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Nutritional Evaluation of Tropical Forage Grass Alone and Grass-Legume Diets to Reduce *in vitro* Methane Production

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Forage grass nutritional quality directly affects animal feed intake, productivity, and enteric methane (CH_4) emissions. This study evaluated the nutritional quality, *in vitro* enteric CH_4 emission potential, and optimization of diets based on two widely grown tropical forage grasses either alone or mixed with legumes. The grasses *Urochloa* hybrid cv. Cayman (UHC) and *U. brizantha* cv. Toledo (UBT), which typically have low concentrations of crude protein (CP), were incubated *in vitro* either alone or mixed with the legumes *Canavalia brasiliensis* (CB) and *Leucaena diversifolia* (LD), which have higher CP concentrations. Substitution of 30% of the grass dry matter (DM) with CB or LD did not affect gas production or DM degradability. After 96 h of incubation, accumulated CH_4 was 87.3 mg CH_4 g^{-1} DM and 107.7 mg CH_4 g^{-1} DM for the grasses alone (UHC and UBT, respectively), and 100.7 mg CH_4 g^{-1} DM and 113.2 mg CH_4 g^{-1} DM for combined diets (70% grass, 15% CB, and 15% LD). Diets that combined legumes (CB or LC) and grass (UHC or UBT) had higher CP contents, gross, and metabolizable energy (GE, ME, respectively) densities, as well as lower concentrations of neutral detergent fiber (NDF) and acid detergent lignin (ADL). The ME and nutritional variables such as NFD, tannins (T), and CP showed a positive correlation with *in vitro* net gas production, while ruminal digestibility was affected by CP, ADL, T, and GE. Optimal ratios of components for ruminant diets to reduce rumen net gas production and increase protein content were found with mixtures consisting of 60% grass (either UHC or UBT), 30% CB, and 10% LD. However, this ratio did not result in a decrease in CH_4 production.

Keywords: ***Canavalia brasiliensis*, *in-vitro* fermentation, *Leucaena* sp., nutritional quality, *Urochloa brizantha* cv. Toledo, *Urochloa hybrid* cv. Cayman**

INTRODUCTION

Cattle and other ruminant livestock are a significant food source for the global human population and are good at converting fibrous species indigestible by humans into highly nutritious food (Wilkinson, 2011). This metabolic conversion is possible due to rumen-dwelling microorganisms that can break down low-quality fibrous plant material, with the formation of gases (methane [CH_4] and CO_2) that are expelled into the atmosphere, plus energy-rich compounds that are required to perform vital functions for both the population of rumen organisms and the host animal (Hyland et al., 2016; Cammack et al., 2018). However, this symbiosis between microorganisms and ruminants is negatively affected by the consumption of diets that are low in protein and high in insoluble fiber (Figueiras et al., 2010).

Therefore, in the search for suitable diets based on tropical forages that simultaneously meet the nutritional needs of livestock and decrease their impact on the environment, mixed production (i.e., agro-pastoral, silvopastoral, and agro-silvopastoral) systems are proposed as a viable option (Arango et al., 2020). In these systems, forage grasses and legumes are combined toward a process of sustainable intensification of livestock production, aiming at not only improving available feed for ruminants but also to restore degraded lands and increase system resilience to more frequent droughts and floods that are associated with climate change (Rao et al., 2015; Ku-Vera et al., 2020a). Furthermore, if properly managed, grass-legume tropical pastures can potentially accumulate large amounts of soil organic carbon; improve chemical, physical, and biological soil health characteristics; fix atmospheric nitrogen; inhibit soil nitrification; improve animal productivity and animal welfare; and reduce CH_4 emissions per unit of livestock product (Peters et al., 2012; Rao et al., 2015; Aynekulu et al., 2020; Ku-Vera et al., 2020a; Vazquez et al., 2020).

Despite the multiple benefits of silvopastoral systems (SPS), the use of grass-legume associations is limited in tropical agricultural systems by several factors. These include reduced plant growth associated with interspecies competition and shading, the potentially low palatability of legumes, the reluctance of farmers to adopt new species due to a general lack of awareness of the benefits of these systems, and the limited availability of legume seeds (Karsten and Carlassare, 2002). However, the specific effects of each association depend on the plant species involved.

A widely studied species in the tropics is the shrub legume *Leucaena* sp., which when planted in SPS provides multiple benefits to grazing livestock, including the provision of high quality protein throughout the year without the need for nitrogen inputs from synthetic fertilizers (Shelton and Dalzell, 2007; Cook et al., 2020), increased forage biomass (Naranjo et al., 2012; Gaviria et al., 2015), improved voluntary forage intake (Cuartas Cardona et al., 2015; Gaviria-Uribe et al., 2015), increased animal productivity (Cuartas Cardona et al., 2014), and reduced CH_4 emissions (Molina et al., 2015; Montoya-Flores et al., 2020). *Canavalia* sp. is a herbaceous legume that can grow in various Latin America locations by direct seeding, alone or in combination with tropical grasses, characterized by high concentrations of protein and high digestibility. However, the relationships between CH_4 emissions (*in vitro*) and nutritional quality of the legumes *Leucaena diversifolia* (more information is available on *Leucaena leucocephala*) and *Canavalia brasiliensis* have been little studied despite their potentials when associated with tropical grasses such as *Urochloa*, which is an important forage grass genus that is widely used in Latin America, Australia and parts of Asia (Low, 2011).

This work aimed to evaluate the effect of mixing different ratios of relevant tropical grasses (*Urochloa* sp. cv. Cayman and Toledo) and legumes (*Canavalia brasiliensis* and *Leucaena diversifolia*) on diet nutritional quality, rumen degradability, and net *in vitro* total gas and CH_4 production. In addition, using optimization analysis, we aimed to find out the ideal proportions of grass and legume(s) to not only reduce net gas production (as a possible indicator of CH_4) at the rumen level but also to increase crude protein (CP) content in the diet.

MATERIALS AND METHODS

Location

Forage samples were collected in the rainy season between April and May of 2016 from a silvopastoral experiment established at the International Center for Tropical Agriculture (CIAT), Palmira, Valle del Cauca, Colombia (3° 30′ 17′′ N and 76° 21′ 24′′ E) at an altitude of 965 meters above sea level. Soils are mollisols, with a pH of 7.2. During sample collection, average temperature was 25.4°C, average relative humidity was 65%, and total precipitation was 231 mm (5.5 mm day^{-1}) and these conditions allowed good regrowth of forage for 56 days.

Forage Samples and Mixed Diets

The tropical forage species evaluated were the two grasses *Urochloa* hybrid (CIAT BR02/1752) cv. Cayman (UHC) and *Urochloa brizantha* (CIAT 26110) cv. Toledo (UBT), the herbaceous legume *Canavalia brasiliensis* (CIAT 17009) (CB), and the shrub legume *Leucaena diversifolia* (ILRI 15551) (LD). Forage materials were planted 2 years before the start of the experiment (2014). The forage crops did not receive any fertilizers, pesticides or irrigation. One kilogram of each of UHC, UBT, and CB were collected at the vegetative stage of development before the beginning of flowering (after 6 weeks of regrowth), by cutting at 10 cm above soil level. Young leaf and stem samples (2:1 ratio) of LD were also manually collected. Two gas production experiments were conducted at two different times: one with UHC, CB, and LD forages, and the other with UBT, CB, and LD. In each experiment the individual forages were evaluated alone (100% UHC or 100% UBT, 100% CB, and 100% LD) and in mixtures with different proportions of DM of grasses and legumes. We used the order (UHC or UBT) - CB - LD, on a DM basis, with the treatment proportions of 0-50-50; 50-50-0 and 50-0-50 which correspond to a mixture in equal proportions (50%) between two species, either a grass with one of the two legumes or with both legumes without UHB or UBT. The treatment denoted 70-30-0 corresponds to the incubated mixture of 70% grass DM plus 30% CB DM, while 70-15-15 refers to the DM proportions 70% UHC or UBT plus 15% CB and 15% LD. Finally, the treatment: 33.3-33.3-33.3, indicates a mixture in equal DM proportions of 33% of the forages (UHC or UBT): CB: LB. A total of nine different treatments were evaluated in each of the two experiments. The proportions of the forages incubated were determined in order to perform a simplex-centroid mixture design.

Nutritional Quality

Samples were evaluated at the Forage Quality and Animal Nutrition Laboratory of CIAT. Samples were dried in a Memmert® UF 750 forced air oven at 60°C for 72 h and until constant weight was achieved. Samples were ground using a cutting mill (Retsch® SM 100, Haan, Germany) with a 1 mm sieve. The content of acid detergent fiber (ADF) and neutral detergent fiber (NDF) was determined using an Ankom 2000 fiber analyzer (Ankom Technology Corp., Macedon, NY, USA) following the method of Van Soest et al. (1991). The ash content was determined using the AOAC method (Association of Official Analytical Chemists, 1990); organic matter (OM) content was calculated as 1,000—ash concentration in g kg^{-1} DM. Gross energy (GE) density was determined using a Parr 6400 (Parr Instrument Company, Illinois, USA) isoperibol calorimeter in accordance with International Standardization Organization, 1998: ISO 9831:1998 specifications. Acid detergent lignin (ADL) content was determined using the method of ANKOM (2016). Total nitrogen content was determined using an autoanalyzer (Skalar Analytical B.V. Breda, Holland) after digestion with sulfuric acid and selenium (Krom, 1980; Searle, 1984). Crude protein (CP) content was estimated as 6.25 × total nitrogen content. Total phenol and tannin contents were determined using Folin-Ciocalteu's method (Makkar, 2003). The metabolizable energy (ME) was calculated according to Lindgren (1983) from the *in vitro* digestibility value obtained at 96 h.

In vitro Gas Production and Dry Matter Degradation

The methodology of Theodorou et al. (1994) was employed for *in vitro* gas production. Rumen fluid was drawn and mixed from two rumen-fistulated *Brahman* steers, grazing on a star grass (*Cynodon plectostachyus*)-dominated pasture with *ad libitum* access to mineralized salt. Briefly, ∼1.0 g of dried/ground samples were placed in individual Wheaton bottles and inoculated with a rumen fluid/buffer solution mixture. After inoculation, all bottles were depressurized (at time 0) and placed in a water bath set at 39°C. Thereafter, pressure and volume measurements were taken at 3, 6, 9, 12, 24, 36, 48, 60, 72, and 96 h of incubation. After each reading, the bottles were gently shaken and placed back in the water bath. Pressure measurements were taken using an 8,40,065 wide-range pressure gauge (Sper Scientific, Arizona, USA) and a PS100 2-bar pressure transducer (Lutron Electronic Enterprise Co. Ltd., Taipei, Taiwan) connected to a three-way valve. The first output was connected to a 1′′ 22 G needle (25 mm × 0.7 mm), the second output to the transducer, the third to a 60 mL syringe, making it possible to record the gas volume removed at each time point required to reduce the bottle internal pressure to atmospheric pressure and to save gas samples for subsequent chromatographic analysis. Upon completion of the test, the contents of the bottles were filtered and dried in a forced-air oven at 105°C for 24 h to determine DM loss. Dry matter degradability (DMD, g kg^{-1}) was calculated for each sample as the change in sample DM weight following incubation, divided by the starting sample DM weight, multiplied by 1,000.

Accumulated gas production (AGP) curves were fitted to the Gompertz model, as proposed by Lavrenčič et al. (1997), using the CurveExpert Professional® software, version 2.4.0 (Hyams, 2016). This model was used to evaluate the gas production points using the following equation:

$$\mathrm{AGP}(\mathrm{mL\ g^{-1}OM}) = \mathrm{a}e^{-e^{\mathrm{b-ct}}} \quad (1)$$

Where a, b, and c are the equation parameters [*a*, maximum gas production; *b*, the difference between initial and final gas at time x; *c*, specific gas accumulation rate; and *t*, time (hours; h)], the accumulated gas production results were expressed on an organic matter (OM) basis. Other biologically significant values were calculated based on parameters a, b, and c. These included the time at inflection point (TIP, h), gas volume at inflection point (GIP, mL), maximum gas production rate (MGPR, mL h^{-1}), and lag phase (LP or microbial settlement, h). These values were estimated using the following formulas:

$$\mathrm{TIP} = \mathrm{b} \times \mathrm{c}^{-1} \quad (2)$$

$$\mathrm{GIP} = \mathrm{a} \times \mathrm{e}^{-1} \quad (3)$$

$$\mathrm{MGPR} = (\mathrm{axc})\,\mathrm{xe}^{-1} \quad (4)$$

$$\mathrm{LP} = \left(\left(\mathrm{bxc}^{-1}\right) - \left(1\mathrm{xc}^{-1}\right)\right) \quad (5)$$

where "e" is Euler's number, ca. 2.72.

Methane Measurements

Methane concentration was quantified in all gas samples collected at the Greenhouse Gas Laboratory of CIAT using a gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector. A three-meter long HayeSep N column was used and the mobile phase was high purity nitrogen at a flow rate of 35 mL min^{-1}. The oven, injector, and detector temperatures were 250, 100, and 325°C, respectively.

Experimental Design and Data Analysis

The nutritional quality, DMD, and CH_4 production data were analyzed using a randomized complete block design, where each treatment had three replicates at each time the readings were taken and three inoculums, the latter being the blocking factor. Mean comparisons were made using Tukey's test when a significant treatment effect ($P < 0.05$) was identified. To check for the normality of data distribution, the Shapiro-Wilk test was conducted on the original residuals using PROC GLM. To determine the correlations among the above variables, type II linear regressions were carried out using the bisector model linear functional relationship procedure of Genstat 18th Edition (VSN International Ltd., Hemel Hempstead, UK). All analyses were conducted using SAS® 9.4 Software (SAS Institute, 2012).

The completely randomized model was:

$$Yij = \mu i + eij \quad (6)$$

Where: *Yij*: observation of the *j*-th repetition of the *i*-th treatment; μi: mean value of the *i*-th treatment, *eij*: experimental error of unit *ij*

The linear regression model employed was:

$$Yi = \beta_0 + \beta_{1*} xi + ei \tag{7}$$

Where: *Yi:* observation of the *i*-th variable response, corresponding with the i-th value xi of the x predictive variable (dependent variable); β_0 *and* β_1 are the regression parameters*;* xi is the independent variable; and *e*: experimental error of unit *i*.

Regression analysis of nutritional quality data (NDF, CP, ADL, GE, and ME) against AGP and DMD parameters was carried out to identify an optimal mixed-diet in which the nutritional quality could be improved (specifically CP) while at the same time reducing gas production. A simplex-centroid mix design was run, using the special cubic model as a response adjustment model using the StatPoint Technologies Inc., 2010: Statgraphics® software (Centurion XVI, version 16.1.18).

The complete simplified special cubic model was:

$$\begin{aligned} y = {} & x_1 G + x_2 C + x_3 L + x_{1,\,2} GC + x_{1,\,3} GL \\ & + x_{2,\,3} CL + x_{1,\,2,\,3} GCL \end{aligned} \tag{8}$$

Where (y) is the crude protein (CP g kg^{-1}) response variable or accumulated gas production (mL g^{-1} OM), x_1, $x_{1,2}$, $x_{1,2,3}$ are the regression coefficients for individual ingredients and mix interactions; G, C, and L are the relative ratios of forage components (grass, CB, and LD).

RESULTS

Nutritional Quality

The CP content of LD was 3.5 times greater than that of both grasses (**Tables 1**, **2** for UHC and UBT, respectively) and it was also greater than that of CB ($P \leq 0.05$). The NDF contents ranged from 492 (CB) to 700 g kg^{-1} DM (100% UBT) ($P < 0.0001$) while the concentrations of ADF were less variable, ranging between 344 and 399 g kg^{-1} DM. For the treatments where different proportions of legumes and grasses were mixed, in both experiments it was observed that the CP content decreased as the proportion of grasses increased, however, the opposite occurred with the NDF content. The lignin content of CB was similar to that of UBT, whereas the lignin content of LD was similar to that of UHC. Legumes, especially LD, have higher GE contents than that reported for 100% grasses treatments or when grasses are replaced up to 30% by legumes ($P = 0.001$), however, this trend is reversed when ME is calculated, since LD treatments or the combination of legumes in equal proportions (50% LD + 50% CB) obtain the lowest values of ME. Much higher concentrations of phenols and tannins were measured in LD compared to both grasses and CB, and the concentrations of both of secondary metabolites were also higher in CB than in both grasses.

Gas Production and Dry Matter Degradation

The total volume of gas produced during the fermentation process ranged from 150 to 255 mL g^{-1} OM (**Tables 3**, **4** for UHC and UBT, respectively). The diet combinations from both systems had a very fast fermentation rate, as evidenced by the low TIP and LP values. The lowest total accumulated gas production values at 96 h occurred with the LD-only treatment in both systems, a value that was almost 0.6 of that from the diet comprising UHC, LD, and CB in a ratio of 70:15:15 and UBT-only diet, respectively (**Figures 1**, **2**).

The highest (inverse) correlation was observed between the content of CP and the AGP values ($R^2 = 0.919$; **Table 5**). In contrast, ME content and gas production were positively related, i.e., the higher the ME content, the higher the gas production ($R^2 = 0.907$). Other strong inverse relationships were observed between the concentration of ADL and DMD g kg^{-1}, GE density and DMD g kg^{-1}, and between T and AGP, and T and DMD.

The correlation analysis results provided the basis for carrying out the optimization objective of selecting the best forage combination for increasing the CP concentration of a dietary mix while decreasing AGP. In the case of the UHC-based treatments, the percentage variance accounted for by these two parameters was 87.9% for CP and 84.3% for AGP. In the UBT-based treatments, the percentage variance accounted for CP and AGP was 87.8 and 87.9%, respectively. **Table 6** shows the restrictions used for obtaining a suitable inclusion of grasses and legumes, as well as the ratio of the best mix found (optimized) and the CP and AGP obtained with the specific mix.

Methane Production

When incubated alone, CH_4 production from CB started declining rapidly after 60 h in measurements of both grasses (**Tables 7**, **8** for UHC and UBT, respectively). The same CH_4 accumulation trend was observed with the other diets for 96 h. It is worth noting that the largest production of CH_4 in the UHC diets came from the 70% UHC: 15% CB: 15% LD diet. The incorporation of legumes into the UBT system contributed to decreased CH_4 production compared to the 100% of UBT diet.

DISCUSSION

Feeds intended for livestock are typically evaluated individually to determine their nutritional values and not integrated with a diet (Tang et al., 2008). Evaluations of individual forages does not allow us to determine interactions with other dietary components in the digestion process (Moss et al., 1992). Although the values of some nutritional parameters of diet components are additive (e.g., CP concentrations), there are possible interactions and synergies between different feeds in a diet and their nutritional values (e.g., energy yield and CP concentrations) that could not be evaluated independently (Tang et al., 2008). This situation can be explained at the rumen level, because depending on the type of diet, some synergy or antagonism may develop due to co-existence of nutrients and their interactions with different microorganisms (i.e., bacteria, protozoa, fungi, and methanogenic archaea) in the rumen (Cammack et al., 2018).

In this investigation, great variability in nutritional composition was found among the different forage diets. For example, the legumes contained twice as much CP as the two grasses evaluated, and the grasses had higher concentrations

TABLE 1 | Mean chemical composition of *Urochloa* hybrid grass cv. Cayman (UHC) and the two forage legumes, *C. brasiliensis* (CB) and *L. diversifolia* (LD), and their mixed proportions used in the study.

Mix UHC-CB-LD	DM	Ash	NDF	ADF	ADL	CP	GE, MJ kg^{-1} DM	ME, MJ kg^{-1} DO	TP	T
100-0-0	199^{de}	122^{a}	642^{a}	365	157^{ab}	68^{i}	17.29^{c}	8.09^{ab}	24.6	1.07
0-100-0	189^{e}	117^{a}	492^{e}	346	98^{c}	195^{c}	17.90^{bc}	7.10^{d}	46.2	15.24
0-0-100	292^{a}	51^{e}	530^{d}	344	176^{a}	256^{a}	19.82^{a}	5.94^{e}	101.1	47.47
0-50-50	240^{bc}	84^{d}	529^{d}	330	132^{bc}	214^{b}	19.32^{a}	6.95^{d}	–	–
50-50-0	194^{de}	117^{a}	565^{c}	368	99^{c}	125^{f}	17.66^{c}	8.02^{ab}	–	–
50-0-50	245^{b}	85^{d}	598^{b}	320	166^{a}	154^{e}	18.49^{b}	7.48^{cd}	–	–
1/3-1/3-1/3	224^{bcd}	96^{c}	566^{c}	317	108^{c}	164^{d}	18.55^{b}	7.66^{cb}	–	–
70-30-0	192^{de}	119^{a}	608^{b}	354	96^{c}	101^{h}	17.76^{c}	8.22^{a}	–	–
70-15-15	206^{cde}	109^{b}	603^{b}	320	91^{c}	110^{g}	17.75^{c}	8.25^{a}	–	–
p-value	0.001	0.001	0.001	0.062	0.001	0.0001	0.001	0.001	–	–
EMS	23.435	1.85	4.68	20.58	15.01	33.8	5.789	0.18	–	–

Data presented as g kg^{-1} DM unless otherwise indicated.
[a,b,c,d,e,f,g,h,i]Mean values in a column with a different letter are statistically different ($P < 0.05$).
UHC, Cayman grass; CB, Canavalia brasiliensis; LD, Leucaena diversifolia; DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; CP, crude protein; GE, gross energy; ME, metabolizable energy; TP, total phenols; T, tannins; EMS, error mean square.

TABLE 2 | Mean chemical composition of *Urochloa brizantha* cv. Toledo (UBT) and the two forage legumes, *C. brasiliensis* (CB) and *L. diversifolia* (LD), and their mixed proportions used in the study.

Mix UBT-CB-LD	DM	Ash	NDF	ADF	ADL	CP	GE, MJ kg^{-1} DM	ME, MJ kg^{-1} DO	TP	T
100-0-0	248.5^{a}	97.2^{d}	700^{a}	399^{a}	83^{cde}	77^{i}	17.69^{e}	8.57^{a}	21.2	0.68
0-100-0	188.7^{f}	117.0^{a}	492^{f}	346^{ab}	98^{cde}	195^{c}	17.78^{de}	7.42^{c}	46.2	15.24
0-0-100	292.0^{bc}	51.4^{i}	530^{e}	344^{ab}	176^{a}	256^{a}	20.06^{a}	6.17^{e}	101.1	47.47
0-50-50	240.3^{cd}	84.4^{g}	529^{e}	330^{b}	132^{b}	214^{b}	19.32^{bc}	6.85^{d}	–	–
50-50-0	218.6^{de}	107.5^{b}	595^{d}	363^{ab}	91^{cde}	129^{f}	17.76^{de}	8.30^{ab}	–	–
50-0-50	270.2^{ab}	73.5^{h}	623^{c}	335^{b}	113^{bc}	158^{e}	18.80^{c}	7.58^{c}	–	–
1/3-1/3-1/3	240.6^{cd}	87.4^{f}	583^{d}	338^{b}	105^{bcd}	167^{d}	18.26^{cd}	7.86^{bc}	–	–
70-30-0	206.6^{ef}	102.2^{c}	655^{b}	383^{ab}	79^{de}	107^{h}	17.74^{de}	8.53^{a}	–	–
70-15-15	213.2^{ef}	92.5^{e}	654^{b}	368^{ab}	67^{e}	116^{g}	18.13^{de}	8.19^{ab}	–	–
p-value	0.001	0.001	0.001	0.0045	0.001	0.001	0.001	0.001	–	–
EMS	6.77	4.3	4.79	19.49	11.01	10.76	3.588	0.16	–	–

Values in g kg^{-1} DM unless otherwise indicated.
[a,b,c,d,e,f,g,h,i]Mean values in a column with a different letter are statistically different ($P < 0.05$).
UBT, Toledo grass; CB, Canavalia brasiliensis; LD, Leucaena diversifolia; DM, dry matter; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; CP, crude protein; GE, gross energy; ME, metabolizable energy; TP, total phenols; T, tannins; EMS, error mean square.

of NDF than the legumes. Similarly, concentrations of phenolic compounds were lower in the grasses than the legumes. These findings concur with data reported in the literature for these tropical species (Lee, 2018; Cook et al., 2020; Gaviria-Uribe et al., 2020), where CP values for grasses can range between 40 and 140 g kg^{-1} DM, and for both legumes studied here, shrub and herbaceous, ranged between 190 and 250 g CP kg^{-1} DM. However, the CP content obtained in the present study was slightly lower than that reported by Peters et al. (2002) for *U. brizantha cv*. Toledo who stated that under optimal conditions CP content ranges between 90 and 120 g kg^{-1} DM. Likewise, the NDF content was within the range of 600 and 800 g kg^{-1} DM reported for *U. brizantha* sp. (Cook et al., 2020; Gaviria-Uribe et al., 2020). However, forage quality has been shown to be closely related to pasture age (Vendramini et al., 2014; Gaviria-Uribe et al., 2020) and the time of the year (Demarchi et al., 2016; Abdalla et al., 2019). The ADL content of *Urochloa* grasses was 86 and 157 g/kg DM, both values were between the ranges reported by Wassie et al. (2018), according to these authors ADL content can vary between 91.2 and 186.9 g/kg depending on ecotype, regrowth age (60, 90 and 120 d) and altitude of the sowing site (1,230, 1,774, and 2,650 masl). It is noteworthy that little information is available on the ADL content of *Urochloa* hybrid cv. Cayman. The ME values found for the legume *L. diversifolia* are slightly lower (8.6 MJ ME kg^{-1} DM) than the results reported by Geleti et al. (2013), while the ME for grasses are above those obtained by Nguku (2015) for 9 grasses of the genus *Urochloa*, whose values ranged between 6.6 and 5.9 MJ ME kg^{-1} DM. However, this variable, as well as the rest of the nutritional components of the diet, can vary according to the

TABLE 3 | Accumulated gas production (AGP; mL g^{-1} OM), dry matter degradability (DMD), and profiles of the adjustment made using the Gompertz model for UHC, CB, LD, and their mixes.

Mix UHC-CB-LD	AGP (mL g^{-1} OM)	DMD (g kg^{-1})	Gompertz model						
			a	*b*	*c*	TIP (h)	GIP (mL)	MGPR (mL h^{-1})	LP (h)
100-0-0	231.5^{a}	712^{a}	215.05^{a}	1.11^{a}	0.06^{c}	17.09^{a}	79.09^{a}	5.12^{cd}	1.63^{a}
0-100-0	180.3^{cd}	638^{bc}	168.63^{bcd}	0.98^{ab}	0.11^{a}	8.66^{f}	62.02^{bcd}	7.05^{a}	–0.15^{b}
0-0-100	150.0^{de}	517^{d}	146.91^{d}	0.88^{b}	0.06^{c}	14.95^{abc}	54.04^{d}	3.18^{e}	–2.06^{h}
0-50-50	167.5^{d}	608^{c}	159.35^{cd}	0.88^{b}	0.08^{bc}	11.07^{e}	58.61^{cd}	4.64^{cd}	–1.56^{g}
50-50-0	210.0^{ab}	703^{a}	200.73^{ab}	0.96^{ab}	0.09^{b}	11.19^{ab}	73.83^{ab}	6.31^{ab}	–0.52^{c}
50-0-50	199.6^{bc}	641^{bc}	196.84^{ab}	0.95^{ab}	0.06^{c}	16.38^{e}	72.40^{ab}	4.21^{de}	–0.83^{e}
33.3-33.3-33.3	193.4^{bc}	662^{b}	185.91^{abc}	0.90^{b}	0.07^{bc}	12.32^{de}	68.38^{abc}	5.00^{cd}	–1.36^{f}
70-30-0	213.5^{ab}	718^{a}	205.75^{a}	0.95^{ab}	0.07^{bc}	12.87^{cde}	75.68^{a}	5.59^{bc}	–0.65^{cd}
70-15-15	234.8^{a}	713^{a}	218.88^{a}	0.95^{ab}	0.07^{bc}	14.02^{bcd}	80.51^{a}	5.45^{bc}	–0.75^{de}
p-value	0.001	0.001	0.001	0.015	0.001	0.001	0.001	0.001	0.001
EMS	8.824	12.01	12.575	0.066	0.007	0.839	4.626	0.402	0.600

[a,b,c,d,e,f,g]Mean values in a column with a different letter are statistically different ($P < 0.05$).

UHC, Cayman grass; CB, Canavalia brasiliensis; LD, Leucaena diversifolia; AGP, accumulated gas production; OM, organic matter; DMD, dry matter degradability; a, maximum gas production (mL); b, difference between initial gas and final gas at an × time; c, specific gas accumulation rate; TIP, time to the inflection point, h; GIP, gas at the inflection point, mL; MRGP, maximum rate of gas production, mL/h; LP, lag phase, h; EMS, error mean square.

TABLE 4 | Accumulated gas production (AGP; mL g^{-1} OM), dry matter degradability (DMD), and profiles of the adjustment made using the Gompertz model for UBT, CB, LD, and their mixes.

Mix UBT-CB-LD	AGP (mL g^{-1} OM)	DMD (g Kg^{-1})	Gompertz model						
			a	*b*	*c*	TIP (h)	GIP (mL)	MGPR (mL h^{-1})	LP (h)
100-0-0	252.9^{a}	726^{ab}	249.84^{a}	1.13^{a}	0.05^{cd}	20.93^{a}	91.89^{a}	4.96^{ab}	2.41^{a}
0-100-0	182.0^{de}	661^{d}	171.64^{cd}	0.98^{ab}	0.09^{a}	10.38^{e}	63.13^{cd}	5.96^{a}	–0.20^{c}
0-0-100	155.6^{f}	532^{f}	153.65^{d}	0.92^{b}	0.05^{cd}	16.87^{bc}	56.51^{d}	3.09^{d}	–1.39^{g}
0-50-50	175.7^{def}	598^{e}	167.20^{cd}	0.94^{ab}	0.07^{ab}	12.34^{de}	61.5^{bc}	4.68^{bc}	–0.79^{d}
50-50-0	225.7^{bc}	715^{ab}	214.91^{ab}	0.92^{b}	0.07^{bc}	13.17^{de}	79.04^{ab}	5.56^{ab}	–1.04^{ef}
50-0-50	202.4^{e}	640^{d}	200.69^{bc}	0.99^{ab}	0.05^{d}	18.71^{ab}	73.81^{cd}	3.91^{cd}	–0.17^{bc}
33.3-33.3-33.3	207.1^{cde}	669^{cd}	199.47^{bc}	0.92^{b}	0.06^{bcd}	14.59^{cd}	73.36^{bc}	4.67^{bc}	–1.11^{f}
70-30-0	230.6^{ab}	727^{a}	230.76^{ab}	0.94^{ab}	0.06^{bcd}	15.16^{cd}	84.87^{ab}	5.28^{ab}	–0.90^{de}
70-15-15	233.7^{ab}	695^{bc}	228.05^{ab}	1.00^{ab}	0.05^{bcd}	17.07^{bc}	83.88^{ab}	4.92^{bc}	0.03^{b}
P	0.001	0.001	0.001	0.028	0.001	0.001	0.001	0.001	0.001
EMS	7.119	1.098	14.784	0.066	0.006	1.045	5.437	0.355	0.076

[a,b,c,d,e,f,g]Mean values in a column with a different letter are statistically different ($P < 0.05$).

UBT, Toledo grass; CB, Canavalia brasiliensis; LD, Leucaena diversifolia; AGP, accumulated gas production; OM, organic matter; DMD, dry matter degradability; a, maximum gas production (mL); b, difference between initial gas and final gas at an × time; c, specific gas accumulation rate; TIP, time to the inflection point, h; GIP, gas at the inflection point, mL; MRGP, maximum rate of gas production, mL/h; LP, lag phase, h; EMS, error mean square.

age of the species and time of year (Givens et al., 1993, Nguku, 2015). In the present investigation, there were differences in ME content between legumes and grasses, contrary to what was reported by Evitayani et al. (2004), who found average values of 7.6 ± 0.14 and 7.3 ± 0.12 MJ ME kg^{-1} DM for grasses and legumes, respectively. Likewise, the highest ME concentrations were for the treatments: 100-0-0, 70-30-0 or 70-15-15, this may favor the synthesis of microbial proteins at the rumen level (Krizsan et al., 2020).

For the *in vitro* analysis, the highest gas production and degradability rates were obtained for samples of both grasses that were individually incubated and when 30% legumes were added to these grasses. Despite this, there was a clear pattern and as the level of inclusion of legumes increased, gas production and degradability decreased. Blümmel et al. (1997) suggested that a feed consisting of a mix of different kinds of ingredients can result in asynchrony in releasing nutrients, thus changing both the biomass of microorganisms produced and gas produced by them. In addition, one factor that can affect the fermentation and gas production of feeds is the configuration of their cell wall polysaccharides (Molina-Botero et al., 2020, Valencia-Salazar et al., 2021). Therefore, the digestibility values depend upon their composition of structural carbohydrates, including the concentration of lignin (Barahona and Sánchez, 2005) and

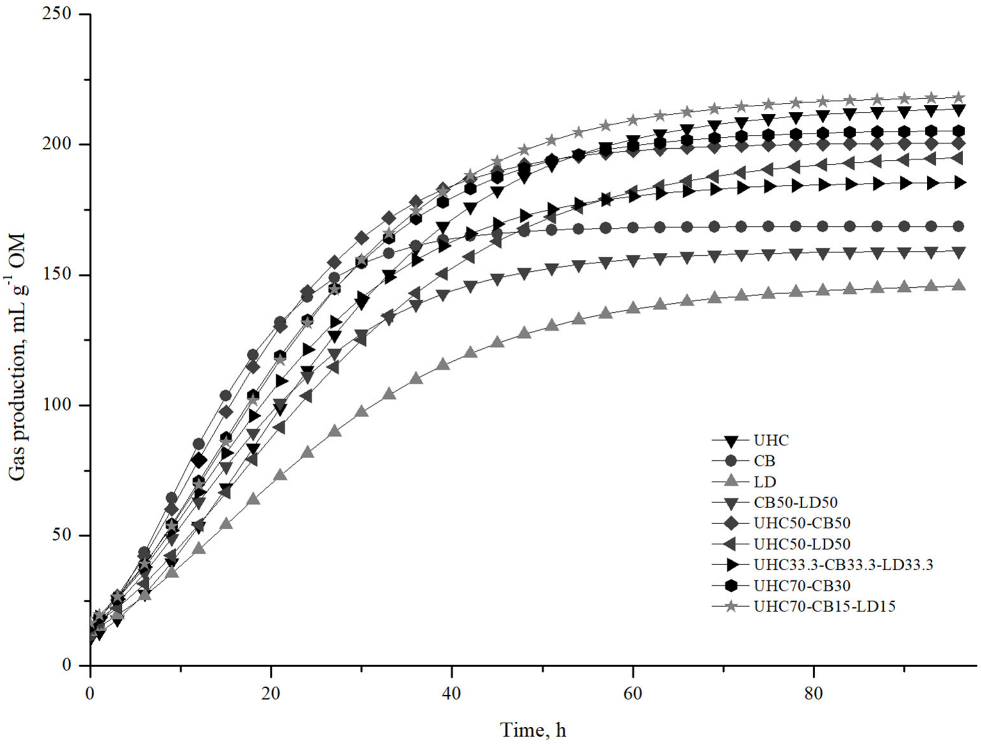

FIGURE 1 | Modeled mean accumulated gas production (mL g^{-1} OM) for UHC, CB, LD, and 6 dietary mixtures. UHC, *Cayman* 100%; CB, *Canavalia* 100%; LD, *Leucaena* 100%; CB50LD50, *Canavalia* 50% + *Leucaena* 50%; UHC50CB50, *Cayman* 50% + *Canavalia* 50%; UHC50LD50, *Cayman* 50% + *Leucaena* 50%; UHC33.3CB33.3LD33.3, *Cayman* 33.3% + *Canavalia* 33.3% + *Leucaena* 33.3%; UHC70CB30, *Cayman* 70% + *Canavalia* 30%; UHC70CB15LD15, *Cayman* 70% + *Canavalia* 15% + *Leucaena* 15%.

the protein included in the diet or treatment evaluated. This postulate agrees with the high correlation values obtained in this study between nutritional compounds such as CP or ADL and variables such as DMD or AGP. Similar results were reported by Lee (2018) where 136 forage plant species or hybrid cultivars grown in 30 countries were evaluated, finding that parameters such as ADF, NDF, ADL content had a correlation >0.7 with DMD or OMD. Although Lee (2018) affirmed that there is a positive correlation (0.62, respectively) between CP and DMD, in the current study there was an inverse correlation between both parameters, perhaps due to the concomitant increase of the content of anti-nutritional compounds associated with the inclusion of CB and/or LD, which could potentially mask the full expression of a diet rich in CP and GE, as was also reported by Jayanegara et al. (2011).

It is clear that to increase our understanding of the nutritive value of forage mixtures composed of tropical forages, the action of the various secondary metabolites (i.e., tannins, saponins) that are present in some legumes must be taken into consideration (Tiemann et al., 2008a,b; Lascano and Cárdenas, 2010). The effect of secondary metabolites depends on their concentration or proportion to the substrate with which they interact. For example, tannins can be found both in the cell wall and inside the cytoplasmic vacuoles of some legumes, primarily in the form of condensed tannins (McAllister et al., 1994; Patra et al., 2017) and their effect depends on their concentration or ratio with the substrate with which they interact. High concentrations of tannins, such as the ones found in diets containing legumes (CB and LD) can delay the digestion of forages by reducing the activity of fibrolytic enzymes (Archimède et al., 2016; Henke et al., 2017; Ku-Vera et al., 2020b). This phenomenon is related to the microbial degradation of structural polysaccharides, and the rate and extent of forage degradation (Archimède et al., 2016; Henke et al., 2017). Likewise, a negative effect has been shown on protein degradation when tannins encapsulate it at low rumen pH (Hess et al., 2003; Archimède et al., 2016). The described tannin effect could explain our results obtained in this study, as in the treatments with an inclusion between 50 and/or 100% of some of these two containing-tannin- legumes (15.2 and 47.5 g kg^{-1} DM for *Canavalia* and *Leucaena*, respectively) and total phenols (46.2 and 101.1 g kg^{-1} DM) a reduction in digestibility variables and therefore in gas production was observed. These results are in contrast to the 100% grass treatments where the values of tannins and total phenols did not exceed 1.07 g T kg^{-1} DM and 24.2 g TP kg^{-1} DM. This observation is consistent with the study of Seresinhe et al. (2012), where a strong inverse relationship was found between tannin concentration and gas production. Tolera et al. (1998) reported condensed tannins content ranging from 7.1 to 13.5% in LD. This concentration of tannins could have bacteriostatic effects on some populations,

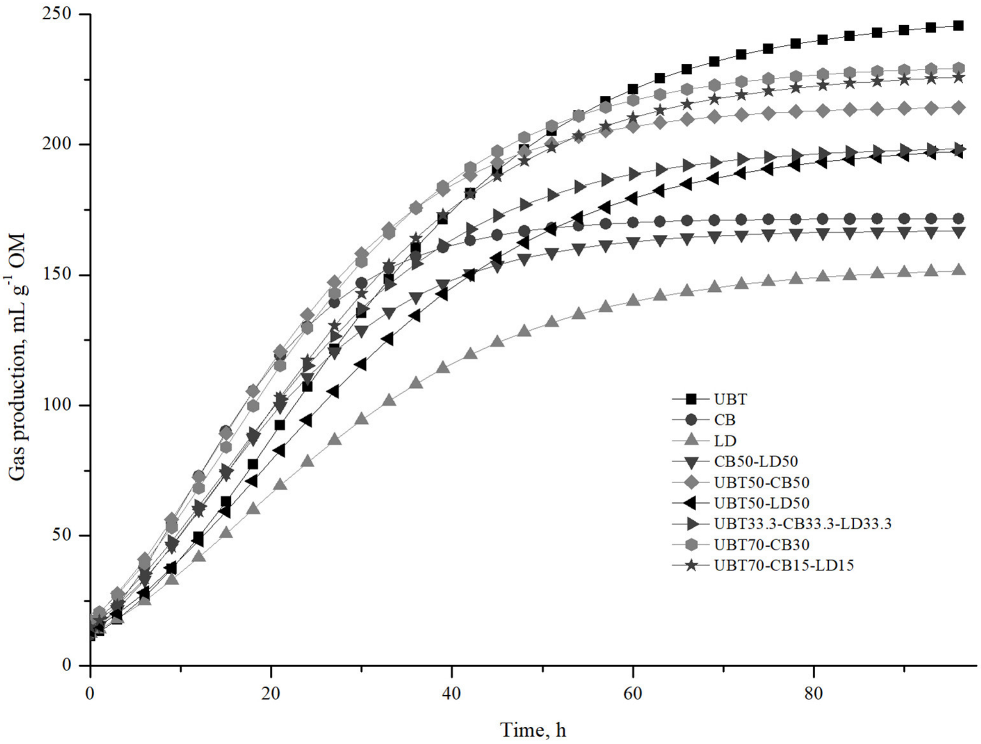

FIGURE 2 | Accumulated gas production (AGP; mL g^{-1} OM) for UBT, CB, LD, mixed diets. UBT, *Toledo* 100%; CB, *Canavalia* 100%; LD, *Leucaena* 100%; CB50LD50, *Canavalia* 50% + *Leucaena* 50%; UBT50CB50, *Toledo* 50% + *Canavalia* 50%; UBT50LD50, *Toledo* 50% + *Leucaena* 50%; UBT33.3CB33.3LD33.3, *Toledo* 33.3% + *Canavalia* 33.3% + *Leucaena* 33.3%; UBT70CB30, *Toledo* 70% + *Canavalia* 30%; UBT70CB15LD15, *Toledo* 70% + *Canavalia* 15% + *Leucaena* 15%.

TABLE 5 | Correlations obtained by type II linear regression analysis.

Correlation	Equation	R^2	SE slope	SE constant
NDF g kg^{-1} (*x*) on AGP mL g^{-1} OM (*y*)	$y = 0.46x - 63.8$	0.743	0.064	37.55
NDF g kg^{-1} (*x*) on DMD g kg^{-1} (*y*)	$y = 1.12x + 7.47$	0.434	0.247	148.7
CP g kg^{-1} (*x*) on AGP mL g^{-1} OM (*y*)	$y = -0.51x + 281.6$	0.919	0.037	6.145
CP g kg^{-1} (*x*) on DMD g kg^{-1} (*y*)	$y = -1.12x + 834.9$	0.876	0.102	16.86
ADL g kg^{-1} (*x*) on AGP mL g^{-1} OM (*y*)	$y = -0.70x + 280.0$	0.622	0.142	16.55
ADL g kg^{-1} (*x*) on DMD g kg^{-1} (*y*)	$y = -1.70x + 847.3$	0.769	0.240	28.07
GE MJ kg^{-1} (*x*) on AGP mL g^{-1} OM (*y*)	$y = -44.9x + 1,025$	0.654	16.56	303.7
GE MJ kg^{-1} (*x*) on DMD g kg^{-1} (*y*)	$y = -83.9x + 2,198$	0.864	26.93	494.1
ME MJ kg^{-1} (*x*) on AGP mL g^{-1} OM (*y*)	$y = -36.8x + 77.7$	0.907	2.936	22.94
T g kg^{-1} (*x*) on AGP mL g^{-1} OM (*y*)	$y = -2.20x + 243.2$	0.791	0.279	6.185
T g kg^{-1} (*x*) on DMD g kg^{-1} (*y*)	$y = -4.56x + 742.5$	0.948	0.266	6.019

NDF, neutral detergent fiber; CP, crude protein; ADL, acid detergent lignin; GE, gross energy; ME, metabolizable energy; T, tannins; AGP, accumulated gas production; OM, organic matter; DMD, dry matter degradation; R^2*, determination coefficient; SE, standard error.*

leading to lower digestibility of the fermented material (Tavendale et al., 2005).

Evaluation of the AGP and CP content in a mix of the three dietary components (grass, CB, and LD) yielded an optimal diet ratio of 60% grass (UHC or UBT), 30% CB, and 10% LD. It should be clarified that although a reduction in gas production was pursued as a measure to reduce CH_4 production and emission, it was never intended to be zero. This expectation is because gas production is of great importance to maintain ideal conditions inside the rumen. For example, in the case of cattle it is important that the formation and utilization of metabolic hydrogen is synchronized (Calsamiglia et al., 2005) in the metabolic pathway that is responsible for glucose oxidation (glycolysis). This is required to regenerate the reducing power

TABLE 6 | Optimization of the crude protein (CP; maximize) and accumulated gas production (AGP; minimize) response variables in the UHC and UBT forage systems.

Systems	Factor	Restrictions		Calculated optimal value	Optimal value (desirability)	Optimized CP ($g\ kg^{-1}$ DM)	Optimized AGP ($mL\ g^{-1}$ OM)
		Minimum	Maximum				
UHC	UHC (%)	60	100	60.0	0.398	147	200
	CB (%)	0	40	30.0			
	LD (%)	10	40	10.0			
UBT	UBT (%)	60	100	60.0	0.420	151	215
	CB (%)	0	40	30.0			
	LD (%)	10	40	10.0			

UHC, Cayman 100%; UBT, Toledo 100%; CB, Canavalia 100%; LD, Leucaena 100%; AGP, accumulated gas production; CP, crude protein; DM, dry matter; OM, organic matter.

TABLE 7 | Methane yield from UHC, CB, LD, and their mixed diets.

Treatment	Methane yield ($g\ CH_4\ kg^{-1}$ DM) at different post-incubation times										$g\ CH_4\ kg^{-1}$ DMD
	3 h	6 h	9 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	96 h
UHC	0.07^{c}	0.98^{b}	1.99^{c}	3.05^{c}	8.98^{a}	11.11^{ab}	13.19^{a}	14.27^{a}	15.55^{a}	18.08^{a}	24.36^{ab}
CB	0.50^{a}	1.99^{a}	4.43^{a}	6.85^{a}	10.42^{a}	13.72^{a}	14.99^{a}	16.34^{a}	11.72^{b}	11.44^{b}	18.52^{b}
LD	0.25^{b}	0.93^{b}	1.77^{c}	2.87^{c}	7.68^{a}	8.90^{b}	9.72^{b}	10.77^{b}	11.16^{b}	12.68^{b}	23.28^{ab}
UHC70CB30	0.49^{a}	1.55^{a}	3.01^{b}	4.65^{b}	10.31^{a}	12.58^{a}	14.68^{a}	15.19^{a}	15.76^{a}	17.84^{a}	23.62^{ab}
UHC70CB15LD15	0.56^{a}	1.66^{a}	3.02^{b}	4.68^{b}	10.68^{a}	12.65^{a}	14.81^{a}	15.97^{a}	17.00^{a}	19.66^{a}	26.29^{a}
p-vale	0.001	0.001	0.001	0.001	0.001	0.0072	0.0013	0.001	0.001	0.001	0.012
EMS	0.054	0.163	0.356	0.546	1.431	1.255	1.184	1.260	0.979	1.232	1.817

a,b,c Mean values in a column with a different letter are statistically different ($P < 0.05$).
DM, dry matter; CH_4, methane; DMD, dry matter degradation; UHC, Cayman 100%; CB, Canavalia 100%; LD, Leucaena 100%; UHC70CB30: Cayman 70% + Canavalia 30%; UHC70CB15LD15: Cayman 70% + Canavalia 15% + Leucaena 15%.

TABLE 8 | Methane yield from UBT, CB, LD, and their mixes.

Treatment	Methane yield ($g\ CH_4\ kg^{-1}$ DM) at different post-incubation times										$g\ CH_4\ kg^{-1}$ DMD
	3 h	6 h	9 h	12 h	24 h	36 h	48 h	60 h	72 h	96 h	96 h
UBT	0.07^{b}	0.46^{c}	1.41^{d}	3.27^{b}	10.71^{a}	14.64^{a}	16.65^{ab}	18.18^{ab}	19.84^{ab}	22.44^{a}	31.57^{a}
CB	0.14^{ab}	0.85^{a}	2.90^{bc}	6.18^{a}	11.85^{a}	13.87^{a}	16.03^{b}	17.13^{b}	16.26^{b}	11.29^{c}	17.69^{c}
LD	0.08^{ab}	0.55^{bc}	1.87^{cd}	3.30^{b}	7.07^{b}	8.93^{b}	10.55^{c}	11.42^{c}	11.32^{c}	12.71^{b}	25.22^{b}
UBT70CB30	0.14^{a}	0.83^{a}	4.00^{a}	5.69^{a}	13.28^{a}	15.71^{a}	18.16^{a}	19.70^{a}	20.81^{a}	21.85^{a}	30.83^{a}
UBT70CB15LD15	0.12^{ab}	0.74^{ab}	3.17^{ab}	5.25^{a}	12.43^{a}	15.04^{a}	17.24^{ab}	18.54^{ab}	19.24^{ab}	21.42^{a}	31.69^{a}
p-vale	0.016	0.0015	0.001	0.001	0.0008	0.001	0.001	0.001	0.001	0.001	0.001
EMS	0.027	0.092	0.405	0.709	1.207	0.741	0.703	0.847	1.350	0.953	1.714

a,b,c,d Mean values in a column with a different letter are statistically different ($P < 0.05$).
DM, dry matter; CH_4, methane; DMD, dry matter degradation; UBT, Toledo 100%; CB, Canavalia 100%; LD, Leucaena 100%; UBT70CB30, Toledo 70% + Canavalia 30%; UBT70CB15LC15, Toledo 70% + Canavalia 15% + Leucaena 15%.

of cofactors such as NAD+ and FAD+, while increasing the synthesis the synthesis of adenosine triphosphate, promoting the growth of other microbial species (e.g., fibrolytic) and helps to regulate the osmotic pressure inside the rumen (Yokoyama and Johnson, 1993; Calsamiglia et al., 2005). Regarding the proportions established in the evaluated diets, this is consistent with the observations of Rojas et al. (2005), who suggested that the percentage of legumes should range from 30 to 40% in mixtures of grasses and legumes to improve the quality of the diet and have an optimal protein:energy balance at the rumen level. Moreover, these proportions coincide with those found in experiments with ruminants fed with tropical legumes that are rich in tannins and whose results affirm that DM intake was reduced when the amount of CT exceeds $50\,g\ kg^{-1}$ DM (Patra and Saxena, 2011). Likewise, cattle systems where the diet is composed of 100% low quality grasses, have low productive indexes due to the low CP concentration, required by ruminal microorganisms for the breakdown of carbohydrates, in addition to a reduction in DM intake due to the high content of structural carbohydrates (Krizsan et al., 2010).

Enteric CH_4 emission rates are associated with the physicochemical characteristics of the diet (e.g., CP and NDF contents), which have a direct impact on diet intake (Gaviria-Uribe et al., 2020) and eating frequency (Grant et al., 2015). Several studies have evaluated the effect of adding a legume to a grass on CH_4 production both *in vitro* (Tope et al., 2013; Molina-Botero et al., 2020) and *in vivo* (Molina-Botero et al., 2019a,b; Gaviria-Uribe et al., 2020; Montoya-Flores et al., 2020). Nevertheless, the conclusions drawn from these studies are unclear, as in some cases the addition of a legume increased *in vitro* CH_4 production (Carulla et al., 2005; Molina-Botero et al., 2020), but in others, it had the opposite effect (Lee et al., 2004). In our case, net CH_4 production per kg of DM did not differ between treatments containing legumes (up to 30% inclusion) and grasses alone, but less gas was produced when 100% legumes were incubated. A similar trend was observed for CH_4 production per unit DMD, being most noticeable for the treatment of 100% CB. When comparing both legumes, we observed that CB was characterized by containing less NDF and ADL than LD, contributing to improved digestibility and therefore higher gas production. This finding coincides with the conclusion reached by Hess et al. (2003), who stated that the difference in *in vitro* CH_4 production among various kinds of forages could be accounted for by the differences between the ratios of digestible carbohydrates and cellulose. Likewise, Patra and Saxena (2010) proposed that the presence of secondary metabolites can affect methanogenesis. However, this was not observed in the present study, because the inclusion of up to 30% of legumes did not reduce *in vitro* CH_4 production. In addition, a greater reduction would be expected with the LD treatment alone, since it contained a greater amount of total phenols and tannins compared to CB alone. These results can be explained by indirect effects of other secondary compounds present in these species, such as mimosine, alkaloids, saponins, steroids, among others that were not evaluated (Hu et al., 2005; Oseni et al., 2011). With our results, it should not be ignored that the *in vitro* technique, despite being an artificial system, is a viable option to initially simulate possible dietary combinations of forages (Danielsson et al., 2017) that can then be validated using ruminants. This is why we highlight the importance of including legumes in cattle diets as a strategy to reduce CH_4 emissions.

Although it was not the primary aim of this study, the use of herbaceous and shrub legumes was shown to have potential positive environmental benefits besides improving nutritive values of diets for ruminants. Vazquez et al. (2020) showed how combining the three types of forages tested here clearly improved chemical, physical and biological soil health characteristics. In addition, the use of shrubs and trees in silvopastoral systems have shown the capacity to sequester greater amounts of carbon at a system level (Aynekulu et al., 2020).

CONCLUSIONS

Diets that combined legumes (CB or LC) with grass (UHC or UBT) had higher protein contents and gross and metabolizable energy densities, as well as decreased concentrations of NDF and lignin. Metabolizable energy and nutritional compounds such as NDF, T, and CP had a high correlation with net gas production, while ruminal digestibility was affected by CP, ADL, GE, T, and other unidentified compounds provided by CB and/or LD.

Optimal ratios of dietary components in both systems were found with mixtures consisting of 60% grass (either UHC or UBT), 30% CB, and 10% LD. The system containing UHC yielded the best combination in terms of an increase in CP and a decrease in AGP. However, this ratio did not result in a decrease in methane production. Therefore, further characterization of the content and activity of other secondary metabolites, perhaps present in both legumes, is required to better explain the behavior response resulting from grass-legume interactions.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Colombian law No. 84/1989 and the Ethics Committee of the International Center for Tropical Agriculture.

AUTHOR CONTRIBUTIONS

SQ-A, IM-B, JR-N, and RB-R: conceptualization. SQ-A and IM-B: methodology. SQ-A, IM-B, and JM: formal analysis. SQ-A: writing—original draft preparation. SQ-A, IM-B, IR, RB-R, NC, JA, and JM: writing—review and editing. RB-R and JA: supervision. JA: project administration. NC, JM, and JA: funding acquisition. All authors contributed to the article and approved the submitted version.

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