



Effect of tropical forage species in silvopastoral arrangements on methane production and *in vitro* fermentation parameters in a RUSITEC system

Efecto de especies forrajeras tropicales en arreglos silvopastoriles sobre la producción de metano y parámetros de fermentación in vitro en un sistema RUSITEC

Efeito de espécies forrageiras tropicais em arranjos silvipastoris sob a produção de metano e parâmetros de fermentação in vitro em um sistema RUSITEC

Aldo J Ibarra-Rondón^{1*}; Pedro J Fragoso-Castilla¹; Luis A Giraldo-Valderrama²; José E Mojica-Rodríguez³.

¹Grupo de investigación Parasitología Agroecología Milenio, Universidad Popular del Cesar, Valledupar, Colombia.

²Departamento de ciencias agrarias, Universidad Nacional de Colombia sede Medellín, Medellín, Colombia.

³Corporación Colombiana de Investigación Agropecuaria (AGROSAVIA), Agustín Codazzi, Colombia.

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Abstract

Background: Supplementation of grazing cattle with native and naturalized forages using silvopastoral systems has been suggested as an affordable strategy to reduce methane production and improve nutrition, diminishing the environmental impact of cattle production. **Objective:** To evaluate the effect of three tropical forage species in a silvopastoral arrangement on methane production and fermentation parameters using an *in vitro* ruminal simulation system (RUSITEC). **Methods:** Four diets were evaluated. The control treatment was a basal diet of colosuana grass (COL; *Bothriochloa pertusa*), while the other diets consisted of 70% COL complemented with 30% shrub forage from either *Leucaena leucocephala* (CL), *Guazuma ulmifolia* (CG), or *Crescentia cujete* (CT). A randomized complete block design with repeated measurements over time was used. **Results:** The inclusion of shrub forage did not affect pH, organic matter degradation (OMD) or volatile fatty acids (VFA). The inclusion of shrub forage affected the degradation of structural components. The concentration of N-NH₃ increased in the CL diet compared to COL ($p < 0.05$). In general, methane production in terms of mL/day, mL/g DMi, mL/g DMd, and mL/g OMD was reduced for CL compared to COL ($p < 0.05$). **Conclusions:** Based on these results, inclusion of *Leucaena leucocephala*, *Guazuma ulmifolia* or *Crescentia cujete* on *B. pertusa*-based diets improves ruminal fermentation parameters and reduces *in vitro* methane production.

Keywords: animal adaptation; cattle; climate change; forages; grazing; methane production; rumen fermentation; ruminants; silvopastoral systems; tropical agriculture.

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*Corresponding author. Carrera 9 Balneario Hurtado vía Patillal, Cesar, Colombia; E-mail: aldoibarra@unicesar.edu.co



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Resumen

Antecedentes: Suplementar ganado en pastoreo con forrajes nativos y naturalizados en sistemas silvopastoriles se ha sugerido como estrategia para reducir la producción de metano y mejorar la nutrición, disminuyendo el impacto ambiental de la ganadería. **Objetivo:** Evaluar el efecto de tres especies forrajeras tropicales en arreglos silvopastoriles sobre la producción de metano y parámetros de fermentación utilizando un sistema de simulación ruminal *in vitro* (RUSITEC). **Métodos:** El tratamiento control consistió en una dieta basal de pasto Colosuana (COL; *Bothriochloa pertusa*), mientras que las demás dietas consistieron de 70% COL complementado con 30% de forraje arbustivo de *Leucaena leucocephala* (CL), *Guazuma ulmifolia* (CG) ó *Crescentia cujete* (CT). Se utilizó un diseño de bloques completos al azar con mediciones repetidas en el tiempo. **Resultados:** La inclusión de forrajes arbustivos no afectó ($p>0.05$) el pH, la degradación de la materia orgánica (DMO), ni los ácidos grasos volátiles (AGV). La inclusión de recursos arbóreos afectó la degradación de los constituyentes estructurales. La concentración de N-NH₃ aumentó en la dieta CL en comparación con el control ($p<0.05$). En general, la producción de metano en términos de mL/día, mL/g DMi, mL/g DMd y mL/g Omd se redujo en CL respecto a COL ($p<0.05$). **Conclusiones:** Con base en estos resultados, la inclusión de *Leucaena leucocephala*, *Guazuma ulmifolia* o *Crescentia cujete* en dietas a base de *B. pertusa* mejora algunos parámetros de fermentación ruminal y reduce la producción *in vitro* de metano.

Palabras clave: adaptación animal; agricultura tropical; cambio climático; fermentación ruminal; forrajes; pastoreo; ganado; producción de metano; rumiantes; sistemas silvopastoriles.

Resumo

Antecedentes: A suplementação do gado em pastejo com forragens nativas e naturalizadas usando sistemas silvipastoris, tem sido sugerida como uma estratégia acessível para reduzir a produção de metano e melhorar a nutrição, diminuindo os impactos ambientais da produção de gado. **Objetivo:** Avaliar o efeito de espécies forrageiras tropicais de arranjo silvipastoril na produção de metano e parâmetros de fermentação usando um sistema de simulação ruminal *in vitro* (RUSITEC). **Métodos:** O controle do tratamento (COL) consistiu em uma dieta basal de Colosuana (*Bothriochloa pertusa*) sozinha, enquanto as outras dietas, CL, CG e CT, foram complementadas com 30% de *Leucaena leucocephala*, 30% *Guazuma ulmifolia* ou 30% *Crescentia cujete*, respectivamente. Um desenho de blocos completos randomizados com medições repetidas ao longo do tempo foi aplicado. **Resultados:** A inclusão de forragem arbustiva não afetou o pH ($p>0,05$), a degradação da matéria orgânica (OMD) e os ácidos graxos voláteis (AGV). A degradação dos componentes estruturais foi afetada pela inclusão de forragem arbustiva. A concentração de N-NH₃ aumentou no CL em comparação ao COL ($p<0,05$). Em geral, a produção de metano em termos de CH₄ (mL/dia), CH₄ (mL/g DMi), CH₄ (mL/g DMd) e CH₄ (mL/g OMD) reduziu ($p<0,05$) no CL em relação ao COL. **Conclusões:** Com base nos resultados e considerando as condições experimentais avaliadas, a inclusão de *Leucaena leucocephala*, *Guazuma ulmifolia* ou *Crescentia cujete* em dietas à base de *B. pertusa* melhora alguns parâmetros de fermentação ruminal e reduz a produção de metano *in vitro*.

Palavras-chave: adaptação animal; agricultura tropical; fermentação ruminal; forragem; gado; mudança climática; pastagem; produção de metano; ruminantes; sistemas silvipastoris.

Introduction

Cattle production is a central activity in Cesar (Colombia, South America), providing socioeconomical services, particularly to small producers. With over 1.4 million cattle, this province holds 6% of the inventory and 4.6% of the national dairy production (Colombian Agricultural Institute [ICA], 2020; Ministry of Agriculture and Rural Development of Colombia [MADR], 2018). Due to market globalization this sector faces serious environmental, social, and commercial challenges. Cattle production in this region requires transformation into a profitable, competitive, carbon neutral and ecologically sustainable activity to guarantee internal food safety and economic growth.

A limitation for this transition is related to animal feeding, given that production relies on grass monoculture (Mojica *et al.*, 2017) with low production, availability, and nutritional quality of forages, mostly during the dry seasons. In consequence, the system poorly adjusts to the nutritional requirements of cattle, thus affecting its productivity and profitability (Murillo *et al.*, 2014; Arce *et al.*, 2013). The low nutritional quality and digestibility of forages, given their high content of structural carbohydrates and low protein, increases ruminal methane (CH₄) emissions (Archimède *et al.*, 2011). The CH₄ has high global warming potential, which is 28 times higher than CO₂ (IPCC, 2014) and represents from 2 to 12% of energy loss (Johnson and Johnson, 1995). Additionally, pasture expansion for cattle grazing has been associated with negative pressure over strategic ecosystems, causing deforestation, biodiversity loss, water contamination, soil compaction and other negative impacts on the environment (Cajas-Girón *et al.*, 2012; Roncallo *et al.*, 2012), with greenhouse gas (GHG) emissions being one of the biggest problems (Herrero *et al.*, 2016).

Given this scenario of the cattle role on GHG emissions, mitigation strategies are required. Silvopastoral systems (SPS) are considered an alternative strategy for ruminant production that has positive effects on methane reduction

(Galindo *et al.*, 2016; Rivera *et al.*, 2015). According to several researchers, the inclusion of legume forages in grass-based diets (such as *Leucaena leucocephala*, clover (*Trifolium* sp.) and other local forage leguminous resources) has a positive effect on methane reduction, ranging from 7 to 54% lower CH₄ emissions (Molina *et al.*, 2013; Navarro-Villa *et al.*, 2011; Klein and Eckard 2008; Possenti *et al.*, 2008).

The objective of this study was to evaluate the effect of tropical forage species in silvopastoral arrangements on methane production and fermentation parameters using an *in vitro* ruminal simulation technique – RUSITEC.

Materials and Methods

Ethical considerations

The Animal Care and Use Committee of Universidad Nacional de Colombia (CICUA-013) approved the use, handling and treatments of animals in the study.

Forage sampling

The forage grass and shrub forage samples were collected from pastoral monocultures and silvopastoral arrangements established by AGROSAVIA (Corporación Colombiana de Investigación Agropecuaria) at its Motilonia Research Center (Agustín Codazzi, Cesar, Colombia; 10°00'07"N, 73°14'51"W; 160 masl). Annual average temperature is 27.8 °C, 70 % relative humidity, and 1,360 mm average rainfall. Sampling was performed during the dry season (April) using the hand-pluck method (Euclides *et al.*, 1992) up to the regular grazing height (45 days regrowth). After collection, samples were analyzed in the Ruminant Biotechnology Laboratory (BIORUM) at Universidad Nacional de Colombia (Medellín, Colombia).

Treatments

Each diet was formulated based on the estimated dry matter consumption of animals grazing on monocultures and SPS (Cuartas *et al.*, 2015) (Table 1). Treatments evaluated

were: COL: 100% colosuana (*Bothriochloa pertusa*), CL: 70% colosuana + 30% Leucaena (*Leucaena leucocephala*), CG: 70% colosuana + 30% guácimo (*Guazuma ulmifolia*), CT: 70% colosuana + 30% totumo (*Crescentia cujete*).

Table 1. Composition of the four evaluated treatments.

Treatment	Colosuana	Leucaena	Guácimo	Totumo
Inclusion (%)				
COL	100	-	-	-
CL	70	30	-	-
CG	70	-	30	-
CT	70	-	-	30

COL: 100% colosuana (*Bothriochloa pertusa*), CL: 70% colosuana + 30% Leucaena (*Leucaena leucocephala*), CG: 70% colosuana + 30% guácimo (*Guazuma ulmifolia*), CT: 70% colosuana + 30% totumo (*Crescentia cujete*).

Analyses of nutritional quality

Samples were dried using a forced air stove at 60°C for 48 hours at constant mass.

Crude Protein (CP) content was determined using the Kjeldhal method (AOAC, 2010). Neutral-detergent fiber (NDF) and acid-detergent fiber (ADF) were estimated using the methodology proposed by Van Soest *et al.* (1991); lignine content (LIG) was determined using sulfuric acid (72% v/v) on the ADF residue. Ash content (AC) was measured by direct incineration of the sample in a muffle at 500°C for 4 hours (adapted from AOAC 942.05), and organic matter (OM) content was calculated as the difference between DM and AC. Ethereal extract (EE) was determined with the Soxhlet method (AOAC, 2010).

In vitro ruminal simulation technique - RUSITEC

For the *in vitro* fermentation, a semi-continuous RUSITEC system (Rumen simulation technique) was used following Czerkawski and Breckenridge (1977).

The ruminal content (liquid and solid) for the initial inoculum was obtained from two Holstein breed cows (700 ± 25 kg live weight) equipped with permanent rumen cannula, adapted to a diet based on angleton grass (*Dichanthium aristatum*)

hay; with CP, NDF, ADF and *in vitro* dry matter digestibility (IVDMD) values of 3.95, 78.5, 52.3, and 45.2%, respectively. Both cows were housed at Paysandú Experimental Farm, property of the National University of Colombia in Medellín (Colombia). The ruminal liquid was collected in thermos, preheated to 39 °C, to guarantee temperature and anaerobiosis conditions during transfer to the laboratory. Once in the laboratory, the liquid was mixed and filtered through two layers of muslin (0.45 mm pore size) under constant gassing with CO₂. The RUSITEC system consisted of eight vessels (fermentation units) with 700 ml effective volume, kept in a 39 °C water-bath. To begin incubation, 80 grams (g) of the solid portion of the ruminal content and 12 g of the treatment to be evaluated were deposited in each fermenter into 100 µm pore size nylon bags, 500 mL of ruminal liquid, and 200 mL of pH = 8.3 artificial saliva (McDougall, 1948). After 24 h, the bag with the solid ruminal content was replaced in each fermenter by one with a new substrate (treatment). In the following days, each bag was replaced at 24-hour intervals, guaranteeing 48-hour incubation per bag. Daily pH was measured immediately after sample collection, using a pH meter (Metrohn model 704, Herisau, Switzerland). During the bag exchange process, the containers were gassed with pure CO₂ to maintain anaerobic conditions. Throughout the experiment, saliva was prepared daily and was continuously supplied in each fermenter at a rate of 535 mL/d (2.47%/h) with a peristaltic pump and constant agitation through a mechanical platform (rate: 10-12 movements/minute) to avoid dilution of the products resulting from the fermentation system. The volume of kitasatos flasks (Glassco, Haryana, India) was measured daily to guarantee the flow of artificial saliva in the fermenters.

During sampling, from days 7 to 13, the effluent liquid was collected in 1L kitasato flasks with a solution 20% v/v of H₂SO₄ (Merck, Whitehouse Station, NJ, USA) to determine VFA profile and ammonia, while the nylon bags with diet residue were used to determine dry matter degradability (DMD). The experiment was carried out in two periods (blocking factor). Incubation in each

period lasted 10 days, with 7 days of adaptation (from day 0 to 7). The measurements were made from day 8 to day 10. The treatments were randomly assigned to the fermenters per period, with four replicates per treatment.

In vitro nutrient degradability, fermentation parameters and methane production

The DMD was calculated as the difference between incubated DM and degraded DM after 48 hours. To determine degradability of cell wall components, NDF and ADF percentage was determined from the residual DM following Van Soest *et al.* (1991). The NDF degradability (NDFD) and ADF degradability (ADFD) were estimated as the difference between incubated NDF or ADF, and degraded NDF or ADF, respectively. The OM digestibility (OMD) was determined considering the incubated OM and the degraded OM. Crude protein degradability (CPD) was determined after a previous detachment of ruminal microorganisms using a methyl-cellulose solution (Whitehouse *et al.*, 1994). The CP was then estimated using Kjeldhal's method (AOAC, 2010). The CPD was obtained from the difference between incubated and degraded CP. Ammoniacal nitrogen (N-NH₃) was quantified following protocols described by the AOAC (1999), using an ammonium selective electrode ISE.NH₃-N (Metrhom model SM703, Herisau, Switzerland). Volatile fatty acids (VFA) were estimated using gas chromatography (GC) (Shimadzu model GC-2014, Kyoto, Japan) equipped with autosampler, auto-injection and an Agilent® HP-FFAP polyethylene glycol capillary column of 25 m length x 0.32 mm internal diameter x 0.5 µm film thickness (Agilent Technologies©, Santa Clara, CA, USA). A flame ionization detector (FID) was used with helium as carrier gas at a constant speed of 42 cm/second. The temperature of the split injection port and detector were 260°C and 280°C, respectively. For quantification purposes, problematic samples were compared to the retention times of established standards.

The volume of gas produced was measured through a dry process gasometer (Shinagawa, Model DC-1C, Tokyo, Japan). The gas produced was collected in 5 L aluminium bags and a

hermetic closure valve, which were connected to the kitasato flasks containing the effluent, while the samples for CH₄ determination were collected in vacutainers. The CH₄ analysis was performed using GC (Shimadzu, model GC- 2014, Kyoto, Japan) equipped with FID and an Agilent® HP-PLOT Molesieve 5Å capillary column of 30 m length x 0.32 mm internal diameter x 12 µm film thickness (Agilent Technologies©, Santa Clara, CA, USA). The carrier gas used was UHP 5.0 grade helium at a linear speed of 35.4 cm/second. Injector and detector temperatures were 100°C and 300°C, respectively.

Statistical Analysis

The fermentation variables were subjected to analysis of variance (ANOVA) using the MIXED procedure of the SAS® program (version 9.1.3; SAS Institute Inc., Cary, NC, USA; 2001) through a randomized complete block (RCB) design with univariate structure and analysis of repeated measures in time (three measurement times) with 4 treatments, 8 repetitions and 2 measurement periods. The model included the fixed effects of diet, the measurement time, and their interaction. The experimental period (blocking factor) and the fermenter (experimental unit) were considered as random effects. Since there were no effects of measurement time or diet x time interactions, these data were not included in the tables. The covariance structures were adjusted by using mixed models with fixed and random effects, which allowed estimating the most appropriate standard errors for the different comparisons, according to the characteristics of each set of values. The minimum values of the Schwarz's Bayesian Information Criterion (BIC) were used to select the covariance structure.

Significant differences were compared using a Tukey test with a 5% significance level ($p < 0.05$), and values between $0.05 \leq p \leq 0.10$ were considered as trends.

The statistical model applied was:

$$Y_{ijk} = \mu + D_i + \beta_j + \epsilon_{ij} + T_k + (DT)_{ik} + \epsilon_{ijk}$$

Where,

Y_{ijk} : reading for the *i*-th treatment, on *j*-th block, at *k*-th time.

μ : overall population average

D_i : *i*-th diet effect.

β_j : *j*-th block effect (1 to 2).

ϵ_{ij}^d : error between diets

T_k : measurement time (1 to 3).

$(DT)_{ik}$: diet and measurement time interaction (1 to 12).

ϵ_{ijk} : experimental error.

Results

Chemical composition and *in vitro* dry matter digestibility

The OM contents were between 88.61 and 89.33%. The CP content was the lowest for COL (7.85%), while inclusion of *Leucaena leucocephala* (CL), *Guazuma ulmifolia* (CG) and *Crescentia cujete* (CT) increased CP (41, 31 and 26%), respectively, compared to the control treatment (COL). Structural cell wall components, ADF and NDF, were higher for COL as compared to other treatments, while LIG was higher in CL, CG and CT, compared to COL (Table 2).

Regarding IVDMD, values were found between 42.43 and 49.05%, with higher digestibility in the three shrub diets (CL, CG and CT). For the forages used in this study, CP contents were between 12.6 for *C. cujete* and 28.4% for *L. leucocephala*, the latter standing out for its high protein content. The OM contents were between 90.8 and 92.4%, and AC were between 7.6 and 9.2%. The NDF contents (between 42.2 and 56.7%) were lower for shrub forage compared to grass-based diets of *B. pertusa*. The ADF content ranged from 24.7 to 36.5%, while LIG ranged from 15.5 to 17.8%, being higher on grass-based diets.

pH, Methane production and *in vitro* fermentation parameters

As shown in Table 3, the pH was not affected ($p>0.05$) by the inclusion of shrub forage in the diets.

Gas production significantly diminished ($p<0.05$) between 9 and 13% in diets with 30% inclusion of forage resources (CL, CG and CT) compared to the control treatment (COL), even when DMD increased between 5 and 9% and OMD not was affected for treatments CL, CG and CT compared to COL.

Table 2. Chemical composition and *in vitro* dry matter digestibility of the diets under evaluation.

Diet	OM	AC	CP	NDF	ADF	EE	LIG	IVDMD
COL	88.6	11.4	7.8	73.9	38.4	1.2	8.3	42.4
CL	89.6	10.4	13.1	58.1	33.2	1.5	9.3	47.9
CG	89.3	10.7	8.2	63.9	36.5	1.8	13.9	43.8
CT	88.8	11.2	10.5	62.7	33.9	2.0	9.5	49.1
L	92.4	7.6	28.4	42.2	24.7	2.7	15.5	49.0
G	91.6	8.4	14.6	52.2	33.7	1.9	17.8	39.7
T	90.8	9.2	12.6	56.7	36.5	1.8	15.7	44.6

COL: 100% colosuana (*Bothriochloa pertusa*), CL: 70% colosuana + 30% *Leucaena leucocephala*, CG: 70% colosuana + 30% guacimo (*Guazuma ulmifolia*), CT: 70% colosuana + 30% totumo (*Crescentia cujete*), L: *Leucaena*, G: guacimo, T: totumo. Chemical composition expressed as percentages of dry matter (DM), organic matter (OM), ash content (AC), crude protein (CP), neutral-detergent fiber (NDF), acid-detergent fiber (ADF), lignine (LIG), ethereal extract (EE), and *in vitro* dry matter digestibility (IVDMD). Different superscript letters (^{a, b, c}) within columns indicate significant statistical difference according to the Tukey-Kramer test ($p<0.05$).

Table 3. *In vitro* ruminal fermentation parameters and nutrient degradation of four treatments after 48 hours of incubation in a semi-continuous ruminal simulation system (RUSITEC).

Parameter	Diet				p-value
	COL	CL	CG	CT	
pH	6.87	6.86	6.86	6.84	0.3763
Total gas (L/day)	2.38 ^a	2.09 ^b	2.17 ^b	2.14 ^b	0.0019
N-NH ₃ (mg/dL)	4.17 ^b	7.50 ^a	4.05 ^b	4.20 ^b	<0.0001
DMD (%)	46.24 ^b	50.27 ^a	48.84 ^a	48.36 ^a	0.0040
OMD (%)	42.42	45.78	44.20	43.68	0.1165
NDFD (%)	34.39 ^a	27.43 ^b	32.30 ^a	28.30 ^b	<0.0001
ADFD (%)	29.52 ^a	29.55 ^a	27.66 ^a	23.10 ^b	<0.0001
CPD (%)	63.39 ^a	64.82 ^a	55.75 ^b	47.83 ^c	<0.0001
Total VFA (mmol/L)	21.62	21.13	22.22	23.24	0.1754
Acetate (mmol/L)	14.08	13.77	13.50	13.80	0.1163
Propionate (mmol/L)	5.47	5.62	5.59	5.70	0.5733
Butirate (mmol/L)	1.71	1.68	1.67	1.98	0.2916
Isobutyrate (mmol/L)	0.10	0.11	0.10	0.10	0.1762
Valerate (mmol/L)	0.31	0.33	0.32	0.30	0.2824
A:P	2.45	2.43	2.41	2.42	0.1000
CH ₄ (mL/day)	12.61 ^a	9.38 ^{bc}	10.47 ^{ac}	11.76 ^{ac}	0.0300
CH ₄ (mL/g DMi)	1.23 ^a	0.96 ^b	0.95 ^b	1.19 ^a	0.0214
CH ₄ (mL/g DMd)	2.70 ^a	1.94 ^{bc}	2.02 ^{ac}	2.30 ^{ac}	0.0345
CH ₄ (mL/g NDFd)	4.89	4.08	4.26	4.62	0.1355
CH ₄ (mL/g ADFd)	10.92 ^a	8.53 ^{ac}	6.47 ^{bc}	8.96 ^{ac}	<0.0001
CH ₄ (mL/g Omd)	3.17 ^a	2.23 ^{bc}	2.20 ^{bc}	2.90 ^{ac}	0.0035

COL: 100% colosuana (*Bothriochloa pertusa*), CL: 70% colosuana + 30% *Leucaena* (*Leucaena leucocephala*), CG: 70% colosuana + 30% guacimo (*Guazuma ulmifolia*), CT: 70% colosuana + 30% totumo (*Crescentia cujete*), DMD: dry matter digestibility, OMD: organic matter degradation, NDFD: neutral-detergent fiber digestibility, ADFD: acid-detergent fiber digestibility, CPD: crude protein digestibility, pH: potential hydrogen, N-NH₃: ammoniacal nitrogen, VFA: volatile fatty acids, A:P ratio: acetate:propionate ratio, Gas: gas production, CH₄: methane, DMi: incubated dry matter, DMd: degraded dry matter, NDFd: degraded neutral detergent fiber, ADFd: degraded acid-detergent fiber, Omd: degraded organic matter. Different superscript letters (^{a, b, c}) within columns indicate significant statistical difference according to the Tukey-Kramer test ($p < 0.05$).

The NDFD significantly diminished ($p < 0.05$) in CL (27.43%) and CT (28.3%) compared to the control (34.49%), but no difference was found with CG (32.3%). The ADF degradation only diminished in CT compared to other treatments. The CPD was higher ($p < 0.05$) for COL (63.69%) and CL (64.82%) in comparison to CG (55.75%) and CT (47.83%). The N-NH₃ concentration increased ($p < 0.05$) by 44 to 46% in CL compared to others.

On the other hand, total VFA production and molar proportions of individual VFA were not affected ($p > 0.05$) by forage inclusion. The acetate:propionate (A:P) ratio showed a tendency to decrease ($p = 0.10$) in diets CL, CG and CT compared to COL.

Methane production expressed in mL/d, mL/g DMd, mL/g DMi, and mL/g Omd, had similar emissions in all diets, showing a significant reduction ($p < 0.05$) for CL compared to COL.

Discussion

Inclusion of shrub forage as a protein source in low quality diets represents an effective alternative to improve the nutritional quality of grass-based diets, increasing animal productivity, particularly in the low tropics (Argüello-Rangel *et al.*, 2019). Additionally, secondary compounds from the plants used in ruminant feeding can be used to manipulate ruminal fermentation dynamics (Anantasook y Wanapat, 2012). Under the present conditions, DMD increased in all diets with inclusion of shrub forage, which could result in increased passage rate and voluntary consumption of forage (Choque *et al.*, 2018). These results agree with Molina *et al.* (2015), who reported that 27% inclusion of *L. leucocephala* foliage in a grass-based diet increased DMD by 12%. The low degradability of fiber fractions (NDFD and ADFD) in diets with shrub forage could have resulted in lower cellulolytic activity due to the effect of secondary compounds, such as tannins and saponins, present in *L. leucocephala* (Soltan *et al.*, 2017), *G. ulmifolia* (López *et al.*, 2004) and *C. cujete* (Pereira *et al.*, 2017) on ruminal microbial populations, particularly protozoa, responsible for up to 25-30% of fiber degradation (Lee *et al.*, 2011), or through the formation of cellulose complexes, reducing carbohydrates degradation (Khiaosa *et al.*, 2015).

The lowest ($p < 0.05$) fiber digestibility was observed in the diet with *L. leucocephala* (NDFD) and *C. cujete* (NDFD and ADFD), possibly due to tannins and phenylpropanoids in different genres of this shrub, which form cellulose complexes and reduce carbohydrate degradation, limiting rumen degradation (Parente *et al.*, 2016; Rojas *et al.*, 2015; Khiaosa *et al.*, 2015). The effect of these compounds may be due to union with sterols of the microbial membranes, particularly cholesterol, forming insoluble complexes and lysing the cells (Ramos-Morales *et al.*, 2017). These compounds can also affect digestibility by binding to proteins and other nutrients, limiting microbial enzymatic activity -for example, α -glycosidase and α amylase (Li *et al.*, 2011)- thereby affecting fermentative processes in the rumen (Jayanegara *et al.*, 2014).

In this context, it is suggested that decreased degradation of structural components may be due to adaptation of microbial populations to the new ruminal environment (Sampaio *et al.*, 2009). According to Bodas *et al.* (2012), the effects of secondary metabolites are more evident in *in vitro* than in *in vivo* studies, probably because the compound is distributed more uniformly in batch or continuous cultures, and microbes are more quickly exposed to the activity of the phyto-chemical.

In this study, CPD was not affected in CL as compared to COL; however, CPD decreased ($p < 0.05$) in CG and CT, which corresponds to the high CP content of this legume. However, the degradation percentage of this nutrient in all diets was greater than the degradability of the wall constituents (NDFD and ADFD), showing that secondary compounds, like tannins and saponins, bind with higher affinity to fiber (NDF and ADF) than to protein. In *L. leucocephala*, 0.16 and 0.22% of condensed tannins were bound to CP and fiber, respectively (La O *et al.*, 2003). These differences may be associated with the type of tannins in the forages. Hydrolysable tannins do not generate a bypass effect as occurs with condensed tannins because the formers do not establish rigid bonds with proteins at pH range of the rumen (McSweeney *et al.*, 1988).

These CP degradability in rumen suggest that the bypass protein would be approximately 40%, which is beneficial since it would improve the synthesis of microbial protein. This guarantees the flow of protein with high biological value to the small intestine (duodenum) favouring an efficient use of forages by ruminants (Rodríguez *et al.*, 2010; Wickersham *et al.*, 2008) and increasing milk protein (Martínez *et al.*, 2004) as well as decreasing nitrogen and ammonia excretion to the environment (Cobellis *et al.*, 2016).

The $N-NH_3$ concentration increased ($p < 0.05$) in CL compared to all other diets. This could be due to the higher CP content and CPD in this diet, supporting Javaid *et al.* (2008), who reported that increased degraded

protein in the rumen raises ammoniacal nitrogen levels in buffalos. Other researchers have also reported a positive association between level of protein supplementation and ruminal ammonia concentrations (Mathis *et al.*, 2000; Wickersham *et al.*, 2008).

Therefore, the synchronization between N-NH₃, CPD, and CP in the diet results in increased microbial protein synthesis (Chanthakhoun *et al.*, 2012). In this regard, Detmann *et al.* (2009) found that the ammonium concentration in rumen where degradation and NDF consumption is optimized, was between 8 to 15 mg/dL. Fiber degradation is limited below that level. However, Slyter *et al.* (1979), evaluating the effect of ruminal ammonium concentration in steers receiving a forage diet with 8% CP, found that 2 to 5 mg/dL of N-NH₃ in the ruminal fluid was sufficient to allow maximum growth of rumen microorganisms.

The low contents of ammoniacal nitrogen observed in treatments CG and CT may be explained by the moderate contents of condensed tannins (<4% DM) in *Guazuma ulmifolia* and *Crescentia sp.* (Rojas *et al.*, 2015). Tannins bind with dietary proteins, inhibit microbial deaminases and reduce proteolysis, diminishing protein degradation in the rumen, consequently diminishing N-NH₃ (Szumacher-Strabel and Cieślak, 2012; Bhatta *et al.*, 2009; Min *et al.*, 2006; Newbold *et al.*, 2004). However, ammonium concentration in LC was higher compared to the other treatments, which is explained by the high CPD, due to a low affinity of tannins and saponins in *Leucaena* with the protein (La O *et al.*, 2003).

The VFA are the main products of ruminal fermentation. They regulate physiological processes in ruminants, such as cholesterol, insulin, and glucagon synthesis (Mao *et al.*, 2016). In the present study, total VFA concentration and individual VFA profiles were not affected by forage inclusion, supporting the pH stability among diets. However, the A:P ratio showed a tendency to diminish in diets supplemented with forages, possibly due to high

DM degradation, also related to the reduction of methane synthesis given that propionate production requires hydrogen (Martin *et al.*, 2010). Patra and Yu (2015) found reductions in the A:P ratio related to decreased protozoal populations, which are associated with methanogenic bacteria. This is a side effect of secondary compounds, given that acetate and butyrate are the main fermentation products of these microorganisms. As an outcome, it contributes to low methane production and high microbial protein availability. Secondary compounds in *L. leucocephala*, *G. ulmifolia* and *C. cujete* could explain the VFA profiles obtained in the present study (Soltan *et al.*, 2017; Galindo *et al.*, 2014; Alvear *et al.*, 2013). These compounds also inhibit growth of Gram-positive bacteria (generally acetate producers) favouring propionate-producing bacterial species through competition (Wallace *et al.*, 2002).

Similarly, Apráez *et al.* (2012) found that inclusion of *Acacia decurrens* (forage with tannins) in Raygrass-based diets decreased acetate production by 54% and methane by 80% compared to an only-Raygrass diet. Variations in total gas and methane production among diets may be explained by the variations in their chemical composition.

The results from the present study are comparable to those of Anantasook and Wanapat (2012) who found total VFA between 22.1 and 34.8 mmol/L after including leguminous resources into the diet.

The diet with *L. leucocephala* had the strongest decrease in methane production, likely due to the presence of tannins and low contents of fiber in this leguminous resource (Soltan *et al.*, 2017). The effects of tannins and saponins over methanogenesis have been demonstrated *in vivo* (Tiemann *et al.*, 2008; Beauchemin *et al.*, 2007) as well as *in vitro* (Jayanegara *et al.*, 2011; Tavendale *et al.*, 2005). The effect of these components is related to direct inhibition of methanogenic archaea and/or depression of microbial metabolic processes implied in methanogenesis (Li *et al.*, 2014; Patra, 2012). However, an antiprotozoal

effect has also been proved through complex formation with sterols in the plasmatic membranes of protozoa (Jouany and Morgavi 2007; Goel and Makkar 2012), consequently reducing methane production given their symbiotic relationship with methanogenic archaea (Kobayashi, 2010). Bodas *et al.* (2008) and Kamra *et al.* (2008) evaluated the potential effect of plant extracts on *in vitro* methanogenesis, reporting reductions in CH₄ between 15 and 25% associated with decreased methanogenic populations without adverse effects on digestibility or VFA production.

From these results, a reduction of ruminal methane synthesis can be inferred, therefore lowering the emission of this greenhouse gas into the atmosphere, which is detrimental to the environment by contributing to global warming and it also represents a loss of energy for the animal (Martínez *et al.*, 2017; Yang *et al.*, 2015).

In conclusion, 30% dietary inclusion of forages such as *Leucaena leucocephala*, *Guazuma ulmifolia* and *Crescentia cujete* in grass-based diets of *B. pertusa* increased protein content and decreased fiber in the diets, improving DMD, ammoniacal nitrogen production, and the dynamics of ruminal fermentation parameters, while decreasing methane production. This suggest *L. leucocephala*, *G. ulmifolia* and *C. cujete* are valuable feed supplements to improve nutrient utilization efficiency in ruminants in the low tropics.

Declarations

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Conflicts of interest

The authors declare they have no conflicts of interest with regards to the work presented in this report.

Author contributions

LAG was responsible for the conception of the study; AJI was responsible for data collection, analysis and writing of the manuscript. PJF was responsible for project administration. All authors reviewed, performed a critical reading, and edited the manuscript.

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