agriculture

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Use of Tropical Legume Tree and Coffee Pulp to Reduce Enteric Methane Emission by Cattle Fed a Low-Quality Forage Diet

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Abstract

Tanniferous forages, leaves and pods from legume trees can be used as feed additives to reduce enteric CH_4 in tropical regions of the world where smallholder farmers cannot afford to purchase commercial anti-methanogenic feed additives. The present work aimed to evaluate the impact of small doses of *Gliricidia sepium* (*G. sepium*) alone or in combination with coffee pulp (COP) on enteric CH_4 production in cattle. A 4 × 4 Latin square experimental design was used, where four Holstein x Charolais heifers of 390 ± 50 kg body weight were used. Four treatments were evaluated, with *G. sepium* (GSep) and COP used as additives. The control treatment (CON) had no additives and was offered ad libitum, the COP treatment contained 1.0 kg DM d^{-1} of COP, the treatment with *G. sepium* contained 0.342 kg DM d^{-1} of this plant, and the treatment with both plants (COP + GSep) had 0.505 and 0.171 kg DM d^{-1}, respectively. The lowest CH_4 production was observed for the COP + GSep treatment, followed by GSep, with 17% and 14.2% less CH_4, respectively, compared to the CON treatment ($p < 0.05$). We concluded that supplementation with *G. sepium*, alone or in combination with COP, can be used as part of a strategy to mitigate enteric CH_4 production in tropical cattle production systems. To the best of our knowledge, this is the first time two natural additives have been used together to reduce enteric methane in cattle fed a low-quality forage.

Keywords: heifers; climate change mitigation; *Gliricidia sepium*; condensed tannins; smallholder farmers; feed additives; maize straw

Academic Editor: Vito Laudadio

Received: 8 December 2025
Revised: 30 December 2025
Accepted: 2 January 2026
Published: 8 January 2026

1. Introduction

Drought, high temperatures, and flooding are now common phenomena in tropical regions worldwide due to climate change, resulting in changes in vegetation patterns [1] and shortages of forage and other foodstuffs for cattle, which will eventually threaten food security. Therefore, it is necessary to shift from traditional cattle production systems to agroecological systems that, on the one hand, adapt to climate change, do not compete with humans for food and water, and on the other hand, contribute to mitigating climate

change through reduction in greenhouse gas (GHG) emissions and fixation of atmospheric carbon in trees, shrubs and grasslands. Especial attention deserves the need to reduce enteric methane (CH_4) production by cattle, which is the primary GHG produced by the agricultural sector worldwide, where cattle account for 2.3 to 2.8 Gt CO_2 eq^{-1} of the sector's CH_4 emissions [1]. The current increment in global temperature has been attributed to CH_4 as its atmospheric levels have risen by ~14% over the last 20 years [2]. Its atmospheric concentration is currently approximately 2.5 times pre-industrial levels, and CH_4 has a higher global warming potential (GWP) than CO_2, particularly over short timescales. According to [3], there is evidence of a strong, coincident relationship between atmospheric CH_4 concentrations and current global temperature trends. The increased atmospheric levels of CH_4 are attributed to the energy sector (oil, coal, and gas) [4], rice production, and the livestock sector; however, cattle production systems dominate the agricultural sector's CH_4 emissions [5] and are a focus of global mitigation targets.

Most global enteric CH_4 emissions from cattle originate in less-developed tropical countries, where the majority of the cattle population is found [6]. Cattle in the tropics are less efficient in terms of feed conversion efficiency and are less productive than cattle in temperate climate regions. Therefore, more CH_4 is produced per unit of milk or beef produced from these regions (CH_4 intensity of emission) [7], although less CH_4 is produced per head. According to [8] the average emission of an adult cow in the United States and Europe is 369 $\pm$ 100 g CH_4 day^{-1} cow^{-1}. In contrast, the average for Latin America is 309 $\pm$ 98 g CH_4 day^{-1} cow^{-1} [9]. So, increasing the sustainable productivity of cattle in the tropics remains a crucial task for animal scientists [4]. Furthermore, tropical countries experienced significantly higher population growth than temperate regions over the past five decades [5]. So, diets in these regions have shifted toward greater consumption of animal-based proteins, thereby increasing demand for beef and milk [10]. Thus, it is crucial to develop sustainable alternative tropical cattle production systems that are low-carbon emitters while continuing to contribute to food security. Silvopastoral systems (SPSs) based on tropical legumes, native trees, and shrubs have demonstrated the potential to improve cattle nutrition by increasing dietary protein availability. The SPS can also improve animal welfare, enhanced nutrient cycling, soil fertility, carbon sequestration, and GHG mitigation [11]. Recent evidence indicates that the presence of secondary metabolites like tannins, saponins, and essential oils in tropical legumes can help to reduce rumen CH_4 production through their adverse effects on rumen protozoa growth and associated methanogenic archaea bacteria [12–15]. Tannins bind to microbial enzymes and cell walls, disrupting methanogen activity [16]. According to [17] high tannin levels can impair fiber digestion by inhibiting cellulolytic bacteria such as *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*. Saponins, plant-derived glycosides, can reduce rumen CH_4 production by suppressing protozoa (which host methanogens) and directly inhibiting methanogenic archaea, though their effectiveness depends on the source, dose, and diet composition. Saponins can disrupt archaeal cell membranes., and moderate supplementation often lowers CH_4 emissions without significant adverse effects on fermentation [18]. Essential oils can reduce enteric methane production in ruminants by inhibiting methanogenic archaea and protozoa, altering fermentation pathways, and shifting volatile fatty acid profiles [19].

Forage and pods from legume trees could be used as feed additives to reduce enteric CH_4 emissions in the tropics, where farmers cannot afford to purchase commercial anti-methanogenic feed additives [20,21]. Furthermore, tropical legumes can also provide rumen-degradable crude protein (CP) that improves rumen function. This, in turn, enhances the degradation of low-quality forages because tropical C4 grasses are the most voluminous part of the diet but are deficient in protein. Some examples include *Leucaena*

leucocephala, *Enterolobium cyclocarpum*, *Brosimum alicastrum*, *Pithecellobium dulce*, *Samanea saman*, *Mimosa bahamensis* and *Gliricidia sepium.* According to García et al. [22], the average CP of leucaena forage is 22% with an apparent digested crude protein (TADCP) ranging from 64.7 to 78.0%, and a tannin content of 1%. Similarly, according to Ekanem et al. [23], the CP in *E. cyclocarpum* leaves is 22%, and the tannin content is 0.15%. Likewise, *G. sepium* leaves are also rich in CP, with an average content of 23% [24]. Moderate levels of condensed tannins (CT) in these tropical legumes also bind to the diet's protein, enhancing the bypass protein flow from the rumen to the duodenum. Increased milk yield and liveweight gain result from increased direct amino acid supply to the animal [25]. In this way, the CH_4 intensity can be reduced as animal productivity increases. In other words, more milk or beef is produced per unit of CH4 emitted to the atmosphere. In southern Mexico, *G. sepium* is widely used, and it has been shown by Bella et al. [26] that it reduces CH_4 production by up to 33% at 1% inclusion in the diet. On the other hand, the tropics are abundant in agricultural by-products, such as coffee pulp (COP), which is a source of valuable secondary metabolites, including phenolic compounds with antioxidant properties and potential antimethanogenic properties [27]. Therefore, COP may be a promising functional ingredient with potential antimethanogenic properties, given its high tannin content, for ruminant diets [28]. Previous studies have explored the use of coffee pulp as animal feedstuff [28–31]. However, no evidence was found regarding the capacity of COP to reduce CH_4 production, nor regarding its association with *G. sepium*.

Consequently, we hypothesised that the enteric CH_4 produced by cattle in tropical regions of southern Mexico may be lower than reported due to unintended effects of plant secondary metabolites, such as those in *G. sepium*, plants, which are regularly browsed and consumed by cattle. The present work aimed to evaluate the impact of low doses of *G. sepium*, alone or in combination with COP, on enteric CH_4 production in cattle.

2. Materials and Methods

This study was conducted 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 (UAEMEX), which is located in Toluca, State of Mexico, at 19°24′15″ north and 99°41′06″ west, and 2632 m. The LABRELE is equipped with three open-circuit respiration chambers (OCRCs): one head-box type and two complete chambers. The use of experimental animals was approved by the Institutional Subcommittee for the Care and Use of Experimental Animals protocol DC2024/2-8 of the UAEMEX.

Additives: Leaves of *G. sepium* were collected during the summer of 2023 from cattle different farms located in the municipality of Tecpatán, 17°23′09″ N and 93°52′35″ W at 320 m, in the state of Chiapas in southern Mexico, where *G. sepium* is used as a live fence. After harvesting, the leaves were dried away from direct sunlight at 25% ambient relative humidity and an average room temperature of 22 °C to prevent the decomposition of secondary metabolites. Afterwards, leaves were milled with a hammer mill to a size of 0.5 cm (Bison model MMRB-20, Aguascalientes, Mexico) and packed for later use. The COP was collected from coffee farms in the lowlands of central Mexico after the green coffee beans were washed, a process that removes fruity material while the coffee cherry is still moist. The COP was also dried away from direct sunlight, and once dried, packed until use. A representative sample was taken from the lots to determine the content of total phenols, total tannins, and condensed tannins.

2.1. Experimental Procedure

A 4 × 4 Latin square experimental design was used, where four heifers (Holstein × Charolais) of 390 ± 50 kg average initial body weight (BW) were used. Before the start of the experiment, the heifers were vaccinated, dewormed, and found to be in good health. The experiment lasted 120 days. The first 16 days were used to adapt the steers to the control diet (CON), to experimental management procedures, and to the environment inside the RECs, using only the two full chambers. During the adaptation period, the heifers were taken in pairs to the OCRCs for up to 8 h day^{-1}, on average, where they were offered the control diet and water ad libitum. With this adaptation period, it was assured that their intake and behavior would not be affected during the measurement periods. The remaining 104 days were divided into four experimental periods of 26 days each, with 16 days allocated to adaptation to the experimental diet and 6 days to measurements (the sampling period). At the end of the sampling period and after the animal left the OCRCs, four days were used to wash out the rumen from the previous experimental diet, during which only the CON treatment was offered.

2.1.1. Treatments

Four treatments were evaluated, with *G. sepium* and COP used as additives; low doses were added to the CON treatment diet. The CON treatment had no additives and was offered ad libitum, the COP treatment contained 1.0 kg DM d^{-1} of COP, the treatment with *G. sepium* (GSep) contained 0.342 kg DM d^{-1} of this plant, and the treatment with both plants (COP + GSep) had 0.505 and 0.171 kg DM d^{-1}, respectively. All treatments were adjusted to provide approximately the same amount of CP (~81 g kg^{-1} DM). This was to prevent CP in the additives from significantly interfering with CH_4 production, given that we used a high-fibrous forage in the basal diet. More CP supply in any of the treatments would have improved microbial efficiency, leading to better fiber digestion and reduced CH_4 per unit of product. According to [32], CH_4 ppm per forage NDF intake linearly decreased ($p \leq 0.005$) with increasing CP from both respiration and eructation. The basal diet was formulated to meet the animals' metabolizable energy and protein requirements for maintenance, as specified by the Agricultural and Food Research Council (AFRC) [33]. The CO treatment consisted of a concentrate composed (on a DM basis) of 46.58 grounded maize, 13.7 molasses, 6.85 wheat bran, 27.4 bakery by-products, alfalfa meal 2.74, and 2.74% soya bean meal. All the heifers received 3.1 kg DM d^{-1} of this concentrate, and chopped maize straw was offered ad libitum. The approximate forage-to-concentrate ratio was 67:33. The additive plants were blended with the concentrate and offered daily at 10:00 h. After all the concentrate was consumed, the maize straw was offered. In this way, we ensured the animals consumed the entire dose of additives. Each animal received each treatment once during each of the four periods. The sequence of treatments was completely randomized.

2.1.2. Measurement of Enteric CH_4 Emissions

We used the two OCRCs of the LABRELE to measure CH_4 emissions from cattle for 48 h, with one animal per chamber and two animals per run. The respiration chambers were designed following the principle of open-circuit indirect calorimetry [34] and were constructed based on the design by Canul-Solis et al. [35]. They were operated as described by Vázquez-Carrillo et al. [12], and all measurement equipment was from Sable Systems International (Las Vegas, NV, USA). The interior of the OCRCs has a metabolic cage, 3 m long × 1.4 m wide × 1.6 m high, made of stainless steel, with bars on the sides, a rear door, and an adjustable front door. Before the experiment, a CH_4 recovery test was conducted as described by Arceo-Castillo et al. [36] for the type of chambers used in the present experiment, yielding a 100 ± 2% recovery rate. Before each assay, two calibrations of the

OCRCs were performed: a zero calibration using high-purity nitrogen (N_2) (Praxair Inc., Toluca, Mexico) and a calibration against a reference gas, known as the span gas. Once the system passed both calibrations, the run started. The *Ym* factor was calculated using the IPCC [37] Tier 2 method for national inventory calculations. This calculation is based on the quotient of the energy lost in the form of CH_4 per animal per day by the total gross energy intake of the same animal per day. The animals had a one-hour rest period between each 24 h measurement, during which they were taken out of the chambers and allowed to walk and drink water. After the resting period, they returned to the chamber, and measurements were resumed to complete 48 h.

2.1.3. Additional Measurements on Heifers

Dry matter intake (DMI, kg d^{-1}) was measured while the animals were in the OCRCs and calculated as the difference between the diet offered and the orts. A sample of the diet was collected from the trough of each heifer at the end of the measurement period in the chambers, and an aliquot from each sample was prepared for chemical analysis. The heifers were weighed weekly and at the beginning and end of each experimental period. A total of four weighing points were recorded per heifer in each experimental period. The heifers were fasted for 12 h of solids and liquids before weighing day, and a livestock scale (WIM SYSTEM, WIM-LP7510, Zhengzhou, China) was used. Feces were collected daily from each animal during the time in the chambers with a shovel directly from the ground of the metabolic cage, placed in a bucket, and weighed with a digital hanging scale (WeiHeng, 45 kg × 10 g, WIM SYSTEMS generic, Zhengzhou, China). Apparent DM digestibility (DMD) was calculated by subtracting the feces DM weight from the DMI. The DM contents of the diet and feces samples were processed on the same day of sampling. Subsequently, each sample was stored individually in plastic bags, identified, and preserved for subsequent chemical analysis. From days 16 to 21 of the sampling period, total daily CH_4 production was measured in OCRCs for 48 h per heifer, with one heifer per chamber. On the second day in the OCRCs, urine production was measured over 24 h. The urine flowed into a stainless-steel tray at the bottom of the metabolic cage, which emptied into a plastic container containing 500 mL of a 20% sulfuric acid solution for preservation. After 24 h, the total urine volume was measured, and an aliquot of 100 mL was collected from each heifer. These urine samples were placed individually in screw-capped beakers, identified, and kept frozen at −5 °C for subsequent N analysis.

2.1.4. Chemical Analysis of Feed, Stools and Urine Samples

Before laboratory analyses, the diet and fecal composite samples were dried in a forced-air oven at 90 °C for 72 h [AOAC Official Method 934.01], ground, and passed through a 1 mm sieve [38]. All samples were processed in a Wiley model 4 mill with a 1 mm sieve. Diet samples from each experimental period were collected, and an aliquot was prepared for analysis. Stool samples were collected individually from each animal daily during their time in the chambers, and an aliquot was prepared for analysis. The ash (ASH, %) [AOAC Official Method 942.05], the CP = [N] × 6. 25% by the Kjeldahl method [AOAC Official method 976.05] [38], gross energy with a Parr calorimetric pump (Parr Instrument Company, Moline, IL, USA) [AOAC Official Method 983.23], neutral detergent fiber (NDF, %) (Using an ANKOM 200® fiber analyzer, Ankom, Technology, Macedon, NY, USA) contents were determined [AOAC Official Method 2002.04] as in [39]. Urinary nitrogen (N) content was determined by the Kjeldahl method [AOAC Official method 976.05] [38]. The total polyphenol concentration of COP and *G. sepium* was determined using the Folin–Ciocalteu procedure. The tannin content was measured according to the polyvinylpolypyrrolidone method [40], and expressed as tannic acid equivalents. The

condensed tannin (CT) content was determined by using the vanillin method [41]. The chemical composition, polyphenols, and tannin content are shown in Table 1.

Table 1. Chemical composition of the control treatment diet (CON) and content of polyphenols and tannins of the *Gliricidia sepium* and coffee pulp used in the experiment.

Variable	CON Treatment	Maize Straw
DM, g kg^{-1}	923.0	89.8
CP, g kg^{-1} DM	79.9	53.7
NDF, g kg^{-1} DM	307.6	754.0
ADF, g kg^{-1} DM	191.6	547.0
ME, MJ kg^{-1} DM	11.6	8.0
OM, g kg^{-1} DM	46.15	93.5
GE, MJ kg^{-1} DM	17.5	17.4
	Gliricidia sepium	Coffee pulp
TP, %	0.06	2.16
TT, %	0.06	1.31
CT, %	2.49	4.12
CP, g kg^{-1} DM	226.0	85
NDF, g kg^{-1} DM	483.0	353.0
ADF, g kg^{-1} DM	416.0	279.1

DM = dry matter, CP = crude protein, NDF = neutral detergent fibre, ADF = acid detergent fibre, ME = metabolizable energy, OM = organic matter, GE = gross energy, TP = total phenols, TT = total tannins, CT = condensed tannins.

2.1.5. Estimation of the Partition of Gross Energy Consumed, *Ym* Factor and Metabolicity (*qm*) of the Diet

The partitioning of the animal's GE intake (GEi, MJ d^{-1}) was estimated based on the gross energy content (GE, MJ) of feed, CH_4, feces, and urine. Thus, DMI, feces production (Ef, MJ d^{-1}), CH_4, and daily urine production (Eu, MJ d^{-1}) were multiplied by their respective GE concentrations to obtain the calorific values of each variable for the different treatments. The calorific value of total CH_4 production per heifer (ECH_4, MJ d^{-1}) was determined by assuming 1 g CH_4 equals 55.5 KJ [42]. The energy content in urine was estimated assuming that 1 g of N is equivalent to 13.4 kcal, as reported in [43]. The digestible energy intake (DEi, MJ d^{-1}) was determined by subtracting the energy lost in feces (Ef) from the gross energy intake (GEi). The metabolizable energy intake (MEi, MJ d^{-1}) 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 [37]. The diet metabolicity (*qm* factor) was calculated according to AFRC [33], as *qm* = MEi/GEi.

2.2. Statistical Model and Data Analysis

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

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

where: Y_{ijkl} was the response variable of the *i-th* animal (i = 1, 2, 3, 4), which received the *j-th* treatment (j = 1, 2, 3, 4) during the *k-th* period (k = 1, 2, 3, 4), μ was the overall mean common to all observations. A_i and P_k were fixed effect and ε_{ijk} was the experimental error common to all observations, assumed independent, normally distributed, with zero mean and unit variance (N, I; $\mu = 0$, $\sigma = 1$). Statistical analysis was performed using Minitab v19. For the variables that were statistically different ($p \leq 0.05$), a Tukey test was conducted.

3. Results

3.1. Dry Matter Intake and Digestibility

Table 2 shows that significant differences were observed for the whole tract digestibility of the neutral detergent fiber (NDFD), where the highest value was observed for treatment GSep followed by COP ($p < 0.02$) in comparison to the CON treatment. Significant differences ($p < 0.1$) were observed in the whole tract DMD, with the highest values observed for the same treatments. No significant differences ($p > 0.05$) were observed for DMI, digestible dry matter intake (DDMI), neutral detergent fiber intake (NDFI) and gross energy intake (GEI).

Table 2. Dry matter and neutral detergent fiber intake, along with their associated digestibility, for the different treatments.

Treatment	DMI, kg d^{-1}	DMD, %	DDMI, kg d^{-1}	NDFI, kg d^{-1}	F-Output kg DM d^{-1}	NDFD, %	GEI, MJ d^{-1}
CON	9.4 ± 0.4	46.4 ± 1.3	4.6 ± 0.5	5.3 ± 0.4	4.8 ± 0.2	46.5 ^{a} ± 1.3	145.6 ± 9.3
GSep	8.9 ± 0.7	52.4 ± 1.3	4.5 ± 0.5	4.9 ± 0.4	4.4 ± 0.1	53.6 ^{b} ± 1.3	144.3 ± 11.1
COP + *GSep*	8.6 ± 0.5	49.7 ± 1.4	4.2 ± 0.5	4.7 ± 0.4	4.4 ± 0.3	45.9 ^{a} ± 1.4	143.5 ± 8.6
COP	9.2 ± 0.4	49.8 ± 1.3	4.7 ± 0.5	5.2 ± 0.4	4.5 ± 0.2	50.4 ^{b} ± 1.3	155.5 ± 7.3
SED	0.85	1.89	0.72	0.58	0.22	1.90	13.4
p value	0.76	0.1	0.87	0.74	0.33	0.02	0.70

Key: CON = control treatment, GSep = *Gliricidia sepium* treatment, COP = coffee pulp treatment, SED = standard error of the difference, DMI = dry matter intake, DMD = digestibility of the dry matter, DDMI = digestible dry matter intake, NDFI = neutral detergent fibre intake, F-ouput = faecal output, NDFD = neutral detergent fibre digestibility, GEI = gross energy intake, ± = Standard error of the mean.

3.2. Methane Production and GE Partitioning

Table 3 presents the results for the variables associated with CH_4 production and the partitioning of GE intake into digestible energy (DE) and metabolizable energy (ME) for the different treatments. For the variable related to CH_4 production, significant differences ($p < 0.05$) were observed in total daily CH_4 emissions, CH_4 yield, and CH_4 yield per kilogram of neutral detergent fiber digested (NDFD). The lowest CH_4 production was observed for the COP + GSep treatment, followed by GSep, with 17% and 14.2% less CH_4, respectively, compared to the CON treatment. These treatments also showed the lowest CH_4 yield (CH_4, g kg^{-1} DMI), and GSep treatment showed the lowest CH_4 yield per kilogram of NDFD. No significant differences ($p > 0.05$) were observed for the *Ym* factor and CH_4 yield per kilogram of DDMI. However, the lowest numerical *Ym* values were also observed for the COP + GSep and GSep treatments.

Table 3. Methane emission and partitioning of the gross energy intake for heifers supplemented with different levels of *Gliricidia sepium*, Pulp of coffee and Pulp of coffee plus *Gliricidia sepium*.

Variable	Treatment				SED	*p*-Value
	CON	GSep	COP	COP + GSep		
			Methane			
CH_4, g d^{-1}	168.4 ^{a} ± 4.3	144.4 ^{b} ± 4.1	152.6 ^{a} ± 4.2	139.9 ^{b} ± 4.3	5.9	0.02
CH_4, g kg^{-1} DMI	17.9 ^{a} ± 0.9	16.3 ^{b} ± 0.8	16.7 ^{a} ± 0.7	16.5 ^{b} ± 1.1	0.52	0.01
Ym, %	6.3 ± 0.4	5.6 ± 0.4	5.4 ± 0.4	5.4 ± 0.4	0.58	0.38
CH_4, g kg^{-1} DDMI	37.5 ± 4.0	31.3 ± 4.0	33.5 ± 4.0	36.0 ± 4.0	5.6	0.72
CH_4, g kg^{-1} NDFD	70.0 ^{a} ± 6.9	50.6 ^{b} ± 1.8	60.0 ^{a} ± 6.9	68.9 ^{a} ± 6.0	12.2	0.01

Table 3. *Cont.*

Variable	Treatment				SED	*p*-Value
	CON	GSep	COP	COP + GSep		
	Partitioning of the gross energy intake					
GE lost in feces, MJ d^{-1}	74.8 ± 7.3	68.1 ± 3.0	70.9 ± 3.9	68.7 ± 5.8	4.4	0.48
F:GE	0.51 ± 0.04	0.47 ± 0.01	0.46 ± 0.03	0.48 ± 0.05	0.05	0.75
GE lost in urine, MJ d^{-1}	7.9 ± 0.5	7.0 ± 0.3	8.0 ± 0.2	7.3 ± 0.2	0.47	0.22
U:GE	0.07 ± 0.007	0.06 ± 0.006	0.08 ± 0.008	0.07 ± 0.007	0.01	0.55
Energy loss as CH_4, MJ d^{-1}	9.3 ^{a} ± 0.7	7.9 ^{b} ± 0.5	8.4 ^{a} ± 0.3	7.7 ^{b} ± 0.1	0.31	0.01
CH_4:GE	0.063 ± 0.004	0.056 ± 0.004	0.054 ± 0.004	0.054 ± 0.004	0.005	0.41
DEi, MJ d^{-1}	70.8 ± 7.4	75.2 ± 8.1	84.5 ± 9.3	74.7 ± 11.5	13.21	0.76
DE:GE	0.48 ± 0.03	0.52 ± 0.03	0.54 ± 0.03	0.51 ± 0.03	0.05	0.74
MEi, MJ d^{-1}	53.6 ± 6.9	60.2 ± 7.5	68.0 ± 9.3	59.6 ± 11.5	12.9	0.745
ME:GE	0.37 ± 0.03	0.41 ± 0.03	0.43 ± 0.03	0.40 ± 0.03	0.05	0.73

CON = Control diet; GSep = *Gliricidia sepium* treatment (0.342 kg DM d^{-1}); COP = Coffee pulp treatment (1 kg DM d^{-1}); COP + GSep = coffee pulp + *Gliricidia sepium* treatment (0.505 and 0.171 kg DM d^{-1}, respectively); CH_4 = methane; CH_4 (g kg^{-1} DMI) = methane yield; Ym factor = CH_4 conversion factor, the energy in CH_4 as a percentage of GEi; GE = gross energy in MJ; F:GE = proportion fecal energy to gross energy; U:GE = proportion urinary energy to gross energy; CH_4:GE = proportion CH_4 energy to gross energy; GEi = gross energy intake; DEi = digestible energy intake; MEi = metabolizable energy intake; DE:GE = proportion digestible energy to gross energy; ME:GE = proportion metabolizable energy to gross energy; SED = standard error of the difference. Values in the same row with different superscript letters a and b are significantly different ($p < 0.05$), ± = Standard error of the mean.

The results for the variables associated with the partitioning of the GE show that significant differences were observed only for energy loss as CH_4, with the highest value observed for the CON treatment and the lowest for COP + GSep, followed by GSep, compared with the CON treatment ($p < 0.01$). No significant differences were observed for the remaining variables in Table 4; however, large numerical differences were observed for the COP and GSep treatments, up to 14.4 MJ d^{-1} in the former and 6.6 MJ d^{-1} in the latter, compared with the CON treatment. Table 4 shows the DE, ME (MJ kg^{-1} DM) and qm factor for the four treatments, it can be observed that no significant differences were observed between treatments for all variables ($p > 0.05$). However, COP and GSep treatments presented the highest ME contents, resulting in a larger proportion of ME in the GE or qm factor.

Table 4. Digestible and metabolizable energy contents and *qm* factor for the experimental heifers supplemented with different levels of *Gliricidia sepium*, coffee pulp and coffee pulp plus *Gliricidia sepium*, MJ kg^{-1} DM.

Variable	Treatment				SEM	*p*-Value
	CON	GSep	COP	COP + GSep		
DE, MJ/kg DM	7.5 ± 0.6	8.3 ± 0.3	9.1 ± 0.6	8.5 ± 0.8	0.80	0.33
ME, MJ/kg DM	5.6 ± 0.6	6.6 ± 0.3	7.3 ± 0.6	6.8 ± 0.9	0.90	0.40
qm Factor	0.37 ± 0.04	0.41 ± 0.02	0.43 ± 0.03	0.40 ± 0.05	0.05	0.73

CON = Control treatment; GSep = *Gliricidia sepium* treatment (0.342 kg DM d^{-1}); COP = Coffee pulp treatment (1 kg DM d^{-1}); COP + GSep = coffee pulp + *Gliricidia sepium* treatment (0.505 and 0.171 kg DM d^{-1}, respectively); DE = digestible energy; ME = metabolizable energy; DM = dry matter; *qm* = metabolisity of the GE, calculated as ME/GE, SEM = standard error of the mean., ± = Standard error of the mean.

4. Discussion

In the present work, we aimed to evaluate the impact of small doses of GSep alone or in combination with COP on enteric CH_4 production in cattle fed a low-quality forage. The use of low-quality forage in our experiment was intentional, as we tested a diet prone to high CH_4 production. This approach allowed us to detect not only the antimethanogenic

properties of the additive plants but also their associative effects on rumen degradation and DM intake [33,44]. We also hypothesized that enteric CH_4 produced by cattle in tropical regions of southern Mexico may be lower than reported due to unintended effects of plant secondary metabolites, such as those in *G. sepium*, a plant regularly browsed and consumed by cattle. Our results show that DMI was not significantly affected by the additives, however, the DMI was moderately low. It ranged from 2.2% to 2.4% of body weight, despite ad libitum access to chopped maize straw and consumption of all concentrate and additive plants offered. This was expected, and can be explained by the high content of low-quality fiber in the maize straw, which resulted in rapid rumen filling and slow passage rate in the experimental animals. So, reduced intake was due to the low quality of the forage, not to the additives used in the present work. Our results are in line with [44], who indicate that dietary fiber concentration is one of the main determinants of DMI in ruminants due to its effects on rumen fill and passage rate, which is slowed down because rumen microbes take longer to colonize and degrade structural carbohydrates in forage. The DMD and NDFD were low but within the expected range for a basal diet based on maize straw or similar low-quality tropical grasses. For example, Ehoche et al. [45] reported a DMD of 47.4% and NDFD of 40.6% in heifers fed ad libitum untreated maize straw supplemented with a concentrate mixture at 2.0% of body weight, which is quite similar to our results. Moreover, according to Indah et al. [46], the average DMD of 62 tropical grasses is 56.7 $\pm$ 11.5%, which is also close to our results for the treatments with *G. sepium* and COP. However, we observed that *G. sepium* and COP significantly increased NDF digestibility (NDFD). This may be explained by the additional CP supplied by *G. sepium* (CP = 226 g kg^{-1} DM) and the fermentable energy provided by the COP, although in small quantities. The CP in *G. sepium* may have improved rumen fermentation in the experimental animals, given that maize straw had low CP content (CP = 53.7 g kg DM^{-1}) [47]. Legumes such as *G. sepium* provide rumen-degradable protein (RDP) that helps correct nitrogen deficiency, stimulating the microbial population and leading to more efficient fermentation and fiber degradation in low-quality roughages [48]. Similar responses regarding low CP levels have been observed in other studies. For example, Chanthakhoun et al. [49] reported that 124 and 181 g kg^{-1} CP in the concentrate supplement resulted in the highest rumen fermentation efficiency in swamp buffaloes fed rice straw. As noted before in the methodology section, the treatments were adjusted to provide ~81 g CP kg^{-1} DM. Together with the concentrate experimental treatments supplied approximately 104.5 g CP kg^{-1} DM. So, it is correct to assume that this extra CP enhanced the degradation of the N.

Furthermore, the additional energy provided by the COP likely increased microbial protein synthesis, which, in turn, increased NDFD [33].

The CH_4 yield of 17.9 g CH_4 kg^{-1} DM obtained in the present study (Table 3) for the CON treatment is similar to that reported in the literature for tropical regions of Mexico. For example, Ku-Vera et al. [50] reported enteric CH_4 emissions a yield of 18.07 g CH_4 kg^{-1} DM intake in heifers (*Bos indicus* $\times$ *B. taurus*) with an average live weight of 288.5 $\pm$ 55 kg fed tropical grasses, and an average intake of 8.2 kg of DM. However, the same parameters obtained in the present work across the experimental treatments are lower than those reported by previous authors. This is probably due to the secondary compounds present in the plant additives (Table 1). The lowest emissions and, notably, yields observed when *G. sepium* and COP were supplemented together could be attributed to the presence of CT in both plants. CTs reduce CH_4 by inhibiting fiber digestion (CT form complexes with lignocellulose) and thereby decreasing H2 production [51]. This indicates that the reduction in CH_4 production was not at the expense of NDFD or DMD, other mechanisms may have been implicated. CT also reduces CH_4 gas production by inhibiting the growth and activity of archaea methanogens. Furthermore, CT exerts an indirect effect by hindering protozoan

symbiosis with methanogens [52] or by inhibiting bacterial cellulase production during fiber digestion. These CT effects better explained the CH_4 reduction observed in the present work. Some studies have hypothesized that tannins promote a shift in VFA production towards propionate rather than acetate, which acts as a hydrogen sink. The reduced availability of H_2, the primary substrate for CH_4 production, reduces methanogenesis [53,54]. According to Cardoso-Gutiérrez et al. [55], a negative weak relationship has been reported between tannin inclusion level and CH_4 emissions, indicating that the effect of CH_4 mitigation increases with increasing tannin inclusion. However, this is not entirely accurate, as several studies have shown that the effect of CT on CH_4 production is quadratic rather than linear [12,56]. Susanto et al. [57] mention that the population dynamics of the rumen microbiome varied depending on tannin levels, tannin type, and tannin source. This means we must not expect the same antimethanogenic activity across tanniferous plants with similar tannin content, even within a single plant species, but grown in contrasting regions. This assumption is corroborated by our results regarding the COP treatment, which, despite having higher CT and total phenols than *G. sepium*, did not result in lower CH_4 production than the previous treatment. According to [58], this variability is attributed to the structural characteristics of the tannins, many of which have been linked to an increased antimethanogenic potential. For example, according to [51], there is a correlation among CT structure, CH_4, and fermentation characteristics. These authors reported that the proportion of prodelphinidins within procyanidins (also known as CT) had the largest effect on fermentation characteristics, followed by average polymer size and the percentage of cis flavan-3-ols. However, Wong-Paz [59] noted that the procyanidins in COP are present only in a minor class of oxidized procyanidins, with important antioxidant capabilities. This suggests that the amount of prodelpinidins in COP is low, because the effect of COP's CT on rumen fermentation was marginal. This explains our results because COP showed a smaller effect on CH_4 production than *G. sepium*.

Unfortunately, there is a lack of precise structural characterization of tannins for many tropical tanniferous plants, which, according to [58], explains the inconsistency in the effect of tannins on CH_4 production. Further variations can arise from differences in the growth conditions of the tested plants, which can affect their secondary metabolite synthesis. For example, according to [60], climate can influence not only the quantity of tannins, but also their molecular composition and cell-wall associations in *Quercus rubra*. This assertion is supported by simultaneous experiments conducted at the Autonomous University of the State of Mexico (UAEMex) and the University of California, Davis (UC Davis). These experiments evaluated the antimethanogenic properties of lemongrass (*Cymbopogon citratus*) in beef cattle. In the former case, a 30% reduction in CH_4 yield was observed ($p < 0.05$) [12,61], whereas in the latter case, no reduction was observed; instead, emissions increased [62]. Later analysis for polyphenols and tannins showed that the Californian lemongrass used in UC-Davis contained no CT at all, whereas the lemongrass produced in Mexico had 52.3 g CT kg^{-1} DM. This difference accounted for the lack of results observed in the UC Davis experiment.

The results of the present work suggest that there is a combined effect when COP and *G. sepium* are supplemented together. This is in line with studies showing that mixtures of tannin-rich species [63] or tanniferous legumes [64] often produce additive or synergistic reductions in CH_4 emissions from ruminants, suggesting a synergistic interaction between tannins and other secondary metabolites. Increased animal productivity has also been reported with the inclusion of two or more tanniferous legumes, resulting in reduced emission intensity (g CH_4 kg^{-1} ADG or kg of milk) [64]. However, other authors report antagonistic effects when several tanniferous plants are used together [63]. Further, excessive tannins can negatively affect digestibility and animal performance. Thus, the quantity

of tanniferous plants must be limited to less than 4% of the dry matter intake. According to [65], dietary tannins in small ruminant diets can improve antioxidant status, modulate ruminal fermentation, and enhance meat quality and fatty acid profiles. Still, high levels may impair growth performance and nutrient digestibility. In cattle, the effects depend strongly on tannin type (condensed vs. hydrolysable), source (plant vs. extract), and dose, with blends often producing the most consistent benefits [66]. Finally, in addition to CH_4 reduction, *G. sepium* offers numerous environmental benefits [67], including carbon sequestration, enhanced soil fertility through atmospheric nitrogen fixation, natural pest control, and the ability to implement silvo-pastoral systems, and gastrointestinal parasites [68]. Due to its flavonoid, saponin, and tannin content, which act as anti-inflammatory agents, it enhances wound healing [69]. However, we recommend cautioning our findings, as it is necessary to replicate the present study with a larger number of animals and possibly different doses of additives.

We anticipate that the use of *G. sepium* as an antimethanogenic additive will be readily adopted among Mexican cattle farmers in southern Mexico. This tree is native to Central America and is already widely cultivated as a living fence and forage. *G. sepium* is easily propagated through cuttings, which are directly planted in the soil; no fertilization is required. It can be harvested just after one year of planting and fed fresh to cattle. The COP is an abundant local byproduct, as Mexico is among the top 10 coffee producers worldwide, but it is not used. It is instead discharged into rivers and lakes, thereby polluting them. Approximately 80% of coffee production is concentrated in the States of Chiapas, Veracruz, and Puebla, where *G. sepium* is also cultivated, particularly in Chiapas and Veracruz, Mexico's southernmost states. All that is needed now is to capacitate farmers on the appropriate use of both additives and the importance of mitigating enteric CH4 emissions. For mitigation, it must be verified that the plants to be used contain the required TAN concentrations. If widely applied, it may be possible to mitigate up to 1060 Gg CH_4 $year^{-1}$, as the cattle population in tropical Mexico accounts for 52%, and the present IPCC-Tier 2 enteric CH_4 inventory for Mexico is 2039 Gg CH^4 $year^{-1}$ [70].

5. Conclusions

We conclude that supplementation with *G. sepium*, alone or in combination with COP, could be used as part of a strategy to reduce enteric CH_4 production in tropical cattle production systems in tropical regions of southern Mexico, where both additives are available. They may also be adequate for farmers who cannot afford antimethanogenic commercial additives. More research is needed to identify additional tropical tanniferous plants and trees browsed by cattle that also reduce CH_4 production. Further investigation is also required into the structural composition of tannins and their antimethanogenic biological activity in both additives.

Author Contributions: Conceptualization, funding acquisition, project administration, validation and supervision, writing, review and editing, O.A.C.-O.; Project administration and supervision, writing—original draft preparation, discussed the results and revised the manuscript, F.A.-N., J.N.-T., J.H.-C. and M.G.-R.; investigation, formal analysis, data curation, C.C.-M. and R.J.T.-V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Secretaría de Ciencia, Humanidades, Tecnología e Innovación, (SECIHTI), grant number 317413.

Institutional Review Board Statement: "The animal study protocol was approved by the Institutional Review Board of Faculty of Veterinary Medicine and Animal Science of the Universidad Autónoma del Estado de Mexico (FMVZ-SICUAE 02042024)." for studies involving animals. Approved Date: 18 November 2024.

Data Availability Statement: The data presented in this study are available on request to the corresponding author.

Acknowledgments: We acknowledge the help of Felix, the farmers who helped to collect the forage of *Gliricidia sepium*. We also thank Marlene Flores Castillo, Cecilia Abundio Eustaquio, Reynaldo Zaragoza Guerrero, and Adriana Atziry Ramos Melesio, for their help in looking after the experimental animals.

Conflicts of Interest: The authors declare no conflicts of interest.

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