences due to planting site, biomass production was two to three times higher in Palmira than in Matazul (interaction planting site legume species $P<0.001$). Independent of planting site and fertilizer treatment, *F. macrophylla* produced between 138 and 180 g of dry matter per plant within nine weeks. Biomass production of the remaining species tended to increase with fertilizer addition in Palmira although the increase was much less pronounced than in Matazul. The largest increase was observed with *L. leucocephala* (+50%) in Palmira. The increase in yield in *C. calothyrsus*, *C. argentea* and *D. velutinum* varied between 5 and 20%.

Fertilization also increased the ratio of leafy biomass to total biomass. Averaged over sites and fertilizer treatments, dry matter production was higher ($P<0.05$) in *C. calothyrsus* (200 g per plant) than in the remaining species (between 133 and 162 g per plant. *L. leucocephala* was not included in this overall comparison because no data were available from Matazul. However, in Palmira, *L. leucocephala* produced considerably more dry

matter (>35% with fertilization and >85% without) than the other species.

In Experiment 2, both P and S fertilization showed clear effects on biomass production but seemed to have little influence on the initial establishment and the survival rate of the experimental species. Although both elements affected biomass production ($P<0.05$), a lack of P resulted in lower dry matter yields than a lack of S ($P<0.05$). Dry matter production among all species averaged 168 g per plant when all elements were supplied. DM production averaged 132 g with no S and 99 g per plant with no P. With *C. calothyrsus* and *C. argentea,* dry matter production decreased by 50% without P and by 36% without S. *D. velutinum* and *F. macrophylla* seemed to be more tolerant to deficiencies in P or S. Without P, biomass production of these species decreased by 35% and without S, by 6 and 10%, respectively.

In summary, these results show that biomass production of most shrub legumes was affected by planting site and fertilization, but the extent of response may vary widely among species. *L. leucocephala* was by far the most affected species, while *F. macrophylla* was not affected at all. On average for both sites, *C. calothyrsus* showed higher biomass production than the other shrub legumes, underlining the good adaptation and high agronomic potential of this species. For production systems on acidic low-fertility soils (e.g. in the Eastern Plains of Colombia) where no fertilizers are applied the most promising species in respect of survival rates, biomass production and development would be *F. macrophylla* and *C. calothyrsus*.

1.3.2 Extraction and characterization of tannins from different legumes

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Rationale

Previous studies conducted at CIAT revealed that differences may exist in the chemical characteristics and the biological activity among condensed tannins from different legume species but also among accessions within a species. In particular, the condensed tannins from *Calliandra calothyrsus* var. Patulul (CIAT 22316) and San Ramon (CIAT 22310) have been shown to differ in their monomer composition and their protein binding capacity. The aim of this activity was to better characterize the chemical properties of the condensed tannins of these two *C. calothyrsus* accessions.

Materials and Methods

Condensed tannins from two accessions of *Calliandra calothyrsus* (CIAT 22310 and CIAT 22316) were extracted using ethyl alcohol (70%) with 0.5% formic acid and 0.05% ascorbic acid as solvent. For that purpose, 40 g of dried and ground leaves were mixed with 400 ml of extraction solution and incubated for 30 minutes, subsequently filtered and centrifuged at 1000g for 15 min. The supernatant was transferred to glass flasks and the alcohol was removed in a rotary evaporator at 40°C. The resulting aqueous solution was filtrated through a Sephadex LH-20 gel column and the different fractions were washed out with ethanol. Subsequently condensed tannins were removed from the column with aqueous acetone (50%), concentrated by evaporation, frozen and lyophilized. For thin layer chromatography (TLC) silica gel was used as stationary phase and butanol: acetic acid: water mixtures or mixtures of ethyl acetate: methyl ethyl ketone: formic acid: water (5:3:1:1) in combination with chloroform: methanol: water (64:33:15) as mobile phase.

Results and Discussion

Tannins from *Calliandra calothyrsus* 22310 showed higher solubility in ethanol than those from *Calliandra calothyrsus* 22316. Tannins did not show displacement in TLC with chloroform: methanol (93:7) which indicates that the components are polar. With the mixture of ethyl acetate: methyl ethyl ketone: formic acid: water (5:3:1:1), *C. calothyrsus* 22310 showed two components of minor polarity with rf 0.86 and 0.58, while *C. calothyrsus* 22316 presented one component with rf 0.86 and one with rf 0.47. Double exposure revealed that *C. calothyrsus* 22310 had a larger proportion of tannins of low polarity than *C. calothyrsus* 22316.

Exposure on bi-dimensional plaques of cellulose with 2-butanol:acetic acid: water (3:1:1) in one direction and acetic acid (30%) in the other, showed that tannins from *C. calothyrsus* 22310 in general have a lower polarity than those of *C. calothyrsus* 22316, probably due to differences in size of the different fractions. In crude extracts, both accessions showed similar proportions of components of low polarity (rf>0.7) but differences in medium polarity between rf 0.7 and 0.5. In *C. calothyrsus* 22310 the 54 detected fractions were run on a polarity gradient silica gel with isopropyl acetate petrol ether, resulting in melting them to 22 new fractions depending on their composition. Two of the major components have been isolated and will be purified for identification as next step.

1.3.3 In vitro assessment of ruminal protein degradability and protein release under conditions resembling acid-pepsin digestion in the abomasum

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Rationale

The inclusion of tannin-free legumes into low-quality grass diets generally increases the ruminal degradation of crude protein. On the other hand, results from in vitro studies conducted last year using a rumen simulation technique demonstrated that supplementation of tannin-rich legumes decreases ruminal protein degradation. It is likely that such a decrease would result in a greater flow of dietary N to the lower digestive tract of ruminants feed tannin-rich legumes. However, given that in previous study only ruminal fermentation processes were considered, the effects of tannin-rich legumes on postruminal protein digestion are unknown. This year, our work focused on the assessment of the potential acid-pepsin digestibility of rumen-undegradable protein from grass-based diets supplemented with contrasting legumes.

Materials and Methods

One grass-alone and 13 legume supplemented diets were incubated for 48 h in vitro in fermentation liquid (McDougall buffer: rumen liquid, 1:4) under CO_2 gasification. *Brachiaria humidicola* (CIAT 6133) was used as grass species. The legume supplement (1/3 of dietary dry matter) consisted either of a single legume (*Cratylia argentea* CIAT 18516, *Calliandra calothyrsus* CIAT 22310 and 22316, *Flemingia macrophylla* CIAT 17403 and *Leucaena leucocephala* CIAT 734) or combinations of *C. argentea* with the other legumes in proportions of 2:1 and 1:2. A total of nine samples were incubated per treatment. Subsequently three samples of every treatment were filtered and the solid residue was analyzed for dry matter (DM) and organic matter (OM) to determine apparent ruminal DM and OM degradabilities. Three samples were filtered, and the solid residue

Table 9. Degradation parameters (mg/g of supply) measured under conditions simulating the rumen and the abomasum (values with same letters in one column are not significantly different).

Treatments		Apparent ruminal degradability		True ruminal degradability	Ruminal + acid-pepsin degradability	True ruminal and total degradability of N	
		OM	DM	DM	DM	Ruminal	Total
B. humidicola 100%		666a	655a	759a	641a	995ab	935ab
C. argentea 18516		576b	565bc	710bc	646a	884abc	954a
C.calothyrsus 22310	3:0	431e	426e	614f	511c	576d	848de
C.argentea	2:1	506cd	499d	654de	581b	n.a.	900abcd
	1:2	538bc	528cd	677cd	625°	787abcd	931abc
C.calothyrsus 22316	3:0	482de	485d	688bcd	467d	677bcd	793e
C.argentea	2:1	497cd	491d	693bc	514c	727abcd	884cbd
	1:2	589b	583b	709bc	557b	789abcd	920abc
F.macrophylla 17403	3:0	500cd	499d	625ef	501cd	605d	870bcd
C.argentea	2:1	540bc	538bc d	656de	499cd	621cd	868cd
	1:2	566b	560bc	682bcd	559b	752abcd	928abc
L. leucocephala 734	3:0	542bc	534bc d	715b	575b	n.a.	906abcd
C.argentea	2:1	572b	557bc	711bc	582b	736abcd	910abcd
	1:2	583b	567bc	711b	584b	801abcd	930abc

a, b, c, d, e means in the same column different ($P < 0.05$)
n.a., data not available due to technical problems

treated with NDF solution and analyzed for DM and N to determine true ruminal DM and N degradability. The remaining three samples were further incubated for 24 h with HCl-pepsin to simulate abomasal digestion. After incubation samples were filtered and the solid residues analyzed for DM and N. Ammonium concentration in the fermentation fluid was measured at 0, 6, 12, 24 and 48 h of incubation using the method of Kjeldahl.

Results and Discussion

The rate of ammonia production during incubation in ruminal fluid was affected by the type of legume supplement and decreased with increasing proportion of tanniniferous legume in the diet. Over all, diets with high proportion of *C. calothyrsus* resulted in the lowest ammonia production. Apparent and true ruminal degradation of DM and of total DM degradation (ruminal + acid-pepsin) as well as nitrogen degradation was inversely related to the proportion of tanniniferous legume in the mixture. This effect varied with species and was not as clear as the relationship between ammonium concentration and legume proportion. Nitrogen degradation was notably improved by incubation with acid-pepsin solution.

Results indicate that the supplementation with tanniniferous legumes increases the proportion of acid-pepsin digestible protein of dietary origin given that the proportion of acid-pepsin digested N (difference between ruminal and total N degradability) was lowest with *C. argentea* alone and increased with the proportion of tanniniferous legume in the mixture (Table 9).

In general, our results show that in mixed diets based on a low-quality grass, the replacement of *Cratylia argentea*, a legume without detectable amounts of tannins, by the tanniniferous

C. calothyrsus (CIAT 22310) decreased apparent ruminal crude protein degradation by 65% (from 884 to 576 mg/g) and increased almost 4 fold (from 70 to 272 mg/g of N supplied) the proportion of dietary crude protein digested by acid-pepsin incubation. This suggests that a high proportion of the tannin-bound protein, which is protected from microbial degradation in the rumen, could be available for acid-pepsin digestion in the abomasums. If these results are validated in vivo the use of mixtures of legumes with and without tannins could effectively improve the supply of available protein in ruminants fed low quality grasses.

The lower ruminal degradation of protein in diets containing a high proportion of *C. calothyrsus* CIAT 22310 as compared with *C. calothyrsus* CIAT 22316 is in good agreement with the results of a feeding trial conducted at CIAT and with observations made in activity 1.3.4 (see below), both of which indicated that tannins from *C. calothyrsus* CIAT 22310 have a higher protein-binding capacity (i.e lower degradability by rumen microbes) than those from *C. callothyrsus* CIAT 22316. This difference in protein-binding capacity between provenances of *Calliandra* has been associated in previous studies with the monomer composition of the extractable CT (ECT) fraction. The ECT from CIAT 22310 have more delphinidin units than the ECT from CIAT 22316, which have more cyanidin units. In temperate legume it has also been shown that tannins from legumes with more delphinidin units (*Lotus pedunculatus*) are more reactive with Rubisco than tannins from legumes with more cyanidin units (*Lotus corniculatus*).

1.3.4 In vitro ruminal fermentation of grass-legume mixtures as affected by PEG and by increasing proportions of purified tannins from different legumes

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Rationale

Low quality of ruminant diets, in conjunction with low feed conversion efficiency leads to low levels of animal production in tropical regions. Protein deficiency is by far the most important cause of low performance of ruminants maintained on low-quality forages, and ensuring adequate ammonia levels in the rumen for microbial growth is the first priority in optimizing fermentative digestion of forage. Promising forage legume species have been identified to overcome these limitations. Many of these legumes contain tannins that could be either advantageous or disadvantageous in terms of feed efficiency and metabolizable protein supply to the animal. The three multipurpose legume species, *Flemingia macrophylla*, *Calliandra calothyrsus* and *Leucaena leucocephala*, have shown good growth performance and could serve as forage supplements for ruminants fed tropical grass-based diets. Results from previous studies showed large differences in the tannin content among these legumes and suggested that there could also be differences in the type of tannins. However, little is known about the relevance of these differences and the effect of different tannins on digestion in ruminants.

Material and Methods

In Experiment 1, seven contrasting grass-legume mixtures were tested using the gas transducer technique. A low quality hay of the tropical grass *Brachiaria humidicola* (CIAT 6133; formerly called *B. dictyoneura*), cut after a growing period of 12 weeks, was used as basal substrate in all treatments. As supplements (1/3 of dry matter), leaves of the shrub legume species *Calliandra calothyrsus* (CIAT 22310 and 22316), *Cratylia argentea* (CIAT 18516), *Flemingia macrophylla* (CIAT 17403), *Leucaena leucocephala* (CIAT 734) and *Desmodium velutinum* (CIAT 33443) and the herbaceous legume *Vigna unguiculata*

(cowpea; CIAT 391) were used. Leaves of shrub legumes were harvested manually eight weeks after the last cut. The material was immediately stored at –20°C and subsequently freeze dried.

The foliage of cowpea (the whole plant, before flowering) was harvested eight weeks after establishment and sun dried for three days. The dried plant material of all species was ground in a laboratory mill to pass a 1 mm screen. All mixtures were incubated without and with polyethylene glycol (PEG), to bind and inactivate soluble condensed tannins. The levels of PEG addition were determined according to the species specific tannin content and were equivalent to 0.33, 0.66 and 1 of the analyzed tannin content. Additionally, all mixtures, including those without any detectable amounts of condensed tannins, were incubated with 240 mg of PEG/g of legume dry matter. This level corresponded to the highest condensed tannin content in legume dry matter observed in the experiment. The experiment was conducted for 144 h with measurements of gas pressure and volume after 3, 6, 9, 12, 24, 36, 48, 60, 72, 96, 120 and 144 h. The solid fermentation residues were analyzed for dry matter and nitrogen content and the fermentation fluid was analyzed for volatile fatty acids.

In Experiment 2, a mixture of *Brachiaria humidicola* (CIAT 6133 and cowpea (2:1) was incubated without or with the addition of increasing levels (25, 50, 75 and 100 mg/g DM) of purified tannins extracted from *C. calothyrsus* (CIAT 22310 and 22316), *F. macrophylla* (CIAT 17403) and *L. leucocephala* (CIAT 734). The measurement of gas production was conducted as described above, but the incubation period lasted for 168 h, to assure that the plateau phase was reached in all treatments.

Results and Discussion

In Experiment 1, PEG addition increased ($P<0.05$) the apparent in vitro dry matter degradation (IVDMD) of the diet containing *C. calothyrsus* 22310, tended to increase ($P<0.1$) IVDMD of the diet containing *F. macrophylla*, had no effect on diets with *C. calothyrus* 22316, *C. argentea* or *L. leucocephala*, and tended to decrease ($P<0.1$) IVDMD of diets containing Desmodium or cowpea. However, it has to be acknowledged that the determination of IVDMD in the presence of PEG is not always free of error, because part of the PEG added may be bound in the solid residue and this could have resulted in an underestimation of the IVDMD in the treatments with PEG. In all mixtures containing tanniniferous legumes, the percentage of crude protein (CP) apparently degraded was increased ($P<0.0001$) by PEG addition. In contrast, in the mixtures with *C. argentea* or *D. velutinum*, the amount of CP degraded tended ($P<0.1$) to decrease, and in the mixture with cowpea significantly less CP was degraded in the presence of PEG ($P<0.05$). The reasons for this unexpected decrease in the amount of CP degraded due to PEG addition are unknown.

The gas production parameters agreed well with the data on IVDMD. Gas production increased with PEG addition in all treatments with tanniniferous legumes. Without PEG addition, treatments with *C. calothyrsus* 22316 and *F. macrophylla* showed a lower initial gas production than the remaining mixtures. This could be related to an inhibition of the initial microbial activity (colonization of forage particles) due to the presence of tannins.

The effects of PEG addition on the concentration of individual volatile fatty acids (VFA) were very variable. In the treatments with *C. calothyrsus* 22310, PEG addition had no effect ($P>0.05$) on the proportion of acetate or propionate but increased ($P<0.05$) the proportion of butyrate. In treatments with *C. calothyrsus* 22316, the total concentration of VFA and the proportion of individual VFA did not show any change due to PEG addition. In the treatments with *F. macrophylla* or *L. leucocephala*, concentration of VFA was not affected ($P>0.05$) by the addition of low or intermediate levels of PEG, but increased with the addition of high levels of PEG.

Overall, there seemed to be a trend for higher VFA concentrations when high levels of PEG were added to mixtures containing tanniniferous

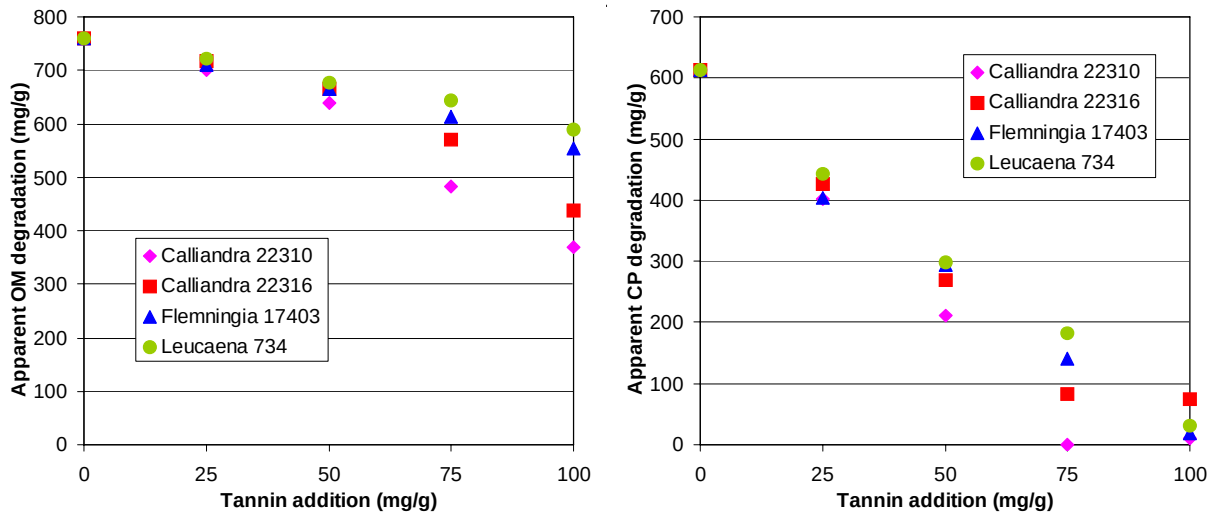

Figure 1. Effect of extracted tannins from different shrub legumes on in vitro apparent organic matter (OM) and crude protein (CP) degradation.

legumes. This trend was more pronounced with butyrate than with acetate or propionate concentrations. In treatments with legumes without detectable amounts of condensed tannins, PEG addition had no effect (P>0.05) on the concentrations of VFA except in the treatment with *C. argentea*, where PEG addition tended to increase (P>0.1) total concentration of VFA.

A comparison of the treatments with tanniniferous legumes incubated with the highest level of PEG (240 mg/g legume dry matter), which is supposed to bind and inactivate all soluble condensed tannins, showed differences in fermentation characteristics of the various diets. The apparent IVDMD was highest with *L. leucocephala,* intermediate with *C. calothyrsus* 22310, and lowest with *C. calothyrsus* 22316 and with *F. macrophylla* (P<0.05). The apparent CP degradation was highest with *C.calothyrsus* 22310 (67%), intermediate with *L. leucocephala* (55%), and lowest with *C. calothyrsus* 22316 (45%) and with *F. macrophylla* (43%) (P<0.05). The concentration of acetate on the fermentation fluid was higher (P<0.05) with *L. leucocephala* than with *C. calothyrsus* 22316, but did not differ significantly with the other two legumes. Concentrations of propionate and butyrate were higher (P<0.05) with *L. leucocephala* than with any other legume. Based on these results, the feeding value of the tested legumes could be ranked as follows: *Leucaena leucocephala* 734 > *Calliandra calothyrsus* 22310 > *Flemingia macrophylla* 17403 > *Calliandra calothyrsus* 22316.

Results from this experiment indicate that a PEG: tannin ratio of 1:1 is not always sufficient to bind and inactivate all soluble tannins. While in mixtures with *C. calothyrsus* 22310 a ratio of 2:3 was adequate to maximize apparent CP degradation, in mixtures with *C. Calothyrsus* 22316 or *F. macrophylla*, CP degradation increased throughout the range of PEG addition tested. This suggests that the PEG: tannin ratio required to maximize ruminal fermentation varies among species and is probably influenced by the type of tannins present in the legumes. To assure a complete inhibition of condensed tannins, the amount of PEG added to the diet should therefore always exceed the estimated amount of tannins in the diet.

Results from Experiment 2 showed that apparent IVDMD decreased with increasing addition of tannins regardless of the origin of the tannins. However, the extent of the reduction varied (P<0.05) among tannins (Figure 1). The largest decrease was observed with tannins from *C. calothyrsus* 22310, followed by tannins from *C. calothyrsus* 22316 and *F. macrphylla*. The smallest decrease was found with tannins from *L. leucocephala.* The apparent CP degradation was drastically decreased with

increasing levels of tannins (61% without tannins, 40-45% with 25 mg, 20-30% with 50 mg, less than 18% with 75 mg and less than 7.5% with 100 mg tannins) ($P<0.001$). With the highest tannin level, CP degradation was almost completely inhibited. Although the effect of tannins on CP degradation was more pronounced than the effect on IVDMD, the differences among types of tannins were smaller. The decrease in CP degradation was larger ($P<0.05$) with tannins from *C. calothyrsus* 22310 than with tannins from the remaining legumes.

Concentrations of acetate, propionate and butyrate in the fermentation fluid decreased ($P<0.001$) with increasing tannin addition and the decrease varied with the origin of the tannins. Acetate concentration was less affected by tannins from *F. macrophylla* than by any other tannins ($P<0.05$). Propionate concentration was higher ($P<0.05$) with tannins from *F. macrophylla* or *L. leucocephala* than with tannins from *C. calothyrsus* 22310 or 22316. Butyrate concentration was highest ($P<0.05$) with tannins from *F. macrophylla*, intermediate with tannins from *L. leucocephala* and lowest ($P<0.05$) with tannins from *C. calothyrsus*.

These results confirm that large differences may exist in the effects of tannins from different legume species on ruminal fermentation. The results further suggest that tannins from *L. leucocephala* and *F. macrophylla* were less effective in decreasing ruminal nutrient degradation and VFA production than tannins from *C. calothyrsus*. Among the two accessions of *C. calothyrsus*, tannins from the accession 22310 were more effective in reducing ruminal nutrient fermentation than those from the accession 22316.

1.3.5 Evaluation of the effects of legume mixtures on rumen microbial populations

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Rationale

The rumen is a complex ecosystem and the population dynamics of ruminal microbes are affected by multiple factors. Recent studies showed that manipulation of ruminal fermentation or a change of the diet may alter the population composition of the whole ecosystem. In respect to the low quality of tropical forages and the increasing demand for more food of high quality, strategies to improve animal nutrition and to increment productivity are required. One possibility could be to raise the proportion of feed protein that escapes rumen fermentation by the inclusion of tanniniferous plants in the diet. However, up to now, relatively little is known about the effects of tanniniferous diets on microbial population dynamics in the rumen. Several modern techniques, including polymerase chain reaction (PCR) and real time PCR (rtPCR), are available to monitor microbial populations, but it is unknown whether these techniques give reliable results with ruminal fluid samples containing tannins. To test the reliability of these techniques, several protocols were applied and compared, and samples of ruminal fluid with and without tannins were analyzed.

Material and Methods

For DNA extraction, three protocols were tested: (i) the protocol recommended by the International Atomic Energy Agency (IAEA) for ruminal fluid; (ii) a protocol for extraction of DNA from gram negative bacteria, adapted for rumen fluid samples; and (iii) a protocol from BioTechniques recommended for ruminal fluid containing tannins. The primers used were made available by Chris McSweeney (CSIRO, Australia) in accordance with IAEA and were specifically designed for this purpose to detect *Archaea*, *Bacteria*, *Fungi*, *Fibrobacter succinogenes* and

Ruminococcus flavefaciens. For conventional PCR, oligonucleotid primers from IAEA and PCR chemicals from Boehringer Mannheim, Germany were used according to manufacturer's recommendations. The PCR-products were separated by agarose-gel electrophoresis (2% gels), stained with ethidium bromide (EB) and visualized under UV. For rtPCR the instructions of the iCycler IQ real-time manual (Biorad, CA) were followed. All reactions were carried out as recommended by the IAEA.

To establish the protocol for rtPCR, DNA was extracted from rumen fluid of a fistulated Holstein bull by the protocol of the IAEA. Concentration and A260:A280 DNA ratios were determined by photospectroscopy, resulting in 250 ng/ì l at a ratio of 1.84. The ratio is based on the fact, that some proteins and phenolic compounds show a peak at 280 nm. This may affect the spectrometric results of DNA measurement at 260 nm. As reference of the impact of such contaminations the ratio allows to calculate the true DNA content resulting from the obtained peak. Based on this DNA, dilutions were prepared of 10, 1, 0.1, 0.01, 0.001 and 0.0001 ng/ì l which were then used to generate a calibration curve using the primer for total bacteria. Subsequently, the amplification of DNA extracted from tannin rich samples was carried out, applying the same protocol as mentioned above.

Results and Discussion

The three protocols evaluated for extraction of DNA from ruminal fluid showed differences in efficiency and manageability (Table 10). The best results were obtained with the protocol recommended by IAEA. Although the quality of the extracted DNA, i.e. purity, was equal with that obtained by the protocol for gram negative bacteria, the efficiency was much higher, yielding the double amount of DNA. The control by electrophoresis showed very little background for samples extracted according to the protocol of IAEA.

Over all, the protocol for gram negative bacteria yielded comparable results. However, a big disadvantage of this technique is the resulting high risk waste of chloroform. Furthermore, it is not known if DNA of all microbial groups of interest was extracted because this technique was specifically developed to extract DNA of gram negative bacteria. Both techniques showed good reproducibility.

The protocol of BioTechniques was less suitable than the other two and yielded a slightly brownish extract instead of a transparent one. This was also reflected by a low ratio value and a higher standard deviation in the concentration of DNA obtained, and resulted in a lower repeatability.

The primers provided by IAEA amplified well for conventional PCR and for rtPCR, and with samples extracted according to the protocol of IAEA and the protocol for gram negative bacteria (Figures 2 and 3).

These results indicate that the method for DNA extraction and amplification was successfully established and suggest that rtPCR could be a reliable tool to monitor microbial populations in ruminal fluid of animals feed tanniniferous diets.

Table 10. Comparison of the three evaluated methods for DNA extraction

Method	Concentration [ng/µl]	STD [ng/µl]	Ratio (260/280nm)	STD (260/280nm)	µl DNA
IAEA	45.5	8.2	1.89	0.34	100
Gram negative	46.8	14.2	1.96	0.69	50
BioTechniques	61.3	72.9	0.98	0.48	50

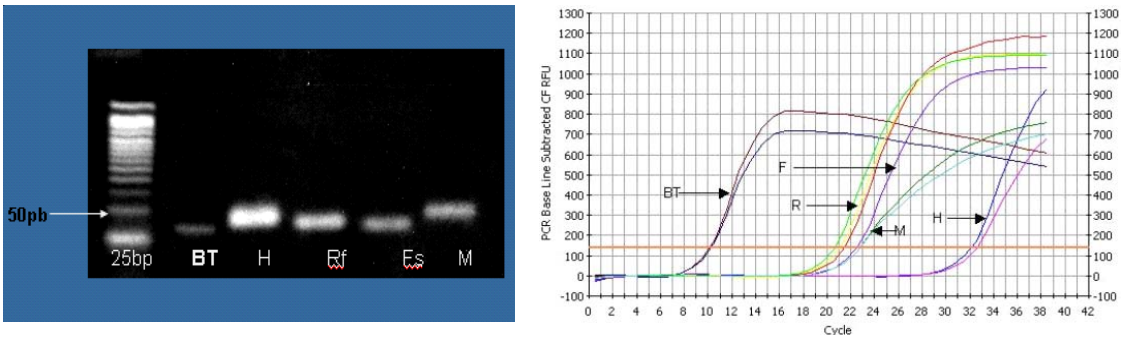

Figure 2. Amplification of PCR products with specific primers for conventional PCR and real time PCR (BT, total bacteria; H, fungi; R, *Ruminococcus flavefaciens*; F, *Fibrobacter succinogenes*; *M. archaea*).

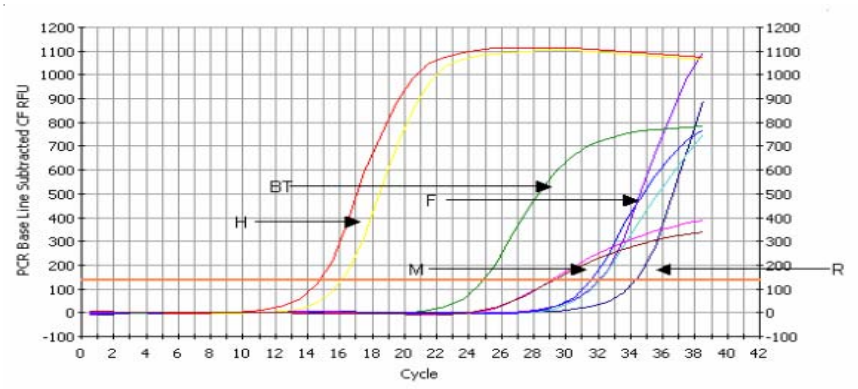

Figure 3. Amplification of DNA of rumen fluid samples rich in tannins (BT, total bacteria; H, fungi; R, *Ruminococcus flavefaciens***;** F**,** *Fibrobacter succinogene***s;** M**,** *Archaea***).**