Agroforest Syst (2024) 98:1967–1984
https://doi.org/10.1007/s10457-024-01046-y

Effect of a silvopastoral system with *Leucaena diversifolia* on enteric methane emissions, animal performance, and meat fatty acid profile of beef steers

Isabel Cristina Molina-Botero · Daniel M. Villegas · Alejandro Montoya · Johanna Mazabel · Mike Bastidas · Alejandro Ruden · Hernan Gaviria · Juan David Peláez · Julián Chará · Enrique Murgueitio · Jon Moorby · Jacobo Arango

Received: 14 December 2023 / Accepted: 22 July 2024 / Published online: 31 July 2024
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Abstract The aim of this research was to evaluate dietary inclusion of *Leucaena diversifolia* into a grass monoculture (GM) diet and its influence on enteric methane emissions, animal live weight gain and meat fatty acid content of steers. Two GM diets comprised *Urochloa* hybrid cv. Cayman or *U. brizantha* cv. Toledo; silvopastoral (SPS) mixed diets included an association of either grass with *L. diversifolia* in a 74:26 (grass:legume) planting ratio. The experiment was conducted over 16 months with 14 Brahman x Angus crossbred steers (374±7.5 kg body weight), divided into two diet groups. Steers were weighed periodically during the experiment, and methane emissions were quantified with the polytunnel technique. The meat quality and fatty acid profile were measured after slaughtering the steers. Energy density, total and digestible dry matter intake and crude protein were higher in steers fed SPS than GM

I. C. Molina-Botero · D. M. Villegas · A. Montoya · J. Mazabel · M. Bastidas · A. Ruden · J. Arango (✉)
International Center for Tropical Agriculture (CIAT), Km 17 Recta Cali-Palmira, A. A, 6713 Palmira, Valle del Cauca, Colombia
e-mail: j.arango@cgiar.org

I. C. Molina-Botero
e-mail: isamo2609@gmail.com

D. M. Villegas
e-mail: d.m.villegas@cgiar.org

A. Montoya
e-mail: alejandro.montoya@cgiar.org

J. Mazabel
e-mail: l.j.mazabel@cgiar.org

M. Bastidas
e-mail: m.bastidas@cgiar.org

A. Ruden
e-mail: d.ruden@cgiar.org

A. Ruden
Agronomy Department, University of Florida, Gainesville, FL 32611, USA

H. Gaviria · J. D. Peláez
Asobrangus Comercial ANGUSAZUL®, Cl. 33AA #78 A 112, Medellín, Antioquia, Colombia
e-mail: abcbogota@angusazul.com

J. D. Peláez
e-mail: davidpelaez@yahoo.com

J. Chará · E. Murgueitio
Centro Para la Investigación en Sistemas Sostenibles de Producción Agropecuaria, CIPAV, Cr 25# 6-62, A. A. 760002 Cali, Valle de Cauca, Colombia
e-mail: julian@fun.cipav.org.co

E. Murgueitio
e-mail: enriquem@fun.cipav.org.co

J. Moorby
Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth SY23 3EE, UK
e-mail: jxm@aber.ac.uk

($P \leq 0.05$). At the end of the experiment, animals from SPS weighed on average 63 kg more than animals offered GM (535 vs. 472 kg; $P=0.025$). Net methane emitted by steers offered SPS was 142 g per day, this is equivalent to 15% less than emissions from animals that were offered GM (168 g; $P=0.007$). The meat fatty acid profile was similar between diets, except for cervoniv-DHA and omega-6: omega-3 ratio, which were higher in steers consuming GM diet ($P \leq 0.05$). The results of this study suggest that the inclusion of *L. diversifolia* improves nutrient intake, increases animal productivity, reduces daily enteric methane emission, and decreases the omega-6: omega-3 ratio in meat.

Keywords Animal productivity · Grass · Meat quality · Agroforestry · Greenhouse gas

Introduction

Bovine livestock production contributes approximately 106 million tons of methane annually from enteric fermentation and manure management (UNEP and CAC 2021). Methane, a greenhouse gas (GHG) has a global warming potential (GWP100) of 28 on a CO_2 equivalent basis (IPCC 2018). Under the business-as-usual scenario, it is estimated that methane emissions will rise three times over the next twenty years (Hristov et al. 2018). However, it is expected that the implementation of mitigation policies during this decade will lead to a 1.3% decrease in GHG emissions from the beef sector by 2030 (OECD/FAO 2022). Nevertheless, methane has a lower impact on radiative forcing and global temperature in the short term compared to other greenhouse gases (Lynch et al. 2020).

Some strategies to reduce enteric methane emissions include modulating rumen fermentation through the supply of antibiotics, probiotics, oils, plant secondary compounds and improving the nutritional quality of the diet by rotational grazing of animals (Ku-Vera et al. 2020; Thompson and Rowntree 2020; de Souza et al. 2021; Arndt et al. 2022). Tropical legumes, fruits, tubers and forages are rich in secondary compounds such as condensed tannins, saponins, flavonoids and/or essential compounds, which can reduce methane emissions by 10-35% (de Souza et al. 2021; Beauchemin et al. 2022; Berça et al. 2023; Magnani et al. 2023), while providing protein and energy in the diet and increasing animal productivity (Caram et al. 2023). In addition, enteric methane emissions are positively associated with DM intake, live weight (LW) and average daily gain (ADG) and have a moderate to low positive correlation with some carcass characteristics such as intramuscular fat (Lakamp et al. 2022). The inclusion of the legume *Leucaena diversifolia* in the diet would likely be beneficial, since it is characterized by a high protein content (250 g/kg DM), medium *in vitro* dry matter (DM) degradability (>55 g/kg DM), high tannin content (323 g/kg DM) (Gaviria-Uribe et al. 2020; Quintero-Anzueta et al. 2021) and the presence of other secondary metabolites such as terpenoids, steroids, saponins and flavonoids (Emitaro et al. 2020).

The inclusion of legume in the diet has promising implications for improving animal productivity while reducing methane emission per unit product or animal. Based on a recent report by the Food and Agriculture Organization of the United Nations (FAO 2023), animal-source foods provide people with essential nutrients that are difficult to acquire from plant-based foods. It emphasizes the importance of animal-derived products, especially during critical life phases like pregnancy, breastfeeding, childhood, adolescence, and elderly (FAO 2023). In addition to high-quality protein, beef provides polyunsaturated fatty acids such as omega 3 and omega 6, which have antioxidant, anti-inflammatory properties among other benefits to human health (Djuricic and Calder 2021). Meat lipid profile, composed mainly of esterified fatty acids, is influenced by the diet consumed by cattle, *e.g.*, pasture and forage-based diets are common sources of omega-3 polyunsaturated fatty acids, while diets rich in grains provide mainly omega-6 (Ponnampalam et al. 2021).

The productivity and environmental benefits from legume feeding (Castro-Montoya and Dickhoefer 2020; Gaviria-Uribe et al. 2020) have been investigated and demonstrated through the use of silvopastoral systems based on the association of *L. leucocephala* with tropical grasses (Chará et al. 2019). But little attention was paid to the nutritional value of *L. diversifolia* for cattle and its impact on methane emissions and meat quality. This work hypothesized that including *L. diversifolia* in cattle feed would enhance LW gain and meat quality, while also reducing methane emissions. Therefore, the present research aimed

to compare enteric methane emissions, animal LW gain, and meat fatty acid profile in cattle grazing grass alone pasture (*Urochloa* hybrid cv. Cayman and *U. brizantha* cv. Toledo) compared to a silvopastoral system composed of grass (Cayman/ Toledo) and legume (*L. diversifolia*).

Methods

Location

The experiment was conducted at the International Center for Tropical Agriculture (CIAT) located at 3°30'7 "N; 76°21'22 "W in the department of Valle del Cauca, Colombia. Soil is categorized as mollic type, Pachic Haplustoll (Corporación Autónoma Regional del Valle del Cauca & Instituto Geográfico Agustín Codazzi, 2021), with a clay-loam texture and a pH of 7.5. During the experimental period, CIAT weather station recorded ambient temperatures ranging from 15.3 to 33.8°C, relative humidity from 65.9 to 100%, and monthly precipitation from zero to 102.6 mm.

Diets evaluated

Throughout the experimental period, beef steers were fed one of two treatments based on a grasses monocultures (GM) with and without the addition of *Leucaena diversifolia* in silvopastoral systems (SPS). For animal performance and meat quality trials, steers on the GM treatment rotated with improved grasses: *Urochloa* hybrid cv. Cayman-CIAT BR02/1752, and *Urochloa brizantha* cv. Toledo (CIAT 26110), while animals on the SPS treatment were grazed on the same two grasses with the addition of *L. diversifolia* ILRI 15551 (in 76.8:23.8 ratio, respectively). These proportions between grasses and legumes consumed were calculated from the difference between the biomass of forage before and after the animals entered the plot for grazing using the method described by Haydock and Shaw (1975) and modified by Montoya et al. (2023) to include shrubs. This legume was previously planted at a density of 2.000 plants per ha (1,500 for animal browsing and 500 for shade).

In the evaluation of enteric methane emissions, the animals were offered the diet of either *U.* hybrid cv. Cayman (GM) or the same grass plus *L. diversifolia* (SPS) in proportions of 74±2.8% grass and 26±2.9% *L. diversifolia* (dry matter basis). These proportions were calculated from the average of the difference between the amounts offered and refused of each forage during the animals' stay in the polytunnel.

During each methane measurement for every period and treatment, a portion of the fresh forage provided to each animal within the polytunnel was extracted and combined to obtain a sample weighing approximately 500 g. Subsequently, all forage samples underwent analysis at CIAT's Forage Quality and Animal Nutrition Laboratory (refer to Tables 1 and 2). The samples were weighed and then dried in a forced-air oven (Memmert UF 750) at 60 °C for 55 h to determine DM content (International Organization for Standardization ISO: Method 6496 (1991)), then ground in a cutting mill (Retsch® SM 100, Haan, Germany) to pass through a 1 mm sieve. Crude protein (CP) content was determined according to the Association of Analytical Communities- AOAC (2001) described in method 2001.11 . Acid detergent fiber (ADF) and neutral detergent fiber (NDF) content were determined using an Ankom 2000® fiber analyzer (Ankom Technology Corp., Macedon, NY, USA) following the method of Van Soest (1970). The ash content was determined using the AOAC (1990) methodology and using this value the organic matter (OM) content was calculated (OM % = 100 - ash content in %). Gross energy (GE) density was determined using a Parr 6400® isoperibolic calorimetry pump (Parr Instrument Company, Illinois, USA) according to ISO 9831:1998 specifications. *In vitro* DM digestibility (IVDMD) was measured using the methodology described by Tilley and Terry (1963) with fresh rumen fluid obtained from steers grazing *Cynodon plectostachyus*, while legume tannin and phenols content determination was performed according to Terrill et al. (1992).

Animal performance

The experiment was conducted with 14 Brangus (Brahman x Angus) crossbred steers of 12±3 months of age and an initial mean weight of 347±7.5 kg (346 kg for animals on the SPS diet and 349 for animals with GM). At the beginning of the experiment, steers underwent a veterinary inspection, as well as internal and external parasite treatments (Ivermectin 1% dosed according to manufacturer recommendations: Vecol®

Table 1 Mean (± SD) biomass production and nutritional composition of the individual diets and mixed diets offered to the steers during the animal performance and meat quality trials

Animal performance and meat quality trials							
Variable	Toledo (GM)[1]	Cayman (GM)	Toledo (SPS)	Cayman (SPS)	*Leucaena diversifolia (Toledo)*	*Leucaena diversifolia (Cayman)*	SPS (76.8:23.8)
Biomass production (DM, Kg ha)							
Months of June -August	1069 ± 266	629 ± 273	1244 ± 288	812 ± 453	94 ± 74	129 ± 116	816
Months of October November	501 ± 473	614 ± 522	695 ± 651	598 ± 471	22 ± 9	30 ± 9	502.7
Nutritional quality – June August							
CP, g/kg DM	96 ± 2	88 ± 1.7	97 ± 1.6	13 ± 1.9	254 ± 4.5	256 ± 5.8	218.9
NDF, g/kg DM	686 ± 2.4	613 ± 1.8	697 ± 1.5	620 ± 5 3	528 ± 5.4	530 ± 4.7	986.7
Nutritional quality – October November							
CP, g/kg DM	79.2 ± 0.6	60.5 ± 0.4	98.3 ± 1.4	133.7 ± 0.6	264 ± 5.8	266 ± 3.9	221.3
NDF, g/kg DM	618.2 ± 2.2	590.6 ± 3.3	606.5 ± 1.8	615.8 ± 3.9	603 ± 4.7	587 ± 3.0	915.6

SPS 76.8% *U. brizantha* cv. Toledo and 23.8% *Leucaena diversifolia*; *DM* dry matter; *OM* organic matter; *CP* crude protein; *NDF* neutral detergent fiber; *ADF* acid detergent fiber; *GE* gross energy; *IVDMD in vitro DM* digestibility. [1]Data obtained from Villegas et al. (2022)

Table 2 Mean (± SD) nutritional composition of the individual diets and mixed diets offered to the steers during the enteric methane emissions trials

SPS 74% U. brizantha cv. Toledo and *26% Leucaena diversifolia; DM* dry matter; *OM* organic matter; *CP* crude protein; *NDF* neutral detergent fiber; *ADF* acid detergent fiber; *GE* gross energy; *IVDMD in vitro DM* digestibility.

Enteric methane emissions trials				
Variable	Cayman (GM)	Cayman (SPS)	*Leucaena diversifolia* (SPS)	SPS (74:26)
DM, g/kg	242 ± 1.6	230 ± 2.5	237 ± 3.7	232 ± 3.4
OM, g/kg DM	873 ± 0.9	863 ± 1.7	933 ± 0.2	881 ± 1.3
CP, g/kg DM	64 ± 1.4	82 ± 0.8	292 ± 0.5	135 ± 0.6
NDF, g/kg DM	699 ± 1.9	714 ± 1.6	614 ± 0.7	688 ± 1.2
ADF, g/kg DM	372 ± 1.9	366 ± 2.2	342 ± 5.2	348 ± 1.9
GE, MJ/kg DM	17.3	17.3	19.8	17.9
IVDMD, g/kg DM	467 ± 2.5	580 ± 3.9	543 ± 5.2	570 ± 2.9
Total phenols, g/kg DM	3.30	3.30	57.6	17.42
Tannins, g/kg DM	0.10	0.03	47.6	12.40

Bogota, Colombia). At their arrival to the experimental site, steers were randomly divided into two groups (n = 7 per treatment group), one group assigned to graze the GM treatment plots, with the other assigned to graze the SPS plots. Over a period of 16 months, each group of animals in the GM or SPS treatment rotated through 6 plots. Each plot was divided into 3 subplots, which were fully grazed within a period of approximately 6 days. Using this rotation pattern, the regrowth age of 37±7 days was always maintained for the forages offered Throughout the experiment's duration, the animals were weighed five occasions: four times during the first 14 months and one more during methane evaluations. Animal weight was consistently performed in the morning hours utilizing a HIWEIGH® scale (Suzhou City, Jiangsu Province, China) with a capacity of one ton, and with a precision of ±200 grams. In addition, steers were provided with mineral salts and water *ad libitum*. The animals were grazing for the duration of the experiment and therefore forage DMI (in kg/d) was estimated using the equation proposed by Azevêdo et al. (2010) and cited by Méo-Filho et al. (2020):

$$DMI = -2.6098 + 0.08844 \times ALW^{0.75} + 4.4672 \times ADG - 1.3579 \times ADG^2$$

Where DMI is the dry matter intake, ALW is the average live weight between the initial and final weight (kg) of last month of the experiment and ADG is the average daily gain (kg/d) over each same period.

Digestible intake of DM or any of the nutrients was obtained from the multiplication between the *in vitro* DM digestibility (%) and its intake (g or kg/d).

Methane emissions

Before concluding the experiment with the animals, methane measurements were conducted every 8 days for four consecutive weeks for each group of animals, spanning from May to June. The measurements were conducted using the polytunnel technique (Lockyer 1997; Murray et al. 2007) based on the procedures outlined by Molina et al. (2016) and Gaviria-Uribe et al. (2020). A total of four polytunnels were used; two of them were polytunnels with fixed structures with an area of 48 m^2 and a volume of 134 m^3, while the other two polytunnels were mobile structures with an area of 24 m^2 and a volume of 62.74 m^3. Each polytunnel was manufactured with a high-strength, waterproof vinyl tarpaulin (IKL® 500 Black Out canvas of 700 g/m^2). Each polytunnel was hermetically divided into two chambers and one animal was housed in each chamber. Each chamber was equipped with a feed trough, an automatic drinking trough, mineral salts, two fans, a thermohygrometer (HTC-2®. Wincom Company Ltd., Hunan, China) and a soil floor with a layer of sawdust. On the opposite side of each chamber from the animal entrance, a 12" exhaust fan was installed with an extraction rate of 0.9±0.15 m^3 per second. The extracted air velocity was measured with an anemometer (BT-866A Pro-Anemometer®, Bestmeter Electronics Technology Co. Ltd. Guangdong, China) each time the extractor fan was turned on for a measurement.

On measurement days, steers were taken to the polytunnels at 06:00 am, and then the polytunnel door flaps were closed. Each animal remained in their chamber for 24 hours, and received the same fresh *ad libitum* diets they had been grazing. Fresh forages offered daily to the animals were harvested at a height of more than 20 cm and 37 days of regrowth; for steers on **SPS**, *L. diversifolia* was manually collected, simulating the harvest height and the bite size of the animals in the silvopastoral system. We conducted 24 consecutive hourly measurements of concentrations of methane in accumulated chamber and ambient air using a Gasmet DX4040® portable FTIR gas analyzer® (Gasmet Technologies OyR, Helsinki, Finland) equipment, previously tested for the presence of no methane inside with ultrapure nitrogen gas grade 5.0 (zero calibration). The ambient concentration of methane gas was also measured hourly. Temperature and relative humidity were simultaneously recorded inside and outside of each of the polytunnels with the thermohygrometer.

Daily methane emission (g/d) for each animal was calculated (Gaviria-Uribe et al. 2020) and was related to diet DM and nutrient consumption of that animal. The proportion of gross energy intake lost in the form of CH_4 (Ym, %) and the emission factor (kg methane per animal per year), were calculated as described in IPCC (2006).

Carcass and meat characteristics

At the end of the experimental period, the steers were transported to a certified slaughterhouse. After slaughter, the weight of the carcass was recorded for each animal (Amador et al. 1995), and it was refrigerated at 2°C for 48h, after which time it was weighed again and deboned. Then two samples (300 and 500-g) were collected from the left portion of the *Longissimus dorsi* muscle, between the 6th and 13th thoracic vertebrae of the muscle following the methodology of Cańeque and Sańudo (2001). Both samples were vacuum packed, refrigerated at 4°C and transported to the laboratories for subsequent analyses. One of the meat samples was sent to the Food Quality Laboratory, of the La Libertad Research Center of AGROSAVIA for shear force testing by Warner Bratzler following the previously published procedures (Font-i-Furnols et al. 2015). The second meat sample was sent to the CIAT Forage Quality and Animal Nutrition Laboratory, where it was minced using a standard kitchen food processor (Black and Decker®. Maryland, United States). After weighing one gram of the ground meat sample, it was diluted in 10 ml of distilled water and the pH was measured with a potentiometer (Hanna HI 9125® pH meter, Woonsocket, USA. Rengifo and Ordóñez, 2010). Calibration of the pH electrode was

performed with standardized buffers (pH 4.0 and 7.0). In the same laboratory, dry matter, CP and ash content of the meat samples were evaluated as described above. The rest of meat sample was freeze-dried for subsequent qualitative quantification fatty acid at the Toxicology Laboratory of the Faculty of Veterinary Medicine and Zootechnics of the National University of Colombia, Bogotá, Colombia.

Extraction and methylation to quantify fatty acids in forages was done according to the methodology described by Mojica-Rodriguez et al. (2017). The measurement of fatty acid, in both forage and meat samples, was performed using a Shimadzu® gas chromatograph (model GC 2014. Shimadzu Corporation®. Kyoto, Japan). The characteristics of the capillary column used, the injector and FID temperatures, the carrier gas and the retention times were described in detail by Mojica-Rodríguez et al. (2017). Individual fatty acids and fatty acid groups as percentage of total fatty acids are reported in the Results section.

Data analysis

Data of feed intake, nutrient digestibility, gross energy densities of diets, animal productivity characteristics and enteric methane emissions underwent analysis using the PROC MIXED procedure of SAS 9.4® software (SAS Institute Inc., Cary, NC, USA, 2012). The analysis employed a repeated measures design over time, with each diet consisting of seven animals (experimental unit) measured at various intervals. For animal performance, measurements were conducted five times over a span of 16 months, while other variables were measured four times within one month. We compared means with Tukey's test when the treatment probability significance level of less than 0.05 was detected under the following statistical model:

$$Y_{ijk} = \mu + \delta_i + \tau_k + \beta_{ji} + (\delta * \tau)_{ik} + \varepsilon_{ijk}$$

where Y_{ijk} is the response of subject j under diet i, during time k; μ is the population mean; δ_i is the effect of the i-th diet (i = with and without *L. diversifolia* intake, 1 or 2 respectively); τ_k is the effect of the k-th time (k = 1..., 4 or 1…,5. The number may vary depending on the specific test being performed); β_{ji}: effect of the j-th steer (j=1.... 14) on i-th diet (i=1 or 2, with and without *L. diversifolia* intake, 1 or 2 respectively); $(\delta*\tau)_{ik}$ is the interaction between i-th diet and k-th time, and ε_{ijk} is the experimental error.

Results of carcass and meat characteristic were analyzed using detrended correspondence analysis (DCA) experimental arrangement with a single factor with the following model:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

where: Y_{ij} is the response of the j-th steer (j=1...14) when consuming the i-th diet (i=with and without *L. diversifolia* intake, 1 or 2 respectively), μ is the global mean, δ_i is the effect of i-th diet (i= 1 or 2), defined as the difference between the mean of the i-th diet and the global mean, ε_{ij} is the random error variable associated to the j-th experimental unit of the i-th diet.

Net methane emissions and their relationship with DM intake or live weight gain were plotted according to the recommendations of Patil (2021) with the R statistical package (R version 4.2.1. R Core Team 2021).

Results

Intake and digestibility of diets

Animals on diet of Cayman grass plus *L. diversifolia* consumed 1.22 times more DM than animals that ingested only grass (8.6 vs. 7.0 kg DM/d, $P \leq 0.05$; Table 2). Differences in mean diet concentrations of CP, together with estimated differences in DM intake, indicated that CP daily intake on the SPS was twice as high as that of the GM ($P \leq 0.05$; Table 2). However, NDF and ADF intake was estimated to be similar for animals on both treatments (Table 3). The GE intake by SPS steers was estimated to be 21% higher than that of GM animals ($P \leq 0.05$). Mean digestibility of DM and CP was higher for the SPS than for the GM ($P \leq 0.05$), while NDF and ADF digestibility did not vary between treatments. Digestible energy ranged between 85.5 and 55.3 MJ/kg DM for SPS and GM respectively ($P \leq 0.05$).

When evaluating the intake of total and digested nutrients (CP, NDF and ADF), differences between measurement periods were observed ($P \leq 0.05$), likewise, there was an interaction between diet and measurement when we evaluated the intake of

Table 3 Mean estimated feed intakes, nutrient digestibility and gross energy densities of the diets available to the animals

Variable	Treatment		SEM	*P-value*		
	SPS	GM		Diet	Measurement	D*M
Intake						
DM (kg/d)	8.57	6.99	0.51	0.0082	0.4294	0.4386
OM (kg/d)	6.29	5.48	0.45	0.0975	0.0001	0.0004
CP (g/d)	847	397	27.6	0.0001	0.0001	0.0001
NDF (kg/d)	5.02	4.44	0.26	0.1241	0.0016	0.0043
ADF (kg/d)	2.61	2.33	0.14	0.1447	0.0039	0.0001
Digestible intake						
DM (kg/d)	4.89	3.26	0.29	0.0005	0.2838	0.3214
OM (kg/d)	3.60	2.59	0.36	0.0834	0.0001	0.1158
CP (g/d)	481	185	32.8	0.0001	0.0002	0.0053
NDF (kg/d)	2.86	2.07	0.39	0.2460	0.0007	0.0516
ADF (kg/d)	1.49	1.09	0.49	0.9289	0.0004	0.0174
Energy						
GE (MJ/d)	150	119	14.6	0.0348	0.3360	0.4457
DE (MJ/d)	85.0	55.0	7.19	0.0009	0.3485	0.4467

D*M= Interaction diet *measurement; *SPS 74% U. hybrid* cv. Cayman and *26% Leucaena diversifolia; GM* 100% *U. hybrid* cv. Cayman*; SEM* Standard error of mean; *DM* dry matter; *OM* organic matter; *CP* crude protein; *NDF* neutral detergent fiber; *ADF* acid detergent fiber; *GE* gross energy; *DE* digestible energy

Table 4 Treatment means animal productivity characteristics and enteric methane emissions for steers in SPS and GM. Live weight gain is the mean of 5 measurements over the 16 months of study. Methane emissions were measured at the end of the feeding

Variable	Treatment		SEM	*P-value*		
	SPS	GM		Diet	Measurement	D*M
Animal productivity[1]						
Final LW (kg)	535	472	10.2	0.0410	n.d.	n.d.
LW gain (g/d)	425	353	35.7	0.1707	0.0001	0.0001
Emissions[2]						
Net methane emissions (g/d)	142	168	5.4	0.0069	0.5951	0.2588
Methane/DMI (g/kg DMI)	16.6	24.4	1.30	0.0012	0.7467	0.4439
Methane/DMID (g/kg DMID)	29.2	52.4	2.63	0.0001	0.8288	0.5413
Methane/OMI (g/kg OMI)	22.8	31.5	1.90	0.0070	0.2098	0.0150
Methane/OMID (g/kg OMID)	39.9	67.6	3.86	0.0003	0.2226	0.0161
Methane/CPID (g/kg CPID)	0.30	0.97	0.05	0.0001	0.0001	0.0002
Methane/NDFID (g/kg NDFID)	50.3	84.2	4.88	0.0004	0.6748	0.0350
Methane/ADFID (g/kg ADFID)	96.8	160	9.6	0.0005	0.5652	0.0117
Ym (%)	5.36	7.98	0.43	0.0007	0.6984	0.4527
EF (kg methane/animal/year)	52.5	61.2	2.01	0.0063	0.5341	0.2463

D*M= Interaction diet *measurement; [1]*SPS* 76.8% *U. brizantha* cv. Toledo or *U. hybrid* cv. Cayman and 23.8% *Leucaena diversifolia*; *GM* 100% *U. hybrid* cv. Cayman or *U. brizantha* cv. Toledo; [2]*SPS 74% U. hybrid* cv. Cayman and *26% L. diversifolia; GM* 100% *U. hybrid* cv. Cayman; *SEM* standard error of mean; *LW* live weight; *DMI* dry matter intake; *DMID* DMI digested; *OMI* organic matter intake; *OMID* OMI digested; *CPID* crude protein intake digested; *NDFID* neutral detergent fiber intake digested; *ADFID* acid detergent fiber intake digested; *Ym* percentage of gross energy consumed lost as methane; *EF* emission factor; n.d.: Not determined

total nutrients and intake of digestible CP and ADF ($P \leq 0.05$). The above was not observed for DM or energy estimates ($P \geq 0.05$).

Animal productivity and enteric methane emissions

Animals grazing the SPS treatment at the end of the experimental period were significantly heavier than

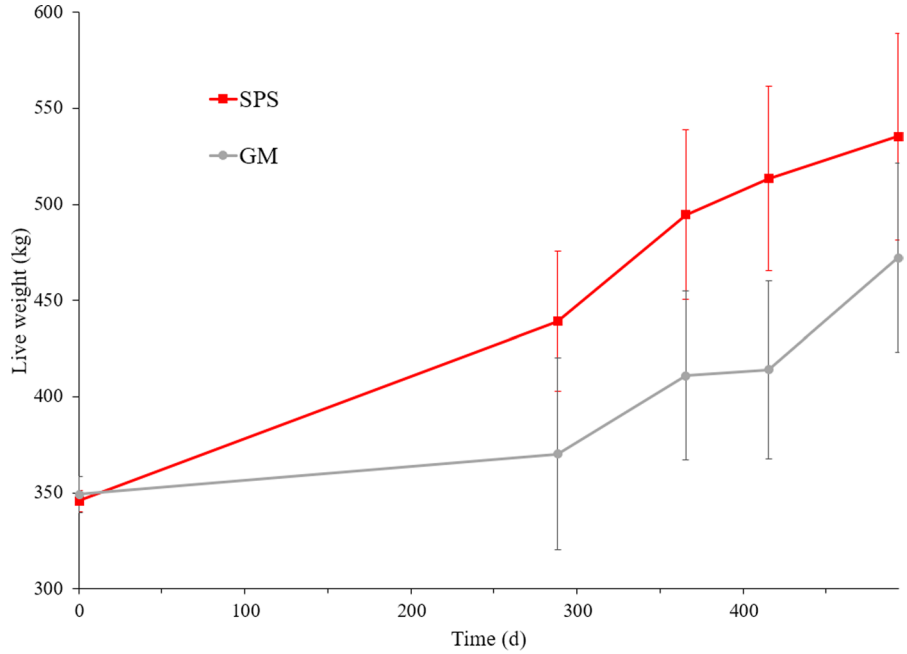


Fig. 1 Live weight of steers during the experiment offered 100% *U. brizantha* cv. Toledo or *U. hybrid* cv. Cayman (GM) or 76.8% *U. brizantha* cv. Toledo or *U. hybrid* cv. Cayman and 23.8% *Leucaena diversifolia* (SPS)

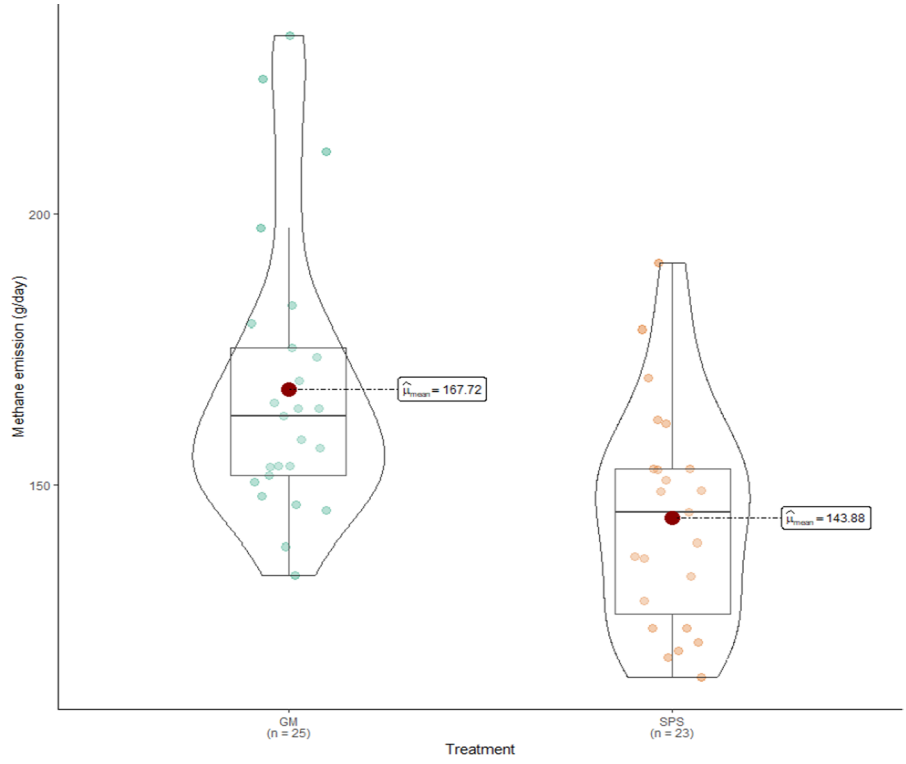


Fig. 2 Daily enteric methane emissions of steers fed grass monocultures (100% Toledo or *U. hybrid* cv. Cayman- GM) or with addition of *Leucaena diversifolia* (74% U. hybrid cv. Cayman and 26% *L diversifolia*- SPS). t_{Welch}(45.08) = 3.6, p= 7.84e-04, $Cl_{95\%}$ [0.42, 1.61], n_{obs}= 48. Where t_{Welch} means unequal variances t-test for unequal sample sizes, Cl means confidence level, and n_{obs} means population size

those grazing GM (Table 4. Fig.1). Despite this, the treatment effect on means daily LW gain for steers on the two treatments was not statistically significant. Mean daily enteric methane emissions were significantly lower for animals that received the SPS diet (143.8 vs. 167.7 g CH_4/day) (Fig. 2), and because

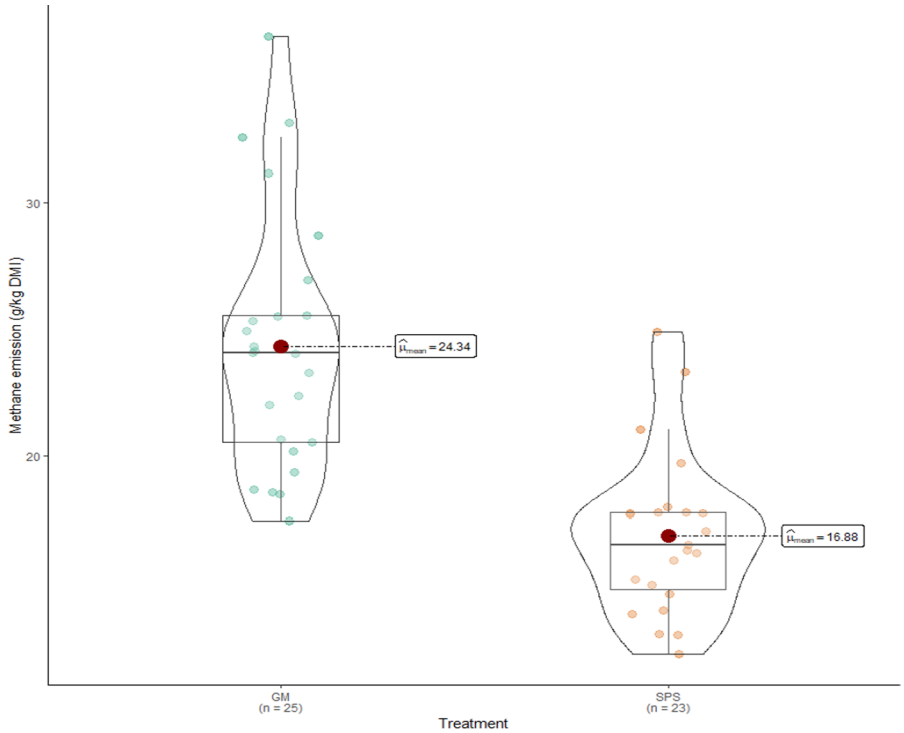


Fig. 3 Daily enteric methane emissions corrected for dry matter intake of steers that consumed only *Urochloa hybrid* cv. Cayman (GM) or a mixture of grass with *L. diversifolia* (SPS) in a 74:26 ratio (grasses:legume). $t_{Welch}(41.05) = 3.23$, p= 2.05e-07, $Cl_{95\%}$ [1.08, 2.41], n_{obs}= 48. Where t_{Welch} means unequal variances t-test for unequal sample sizes, Cl means confidence level, and n_{obs} means population size

these animals had a higher estimated DMI than GM steers, differences in methane emissions expressed per unit of feed intake were even greater (16.8 vs. 24.3 g CH_4/kg DMI) (Fig. 3). The Ym was significantly lower, by approximately 34%, for those animals offered the **SPS** treatment diet, as were the calculated annual emission factors.

Carcass and meat characteristics

Hot and cold carcass weights differed significantly between treatment diets (Table 5); in both parameters, carcasses from steers that consumed diets of SPS weighed 18% more than steers that consumed diets of GM ($P \leq 0.05$). Cold and hot carcass yields both averaged approximately 60%, with a tendency for a higher yield from the SPS-treated animals. Meat tenderness, pH and meat protein concentration values were not significantly different between treatments.

Table 6 shows the fatty acid profile of meat obtained from the *Longuissimus dorsi* meat of steers fed with diets from GM or SPS. The dietary forage fatty acids found in higher proportions were palmitic, linoleic and α-linolenic acids (ALA), while the meat fatty acids with the highest presence were palmitic, stearic and oleic. The fatty acid profile in meat was similar between both diets except for cervonic-DHA and omega-6: omega-3 ratio, which were higher in steers consuming diets of only grasses ($P \leq 0.05$).

Table 5 Mean carcass and meat characteristics from steers grazed on diets with and without access to (or inclusion of) *L. diversifolia*

Variable	Treatment		*SEM*	*P-value*
	SPS	GM		
Hot carcass weight (kg)	309	255	33.6	0.0115
Hot carcass yield (%)	62.7	60.3	2.02	0.0645
Cold carcass weight (kg)	300	248	32.6	0.0109
Cold carcass yield (%)	60.7	58.5	1.96	0.0556
Warner-Bratzler Shear Force (kgF)	4.20	3.56	1.12	0.3068
pH	5.99	5.93	0.31	0.6784
Protein (%)	22.8	22.1	0.85	0.1329

SPS 76.8% *U. brizantha* cv. Toledo or *U. hybrid* cv. Cayman and 23.8% Leucaena diversifolia; *GM* 100% U. hybrid cv. Cayman or *U. brizantha* cv. Toledo; *kgF* kg of force; *SEM* standard error of mean

Table 6 Fatty acid profile of forages offered, and meat obtained from the *Longuissimus dorsi* muscle of steers fed with and without *L. diversifolia*

Fatty acids (% total fatty acids)		Grass	*L. Diversifolia*	Diet			Meat		
Name	*Nomenclature*			SPS	GM	SPS	GM	*SEM*[1]	*P-value*[1]
Miristic	C14:0	1.10	0.20	0.87	1.30	2.37	2.56	0.427	0.4329
Miristoleic	C14:1	0.00	0.00	0.00	0.00	0.29	0.33	0.123	0.5282
Pentadecanoic	C15:0	0.50	0.10	0.40	0.60	0.69	0.66	0.103	0.6117
Palmitic	C16:0	28.5	22.0	26.8	26.8	26.2	27.4	1.398	0.1484
Palmitoleic	C16:1	0.30	0.30	0.30	0.00	2.26	2.33	0.366	0.7225
Margaric	C17:0	0.60	0.20	0.50	0.60	1.34	1.39	0.184	0.6710
Heptadecenoic	C17:1	0.00	0.00	0.00	0.00	0.97	0.83	0.373	0.4877
Stearic	C18:0	4.40	5.80	4.76	4.60	20.9	21.4	2.921	0.7684
Oleic	C18:1n-9c	3.90	2.90	3.64	4.80	32.8	33.0	2.764	0.8355
Cis-vaccinic	C18:1n-7c	0.60	0.50	0.57	0.70	4.10	3.64	0.420	0.0640
Linoleic	C18:2n-6c	21.8	18.6	20.9	23.0	3.81	3.26	1.389	0.4681
g-linoleic (SBA)	C18:3n-6	0.00	0.00	0.00	0.00	0.00	0.01	0.027	0.3377
α-linolenic (ALA)	C18:3n-3	29.6	45.2	33.6	28.6	1.16	0.89	0.541	0.3663
Araquidic	C20:0	1.60	1.40	1.55	1.60	0.14	0.17	0.051	0.3178
Eicosenoic	C20:1n-9	0.00	0.00	0.00	0.00	0.09	0.10	0.027	0.3373
Henecosanoic	C20:2n-6	0.00	0.00	0.00	0.00	0.04	0.06	0.053	0.6264
Eicosatrienoic	C20:3n-6	0.00	0.00	0.00	0.00	0.00	0.03	0.034	0.1474
Araquidonic	C20:4n-6	0.00	0.00	0.00	0.00	0.96	0.79	0.523	0.5512
Timmodonic	C20:5n-3	0.00	0.00	0.00	0.60	0.29	0.20	0.165	0.3521
Heneicosanoic	C21:0	0.00	0.00	0.00	0.00	0.17	0.16	0.087	0.7654
Behenic	C22:0	2.30	0.90	1.94	2.50	0.10	0.01	0.189	0.4132
Erucic	C22:1n-9	0.60	0.10	0.47	0.30	0.01	0.00	0.027	0.3377
Adrenic	C22:4n-6	0.00	0.20	0.05	0.00	0.27	0.17	0.405	0.6527
Thymnodonic-EPA	C22:5n-6	1.10	0.00	0.82	0.00	0.14	0.00	0.267	0.3375
Clupanodonic – DPA	C22:5n-3	0.00	0.00	0.00	0.00	0.53	0.37	0.202	0.1713
Cervonic-DHA	C22:6n-3	0.00	0.00	0.00	0.50	0.01	0.07	0.044	0.0312
Tricosanoic	C23:0	0.6	0.1	0.5	0.7	0.0	0.0	n.d.	n. d.
Lignoceric	C24:0	2.50	1.40	2.22	2.90	0.00	0.00	n. d.	n. d,
Nervonic	C24:1n-9	0.00	0.00	0.00	0.00	0.34	0.21	0.571	0.6811
Fatty acids groups (% of total fatty acids)									
Saturated fatty acids		42.1	41.4	32.3	41.4	52.0	53.7	4.063	0.4453
Monounsaturated fatty acids		4.80	5.90	3.90	5.90	40.8	40.5	2.868	0.8557
Polyunsaturated fatty acids		53.1	52.7	63.8	52.7	7.29	5.83	3.160	0.4062
Omega 3 fatty acids		30.2	29.7	45.2	29.7	2.03	1.51	0.828	0.2681
Omega 6 fatty acids		22.9	23.0	18.6	23.0	5.27	4.33	2.340	0.4655
Omega-6: omega-3 ratio		0.80	0.80	0.40	0.80	2.59	2.91	0.223	0.0173

SPS 76.8% *U*. brizantha cv. Toledo or U. hybrid cv. Cayman and 23.8% *Leucaena diversifolia*; *GM* 100% U. hybrid cv. Cayman or *U.* brizantha cv. Toledo; *SEM* standard error of mean. n.d.: not determined

[1]Statistical analysis carried out for meat samples only

Discussion

The hypothesis that incorporating *L. diversifolia* into cattle diets can offer both productivity and environmental benefits was accepted. Specifically, the inclusion of this legume resulted in higher live weight, improved meat quality (with a reduced omega-6 to omega-3 ratio), and decreased methane emissions from cattle. Each of these outcomes is discussed in detail below.

Intake and digestibility of diets

Silvopastoral systems (SPS) offer multiple benefits to the ecosystem by enhancing biodiversity potential, increasing livestock yield, increasing carbon sequestration and potentially reducing GHG emissions (González-Quintero et al. 2022). This is largely due to the inclusion of forage legume trees or shrubs as part of the SPS (Domínguez-Núñez 2022; Monjardino et al. 2022; Ruiz 2023) in association with improved grass pastures (Villegas et al. 2020). In addition, the use of SPS also offers benefits to animals such as improved welfare (Améndola et al. 2016), and better nutritional quality of the diet (Gaviria-Uribe et al. 2020; Quintero-Anzueta et al. 2021). Results from this study also indicate that diets including *L. diversifolia* contribute to a higher CP concentration and GE intake.

Dry matter (DM) intake was observed to be higher in steers grazing on Cayman plus *L. diversifolia,* consistent with prior studies involving *Zebu* steers under comparable management and environmental conditions (Gaviria-Uribe et al. 2020). This increase in consumption is likely attributed to the greater availability of forage biomass, as indicated by preliminary investigations conducted at the CIAT campus. These studies found that the SPS offered significantly more biomass during both rainy and dry seasons compared to the traditional system (Villegas et al. 2022). Moreover, environmental factors such as temperature (33°C), humidity, and solar radiation may have influenced DM consumption rates. Specifically, during the dry season, animals within the SPS grazed for more extended periods than those in treeless systems, potentially due to enhanced thermal comfort provided by tree cover (Nascimento et al. 2022; Carvalho et al. 2020).

Regarding the effect of the nutritional quality of the diet on intake, authors such as Arelovich et al. (2008), state that the NDF content affects DM intake through physiological regulation at digestive and metabolic levels. However, in the present investigation, NDF and ADF in both diets presented similar values. In addition to the above, no apparent negative effect on DM intake due to the presence of tannins in *L. diversifolia* was observed in the present investigation.

There was a significant effect of time of measurement (1 every 8 days for one month) on the estimated consumption of CP, NDF and ADF, probably due to changes in animal age and, therefore, in steer weight. There is likely also a confounding effect with season, because the experiment was carried out over the course of 16 months. Steers that grazed the SPS treatment (Cayman plus *L. diversifolia*) were estimated to consume more CP than the animals that grazed the grass-only treatment plots, because of the high CP content of the legume. This is likely to have had positive nutritional effects such as an increase total bacteria count in rumen and subsequent increases in microbial nitrogen flow to the duodenum (Hindrichsen et al. 2002). Differences between DM intake and digestible intake of CP between diets might be due to the difference that existed between the cell wall content of forages that indirectly affects digestibility (Lee 2018; Beauchemin et al. 2022). In addition, Rira et al. (2022) found that the total amount of hydrolyzable and condensed tannins contained in a plant can negatively affect degradability, which was not observed in the present study. This observed effect may be due to the low contribution made by *L. diversifolia* to the total diet (32.6 g/kg DM). According to Ugbogu et al. (2019), a low to medium concentration (<50 g/kg DM) of tannins in the diet does not alter nutrient intake and feed DM degradation. Tannins can form complexes with proteins in the rumen pH and protect them from fermentation. As these complexes are unstable at the acidic pH of the abomasum, the proteins remain available for digestion in the small intestine (Besharati et al. 2022; Addisu 2016).

Methane emissions

Steers consuming diets containing *L. diversifolia* emitted the lowest quantities of methane, expressed on a g/d basis, and when related to parameters such as nutrient intake, digestible intake of nutrients and

animal productivity. For example, with the inclusion of 26% *L. diversifolia*, net emissions were reduced by 15.5% consistently over the measurement period. This agrees with the study of Arndt et al. (2022), who found that increasing DM intake, decreasing forage maturity, or increasing the proportion of concentrate feed in the diet, led to a reduction in GHG emissions per unit product, while supplementing with tannin-rich forages can reduce daily GHG emissions. This is because these polyphenol compounds negatively affect gram-positive fibrolytic bacteria, archaea and protozoa, which are largely responsible for fiber digestion in the rumen, thus reducing the production of volatile fatty acids, especially acetate, and net methane production (Vasta et al. 2019). This has been corroborated by several authors who have investigated the influence of legumes and tropical fruits that contain tannins on methane emission (Ku-Vera et al. 2020; Cardoso-Gutierrez et al. 2021; Berça et al. 2023).

Our findings on methane emissions per unit of DMI or OMID, support those reported for an experiment with fewer replicates conducted and shorter time (one single measurement of 24h) by Gaviria-Uribe et al. (2020) where *Bos indicus* steers were supplemented with *L. diversifolia* (19.8 g CH_4/kg DMI and 38.3 g/kg OMID). Also, these findings agree with Min et al. (2020), who reported a methane yield range between 15 and 25 g CH_4 per kg of DMI. The Ym values found in this study are similar to those reported elsewhere that investigated diet containing tropical legumes (Gaviria-Uribe et al. 2020; Montoya-Flores et al. 2020; Stifkens et al. 2022), and the grand mean value was very close to that reported by the IPCC (2006) for grazing cattle (*i.e.,* 6.5%).

Animal productivity

Previous research conducted at CIAT under the same management conditions, but different animal genetics reported LW gains for animals grazing Cayman plus *L. diversifolia* of 657±73 g/d, which was 33% more than reported for animals grazing Cayman grass alone (Enciso et al. 2019). This is likely due to increased feed protein intake, or an improvement in the balance of dietary protein and energy leading to improved ruminal fermentation, microbial protein synthesis, and feed utilization (Chumpawadee et al. 2006; Bhatt et al. 2023; Min et al. 2020). In the current study, despite improvements in CP intake of SPS-treated animals, no significant differences in daily gains were found with respect to the control treatment, perhaps because nutrient intake of cattle was not constant throughout the experiment. This can be corroborated in Table 1, where variability was observed in biomass availability and in the NDF and CP content of the pastures in the two seasons of the year.

Beef yield and quality

The animals offered the diet including *L. diversifolia* exhibited higher hot and cold carcass weights, which aligns with their higher final LW. The crude protein (CP) content in the meat obtained from the *Longuissimus dorsi* muscle average 22% for both diets, consistent with values reported for the meat of the Angus breed by several authors (Jiu et al. 2019). These findings suggest that CP content is primarily influenced by animal breed rather than diet. Mean meat pH was 5.9, falling within the established range for meat pH (5.8-6.0; Wulf et al. 2002). Li et al. (2014) assert that meat pH is generally unaffected by animal sex or dietary protein content but is influenced by energy intake, which may explain the lack of differences observed between treatments. Additionally, pH levels are directly linked to glycogen levels and animal stress in the meat at the time of slaughter (Geletu et al. 2021). This discussion provides valuable insight into the multifactorial nature of meat quality, highlighting the intricate interplay between dietary factors, animal physiology, and stress levels during slaughter.

The myristic (C:14:0), palmitic (C:16:0) and stearic (C18:0) acid values in this study closely resemble those reported by Montoya et al. (2015) for forage-fed animals (3, 30 and 22 g/100 g of fatty acid respectively). However, Montoya et al. (2015) found significant variations in fatty acid content among animals grazing in different systems, such as a SPS, improved grassland, or a traditional system. This contrasts with our research findings, possibly due to various factors, including differences in diets, breed, LW, animal age or the duration of rearing and finishing

periods (Wood et al. 2008; Monteiro et al. 2022; Pavan et al. 2023).

There is little research evaluating the effects of *L. diversifolia* inclusion on the lipid profile of meat. However, in the literature, it is reported that the incorporation in the diet of shrubs and trees rich in secondary compounds can increase more than 50% the α-linolenic acid content, while oleic and linoleic acids can remain the same or increase slightly (15%) in products such as meat or milk (Toral et al. 2018). More specifically, consumption between 20 and 40 g/kg of tannins can change ruminal lipid metabolism and, consequently, increase the content of polyunsaturated acids (18:3n-3, 18:2n-6, CLA, or vaccenic acid) in milk or meat (Morales and Ungerfeld 2015; Mwangi et al. 2019; Frutos et al. 2020). However, in the present study, it was observed that the profile of polyunsaturated acids contained in the meat was the same for both diets, except for cervonic-DHA, perhaps due to intrinsic differences in each experiment such as the chemical and structural diversity of the tannins used, interaction with other dietary ingredients, experimental approaches, ruminant species or variations over time that make that there is no effect of legume consumption on the fatty acid profile in meat (Toral et al. 2018; Frutos et al. 2020).

Durand et al. (2005) reported that beef fatty profile is composed by saturated fatty acids (50%), followed by monounsaturated fatty acids (44%) and, polyunsaturated fatty acids (6%) and these proportions coincide with those obtained in the present investigation. This distribution of fatty acids may vary according to the diet, *e.g.*, beef from grain-rich diets is characterized by low amounts of saturated fatty acids and high monounsaturated proportion, while beef from forage- rich diets have a higher proportion of phospholipids rich in polyunsaturated fatty acids (Hwang and Joo 2017), particularly n-3 fatty acids (linolenic acid, EPA, DPA, DHA) and therefore reducing the omega 6: omega 3 ratio (Duckett et al. 2013; Vahmani et al. 2015; Jacques et al. 2016). Although the content of cervonic-DHA acid in the meat was different among treatments, no differences were observed in the sum of omega-3 acids (ALA, EPA and DHA), perhaps because DHA acid has a very small contribution to the sum.

In our study, the omega 6: omega 3 ratio was lower for animals consuming *L. diversifolia*, which can represent a benefit to human health by reducing the risk of diseases such as diabetes, coronary heart disease, hypertension, diabetes and arthritis (Vahmani et al. 2015). The difference in the content of docosahexaenoic acid between diets (DHA- C22:6n-3. 0.01 vs. 0.07) may be mainly due to the low contribution made by *L. diversifolia* to the diet.

Conclusions

The present study suggests that the inclusion of 26% of *L. diversifolia* in a diet based on *Urochloa* hybrid cv. Cayman or *U. brizantha* cv. Toledo is a promising strategy to generate multiple benefits (environmental, productivity and meat quality). Dietary inclusion of *L. diversifolia* consistently reduced in repeated measurements daily enteric methane emissions by 15% as well as significantly lowering carbon emission intensity per unit of product. This strategy improves dry matter and nutrient intake such as CP and crude energy, when consumed by steers. Inclusion of *L. diversifolia* increased final live weight of steers by 63 kg, with a subsequent increase in hot and cold carcass weights. It was also found to reduce the omega 6: omega 3 ratio in meat of those animals.

Future research should include more forage legume options in different livestock systems to encourage their use and thus support the premise that animal-derived foods can help achieve the global goals of eradicating human malnutrition and reducing GHGs. Additionally, more measurements are needed to evaluate human and animal welfare aspects under different silvopastoral arrangements compared to grass alone pastures.

The analysis presented here shows the relevance of integrating legume germplasm into traditional monoculture pastoral systems to confront the climate crisis (*i.e.*, reducing enteric methane emissions) while increasing resilience of the livestock systems.

Acknowledgments We thank Angus Azul® for acquisition of animals and facilities. This work was conducted as part of the CGIAR Initiative on Livestock and Climate. We thank all donors who globally support our work through their contributions to the CGIAR System. We thank Dr. Idupulapati Rao for technical and English editing of this manuscript.

Author contributions Conceptualization: IC.M-B., H.G, J.D.P., J.C., E.M., J.A.; Methodology: IC.M-B, H.G., J.D.P.; Formal analysis: I.C. M-B.; Research: I.C.M-B., D.V., A.M., J.M., M.B.; Resources: J.A., J.M.; Data curation: I.C.M-B.,

D.V., A.M., J.M., M.B.; Writing-preparation of original draft: I.C.M-B.; Writing-revising and editing: I.C.M-B., D.V., A.M., J.M., M.B., A.R., H.G., J.D.P., J.C., E.M., J.M., J.A.; Supervision: J.A.; Project administration: J.A.; Fundraising: J.A., J.M.

Funding This research was funded by the OneCGIAR initiative on Livestock and Climate, Advancing sustainable forage-based livestock production systems in Colombia (CoForLife), and The Low-Methane Forages project funded by the Bezos Earth Fund and The Bill and Melinda Gates Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data availability No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval The study was conducted according to the regulations and ethical guidelines set by the Centro para la Investigación en Sistemas Sostenibles de Producción Agropecuaria (CIPAV). Ethical approval for animal experimentation was authorized by the Ethics Committee for Scientific Research No. 019, approved on March 1st, 2022.

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