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The Effects of Two Species of Leucaena on In Vitro Rumen Fermentation, Methane Production and Post-Ruminal Protein Supply in Diets Based on *Urochloa hybrid* cv. Cayman

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Abstract: This study evaluates the effects of the inclusion of two different *Leucaena* species as a source of condensed tannins based on in vitro fermentation, methane production and post-ruminal protein supply in a diet based on *Urochloa hybrid* cv. Cayman CIAT BR02/1752 grass. Under in vitro conditions, *Leucaena leucocephala* CIAT 17263 and *Leucaena diversifolia* ILRI 15551 were incubated in two proportions (25% and 50% w/w) with Cayman grass and with/without polyethylene glycol as a tannin-binder. The results show that substrates with *Leucaena diversifolia* produced less gas and methane than those with *Leucaena leucocephala* with and without polyethylene glycol. The mass in undegraded feed + solid associated microbes fraction decreased linearly with increasing level of inclusion of both species of *Leucaena*, while increasing its nitrogen content. It is concluded that the condensed tannins of *Leucaena diversifolia* possess the superior activity and that the tannin content of both evaluated *Leucaenas* did not affect the diet degradation, and reductions in post-ruminal protein did not occur in the present study. Further studies are needed to differentiate the tannins present in different *Leucaena* species and their dietary effects.

Keywords: condensed tannin; microbial crude protein; ruminant; legumes

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1. Introduction

Feed becomes a substrate for rumen microbes that grow and eventually flow to the abomasum, together with undegraded feed particles, becoming an important source of nutrients for the animal [1]. The efficiency of ruminant production depends mainly on the quality and quantity of feed consumed which are limited in the tropics. Therefore, there is a need to incorporate forages in ruminant diets that improve animal productivity while mitigating greenhouse gas emissions such as enteric methane (CH_4).

A promising strategy to increase animal productivity in tropical livestock production systems is the dietary inclusion of legumes and fodder of high nutritional value, which often results in additional benefits such as the reduction of greenhouse gas emissions such as CH_4 [2,3] and also in carbon capture in trees and shrubs included in silvopastoral

systems [4]. For example, the dietary inclusion of the shrub legume *Leucaena leucocephala* (leucocephala), a plant known for its tannins content, has been associated with increases in diet digestibility, dry matter intake, better use of intake protein and decreased CH_4 emissions [5–7]. The presence of tannins in the diet can be beneficial or detrimental depending on their level and nature [8] and their molecular weight and proanthocyanidin content [9]. High tannin levels are often associated with decreased forage intake, dry matter digestibility, and nitrogen (N) utilization [10]. On the other hand, low levels of tannins can benefit protein usage and reductions in CH_4 emissions [11,12].

It has been shown that condensed tannins (CT) can precipitate and bind to proteins [13,14]. The formation of tannin-protein complexes may result in proteins not being degraded by rumen microorganisms, and thus, the dietary flow of proteins to the abomasum and small intestine increases [15]. Hence, CT can improve the efficiency of N utilization in ruminants [16]. At the same time, their presence can lead to reduced ruminal methanogenesis through their effect on methanogens and indirectly through decreases in fiber degradation [17]. Montoya et al. [18] showed an inverse relation between tannins concertation in a diet containing leucocephala leaves and CH_4 emissions, suggesting a dose-effect. However, other important parameters, such as dry matter and organic matter intakes, and gross energy intake remained unchanged.

In ruminant feeding systems, leucocephala is the most commonly studied tropical shrub legume. However, another *Leucaena* species, *Leucaena diversifolia* (diversifolia), is widely distributed and has also been proposed as a forage that can increase the nutritional quality of cattle diets and reduce enteric CH_4 emissions [19]. The main difference between both species is their adaptive characteristics; *leucocephala* grows best at altitudes up to 2000 m.a.s.l., in alkaline soils (pH 7.0–8.5) and tolerates long dry seasons, whereas *diversifolia* can grow at a maximum altitude of 1500 m.a.s.l., tolerates better low temperatures and flourishes in more acidic soil conditions (pH 5.5–6.5) which are the most common in the tropics [20].

We hypothesize that legumes improve the nutritional quality of the diet and can potentially reduce CH_4 emissions due to the presence of CT. To test this hypothesis, this research aims to evaluate the effects of including two *Leucaena* species as a source of CT based on in vitro fermentation, CH_4 production, and post-ruminal protein supply in a diet based on *Urochloa hybrid* cv. Cayman (CIAT BR02/1752) (Cayman) grass.

2. Materials and Methods

2.1. Treatments and Forages Evaluated

Treatments consisted of the inclusion of *Leucaena leucocephala* CIAT 17263 (Leucocephala) and *Leucaena diversifolia* ILRI 15551 (diversifolia) in two different proportions of inclusion as dry matter (DM) basis (25% and 50%), with and without polyethyleneglycol (PEG) (molecular weight 4000) to a control treatment of *Urochloa hybrid* cv. Cayman CIAT BR02/1752 grass (Cayman). Forages were collected from experimental plots established at the International Centre for Tropical Agriculture (CIAT), Palmira, Valle del Cauca, Colombia (3°30′7″ N 76°21′22″ W) at an altitude of 1050 m.a.s.l, with an average annual temperature and rainfall of 27 °C and 1008 mm, respectively. *Leucocephala* and *diversifolia* were collected after 60 days of regrowth, and Cayman was collected 42 days after regrowth. After drying (55 °C until constant weight), the forage samples were ground (Wiley mill, Thomas®, Swedesboro, NJ, USA) to a particle size of 1 mm for subsequent bromatological analysis and in vitro experiments.

Forages were analyzed at the Chemical and Bromatological Analysis laboratory of the Faculty of Agricultural Sciences, Universidad Nacional de Colombia, Medellín, Colombia. The forages were analyzed for their concentrations of crude protein (CP) [(CP = N concentration × 6.25) Kjeldahl method [21]; neutral detergent fiber (NDF), and acid detergent fiber (ADF) by the methodologies proposed by Van Soest et al. [22]; ether extract (EE) through Soxhlet extraction by immersion, based on the NTC 668 method [23] and organic matter (OM) content was calculated as the 1000-ash concentration in g kg^{-1} DM. Condensed tannins (CT) were determined only in the legumes, according to Terrill et al. [24] (Table 1).

Table 1. Nutrients and condensed tannins content in the diets utilized for the in vitro incubations. Cayman: *Urochloa hybrid* cv. Cayman (CIAT BR02/1752); *leucocephala*: *Leucaena leucocephala* CIAT 17263; *diversifolia*: *Leucaena diversifolia* ILRI 15551.

Variable	Cayman	*Leucaena leucocephala*	Cayman + *Leucocephala*		*Leucaena diversifolia*	Cayman + *Diversifolia*	
	100%	100%	25%	50%	100%	25%	50%
Organic matter, g kg^{-1} DM	918.6	933.2	918.4	918.2	934.7	921.3	923.9
Crude protein, g kg^{-1} DM	88.1	280.1	136.2	184.2	310.2	143.6	199.1
Neutral detergent fiber, g kg^{-1} DM	589	354	484.5	380	366	492.3	395.5
Acid detergent fiber, g kg^{-1} DM	296	246	278	260	252	267.8	239.5
Ether extract, g kg^{-1} DM	19.6	21.2	20.3	20.9	22.3	19.7	19.8
Condensed tannins *, g kg^{-1} DM	-	32.2	-	-	37.8	-	-

* Content of condensed tannins was determined only for the *Leucaena* forage samples. Each of the forages components in the diets was analyzed individually. Values reported for the diets were calculated from the respective percentages of inclusion of each forage.

2.2. In Vitro Experiments

Two in vitro experiments were carried out at the Institute for Animal Nutrition and Rangeland Management in the Tropics and the Subtropics of the University of Hohenheim, Stuttgart, Germany. Both experiments were conducted using the Hohenheim gas test (HGT) method, following the official method 25.1-VDLUFA, 2007 (Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten). In each experiment, three different runs were performed on separate days, with samples accurately weighed (380 ± 1 mg) into 100-mL glass syringes (three syringes for each treatment in experiment 1 and six syringes for each treatment in experiment 2). Six syringes were included as blanks containing only the incubation media and a standard substrate to correct gas production.

Rumen fluid was collected from two rumen-fistulated Jersey cows fed *ad libitum*, a total mixed ration containing corn silage, grass silage, grass hay, barley straw and a commercial concentrate. The total mixed ration had a forage to concentrate ratio of 62:38 (DM basis) and contained 140 g CP/kg DM. Drinking water was offered *ad libitum*.

2.2.1. Experiment 1: In Vitro Fermentation Kinetics, Organic Matter Digestibility (*IVDOM*) and Metabolizable Energy (*ME*)

An in vitro experiment was carried out to study the fermentation kinetics and to calculate metabolizable energy (*ME*; Equation (1)) and in vitro digestible organic matter (*IVDOM*; Equation (2)) according to Menke and Steingass [25].

$$ME_{(\text{MJ kg/DM})} = 1.242 + (0.146 \times GP) + (0.007 \times CP) + (0.0224 \times EE) \quad (1)$$

$$IVDOM_{(\text{g/100g DM})} = 14.88 + (0.889 \times GP) + (0.0045 \times CP) + (0.065 \times Ash) \quad (2)$$

where, *GP*: Gas production (mL for 200 mg *DM* of the incubated sample) after 24 h of in vitro incubation *CP*: Crude protein (g/kg *DM*); EE: Ether extract (g/kg *DM*)

Gas production was recorded at 2, 4, 6, 8, 12, 24, 36, 48, 72 and 96 h of incubation. The accumulated gas production curves were adjusted through non-linear procedures with the CurveExpert Professional 2.1.0 program [26]. The model used was a modification of the Gompertz expression (Equation (3)) [27]:

$$y = e^{a - be^{-cx}} \quad (3)$$

where, y = cumulative gas production at a time x; $a > 0$ is the maximum gas production; $b > 0$ difference between the initial gas and the final gas at a time x; $c > 0$ specific rate of gas accumulation.

The following fermentation indicators were calculated:

HIP: hour at the inflection point (h); $HIP = {}^{b}/_{c}$

GIP: gas at the inflection point (mL); $GIP = {}^{a}/_{e}$
MGPR: maximum gas production rate (mL/h); $MGPR = {}^{(a \times c)}/_{e}$
LP: Lag phase or microbial establishment (h); $LP = {}^{b}/_{c} - {}^{1}/_{c}$
Note: The value of e corresponds to the Euler number ≈ 2.718281828459

2.2.2. Experiment 2: Fermentation Parameters, Methane Production, Dietary CP Degradation and Microbial Growth

A second in vitro experiment was carried out to study the effect of both *Leucaenas* on different fermentation parameters, CH_4 production, dietary CP degradation, and microbial growth following the same incubation procedure described above. After 24 h of incubation, GP was measured, and syringes were transferred to a fridge with a temperature set at 4 °C to stop the fermentation process. After that, the concentration of CH_4 in the gas was measured using an infrared CH_4 analyzer (Pronova Analysentechnik GmbH and Co. KG, Berlin, Germany) calibrated against 10.6 mL CH_4/100 mL gas [28].

For determination of microbial mass, the contents of each syringe were transferred to falcon tubes of 50 mL and centrifuged at 500× *g* for 10 min at 4 °C (Hettich Rotanta RPC, Andreas Hettich GmbH and Co., KG, Tuttlingen, Germany). The solid fractions after this process correspond to undegraded feed particles and solid-associated microbes (Ufeed+SAM). Next, the supernatant was transferred in another falcon tube 50 mL and centrifuged at 20,000× *g* for 10 min at 4 °C (Avanti™ J25, Becker Coulter™, Indianapolis, IN, USA). The solid fraction after this process corresponds to liquid-associated microbes (LAM) [29]. Recovered UFeed+SAM and LAM samples were lyophilized, weighed, ground using a ball mill (Retsch, MM200, Haan, Germany) for 2 min at a frequency of 30/s, and then stored until further analysis.

After the second centrifugation, 15 mL of the final supernatant was used for NH_3-N (mg/mL) analysis by the indophenol reaction [30]. 20 µL of supernatant collected during centrifugation of samples was transferred into 2 mL vial and was added 900 µL of reagent A (2.5 g phenol + 12.5 mg sodium-nitroprusside dissolved in 250 mL distilled water). The mixture was then centrifuged for 45 s at 10,000× *g* for 10 min at 4 °C (Biofuge, Heraeus, Hanau, Germany). After 900 µL of reagent B ((2.5 g sodium hydroxide + 2.1 mL sodium hypochlorite (containing 12% chlorine)) was added and then incubated for 20 min at 35 °C. After incubation, the solution was transferred to a semi-micro cuvette, and the samples were read at 625 nm using a spectrophotometer (Varian Cary 50 Bio, UV–vis, Palo Alto, CA, USA).

Nitrogen concentrations in LAM and Ufeed+SAM fractions were determined using a C/N analyzer system (Vario Max cube CN, Elementar Analysensysteme GmbH, Frankfurt am Main, Germany). The microbial N yield in both fractions was calculated by multiplying the N concentrations by the weight of the LAM or Ufeed+SAM fractions. The microbial crude protein synthesis was expressed as a sum of microbial N yield of LAM and Ufeed+SAM. Finally, N post-ruminal recovery efficiency was calculated as the difference between the initial N content of forage samples and N content recovered after the fermentation process.

2.3. Statistical Analysis

Effects of the *Leucaena* species, level of inclusion and presence of PEG (main effects) on the parameters studied were tested using a mixed procedure (PROC MIXED) by SAS (version 9.2, SAS Institute, Cary, NC, USA). Significant effects of the level of inclusion of *Leucaena* were analyzed using orthogonal polynomial contrasts, estimating the probability of linear or quadratic responses. All significant differences were declared at $p < 0.05$ using the following model:

$$Y_{ijkl} = \mu + S_i + L_j + P_k + (SL)_{ij} + (SP)_{ik} + (LP)_{jk} + (SLP)_{ijk}R_l + \varepsilon_{ijkl} \quad (4)$$

where, Y_{ijkl} is the dependent variable; μ is the overall mean; S_i is the effect of species i; L_j is the effect of level of *Leucaena* inclusion j; P_k is the effect of PEG k; $(SL)_{ij}$ is the interaction

effect between specie i and level of *Leucaena* inclusion j; $(SP)_{ik}$ is the interaction effect between specie i and PEG k; $(LP)_{jk}$ is the interaction effect between level of *Leucaena* inclusion i and PEG k; $(SLP)_{ijk}$ is the interaction effect between specie i, level of *Leucaena* inclusion, and PEG k; R_l is the effect of run l (random effect); ε_{ijkl} is the error term.

3. Results

Parameters estimated by applying the Gompertz model to the in vitro fermentation data are shown in Table 2. In this study, the different treatments had a phase of establishment or colonization (lag phase) that ranged from 1.17 to 1.66 h. The lag phase increased with the inclusion of PEG and *leucocephala* but decreased linearly with increasing levels of *diversifolia* ($p = 0.01$). The effects of PEG addition on the lag phase were more evident at high levels of *Leucaena* inclusion ($p = 0.04$). Thus, PEG addition led to a 3.37% increase in the lag phase in the 25% *leucocephala* diet and 6.41% in the 50% *diversifolia* diet. For *diversifolia*, PEG addition led to an 11% increase in lag phase in the 25% *diversifolia* diet and 18.8% in the 50% *diversifolia* diet.

The maximum gas production rate was higher in treatments with *L. leucocephala* than in those with *diversifolia* ($p < 0.0001$), and MGPR increased with increasing dietary inclusion of *leucocephala* but decreased with increasing inclusion of *diversifolia* ($p = 0.01$). Adding PEG increased MGPR ($p < 0.0001$) which was more evident at the highest levels of *Leucaena* inclusion (50%), especially for *diversifolia*. Thus, with the 25% *diversifolia* diet, MGPR increased by 2.80%, while with the 50% *diversifolia* diet, MGPR increased by 18.2%.

The highest gas at the inflection point occurred between 18 and 19 h and was observed in those treatments that accumulated the most gas (Tables 2 and 3). Gas production at the inflection point was higher for PEG treatments than those where PEG was absent ($p = 0.04$). Gas production at the inflection point increased as the inclusion level of *leucocephala* increased from 0% to 25%, but GIP decreased when *leucocephala* was included at 50% of the diet. In the case of *diversifolia*, GIP decreased linearly with increasing legume inclusion levels ($p = 0.01$).

Fermentation parameters and CH_4 production are shown in Table 3. In general, *leucocephala* had greater gas production than *diversifolia* ($p = 0.01$). In the presence of PEG, gas production increased linearly with an increasing level of inclusion of both legumes ($p = 0.05$). There was a three-way interaction for CH_4 emissions (g/g DM incubated ($p = 0.04$) where PEG did not have a major effect in treatments with different levels of *leucocephala*; while in *diversifolia* treatments, PEG caused an increase in CH_4 (g/g incubated DM) at the highest level of inclusion. Nevertheless, the addition of PEG had a strong effect on CH_4 emissions expressed as g/g incubated DM, particularly at the highest inclusion level of *diversifolia* ($p < 0.01$). When expressed as g/g degraded DM, CH_4 emissions aumented with increased inclusion of *leucocephala* CH_4 and decreased slightly with increasing inclusion levels of *diversifolia* ($p = 0.04$). In terms of ME and IVDOM estimates, *leucocephala* was higher than *diversifolia*, and PEG increased ME and IVOMD.

The effects of the inclusion of both *Leucaena* species on post-ruminal protein supply are shown in Table 4. In treatments with *leucocephala*, N yield in LAM fraction increased linearly with increasing level of inclusion ($p = 0.01$). However, for treatments including *diversifolia*, these variables decreased with an increasing level of inclusion. In the case of the Ufeed+SAM fraction, an increased level of inclusion from 25% to 50% of both *Leucaenas* seem to have no evident effect on the amount of mass in either of the *Leucaena* species evaluated ($p = 0.09$). However, Ufeed+SAM in *leucocephala* tends to be lower than that Ufeed+SAM in *diversifolia* ($p = 0.01$). The N content in this fraction increased linearly with increasing inclusion of both *Leucaena* species ($p < 0.01$), and in general, N content is higher in treatments with *diversifolia* ($p < 0.01$). Nevertheless, when comparing treatments with and without PEG, this had a different effect for both species of *Leucaena* ($p < 0.01$). In treatments with *leucocephala*, N content increased with PEG addition at the same level of inclusion. However, in treatments with *diversifolia*, N content decreased with PEG addition at the same level of inclusion.

Table 2. Effect of *Leucaena* inclusion on the degradation kinetics of *Urochloa hybrid* cv Cayman grass-based treatments. Cayman: *Urochloa hybrid* cv. Cayman (CIAT BR02/1752); *leucocephala*: *Leucaena leucocephala* CIAT 17263; *diversifolia*: *Leucaena diversifolia* ILRI 15551.

Variable	Cayman + *Leucocephala*					Cayman + *Diversifolia*				SEM	*p* Values								
	−PEG			+PEG		−PEG		+PEG			Species	Level	PEG	S×L	S×P	L×P	S×L×P	Linear	Quadratic
	0% *	25%	50%	25%	50%	25%	50%	25%	50%										
LP (h)	1.34	1.48	1.56	1.53	1.66	1.27	1.17	1.31	1.39	0.09	<0.0001	0.005	<0.0001	0.01	0.52	0.04	0.61	0.001	0.85
TIP (h)	10.63	9.97	8.66	10.02	8.41	10.16	8.77	10.61	8.46	1.15	0.58	0.001	0.90	0.96	0.31	0.80	0.20	0.001	0.01
GIP (mL)	18.82	18.87	18.10	19.20	18.60	17.46	16.41	18.22	17.55	0.75	0.003	0.001	0.04	0.01	0.97	0.10	0.46	<0.01	0.73
MGPR (mL)	1.44	1.59	1.67	1.64	1.77	1.38	1.26	1.42	1.49	0.09	<0.0001	0.011	<0.0001	0.01	0.51	0.04	0.59	0.003	0.68

* Treatment only with *Urochloa hybrid* cv Cayman grass; −PEG: Without polyethyleneglycol; +PEG: With polyethyleneglycol; S×L = Specie×Level; S×P = Specie×PEG; L×P = Level×PEG; S×L×P = Specie×Level× PEG; LP = Lag phase; TIP = Time to inflection point; GIP = Gas at the inflection point; MGPR = Maximum gas production rate.

Table 3. Effect of the inclusion of *Leucaena* on fermentation parameters and methane production of *Urochloa hybrid* cv Cayman grass-based treatments. Cayman: *Urochloa hybrid* cv. Cayman (CIAT BR02/1752); *leucocephala*: *Leucaena leucocephala* CIAT 17263; *diversifolia*: *Leucaena diversifolia* ILRI 15551.

Variable	Cayman + *Leucocephala*					Cayman + *Diversifolia*				SEM	*p* Values								
	−PEG			+PEG		−PEG		+PEG			Species	Level	PEG	S×L	S×P	L×P	S×L×P	Linear	Quadratic
	0% *	25%	50%	25%	50%	25%	50%	25%	50%										
Gas production (mL/mg DM)	266.5	309.2	310.4	314.6	316.1	290.4	280.8	296.2	297	1.27	<0.01	<0.01	<0.01	0.00	0.32	0.05	0.42	<0.01	<0.01
CH_4 (g/g DM incubated)	16.3	16.8	18.2	16.7	17.9	15.6	14.5	16.3	16.8	0.77	<0.01	0.07	0.06	<0.01	<0.01	0.18	0.04	0.03	0.38
CH_4 (g/g DM degraded)	38.1	36.9	38.7	35.7	37.7	36.0	35.2	37.9	39.1	2.01	0.88	0.04	0.80	0.75	0.03	0.45	0.23	0.50	0.01
ME (MJ/kg DM)	5.67	6.2	6.36	6.31	6.44	5.73	5.41	5.72	5.75	0.20	<0.01	<0.01	<0.01	0.15	0.67	0.33	0.38	<0.01	0.01
IVDOM (g/100 g OM)	42.15	45.28	46.56	46.25	46.86	42.71	40.47	42.87	42.57	1.25	<0.01	<0.01	<0.01	0.17	0.74	0.31	0.35	<0.01	0.02

* Treatment only with *Urochloa hybrid* cv Cayman grass; S×L = Specie×Level; S×P = Specie×PEG; L×P = Level×PEG; S×L×P = Specie×Level×PEG; CH_4 = Methane; ME = metabolizable energy; DM = dry matter; IVDOM = organic matter digestibility; OM = organic matter.

Table 4. Effect of the inclusion of *Leucaena* on the content of liquid-associated microbes (LAM) and undegraded feed particles+solid-associated microbes (Ufeed+SAM) after 24 h of incubation of *Urochloa hybrid* cv Cayman grass-based treatments. Cayman: *Urochloa hybrid* cv. *Cayman* (CIAT BR02/1752); leucocephala: *Leucaena leucocephala* CIAT 17263; *diversifolia*: *Leucaena diversifolia* ILRI 15551.

Variable	Cayman + *Leucocephala*					Cayman + *Diversifolia*				*p* Values									
	−PEG			+PEG		−PEG		+PEG		SEM	Species	Level	PEG	S×L	S×P	L×P	S×L×P	Linear	Quadratic
	0% *	25%	50%	25%	50%	25%	50%	25%	50%										
LAM- mass (mg DM/syringe)	188	282	364	311	341	323	273	309	388	0.004	0.93	<0.01	0.44	0.17	0.22	0.27	0.01	0.02	0.56
LAM-N (mg/100 mg DM)	8.26	9.72	9.79	9.64	9.77	9.64	9.07	9.77	9.40	0.04	0.60	<0.01	0.33	0.29	0.31	0.67	0.04	<0.01	0.03
Ufeed+SAM-mass (mg DM/syringe)	2620	2550	2530	2570	2500	2710	2640	2590	2600	0.01	<0.01	0.06	0.04	0.09	0.16	0.44	0.18	<0.01	<0.01
Ufeed+SAM-N (mg/100 mg DM)	3.51	4.23	5.03	4.28	5.03	4.18	5.45	4.33	5.10	0.03	<0.01	<0.01	<0.01	0.08	<0.01	0.02	0.01	<0.01	0.04
LAM+UFeed+SAM N (mg/syringe)	107	135	163	140	161	143	171	141	168	0.05	0.01	<0.01	0.17	0.90	0.36	0.63	0.65	<0.01	0.03
NH_3-N (mg/mL)	31.2	36.8	41.6	39.7	43.8	36.7	39.9	38.5	44.7	1.58	0.46	<0.01	0.85	<0.01	0.55	0.01	0.24	<0.01	<0.01
NRE (%)	78.3	62.10	55.30	64.40	54.90	62.50	53.70	61.60	52.70	5.78	0.11	0.01	0.14	0.11	0.12	0.11	0.10	0.008	0.11

* Treatment only with *Urochloa hybrid* cv Cayman grass; S×L = Specie×Level; S×P = Specie×PEG; L×P = Level×PEG; S×L×P = Specie×Level× PEG; NRE = Total N post-ruminal recovery efficiency.

In contrast, as the proportion of *Leucaena* inclusion increased, the N recovered after the ruminal fermentation process decreased ($p = 0.01$) (Table 4). Thus, the highest N recovery efficiency was for treatment without *Leucaena* inclusion (78.3%), and this variable did not present significant differences for species (*leucocephala or diversifolia*) or PEG presence effects ($p = 0.12$). However, there was a significant interaction between species, the inclusion level (percentage) of *Leucaena* inclusion and PEG addition for the following variables: the content of liquid-associated microbes (LAM) ($p = 0.01$) and N concentrations in Ufeed+SAM fractions ($p = 0.01$).

4. Discussion

Including legumes in animal diets can improve animal production parameters and mitigate greenhouse gases such as CH_4 [6,7]. Legumes are included in cattle diets mainly because they are a source of protein when animals are fed low-quality pastures, as legumes typically contain between 120 and 298 g CP/kg DM [31,32]. In addition, the dietary inclusion of legumes such as *leucocephala* may improve the diet's nutrients profile, increases forage intake and provides valuable amounts of rumen non-degradable protein [5,19,33,34]. Moreover, tannins and their ability to bind to proteins render the latter more resistant to ruminal degradation than other tropical forages [35].

In our study, the addition of forage from both species of *Leucaena* increased protein and decreased NDF concentrations in the diet (Table 1), which modified the fermentation parameters. The decreased NDF concentration, and its concomitant increase in fermentable carbohydrates, was probably the cause of the higher GP and CH_4 in the treatments that included *Leucaena*, as also reported in other studies [34,36]. The present study results show a lower CH_4 production when both *Leucaena* species are included at 25% compared to the grass alone treatment. Similar results were reported by Molina et al. [37] in an in vitro study with the inclusion of *leucocephala* 30%.

leucocephala has been widely studied and has been successfully used in cattle feeding systems in the tropics and subtropics. However, recent studies have suggested that *diversifolia* may have additional advantages. For example, a recent in vivo study in which both *Leucaena* species (*leucocephala* and *diversifolia*) were used in association with Cayman grass showed no significant differences between the two species but showed a tendency for *diversifolia* to cause a greater reduction in CH_4 emissions and greater nutrient intake [19]. The authors suggested that the higher tannin content in *diversifolia* could explain the registered CH_4 reductions.

When the effect of CT was evaluated (as per the inclusion of PEG), there was a linear decrease in CH_4 production only in *diversifolia* in the absence of PEG. However, in the case of treatments containing *leucocephala*, these values augmented with increasing inclusion levels. The results in this study highlight the differences between *Leucaena* species in modulating enteric CH_4 production. Although the CT content was higher for *diversifolia* (Table 1), differences are not probably high enough to conclude that observed effects are associated only with quantity, and therefore these differences may be associated with the activity of those tannins. A theory for this effect could be that the CT present in *diversifolia* forage prevented the degradation of organic matter so that the production of gas and CH_4 was lower, an effect not observable for *leucocephala*. Once the action of tannins is inhibited (PEG treatments), CH_4 production increases with the inclusion of both *Leucaena* species, so one can conclude that the activity of *Leucaena* tannins has an effect in reducing CH_4 production and that *diversifolia* tannins have a greater activity. Our results are consistent with those reported by García et al. [38], who characterized the nutritional quality of 53 accessions of the genus *Leucaena*. These authors determined that the edible biomass of *leucocephala* had better nutritional quality than that of *diversifolia*, but the latter presented a higher amount of phenols and CT and lower rumen degradability.

The mechanism by which tannins can modify the ruminal degradation of different diet components is diverse and unclear to some extent. However, it is widely accepted that tannins reduce the amount of degraded substrate, inhibit gut enzymes [39], and affect

some ruminal microorganisms [40,41]. Tannins can have beneficial or detrimental effects on livestock depending on various factors such as quantity, nature and molecular weight [8]. One of the most important adverse effects of tannins is their ability to combine with proteins, cellulose, hemicellulose and pectin, decreasing their digestion [42]. However, these effects on digestibility usually occur with the high dietary presence of tannins, which was more evident in this study when incubating *diversifolia*. Indeed, in this study, only tannins from *diversifolia* affected the diet digestibility (Table 3), perhaps because this species has a higher content of CT, and their activity might differ. The anti-methanogenic effect of tannins depends on the number of hydroxyl groups in their structure, and in the case of CT, reduced CH_4 emissions are more due to the inhibition of fiber digestion. In contrast, hydrolyzable tannins tend to act by directly inhibiting methanogenic microorganisms [43,44]. In addition, reductions in CH_4 production also depend on the molecular weight of CT contained in the forage biomass, so that the higher molecular weight of tannins is related to greater reductions in CH_4 production [45,46]. From the above it can be deduced that *diversifolia* tannins may probably have a higher molecular weight, suggesting that further studies should be conducted to provide a more precise explanation for the observed reduction in CH_4 production.

Piñeiro-Vásquez et al. [47] reported that the inclusion of 80% *leucocephala* in the diet of heifers, fed low-quality tropical forages, reduced CH_4 emissions by up to 61.3% without affecting dry matter and organic matter intake. In addition, neither was the protozoan population nor the molar volatile fatty acids concentration affected. However, more rumen-fermentable N was provided for microbial population growth, which partially confirms our findings in decreased CH_4 production without affecting digestibility when *leucocephala* is included at a level of up to 50%. Similarly, Tan et al. [48] reported that CT of *leucocephala*, with a relatively low CT content of 30 mg/g DM, reduced CH_4 production, decreased methanogen and protozoal populations and reduced N disappearance with only a 7% reduction of in vitro DM digestibility.

One key factor of ruminants' productivity is the supply of protein into the duodenum, both dietary and microbial protein. When analyzing the undegraded feed particles and solid-associated microbes (Ufeed+SAM), there was no evidence of an increment of the mass of this fraction with an increasing level of inclusion of both *Leucaena* species with no PEG added (Table 4). This result suggests that tannins did not affect diet degradation, therefore the inclusion of *Leucaena* in the diet could be advantageous to protect nutrients from digestion at the rumen level with the potential to increase the total nutrient supply available for absorption in the lower gut. Clearly, the inclusion of *Leucaena* in the diet can generate significant changes in nutrient metabolism, independent of its tannin content. More importantly, the total N (LAM+Ufeed+SAM) increased linearly with incresases in *Leucaena* level irrespective of the species evaluated, and this was the result of a marginally greater dietary undegraded N (N in Ufeed+SAM) and a clear increase in N from LAM. It is not clear if the presence of CT directly caused this because the effect of PEG on total N was not significant. However, it is important to consider that a fraction of CT in plants are bound to the substrate (DM or fiber) [35] and do not interact with PEG. Hence, if the proportion of bound CT is greater than that of soluble tannins, the addition of PEG might not result in changes in post-ruminal protein availability. In addition, legumes such as *leucocephala* have higher protein contents of fractions A, B1 and B2 that are of rapid degradation and intermediate degradation in the rumen [34], while also having higher concentrations of NDF-N [31], which have the potential to escape the rumen and be utilized in the duodenum. If the protein flow (dietary and microbial) increases in vivo when feeding legumes, such as this study suggests, the critical feature to prove this protein supply is available for absorption in future duodenum studies.

For rumen N retention, a decreased level was associated with increased inclusion of *Leucaena*. It is known that the amount of protein reaching the intestine depends mainly on the protein-energy balance of the diet. Ruminal microorganisms need carbohydrates and an N source to synthesize somatic proteins, and an imbalance between the two substrates

can affect rumen microbial protein production [49]. It has been reported that *Leucaena* levels above 30% do not cause negative impacts on productivity parameters or problems in using the protein for metabolic processes [50]. The LAM-mass and the LAM-N, both indicators of microbial growth in the in vitro system, increased with augmenting levels of inclusion of both *Leucaena* species evaluated, and no effect was evident with PEG addition. These observations would indicate the role of *Leucaena* in the promotion of microbial protein synthesis regardless of the tannins activity. However, more studies are needed to know precisely the metabolic processes by which this N is incorporated.

In addition to the points discussed above, these *Leucaena* species evaluated have other secondary compounds such as mimosine, alkaloids, saponins, steroids, among others [51,52] that were not quantified in the present work. These missing informationmay have help to better explain the response from the interactions between grasses and legumes.

5. Conclusions

In this study, the addition of forage from both species of *Leucaena* increased available protein and decreased the amount of NDF in the diet, which modified the fermentation parameters of the treatments evaluated. The *Leucaena* species evaluated showed different responses, with *diversifolia* showing a higher inhibition of CH_4 and total gas production, likely due to a higher activity of their CT. Since there was no increase in the mass of the Ufeed+SAM fraction when the inclusion level of *Leucaena* was increased, we can conclude that the tannin content of *Leucaena* did not affect the DM degradation of the diet. However, rises in the N amount available after the incubation with increasing levels of both *Leucaena* species indicate a potential to enhance the supply of protein when feeding legumes compared with tropical grass. Further studies are needed to differentiate the nature of tannins present in different *Leucaena* species, their dietary effects and the fate of the increased N supply after the rumen fermentation.

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