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Effect of *Cymbopogon citratus* on Enteric Methane Emission, Nutrients Digestibility, and Energy Partition in Growing Beef Cattle

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Abstract: Methane production is a waste of energy for ruminants and contributes to greenhouse gas emissions. The objective of the present study was to evaluate the anti-methanogenic effect of increasing the supplementation levels of *Cymbopogon citratus* (CC) on the dry matter intake (DMI), digestibility, methane (CH_4) production, and partitioning of the gross energy intake in growing beef heifers fed with a diet high in forage (68.6% forage: 31.4% concentrate). An experiment was conducted using Holstein × Charolais heifers distributed in a 4 × 4 Latin square design. The experimental treatments were: (1) control diet (CO), (2) CO + 30 g CC DM/d, (3) CO + 60 g CC DM/d CC, and (4) CO + 90 g CC DM/d. A reduction of 22.4% in methane yield (CH_4 g/kg DMI) and a reduction of 21.2% in the *Ym* factor was observed with the 30 CC treatment ($p \leq 0.05$). However, no significant differences ($p > 0.05$) were observed for the total daily CH_4 production, DMI, nutrient digestibility, and gross energy intake partitioning in the heifers. Therefore, we concluded that the supplementation of 30 g CC DM/d reduced the CH_4 yield without affecting the animal performance. However, the anti-methanogenic properties of *Cymbopogon citratus* deserve more investigation.

Keywords: *Cymbopogon citratus*; beef cattle; methane; mitigation; energy partitioning

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1. Introduction

Methane (CH_4) is a potent greenhouse gas (GHG) that contributes to global warming. In 2019, the CH_4 produced by enteric fermentation contributed 27% of the total CH_4 produced by the agriculture, forestry, and other land use (AFOLU) sector and, in turn, that sector accounted for 24% of the total GHG produced worldwide [1]; representing about 3 to 5% of the total GHG emissions [2]. Furthermore, this gas is one of the final by-products of rumen fermentation and is an energy loss for the animal, representing up to 12% of the total gross energy intake [3]. In this sense, reducing its production would lead to more efficient use of the energy transformed into the final product (meat or dairy) [4] and reduce CH_4 emissions to the environment [5]. Further, to improve the productive efficiency of cattle, it is essential to define their nutritional requirements, especially energy, to satisfy their maintenance and production needs. For this purpose, tools such as animal calorimetry are used. Furthermore, it is also essential to determine the partition of the gross energy intake (GEi) to predict the animal's energy balance [6–8]. Recently, several authors [6–15] raised the importance of the development of reference values (such as the ME:DE value) for the development of country-specific energy systems given the particularities of each region and the various factors that influence the calculation of cattle energy requirements in the context of climate change and the need to mitigate enteric CH_4 emissions. Therefore,

the study of GEi partitioning offers the possibility of knowing and identifying the stages where energy loss, such as via CH_4, can be reduced and channelled towards forming substrates (milk and meat), thus increasing energy efficiency [16] with the use of an anti-methanogenic feed additive. Different strategies to mitigate enteric CH_4 in cattle were evaluated recently, such as incorporating feed additives into the diet. However, the inclusion of chemical compounds (such as halogenated CH_4 analogues and related compounds, such as chloroform and chloral hydrate) as feed additives has been controversial due to the toxic effects they may cause to the animal [17], and the use of antibiotics has reduced social acceptance due to the appearance of residues in the final product (meat and dairy) [18]. Currently, the use of plants or plant extracts as feed additives was studied as a natural, environmentally friendly, and human-safe alternative measure [19,20]. These plants are characterized by their high content of secondary metabolites, such as condensed tannins (CT), essential oils (EOs), saponins, and flavonoids [20]. These compounds have the ability to reduce CH_4 synthesis [19,21] by acting directly on rumen methanogenic archaea [22] or indirectly by suppressing the population of protozoa [23,24], fungi [25], or enzymatic activity [26], thus modifying fermentation and reducing the digestibility of organic matter [22,25]. *Cymbopogon citratus* (CC) is an example of these plants and is known as lemongrass, which is a perennial grass cultivated in sub-tropical and tropical regions of the Asian, American, and African continents [27]. Its diverse and rich bioactive compounds, such as phenols, saponins, tannins, flavonoids, alkaloids, steroids, anthraquinones [28], and high concentration of essential oils (EOs) [29], make it a good candidate for reducing CH_4 production in ruminants. However, few published studies evaluated the in vivo anti-methanogenic effect of CC on cattle. Existing studies only study its effect on digestibility, rumen ecology, nitrogen (N) balance, and volatile fatty acids (VFAs) concentration. The few existing studies on its anti-methanogenic effect were conducted primarily in in vitro conditions.

Moreover, none of the previous studies reported the partitioning of GEi by cattle and how it is affected by CC. Likewise, previous studies focused on CC's effects on metabolism, digestibility, rumen ecology, and antioxidant activity. For example, Hosada et al. [30] evaluated the effect of including 5% peppermint, clove, and CC on blood metabolites, hormones, antioxidant activity, immunoglobulin (Ig) G concentration, and ruminal fermentation in steers. They found that peppermint and CC increased ruminal ammonia concentrations. Wanapat et al. [31] evaluated increasing levels (0, 100, 200, and 300 g CC DM/d) of CC on rumen ecology, microorganisms, VFAs concentration, and nutrient digestibility, observing that supplementation with 100 g CC DM/d improved the digestibility, rumen microbial population, and efficiency of microbial protein synthesis. Finally, the study conducted by Wanapat et al. [32] is one of the few works that evaluated the effect of CC supplementation (100 g DM/d) alone or combined with mint powder and garlic powder on CH_4 production (calculated from the VFAs concentration). The authors found that CC and its combination with other herbs decreased the protozoan population and CH_4 production and increased propionate and N utilization.

On the other hand, Nanon et al. [18] evaluated (in vitro) the effect of increasing CC essential oil (EO) supplementation (0, 100, and 200 mg CC EO DM/kg substrate DM) on ruminal fermentation characteristics and diet digestibility of dairy cows. These authors observed that CC EO supplementation increased large and small peptide N and reduced ammonia N concentrations. They also reported that adding 200 mg CC OE increased microbial N production and diet digestibility. Similarly, another experiment by Nanon et al. [33] evaluated CC EO and a ginger–garlic EO mixture on gas production and in situ digestibility. They observed that forage DM, NDF digestibility, and TMR improved at 24 or 48 h after incubation with the CC EO. Further, the CH_4 production presented a quadratic effect with increasing CC EO levels. Likewise, Temmar et al. [34] evaluated twelve herbs' EOs alone or a combination of them, observing that CC increased the propionate ratio and decreased the acetate-to-propionate ratio. They also observed a synergy between CC and coriander EOs that increased the total VFAs production and propionate ratio and decreased the acetate-to-propionate ratio.

The first ever in vivo study on fattening beef cattle to investigate the anti-methanogenic properties of CC with promising results was conducted by Vázquez-Carrillo et al. [35]. These authors observed that the inclusion of 100 g CC DM/d significantly reduced the CH_4 yield (g/kg DMI) by 32.5% compared with the control diet ($p < 0.05$) without affecting the nutrient digestibility or total daily CH_4 production. These authors also reported that in a second study, the inclusion of 280 g and 411 g of CC/d (on a DM basis) in the diet (F:C = 50.7:49.3) significantly reduced the total daily CH_4 production (g/d) by 26.0% and 26.3%, respectively, compared with the CO diet ($p = 0.05$), also in beef cattle. However, the digestibility of the DM, neutral detergent fibre (NDF), and acid detergent fibre (ADF) were depressed due to the CC supplementation levels used [35]. Therefore, in the present work, we hypothesized that the anti-methanogenic properties of CC are maintained in a high-in-forage diet and lower doses of CC ($\leq$100 g CC DM/d) without an adverse effect on digestibility. Therefore, the objective of the present study was to evaluate the in vivo anti-methanogenic effects of low supplementation levels of CC on DMI, nutrients digestibility, the concentration of VFAs, and partitioning of the gross energy intake in growing beef cattle fed a high-forage diet.

2. Materials and Methods

This study was conducted from September 2020 to October 2021 at the Laboratory for Research on Livestock, Environment, and Renewable Energy (LABRELE) of the Faculty of Veterinary Medicine and Animal Science of the Autonomous University of the State of Mexico, which is located in El Cerrillo Piedras Blancas, Toluca, State of Mexico, at 19°24′15″ north and 99°41′06″ west, and at an altitude of 2632 m above sea level. The use of animals in the experiment was approved by the Institutional Subcommittee for the Care and Use of Experimental Animals protocol DC2018/2-8 of the National Autonomous University of Mexico.

2.1. Experimental Procedure

Four heifers (3/4 Holstein 1/4 Charolais) of 225 $\pm$ 64 kg average initial BW were used and distributed in a 4 $\times$ 4 Latin square experimental design. The heifers were dewormed, vaccinated, and found to be healthy before beginning the experiment. The experiment had a duration of 184 days. The first 31 days were for the adaptation of the heifers to the control diet, management, and open-circuit respiration chambers (RCs). Twice a week during the adaptation period, the heifers were taken in pairs to an RC for eight hours, on average, where they were offered the control diet and water ad libitum. With this adaptation period, it was assured that their intake and behaviour would not be affected during the measurement periods. The remaining 150 days were divided into four experimental periods of 33 days each, with a washout period of 7 days between each. Each experimental period was divided into 25 days of adaptation to the experimental diet and eight days for measurements (sampling period).

2.2. Experimental Treatments

Four treatments were evaluated: (1) control diet (CO), (2) CO + 30 g CC DM/d (30 CC), (3) CO + 60 g CC DM/d (60 CC), and (4) CO + 90 g CC DM/d (90 CC). The CO was a TMR offered ad libitum and was formulated to meet the heifers' metabolizable energy and metabolizable protein requirements given in the Agricultural and Food Research Council System [36]. The CO had a forage: concentrate ratio of 68.6:31.4. The CO consisted of 7.3% alfalfa hay, 61.3% oat hay, 3.0% soybean meal, 10.2% ground corn, 12.4% cookie waste, and 5.8% wheat bran.

Preparation of Cymbopogon citratus

The CC used in the present experiment was purchased in a single exhibition from a local supplier. The CC was dried on metal grids under shade, with 25% ambient relative humidity, an average temperature of 22 °C, and adequate ventilation to pre-

vent the denaturation of secondary metabolites [37]. Each week, the dry matter (DM) content was determined, and when the average DM content was above 90%, it was ground with a hammer mill to a size of 0.5 cm (Bison model MMRB-20, Aguascalientes, Mexico). A representative sample was taken from the lot to determine the content of condensed tannins.

2.3. Measurements on Heifers

The heifers were fed twice daily, namely, at 10:00 h and 17:00 h. The TMR offered to each heifer was weighed daily, and the dry-milled CC was incorporated into each animal's diet. The DMI (kg/d) was calculated from the difference between the diet offered minus the ort. The heifers were weighed weekly and at the beginning and end of each experimental period. A total of 6 weighing points per heifer were recorded in each experimental period. The heifers were fasted of solids and liquids for 14–16 h before weighing, where a livestock scale (model WIM-LP7510, Wim Systems, Shangai, China) was used. Dry matter intake (DMI) and total faeces production were measured during the sampling period. An aliquot of the TMR was taken from the trough of each heifer. Faeces were collected daily with a shovel directly from the ground of the RC, placed in a bucket, and weighed with a digital hanging scale (model WH-A05, WeiHeng, accuracy 45 kg × 10 g, Guangzhou Weiheng Electronic Technology Co., Ltd., Guangdong, China). A representative sample of 10% of the total faeces of each heifer was taken directly from the bucket. The DM content of the TMR and faeces samples was determined on the same day of sampling. Subsequently, each sample was stored individually in plastic bags, identified, and preserved for subsequent chemical analysis. From days 5 to 7 of the sampling period, the total daily CH_4 production was measured in an open-circuit RC for 72 h per heifer and one heifer per chamber [35]. Simultaneously, the DMI and total faeces production were measured daily. On day eight, urine was collected for 24 h, and rumen fluid was sampled from each heifer for the VFAs analysis. A metabolic cage within the RC was used for urine collection. The urine fell into a stainless-steel tray at the bottom of the cage, which flowed into a plastic container containing 500 mL of 20% sulfuric acid solution. After 24 h, the total urine volume was measured, and an aliquot of 100 mL per heifer was taken. These samples were placed individually in screw-capped beakers, identified, and kept frozen at −5 °C for subsequent analysis. Rumen liquor from each heifer was collected via oesophagal probing at 6 h post-feeding. Upon sample collection, the pH was determined with a potentiometer (Hanna Instruments, HI 98128, Padova, Italy). Each sample was filtered through triple gauze and poured into previously identified screw-capped beakers. A 40 mL sample of rumen liquor was separated and homogenized with 10 mL of 25% (w/v) metaphosphoric acid. The samples were frozen at −10 °C for subsequent gas chromatography analysis of VFAs.

Methane Measurement

The LABRELE is equipped with two open-circuit RCs for whole-animal measurement. The RCs are made of metal and measure 4 m long × 2 m wide × 2 m high. They have a front and rear door, an air inlet valve at the top, a fan, air conditioning, and artificial light inside. The heifers were kept at an average temperature of 18 °C. The interior has a metabolic cage, 3 m long × 1.4 m wide × 1.6 m high, made of stainless steel, delimited with bars on the sides, rear door, and adjustable front door. The floor was covered with a non-slip plastic mat at the front. It has a canoe-type feeder and an automatic drinker. The feeder measured 91 cm long × 74 cm wide × 50 cm deep, and the drinking trough measured 40 cm long × 75 cm wide × 40 cm deep. The tap that supplied water to the drinking trough was outside the RC. At the back of the cage, there was a 1.25 m long × 90 cm wide × 20 cm deep container for collecting faeces and urine. Methane was measured over 72 h. All measurement equipment was from Sable Systems International (Las Vegas, NV, USA). Before starting each measurement, the equipment was calibrated using high-purity nitrogen (Praxair Inc., Toluca, Mexico) and a gas with a known concentration of CH_4 (1000 ppm CH_4 in high-purity N_2). The gas flowed at a flow rate of 0.3 L/min into the CH_4

analyzer (model MA-10). First, the high-purity N_2 gas was released; once the analyzer was set to zero and remained constant, the mixture with the known concentration of CH_4 gas was released. Once the CH_4 analyzer detected the known concentration, the equipment was considered calibrated. Subsequently, the N_2 was released so that the analyzer returned to the 0.000% reading and the measurement with the heifers was started. Air was drawn from the RC using a flow generator (Model FK2K) at a flow rate of 800 L/min; the air first passed through a filter that collected particles from the filter. At a controlled flow rate of 0.3 L/min, a subsample was passed every second through a drierite desiccator before being delivered to the CH_4 analyzer. The CH_4 readings were sent to a computer via a universal interface (Model UI2), and the data were analyzed with ExpeData software (Sable Systems v.1.9.11). All data from the 72 h measurement were used to calculate the daily CH_4 emissions in Excel as in [35].

2.4. Chemical Analysis of Samples

The TMR and faeces samples were dried in a forced air oven at a constant temperature of 60 °C for 72 h or until they maintained a constant weight [38]. All samples were processed in a Wiley model 4 mill with a 1 mm sieve. The dry matter (DM, %), ash (ASH, %), crude protein (CP = [N] × 6.25%) using the Kjeldahl method [38], gross energy with a Parr calorimetric pump (Parr Instrument Company, Moline, IL, USA), neutral detergent fibre (NDF, %), and acid detergent fibre (ADF, %; using an ANKOM 200® fibre analyzer(ANKOM Technology, 2052 O'Neil Road, Macedon, NY, USA) contents were determined [39]. The condensed tannin content of CC was determined according to the vanillin method [40]. The chemical composition of the CO and condensed tannins content of CC is shown in Table 1. The concentration of VFAs was determined using gas chromatography. Briefly, each heifer's sample of rumen liquor was thawed, and a 25 mL subsample was taken and centrifuged at 17,000× *g* for 20 min. Then, 5 mL of the supernatant obtained was filtered using a hydrophilic Nylon acrodisc (Nylon Syringe filter, membrane solutions, model MS PES, Zhejiang Airjiren, Inc., Quzhou, China) with a 25 mm diameter and 45 µm pores. The filtered rumen liquid prepared this way was kept refrigerated for subsequent injection into the gas chromatograph (GC). A calibration curve was performed with a WSFA-2 standard (SUPELCO 4-7056, Merck KGaA, Darmstadt, Germany) of each VFA with a concentration range of 0.2 to 1.0 µg/µL. Afterwards, 2 µL (split mode) of the filtrate was injected into the GC (AutoSystem XL, PerkinElmer Instruments, Woodland, CA, USA). The working conditions of the GC were as follows: injector temperature, 190 °C; flame ionization detector (FID) temperature, 250 °C; oven at 80 °C, with an initial temperature gradient program of 80 °C for 1 min, and increased at 15 °C/min to 200 °C for 4 min, with a total run time of 13 min; carrier gas pressure (Nitrogen 4.8 chromatographic, 32135, INFRA, Mexico City, Mexico), 5.0 psi (7 mL/min); hydrogen chromatographic flow (4.8b, 32100, INFRA, Mexico), 45 mL/min; and chromatographic extra dry air (32015, INFRA, Mexico), 450 mL/min. VFAs were quantified using a DB-FFAP column (model PN-125-3232, Agilent Technologies, Agilent J & W GC Columns, Santa Clara, CA, USA) that was 30 m long and 0.53 mm diameter (Megaboron) with a 1.00 µm film. The acid retention times obtained were as followed: acetic (5.16 m), propionic (5.84 m), isobutyric (6.01 m), butyric (6.54 m), isovaleric (6.85 m), and valeric (7.42 m). For the preparation of the curve, the standard contained 0.1% of each VFA equivalent to 1 µg/µL; six calibration points (0.1, 0.2, 0.4, 0.6, 0.8, and 1.0 µg/µL) were performed; aliquots of 50, 100, 200, 300, 400, and 500 µL, respectively, of the WSFA-2 standard (SUPELCO 4-7056) were taken and were gauged to 500 µL; and, finally, 1 µL of each point was injected.

2.5. Estimation of the Partition of Gross Energy Consumed, Ym Factor and Metabolicity of the Diet

The partitioning of the animal's GE intake (GEi, MJ/d) was estimated using the gross energy content (GE, MJ) in the feed, faeces, and urine. Thus, the daily dry matter intake, daily faeces production (Ef, MJ/d), and daily urine production (Eu, MJ/d) were multiplied by their respective GE concentration values to obtain the calorific value of each variable.

The calorific value of the total CH_4 production per heifer (ECH_4, MJ/d) was determined by assuming that 1 g CH_4 equalled 55.5 kJ [41]. The energy content in urine was estimated by assuming that 1 g of N was equivalent to 13.4 kcal [42], and the N content in urine was determined using the Kjeldahl method. The digestible energy intake (DEi, MJ/d) was determined by subtracting the Ef loss from GEi. The metabolizable energy intake (MEi, MJ/d) was determined by subtracting ECH_4 and urine energy losses from DEi. The CH_4 conversion factor (*Ym*, %) was calculated as the percentage of the GEi converted to CH_4 [5]. The diet metabolicity (*qm*) was calculated according to the AFRC method [36].

Table 1. Chemical composition of the control diet (CO) and content of the condensed tannins of the *Cymbopogon citratus* used in the experiment.

Variable	CO
DM, g/kg	942.7 ± 2.0
CP, g kg/DM	93.51 ± 0.58
CF, g kg/DM	264.3 ± 1.8
NFE kg/DM	493.7 ± 35.4
NDF, g kg/DM	491.3 ± 16.8
ADF, g kg/DM	306.1 ± 7.2
TDN, g kg/DM	817.8 ± 24.8
OM, g kg/DM	912.3 ± 28.3
GE, MJ/kg DM	16.2 ± 0.14
	C. citratus
CT, g/kg DM	44.5

DM—dry matter, CP—crude protein, CF—crude fibre; NFE—nitrogen-free extract, NDF—neutral detergent fibre, ADF—acid detergent fibre, TDN—total digestible nutrients; OM—organic matter, GE—gross energy, CT—condensed tannins.

2.6. Statistical Model and Data Analysis

The results were analyzed using analysis of variance with the following linear and additive model for a Latin square experimental design:

$$Y_{ijk} = \mu + A_i + T_j + P_k + \varepsilon_{ijk}$$

where Y_{ijk} was the response variable of the ith animal (i = 1, 2, 3, 4), which received the jth treatment (j = 1, 2, 3, 4) during the kth period (k = 1, 2, 3, 4); μ was the overall mean common to all observations; A_i was the random effect of the animal; T_j was the fixed effect of the treatment; P_k was the fixed effect of the period. and ξ_{ijk} was the experimental error common to all observations, which was assumed independent and normally distributed with zero mean and unit variance (N, I; $\mu = 0$, $\sigma = 1$).

The statistical analysis was performed with R Software v.1.3.1073. For the variables that were statistically different ($p \leq 0.05$), an orthogonal polynomial analysis was conducted to determine whether there was a linear, quadratic, or cubic effect of the experimental treatments. For the latter, the JMP v.11.0.0 software was used. Subsequently, a regression analysis was performed on the statistically significant variables to obtain the minimum dose from the derivation.

3. Results

Our results for the average daily intake of DMI, OM, NDF, ADF, CP, and GE showed no significant differences ($p > 0.05$) at any CC supplementation levels tested (Table 2). Furthermore, the digestibility of DM and the feed fractions depicted in Table 2 also showed no adverse effects due to CC supplementation.

Table 2. Effect of increasing *Cymbopogon citratus* levels on the dry matter, organic matter, fibre fractions, crude protein, and gross energy intakes, and their digestibilities observed in the experimental heifers fed a high-forage diet.

Variable	Treatment				SEM	*p*-Value
	CO	30 CC	60 CC	90 CC		
			Intake, kg/d			
DMI	9.23	8.86	9.48	8.22	0.54	0.444
NDF	4.49	4.35	4.63	4.06	0.27	0.531
ADF	2.81	2.71	2.89	2.53	1.16	0.498
CP	0.86	0.83	0.89	0.77	0.51	0.449
OM	8.41	8.09	8.60	7.51	0.51	0.505
GE (MJ/d)	149.50	143.70	153.50	133.40	8.83	0.464
			Digestibility, %			
DM	60.09	60.40	64.12	58.98	2.22	0.449
NDF	52.60	53.98	57.07	51.78	3.52	0.736
ADF	51.33	51.78	56.95	49.66	3.61	0.556
OM	64.12	64.17	67.84	62.88	2.08	0.431
CP	58.79	56.39	60.23	59.09	2.84	0.809
GE	61.82	63.49	66.92	61.78	2.41	0.449

Nutrient intake and digestibility were determined from samples taken when the heifers were in the RC. CO—control diet; 30 CC—*Cymbopogon citratus* (30 g DM/d); 60 CC—*Cymbopogon citratus* (60 g DM/d); 90 CC—*Cymbopogon citratus* (90 g DM/d); DM—dry matter; NDF—neutral detergent fibre; ADF—acid detergent fibre; OM—organic matter; CP—crude protein; GE—gross energy, SEM—standard error of the mean.

Table 3 shows the results for the CH_4 production variables. The inclusion of CC in the diet presented a quadratic effect for the CH_4 yield (CH_4 g/kg DMI), *Ym* factor, and CH_4:GE ratio, with the lowest point being found for the 30 CC treatment ($p < 0.05$). However, the optimal minimum doses, according to the regression analysis and derivation, to obtain the lowest CH_4 yield, *Ym* factor and CH_4:GE ratio were 53.06 g CC DM/d ($R^2 = 0.899$), 54.9 g CC DM/d ($R^2 = 0.909$), and 50.0 g CC DM/d ($R^2 = 0.933$), respectively. It can be observed that the daily CH_4 production (CH_4 g/d) did not differ significantly ($p = 0.18$) between treatments. However, large numerical differences were observed between the CO and the CC treatments. For example, compared with the CO treatment, the 30 CC, 60 CC, and 90 CC treatments reduced the CH_4 production by 21.8%, 17.3%, and 20.7%, respectively ($p > 0.05$). In contrast, compared with the CO treatment, a significant reduction of 22.4% in the CH_4 yield was observed with the 30 CC treatment ($p < 0.05$, $Q = 0.016$). Likewise, a significantly lower *Ym* factor was observed with the 30 CC treatment compared with the CO treatment ($p < 0.05$), being 21.2% lower. No significant differences ($p > 0.05$) existed between the treatment means for gross, digestible, and metabolizable energy intakes or losses of energy in urine, faeces, and CH_4 production ($p > 0.05$) (Table 3). The same applied to the F:GE, U:GE, DE:GE, ME:GE, and ME:DE ratios ($p > 0.05$). However, as expected, there was a significant difference in the CH_4:GE ratio ($p < 0.05$), with the 30 CC treatment, with 21.4% less GEi lost as CH_4 compared with the CO treatment. No significant differences ($p > 0.05$) were observed for the ADWG and CH_4 intensities of production at any of the CC levels tested.

Table 4 shows no significant differences for the means of the experimental treatments' energy densities and *qm* factors ($p > 0.05$). Similarly, Table 5 shows that the pH and VFA concentrations were not negatively affected by any of the CC supplementation levels tested ($p > 0.05$).

Table 3. Methane emission and partitioning of the gross energy intake for heifers supplemented with different levels of *Cymbopogon citratus* supplementation.

Variable	Treatment				SEM	*p*-Value	Statistical Significance	
	CO	30 CC	60 CC	90 CC			L	Q
Methane								
CH_4 g/d	184.50	144.30	152.50	146.30	12.48	0.182	NS	NS
CH_4 g/kg DMI	20.81 ^{a}	16.15 ^{b}	16.90 ^{ab}	18.04 ^{ab}	0.87	0.037	NS	0.016
ADWG, kg/d	0.70	1.01	1.00	0.83	0.14	0.428	NS	NS
CH_4 g/kg ADWG	268.80	150.70	199.60	257.30	48.47	0.365	NS	NS
Ym factor, %	7.02 ^{a}	5.53 ^{b}	5.74 ^{ab}	6.11 ^{ab}	0.30	0.047	NS	0.020
Partitioning of the Gross Energy								
Energy in faeces, MJ/d	57.53	52.38	47.78	50.09	3.36	0.299	NS	NS
F:GE	0.38	0.37	0.33	0.38	0.02	0.424	NS	NS
Urinary energy, MJ/d	2.87	3.46	4.26	4.09	0.40	0.161	NS	NS
U:GE	0.020	0.025	0.025	0.030	0.004	0.455	NS	NS
CH_4 energy, MJ/d	10.19	7.97	8.42	8.08	0.69	0.182	NS	NS
CH_4: GE	0.070 ^{a}	0.055 ^{b}	0.057 ^{ab}	0.061 ^{ab}	0.003	0.041	NS	0.020
GEi, MJ/d	149.5	143.7	153.5	133.4	8.83	0.464	NS	NS
DEi, MJ/d	92.01	91.37	105.69	83.32	8.45	0.385	NS	NS
MEi, MJ/d	78.95	79.95	93.02	71.15	7.75	0.339	NS	NS
DE:GE	0.62	0.64	0.67	0.62	0.02	0.424	NS	NS
ME:GE	0.53	0.56	0.59	0.53	0.02	0.265	NS	NS
ME:DE	0.86	0.88	0.88	0.86	0.01	0.162	NS	NS

CO—control diet; 30 CC—*Cymbopogon citratus* (30 g DM/d); 60 CC—*Cymbopogon citratus* (60 g DM/d); 90 CC—*Cymbopogon citratus* (90 g DM/d); CH_4—methane; CH_4 g/kg DMI—methane yield; ADWG—average daily live weight gain; CH_4 g/kg ADWG—intensity of methane emission; *Ym* factor—methane conversion factor, i.e., the energy of CH_4 as a percentage of GEi; GE—gross energy; F:GE—proportion faecal energy: gross energy; U:GE—proportion urinary energy: gross energy; CH_4: GE—proportion methane energy: gross energy; GEi—gross energy intake; DEi—digestible energy intake; MEi—metabolizable energy intake; DE: GE—proportion digestible energy: gross energy; ME: GE—proportion metabolizable energy: gross energy; ME: DE—proportion metabolizable energy: digestible energy; SEM—standard error of the mean. Values in the same row with different superscript letters ^{a,b} were significantly different ($p < 0.05$). L and Q—linear and quadratic effects, respectively; NS—non-significant ($p < 0.05$).

Table 4. Digestible and metabolizable energy contents and q*m* factors of the treatment diets supplemented with different levels of *Cymbopogon citratus*.

Variable	Treatment				SEM	*p*-Value
	CO	30 CC	60 CC	90 CC		
DE, MJ/kg DM	10.03	10.30	10.85	10.02	0.39	0.453
ME, MJ/kg DM	8.56	9.01	9.53	8.54	0.35	0.248
qm factor	0.53	0.56	0.59	0.53	0.22	0.265

CO—Control; 30 CC—*Cymbopogon citratus* (30 g DM/d); 60 CC—*Cymbopogon citratus* (60 g DM/d); 90 CC—*Cymbopogon citratus* (90 g DM/d); DE—digestible energy; ME—metabolizable energy; DM—dry matter; SEM—standard error of the mean.

Table 5. Concentrations of VFAs in and pHs of rumen liquor from heifers supplemented with different levels of *Cymbopogon citratus*.

Variable	Treatment				SEM	*p*-Value
	CO	30 CC	60 CC	90 CC		
pH	6.56	6.76	6.57	6.96	0.127	0.276
VFA Concentrations, mM						
Acetic	50.62	56.85	48.99	50.55	6.152	0.818
Propionic	16.10	17.83	16.12	17.58	2.389	0.926
Butyric	11.31	13.58	12.59	11.99	2.167	0.896
Isobutyric	1.05	1.10	0.96	1.00	0.063	0.549
Isovaleric	1.26	1.41	1.21	1.25	0.095	0.550
Valeric	2.97	3.02	2.50	2.71	0.313	0.659
Total	83.31	93.79	82.36	85.08	10.84	0.276

Table 5. *Cont.*

Variable	Treatment				SEM	*p*-Value
	CO	30 CC	60 CC	90 CC		
Molar Proportion of VFAs, %						
Acetic	60.68	60.62	59.82	59.62	0.576	0.545
Propionic	19.28	18.97	19.67	20.52	0.445	0.264
Butyric	13.52	14.52	14.87	13.89	0.778	0.658
Isobutyric	1.33	1.17	1.16	1.23	0.160	0.866
Isovaleric	1.61	1.50	1.45	1.52	0.199	0.947
Valeric	3.59	3.22	3.03	3.22	0.350	0.743
Acetic-to-propionic ratio	3.15	3.20	3.04	2.10	0.087	0.269

CO—control diet; 30 CC—*Cymbopogon citratus* (30 g DM/d); 60 CC—*Cymbopogon citratus* (60 g DM/d); 90 CC—*Cymbopogon citratus* (90 g DM/d); SEM—standard error of the mean.

4. Discussion

We aimed in the present work to evaluate the in vivo anti-methanogenic effects of low supplementation levels of CC on the DMI, nutrients' digestibility, concentrations of VFAs, and partitioning of the GEi in growing beef cattle fed a high-forage diet. Our results indicate that of the doses evaluated, the supplementation with 30 g CC DM/animal/d reduced the CH_4 yield and the *Ym* factor compared with the CO diet ($p < 0.05$). This suggests that the anti-methanogenic effect of CC was maintained at low supplementation levels in a diet prone to producing a large amount of CH_4. The minimum dose derived from the regression analysis (53.06 g CC DM/d) was not distant from the previous figure. A similar anti-methanogenic effect was reported by Vázquez-Carrillo et al. [35]; these authors found a 33% reduction in CH_4 yield with the inclusion of 100 g CC DM/d relative to their control diet in finishing-period beef cattle fed a diet high in concentrate (19.4 forage: 80.6 concentrate (F:C ratio)). Likewise, a second experiment by Vázquez-Carrillo et al. [35] reported a reduction in individual CH_4 emissions (CH_4, g/d) of 26.0%, 26.2%, and 15% but using higher doses of CC (280, 411, and 572 g CC DM/d) and at the expense of depression of the digestibility of DM, NDF, and ADF ($p < 0.05$).

In contrast, in the present study, none of the digestibility variables was affected at the CC supplementation levels tested. Similarly, Guggenberger et al. [43] observed a reduction of 14.6% in daily CH_4 emissions when supplemented with 100 g CC DM/d to Fleckvieh beef cattle. The same authors tested increasing levels of CC in an in vitro study and obtained a 15.8% reduction in gas formation by adding 1% CC DM to the diet [43]. Likewise, Wanapat [32] evaluated the anti-methanogenic effects of CC, peppermint, and garlic meal, as well as a combination of the three herbs, and observed that the CH_4 production was the lowest with supplementation CC meal at 100 g DM/d plus peppermint powder at 10 g DM/d. These authors also observed that it reduced the CH_4 concentration with CC meal at 100 g DM/d dose plus peppermint powder at 10 g DM/d and 40 g DM/d garlic powder doses [32]. Finally, Abrar et al. [44] compared dry and ensiled *Cymbopogon Nardus* L. and reported that this lemongrass variety decreased CH_4 production (in vitro) by 44% in the ensiled treatment compared with the dried *Cymbopogon*. The CH_4 yield observed in the present work for the CO diet (20.8 CH_4 g/kg DM) was similar to the mean value reported by Van Lingen et al. [45] for a meta-analysis of an intercontinental database of individual measurements of CH_4 production, who reported a mean CH_4 yield of 20.7 g/kg DM for animals fed high-forage diets. This similarity in CH_4 yield for the CO diet supports our hypothesis that the lower yield found with the 30 CC treatment may be attributed to the CC in the diet ($p < 0.05$). Additionally, the reduction in the *Ym* factor with 30 CC treatment suggests that the energy not lost in the form of CH_4 could have been channelled to the daily weight gain of the heifers, contributing to reducing emissions of this GHG to the environment. However, the effect of CC on the ADWG requires exhaustive evaluation since it is necessary to use a more extended experimental period than those used in the present work and an experimental design that is more suitable for measuring this variable.

According to Vázquez-Carrillo et al. [35] and Guggenberger et al. [43], the anti-methanogenic activity of CC can be attributed to its tannins, particularly condensed tannins and essential oils content. This statement agrees with Cardoso-Gutiérrez et al. [46], who indicated a negative linear relationship between the level of tannin inclusion in the diet and CH_4 emission. In contrast to the former authors, we found a quadratic response, and thus, the greatest reduction in CH_4 yield was observed with the lowest inclusion of CC and not with the highest level. Several theories on the anti-methanogenic effects of condensed tannins (CT) were presented. For example, Ng et al. [47] reported the existence of a protein-based adhesin in the rumen protozoa's fimbriae that facilitate symbiosis between methanogenic archaea and protozoa. According to these authors, CT bind to the adhesin, inhibiting the necessary symbiosis for CH_4 production. In addition, parts of the cell envelope also contain proteins, which may facilitate the binding of CT to such structures, interfering with symbiosis and inhibiting or reducing the H_2 transfer between protozoa and archaea, negatively affecting the population of ciliate protozoa and methanogenic archaea [48,49]. Another hypothesis is that CT produce an indirect inhibition of microbial growth by decreasing the nutrient availability through substrate deprivation, enzymatic inhibition, or as chelating agents, decreasing the adherence of rumen microorganisms to plant cell walls and inhibiting fibrolytic enzymes, such as hemicellulases and cellulases [50]. Since in the present work, there were no significant differences ($p > 0.05$) in the NDF and ADF digestibilities, it is suggested that the anti-methanogenic activity was due to the direct reduction of methanogenic archaea. Furthermore, if the protozoan population had been inhibited, a reduction in the NDF and ADF digestibilities would probably have been observed [51]. As in Vázquez-Carrillo et al. [35], the anti-methanogenic effect of CC was likely due to the presence of CT and essential oils. Bhatta [22] argued that the most promising enteric fermentation CH_4 mitigation strategies are based on modifying diet composition and using local and low-cost resources. However, although secondary plant metabolites, such as tannins, saponins, and essential oils, seem very promising for reducing enteric CH_4 emissions, the results are inconsistent in the different studies because of the significant variation in the concentration of secondary compounds, doses, and feed compositions. Several authors emphasized that the content of phytochemicals in plants is highly influenced by the geographical region, genetics of herbs, environment, part of the plant used, preservation or extraction method, age, and cutting season [22,28,52,53]. Our results align with the former authors because the most significant anti-methanogenic effect of CC was observed when the concentration of CT was the highest, as in Vázquez-Carrillo et al. [35], and then declined as the concentration of CC declined.

In our experience, the variation in the concentration of CT in tropical tanniferous plants is crucial when using them as an anti-methanogenic additive. For example, we observed that CT concentration in CC varies from nil to 61 g/kg DM depending on the harvest time, the season of the year, and the cultivation place. This variation explains why no anti-methanogenic effect was observed in some experiments, such as in Honan et al. [54], where the CC used had no CT, whereas, in other experiments [35,43], a significant reduction of up to 32% was observed. Therefore, if CC or other similar tropical tanniferous plants will be used to mitigate enteric fermentation CH_4 production, it is necessary to identify those varieties that produce the highest concentration of CT and ideally breed them to obtain a constant and stable production of tannins, particularly CT. Further, our results suggest that the energy not lost as CH_4 was likely channelled to weight gain. For example, we observed a numerical reduction in the CH_4 emission intensity. According to Naumann [49], using tanniferous plants can improve energy utilization efficiency in the diet, resulting in more milk or meat produced by the animal. It was reported that protein–CT binding has some benefits for ruminants due to the complexes formed with essential amino acids establishing stable hydrogen bonds at a pH of 3.5–8.0, which prevents their degradation in the rumen and causes them to dissociate in the posterior tract, and thus, become absorbed directly by the animal [49,50]. Kongphitee et al. [55] mentioned that this binding affects the rumen

microbial population, digestion, and utilization of nutrients and energy, which may result in higher animal performance and increased meat or milk production [46,50].

On the other hand, our results for DMI and digestibility show that including small amounts of CC in the diet of the heifers did not influence the intake or digestibility of DM, OM, CP, NDF, ADF, and GEi ($p > 0.05$). These results differ from what was found by Vázquez-Carrillo et al. [35]; these authors reported a quadratic effect on the digestibility of DM, NDF, ADF, and GE, presenting the lowest digestibility of DM and nutrients with the inclusion of 411 g CC DM/d compared with the CO treatment. However, with the inclusion of 100g DM/d, the same authors found no effects on nutrient intake and digestibility compared with the CO treatment. Therefore, Vázquez-Carrillo et al. [35] concluded that 100 g of CC DM/d could be an adequate amount to supplement fattening beef cattle without affecting nutrient intake and digestibility. We agree with this conclusion because our results indicate that a dose as low as 30 g DM/d of CC reduced the CH_4 yield without adversely affecting animal performance, but for beef cattle not in the fattening period and with a diet high in forage. In fact, the supplementation level of CC in the present work never exceeded 1% of daily DMI. Furthermore, similar results were reported by Wanapat et al. [31]; they found that with the inclusion of 100 g CC DM/d, the DM digestibility of the diet was not affected, being even higher (74.8%) than the rest of the experimental treatments (0 g CC DM = 64.7%, 200 g CC DM = 66.8%, and 300 g CC DM = 62%). Similarly, in another study conducted by Wanapat et al. [32] using four different treatments (CO, CC, a combination of CC meal and peppermint powder, and CC meal plus peppermint and garlic powder), they reported the highest DM digestibility with the inclusion of 100 g CC DM/d compared with the rest of their experimental treatments. Likewise, Pawar et al. [56] evaluated different essential oils in buffalo rumen liquor, including CC, and found that the lowest level tested in this experiment (167 µL L^{-1} of incubation medium) was the most appropriate level of inclusion, as higher doses were detrimental to feed digestibility and fermentation.

Regarding the GEi partitioning, we observed that CC did not affect these variables significantly ($p > 0.05$) at any CC supplementation level, except for the CH_4:GE ratio, where a significant quadratic decline was observed with the inclusion of CC. Furthermore, we observed that supplementing CC at all levels tested reduced the amount of energy loss in faeces numerically. This effect resulted in 5.6% and 11.32% more ME available (as a percentage of GEi (ME:GE or *qm* factor)) than the CO treatment for the heifers with the 30 CC and 60 CC treatments, respectively. The former assumption was confirmed with the 60 CC treatment when we calculated, using the AFRC [36] system and *qm* factor in Table 4, that 10.2 MJ of ME was necessary to gain the extra 0.30 kg of live weight gain observed in heifers with the 60 CC treatment in comparison with heifers in the CO treatment. Therefore, this calculated MEi was similar to the 14 MJ MEi listed in Table 3 for the 60 CC treatment. Unfortunately, the same pattern was not observed with the 30 CC treatment, where 0.30 kg of extra live weight gain was also observed compared with the CO treatment, but the MEi was similar to the control. We cannot explain why this response was only observed with the 60 CC treatment. We also observed that the ME:DE ratio ranged from 0.86 to 0.88 for all treatments, which differs from the values established by the different feeding systems. For example, the NASEM [57] recommends a value of 0.82 for beef cattle; however, NASEM suggests updating and generating prediction equations to convert DE to ME according to the context of each country. This indicates that NASEM [57] acknowledges that using a fixed ME/DE ratio is not advisable across all countries and situations. Further, in a study conducted on F1 cattle (Holstein × Gyr) fed a diet with 50% tropical forage and 50% concentrate diet, Da Fonseca et al. [14] found a value of 0.86 for the ME:DE ratio for their CO. This value equals our ME:DE ratio for our CO treatment. Posada-Ochoa et al. [58] found ME:DE values ranging from 0.85 to 0.87 in growing Nellore cattle fed high-forage diets. Similarly, Ibihi et al. [8] developed a series of equations for Korean Hanwoo beef cattle to define ME:DE with a wide range of diets, sex, and intake levels, obtaining ME:DE values ranging from 0.83 to 0.89. The former authors concluded that the ME/DE conversion

factor should be reevaluated according to geographic region, breed specificity, and feed quality to improve animal productivity and maximize economic performance for beef cattle farmers. Likewise, Galyean et al. [11] reported a strong linear relationship between DE and ME and found that ME was approximately 0.86 of DE instead of the fixed value of 0.82 normally used [57]. It appears that increased values of the ME:DE ratio are related to the age of the animals, especially young animals. For example, Hales [59] mentioned an increase in the ME:DE ratio in response to greater inclusion of concentrate in the diet in growing ruminants relative to mature ruminants since CH_4 and urinary energy losses are lower in growing animals. Finally, the increase in CH_4 production in mature versus growing cattle could be related to a larger rumen size in mature cattle and a slower rate of passage, which allows for greater CH_4 production [59]. Since ME is the starting point for the NE system and the prediction of ME is made using DE, the NE requirements could be affected, which is why a thorough review and future research are needed to better understand the underlying mechanisms that affect the conversion of DE to ME [59,60]. This review is essential for cattle systems in tropical regions where cattle are fed tanniferous herbs, forages, and trees.

Therefore, Da Fonseca et al. [14] highlighted the importance of performing energy-partitioning studies. These studies will offer the possibility of knowing the specific points to implement solutions to energy losses during its flow in the ruminant [6,61], and of particular relevance when cattle receive anti-methanogenic additives. Additionally, accurate estimation of energy requirements for current genotypes and feeding conditions is crucial for improving the profitability and reducing the environmental impact of the beef industry. More importantly, the outdatedness of some energy systems can have consequences on the efficiency of dietary energy use in the productive functions of cattle [62].

5. Conclusions

It was concluded that the anti-methanogenic effect of CC was maintained at low supplementation levels, and this effect showed a quadratic response. We also concluded that 30 g CC DM/d reduced the CH_4 yield without affecting the DMI, digestibility, and gross energy intake partitioning. However, the minimum calculated dose for a lower CH_4 yield was 53.06 g CC DM/d. Therefore, further research is guaranteed.

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