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Feeding Strategies to Increase Nitrogen Retention and Improve Rumen Fermentation and Rumen Microbial Population in Beef Steers Fed with Tropical Forages

Sandra Guisela Durango [1,*], Rolando Barahona [2], Diana Bolívar [2], Ngonidzashe Chirinda [3] and Jacobo Arango [1]

[1] International Center for Tropical Agriculture (CIAT), Cali 760001, Colombia; j.arango@cgiar.org
[2] Departamento de Producción Animal, Facultad de Ciencias Agrarias, Universidad Nacional de Colombia, Medellín 050034, Colombia; rbarahonar@unal.edu.co (R.B.); dmboliva@unal.edu.co (D.B.)
[3] AgroBioSciences (AgBS), Agricultural Innovations and Technology Transfer Centre (AITTC), Mohammed VI Polytechnic University (UM6P), Benguerir 43150, Morocco; nchirinda@gmail.com
* Correspondence: s.durango@cgiar.org

Abstract: The effect of the inclusion of *Leucaena leucocephala* and *Tithonia diversifolia* in Zebu steers receiving a diet based on improved pastures such as *Brachiaria decumbens* and *Brachiaria hybrid* cv Cayman on nitrogen (N) excretion, urinary volume and rumen microbial population was evaluated. To determine the dry matter intake and nutrient excretion, eight steers were used in a 4 × 4 Latin square design consisting of four periods and four diets. Four of them were cannulated for ruminal fluid extraction and quantification of ruminal microorganisms in three times of grazing (T0, T7 and T15). Forage intake was calculated through the external marker titanium dioxide. Diet including forages with superior protein content generated an increase in the gene copy numbers of *Prevotella ruminicola* and total bacteria on 15 sampling day ($p < 0.001$). Animals receiving diets with the dietary inclusion of *Leucaena* and *Tithonia* had daily N intakes of 228 and 113.5g N intake d^{-1}, of which they excreted 42% and 61%, respectively. Inclusion of both protein forages increased daily urinary volume (9% and 7% d^{-1}), with respect to the pasture-based diet. This study revealed that the inclusion of 18% Leucaena in a pasture-based diet improves the dry matter intake and N retention in Zebu steers under tropical conditions.

Keywords: dry matter intake; feed resources; *Leucaena leucocephala*; nitrogen balance; *Tithonia diversifolia*; total bacteria; urine volume

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1. Introduction

Improving productivity is a challenge for the entire agricultural sector, including livestock production, since the growth of the world's population requires meeting the growing demand for more food with fewer resources. Latin America and the Caribbean contribute 30% of the global demand for beef with South American countries (i.e., Brazil, Argentina, Uruguay, Paraguay and Colombia) contributing 80%, and the remaining 20% coming from Mexico and Central America [1]. Although it has all the potential to constitute a sustainable productive system, since its inception cattle production has developed as a productive activity based on grass monocultures, commonly offering diets characterized by high-fiber and low crude protein (CP) contents, specifically in the tropical region [2]. In cattle production systems, a low-protein diet reduces the efficiency of the productive system, since it can be unfavorable for microbial growth, ruminal digestion, availability of nitrogen (N) and carbohydrates by the animals [3]. Ruminants depend mainly on the essential amino acids obtained from the microbial protein and the energy provided by the organic acids produced during ruminal fermentation [4]. A deficit of metabolizable energy and the low CP content of the diets usually consumed by livestock generate losses of N and excretion of nutrients that lead to great economic and environmental costs. The excess

of N excreted as ammonium (NH_4) through the urine or dung leads to the accumulation of nitrates that can contaminate the aquifers or become a source of nitrous oxide (N_2O) emission from soil [5]. At the same time, a part of the NH_4 can be volatilized into the atmosphere as ammonia (NH_3) [6] generating environmental pollution. Protein is one of the most expensive nutritional source that must be covered in livestock feed in the tropics, therefore offering it in rich diets to be later lost through excreta as NH_4, reduces the efficiency and profitability of the production system.

An option that should be considered in the efforts to mitigate N_2O and CH_4 from livestock systems should contemplate the improvement of N-use efficiency and energy availability in the fodder diets offered. Further research is required into the potential of different local forage resources, to improve the nutritional status of animals in the tropics. Dietary changes can improve microbial fermentation and help reduce the loss of nutrients through excreta and the energy cost that this represents. Implementing feeding strategies that improve the efficiency of meat and milk production should lead to greater farmer profitability and reduced environmental impact.

The inclusion of legumes in ruminant diets can have positive results in terms of microbial activity and final fermentation products [7], which can contribute to N flow improvement in the cattle. Legumes are a good source of protein; they are rich in amino acids, vitamins and minerals and are good substrates of cellulolytic microorganisms for growth and enzymatic function [8].

Leucaena leucocephala (*L. leucocephala*) is a forage tree widely studied and is considered to be a high nutritional value forage legume that contains high levels of CP, favors dry matter degradation, and yields high amounts of biomass when grown in appropriate soils [9,10]. When sown in rows associated with tropical grasses, it constitutes a profitable and sustainable productive system [11,12]. This legume is often used in silvopastoral systems, which represent an important option for mitigation and adaptation since this system contributes to improve the resilience of livestock systems to the climate variability [1].There are non-leguminous forage resources that are also characterized by high protein levels, which can enrich tropical grasses-based diets. *Tithonia diversifolia* (*T. diversifolia*) is a shrubby plant, with a high potential for feeding ruminants and monogastric species [13–15]. This plant has been evaluated in a variety of investigations whose purpose has been to find feeding strategies to reduce the use of grains in dairy cattle [13], to evaluate the potential intake [16] as well as its agronomic behavior to identify its grazing potential in livestock system [17]. However, research on the effect of feeding with these species on N use in steers is still scarce. Finding feeding strategies that reduce the excretion of nutrients in beef production systems is a main objective to reduce their negative impact on the environment around the world.

This study evaluated the different feeding strategies including *L. leucocephala* and *T. diversifolia* as planted shrubs of beef steers fed with a diet based on the tropical grasses *Brachiaria hybrid* CV CIAT BR02/1752 (Cayman) and *Brachiaria decumbens* CIAT 606 (*B. decumbens*). These improved pastures have a high potential for adaptation to poor, low-aerated soils and to withstand periods of drought, while maintaining a good level of biomass production [18] and can constitute a good forage association with the legume species. However, its potential for N retention is relatively unknown. Therefore, the objective of this study was to evaluate four diets: (i) a silvopastoral system based on Cayman associated with *L. leucocephala*; (ii), *B. decumbens* pasture with *T. diversifolia*; (iii) a Cayman grass monoculture system; and (iv) *B. decumbens* monoculture system. These diets were evaluated for their effect on some parameters of ruminal fermentation, representative ruminal microorganisms, as well as on nutrient excretion.

2. Materials and Methods

2.1. Location

This study was conducted at the International Center for Tropical Agriculture (CIAT), located at 3°30′17″ N and 76°21′24″ W, in the rural area of Palmira, Colombia. According

to the Köppen–Geiger classification, this sub-region is classified as Climate Tropical dry (As) [19]. The study site was situated in the context of a livestock agroecosystem corresponding to the agroecosystem of mosaic of crops and pastures of temperate semi-arid climate in fans with very severe erosion soil and ustic regime [20].

The average temperature in the research center and the annual rainfall are 28.4 °C and 953 mm, respectively (CIAT meteorological station). In Palmira the average temperature is 24.5 °C and the annual rainfall is in the range of 1000–1500 mm, with the rainiest months occurring between April and October [21]. This study was conducted between the months of February 2017 and January 2018. During the experimental feeding period, the average temperature registered was 25.3 °C (max 30.5 and min 20.2 °C), and the average rainfall was 169.1 mm.

2.2. Animals and Experimental Design

This experiment was conducted following protocols that ensured animal welfare. All experimental protocols were reviewed and approved by an external Ethics Committee for Scientific Research of the Center for Research on Sustainable Agricultural Production Systems—CIPAV.

Eight Zebu 20-month-old steers with an initial weight of 282 ± 16.4 kg were used, for the quantification of dry matter intake and nutrient excretion in dung and urine. Four of them were cannulated for extraction of ruminal fluid and quantification of the abundance of ruminal microorganisms.

A 4 × 4 Latin square design was used, with four treatments and four periods. The diets consisted of two grasses: *Brachiaria hybrid* cv CIAT BR02/1752 (Cayman) and *Brachiaria decumbens* CIAT 606 cv both in monoculture. Other treatment consisted of a silvopastoral system of Cayman associated with *L. leucocephala,* and the last treatment consisted of a mix of *B. decumbens* with *T. diversifolia*. The evaluation periods for variables such as fecal production, urinary volume, N excretion and dry matter intake consisted of 15 days of grazing, of which 12 days were an adaptation phase to the different diets and 3 days for sample collection. To register microbial changes at the rumen level, the sampling days included day 0, where the animals entered each treatment (plot), followed by day 7 of grazing and finally to day 15 of grazing.

Four experimental plots of 46 × 63 m ha were established, which were directly grazed by steers. In the silvopastoral system, the *L. leucocephala* was established at a planting density of 1428 plants per hectare. As a contrast plot, Cayman grass was established in monoculture. In another two plots, *B. decumbens* grass was established in monoculture through sexual seed. A fifth plot of 40 × 46 m ha with *T.diversifolia* next to the others treatments was established. The *T.diversifolia* was sown through vegetative material at a planting density of 2 plants per m^2. The mixed diets were offered in an 80:20 and 65:35 ratio for Cayman/*L. leucocephala* and *B. decumbens*/*T. diversifolia*, respectively. Both leaves and stems of the *T. diversifolia* forage were supplied in individual feeders every day within the grassland. Each plot with the treatment was divided into strips using electrical fence tape to manage the grazing of the animals. Every three days the animals moved to a new strip.

All plots were fertilized during establishment with di-ammonium phosphate and 46%N-18%P-0%K. Grasses were offered to steers with a regrowth period between 50 and 55 days, while *T. diversifolia* and *L. leucocephala* were offered with a regrowth between 50 and 60 days, respectively.

2.3. Dry Matter Intake

2.3.1. Dry Matter Intake Measured Using Titanium Dioxide (TiO_2) as External Marker

Dry matter intake was calculated using the external marker titanium dioxide (TiO_2) and the diet digestibility was determined through the internal marker indigestible neutral detergent fiber (iNDF). For 15 consecutive days, animals received 10 g of the external marker in the morning and in the afternoon (20 g TiO_2 per day) with an interval of eight hours between AM and PM supply [22]. In the last three days of marker supply, individual

samples of feces were collected twice per day from each animal, which were immediately weighed on a digital scale (SF-400) to determine their fresh weight. They were oven-dried at 60 °C for three days and ground using a 2 mm sieve. All samples per animal were mixed to deliver the marker; a mixture of wheat flour, vegetable oil and water was used, with which a malleable mass was obtained that allowed the wrapping of the 10 g of TiO_2 for each dose. The final weight of the mixture (mass/TiO_2) was 32.5 ± 6.5 g. The concentration of the marker in feces was performed through the H_2O_2 digestion method [18], with amounts of 0, 2, 4, 6, 8 and 10 mg of TiO_2 used as standard concentrations. The analysis obtained a determination coefficient of 0.9951 between TiO_2 added (mg) and TiO_2 analyzed (mg), and the model of the linear equation was Y = 0.1346 × −0.0729.

A first test was carried out where the dung samples were collected in the morning and afternoon, as well as the mixture of both being analyzed separately. This allowed the determination that the level of variation and concentration of the marker was stable with the analysis of the mixture of the samples obtained daily in the morning and in the afternoon. Therefore, three samples per animal in each diet were used to quantify the marker and each sample was quantified in triplicate. The TiO_2 concentration was estimated by calorimetrically measuring absorbance at a wavelength of 410 nm. Likewise, a TiO_2 recovery test to confirm the quantification capacity of the laboratory technique (96.7–98.5% TiO_2) was carried out [18]. For the recovery percentage of the marker in the field, an average of 96% was assumed based on the previous reports [23,24].

2.3.2. INDF Determination

The iNDF was determined by means of the in vitro DaisyII technique (ANKOM Technology-08/05), in forage samples of the different treatments, which were collected before the animals were admitted to the pasture. Likewise, the iNDF was determined in the dried dung samples collected during the grazing of the animals within the period of the marker supply. 0.5 g of each sample was deposited in Fiber Filter Bags of 25 micron porosity (F57 filter bags), and incubated for 144 h in triplicate [25]. For the in vitro technique, 400 mL of ruminal fluid were collected from animals grazing on diets corresponding to the diet to be incubated. The DaisyII incubator consists of four jugs with a capacity of two liters each, where each jug was inoculated with the ruminal fluid (filtered twice with gauze) corresponding to each of the four animals that grazed the treatment. The culture medium for in vitro incubation consisted of buffer solution A (10 g L^{-1} KH_2PO_4; 0.5 g L^{-1} $MgSO_4$ $7H_2O$; 0.5 g L^{-1} NaCl; 0.1 g L^{-1} CaCl $2H_2O$; 0.5 g L^{-1} Urea), buffer solution B (15 g L^{-1} $C_6Na_2CO_3$ and 1.0 g L^{-1} Na_2S $9H_2O$) and neutral detergent solution. In each digestion jar, solutions A and B were added in a ratio of 1:5 (1330 mL:266 mL) for a total volume of 1600 mL of combined A/B mixture in each digestion jar. After the incubation process was completed, the bags were rinsed in water and the NDF content was subsequently analyzed.

To calculate the total feces daily production, the following equation was used [26] (Equation (1)):

$$Fecal\ excretion\left(\text{kg d}^{-1}\right) = \frac{\left(Amount\ of\ indicator\ administered\ (TiO_2)\ \left(\text{g d}^{-1}\right)\right)}{Concentration\ of\ indicator\ (TiO_2)\ in\ dung\ \left(\text{g kgDM}^{-1}\,\text{d}^{-1}\right)} \quad (1)$$

For the dry matter intake estimation from iNDF, the following equation was used [27] (Equation (2)):

$$DMI\left(\text{kg d}^{-1}\right) = \left[\frac{((FE \times ID) - IS)}{CIP}\right] + DMIS \quad (2)$$

where:

DMI = dry matter intake; *FE* = fecal excretion (kg d^{-1}); *CID* = concentration of the indicator (iNDF) in dung (kg d^{-1}); *IS* = indicator (iNDF) in the protein sources (kg d^{-1}); *CIP* = concentration of the indicator (iNDF) in pasture (kg d^{-1}); *DMIS* = dry matter intake of shrubs (*Leucaena leucocephala* or *Tithonia diversifolia*).

2.4. Forage Quality Parameters

The availability and collection of forage samples was made through the double sampling methodology [28] using a quadrant of 0.25 m^2 and 0.5 m × 1 m for pasture and shrub forages, respectively. The estimation of the Cayman/*L. leucocephala* ratio offered to animals was carried out using the agronomic method [16], where the difference between the amount of fodder offered and the amount remaining at the end of each grazing was quantified. On the other hand, the level inclusion of *T. diversifolia* was estimated by employing the difference between the amounts of fodder offered to animals and the amount remaining at the feeder at the end of each day. The green biomass supply based on the average forage production was 4,2-ton ha^{-1} for the *B. decumbens* per grazing period; 4,3-ton ha^{-1} for the Cayman in monoculture; 4-ton ha^{-1} of *T. diversifolia* and 8.7-ton ha^{-1} on the silvopatoral system (Cayman 4.5-ton, *L. leucocephala* 4.2-ton).

Forage samples were collected one day before the animals were allowed to graze of each treatment and were subsequently weighed and dried at 60 °C for 72 h in a forced-air oven to calculate the dry matter content (DM). The dried samples were ground in a 1 mm sieve, for later ash analysis (4 h at 500 °C; ([29]: method 942.05)), crude protein (CP = N × 6.25; Kjeldahl AN 3001 FOSS; ([30]: method 984.14)). Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the methodologies proposed by Van Soest et al. [31], adapted to an Ankom Fiber Analyzer AN 3805 (Ankom® Technology 2052 O'Neil Rd, Macedon, NY 14502, USA). Forage digestibility was determined as described by Goering and Van Soest [32]. Lignin was analyzed following the procedure described in ISO 13906:08, AOAC 973.18, AN 3804 ANKOM. The main forage nutritional components of diets are showed in Table 1. All laboratory analyses were developed at the Animal Nutrition and Tropical Forages Quality Laboratory of the International Center of Tropical Agriculture (CIAT).

Table 1. Chemical composition of different experimental diets.

Item	Bd		Bd *		Td		Cy		Cy *		Ll	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
DM %	33.9	0.8	34.1	2.7	21.2	0.10	24.1	4.0	27.0	0.3	30.5	2.1
IVDDM %	66.45	1.2	66.71	1.8	50.64	2.1	62.25	1.3	65.13	1.0	56.33	1.6
CP %	5.0	0.8	7.1	0.7	18.5	1.3	11	0.9	12.1	0.9	27.0	0.7
NDF %	71.4	3.7	72.1	4.4	46.2	4.0	68.6	0.9	67.7	0.3	48.05	1.3
IVDFDN %	73.6	2.4	73.5	2.7	68.16	1.4	78.43	3.1	76.53	1.2	64.14	1.0
ADF %	35.3	3.6	35.6	5.7	34.3	1.0	33.8	1.3	31.7	1.0	34.6	5.2
Ash %	9.5	1.1	9.5	1.3	14.6	0.9	14.6	1.0	14.0	0.1	7.1	0.6
Lignin %	6.1	0.1	6.4	0.5	10.3	2.1	7.6	0.5	7.1	0.3	9.3	1.2

DM = Dry matter; CP = crude protein; NDF = Neutral detergent fiber; ADF = Acid detergent fiber; IVDDM = invitro digestibility of dry matter; IVDFDN = invitro digestibility of neutral detergent fiber; Bd = *Brachiaria decumbens*; Td = *Tithonia diversifolia*; *Cy* = Cayman; Ll = *Leucaena leucocephala*. * Graminea in association.

2.5. Urine Volume and Nitrogen Balance

A manual collector was used to obtain urine samples from steers during grazing. During the first 12 days of grazing, the steers were accustomed to the manual urine collector and to the presence of humans. On days 13, 14, and 15 urine sub-samples (50 mL) were collected every four hours for a period of 12 h per day to calculate urine volume through creatinine quantification in urine (Creatinine-Kit Biosystems®, Costa Brava 30, 08030 Barcelona, Spain) [33]. The sub-sample of urine was acidified with 0.5 mL sulfuric acid (1 M) and frozen at −20 °C. The dung samples collected for quantification of total excreta production (item 2.4.1) were also used for the quantification of total N and carbon content. The urine and dung N content was determined by Kjeldahl method [34]. The carbon content was calculated through a dry combustion technique. The nitrogen balance was calculated by the difference between the N intake and the N excreted in dung and urine.

All laboratory analyses were developed at the CIAT Forage Quality and Animal Nutrition Laboratory.

2.6. Rumen Fermentation Parameters

On days 0, 7 and 15 of each grazing event, a liter of solid and liquid content mixture of the rumen of each animal was collected through the fistula of four cannulated steers. The collection was carried out 4 h (at 10:30 am) after introducing the animals to a new grazing strip. The ruminal fluid was stored in thermoses preheated to 38 °C, which were transported to CIAT's Forage Quality and Animal Nutrition Laboratory for further processing. The content of each thermos was filtered using five layers of mesh and subsequently the pH was measured. Approximately 50 mL were stored in sterile falcon tubes at −80 °C for subsequent DNA extraction. On the other hand, 10 mL of ruminal fluid were stored in sterile falcon tubes at −80 °C, in order to quantify the ammoniacal concentration N (NH_3). Additionally, 50 mL subsamples of ruminal fluid (stored in falcon tubes) were centrifuged at 13,000× *g* for 30 min. From the resulting supernatant, 4 mL + 1 mL of 25 % metaphosphoric acid were taken. These samples were stored in sterile falcon tubes at −20 °C for subsequent volatile fatty acids (VFA) analysis. The VFA ratios were determined by high-performance liquid chromatography (HPLC; Shimadzu® series 20 A, 7102 Riverwood Drive, Columbia, MD 21046, U.S.A.) equipped with a UV/VIS detector at a wavelength of 210 nm (SPD-20 AV), and a chromatography column (BIO-Aminex RAD HPX-87 H). The mobile phase was prepared for analysis with 0.005 M H_2SO_4 with a flow rate of 0.7 mL min^{-1} and an injection volume of 20 µL. The molar proportions of volatile fatty acids were calculated by retention times and peak areas of commercial standards of acetic, butyric and iso-butyric acids (10, 100, 250, 1000, 1500, 2000 and 3000 ppm) and corrected by molar mass of each acid.

2.7. Microbial Analyzes

2.7.1. DNA Extraction from Rumen Digesta

The DNA was extracted using 1 mL of ruminal fluid [35]. Once the DNA was extracted, it was treated with RNase (®Fair Lawn, NJ, USA) according to the Joint Genome Institute: (i) RNase A treatment: bring the DNA volume to 200 µL in TE buffer (pH 8.0); add 10 µL of RNase A; incubate at 37 °C for 1 h; check small aliquot (5 µL) on an agarose gel with no treatment control; run gel 10–15 min. (ii) Ethanol precipitation: add 1/10 volume of 3 M sodium acetate to RNase A treated DNA; add 2.5 volumes of 100 % ethanol; mix and spin down simply; place at −80 °C for 30 min; spin sample at 4 °C for 20 min to pellet DNA; carefully pour off supernatant; wash pellet with 70% ethanol (cold); spin sample at 4 °C for 3–4 min; take out all ethanol with pipet tips; air-dry the pellet; resuspend pellet with 100 µL of TE. The concentration and purity of extracted DNA was measured with the Nanodrop 2000 spectrophotometer (Thermo Scientific®, Wilmington, DE, USA). The DNA was diluted, and 20 ng were used as template in quantitative real-time polymerase chain reactions (qPCR). The DNA was stored at −80 °C.

2.7.2. Quantitation of Microbial Populations

SYBR® Green-based real-time PCR was used for absolute quantification of *Fibrobacter succinogenes*, *Prevotella ruminantium*, *Selenomonas ruminantium*, Protozoa and total bacteria populations. The primer sequences used and the amplification conditions are described in Table 2. Each reaction mix was characterized by containing 10 µL SYBR® Green (QIAGEN®, 19300 Germantown Road, Germantown, MD 20874, USA), 1 µL of each primer, 2 µL of DNA samples plus 6 µL of ultrapure water for a total volume of 20 µL per reaction. Standard curves for each microorganism were generated with 101 to 108 copies of recombinant plasmids per µL from the cloning and transformation of competent cells of Escherichia coli, where the Promega commercial purification kit was used (Wizard® Plus SV Minipreps DNA Purification System, Promega Corporation, 2800 Woods Hollow Road, Madison, WI 53711 USA). Number of copies of the DNA template were calculated using the equation

developed by Faseleh et al. [36], and the absolute abundance was expressed as copies mL^{-1} of culture sample. Estimation of gene copy number for the samples was made from the linear relationship between the threshold amplification (Ct) and the logarithm of DNA copy numbers from the standard (R^2 0.991, with a mean efficiency 97 ± 1.2 %, and a slope value of 3.2).

Table 2. Target rumen microorganisms and primers used in real-time quantitative and amplification conditions.

Target Species	Primer Set	Primer Sequences (5′–3′)	Process	T(°C)	Time	Cycle	Product Size (bp)	Reference
Fibrobacter succinogenes	Fibro_succ_1F	GGTATGGGATGAGCTTGC	-	95	10′	-	446	[37]
			Denaturation	95	15″	45		
	Fibro_succ_2R	GCCTGCCCCTGAACTATC	Annealing	62	15″	-		
			Extension	72	35″	-		
Prevotella ruminicola	P1prevo_rum	GGTTATCTTGAGTGAGTT	-	95	3′	-	484	[38]
			Denaturation	95	30″	35		
	P2prevo_rum	CTGATGGCAACTAAAGAA	Annealing	53	30″	-		
			Extension	72	1′	-		
Selenomona ruminantium	SelRum2F	CAATAAGCATTCCGCCTGGG	-	94	4′	-	71	[38]
			Denaturation	94	30″	40		
	SelRum2R	TTCACTCAATGTCAAGCCCTGG	Annealing	58	60″	-		
			Extension	72	90″	-		
Total Protozoa	P-SSU-316F	GCTTTCGWTGGTAGTGTATT	-	94	4′	-	223	[39]
			Denaturation	94	30″	40		
	P-SSU-539R	CTTGCCCTCYAATCGTWCT	Annealing	55	30″			
			Extension	72	2′	-		
Domain Bacteria	BAC338F	GCTTTCGWTGGTAGTGTATT	-	94	4′	-	130	[38]
			Denaturation	94	30″	40		
	BAC805R	CTTGCCCTCYAATCGTWCT	Annealing	55	30″			
			Extension	72	2′	-		

2.8. Statistical Analysis

Following a Latin square design with crossover arrangement, with four diets and four periods, the variables dry matter intake, dung and urine production, N concentration in urine and dung, N balance, and volatile fatty acid production were analyzed, as well as the change in the abundance of ruminal microorganisms.

The variable dry matter intake, dung and urine production, N level in urine and dung, and N balance were evaluated through the following model (Equation (3)):

$$yijK = \mu + \delta i + Pj + \beta k + \varepsilon ij \quad (3)$$

where:

yijk = response of steer k under diet i during period j
μ = the general mean
δi = effect of the i-th diet (I = Cayman, Cayman + *L. leucocephala*, *B. decumbens*, *B. decumbens* + *T. diversifolia*)
Pj = effect of the j-th period (j = 1 . . . , 4)
βk = effect of the k-th steer (k = 1 . . . , 4),
εij = experimental error

The variables, volatile fatty acid production, and microorganism population were analyzed through the following model (Equation (4)):

$$yijk = \mu + [\delta i + \beta k\acute{\imath}] + Dj + (\delta D)ij + \varepsilon ijk \quad (4)$$

where:

yijk = response of the steer k, under diet i and day j
μ = the general mean

δi = effect of the i-th diet (I = Cayman, Cayman + *L. leucocephala*, *B. decumbens*, *B. decumbens* + *T. diversifolia*)
βk/i = effect associated with the steer k within the diet i
Dj = day effect j (j = 0, 7, 15)
(δD)ij = effect of diet i interaction and day j
εijk = experimental error

All data were subjected to analysis of variance and when significant treatment effects occurred, differences between means were evaluated using the Bonferroni test. Pearson correlation coefficients were determined to evaluate linear relationships between variables. Programing language R was used with package 'agricolae' for statistical analyses [40].

3. Results

3.1. Dry Matter Intake and Nitrogen Excretion

The inclusion of *L. leucocephala* and *T. diversifolia* in pasture-based diets increased the total dry matter intake ($p < 0.0001$), and the silvopastoral system resulted in being the diet with the highest dry matter intake from the evaluated treatments. Intake was not different between monoculture grass diets (Table 3). The inclusion of high protein species did not increase dung production compared to treatments in which the same pasture grass was included. However, the silvopastoral system generated a greater dung production with respect to the *B. decumbens* and *B. decumbens* + *T. diversifolia* diet. Nitrogen intake by steers on diet *B. decumbens* based increased when *T. diversifolia* was included in the diet, but there was no difference when steers were fed with Cayman in monoculture.

Table 3. Dry matter intake and flow of nitrogen in steers fed with *Brachiaria decumbens* (Bd); *Tithonia diversifolia* (Td); Cayman (Cy) and Cayman + *Leucaena leucocephala* (Ll).

Item	Bd	Bd + Td	Cy	Cy + Ll	SE
Dry matter intake (kg d^{-1})	6.52b	8.40a	7.05b	9.69a	0.22 ***
Dung (kg DM d^{-1})	2.84b	2.96b	3.35a	3.4a	0.07 ***
Urine (L d^{-1})	8.24b	8.83a	8.30b	9.07a	0.09 ***
N intake (g d^{-1})	63.23c	113.48b	116.93b	228.40a	3.8 ***
N urine (g d^{-1})	22.45c	30.26b	32.74b	55.56a	1.02 ***
Ratio N urine: N intake	0.35	0.26	0.27	0.24	
N dung (g d^{-1})	30.94c	39.03b	59.24a	42.37b	1.9 ***
Ratio N dung: N intake	0.48	0.34	0.50	0.18	
Ratio N excretion: N intake	0.84	0.61	0.78	0.42	
N Balance (g d^{-1})	9.83d	44.19b	24.94c	130.46a	1.76 **

*** $p \leq 0.001$, ** $p \leq 0.01$. SE = standard error of the mean. Means followed by the same letters in rows are not statistically different ($p > 0.05$).

The urine volume changed between diets with higher protein content led to higher urine synthesis (Table 3). The N excretion in urine and dung changed across diets. The dry matter intake in the silvopastoral system generated higher N excretion in urine, while the diet *B. decumbens* + *T. diversifolia* generated a similar N excretion to the Cayman in monoculture. Between the two diets in monoculture, the *B. decumbens* diet excreted less N in urine and dung, but this diet offered less N as well. There was no difference in the N excretion in dung by the *T. diversifolia* and *L. leucocephala* diets that were pasture based. Steers receiving the Cayman diet had higher N excretion in dung (Table 3).

The *T. diversifolia* + *B. decumbens* diet was associated with an increase in N balance compared to the *B. decumbens*. This same mixture led to greater N balance compared with the Cayman diet, even though N intake with these two diets was the same (Table 3). In terms of the N excreted/N ingested ratio, the silvopastoral system showed a higher N retention. Likewise, the balance (N intake-urine N-dung N) was significantly higher for the steers that grazed on Cayman associated with *L. leucocephala*. The pastures in monoculture showed a low N balance, and the N balance with *B. decumbens* was significantly lower

Cayman. With both grasses there was improved N balance when a protein source was included (Table 3).

Table 4 presents the Pearson correlations between variables related with N flows. Strong correlations were observed among a group of variables, including dry matter intake, N intake, urine volume, N urine and N balance. The urine volume was correlated with dry matter intake, N intake and N balance (Table 4). The N intake was correlated with dry matter intake (0.76 $p < 0.001$) and the same time, and the N urine and N balance were highly correlated with N intake (0.95 $p < 0.001$; 0.96 $p < 0.001$). The N balance was also correlated with urine volume (0.82 $p < 0.001$) and N urine (0.87 $p < 0.001$). The N dung was correlated only with dung production (0.6 $p < 0.05$), while this was correlated only with the N intake (0.65 $p < 0.05$).

Table 4. Correlation matrix between dry matter intake, excretes and nitrogen flow (n = 8).

Variable	DMI kg d^{-1}	N Intake g d^{-1}	Urine L d^{-1}	Dung kg DM d^{-1}	N Urine g d^{-1}	N Dung g d^{-1}
DMI kg d^{-1}	_____					
N intake g d^{-1}	0.76 ***	_____				
Urine Lt d^{-1}	0.79 ***	0.77 ***	_____			
Dung kg DM d^{-1}	0.23	0.65 *	0.29	_____		
N urine g d^{-1}	0.60 *	0.95 ***	0.64 *	0.68 *	_____	
N dung g d^{-1}	−0.17	0.20	−0.1	0.60 *	0.22	_____
Balance g d^{-1}	0.84 ***	0.96 ***	0.82 ***	0.49	0.87 ***	−0.06

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2. Fermentation Parameters and Microbial Populations

None of the diets evaluated affected either the pH or the production of propionic, butyric and isobutyric acid in the rumen (Table 5). On the contrary, the N-NH_3 production, acetic acid, and the acetic:propionic ratio were affected by the different diets and an interaction between measurement time and diets was observed. The N-NH_3 production in the *B. decumbens* diet decreased at days 7 and 15, with the N-NH_3 value at day 15 being lowest compared with the other diets on day 15. When *T. diversifolia* was combined with B. *decumbens*, the N-NH_3 level of the diet increased on day 7 with no difference to day 15. The Cayman-alone diet had an increase on day 7 and remained unchanged on day 15. The concentration of N-NH_3 in the rumen was greater with time in the silvopastoral system diet, showing higher N-NH_3 production at day 15 compared with the other diets on the time point (day). The inclusion of *T. diversifolia* to the *B. decumbens* diets reduced the acetic acid levels on days 7 and 15 compared with the *B. decumbens*-alone diet and presented similar levels to the Cayman-alone diet. On day 15, the silvopastoral system diet generated higher acetic acid levels in the rumen compared with the other diets (Table 5).

The acetic:propionic ratio was lower in the *B. decumbens* + *T. diversifolia* and Cayman-alone diets, while it was higher under the silvopastoral system and *B. decumbens* diets (Table 5).

The inclusion of shrub forages high in protein generated an increase in the gene copy numbers of *Prevotella ruminicola* and total bacteria on day 15 of sampling (Figure 1). Meanwhile, with the *B. decumbens* diet, the population of *Prevotella ruminicola* was reduced on day 7 of measurement. However, animals grazing on the *B. decumbens*-alone diet did not show changes in the abundance of total bacteria during the different sampling days. On day 7 of measurement, the total bacterial population decreased compared to the start day of the experiment under the Cayman monoculture diet but increased on day 15 of measurement.

Table 5. Molar proportions of volatile fatty acids (VFA) in the rumen of steers fed *Brachiaria decumbens* (Bd), *Brachiaria decumbens* + *Tithonia diversifolia* (Bd + Td), Cayman (Cy), and Cayman + *Leucaena leucocephala* (Cy + Ll), during three different sampling days.

	Bd			**Bd + Td**			**Cy**			**Cy + Ll**			***p*-Value**		
							Day								
Item	**0**	**7**	**15**	**0**	**7**	**15**	**0**	**7**	**15**	**0**	**7**	**15**	**Diet**	**Day**	**Diet * Day**
Rumen pH	6.3	6.3	6.5	6.5	6.6	6.6	6.7	6.6	6.6	6.6	6.6	6.6	0.247	0.939	0.138
N-NH_3 mg L^{-1}	33.6 ^{aB}	24.5 ^{bC}	19.6 ^{cC}	26.1 ^{bC}	47.2 ^{aB}	48.6 ^{aB}	28.3 ^{bC}	45.3 ^{aB}	49.5 ^{aB}	38.9 ^{cA}	75.9 ^{bA}	91.2 ^{aA}	0.002	0.001	0.001
Acetic acid (mmol L^{-1})	48.3 ^{cA}	64.1 ^{bA}	75.0 ^{aB}	47.2 ^{bA}	55.1 ^{aB}	57.7 ^{aC}	48.8 ^{A}	49.3 ^{B}	52.8 ^{C}	47.2 ^{cA}	61.1 ^{bA}	86.1 ^{aA}	0.011	0.045	0.032
Propionic acid (mmol L^{-1})	15.7	18.2	19.3	14.7	21.2	24.2	13.4	15.8	14.8	15.3	27.4	25.7	0.053	0.075	0.438
Butyric acid (mmol L^{-1})	8.3 ^{B}	10.3 ^{A}	11.1 ^{B}	8.8 ^{B}	9.5 ^{B}	10.1 ^{B}	9.2 ^{bA}	11.2 ^{bA}	13.9 ^{aA}	8.1 ^{B}	11.6 ^{A}	10.8 ^{B}	0.947	0.049	0.095
Isobutyric acid (mmol L^{-1})	0.1	0.3	0.3	0.1	0.6	0.3	0.1	0.2	0.2	0.1	0.3	0.5	0.546	0.764	0.999
Acetic:propionic	3.0 ^{B}	3.5 ^{A}	3.8 ^{A}	3.2 ^{B}	2.6 ^{B}	2.3 ^{B}	5.8 ^{aA}	2.9 ^{bB}	2.5 ^{bB}	3.3 ^{aB}	2.2 ^{bB}	3.3 ^{aA}	0.001	0.060	0.072

Lowercase letters indicate statistical differences between the different measurement days within the same treatment. Capital letters indicate statistical differences on the same measurement day between the different diets (Bd, Bd + Td, Cy, and Cy + Ll). * means to depict an interaction.

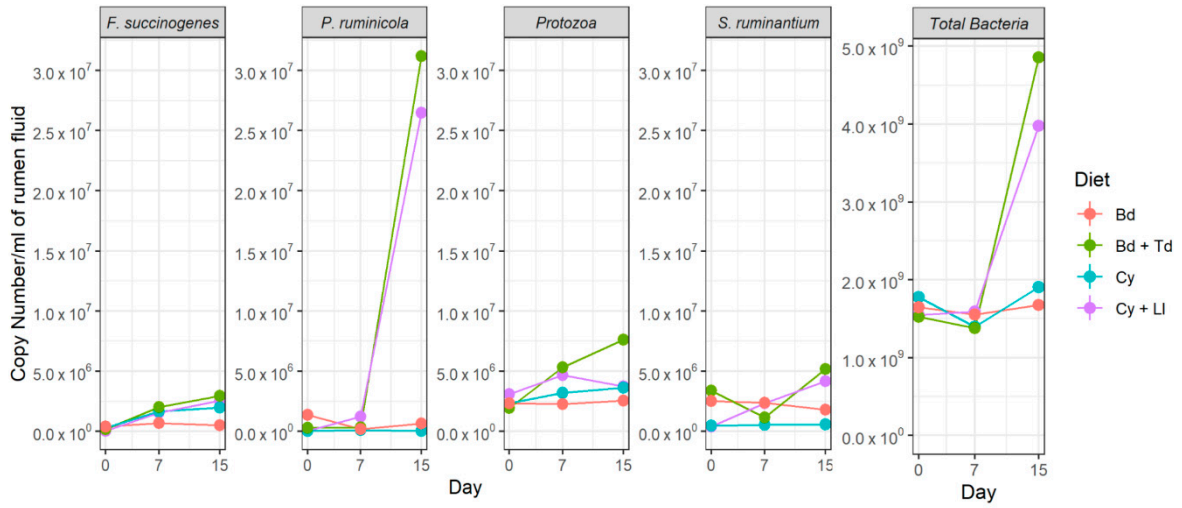

Figure 1. Gene copy numbers for *Fibrobacter succionogenes*, *Prevotella ruminicola*, *Protozoa*, *Selenomonas ruminantium* and total bacteria in liquid fraction of rumen contents at different sampling days with four different diets. *Brachiaria decumbens* (Bd), *Brachiaria decumbens* + *Tithonia diversifolia* (Td), Cayman (Cy), and Cayman + *Leucaena leucocephala* (Ll). Effect of diets, day, and day * diet interaction, on the abundance of ruminal microorganisms.

The protozoa population was different in each sampling period, where in all diets there was an increase on the last time point (day 15) of measurement. However, there was no significant difference in the protozoa abundance between different diets. Neither the diets nor the measurement periods affected the population of *Fibrobacter succinogenes* and *Selenomonas ruminantium*. (Figure 1, Table 6).

Table 6. Effect of different diets on the ruminal microorganism populations. ANOVA *p*-value.

	Microbial Species				
	F. succinogenes	*P. ruminicola*	Protozoa	*S. ruminantium*	Total bacteria
Diet	0.136	0.855	0.825	0.996	0.833
Day	0.629	*	***	0.112	***
Diet × day	0.297	***	0.258	0.189	***
SE	1179.25	213.96	1254.45	1345.36	111,134.69

* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. n = 4. SE = standard error of the mean. *F* = *Fibrobacter*, *P* = *Prevotella*, *S* = *Selenomona*.

4. Discussion

4.1. Dry Matter Intake

Cattle systems in the tropics are characterized by meat and milk production under the use of monoculture pastures of low nutritional value, affecting diet consumption parameters and efficiency in the use of the nutrients consumed. In the present study, the inclusion of species such as *T. diversifolia and L. leucocephala* in diets based on improved pastures contributed to higher food intake and greater N retention. The steers increased their dry matter intake when the diets offered included species with a high protein value and low NDF content, thus achieving an average intakes of 3% and 3.2% of live weight in the *B. decumbens* + *T. diversifolia* and in the Cayman + *L. leucocephala diets*, respectively. These results are in line with previous information reported by other authors [41], who observed higher intake levels as the CP level increased in the diet and aligned with other studies that reported increased intake in diets with added *L. leucocephala* [42,43].

The inclusion level reached in the *T. diversifolia* diet was 32.7%, while the *L. leucocephala* reached an 18.4% inclusion level. These results are consistent with other studies that have reported levels of inclusion of *T. diversifolia* between 15% and 35% in grazing of lactating cows, without altering the animals' voluntary intake [10,13]. The NDF and ADF values

reported in this study for *T. diversifolia* are below those reported in other studies [44] (NDF: 53%; ADF: 48.18%) and above those reported by other investigations [45] (NDF: 33.35% and ADF: 12.21%) for similar regrowth ages (56 and 60 days), and establishment of the shrubby cuttings. Both investigations were carried out in different environmental conditions than those of this study (2456 m.a.s.l and 2905 m.a.s.l, respectively). Chemical composition of this shrub fluctuates in response to altitude and, according to other authors [46], the composition of *T. diversifolia* is mainly related to factors such as soil fertility, environmental temperature, and the level of solar radiation, which can influence the plant maturation process and structural carbohydrates. This composition of the *T. diversifolia* did not represent a limiting factor to forage intake with respect to the *B. decumbens* and Cayman diets.

The *L. leucocephala* intake values reported in our study are consistent with those reported [47]. A diversified supply of fodder stimulates voluntary intake in ruminants during grazing, since they can select and consume plant materials of the best nutritional quality [15]. It has been reported that increases in voluntary intake by providing *L. leucocephala* in the diet is associated with a higher DM rate degradation and a higher passage rate [47]. In our study, the forages offered in the Cayman grassland, by including *L. leucocephala* in rows, was 1.7 times greater (2591 kg DM ha^{-1}) than in the Cayman grassland and *B. decumbens* in monoculture (1513 kg DM ha^{-1}; 1481 kg DM ha^{-1}, respectively), as well as 1.3 times greater than in *B. decumbens* + *T. diversifolia* grassland (1965 kg DM ha^{-1}). Likewise, the DM and CP *L. leucocephala* content values are consistent with the quality parameters referenced for this type of legume [48] and with those reported by other studies under similar conditions of the tropics [49,50]. However, the NDF and ADF values found in our study for both *L. leucocephala* and *T. diversifolia* are above those reported in other studies at similar sampling ages [43,45,49,50]. According to several studies [51,52] high levels of fiber constitute physical factors that limit the DM intake and degradation rate. However, similar values of NDF and ADF in *L. leucocephala* to those found in our study have been reported [47], and when included at 20% in the diet did not generate any effect on the DM intake. However, the same level generated a reduction in NDF digestibility. In our experiment, the greater protein contribution in the diet by the *L. leucocephala* inclusion favored the protein degradation, in part due to supply of adequate energy levels at the rumen, not limiting total DM degradation and DM intake. However, individually, both *L. leucocephala* and Cayman grass presented acceptable in vitro DM and NDF digestibility values.

The inclusion of *L. leucocephala* in silvopastoral systems contributes to improving soil fertility and, therefore, pasture productivity through biological N_2 fixation [53]. At the same time, other studies [12,54] indicate that the arrangement in rows can generate a better distribution of the N_2 fixed by *L. leucocephala*, as well as provide better microclimatic conditions where the grass is less exposed to direct solar radiation and wind effects. The above contributes to the increase in the nutritional quality of the grass in terms of CP and gross fiber [49]. In our study, no differences were found in the nutritional quality of Cayman associated with *L. leucocephala*, compared to the Cayman in monoculture. This may be due to the *L. leucocephala* low planting density (1428 seedlings ha^{-1}), and to the early age of the crop (one year), due to a not yet significant N contribution by the legume to the grassland, as well as a less homogeneous N distribution in the pasture. The N levels are significantly higher below the rows of *L. leucocephala* (0.9 t N ha^{-1} at 0.2 m deep), being 27% more than in grassland [55].

On the other hand, in our study the soil was characterized as a compact soil of low aeration. Additionally, although the soils in this study corresponded to neutral soils [56], and phosphorus fertilization was performed, the P levels in the soil were low compared to other studies in plots established in the same research center [57], and which indicate a moderate P deficiency (5–15 mg kg^{-1}) [56]. P deficiency in the soil can directly affect *L. leucocephala* growth, limit nodular development, and therefore have an indirect effect on the symbiotic N_2 fixation [58]. Maybe, in our study, these physical and chemical characteristics would influence the root development of the legume and a higher planting density will be

needed to generate a greater impact on the physical soil structure and the nutritional grass quality associated.

The DM and CP levels found in our study for both plots of *B. decumbens* are consistent with those reported by other authors at similar ages and under tropical conditions [41]. However, CP levels are low, especially in consideration of the high NDF content found. Therefore, energy may have been a limiting factor to ensure an intake level for these pastures. In general, the growth of grasses in all plots was slow and this led to long resting periods (50 days), which could explain the high NDF values reported in this study. Inclusion of both protein sources in this study generated a greater volume of urine, which is consistent with reports by other authors, who attributed this effect to the greater N ingestion [59], given the increased water intake that is required to digest the proteins and for N metabolism. Furthermore, the contribution of N supplied with the diet of the silvopastoral system, in turn, increased the concentration of N-NH_3 in the rumen and at the same time increased the amount of urine N compared to the other treatments. However, the daily contribution of CP for treatments that included species with high protein value was 84 and 147 g kg DM^{-1}. These CP values do not exceed the range of requirements for ruminants (100 to 170 g kg DM^{-1}; [8]). In this sense, the total amounts of N excreted per N consumed is low. Therefore, the inclusion of *T. diversifolia* and *L. leucocephala* in the diet leads to better dietary N use and, in turn, can improve the use of the low-quality forage and lead to better performance in terms of live weight gain [13,16,60,61]. Additionally, other studies have reported that the exponential increase in N excretion through urine is generated with N intakes above 400 g d^{-1} [62,63]. In our study, the highest N consumed corresponded to 228 g d^{-1}, and the N excreted/N consumed ratio was 61% and 42% for the *T. diversifolia* and the silvopastoral system, respectively. Therefore, our results suggested that the mixture of Cayman + *L. leucocephala* has the potential to favor N retention and thus favor DM degradation under tropical conditions. Finally, this can contribute to improving N use efficiency at the ruminal level [64].

Use of the silvopastoral system and the inclusion of *T. diversifolia* in the *B. decumbens* diet generated a lower N excretion in dung compared to the monoculture diets. This is consistent with several studies [62,65] where no direct relationship was found between N ingestion and N excretion in dung. These results suggest that protein forages could have contributed to better amino acid digestibility at the intestinal level since the fecal N corresponds largely to the undigested N of the food [66].

The *B. decumbens* diet in monoculture had the lowest N retention, which is characteristic of the low-quality diets used in the tropics [67]. The N excretion in dung and urine as a proportion of the ingested N decreased with *T. diversifolia* inclusion, generating a better N balance (Table 3). These results are consistent with data reported by other authors [68], who found a higher percentage of N retention (35.5%) resulting from the inclusion of 35% *T. diversifolia* with respect to the 100% grass-based diet. Likewise, other studies found that N excretion in dung, as a proportion of the N ingested, showed linear and quadratic reductions [69].

4.2. Ruminal Parameters

In our study, rumen fluid pH values remained within an optimal range for the degradation of dry matter, ranging between 6.3 and 6.7, without being affected by the different treatments. These results are consistent with other studies where animal diets are based on forages [70]. Likewise, in another study where the inclusion of different CP levels were evaluated through the supply of *L. leucocephala* flour, pH levels were not affected [71]. On the other hand, the ruminal N-NH_3 concentration was affected by the different diets; where the inclusion of protein forages increased the amount of N-NH_3 in the rumen over the days, the silvopastoral system diet presented the larger increase on the 15th day of measurement. The minimum required N-NH_3 concentration for maximum microbial growth in the rumen is 50 mg L^{-1} [72]. Likewise, another study reported that levels of 50–80 mg L^{-1} appear to be sufficient to favor fiber digestion, because when they obtained higher N-NH_3 levels

they did not significantly increase the degradation characteristics of food in the rumen [73]. According to this information, the N-NH_3 levels reported in our study under *L. Leucaena* in the Cayman diet would be meeting the minimum requirement at the ruminal N level, which could be used in the synthesis of microbial proteins at the level ruminal [74]. In the same way, the N-NH_3 concentration in the rumen is a gross predictor of the efficiency of the dietary N conversion into microbial N [73]. Although this same diet has generated a higher concentration of urine N, it also generated a greater N retention. Despite this, it is not possible to infer that under the *L. Leucocephala* inclusion in the diet it has contributed to efficient microbial protein synthesis, since predictive factors such as the DPR and UDPR level, as well as the passage rate, were not quantified in this study.

The production of VFA in the rumen is influenced by dietary carbohydrate fraction and degradability. In our study, the molar acetate concentration was higher with the *L. leucocephala* inclusion, which could relate to a greater CP availability in the diet, since the CP promotes the growth of ruminal bacteria and with it greater acetate production arising from cellulose degradation [75]. Similar results were reported in the other studies, where an increase in the protein level supplied to lambs increased acetic acid concentration and the acetic:propionic ratio, as well as higher N-NH_3 concentration [76]. In this sense, in our study, with the inclusion *T. diversifolia* in the diet of *B. decumbens*, a greater total bacteria production was found at the 15th day of measurement. This was followed by the diet including *L. Leucocephala*, both of which were the diets with greater CP contribution supplied in this trial. These results are consistent with those reported in other studies [77,78], where a greater total bacteria production in the rumen was evidenced by increasing CP in the diet. On the other hand, *L. leucocephala* has a non-protein amino acid called mimosine (α-amino, 3-hydroxy-4-pyridinepropanoic acid). This component fluctuates between 40 to 120 g kg DM^{-1} according to its physiological state and plant fraction [11] and, during ruminal fermentation, can contribute to the increase in acetate production as well as to an increase in the N-NH_3 content and organic matter degradation [79]. On the other hand, if the higher N-NH3 level found in this study under the silvopastoral system would influence the higher growth of ruminal microorganisms, would be promoting cellulose degradation [80], which would contribute to acetic acid synthesis. However, the acetic:propionic ratio in the ruminal fluid was higher in *B. decumbens* grass in monoculture, but this diet presented quantitatively lower production of propionate.

Other populations quantified in our study, such as *F. succinogenes*, *S. ruminantium*, and Protozoa, were not significantly affected by the different diets ($p > 0.05$), although the Protozoa populations showed a significant increase during 15th day of measurement under the *T. diversifolia* inclusion in the *B. decumbens*-based diet ($p < 0.001$). This result could trigger higher emissions of enteric CH_4, since, methanogens associated with ciliated protozoa are responsible for 9% to 37% of methane production in the rumen [79]. Therefore, by increasing the population of protozoa, the activity of the methanogenic bacteria associated with them could be increased. These results are contrary to those reported by various studies where *T. diversifolia* was included and reduced the protozoa population significantly in in vitro conditions [81,82], which is mainly attributed to the content of secondary metabolites such as condensed tannins (CT) and total phenols [83–85]. However, in another study that evaluated *T. diversifolia* between 30 and 60 days of regrowth, the presence of CT and total phenols was low, both in leaves and in the mixture of leaves and stems, mainly finding alkaloid type metabolites [86].

On the other hand, the presence of CT in the plant negatively affects its palatability, thus reducing DM intake [87]. However, in our study, the results indicate an increase in the DM intake with respect to the diet offered in *B. decumbens* and the Cayman in monoculture, despite there being high levels of the shrub in the diet (35%). Therefore, according to the previous studies, it may be that the concentrations of CT and phenols in the ration were not high enough to generate an effect in reducing the population of protozoa. In contrast, the diet offered has high levels of fiber and did not show variation in pH, which could be a reason to stimulate the population of protozoa in the rumen [88,89].

Better physical and chemical characteristics of the soil than those observed in this study are necessary to improve the performance and the nutritional quality of *B. decumbens* grass, and thereby lead to better results on ruminal parameters when this grass is associated with protein forages such as *T. diversifolia*. Likewise, the concentration of tannins and phenols in the plant depends on edaphoclimatic factors [83]. Therefore, further research is necessary to evaluate the tannin concentration in *T. diversifolia* and its relationship with the population of protozoa and methanogenic bacteria, to identify the role of this shrub more accurately on the population of protozoa and methanogenic bacteria in the rumen.

Prevotella ruminicola 23 is one of the most abundant and important microorganisms for ruminal fermentation; 50% of the population grows well under cultivation in the lab and has a high frequency within the 16S gene [37,38]. Its population was affected by the inclusion of protein forages in the diet, showing an increase on the 15th day of measurement, being slightly higher with the *T. diversifolia* inclusion for the same period. This result suggests that the diet is providing enough energy for multiplication and microbial growth [34]; this benefit is only evidenced up to 15 days after consuming protein forages (*T. diversifolia* and *L. leucocephala*). This result is consistent with those reported by another study [76], which found greater abundance of *Prevotella* when lactating cows were fed legume hay (*Medicago sativa*). Likewise, other authors found that the total count of viable bacteria, cellulolytic bacteria, and proteolytic bacteria counts increased when *L. leucocephala* leaf meal was supplied without thermal processing [71].

The higher acetate production on the 15th day of measurement found that *L. leucocephala* may be related to the greater abundance of *Prevotella,* since acetate corresponds to one of its main fermentation products [90]. However, it is known that *L. leucocephala* has secondary compounds such as condensed tannins whose total concentration can fluctuate between 1.8–4% [91] and can affect the DM intake [50], as well as causing rumen defaunation [92]. In our study, the tannin content in *L. leucocephala* was not quantified; therefore, the level of this secondary metabolite ingested by the animals during this test is not known. However, under the inclusion *L. leucocephala* level (18%) obtained, neither the intake parameters nor the total protozoa population were affected. Likewise, in a study where the CT effect extracted from *L. leucocephala* (CIAT 734) and *Desmodium ovalifolium* on the parameters of fermentation and cellulolytic bacteria was evaluated, *L. leucocephala* had a lower effect on digestibility, fibrolytic population bacteria, and total AVF production [93].

5. Conclusions

The silvopastoral system, with the inclusion of 18% *L. leucocephala* as a fresh matter basis, provides a source of protein widely accepted by cattle, which led to increased DM intake, greater rumen total bacteria populations and greater N retention in steers. This is a superior advantage for tropical beef production systems, given that greater retention of dietary N may contribute to improving productive parameters such as live weight gain, thereby reducing age at slaughter. This has environmental implications, as reduced grazing time would lead to both reduced lifetime of enteric methane emissions and reduced excreta deposition to the soil, also a major source of nitrous oxide emissions from pastures.

The N-NH_3 rumen concentration in this study when feeding steers with *T. diversifolia* showed the potential for increased rumen protein degradation, and thus provided the N necessary for the generation of rumen microbial protein.

The incorporation of different species and plant strata in livestock feeding constitutes an effective strategy to generate economically efficient beef production systems with low environmental impact (i.e., GHG emissions). Further studies of the potential of plant protein sources as only food in livestock feeding should undertake to contribute to the environmental sustainability of beef and milk production systems.

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