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In vitro gas production and fatty acids biohydrogenation of diets containing different unsaturated fatty acids sources plus crude glycerin

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Abstract: An in vitro trial was conducted to evaluate the effects of different unsaturated fatty acids (UFA) sources plus crude glycerin (CGL) on gas production and rumen biohydrogenation (RBH). Incubated diet corresponded to diets containing corn silage (30%) and concentrate (70%) composed of corn, urea, mineral salts, CGL, and different UFA sources as follows: no additional fat (NAF), rumen protected fat (RPF), soybean oil, linoleic acid (LA) or alpha-linolenic acid (LN). Methane concentrations in LA and LN were lower compared to NAF ($p < 0.001$), but when CH4 was expressed as a proportion of total gas, NAF and RPF showed the greatest values among evaluated UFA sources ($p = 0.001$). 18:1 t11 concentration showed a faster increase from 0 to 1 h and a slower decrease from 1 to 36 h of incubation, irrespective of UFA source. A higher C18:1 t11 production rate was observed from 1 h to 5 h of incubation, where LA and LN have higher values compared to NAF and RPF diets ($p < 0.001$). Diets with a high content of LA may be efficient as a nutritional approach to reduce methane production and RBH, resulting in positive effects on vaccenic acid concentrations.

Key words: Cattle, conjugated linoleic acid, glycerol, greenhouse gas, protected fat, soybean oil

1. Introduction

Ruminants significantly contribute to global food security because they can transform plant forage into high-quality foods such as milk and meat, due to their complex digestive system and microbial fermentation. To meet the increasing demand for food of animal origin in the world, the livestock sector should increase the production efficiency while reducing the environmental impact. In this sense, unsaturated fatty acids (UFA) incorporation in cattle diets has increased the diet energy density and levels of these fatty acids in both meat and milk, which allows ruminant products to decrease the risk of cardiovascular diseases and obesity in humans [1].

The conjugated linoleic acid (CLA; 18:1 c9, t11) is considered an important fatty acid (FA), which is linked to human health [1]. Several studies have indicated that increasing the rumen production of vaccenic acid (18:1 t11) is a suitable method for enhancing CLA concentrations in ruminant products [2,3].

However, a high percentage of UFA supplemented is transformed into saturated fatty acids (SFA) by the rumen microbiota during the biohydrogenation process (RBH), which also results in various cis/trans FA isomers [4]. Most of the dietary UFA in ruminants are 18-carbon FA, such as oleic acid (18:1 c9), linoleic acid (18:2 c9, c12), and alpha-linolenic acid (18:3 c9, c12, c15) [5], and the main rumen RBH pathways of 18:2 c9, c12 and 18:3 c9, c12, c15 could result in 18:1 t11 as intermediate [2,6].

Crude glycerin (CGL) is the principal byproduct during biodiesel production, which has been used in cattle diet as an energy source with positive effects on both ruminal fermentation and RBH [7, 8] because it decreases the production of SFA [9,10] and increases the duodenal flow of 18:1 t11 when associated to vegetable oils in beef cattle [9].

In addition, this association may reduce the enteric CH_4 production [8], a greenhouse gas that has turned into a predilection in ruminant nutrition research. In

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vivo [8] and in vitro [11] studies have shown that CGL and UFA in the diet may reduce CH_4 production by reducing H_2 accumulation through RBH and inhibiting the activity of ruminal methanogens. However, there remains a lack of knowledge on the mitigation potential of different UFA sources in basal diets containing CGL since this association may limit ruminal RBH [10,12,13]. In consequence, the in vitro evaluation of the effects of diets containing different UFA sources associated with CGL on greenhouse gas production (i.e. CH_4, CO_2, and N_2O), RBH, and rumen FA profile may give us insight into how to incorporate such diets in cattle nutrition, optimizing the quality of ruminant products (meat or milk) and mitigating greenhouse gas production. Thus, the current in vitro study investigated the effect of different sources of UFA associated with CGL on rumen gas production and rumen FA profile and RBH rates. We hypothesized that UFA sources with a high content of linolenic acid would produce less greenhouse gases and improve the FA profile during ruminal fermentation.

2. Materials and methods

The experimental procedures used in this study was in agreement with the Ethical Principles of the Brazilian College of Animal Experimentation and were approved by the Ethics Committee of Animal Experimentation of São Paulo State University–UNESP, Jaboticabal campus, Brazil (Protocol 07784/14).

2.1. Diets and fluid donors

Incubated diets contained corn silage (30% of dry matter (DM)) and concentrate (70% of DM) composed of ground corn, urea, mineral salts, CGL, and the different sources of UFA as follows: no additional fat (NAF); rumen-protected fat from soybean oil (RPF) (Megalac-E, Church and Dwight, affiliate Química Geral do Nordeste S.A); soybean oil (SO); 18:2 c9, c12 (LA; chemical purity 99%, Sigma, Aldrich Chemical Company) or 18:3 c9, c12, c15 (LN; chemical purity 99%, Sigma, Aldrich Chemical Company) were added according to treatments (Table 1). The CGL was composed of 83.9% glycerol, 1.7% EE, 4.3% ash, and 12.0% water (Cargill, Brazil).

Feed chemical analysis for DM (934.01), crude ash (MM; 942.05) and ether extract (EE; 920.39) quantification were following the AOAC [14]. Nitrogen was measured with a LECO FP-528 nitrogen analyzer (LECO Corp., St. Joseph, MI, USA). Neutral detergent fiber was determined in an Ankom200 Fiber Analyzer (Ankom Technology, Fairport, NY) with α-amylase and without the addition of sodium sulphite [15]. Sequentially, the acid detergent fiber was determined as described by Goering and Van Soest [16].

As rumen fluid donors, nine castrated Nellore steers (mean body weight 540 ± 20 kg), provided with a ruminal silicone cannula, allocated into individual stables (12 m^2) equipped with feed troughs and automatic waterers were used. Steers were randomly distributed into three group diets (treatments) and fed twice daily (at 07:00 and 17:00) ad libitum during 14 days of adaptation, with diets NAF, SO, and RPF.

After the diet adaptation, the rumen liquid was sampled before the morning feeding and tensed through two layers of cheesecloth to obtain 500 mL of rumen fluid, being immediately placed into a warm (39 °C) insulated flask. Rumen fluid was transferred to the laboratory for in vitro incubations [17]. The initial concentrations (Δ0) of the principal FA of flask content included the ruminal fluid donor were analyzed (Table 2) and three incubation runs were completed on different days as experimental replicates.

2.2. In vitro incubations

For each incubation day, five sets of flasks were prepared combining NAF diet with the ruminal fluid of steers fed NAF, RPF diet with the ruminal fluid of steers fed RPF, and SO, LA, and LN diets with the ruminal contents of steers fed SO, respectively. Each set contained six control flasks (i.e. without experimental diet) and six experimental flasks (i.e. with experimental diet), corresponding to six incubation times (0, 1, 5, 12, 24, and 36 h).

Each volumetric flask was 120 mL, containing 1000 mg of experimental diet, 25 mL of buffer solution [16], and 15 mL of ruminal fluid (prewarmed to 39 °C, prestrained through a 250 µm nylon stuff, and pregassed with CO_2 for 10 min). The flasks were sealed and incubated anaerobically in a shaking water bath (Tecnal TE-056-MAG, Piracicaba, Brazil) at 39 °C. Six control incubators from each series were removed after 0, 1, 5, 12, 24, and 36 h of incubation.

2.3. Gas production

Every 2 h, gas pressure inside the bottles was determined using a digital pressure sensor (Datalogger pressure-press DATA 800, MPL, Piracicaba, Brazil) to calculate the gas production as per Theodorou et al.'s [18] method and adapting the semiautomatic system proposed by Mauricio et al. [19]. Control incubators (without experimental diet) were used to adjust gas production of nutrients present in the buffered rumen fluid. The pressure values were transformed to volume of gas using the equation previously determined for our laboratory conditions: $GV = [(4.25 \times PR) - 0.1]$, where the GV corresponds to the gas volume (mL) and PR corresponds to the measured pressure (psi).

For each incubation time, the flasks were taken out from the shaking water bath and immersed in ice water to inhibit microbial activity. A gas sample (5 mL) was taken with a syringe and injected into a chromatograph Shimadzu CG-2014 with flame ionization detector (Greenhouse gas analyzer, packed column Hayesep D 80/100 mesh, 4 m, 1/8), to determine gas composition (i.e.

Table 1. Experimental diets (treatments), proportion of ingredients used, and bromatological composition of incubated diets.

	Diets †				
	NAF	RPF	SO	LA	LN
Ingredients, % of DM					
Corn silage	30.00	30.00	30.00	30.00	30.00
Ground corn	47.89	45.40	45.71	45.71	45.71
Soybean meal	8.11	8.63	8.56	8.56	8.56
Soybean oil	0.00	0.00	1.73	0.00	0.00
Rumen-protected fat	0.00	1.97	0.00	0.00	0.00
Linoleic acid	0.00	0.00	0.00	1.73	0.00
Alpha-linolenic acid	0.00	0.00	0.00	0.00	1.73
Crude glycerin	10.00	10.00	10.00	10.00	10.00
Urea	1.00	1.00	1.00	1.00	1.00
Mineral salt ‡	3.00	3.00	3.00	3.00	3.00
Bromatological composition					
Dry matter, % DM	69.03	67.65	68.03	68.03	68.03
Organic matter, % DM	94.15	94.18	94.15	94.15	94.15
Crude protein, % DM	16.44	15.91	15.52	15.52	15.52
Ether extract, % DM	3.74	4.79	4.98	4.90	4.92
aNDFom, % DM	25.85	24.09	24.11	24.10	24.10
Acid detergent fiber, % DM	9.03	8.90	8.77	8.77	8.77
Non-fibrous carbohydrates, % DM	48.12	49.39	49.55	49.50	49.58
Metabolizable energy, MJ/kg DM	12.17	12.42	12.46	12.51	12.51

† NAF: no addition of fat, RPF: rumen protected fat, SO: soybean oil, LA: linoleic acid (18:2 c9, c12), and LN: alpha-linolenic acid (18:3 c9, c12, c15).
‡ Composition per kg of supplement: calcium, 210 g, phosphorus 20 g, sulfur 37 g, sodium 80 g, copper 4.90 g, manganese 1.4 g, zinc 1.8 g, iodine 36 mg, cobalt 29 mg, selenium 9 mg, fluorine ≤ 333 mg.
SFA = saturated fatty acids as sum of C8:0, C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0 and C22:0. UFA = unsaturated fatty acids as sum of C14:1 cis 9, C16:1 cis 9, C18:1 cis 9, C18:2 cis 9 cis 12, C18:3 cis 9 cis 12 cis 15, C20:1 cis 11 and C22:1 cis 13.

CH_4, CO_2, and N_2O). In addition, the pH of the rumen fluid was determined and the whole content of each volumetric flask was lyophilized in a ModulyoD Freeze Dryer (Thermo Electron Corporation, Nepean, ON, Canada) to determine the DM of the incubation system. Finally, the dried incubation contents were ground in a hammer mill through a 1-mm sieve (Wiley mill, Thomas Scientific, Swedesboro, NJ), and stored until FA analysis.

2.4. Fatty acid analysis

Total FA were extracted and methylated according to Palmquist and Jenkins' [20] method, using the methyl nonadecanoate (19:0; Sigma-Aldrich, Supelco, USA) as internal standard and gas chromatography as described by Granja-Salcedo [10].

Both production and RBH rates were calculated for each FA, in accordance with Troegeler-Meynadier et al.'s [21] method. Thus, production or RBH rates were estimated using the following equation: Production or RBH of FA = (FACi – FACt) / Δt, where FACi and FACt represent the concentrations of FA at the beginning and at the end of the incubation time (Δt), respectively.

2.5. Statistical analysis

All data were analyzed by considering a completely randomized block design in a factorial arrangement 5 × 6, treatment, and incubation time, respectively, by the MIXED procedure of SAS (Statistical Analysis System for Windows 9.3, SAS Institute Inc., Cary, USA). The statistical model included the fixed effects of treatment,

Table 2. Initial concentrations (Δ0) of the principal fatty acids of flasks content.

	Diets †				
	NAF	RPF	SO	LA	LN
Fatty acids initial concentration					
Total, mg/dL	219.13	333.01	345.09	331.16	336.21
< C15:0	27.92	24.22	27.54	23.65	28.00
C16:0	28.24	25.37	28.74	25.53	26.92
C17:0	4.47	3.55	4.22	4.04	4.24
C18:0	8.77	12.34	12.08	11.87	11.20
C18:1 c-6	1.87	2.11	1.80	1.65	1.51
C18:1 t-6	5.47	2.88	3.58	3.71	3.04
C18:1 c-9	37.70	58.22	59.75	14.08	11.63
C18:1 t-9	4.58	12.82	18.34	2.28	2.29
C18:1 t-11	4.69	10.33	8.69	11.10	10.42
C18:2 c-9, c-12	63.41	147.47	141.62	197.69	77.52
C18:3 c-,9, c-12, c-15	9.71	13.46	12.46	12.94	122.48
> C20:0	18.82	17.58	23.10	20.06	34.13
SFA	69.51	67.81	74.26	67.23	73.13
UFA	149.62	265.20	270.83	263.93	263.07

† NAF: no addition of fat, RPF: rumen protected fat, SO: soybean oil, LA: linoleic acid (18:2 c9, c12), and LN: alpha-linolenic acid (18:3 c9, c12, c15). SFA = Saturated fatty acids as sum of C8:0, C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0 and C22:0. UFA = unsaturated fatty acids as sum of C14:1 cis 9, C16:1 cis 9, C18:1 cis 9, C18:2 cis 9 cis 12, C18:3 cis 9 cis 12 cis 15, C20:1 cis 11 and C22:1 cis 13.

incubation time, and all interactions. Random effects were experimental runs from each different day (blocks) and residual error. When the ANOVA indicated a significant difference, the Tukey's post hoc test was used considering statistical significance when $p \leq 0.05$.

3. Results

3.1. Gas production

Total gas, CH_4, and CO_2 productions were affected by both UFA source and incubation time ($p < 0.05$), without interaction effect (Table 3; $p > 0.05$). Both RPF and LN had the lowest values of total gas production among the evaluated diets ($p = 0.026$). The CH_4 concentrations (expressed as mL per bottle) in LA and LN were lower compared to NAF ($p < 0.001$). However, when CH_4 concentrations were expressed as a proportion of total gas, NAF and RPF showed the greatest values among evaluated UFA sources ($p = 0.001$).

Carbon dioxide expressed as CO_2 % of total gas was lower in SO and Linoleic acid diets ($p = 0.036$), whereas no difference between UFA sources in CO_2 concentration was detected, when CO_2 was expressed as mL per bottle ($p = 0.107$). Nitrous oxide (N_2O) concentration (expressed as ppm per bottle) was not different among lipid sources ($p = 0.624$).

The lowest values of total gas production were observed at 1 h of incubation, whereas the highest values were observed from 12 to 36 h of incubation ($p < 0.001$; Figure 1A). Both total CH_4 and CO_2 production increased in each incubation time ($p < 0.001$; Figures 1B and 1C), and N_2O concentration was higher at 36 h compared to 1 and 5 h of incubation ($p < 0.001$; Figure 1D).

3.2. Kinetics of fatty acid biohydrogenation

Total FA and C18:1 c-6 concentration were affected by UFA sources ($p < 0.05$; Table 4). Thus, the lowest total FA concentration was observed in NAF incubated diet, while LA and SO had higher total FA concentration compared to RPF ($p < 0.001$), and higher C18:1 c-6 concentration were observed in RPF, SO, and LA in relation to NAF diet ($p < 0.001$).

The FA C16:0, C17:0, C18:1 t-6, C18:1 t-9 were affected by the incubation time ($p < 0.05$). Thus, C16:0 concentration was higher after 24 h (30.43 mg/dL) compared to 0 to 5 h (26.29 mg/dL) of incubation ($p <$

Table 3. Mean and standard error of mean (SEM) of in vitro gas production and gas profile of diets containing different sources of unsaturated fatty acids associated with crude glycerin.

	Diets †					SEM	p-value‡	
	NAF	RPF	SO	LA	LN		Diet	Time
Total gas, mL	110.12[a]	100.96[b]	107.71[a]	110.58[a]	101.68[b]	6.585	0.026	<0.001
CH_4, mL	26.17[a]	24.45[ab]	22.87[ac]	21.41[bc]	20.45[c]	2.229	<0.001	<0.001
CO_2, mL	39.91	35.03	33.24	34.03	35.70	3.265	0.107	<0.001
N_2O, ppm	0.57	0.42	0.49	0.46	0.46	0.041	0.624	<0.001
CH_4, % total gas	18.59[a]	18.78[a]	16.70[b]	15.24[b]	15.35[b]	1.231	0.001	<0.001
CO_2, % total gas	27.64[a]	27.59[a]	24.96[b]	24.60[b]	27.26[a]	1.704	0.036	<0.001

† NAF: no addition of fat, RPF: rumen-protected fat, SO: soybean oil, LA: linoleic acid (18:2 c9, c12), and LN: alpha-linolenic acid (18:3 c9, c12, c15).
‡ No interaction Diet × Time ($p > 0.05$) to any variable analyzed.
Mean values followed with different superscript letters on the same row mean a significant difference ($p < 0.05$) among source UFA in the diet.

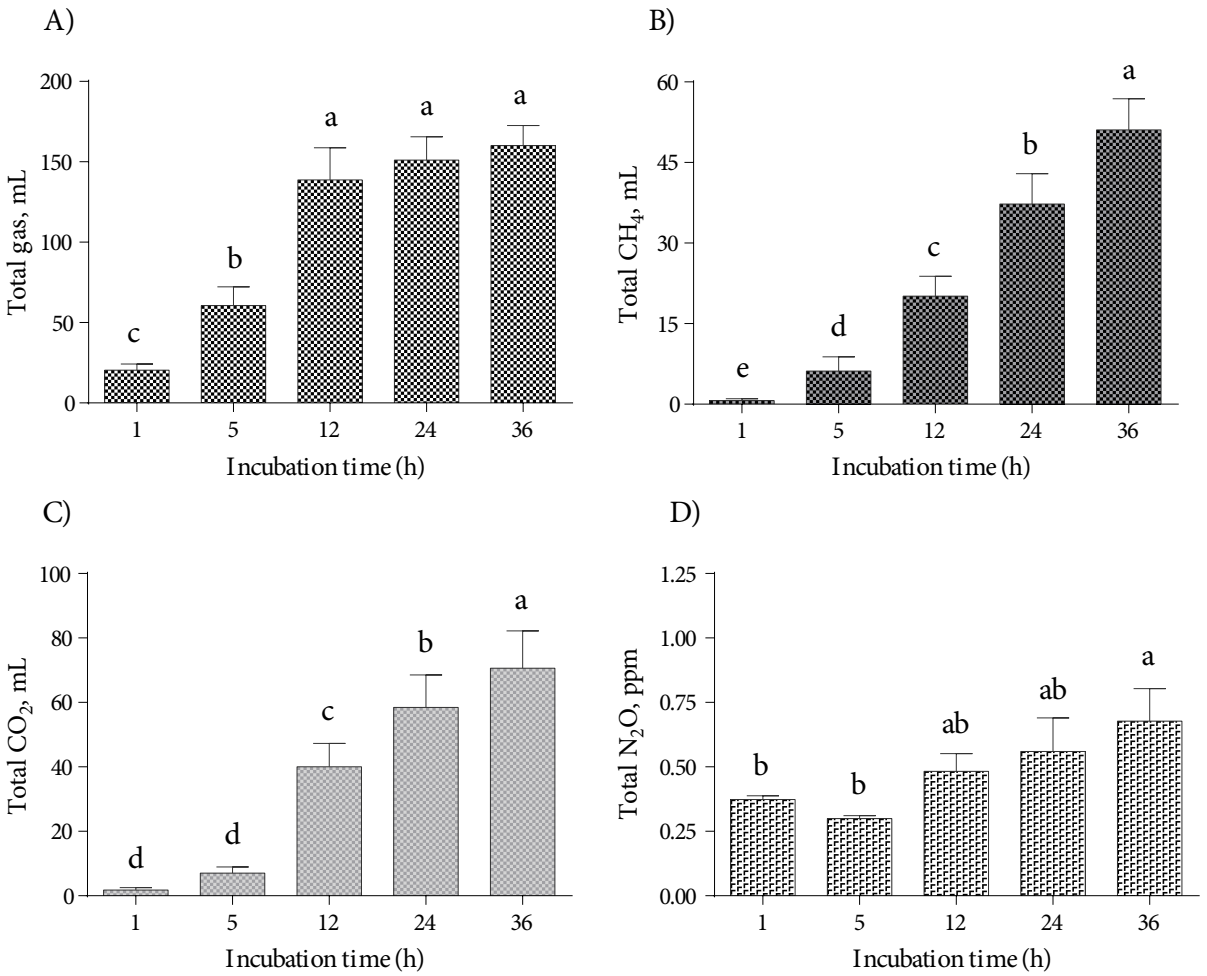

Figure 1. Effect of incubation time on total in vitro gas (A), methane (B), carbon dioxide (C), and nitrous oxide (D) production. Different letters represent a significant difference ($p < 0.05$), as obtained using Tukey's test.

Table 4. Mean and standard error of mean (SEM) of FA concentrations from rumen fluid incubated in vitro with diets containing different sources of unsaturated fatty acids associated to crude glycerin.

	Diets †					SEM	p-value		
	NAF	RPF	SO	LA	LN		Diet	Time	D×T‡
Fatty acids, mg/dL									
Total	211.71^{c}	320.73^{b}	337.22^{a}	334.89^{a}	331.39^{ab}	7.203	<0.001	0.251	0.135
16:0	29.12	30.20	27.15	26.08	27.13	1.201	0.134	0.025	0.917
17:0	4.81	4.36	4.32	4.09	4.10	0.209	0.119	0.009	0.878
18:0	25.83^{c}	25.87^{c}	35.08^{b}	44.58^{a}	43.98^{a}	2.571	<0.001	<0.001	<0.001
18:1*c6*	1.58^{c}	2.08^{a}	1.96^{a}	1.92^{ab}	1.64^{bc}	0.079	<0.001	0.567	0.707
18:1*t6*	3.94	3.58	4.40	4.59	3.57	0.291	0.222	<0.001	0.933
18:1c*9*	31.42^{c}	47.58^{b}	62.37^{a}	20.41^{d}	21.23^{d}	3.184	<0.001	0.008	<0.001
18:1*t9*	0.74	0.79	0.76	0.76	0.76	0.020	0.616	<0.001	0.919
18:1t*11*	9.65^{d}	16.18^{c}	23.90^{b}	38.06^{a}	37.87^{a}	1.256	<0.001	<0.001	<0.001
18:2*c9,c12*	38.24^{d}	117.88^{a}	97.58^{b}	113.02^{a}	48.95^{c}	5.901	<0.001	<0.001	<0.001
18:3c*9,c12,c15*	5.13^{c}	8.50^{b}	6.52^{bc}	7.30^{bc}	61.46^{a}	1.374	<0.001	<0.001	<0.001
SFA	95.12^{b}	91.67^{b}	99.39^{b}	117.88^{a}	125.37^{a}	3.641	<0.001	<0.001	0.020
UFA	115.05^{c}	227.65^{a}	236.59^{a}	215.77^{b}	208.80^{b}	3.043	<0.001	<0.001	0.001

† NAF: no addition of fat, RPF: rumen-protected fat, SO: soybean oil, LA: linoleic acid (18:2 c9, c12*)*, and LN: alpha-linolenic acid (18:3 c9, c12, c15).

‡ Diet × time incubation interaction effect.

Mean values followed with different superscript letters on the same row mean a significant difference ($p < 0.05$) among source UFA in the diet.

SFA = saturated fatty acids as sum of C8:0, C10:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, C18:0, C20:0 and C22:0. UFA = unsaturated fatty acids as sum of C14:1 cis 9, C16:1 cis 9, C18:1 cis 9, C18:2 cis 9 cis 12, C18:3 cis 9 cis 12 cis 15, C20:1 cis 11 and C22:1 cis 13.

0.001). C17:0 concentration increased by time incubation, resulting in higher values after 24 h (4.75 mg/dL) compared to times 0, 1, and 5 h (4.02 mg/dL). C18:1 t-6 concentration increased after 12 h of incubation from 3.20 to 4.84 mg/dL, and C18:1 t-9 showed high values after 12 h of incubation (0.82 mg/dL) in relation to times 0 to 5 h (0.70 mg/dL).

There was an interaction effect between UFA source and time of incubation on the means of concentrations of C18:0, C18:1 c9, C18:1 t11, C18:2 c9 c12, C18:3 c9 c12 c15, SFA, and UFA ($p < 0.001$; Table 4). In all UFA sources, C18:0 concentration increased by incubation time, after 1 h of incubation LA and LN had higher concentrations than NAF and RPF, and until 12 h of incubation, NAF, RPF, and SO had similar concentrations (Figure 2A).

The concentration of C18:1 c9 from SO was higher than NAF, LA, and LN during all incubation times, and at 5 and 12 h of incubation SO showed the highest values (Figure 2B). C18:2 c9 c12 and C18:3 c9 c12 c15 concentration decreased by incubation time (Figures 2C and 2D), and LN had the highest values of C18:3 c9 c12 c15 during all tested times (Figure 2D). Across all treatments, SFA concentrations (Figure 2E) tended to increase, whereas UFA concentrations (Figure 2F) tended to decrease, as the incubation time increased. At 24 h and 36 h of incubation, the total SFA concentration was higher in LA and LN diets compared to other tested diets (Figure 2E; $p < 0.05$). Furthermore, after 12 h of incubation RPF and SO showed higher UFA concentrations (Figure 2F; $p < 0.05$).

The 18:1 t11 concentration showed a faster increase from 0 h (9.04 mg/dL) to 5 h (37.64 mg/dL) and then decrease from 12 h (32.28 mg/dL) to 36 h (16.84 mg/dL) of incubation, irrespective of UFA source, and its concentrations were higher in LA and LN from 1 h to 36 h of incubation (1 h = 55.97; 36 h = 24.14 mg/dL) in relation to NAF and RPF (1 h = 10.34; 36 h = 9.01 mg/dL) ($p < 0.05$).

Both production (i.e. C18:0 and C18:1 t11) and RBH (i.e. C18:1 c9, C18:2 c9 c12, 18:3 c9 c12 c15, and UFA) rates of FA were affected by the interaction among UFA source and incubation time ($p < 0.01$; Table 5). RPF presented the lowest C18:0 production rate at all incubation times, while SO, LA, and LN had a higher rate than NAF and RPF diets ($p < 0.001$). A higher C18:1 t11 production rate was observed from 1 h to 5 h of incubation, where LA and

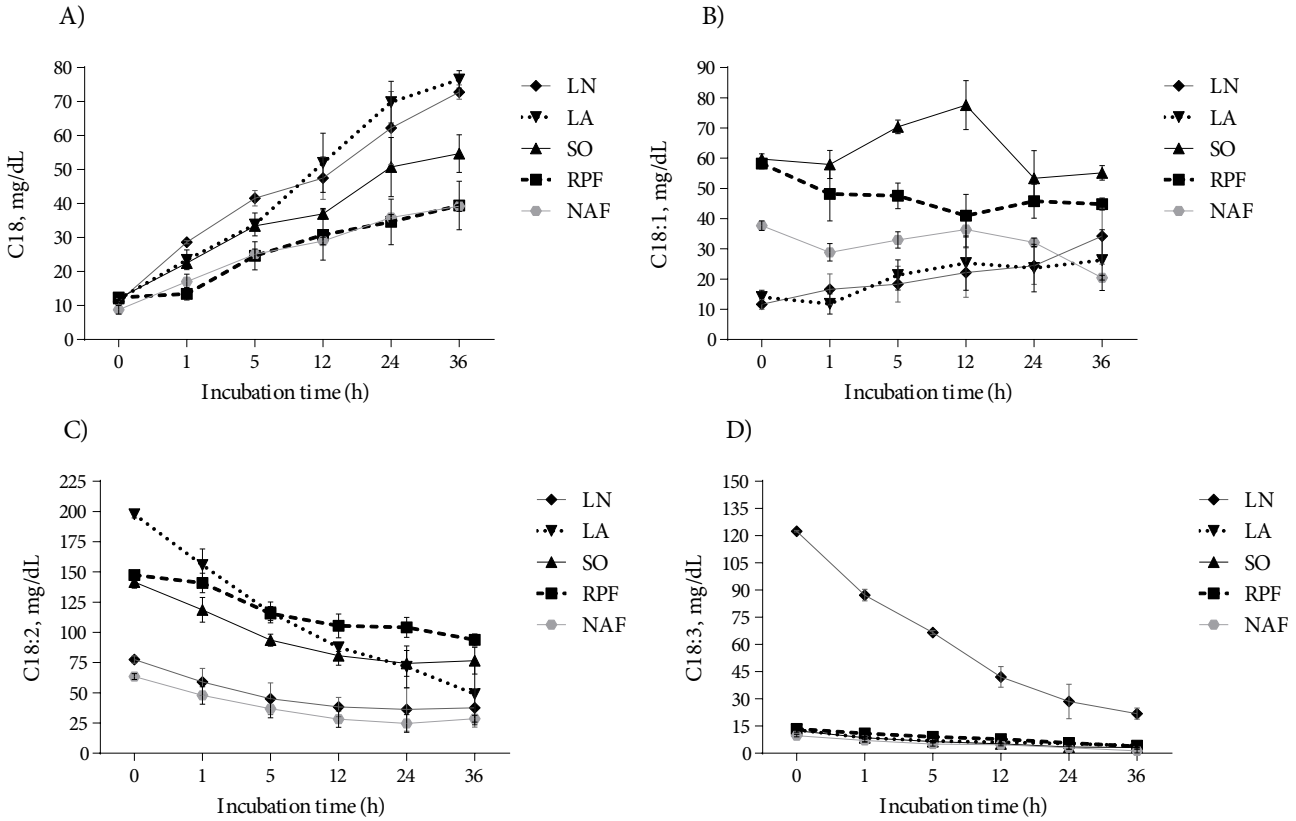

Figure 2. Concentrations (mg/dL) of stearic acid (18:0; A), oleic acid (18:1c9; B), linoleic acid (18:2c9,c12; C), alpha-linolenic acid (18:3,c9,c12,c15; D), total saturated fatty acids (SFA; E), and total unsaturated fatty acids (UFA; F) in rumen fluid incubated in vitro with diets containing different sources of unsaturated fatty acids associated to crude glycerin. LN: linolenic acid, LA: linoleic acid, OS: soybean oil, RPF: rumen-protected fat, NAF: no addition of fat. SFA = sum of 8:0, 10:0, 12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, and 22:0). UFA = sum of 14:1c9, 16:1c9, 18:1c9, 18:2c9,c12, 18:3c9,c12,c15, 20:1c11, and 22:1c13.

LN have higher values compared to NAF and RPF diets ($p < 0.001$).

The NAF and RPF showed the greatest 18:1 c9 RBH rates at 1 h of incubation and lower values were observed in LN compared to SO and LA ($p < 0.001$), whereas from 5 to 36 h of incubation C18:2 c9, c12 and 18:3 c9, c12, c15 RBH rates were similar between NAF, RPF, and SO diets. The LA showed the greatest 18:2 c9, c12 RBH rates, while the greatest 18:3 c9, c12, c15 RBH rates were observed in LN, across whole incubation times ($p < 0.05$) and at 1 h linolenic acid RBH rate were higher in LA than RPF diet.

With respect to UFA RBH rates, the greatest values were observed for NAF, RPF, and LN treatments at 1 h of incubation ($p < 0.05$) and LA had lower values in relation to SO diet ($p < 0.001$).

4. Discussion

We hypothesized that UFA sources with high content of linolenic acid would produce less greenhouse gases and improve the FA profile during the ruminal fermentation. However, even LN diet resulted in lower total gas and methane production, the production rate of 18:0 in LN tended to be greater compared to LA, whereas production rate of 18:1 t11 in LA was greater than in LN; thus, our hypothesis was rejected.

Our results indicate that the inclusion of lipids (i.e. oils or pure fatty acids) associated with CGL influences the total gas production in the rumen. Diets with SO and LA produced the highest amount of total gas, whereas they induced the lowest amount of both CH_4 and CO_2 production (i.e., when expressed as a percentage of total gas). Conversely, NAF and RPF diets produced the highest values of CH_4 production, while CO_2 production did not differ among them. These results are in accordance with Castagnino et al. [11], who, in an in vitro trial, showed that the use of lipid sources (soybean oil and flaxseed oil) associated with glycerol in the diet reduces the methane production in relation to no lipid added diet.

It is known that UFA competes with methanogenesis for reducing equivalents during RBH in the rumen [22]. However, in vitro and in vivo studies support that CGL can decrease the RBH of UFA [9,10,12]. According to

Table 5. Production and biohydrogenation rates of the main rumen fatty acids derived from treatments containing different sources of unsaturated fatty acids associated to crude glycerin.

	Diets †					SEM	p-value		
	NAF	RPF	SO	LA	LN		Diet	Time	D× T‡
Production rate, %/h									
18:0						0.219	<0.001	<0.001	<0.001
1 h	8.27^{b}	1.12^{c}	10.43^{ab}	11.58^{ab}	17.42^{a}				
5 h	3.26^{ab}	2.46^{b}	4.27^{ab}	4.40^{ab}	6.07^{a}				
12 h	1.69^{ab}	1.54^{b}	2.07^{ab}	3.34^{a}	3.02^{ab}				
24 h	1.13^{bc}	0.93^{c}	1.61^{ac}	2.41^{a}	2.13^{ab}				
36 h	0.85^{bc}	0.75^{c}	1.18^{ac}	1.80^{ab}	1.71^{a}				
18:1t11						0.561	<0.001	<0.001	<0.001
1 h	5.85^{d}	0.16^{e}	28.85^{c}	52.06^{a}	38.38^{b}				
5 h	2.33^{b}	2.11^{b}	6.00^{ab}	9.04^{a}	9.11^{a}				
12 h	0.77	1.57	1.11	3.09	3.14				
24 h	0.13	0.11	0.54	0.60	1.23				
36 h	–0.03	0.09	0.25	0.36	0.38				
Biohydrogenation rate, %/h									
18:1c9						1.404	<0.001	<0.001	0.001
1h	8.85^{a}	10.06^{a}	1.78^{b}	2.30^{b}	–4.97^{c}				
5h	0.94	2.13	–2.12	–1.45	–1.34				
12h	0.11	1.44	–1.49	–0.93	–0.88				
24h	0.23	0.52	0.26	–0.40	–0.53				
36h	0.48	0.37	0.13	–0.34	–0.62				
18:2 *c9, c12*						1.067	<0.001	<0.001	<0.001
1h	15.58^{b}	6.54^{c}	22.95^{b}	41.99^{a}	18.53^{b}				
5h	5.32^{b}	6.38^{b}	9.61^{ab}	16.21^{a}	6.49^{b}				
12h	2.94^{b}	3.51^{b}	5.08^{ab}	9.16^{a}	3.27^{b}				
24h	1.62^{b}	1.81^{b}	2.80^{ab}	5.26^{a}	1.72^{b}				
36h	0.97^{b}	1.49^{ab}	1.81^{ab}	4.13^{a}	1.11^{b}				
18:3 *c9, c12, c15*						0.470	<0.001	<0.001	<0.001
1h	2.68^{bc}	2.47^{c}	3.93^{bc}	4.40^{b}	35.27^{a}				
5h	0.93^{b}	0.88^{b}	1.21^{b}	1.22^{b}	11.19^{a}				
12h	0.42^{b}	0.47^{b}	0.61^{b}	0.57^{b}	6.70^{a}				
24h	0.28^{b}	0.32^{b}	0.38^{b}	0.32^{b}	2.91^{a}				
36h	0.23^{b}	0.27^{b}	0.26^{b}	0.24^{b}	2.78^{a}				
UFA						2.348	0.001	<0.001	<0.001
1h	30.10^{a}	19.06^{a}	6.92^{b}	–4.62^{c}	22.76^{a}				
5h	6.41	7.28	4.07	5.85	9.16				
12h	2.78	3.77	2.95	4.56	6.43				
24h	2.16	2.29	3.02	3.86	4.06				
36h	1.67	1.93	1.95	3.25	2.95				

† NAF: no addition of fat, RPF: rumen-protected fat, SO: soybean oil, LA: linoleic acid (18:2 c9, c12), and LN: alpha-linolenic acid (18:3 c9, c12, c15). ‡ diet × time incubation interaction effect. Mean values followed with different superscript letters on the same row mean a significant difference ($p < 0.05$).

Bayat et al. [23], supplementing lipids inhibit the activities of methanogens and protozoa and thus decrease CH_4 production. Granja-Salcedo et al. [8], in an in vivo study, observed that soybean oil plus CGL in the diet have high potential to reduce methane emission, by reduction in the total number of protozoa and decrease in the abundance of Archaea in the rumen. Therefore, these results suggest that the use of SO or LN associated to GC may be effective for improving the energy use of diet in ruminants, considering that the excretion of CH_4 represent about 2% to 12% of energy loss of the ingested feed energy [24]. Moreover, it may be a potential strategy for decreasing the environmental damaging effects of ruminant production, considering that CH_4 is a greenhouse gas with nearly 25 times greater warming potential than CO_2 [25].

The UFA source influenced the concentration of the main FA intermediates and products from RBH of 18:2 c9,c12 and 18:3 c9,c12,c15 (i.e. 18:0, 18:1 c9, 18:1 t11, SFA and UFA), as well as their RBH or production rates. We observed that LA and LN showed a greater concentration and production rates of 18:0 and 18:1 t11 than RPF. However, production rate of 18:0 in LN tended to be greater than in LA, whereas production rate of 18:1 t11 in LA was greater than in LN. The difference in 18:0 and 18:1 t11 production between protected and nonprotected lipid sources may be explained by the fact that lipid protection decreases the access of ruminal microorganisms to FA [26] and hence may decrease the production of 18:1 t11and 18:0. These FA are the main rumen intermediary and product of the RBH of 18:2 c9,c12, and 18:3 c9,c12,c15 [5]. Nevertheless, a greater and lower production rate of 18:0 and 18:1 t11, respectively, in LN diet compared to LA diet, may suggest that unprotected lipid sources with a high content of 18:2 c9,c12 may promote the accumulation of FA intermediates with special interest, such as 18:2 c9,t11 (CLA) and 18:1 t11, and may be desirable for reducing the SFA in ruminant derived products compared to lipid sources with a high content of 18:3 c9,c12,c15. The enhancement of rumen 18:1 t11 in the rumen is one of the most important topics on the quality of ruminant products due to the positive correlation between rumen 18:1 t11 and endogenous CLA production [27], which has shown positive effects on human health [28].

As expected, SO diet produced the highest levels of 18:1 c9 since SO lipid source had the greatest 18:1 c9 concentration among the lipid sources tested. In addition, the lowest RBH rate observed in 18:1 c9 is in line with previous in vivo and in vitro studies [6,29], which suggests that 18:1 c9 tends to be bio hydrogenated more slowly than another UFA due to the lowest affinity with enzymatic systems. Previous in vitro studies observed a slower rate of conversion of 18:1 c9 into 18:1 t11 of 5.6 %/h compared to other RBH steps [30], and 18:1 c9 conversion to 18:0 is less spontaneous (ΔG_{rxn} = -2.90 kJ/mol) than other RBH steps [6].

The greater concentration of 18:2 c9,c12 in SO and LA than LN, and greater concentrations of 18:3 c9,c12,c15 in LN than SO and LA was expected, considering that SO and LA lipid sources are rich in 18:2 c9,c12 while LN is rich in 18:3 c9,c12,c15. However, we observed that RBH rate of 18:2 c9, c12 in LA treatment was greater than the RBH rate of 18:3 c9, c12, c15 in LN treatment. This is in line with in vitro studies from Ribeiro et al. [30] and Vargas et al. [6] who showed that 18:2 c9, c12 bio hydrogenate faster than 18:3 c9, c12, c15. Kepler and Tove [31] reported that linoleate isomerase (i.e. an enzyme that participates in the first RBH step of 18:2 c9,c12 and 18:3 c9,c12,c15) presents a greater affinity for 18:2 c9, c12 than for 18:3 c9,c12,c15.

We observed that independently of lipid source, SFA concentration tended to increase, whereas UFA tended to decrease, as the incubation time increased. The kinetics of FA with a decrease in C18 unsaturated FA and an increase in C18:0 over time agree with the literature findings [6,17] where the authors observed a similar response when LA and LN were incubated in vitro. Additionally, NAF, RPF, and LN showed greater rumen BH rates of UFA than those observed in SO and LA. This may be due to lipids in NAF and RPF treatments mainly come from forages, and these are rich in 18:3 c9, c12, c15 [32] the main FA in LN lipid source. The UFA in plants are located mainly into the chloroplast in the chemical form of mono- and digalactosyldiglycerides (i.e. galactolipids) [33] and the hydrolysis of plant galactolipids is almost complete (between 78% and 95%) after 4 h of incubation [34].

The lower total gas production observed in RPF diet compared to NAF may be a result of the partial dissociation of calcium salts allowed by the acid rumen pH conditions, since tested diets contained a high proportion of concentrate and it may permit the releasing FA from RPF [9,35] and negatively affected gas production [36].

5. Conclusion

The inclusion of vegetable oils or pure fatty acids associated with crude glycerin influences the total gas production, the concentration of the main fatty acid intermediates, and products from ruminal biohydrogenation. Diets with a high content of linoleic acid may be efficient as a nutritional approach to reduce methane production and rumen biohydrogenation in beef cattle, resulting in positive effects on vaccenic acid concentrations during the ruminal fermentation, which could improve the content of unsaturated fatty acids in milk and meat.

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Conflict of interests

The authors declare that are no conflicts of interest regarding the current manuscript.

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