Effects of partial replacement of maize in the diet with crude glycerin and/or soyabean oil on ruminal fermentation and microbial population in Nellore steers

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Abstract

The objective of this study was to determine whether a combination of crude glycerin (CG) and soyabean oil (SO) could be used to partially replace maize in the diet of Nellore steers while maintaining optimum feed utilisation. Eight castrated Nellore steers fitted with ruminal and duodenal cannulas were used in a double 4×4 Latin square design balanced for residual effects, in a factorial arrangement ($A \times B$), when factor A corresponded to the provision of SO, and factor B to the provision of CG. Steers feed SO and CG showed similar DM intake, DM, organic matter and neutral-detergent fibre digestibility to that of steers fed diets without oil and without glycerine ($P > 0.05$). Both diets with CG additions reduced the acetate:propionate ratio and increased the proportion of *iso*-butyrate, butyrate, *iso*-valerate and valerate ($P < 0.05$). Steers fed diets containing SO had less total N excretion ($P < 0.001$) and showed greater retained N expressed as % N intake ($P = 0.022$). SO and CG diet generated a greater ruminal abundance of *Prevotella*, *Succinivibrio*, *Ruminococcus*, *Syntrophococcus* and *Succiniclasticum*. Archaea abundance ($P = 0.002$) and total ciliate protozoa were less in steers fed diets containing SO ($P = 0.011$). CG associated with lipids could be an energy source, which is a useful strategy for the partial replacement of maize in cattle diets, could result in reduced total N excretion and ruminal methanogens without affecting intake and digestibility.

Key words: Digestibility: Glycerol: Lipid supplementation: Protozoa: Rumen bacteria

The increase in global biofuel production and human utilisation, although controversial, has resulted in high prices of agricultural products, including maize[1,2], an important feed grain for livestock, which has increased the demand for alternative energy sources in animal feed.

Crude glycerin (CG), a co-product of biodiesel production, is an ingredient that can replace maize in ruminant diets, and its production has the potential for continuous growth in the future[3,4]. This by-product could increase the available energy for use by the animal, as it would be absorbed through the ruminal epithelium and used as a precursor of gluconeogenesis in the liver[5]. The main effect of CG on ruminal fermentation is characterised by a reduction of the acetate:propionate ratio[6,4] and, when used up to 100 g/kg in Nellore steer diets, CG does not influence DM intake (DMI)[7].

Another alternative source used to increase the energy density in diets for increased animal production is lipids[8,9]. Lipid supplementation in Nellore has consistently been associated with an increase in microbial protein synthesis[10,11]. Currently, improved N use efficiency in ruminants has received increased attention owing to the high cost of protein sources in animal feed and the environmental impact of excessive excretion of N from livestock production[12].

However, lipid supplementation could cause negative effects on the DMI and diet digestibility when the diethyl ether extract (EE) content exceeds 70 g/kg[11]. Adverse effects of lipids on ruminal fermentation are mainly owing to the toxicity of unsaturated fatty acids (UFA) to ruminal microbiota, especially cellulolytic bacteria[13]. However, CG could partially inhibit ruminal lipolysis and modulate the release of UFA in the

rumen[14–16], and could thereby reduce the deleterious effects of UFA on ruminal fermentation. Thus, CG associated with lipids could be an alternative energy source and could constitute a useful strategy for the partial replacement of maize in cattle diets.

Previous studies have reported beneficial effects of CG associated with soyabean oil (SO) in Nellore bull diets, such as greater daily gain and feed efficiency[17], without adversely affecting the DMI and ruminal fermentation parameters[9] and increased UFA duodenal flow[16]. However, few studies have examined the effects of CG and lipid combination on ruminal fermentation, the ruminal microbial population and N utilisation in high-lipid-content diets (>70 g EE/kg MS).

Thus, the objective of this study was to determine whether a combination of CG and SO could be used to partially replace maize in the diet of Nellore steers while maintaining optimum feed utilisation. Our hypothesis was that diets with high lipid content and the addition of CG could partially replace maize without compromising adversely the ruminal microbiota, DMI, digestibility and ruminal metabolism.

Methods

Animals, experimental design and diets

This study was carried out in strict accordance with the recommendations in the Brazilian College of Animal Experimentation (Colégio Brasileiro de Experimentação Animal) guidelines. The protocol was approved by the Ethics, Bioethics and Animal Welfare Committee (Comissão de Ética e Bem Estar Animal) of Facultade de Ciencias Agrarias e Veterinarias (FCAV) of the Universidade Estadual Paulista (UNESP), Jaboticabal campus, Brazil (protocol no. 07784/14). Ruminal and duodenal cannulation was conducted 8 months before the experiment under xylazine sedation and local anaesthesia with lidocaine hydrochloride, and all efforts were made to minimise suffering.

The experiment for the evaluation of ruminal fermentation and microbial population was conducted at the Experimental Feedlot of the Animal Science Department in the UNESP, Jaboticabal, Brazil.

Eight Nellore steers (mean body weight 501·3 (SEM 18) kg), castrated and fitted with ruminal silicone-type ruminal cannulas and duodenal T-type cannulas (Kehl®), were used in a double, simultaneous, Latin square design 4×4 with a 2×2 factorial arrangement of treatments (with or without SO and with or without CG). The experiment consisted of four consecutive periods of 21 d each. Each period consisted of 14 d adaptation, 5 d faeces and urine collection and 1 d sampling of ruminal fluid.

The animals were fed with experimental diets (Table 1) and a mineral supplement at 100 g/animal (containing per kg: Ca, 146 g; P, 40 g; S, 40 g; Na, 130 g; Cu, 1·35 g; Mn, 1·04 g; Zn, 5 g; iodine, 100 mg; Co, 80 mg; Se, 26 mg; F, ≤800 mg) twice per d at 06.00 and 16.00 hours as total mixed ration. Throughout the entire experimental period, the allowance was adjusted to allow refusals of approximately 5 % of the total amount consumed on the previous day.

Experimental diets contained Tifton 85 hay, ground maize, soyabean meal, urea and 60 g/kg DM of SO combined with 100 g/kg DM of CG; or 60 g/kg DM of SO without CG; or 100 g/kg DM CG without SO; or without CG and SO. Experimental diets were formulated according to recommendations of the Agricultural and Food Research Council[18], had a roughage: concentrate ratio of 30:70, were isonitrogenous and were prepared to be consumed within 4 d to prevent the oxidation of dietary ingredients. The CG (glycerol: 803·4 g/kg; EE: 15·9 g/kg; ash: 50·3 g/kg; water: 120·2 g/kg) was obtained from biodiesel

Table 1. Proportion of ingredients and bromatological composition of experimental diets

	Diets			
	SO and CG*†	SO and no CG*	No SO and CG†	No SO and no CG
Ingredients (% of DM)				
Tifton 85 hay	300·0	300·0	300·0	300·0
Ground maize	362·0	485·0	434·0	560·0
Soyabean meal	168·0	145·0	156·0	130·0
Urea	10·0	10·0	10·0	10·0
SO	60·0	60·0	–	–
CG	100·0	–	100·0	–
Bromatological composition				
DM (g/kg)	858·3	896·4	849·5	839·9
Ash (g/kg DM)	54·1	57·4	56·1	59·4
Crude protein (g/kg DM)	153·7	152·4	153·5	151·2
RDP (g/kg DM)	102·0	98·0	100·0	95·2
Neutral-detergent fibre (g/kg DM)	404·6	430·5	420·1	446·0
Acid-detergent fibre (g/kg DM)	155·4	156·3	156·1	156·8
Lignin (g/kg DM)	15·9	16·0	16·0	16·0
NFC (g/kg DM)	311·1	283·7	352·0	325·7
Starch (g/kg DM)	190·0	248·0	224·1	283·3
Gross energy (MJ/kg DM)	17·3	17·0	17·6	17·3
ME (MJ/kg DM)	10·7	10·3	10·4	10·7
Diethyl ether extract (g/kg DM)	86·4	89·2	30·0	33·0

SO, soyabean oil; CG, crude glycerine; RDP, rumen degradable protein; NFC, non-fibrous carbohydrates; ME, metabolisable energy.
* 60 g/kg of SO in DM diet.
† 100 g/kg of CG in DM diet.

production based on SO (Cargill). The SO used contained 121·6 g/kg stearic acid; 301·6 g/kg oleic acid; 347·83 g/kg linoleic acid; and 2·29 g/kg linolenic acid.

Feed intake and apparent digestibility

On days 15–21 of each experimental period, before the morning feeding when subsamples of approximately 100 g/kg were obtained and frozen at −20°C, the feed refusals were collected and weighed daily. At the end of each experimental period, composite samples were collected for each animal based on the feed refusal weight.

Faeces were collected on days 16–20 of each experimental period to estimate the apparent digestibility of dietary constituents. Samples were collected daily immediately after each spontaneous defecation, and stored in 20-litre buckets. After each 24-h collection period, the total amount of faeces produced per animal was weighed and homogenised; from this, a sample (15 % of daily faecal production; approximately 200 g) was removed and dried. For each animal, in each experimental period, we formed a faecal-composite sample based on the pre-dried weight.

To measure organic matter (OM) apparently digested in the rumen (OMDR), duodenal samples (150 ml) were obtained by duodenal cannula collected at 6-h intervals on days 19 and 20 of each experimental period. The sample collection during the 2nd day was delayed for 3 h to ensure that every 3-h slot in a 24-h period was properly represented. Indigestible neutral-detergent fibre (NDF), an indicator of the daily flow of DM in the duodenum[19], was assessed via *in situ* incubation of feeds offered, feed refusals, faeces and duodenum samples for 288 h[20]. After the incubation time, bags were removed and washed in running water until the water was clear. Next, samples were pre-dried at 55°C for 72 h and used for NDF determination, and duodenal DM flow was calculated according to the following equation: (faecal DM × g/kg indigestible NDF in faecal DM)/g/kg indigestible NDF in duodenal DM.

Feeds, feed refusals, faeces and duodenal samples were dried in a forced-air-circulation oven at 55°C for 72 h and ground in a Wiley mill (Thomas Scientific) to pass a 1-mm screen. Samples were analysed for DM (Method 934.01), ash (Method 942.05) and EE (Method 920.39) according to Association of Official Analytical Chemists[21]. N was determined using a LECO FP-528 N analyzer (LECO Corp.) and the N values were converted to crude protein (CP) by multiplying by 6·25. Gross energy was obtained by the combustion of samples in an adiabatic bomb calorimeter (IKA model 2000 Basic; IKA Corp.). NDF determination was conducted using α-amylase without the addition of sodium sulphite, following the recommendations of Van Soest *et al.*[22], and adapted for the ANKOM 200 Fiber Analyzer (ANKOM Technology Corporation). Acid-detergent fibre was determined using the method described by Goering & Van Soest[23] and adapted for the ANKOM 200 Fiber Analyzer. Acid-detergent lignin was determined by solubilisation of cellulose with sulphuric acid according to Van Soest & Robertson[24]. Starch was determined enzymatically according to Bach Knudsen *et al.*[25]. Total carbohydrates and non-fibrous carbohydrate (NFC) values were calculated as described by

Sniffen *et al.*[26]. Ruminal degradable protein was calculated as described by Ørskov & McDonald[27].

Ruminal fermentation parameters

Ruminal fluid was sampled at day 21 of each experimental period at 0, 3, 6, 9, 12, 15 and 18 h after the morning feeding. An amount of 50 ml of ruminal fluid was taken after filtration through cotton double fabric, and then pH was measured. Two aliquots of 25 ml were stored at −20°C and later used to determine ammonia N (NH_3-N) and volatile fatty acid (VFA) concentration. NH_3-N was determined in duplicate following the methodology of Fenner[28] adapted for use in Kjeldahl distillation. The VFA concentration was quantified by GC (GC Shimatzu model 20−10, with automatic injection; Shimatzu Corporation) using a SP-2560 capillary column (30 m × 0·25 mm diameter, 0·02 mm thick; Supelco) according to the methodology described by Palmquist & Conrad[29].

Ruminal micro-organisms

On day 21 of the trial period, samples of ruminal content were collected before the morning feeding. Samples of approximately 60 g/animal (a mix of liquid and solid) from the dorsal, central and ventral regions of the rumen were collected through the ruminal cannula, immediately frozen and stored at −80°C for DNA extraction.

A Fast DNA SPIN Kit for Soil (MP Bio®; Biomedicals) extraction kit was used to extract metagenomic DNA from 200 mg of sample according to the manufacturer's instructions. DNA concentrations were measured fluorometrically (Qubit® 3.0, kit Qubit® dsDNA Broad Range Assay Kit; Life Technologies) and DNA purity was assessed spectrophotometrically (NanoDrop® ND-1000 Spectrophotometer; Thermo Fisher Scientific) at A260:A230 nm and A260:A280 nm ratios. Integrity was determined by agarose gel electrophoresis using a 0·5 % (w/v) gel, and subsequent staining with ethidium bromide (5 mg/ml).

Bacterial diversity analysis was performed on selected samples from animals of the first Latin square 4 × 4 (a total of sixteen samples; four of each experimental group). Primers for PCR (515F = 5'-GTGNCAGCMGCCGCGGTAA-3' and 926R = 5'-CCGYCAATTYMTTTRAGTTT-3') and sequencing used in this analysis were described by Caporaso *et al.*[30]. Each sample was amplified in triplicate, and each PCR reaction mixture (20 μl final volume) contained 20 ng of DNA, 10 μM of each forward and reverse primer, 1·25 mM magnesium chloride, 200 μM dNTP mix (Invitrogen), 1·0 U platinum Taq DNA polymerase high fidelity (Invitrogen), a high-fidelity PCR buffer (1×) (Invitrogen) and Milli-Q water (Milli-Q® Type 1 Ultrapure Water Systems). Reactions were conducted at 95°C for 3 min to denature the DNA, with amplification proceeding for forty cycles at 95°C for 30 s, 53·8°C for 30 s and 72°C for 45 s; a final extension of 10 min at 72°C was added to ensure complete amplification.

The purity of PCR products was verified by agarose gel (1 %) electrophoresis and the amplicon size was estimated by comparison with a 1 Kb plus DNA ladder (Invitrogen). The PCR fragments were purified using the Zymoclean™ Gel DNA Recovery kit (Zymo Research) according to the manufacturer's instructions. Composite samples for sequencing were created

by combining equimolar ratios of amplicons from the triplicate samples. Sequencing was performed using the Ion Torrent Personal Genome Machine (PGM™; Thermo Fisher Scientific) with the Ion 314™ Chip Kit v2 (Thermo Fisher Scientific) at the Sequencing Facility, Department of Technology of FCAV, Jaboticabal, Brazil.

Sequence trimming was carried out by *Prinseq* version 0.20.3 selecting sequences over 200 bp in length with an average quality score greater than 25 based on *Phred* quality. Sequences were clustered using *Mothur* 1.36.1[31] and taxonomic assignment of sequences was performed against the SILVA database[32]. Sequence data were grouped into operational taxonomic units that shared >97 % sequence similarity and were summarised at phylum and genera levels.

Quantitative PCR (qPCR) was used to identify and quantify Archaea abundance, with a total bacterial primer set recommended by Denman & McSweeney[33] (forward 5'-CGGCAACGA CAACCC-3' and reverse 5'-CCATTGTAGCACCTGTGTAGCC-3'); a different set of primers was used for Archaea (forward 5'-TTCGGTGGATCDCARAGRGC-3' and reverse 5'-GBARGTCG WAWCCGTAGAATCC-3') as used by Denman *et al.*[34].

All forward and reverse primers used were tested in four concentrations (200, 400, 600 and 800 nM) to determine the minimum concentration of primer with the lowest threshold cycle (C_t), and reduce non-specific amplifications. We determined the slope value and calculated the efficiency. The validation of the selected primer concentrations was performed with different concentrations of DNA (150, 125, 100, 50, 25 and 12·5 ng).

The qPCR (final volume reactions 12·5 µl) contained 6·25 µl of SYBR Green PCR Master Mix (Bio-Rad), 200 (Archaea) or 600 nM (total bacteria) of each primer pair, ultrapure water (Milli-Q; Millipore Corporation) and 100 ng of the DNA. Reactions were performed in triplicate in an Applied Biosystems 7500 Real-time PCR System (Thermo Fisher Scientific). Negative controls contained all reaction components except DNA. ROX (ROX™/Texas Red®; Thermo Fisher Scientific) was used as a passive reference dye.

PCR conditions were as follows: amplification at 50°C for 2 min, 95°C for 10 min; 35 denaturing cycles at 95°C for 15 s; annealing at 60°C for 60 s; and extension at 78°C for 1 min. After the amplification cycle, a step was added, increasing the temperature from 60 to 95°C, to obtain a dissociation curve of the reaction products, and analyse the specificity of the amplification.

Changes in targeted populations were calculated using a relative quantification calculation and the $2^{-\Delta\Delta C_t}$ method, with the control period used as the calibrator and total bacterial[33] C_t (cycle threshold) values used as the reference value[35]. Thus, results were expressed as a proportion of the 16S rRNA associated with total bacteria.

For the quantification of ruminal ciliate protozoa, samples of ruminal content were collected via the cannula on day 21 of the trial period 3 h after the morning feeding, and ruminal content aliquots were preserved in formalin (a 1:1 solution of water and 370 ml/l formaldehyde) as described by D'Agosto & Carneiro[36]. Direct identification and counts of protozoa were performed in a Sedgewick Rafter counting chamber[37]. The samples were diluted with 200 ml of glycerol/l and stained using Lugol's solution before counting the cells[36].

Statistical analyses

Statistical analyses were performed using R Software version 3.2.2 (2015; R Core Team). Initially, the mathematical assumptions of data were tested (Shapiro–Wilk test and Bartlett test).

Data of intake, digestibility, OMRD and ruminal protozoa were compared between treatments by ANOVA as a double 4 × 4 Latin square design (four treatments and four periods), balanced for residual effects, in a factorial arrangement (A × B). The fixed effect of factor A corresponded to the SO addition (yes/no) and factor B to the CG addition (yes/no), factors interactions, treatments error, the random effects of Latin square, period, animal and residues corresponding to the model.

Data regarding pH, NH_3-N and VFA were compared between treatments and time by repeated-measures ANOVA in a double 4 × 4 Latin square design (four treatments and four periods), balanced for residual effects, in a split-plot factorial arrangement (A × B). The model included the fixed effects of factor A (corresponded to the SO addition, yes/no) and factor B (corresponded to the CG addition, yes/no) that were considered as independent variables; the sampling times considered as dependent variable (covariate), factors interactions, treatments residues, factors and time interactions; and the same random effects of the previous model.

Tukey's *post hoc* test was applied when ANOVA indicated a significant difference, considering statistical significance when $P \leq 0.05$.

Bacterial and Archaea abundance was compared between experimental groups (oil and glycerin; oil and no glycerin; no oil and glycerin; and no oil and no glycerin) using Kruskal–Wallis and Dunn's *post hoc* tests.

Results

Feed intake and digestibility

All diets allowed a similar intake of DM, OM, CP and NDF when expressed as % of animal body weight ($P > 0.05$; Table 2). However, when the intake was expressed as kg/d, there was an interaction ($P < 0.05$) between SO and CG on DMI, CP, NFC and GE intake; thus, steers supplemented with oil without glycerin showed the lowest intake of DM and CP ($P < 0.05$). Therefore, steers fed with oil and glycerin in the diet presented similar DMI and CP intake than steers fed a no oil and no glycerin diet ($P = 0.216$). Diets with an oil addition reduced the NDF intake ($P = 0.049$) but increased the EE intake ($P < 0.001$). The highest NFC intake was presented by animals fed a no-oil and no-glycerin diet ($P < 0.05$), and the no-oil and glycerin addition diet generated the lowest GE intake ($P < 0.001$).

There was an interaction effect of SO and CG on DM, NDF and EE total apparent digestibility. Thus, diet containing oil and no glycerin presented the lowest DM and NDF digestibility ($P < 0.05$). EE digestibility was lowest for the diet with no oil and no glycerin when compared with diets containing SO ($P = 0.001$). Steers fed diets containing SO showed greater CP digestibility ($P < 0.001$).

Steers fed diets containing SO had less total faecal N excretion expressed as g/d ($P = 0.002$; Table 3) and % of N intake ($P = 0.003$).

Table 2. Nutrient intake, total apparent digestibility and nitrogen faecal outputs in feedlot Nellore steers fed diets containing or not containing soyabean oil (SO) with or without crude glycerine (CG)

	Diet					P		
	SO and CG*†	SO and no CG*	No SO and CG†	No SO and no CG	SEM	O	G	O × G
Intake (% BW)								
DM	1·84	1·51	1·90	1·79	0·071	0·063	0·117	0·052
Organic matter	1·69	1·40	1·75	1·52	0·066	0·426	0·035	0·733
Crude protein	0·30	0·24	0·29	0·27	0·012	0·718	0·107	0·450
NDF	0·83	0·73	0·86	0·83	0·038	0·257	0·283	0·405
Intake (kg/d)								
DM	9·38^b	8·25^c	10·62^a	9·61^{a,b}	0·396	0·046	0·129	0·045
Organic matter	8·64	7·29	9·77	8·90	0·393	0·045	0·110	0·070
Crude protein	1·55^a	1·26^b	1·56^a	1·52^a	0·091	0·196	0·357	0·040
NDF	4·46	3·94	4·73	4·90	0·208	0·049	0·561	0·421
NFC	2·01^b	2·56^b	2·70^b	3·06^a	0·154	0·005	0·007	0·048
Diethyl ether extract	0·94	0·88	0·36	0·35	0·058	<0·001	0·567	0·789
Gross energy (MJ/kg DM)	17·57^a	17·74^a	17·11^b	17·74^a	0·176	0·025	0·001	0·040
Total apparent digestibility (%)								
DM	76·30^a	70·17^b	76·04^a	74·46^a	0·887	0·265	0·042	0·043
Organic matter	85·07	79·41	87·31	84·67	0·768	0·131	0·118	0·057
Crude protein	78·86	78·50	74·54	74·68	2·266	0·006	0·969	0·939
NDF	56·33^{a,b}	51·48^b	58·90^a	58·84^a	1·361	0·047	0·165	0·025
Diethyl ether extract	91·39^a	89·78^a	87·44^{a,b}	83·23^b	0·814	0·002	0·066	0·032
Gross energy	75·21	71·68	73·99	75·28	0·730	0·534	0·546	0·220
N faecal outputs								
Faecal N (g/d)	27·39	22·25	37·58	36·28	8·032	0·002	0·055	0·964
Faecal N (% of N intake)	11·05	10·98	14·87	14·87	0·727	0·003	0·752	0·735

O, SO addition effect; G, CG addition effect; BW, body weight; NDF, neutral-detergent fibre; NFC, non-fibrous carbohydrates.
^{a,b,c} Values on the same row with unlike superscript letters were significantly different ($P < 0.05$), as obtained with Tukey's test.
* Addition of 60 g/kg of SO in DM diet.
† Addition of 60 g/kg of SO for 100 g/kg of CG in DM diet.

Table 3. Rumen fermentation parameters in feedlot Nellore steers fed diets containing or not containing soyabean oil (SO) with or without crude glycerine (CG)

	Diet					P						
	SO and CG*†	SO and no CG*	No SO and CG†	No SO and no CG	SEM	O	G	O × G	T	O × T	G × T	O × G × T
pH	6·49^a	6·37^b	6·37^b	6·34^b	0·022	0·029	0·038	0·036	<0·001	0·061	0·348	0·728
NH$_3$-N (g/l)	0·24^b	0·30^a	0·29^a	0·28^a	0·002	0·035	0·007	0·001	<0·001	0·582	0·404	0·044
Total VFA (mmol/l)	81·31	83·98	88·58	85·41	0·528	0·075	0·846	0·202	0·181	0·206	0·719	0·545
Acetate‡	67·01	71·04	67·97	71·28	0·412	0·064	0·026	0·086	0·092	0·811	0·341	0·966
Propionate‡	18·42	17·84	17·71	17·01	0·135	0·838	0·398	0·562	0·135	0·543	0·562	0·221
Iso-butyrate‡	0·44	0·41	0·42	0·41	0·001	0·383	0·015	0·249	0·169	0·434	0·910	0·340
Butyrate‡	12·83	10·52	13·19	10·59	0·104	0·162	0·001	0·266	0·059	0·306	0·458	0·301
Iso-valerate‡	0·86	0·78	0·80	0·76	0·003	0·773	0·002	0·790	0·154	0·696	0·731	0·128
Valerate‡	0·81	0·75	0·85	0·78	0·005	0·008	0·010	0·444	0·094	0·391	0·253	0·093
A:P	3·72	4·22	4·06	4·45	0·078	0·111	0·011	0·483	0·485	0·581	0·611	0·562
OMDR (kg/d)	4·06^a	3·49^b	4·39^a	4·14^a	0·276	0·045	0·051	0·015	–	–	–	–

O, SO addition effect; G, CG addition effect; T, time effect; VFA, volatile fatty acid; A: P, acetate:propionate ratio; OMDR, organic matter degraded into rumen.
^{a,b} Values on the same row with unlike superscript letters were significantly different ($P < 0.05$), as obtained with Tukey's test.
* Addition of 60 g/kg of SO in DM diet.
† Addition of 60 g/kg of SO for 100 g/kg of CG in DM diet.
‡ Expressed as mmol/100 mol of total VFA.

Ruminal fermentation parameters

The addition of SO, CG and sampling time affected ruminal NH$_3$-N concentration ($P < 0.05$; Fig. 1); steers fed SO and glycerin addition diet had the lowest ruminal NH$_3$-N concentration at 3, 6 and 12 h after feeding. In addition, SO and CG affected the average ruminal pH and NH$_3$-N concentration (Table 3); steers fed an oil and glycerin diet had the highest ruminal pH and lowest ruminal NH$_3$-N concentration ($P < 0.05$). The total VFA concentration and propionic (P) proportion was not influenced by the diets ($P > 0.05$). However, diets containing CG led to a less proportion of acetate (A) and A:P ratio and greater proportions of iso-butyrate, butyrate, iso-valerate and valerate ($P < 0.05$).

Ruminal micro-organisms

The number of the generated sequences was not affected by the diet ($P = 0.639$). In all, fifteen phyla were identified and 13 % of the

sequences could not be classified at the phylum level. Firmicutes and Bacteroidetes were the most abundant phyla and accounted for >76 % of the total bacterial community in all samples sequenced.
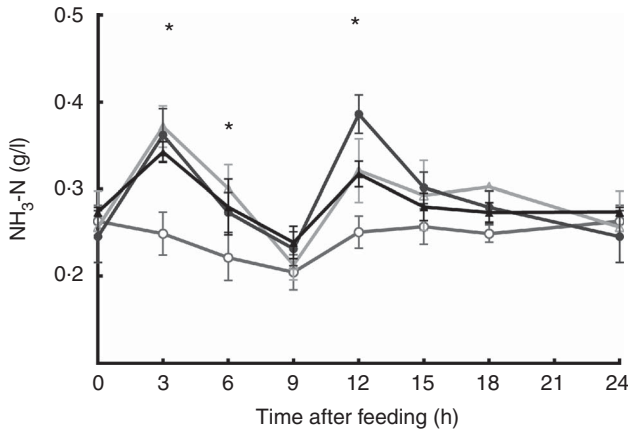
Fig. 1. Ruminal ammonia N (NH₃-N) concentration during a 24-h period in Nellore steers fed diets containing or not containing 60 g/kg of soyabean oil in DM diet (SO) with or without 100 g/kg of crude glycerine in DM diet (CG). Values are means with their standard errors. ─○─, SO and CG; ─●─, SO and no CG; ─△─, no SO and CG; ─▲─, no SO and no CG. * SO and CG diet results in a lowest ruminal NH3-N concentration (P < 0·05) as obtained with Tukey's test.

The abundance of the genera *Fibrobacter*, *Butyrivibrio*, *Ruminococcus*, *Lachnospiracea incertae sedis* and *Pseudo-butyrivibrio* was less in the rumen of steers fed diet with oil without glycerine (Table 4; P < 0·05). In contrast, *Acinetobacter* was most abundant in steers fed diets containing SO (P = 0·001).

Independently of the addition of oil, CG negatively affected *Treponema*, *Kurthia*, *Roseburia* and *Clostridium_IV* populations (P < 0·05), but stimulated *Selenomonas* abundance (P = 0·001). Animals fed no oil and CG in the diet showed greater ruminal populations of *Pseudobutyrivibrio* (P = 0·025).

Steers fed oil and glycerin as partial replacement of maize in the diet had a greater ruminal abundance of *Prevotella*, *Succinivibrio*, *Ruminococcus* and *Succiniclasticum* (P < 0·05).

Ruminal Archaea abundance was affected by diets containing SO (P = 0·012). Thus, the diets of oil and glycerin and oil and no glycerin addition decreased the Archaea proportion by four and three times, respectively.

Total ruminal ciliate protozoa were less in steers fed diets containing SO (Table 5; P = 0·011). The addition of oil decreased the abundance of *Entodinium*, *Dasytricha*, *Isotricha*, *Diploplastron*, *Eudiplodinium* and *Polyplastron* genera. In contrast, when CG was used, the abundance of *Diploplastron* increased (P = 0·034).

Table 4. Ruminal bacteria composition and methanogen (Archaea) abundance in feedlot Nellore steers fed diets containing or not containing soyabean oil (SO) with or without crude glycerine (CG)
(Medians and interquartiles)

		SO and CG†‡		SO and no CG†		No SO and CG‡		No SO and no CG		
Phylum	Genera	Median	Interquartile	Median	Interquartile	Median	Interquartile	Median	Interquartile	P
Bacteroidetes	*Barnesiella*	0·29	0·06	0·54	0·11	0·38	0·04	0·25	0·01	0·087
	Prevotella	17·30ᵃ	1·20	11·40ᵇ	1·05	11·68ᵇ	1·19	9·12ᶜ	0·81	0·007
Fibrobacteres	*Fibrobacter*	0·69ᵃ	0·05	0·16ᵇ	0·02	0·72ᵃ	0·13	1·12ᵃ	0·06	0·013
Proteobacteria	*Ruminobacter*	1·18	0·02	1·00	0·05	1·27	0·04	1·75	0·11	0·327
	Succinimonas	0·03	0·01	0·02	0·01	0·05	0·02	0·01	0·01	0·384
	Succinivibrio	4·54ᵃ	0·95	0·83ᵇ,ᶜ	0·31	0·29ᶜ	0·07	1·62ᵇ	0·58	0·024
	Acinetobacter	0·19ᵃ	0·04	0·94ᵃ	0·12	0·01ᵇ	0·01	0·01ᵇ	0·00	0·001
Spirochaetes	*Treponema*	0·39ᵇ	0·03	0·71ᵃ	0·18	0·17ᵇ	0·06	1·00ᵃ	0·06	0·017
Firmicutes	*Kurthia*	0·43ᵇ	0·10	10·86ᵃ	2·31	0·14ᵇ	0·05	13·98ᵃ	1·52	0·001
	Aerococcus	0·92ᵇ	0·14	2·97ᵃ	0·17	0·03ᶜ	0·01	1·00ᵇ	0·08	0·001
	Streptococcus	0·04	0·02	0·02	0·01	0·07	0·03	0·01	0·00	0·537
	Mogibacterium	0·10	0·01	0·16	0·05	0·12	0·03	0·50	0·08	0·226
	Blautia	0·10	0·08	0·12	0·09	0·11	0·05	0·25	0·10	0·171
	Butyrivibrio	0·22ᵇ	0·01	0·11ᵇ	0·03	0·27ᵇ	0·08	0·75ᵃ	0·12	0·037
	Lachnospiracea incertae sedis	0·19ᵃ,ᵇ	0·06	0·09ᶜ	0·04	0·15ᵇ	0·01	0·25ᵃ	0·07	0·022
	Pseudobutyrivibrio	0·35ᵇ	0·12	0·45ᵇ	0·03	2·02ᵃ	0·88	0·87ᵇ	0·32	0·025
	Roseburia	0·11ᶜ	0·02	1·25ᵇ	0·53	1·04ᵇ	0·46	8·86ᵃ	0·83	0·014
	Syntrophococcus	0·01	0·00	0·02	0·01	0·05	0·01	0·12	0·02	0·161
	Clostridium_IV	0·08ᵇ	0·01	0·29ᵃ	0·07	0·00ᵇ	0·00	0·25ᵃ	0·09	0·011
	Ruminococcus	1·09ᵃ	0·01	0·22ᵇ,ᶜ	0·08	0·40ᵇ	0·10	0·12ᶜ	0·05	0·034
	Saccharofermentans	0·18ᶜ	0·04	0·47ᵇ	0·07	0·93ᵃ	0·17	0·12ᶜ	0·03	0·016
	Anaerovibrio	0·03	0·01	0·01	0·00	0·03	0·01	0·12	0·07	0·206
	Succiniclasticum	0·94ᵃ	0·25	0·56ᵇ	0·11	0·64ᵇ	0·04	0·01ᶜ	0·00	0·038
	Selenomonas	0·56ᵃ	0·06	0·01ᵇ	0·00	0·84ᵃ	0·13	0·01ᵇ	0·00	0·031
Total sequences		918·211	8·941	790·451	7·339	888·225	8·752	840·682	5·429	0·639
Total Archaea§		0·51ᵇ	0·10	0·44ᵇ	0·04	1·83ᵃ	0·31	1·78ᵃ	0·18	0·012

ᵃ,ᵇ,ᶜ Median values on the same row with unlike superscript letters were significantly different (P < 0·05), as obtained using Dunn's test.
* Expressed as a proportion of operational taxonomic units identified.
† Addition of 60 g/kg of SO in DM diet.
‡ Addition of 60 g/kg of SO for 100 g/kg of CG in DM diet.
§ Measured based on the proportion of the specific 16S rRNA associated with total bacteria.

Table 5. Ruminal ciliate protozoa concentration in feedlot Nellore steers fed diets containing or not containing soyabean oil (SO) with or without crude glycerine (CG)

	Diet					P		
	SO and CG*†	SO and no CG*	No SO and CG†	No SO and no CG	SEM	O	G	O × G
Total protozoa‡	70·8	76·7	118·0	123·3	1·049	0·011	0·739	0·990
Entodinium	70·7	76·4	113·4	121·1	1·031	0·005	0·439	0·725
Dasytricha	0·00	0·00	0·20	0·24	0·066	0·026	0·302	0·303
Isotricha	0·00	0·00	0·40	0·16	0·055	0·006	0·196	0·196
Charonina	0·04	0·12	0·20	0·00	0·038	0·777	0·400	0·595
Eremoplastron	0·00	0·00	0·44	0·00	0·076	0·103	0·153	0·113
Diploplastron	0·04	0·00	0·89	0·04	0·131	0·032	0·034	0·304
Polyplastron	0·00	0·12	1·84	1·29	0·199	0·002	0·508	0·306
Eudiplodinium	0·04	0·00	0·25	0·40	0·066	0·030	0·634	0·479

O, SO addition effect; G, CG addition effect.
* Addition of 60 g/kg of SO in DM diet.
† Addition of 60 g/kg SO for 100 g/kg of CG in DM diet.
‡ Expressed as no. of protozoa × 10^4/ml of rumen liquid.

Discussion

In this study, we evaluated the ability of diets with high lipid content and CG to partially replace dry ground maize for Nellore steer feed by examining the effects of this diet on nutrient intake and digestibility, ruminal fermentation parameters and ruminal microbiome. Steers fed SO and CG to partial replacement of ground showed similar DMI, DM, OM and NDF digestibility to that of steers fed a diet without oil or glycerin. Thus, the hypothesis that diets with high lipid content (>70 g EE/kg DM) and supplemented with CG could partially replace maize diets without compromising intake, digestibility and ruminal fermentation was accepted.

In ruminants, negative effects on DMI and ruminal fermentation are often reported when they are fed diets including vegetable oils[38,39,11]. As expected, the diet with oil and no glycerin addition (89 g EE/kg DM) decreased the DMI and NDF intake and digestibility. These observations may be linked to the high content of EE of this diet, especially the high proportion of UFA with the SO that negatively affected the ruminal microbes, including fibre-degrading (e.g. *Fibrobacter*, *Butyrivibrio*, *Lachnospiracea incertae sedis* and *Ruminococcus*) and starch-degrading (e.g. *Ruminobacter*, *Pseudobutyrivibrio*, *Roseburia* and *Syntrophococcus*) bacteria, as well as total ruminal ciliate protozoa. UFA could directly disrupt microbial cell membranes and cellular function[13], coat bacteria and feed particles in the rumen in lipids[40], and directly inhibit ruminal protozoa[41,38] and Archaea[42]. Consequently, animals fed the SO and no glycerin addition diet showed a marked reduction in OMDR, which represented the lowest DMI and NDF intake and digestibility.

However, animals fed oil and glycerin addition (86 g EE/kg DM) showed similar DMI, DM and NDF digestibility to that of animals fed without SO or CG supplementation (no oil and no glycerin diet: 33 g EE/kg DM). These findings suggest the potential of glycerin as a partial inhibitor of ruminal lipolysis[14–16]; consequently, the modulation of UFA liberation in the rumen could reduce the adverse effects of UFA on ruminal micro-organisms and ruminal fermentation. It is possible that glycerin has a protective effect in diets with high lipid content and ameliorated the effects of lipids on ruminal fermentation[43,11].

The similar OMDR between steers fed oil and glycerin to partial replacement of ground and steers fed a diet without oil or glycerin, and the ruminal microbial profile of animals fed oil and glycerin diet, supports these results; SO and CG addition did not affect the abundance of important cellulolytic bacteria (*Lachnospiracea incertae sedis* and *Ruminoccocus*) and increased the abundance of some amylolytic/proteolytic bacteria (*Prevotella*, *Succinivibrio*, *Succiniclasticum* and *Selenomonas*).

Supplementation with oil and glycerin could replace 35 % of the ground maize used in the unsupplemented diet. Clearly, the lowest dietary starch (190 g starch/kg DM) resulted in a greater mean ruminal pH. However, although all diets contained a high proportion of concentrate, the average pH was adequate (pH > 6·0) for the maintenance of cellulolytic bacteria and ciliated protozoa in the rumen[44,45].

Inclusion of CG in the current study altered the ruminal VFA proportions, decreased acetate and increased butyrate ruminal concentrations. These results are consistent with those of other studies on cattle fed CG[46,47], suggesting that glycerin is associated with shifts in the microbial genera in the rumen. For example, it increases the abundance of butyrate-producing bacteria (e.g. *Pseudobutyrivibrio*)[48] and *Selenomonas*, which is a bacteria capable of fermenting glycerin that is considered a secondary fermenter, converting lactate and glucose to propionate or valerate[49,50].

Although the ruminal propionate proportion did not differ significantly between the diets, the A:P ratio was less in animals fed an oil and glycerin diet, as a reflection of changes on microbial profiles that reduced ruminal acetate. Interestingly, when animals were fed an oil and glycerin diet to partial replacement of ground, the abundance of three gram-negative bacteria (e.g. *Prevotella*, *Succinivibrio*, and *Succiniclasticum*), involved in ruminal propionate production, increased. The cooperative interaction between these three bacterial genera may have a synergistic effect on digestion and reduce acetate. The genus *Prevotella* can degrade polysaccharides such as xylan, hemicellulose, pentose and arabinose; has proteolytic

and amylolytic abilities; and produces acetate, succinate and propionate as ruminal fermentation products[50]. In addition, *Succinivibrio* attacks dextrin, pectin and some sugars and forms succinate, lactate, acetate and formate[51], and *Succiniclasticum* specialises in fermenting succinate and converting it to propionate[52].

Moreover, both diets with CG addition induced a greater ruminal concentration of valerate, iso-valerate and iso-butyrate–fatty acids resulting from ruminal degradation of branched amino acids that are essential growth factors for cellulolytic bacteria[53], as these fatty acids are important precursors of microbial odd and branched-chain fatty acids that characterise ruminal bacterial composition[54].

The greater ruminal ammonia concentration observed at the 1st hours are likely related to dietary urea used in all diets, once urea is quickly converted to NH_3-N in the rumen, which can increase NH_3-N levels faster than its use by rumen microbiota[4]. However, ruminal ammonia variations by lipid supplementation can be attributed to changes in protein degradation or synthesis of microbial protein[55]. In our study, diets with SO supplementation showed similar CP digestibly, but oil and glycerin diet allowed a greater CP intake than the diet with oil and without glycerin, and lowest ruminal NH_3-N concentration was observed in animals fed oil and glycerin diet at the 1st hours after morning and afternoon feeding. These findings suggest that oil and glycerin addition in the diet allowed a better N: energy synchrony at 1st hours after feeding; this could result in enhanced assimilation of N by the ruminal ammonia-utilising species; consequently, oil and glycerin combination could enable a greater microbial protein flow.

The great decrease in total ruminal protozoa with diets containing SO was expected, because UFA have been reported to directly inhibit ruminal protozoa[41]. In addition, oil supplementation improves CP digestibility, which may be associated with the low protozoa counts in these diets, as ruminal protozoa populations have a negative effect on N utilisation in ruminants[56]. Results of previous studies in bovines showed that oil supplementation can decrease the protozoa abundance and increase the proteolytic bacteria[57,38].

On the other hand, hindgut fermentation in cattle has about 14 % of the capacity for fermentation of the rumen, and becomes more important with high grain feeding owing to increased flow of undigested substrate into the hindgut[58]. Therefore, N could also be recycled into the hindgut contributing to improve CP digestibility in steers fed diets containing SO. Once in ruminants fed high-concentrate diets, the depression of diet degradation in the rumen could be balanced by compensatory action of the hindgut[45,59].

It is possible that greater CP digestibility was the determining factor that reduced the faecal N excretion in animals fed both diets containing SO. Nevertheless, from an environmental point of view, an important difference between the N lost with urine and that lost with faeces is that the main form of N in urine is urea[60]; the ammonia that is emitted from livestock manure is mainly a breakdown product of urinary urea. The faecal N is more stable and contributes little to N emissions[61].

The ruminal Archaea abundance between both no-oil diets was similar, in accordance with other studies on cattle, which have reported no effects on Archaea abundance when CG was added to diets[62,63]. However, when SO was added, the ruminal Archaea abundance decreased; this may be owing to different factors. Some of these factors could be as follows: Archaea are susceptible to UFA[42]; as oil can also reduce the H concentration in the rumen by biohydrogenation, it could act as a H sink and thereby reduce Archaea activity[64]; and the reduction in the total number of protozoa could have led to the decrease in the abundance of Archaea, as Archaea works symbiotically with protozoa to participate in H transfer.

A certain proportion of methanogens are associated with protozoa[65] and a reduction in protozoa abundance is in most cases indicative of a reduction in methane emission[66]. Together, these results suggest that such dietary supplementation might lead to a reduction in methane emissions; future studies are required to confirm this inference.

Conclusion

CG associated with SO could be used as an energy source, and is a useful partial replacement for maize in cattle diets, resulting in a reduction of the faecal N excretion and ruminal methanogen abundance. This is environmentally beneficial, as methane emissions could be reduced without affecting dietary intake and digestibility, even when high lipid content is added to the diet.

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