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Reduction of Enteric Methane Emissions in Heifers Fed Tropical Grass-Based Rations Supplemented with Palm Oil

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Abstract: Vegetable oils have been shown to reduce enteric methane (CH_4) production by up to 20%. However, when the level of incorporation exceeds the threshold of 70 g/kg DM, dry matter intake (DMI) and nutrient digestibility may be reduced. The objective of this study was to determine the effects of the incorporation of three levels of palm oil (PO) on enteric CH_4 emissions, rumen fermentation and apparent digestibility in heifers fed low-quality grass. Four rumen-cannulated heifers (*Bos taurus* × *Bos indicus*) were randomly assigned to four treatments: control (CON) and three increasing PO levels: 20, 40 and 60 g/kg in a 4 × 4 Latin square design with four periods of 22 days (14 days of adaptation to the ration), 5 days of feces and rumen fluid sampling (day 18, 4 h postprandial) and the last 3 days for measurements of CH_4 in respiration chambers. With the exception of CP ($p = 0.04$), starch ($p = 0.002$) and EE ($p < 0.001$), the intake of nutrients was not affected by the inclusion of PO ($p > 0.05$). The apparent digestibility (AD) of nutrients was not affected by the inclusion of PO ($p > 0.05$), except for starch, which reduced its AD as the PO level was increased ($p < 0.05$). The gross energy intake was higher in PO-containing rations ($p = 0.001$), on the other hand, the digestible energy intake was similar between treatments ($p > 0.05$). In situ ruminal digestion kinetics and the potential degradability remained unchanged ($p > 0.05$), however, the effective degradability decreased with the inclusion of PO in the rations ($p < 0.05$). The ruminal pH and molar proportions of acetic, isovaleric and valeric acid were not different between treatments ($p > 0.05$). The ruminal concentration of propionic acid increased as the PO level increased, reaching its highest molar proportion with 60 g/kg PO ($p < 0.05$), however, the acetic/propionic ratio and the molar proportions of butyric acid and isobutyric acid decreased as the PO level increased ($p < 0.05$). The total daily CH_4 production was lower in diets containing 20, 40 and 60 g/kg PO compared to the CON diet ($p < 0.001$). The production of CH_4 per kg DMI and DOMI was greater ($p < 0.05$) for the CON diet compared to all three rations containing PO. The emission intensity, Y_m, energy lost as CH_4, emission factor (EF) and kg CO_2 eq/year were reduced as an effect of the inclusion of PO ($p < 0.05$). Based on the results obtained, it is concluded that the incorporation of PO in cattle rations has the potential to reduce enteric methane emissions by 4% for every 10 g/kg PO in the ration, without affecting DMI, apparent digestibility or the consumption of digestible nutrient fractions.

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1. Introduction

Methane (CH_4) is a greenhouse gas (GHG) emitted by livestock [1] and represents 19% of total emissions [2], of which 45% are attributed to enteric fermentation [3]. Cattle production contributes 4.6 Gt CO_2-eq, from which 54.3% and 45.7% correspond to beef and milk production, respectively [4]. In the tropical regions, cattle consume and transform C4 type grasses of medium-low quality into high-quality proteins for human consumption through anaerobic fermentation in the rumen carried out by bacteria, protozoa and fungi with concomitant synthesis of volatile fatty acids [VFA), carbon dioxide (CO_2) and hydrogen (H_2). Hydrogen available in the rumen is used by methanogenic archaea to reduce CO_2 to CH_4 through methanogenesis [5], the main pathway to obtain CH_4 as the final product of the anaerobic fermentation of digestible organic matter (OM) in the rumen and the hindgut, resulting in the loss of 2 to 12% of the gross energy (GE) ingested in the feed [6]. Therefore, when CH_4 production is mitigated, it is possible to increase the quantity and quality of production [7], as carbon sources are utilized for meat and milk production and not for CH_4 synthesis, thus benefiting the environment by reducing the carbon footprint.

Methane mitigation can been achieved in different ways. One option is the incorporation of lipids in ruminant rations. Usually, lipid supplementation is used to increase the energy density of the ration [8]. There is considerable evidence that shows rations containing supplementary oils reduce the production of enteric CH_4 [9]. It has been demonstrated that the change from carbohydrates to lipids in ruminant rations modifies gas production in the rumen, as fermentation and CH_4 production are reduced [10,11].

Recent work has reported reductions from 7.2 to 21.4% [9,12–15] when 20 to 60 g/kg of lipids were included in the rations of dairy cows and beef cattle. The mechanisms for the suppression of methanogenesis consist of replacing rumen fermentable organic matter in the diet, decreasing the numbers of ruminal methanogens and protozoa, and the biohydrogenation of unsaturated fatty acids [16]. The experiment hereby described was designed to investigate the effect of the inclusion of palm oil (PO; *Elaeis guineensis* Jacq.) on enteric CH_4 emissions, rumen fermentation and apparent digestibility in cattle fed a 70:30 forage–concentrate ratio. The hypothesis behind this experiment was that palm oil in the rumen would the change pattern of fermentation, and thus, CH_4 emissions would be reduced.

2. Materials and Methods

The experiment was approved by the Bioethics Committee and Manual for Research with Living Organisms and Environmental Conservation of the Faculty of Veterinary Medicine and Animal Science, University of Yucatan, Mexico.

2.1. Heifers, Experimental Design, and Dietary Treatments

Four crossbred (*Bos taurus* × *Bos indicus*) heifers cannulated in the dorsal sac of the rumen (10 cm, Bar Diamond Inc.) were randomly allotted to four treatments: control (CON) and three increasing levels of palm oil (PO) [20, 40 and 60 g/kg DM] in a 4 × 4 Latin square [17] for four periods of 22 days (14 days for adaptation to the ration, 5 days for feces sampling and 3 days for enteric CH_4 measurements in open-circuit respiration chambers (OCRC); rumen fluid sampling was performed on the 18th day of each period, 4 h postprandial). Twenty days before the start of the experiment, heifers were fed the CON ration for adaptation and to obtain reference values on dry matter intake (DMI); furthermore, all heifers were given an intramuscular injection of Vigantol ADE Bayer® (1 mL/50 kg LW; 1 mL contains = vit. A: 500,000 IU; vit. D3: 75,000 IU; vit. E1: 50 mg) and were dewormed with Ivermectin Sanfer® (1 mL/50 kg LW). At the beginning of the

experiment, the heifers averaged (μ ± SD) 7.6 ± 0.45 kg/d of DMI and 334 ± 10.26 of live weight (LW).

During the experiment, the heifers were housed in individual metabolic crates and had free access to water. The heifers were fed *ad libitum* at 8:00 h (10% orts, on as-fed basis). On a DM basis, the experimental treatments consisted of totally mixed rations (TMR) consisting of 70% of *Megathyrsus maximus* cv. Mombasa (120 day regrowth) and 30% of a concentrate (ground corn, soybean meal, urea and minerals) formulated according to the energy and protein requirements for maintenance and growth of heifers [18]. In the TMR, different proportions (20, 40 and 60 g/kg DM) of ground corn were replaced by equal proportions of PO in order to establish experimental treatments. The proportions and chemical composition of the TMR are shown on Tables 1 and 2. The heifers were weighed at the end of each experimental period.

Table 1. Proportion of ingredients of experimental diets.

Item	Treatments			
	CON	**20**	**40**	**60**
Ingredients (g/kg DM)				
Megathyrsus maximus cv Mombasa	700	700	700	700
Ground corn	190	170	150	130
Soybean meal	85	85	85	85
Palm oil	0	20	40	60
Urea	10	10	10	10
Minerals [1]	15	15	15	15

CON, control treatment; Treatments, 20, 40 and 60 PO level inclusion in diet; [1] Mineral premix contained (minimum values per kg): 40 g P, 120 g Ca, 0.74 g Fe, 10 g Mg, 400 g NaCl, 1.50 g Mn, 1.5 g Zn, 0.15 g Cu, 0.0018 g I, 0.001 Co.

Table 2. Chemical composition of experimental diets.

Item	Treatments			
	CON	**20**	**40**	**60**
Chemical composition (g/kg DM)				
Dry Matter (DM)	937 ± 0.50	946 ± 0.50	946 ± 0.50	946 ± 0.96
Organic Matter (OM)	928 ± 2.06	935 ± 2.38	930 ± 1.15	933 ± 1.50
Crude Protein (CP)	109 ± 1.00	104 ± 1.50	104 ±1.26	103 ± 2.01
Neutral Detergent Fiber (NDF)	588 ± 0.58	588 ± 9.98	586 ± 7.44	585 ± 9.90
Acid Detergent Fiber (ADF)	374 ± 1.73	378 ± 1.73	376 ± 5.26	377 ± 7.94
Starch	216 ± 3.10	207 ± 7.54	185 ± 6.38	170 ± 11.6
Crude Ash (CA)	71.8 ± 2.01	65.7 ± 2.04	70.2 ± 1.05	67.4 ± 1.54
Ether Extract (EE)	16.5 ± 1.53	35.7 ± 1.41	54.9 ± 1.73	74.2 ± 1.31
Non-Fibrous Carbohydrates (NFC) [1]	248 ± 3.60	239 ± 6.70	218 ± 6.02	203 ± 10.8
NDF/NFC	2.37 ± 0.03	2.46 ± 0.11	2.70 ± 0.10	2.90 ± 0.21
Total Digestible Nutrients (TDN) [2]	598 ± 2.50	627 ± 2.16	646 ± 3.30	672 ± 2.22
Gross Energy (MJ/kg DM)	15.7 ± 0.21	16.5 ± 0.18	16.9 ± 0.28	17.6 ± 0.20
Digestible Energy (MJ/kg DM)	11.0 ± 0.05	11.6 ± 0.06	11.9 ± 0.08	12.4 ± 0.01
Metabolizable Energy (MJ/kg DM) [3]	10.3 ± 0.08	10.8 ± 0.05	11.2 ± 0.06	11.6 ± 0.05

CON, control treatment; Treatments, 20, 40 and 60 PO level inclusion in diet; [1] Determined according to Detmann & Valadares-Filho [19]. [2] Estimated by the National Research Council [20]. [3] Determined according to Galyean et al. [21].

2.2. Data Collection and Chemical Analyses of Feeds

During each data collection period, offered and refused feed samples were taken daily per treatment for DM determination in a forced-air oven at 60° during 72 h. Then, each sample was kept in plastic bags and stored in a dry shelf. At the end of each experimental period, feed samples and refusals by treatment were grouped and an aliquot (10%) was

taken for later analysis. Samples were ground through a 2 mm sized mesh in a Wiley® mill (Arthur H. Thomas Co., Philadelphia, PA, USA). Each composite sample was analyzed in triplicate for DM determination by drying at 100° for 24 h (method 990.03; [22]), ash at 600° during 2 h to OM (method 942.05; [22]), total nitrogen by the combustion method (LECO® series CN-2000 3740, LECO® Instruments Inc., St. Joseph, MI, USA), ether extract by the method of acid hydrolysis using petroleum ether as a solvent (EE; method 922.06; [22]), GE using a bomb calorimeter (C200, IKA Works® Inc., Staufen, Germany), neutral detergent fiber (NDF) and acid detergent fiber (ADF) by the procedures described by Van Soest [23], and results were expressed with residual crude ash. NDF analysis was performed using α-amylase with no sodium sulfite added to the detergent. Nutrient intake was calculated by multiplying DMI by nutrient concentration in the TMR. Daily intake values were corrected for the concentrations of nutrients in refusals. CON, control treatment; Treatments, 20, 40 and 60 PO level inclusion in diet; Determined according to Detmann & Valadares-Filho [19].

Non-fibrous carbohydrates (NFC) were determined according to following equation [19]:

$$\text{NFC } (\%): \ 100\% - \text{CA} - \text{EE} - \text{NDF} - (\text{CP} - \text{CPu} + \text{U}) \quad (1)$$

in which CA is crude ash, EE is ether extract, NDF is neutral detergent fiber, CP is crude protein and the CPU is the CP from urea and U is the urea content. All terms are expressed as % DM. Total digestible nutrients (TDN) were calculated according to equation [24]:

$$\text{TDN } (\%) = \text{DCP} + \text{DNDFap} + \text{DNFC} + (2.25 \times \text{DEE}) \quad (2)$$

where: DCP = digestible CP; DNDFap = digestible NDFap; DNFC = digestible NFC and DEE = digestible EE [20]. TDN contents of the ingredients and total diets were calculated according to equations described by the National Research Council [20]. For metabolizable energy (ME), it was considered that 1.0 kg of TDN is equivalent to 18.447 MJ of digestible energy (DE), and for transformation into ME [21] the following equation was used:

$$\text{ME} = 0.9611 \times \text{DE} - 0.2999 \quad (3)$$

For fatty acid (FA) profile determination, PO was converted into the fatty acid methyl ester (FAME) according to the procedures described by Danish and Nizami [25] using methanolic NaOH 0.5 N (Table 3). One μL of FAME extract was injected into a capillary column TR-FAME [20 m × 0.25 mm i.d., 0.25 μm film thickness Thermo P/N: 260M166L] connected to GC system Agilent Technologies 7820A fitted with a flame ionization detector. The temperature of the oven was programmed as follows: initial temperature was kept at 150° for 5 min, increased to 180° at a speed of 10°/min, then increased at 210° at a speed of 1.5°/min and kept like this for 10 min. The injector and detector temperatures were 250° and 280°, respectively. Helium was used as the carrier and make-up gas at 1.3 mL/min and 25 mL/min, respectively. The split ratio was 1:100. Methyl esters of fatty acids were identified by comparing retention times with standards: 37 Component FAME Mix (Sigma-Aldrich 47885); α-linolenic acid (Fluka 62160), Eicosapentaenoic acid methyl ester (Fluka 17266) and Docosahexaenoic acid methyl ester (Fluka 05832); and quantified in relation to the internal standard Heptadecanoic acid (C17:0), which was prepared with a concentration of 10 mg/mL in Hexane (200 μL of C17:0 added to 50 μL extracted lipid before FAME preparation).

Table 3. Fatty acids with their relative percentage in the total fat of the crude palm oil.

Fatty Acids in Palm Oil	Fatty Acids Group	RT	Peak Area (FAME)	% Fat (of Total Fat)
C12:0–Lauric acid	SAFA	11.8	37.6	0.19
C14:0–Myristic acid	SAFA	13.3	198	1.02
C16:0–Palmitic acid	SAFA	15.7	8574	44.1
C16:1–Palmitoleic acid	MUFA	16.3	27.7	0.14
C18:0–Stearic acid	SAFA	18.7	1001	5.15
C18:1 (n−9)–Oleic acid	MUFA/ω9FA	19.7	7372	37.94
C18:2 *trans*-(n−6)–Linolelaidic acid	TFA	19.8	143	0.73
C18:2 (n−6)–Linoleic acid	PUFA	21.0	1963	10.1
C18:3 (n−6)–*g*-Linolenic acid	PUFA/ω6FA	22.8	63.7	0.33
C21:0–Heneicosanoic acid	SAFA	23.7	24.2	0.12
C22:1–(n−9)–Erucic acid	MUFA/ω9FA	27.4	12.7	0.07
C24:0–Lignoceric acid	SAFA	32.8	15.6	0.08
Monounsaturated Fats	MUFA	–	–	38.15
Polyunsaturated Fats	PUFA	–	–	11.16
Total Unsaturated Fats	TUFA	–	–	49.39

RT = Retention Time, FAME = Fatty Acid Methyl Ester, ω6FA = Omega-6 Fatty Acids, ω9FA = Omega-9 Fatty Acids, SAFA = Saturated Fatty Acids.

The fatty acid profile of PO was used to estimate the grams of CH_4 that were not produced by redirection of metabolic H_2 for biohydrogenation of unsaturated fatty acids [26]. The calculation of H_2 utilization following the biohydrogenation of ingested lipid (UHyLi) was carried out with equation [27]:

$$\text{UHyLi } (\text{mol}H_2/\text{d}) = \text{PLi} \times \text{Liferm} \times 1.805 \times 2 \quad (4)$$

where PLi is the intake of feed lipid (mol/d), Liferm is the proportion of feed lipid subject to lipolysis within the rumen, 1.805 is the coefficient describing the moles of UFA per mole of feed lipid, and 2 is the moles of H_2 utilized per mole of UFA [28].

2.3. Apparent Total-Tract Digestibility

The apparent digestibility (AD) was calculated by the procedure described by Schneider and Flatt [29] by collecting and weighing daily total fecal production during 5 consecutive days, starting at 8:00 h of day 14 and finishing at the same time on day 19 of each experimental period. The equation used was the following:

$$\text{AD } (\%) = \frac{\text{Ingested nutrient} - \text{Excreted nutrient}}{\text{Ingested nutrient}} \times 100 \quad (5)$$

The feces production was determined gravimetrically, giving rise to 5 samples of (1 kg) by heifer for each period. Metabolic crates were provided with an individual tray (1 m^2) fixed below the grid for feces collection. The trays had a steel mesh (2 mm × 2 mm × 2 mm) of 1 m^2 in the upper part that works as a urine filter to keep the feces with a low percent of humidity. Fecal samples were dried at 60° in a forced air oven during 96 h, they were ground through a 2 mm sized mesh in a Wiley® mill (Arthur H. Thomas Co., Philadelphia, PA, USA), then pooled for each period and treated for chemical analysis at the end of the experiment as described above for feed samples. The nutrient concentration in the feces was multiplied by the percentage of fecal dry matter to determine the amount of nutrients excreted.

2.4. In Situ Degradation

The same four cannulated heifers used in the in vivo experiment were used to estimate DM in situ degradation in each period [30] using the nylon bag rumen degradation technique [31]. Before commencing measurements, nylon bags (5 × 10 cm; pore size: 5 µm)

were dried in a forced air oven at 55° for 24 h, then left in a desiccator to constant weight and weighed. Five grams of experimental rations (Table 1) were introduced in the nylon bags by duplicate. The feed samples for rumen incubation were ground through a 2 mm sized mesh in a Willey® mill (Arthur H. Thomas Co., Philadelphia, PA, USA). Nylon bags were introduced into the ventral sac of the rumen for incubation times of 0, 12, 24, 48, 72 and 96 h, in inverse order, in order to allow for all bags to be removed simultaneously. In addition, a bag without a sample was incubated for each sampling time as a correction factor. After the incubations were completed, the nylon bags were manually washed under a running tap until the water from each bag was observed to be colorless and particle-free. The bags were then dried at 60° for 48 h and weighed.

The percent rumen degradability ($Deg_{(t)}$) was obtained with the equation [30]:

$$Deg_{(t)} = a + b * \left(1 - e^{-kd \times t}\right) \quad (6)$$

where: 'a' represents the soluble and quickly degradable fraction at t = 0; 'b' represents the fraction potentially degradable at a given time; 'kd' is the constant trate of disappearance (/h); 't' is the time of incubation (h) and 'e' is the base of natural logarithm (2.71828). Potential degradability values (PD, %) were estimated as the sum of a + b. The effective rumen degradability (ERD, %) was calculated using the following equation:

$$ERD = a + ((b \times kd)/kd + kp) \quad (7)$$

where the parameters: a, b and kd have the above described meaning, and 'Kp' is the estimated rate of passage of particles through the rumen, which is 0.05 per hour for ruminants fed at low levels of production [32].

2.5. Rumen Fermentation Parameters

The rumen liquor was sampled 4 h postprandial. A total of 100 mL was collected from several locations (anterior dorsal, anterior ventral, medium ventral, posterior dorsal, and posterior ventral) within the rumen using a 50-mL syringe attached to a stainless tube ending with a probe covered by a fine metal mesh (RT Rumen Fluid Collection Tube, Bar Diamond™ Inc., Parma, ID, USA). The rumen pH was measured immediately after sampling with a portable potentiometer (Hannah® Instruments, Woonsocket, RI, USA), previously calibrated with buffers with pH 4, 7 and 10. Then, a 4 mL aliquot of rumen liquor was transferred for centrifugation to a conic tube containing 1 mL of a deproteinizing solution made of metaphosphoric acid 25% (wt/vol) and 3-methylvaleric acid to obtain a ratio 4:1, then the sample was kept in a freezer at −20°. The VFA proportions in the rumen liquid were determined by gas chromatography (7890A GC system Agilent Technologies Inc., Santa Clara, CA, USA) equipped with a flame ionization detector [33].

2.6. Enteric Methane Measurement

Enteric methane (CH_4) measurements were carried out over the last three days of each experimental period during a 23 h window in which the heifers were housed in individual OCRC. Details describing the construction, operation and calibration of the chambers have been described by Canul-Solis et al. [34] and Arceo-Castillo et al. [35]. The chambers were equipped with air conditioning and dehumidifier units which kept the temperature at 23 ± 1° and the relative humidity (RH) at 55% ± 10%. A small fan fitted inside the chambers kept the air circulating for good sampling. Air from the chambers was pulled with mass flowmeters (Flow Kit 50-500; Sable Systems International®, Las Vegas, NV, USA) at a rate of 1.0 L/min for each kg live weight [36], thus generating a slight negative pressure of −276 Pa inside the chambers. The methane concentration in the air samples was measured with an infrared analyzer (MA-10, Sable Systems International®, Las Vegas,

NV, USA). The data were extrapolated to 24 h using Expe Data® software (Sable Systems International®, Las Vegas, NV, USA) and calculated according to following equation:

$$P(CH_4; L/d) = \frac{M * 60 \times 24 * AE}{100} \quad (8)$$

where P = CH_4 production in 24 h; M = Mean rate of CH_4 production; AE= Air extraction rate (L/min) by the mass flowmeters. The infrared CH_4 analyzer was calibrated before each experimental period. The results obtained from chambers 1 and 2 were replaced in the proposed correction equations by Arceo-Castillo et al. [35]:

$$Y_1 : \ 0.6312\ (x) + 152.89\ (R2 = 0.9821) \quad (9)$$

$$Y_2 : \ 0.6107\ (x) + 150.46\ (R2 = 0.9906) \quad (10)$$

The percent gross energy intake converted to CH_4 (Ym) was calculated by applying the factor 55.65 MJ/kg CH_4 [37]. Additionally, the estimate of the emission factor (EF) of CH_4 kg animal^{-1} year^{-1} was calculated according to IPCC [37].

2.7. Statistical Analysis

The nutritional composition data, DMI, production of enteric CH_4, pH and molar proportions of VFA were subjected to analysis of variance for a 4 × 4 Latin square design using the PROC MIXED procedure of SAS 9.4 [38]. The statistical model employed was as follows:

$$Y_{ijk} = \mu + T_i + P_j + \beta_k + E_{ijk} \quad (11)$$

where Y_{ijk} is the response variable, μ is the population mean, T_i is the effect of *i*-th treatment (i = 1 . . . , 4), P_j is the effect of j-th period (j = 1 . . . , 4), β_k is the effect of k-th heifer (k = 1 . . . , 4; random effect), E_{ijk} is the effect of the random error and $E_{ijk} \sim N(0, \delta^2)$. The mean comparison was carried out by means of the Tukey test ($\alpha = 0.05$).

The rumen degradation kinetics were obtained from the equation proposed by Ørskov & McDonald [30] for each treatment using the non-linear regression procedure of SAS [38], and the parameters of in situ degradation kinetics were analyzed with the mixed procedure of SAS where treatments were considered as fixed effects, and incubation replicates in the rumen were assumed to be a random effect. The model used for the analysis was as follows:

$$Y_{ij} = \mu + F_i + R_j + E_{ij} \quad (12)$$

where Y = the observation of the dependent variable ij; μ = the overall mean of Y; F_i = the effect of treatment (i = 4), R = the effect of incubation run as replicate (j = 4 animal); and E_{ij} = the random error associated with the observation ij. The standard error of the difference among means was carried out using least square means. Treatment differences were considered significant at $p < 0.05$.

3. Results

3.1. Diet Composition and Fatty Acid Profile of Palm Oil

The chemical composition of the dietary treatments and the fatty acid profile of PO are shown on Tables 2 and 3. A statistical comparison of the diets was not conducted because the nutrient composition of the diets was based on pooled samples. However, the inclusion of 20, 40 and 60 g/kg PO in the experimental diets favored energy density, increasing the concentration of GE (5.1 > 7.6 > 12.1%), DE (5.5 > 8.2 > 12.7%) and ME (4.9 > 8.7 > 12.6%; $p < 0.0001$). On the other hand, the FA profile of the PO used contains 50.7% SAFA and 49.7% PUFA (Table 3). Palmitic acid (C16:0; 87.1%) and stearic acid (C18:0;10.2%) were the main SAFA present. Among the UFA, oleic (C18:1, 76.8%) and linoleic acids (C18:2, 20.4%) were the main MUFA and PUFA, respectively.

3.2. In Vivo Experiment

Feed Intake and Apparent Digestibility

With the exception of CP ($p = 0.04$), starch ($p = 0.002$), EE ($p < 0.001$) and GE ($p = 0.001$), no significant differences were found in the intake of nutrients by the inclusion of 20, 40 and 60 g/kg of PO ($p > 0.05$; Table 4), while the consumption of CP was 5% lower in the ration that contained 60 g/kg of PO compared to the control treatment ($p < 0.05$). The apparent digestibility of nutrients (except starch; $p = 0.001$) was not affected by the inclusion of PO ($p > 0.05$. Intake and apparent digestibility of starch was lower by 21.3% and 8.3%, respectively, with the inclusion of 60 g/kg PO compared to CON ration ($p < 0.05$. The EE consumption was different between treatments, increased by 2.26, 3.41 and 4.49 times its concentration in the experimental diets by the addition of 20, 40 and 60 g/kg PO, respectively ($p < 0.0001$). An increase in the intake of highly digestible EE increased the intake of GE ($p = 0.001$) in response to PO supplementation.

Table 4. Effect of palm oil supplementation on intake and apparent digestibility in heifers.

Items	Treatments				SE	*p*-Value
	CON	20	40	60		
LW (kg)	349 ± 19.9	350 ± 23.0	348 ± 21.7	347 ± 2.50	6.534	0.491
ADG (g/d)	545 ± 158	597 ± 146	549 ± 34.9	588 ± 132	56.40	0.478
Intake						
DM (kg/d)	7.80 ± 0.38	8.18 ± 0.63	8.02 ± 0.71	7.80 ± 0.31	0.254	0.132
OM (kg/d)	7.24 ± 0.36	7.64 ± 0.57	7.46 ± 0.68	7.28 ± 0.30	0.237	0.096
CP (g/d)	846 ± 42.1 ^{ab}	852 ± 60.2 ^{a}	832 ± 82.6 ^{ab}	804 ± 40.7 ^{b}	26.39	0.040
NDF (kg/d)	4.58 ± 0.22	4.80 ± 0.34	4.70 ± 0.36	4.57 ± 0.23	0.143	0.096
ADF (kg/d)	2.90 ± 0.14	3.09 ± 0.24	3.01 ± 0.26	2.94 ± 0.18	0.102	0.057
Starch (kg/d)	1.69 ± 0.08 ^{a}	1.70 ± 0.13 ^{a}	1.54 ± 0.23 ^{a}	1.33 ± 0.05 ^{b}	0.067	0.002
NFC (kg/d)	2.24 ± 0.11	2.37 ± 0.23	2.32 ± 0.26	2.26 ± 0.09	0.087	0.276
TDN (kg/d)	4.31 ± 0.28	4.62 ± 0.49	4.52 ± 0.58	4.38 ± 0.22	0.197	0.149
EE (g/d)	12.9 ± 0.58 ^{d}	29.2 ± 2.08 ^{c}	44.0 ± 3.63 ^{b}	57.9 ± 2.14 ^{a}	1.137	<0.001
Apparent nutrient digestibility (g/kg)						
DM	631 ± 42.8	600 ± 39.5	632 ± 28.5	642 ± 69.4	21.60	0.241
OM	643 ± 38.8	615 ± 42.0	646 ± 26.4	655 ± 67.2	21.28	0.264
CP	750 ± 41.4	699 ± 10.0	740 ± 16.7	760 ± 55.5	19.00	0.146
NDF	536 ± 45.1	506 ± 74.1	551 ± 31.9	553 ± 105	31.91	0.499
ADF	715 ± 30.7	697 ± 51.3	719 ± 25.9	718 ± 58.4	20.07	0.646
Starch	865 ± 15.6 ^{a}	822 ± 17.4 ^{b}	803 ± 17.3 ^{b}	793 ± 39.9 ^{b}	11.12	0.001
NFC	842 ± 41.3	816 ± 35.7	816 ± 29.0	823 ± 41.1	15.70	0.596
TDN	634 ± 40.9	609 ± 48.8	645 ± 29.2	652 ± 66.0	21.19	0.126
EE	998 ± 2.00	999 ± 0.58	1000 ± 0	1000 ± 0	0.493	0.082
Energy intake (MJ/d)						
GE	123 ± 6.88 ^{b}	135 ± 9.84 ^{a}	135 ± 13.1 ^{a}	137 ± 4.04 ^{a}	4.227	0.001
DE	79.5 ± 5.16	85.2 ± 9.06	83.4 ± 10.5	80.7 ± 4.03	3.630	0.149

CON, control treatment; 20, 40 and 60 PO level inclusion in treatments (g/kg DM); SEM, standard error of the mean; a, b, c, d, means in the same column with different letters are statistically different according to Tukey's test ($p < 0.05$).

3.3. In Situ Study

In the present trial, the soluble fraction (a), the potentially degradable fraction (b), the constant rate of disappearance (kd) and the degradation potential (DP) were not different among treatments as a result of the inclusion of PO ($p > 0.05$) in the experimental rations after 0, 12, 24, 48, 72 and 96 h of incubation (Table 5). Nonetheless, the effective rate of degradation was superior for CON and it was reduced 14.7, 18.5 and 20.6% by the inclusion of 20, 40 and 60 g/kg PO, respectively ($p < 0.001$).

Table 5. In situ rumen degradation kinetics, potential degradability and effective degradability of rations.

Item	Treatments				SEM	*p*-Value
	CON	20	40	60		
a (%)	21.7 ± 4.66	19.9 ± 4.47	20.2 ± 4.49	19.7 ± 4.43	0.666	0.199
b (%)	42.8 ± 6.54	44.4 ± 6.67	45.8 ± 6.77	44.6 ± 6.68	1.842	0.572
kd (h^{-1})	0.044 ± 0.21	0.030 ± 0.17	0.025 ± 0.15	0.025 ± 0.15	0.004	0.113
PD (%)	64.5 ± 8.03	64.4 ± 8.02	66.0 ± 8.12	64.2 ± 8.01	2.215	0.795
ERD (%)	42.2 ± 6.49 [a]	36.0 ± 6.00 [b]	34.4 ± 5.86 [b]	33.5 ± 5.79 [b]	0.372	0.001

a, soluble fraction; b, fraction potentially degradable at a given time; kd, constant rate of disappearance; PD, potential degradability values and ERD, Effective rumen degradability (considering a passage rate of 5% per hour); CON, control treatment; Treatments: 20, 40 and 60 PO level inclusion in diet; SEM, standard error of the mean. a, b, means in the same column with different letters are statistically different according to Tukey's test ($p < 0.05$).

3.4. Rumen Fermentation Parameters

The rumen pH and acetic, valeric and isovaleric acids were not affected by the incorporation of PO in the rations ($p > 0.05$; Table 6). The molar proportion of propionic acid was highest in 60 g/kg PO treatment, intermediate in CON and 40 g/kg PO treatments, and lowest when 20 g/kg PO were included ($p = 0.014$). In contrast, the molar proportions of butyric and isobutyric ($p = 0.024$) acids decreased by 14.4 and 12.8%, respectively, when the PO level was 60 g/kg DM compared to CON treatment. The acetic/propionic acid ratio was highest with 20/kg PO treatment, intermediate with CON and 40 g/kg PO treatments and lowest when 60 g/kg PO was included.

Table 6. Effect of palm oil supplementation on rumen fermentation parameter in heifers.

Item	Level of Incorporation of Palm Oil in the Ration (% DM)				SEM	*p*-Value
	CON	20	40	60		
Rumen pH	6.43 ± 0.05	6.35 ± 0.17	6.33 ± 0.17	6.25 ± 0.19	0.067	0.401
Acetic acid (%)	50.8 ± 1.00	50.9 ± 0.71	50.5 ± 0.77	52.1 ± 0.83	0.424	0.061
Propionic acid (%)	24.4 ± 0.36 [ab]	23.4 ± 1.16 [b]	25.1 ± 1.36 [ab]	26.4 ± 1.64 [a]	0.440	0.014
Butyric acid (%)	17.4 ± 0.70 [a]	18.2 ± 0.42 [a]	17.0 ± 1.20 [ab]	14.9 ± 1.99 [b]	0.482	0.012
Isobutyric acid (%)	2.10 ± 0.16 [ab]	2.28 ± 0.15 [a]	1.98 ± 0.15 [ab]	1.85 ± 0.06 [b]	0.069	0.022
Isovaleric acid (%)	3.30 ± 0.64	3.38 ± 0.42	3.23 ± 0.12	3.03 ± 0.38	0.198	0.630
Valeric acid (%)	2.08 ± 0.47	1.98 ± 0.28	2.15 ± 0.23	1.85 ± 0.23	0.112	0.346
Acetic:propionic ratio	2.08 ± 0.05 [ab]	2.18 ± 0.13 [a]	2.00 ± 0.14 [ab]	1.98 ± 0.10 [b]	0.040	0.034

CON, control treatment; 20, 40 and 60 PO level inclusion in treatments (g/kg DM); SEM, standard error of the mean; a, b, means in the same column with different letters are statistically different according to Tukey's test ($p < 0.05$).

3.5. Enteric Methane Emissions

The total daily CH_4 production was lower in rations containing 20, 40 and 60 g/kg PO compared to the CON ration ($p < 0.001$; Table 7). The production of CH_4 per kg DMI, DOMI and DNDF was greater ($p < 0.05$) for the CON diet compared to all three rations containing PO. This directly influenced the intensity of CH_4 emissions (kg)/GMD(kg)/year, Ym, energy losses as enteric CH_4, the emission factor, and kg CO_2 eq/year that decreased compared to control treatment by increasing the PO level in the ration ($p < 0.05$).

Table 7. Effect of palm oil supplementation on enteric methane emissions in heifers.

Items	Treatments				SEM	*p*-Value
	0	2	4	6		
CH_4 (g)/d	154 ± 7.18 [a]	138 ± 12.2 [b]	128 ± 5.20 [b]	129 ± 1.26 [b]	3.318	0.001
CH_4 (g)/DMI (kg	19.3 ± 0.50 [a]	17.5 ± 1.29 [ab]	16.3 ± 0.50 [a]	16.0 ± 0.82 [a]	0.402	0.003
CH_4 (g)/DOMI (kg)	30.8 ± 2.23 [a]	28.0 ± 1.40 [ab]	24.8 ± 1.86 [b]	25.8 ± 3.71 [b]	1.246	0.004
CH_4 (g)/DNDF (kg)	62.1 ± 5.84	57.7 ± 10.3	48.5 ± 2.84	52.5 ± 13.9	4.414	0.075
CH_4 (kg)/ADG (kg)/year	0.30 ± 0.06 [a]	0.24 ± 0.05 [b]	0.23 ± 0.02 [b]	0.23 ± 0.05 [b]	0.022	0.013
Ym (%)	6.61 ± 0.54 [a]	5.56 ± 0.28 [b]	5.20 ± 0.43 [b]	5.23 ± 0.29 [b]	0.163	0.003
Energy loss as CH_4 (% GE)	8.57 ± 0.41 [a]	7.67 ± 0.67 [b]	7.08 ± 0.28 [b]	7.17 ± 0.08 [b]	0.183	0.001
EF (kg/year)	56.2 ± 2.69 [a]	50.3 ± 4.37 [b]	46.5 ± 1.86 [b]	47.0 ± 0.53 [b]	1.183	0.001
kg eq CO_2 /year	1574 ± 75.3 [a]	1408 ± 123 [b]	1301 ± 52.1 [b]	1317 ± 15.0 [b]	33.55	0.001

CON, control treatment; 20, 40 and 60 PO level inclusion in treatments (g/kg DM); SEM, standard error of the mean; a, b, means in the same column with different letters are statistically different according to Tukey's test ($p < 0.05$).

4. Discussion

4.1. Diet Composition and Fatty Acid Profile of Palm Oil

Even when the content of PO in the rations was different, only treatment with 60 g/kg PO exceeded the recommended maximum of 70 g/kg DM (Table 2). However, the levels of inclusion of PO in the rations were enough to increase the energy density due to the high content of long chain fatty acids [32]; which is in agreement with previous reports in which vegetable FAs were used as an energy source [8,39,40]. On the other hand, the composition of PO presented a similar balance between SAFA and UFA (50.7 vs. 49.3; Table 3). In this regard, previous studies reported a higher concentration of UFA than SAFA [41]. In that respect, a high concentration of UFA in PO may interfere with microbial fermentation because the dietary esterified FAs are rapidly hydrolyzed by lipolytic microorganisms within the rumen to yield free FA [32,42]. These FAs, with their surfactant properties, can disrupt membrane integrity, impair nutrient uptake and inhibit membrane enzyme activity and energy production, leading to cell death [43–45], thereby reducing the digestibility of the diet and the DM intake [46]. However, rumen bacteria possess an inherent protective mechanism called biohydrogenation, which allows for a reduction in the concentration of those fatty acids in the rumen, thus minimizing its effects on the fermentation of feeds and digestibility [47].

4.2. In Vivo Experiment

Feed Intake and Apparent Digestibility

Even when lipids may interfere with DMI [18,32], the feed intake reported in this study was not different between experimental treatments (Table 4). These findings agree with previous work in which different sources of lipids of vegetal and animal origin were included, where the ether extract content of a dairy cow ration varied from 3% ± 0.24 to 5.5% ± 0.33 [9,13–15,39,48,49]. In contrast, Chilliard et al. [50] found that the inclusion of extruded linseed and linseed oil in the feeding of dairy cows decreased DMI when the ether extract in the ration was increased from 2.6% (control) to 7.0 and 8.4%, respectively. This finding agrees with Gouvêa et al. [51], who reported a reduction in dry matter intake of 18.74% when the ether extract (per kg DM) in the ration was increased from 3.21 to 6.39% with the incorporation of 320 g/kg DM of cottonseed. Furthermore, the meta-analysis carried out by Eugène [52] and Rabiee et al. [53] confirmed a reduction in DMI of 6.4 and 4%, respectively, by supplementation with oils and seeds. Nonetheless, the variation in CP consumption can be explained by the variation in the chemical composition of the experimental diets (Table 2). On the other hand, the high energy density of PO allowed a greater consumption of GE [8,18,32,46]. Consequently, in appropriate proportions, fats and oils enhance the energy density in diets. These changes in gross energy may influence the productive response observed when palm oil is supplemented [32], maintaining an average

live weight gain of 570 g/day (Table 2), 175 g above the average weight gain of 395 g/day under tropical grazing conditions for cattle in Mexico [54].

Based on an analysis of the literature regarding the effects of vegetable oils on digestibility, Weld and Armentano [55] found a modest negative effect of supplemental SAFAs (lauric and myristic) on total NDF digestibility. Furthermore, plant oils rich in unsaturated FA have previously been purported to have a markedly negative effect on fiber digestibility [56]. With the exception of starch, in the present study, the inclusion of PO had no adverse effect on the apparent digestibility of nutrients, since the long-chain saturated fatty acids released in the hydrolysis of PO are considered inert in the rumen [47,57]. On the other hand, PUFA contained in PO may have undergone significant structural changes as they passed through the rumen, since lipolysis occurs at a high rate [32,58], followed by biohydrogenation, which may occur quickly because of the excess metabolic hydrogen product of the anaerobic fermentation of fiber [40]. This reduces the effects on cellulolytic bacteria in the rumen and on the apparent digestibility of the ration while holding the dry matter intake level steady [47].

4.3. In Situ Study

The potential response to supplemented lipids in ruminants is still not fully understood and some of the results are inconsistent [48]. Lipids may affect the dynamics of rumen fermentation and nutrient metabolism, and reduce dry matter intake [59] by inhibiting the adhesion of cellulolytic bacteria to the substrate, and by reducing the activity of fibrolytic enzymes. In the present work, the difference observed in ERD may have been due to the intrinsic characteristics of the rations, such as particle size, molecular bonds, DM and NDF contents and soluble carbohydrate content, which may contribute to a reduction in the rate of hydrolysis, resulting in different rates of rumen degradability effectiveness [60,61]. Additionally, it has been suggested that the lipid cover of ration particles may have limited the immediate access of cellulolytic bacteria, consequently lowering the effective degradation of rations with PO [16].

4.4. Rumen Fermentation Parameters

Anaerobic fermentation of fiber in the rumen yields diverse final products [62] such as volatile fatty acids (VFA), which ruminants can absorb and utilize as an energy source via various tissues and organs [63]. However, the level, the source of lipids and the TMR may affect rumen fermentation [47]. Therefore, it is essential to guarantee an appropriate symbiosis among the rumen microorganisms and the host [64] in order to maintain an adequate functioning of the rumen [65]. Optimal rumen pH is critical for the maintenance of rumen function and the bacterial population [66]. The results hereby presented partially agree with other trials where no changes in rumen pH were reported. The individual proportion of VFAs (acetic, valeric and isovaleric acids; $p > 0.05$) when lipids were included in the ration of dairy cows and EE in the ration varied from 4.77 $\pm$ 0.12 to 6.9 $\pm$ 0.57 [67,68]. However, Alvarez–Hess et al. [9] reported a reduction in rumen pH when they supplemented 0.8 kg of canola oil to Holstein-Friesian cows ($p < 0.05$). Besides, Costa et al. [69] reported that steers supplemented with fish oil produced a smaller amount of acetic acid and increased the concentration of propionic acid ($p < 0.05$) compared to the other lipid sources, but they were not different from the control treatment ($p > 0.05$). It is suggested that the results obtained in the present trial are due to the fact that a rumen pH above 6.1 is adequate for a good fermentation of fiber in the rumen [70] and a high content of structural carbohydrates favor acetic acid synthesis [71]; an observation which agrees with the present trial where mean rumen pH was 6.3 and the content of NDF in the rations was 587 g/kg DM, thus promoting an acetic type of fermentation [32]. On the other hand, previous studies reported increases in the molar proportions of propionic, butyric and isobutyric acids when including fish oil [16,69]. The results presented here are in partial agreement with these studies; the highest concentration of propionic acid was evident by the PO level of 60 g/kg in the diet, which may be explained by PO lipolysis releasing glycerol in

the rumen, which can be quickly fermented [56] and converted to propionic acid [72] in amounts of 35 − 69% [73]. In addition, the conversion of glycerol to propionic acid reduced the acetate:propionate ratio [16]. Furthermore, during lipolysis, the release of unsaturated fatty acids may have exceeded the capacity of biohydrogenation at times, without affecting rumen fermentability, allowing for an increase in the production of propionic acid [74] competing for metabolic hydrogen a byproduct of fermentation [64], thus reducing the concentration of butyric and isobutyric acids as observed in the trial hereby described.

4.5. Methane Emissions

The results recorded in the present trial show potential mitigation of CH_4 by PO, potentially being able to decrease emissions by up to 16.9% (with 40 g/kg of PO), as well as the emissions by kg DMI (−17.1%, with 60 g/kg of PO), DOMI (−19.5%, with 40 g/kg of PO) and DNDF (−21.9%, with 40 g/kg of PO) when the incorporation of PO was of 40—60 g/kg in the ration (EE = 5.49 to 7.42%, Table 2). In this respect, Drehmel et al. [13] fed Jersey dairy cows and reported a reduction in CH_4 emissions per day (7.2%) and by kg DMI (10.5%) when the content of EE of the ration was 4.98% ± 0.47. On the other hand, Judy et al. [15] reported that the incorporation of corn oil plus dried distillery grains in the ration of dairy cows (Holstein and Jersey) mitigated 10.8% of CH_4 emissions per kg dry matter intake, which agrees with the trial by Alvarez-Hess et al. [9] who reported a lower production of CH_4 (12.4%) per kg dry matter intake in dairy cows when fat concentration was raised from 20 to 60 g/kg DM. Grazing trials have also shown that the CH_4 suppressing effect of lipids incorporating soybean oil and whole cottonseed in supplements for grazing cattle reduced CH_4 yield by 20.8% [12]. According to different meta-analysis published in the scientific literature, there have been reports of reductions from 3.8 to 5.6% for each 10 g/kg of lipid supplemented in a ration [7,75], which agrees with our results, which, on average, reduced CH_4 emissions by 4% for each 10 g/kg of PO added to the ration. Additionally, other studies reported that complementing cattle rations with lipids containing different contents of fatty acids may reduce CH_4 emissions, as the molar proportion of propionic acid is increased, which gives an hydrogen sink, as its utilization by methanogenic archaea is reduced for the production of CH_4 [76]. This is likely to have happened in the present study where the molar proportion of propionic acid was higher while CH_4 emissions were reduced, as the PO level in the diet increased. On the other hand, it is suggested that cows fed PO could experience a surge in the ruminal concentration of unsaturated fatty acids immediately after feeding that could be enough to cause antibacterial effects in the rumen [47], which in turn could result in a reduction of CH_4 emissions.

Dewanckele et al. [58] pointed out that the biohydrogenation of UFA can be carried out by different microbial species in the rumen, and this pathway can provide an alternative sink of [H] to compete with methanogenesis [5], but this is quantitatively small (1% to 2.6% of [H] used for this reaction) [26,27,77]. In the current study, the potential reduction of CH_4 through competition for redirected metabolic hydrogen for biohydrogenation of UFA was of 0.7, 1.36 and 2.1% with 20, 40 and 60 g/kg PO, respectively. In general terms, lipids inhibit methanogenesis by replacing rumen fermentable organic matter in the diet, decreasing the numbers of ruminal methanogens and protozoa and through the biohydrogenation of unsaturated fatty acids [5,16,78].

On the other hand, cattle lose energy as heat, a third of their gross energy through feces, 3% through urine [79] and from 2 to 12% as enteric CH_4 [6]. In the present work the reduction in CH_4 emissions observed was a key factor in reducing energy loss as CH_4, kg CO_2 eq/year, intensity of emissions CH_4 kg/ADG (kg)/year, Ym and EF. In the present trial, the percentage of gross energy lost as CH_4 went from 8.57% with the CON ration to 7.08% when PO was included at 40 g/kg DM. Those values are within the range suggested by Gunter et al. [80] and Johnson and Johnson [6]. In general, a reduction in daily emissions of CH_4 as a result of supplementation with PO also allowed a reduction in the loss of gross energy as CH_4 by 10.5, 17.4 and 16.3% when the incorporation of PO in the ration increased

by 20, 40 and 60 g/kg DM, respectively. The intensity of CH_4 emission showed a positive response by a decrease ranging from 20 to 23.3% as a response to supplementation with PO.

The Ym recorded for treatment CON was 6.89%, which was above the value of 6% reported by Niu et al. [81] when an intercontinental database was analyzed (n = 2556), and similar to the 6.5% proposed by IPCC [37] for grazing cattle. The emission factor for the treatment CON was 56.2 kg CH_4/head/year, similar to the average of 57 kg CH_4/head/year reported for Brazil and Australia [82], and 10.8% lower than the 63 kg CH_4/head/year for dairy cattle in Latin America [83]. As a response to supplementation with 20, 40 and 60 g/kg of PO in the rations, Ym and EF in the present trial were decreased by a range of 15.2 to 26.6% and 10.5 to 16.4%, respectively. The reduction in energy losses as CH_4 was an indirect effect related to the lower methane synthesis per day and per kg of dry matter intake observed in the present trial, along with the increase in the concentration of propionate and a possible change in the rumen microbial population. Biohydrogenation of unsaturated fatty acids from PO was another factor that probably favored a reduction in energy loss as CH_4.

5. Conclusions

On the basis of the results hereby described, it can be concluded that incorporation of PO in cattle rations has the potential to mitigate enteric methane emissions by 4% for each 10 g/kg of PO included, without affecting DMI, apparent digestibility or intake of digestible nutrients. Generally speaking, the dietary strategy to mitigate enteric CH_4 through the addition of PO may also improve the energy density of a ration, favoring propionate synthesis and reducing the intensity of emissions per kg of daily weight gain in cattle. It is recommended to focus subsequent research on the assessment of the persistence of the methane mitigation effect of PO for longer periods of time.

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