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Crude glycerin changes ruminal parameters, *in vitro* greenhouse gas profile, and bacterial fractions of beef cattle

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ABSTRACT

As the biodiesel industry produces a large surplus of crude glycerin, this by-product is increasingly being considered as an attractive source of energy to replace corn in livestock diets. The objective of this study was to evaluate how the inclusion of up to 30% crude glycerin in Nellore cattle diets affects ruminal parameters such as pH, ammonia, and volatile fatty acids as well as greenhouse gas production, and concentration of the protozoal and bacterial fractions. Five ruminally cannulated Nellore steers were randomly assigned in a 5×5 Latin square design and fed diets containing 30% corn silage and 70% concentrate composed of sunflower meal, corn grain, soybean hulls, minerals, and 0, 7.5, 15, 22.5, or 30% crude glycerin (860 g glycerol/kg). After 14 d of adaptation, animals were submitted to rumen content sampling for 7 d. With the supplementation of glycerin in the diets, total VFA and acetate concentrations decreased (linear, $P=0.03$, $P<0.0001$, respectively), and propionate concentrations increased (linear, $P=0.007$; quadratic, $P=0.008$), leading to an acetate to propionate ratio decrease (linear, $P<0.0001$). The rumen ammonia was not affected while pH was quadratically affected and was lesser for glycerin treatments (quadratic, $P=0.04$). Methane production was reduced (linear, $P<0.0001$) when glycerin was added, as well as the CO_2 (linear, $P<0.0001$; quadratic, $P=0.0001$; cubic, $P<0.0001$). The concentration of liquid phase microorganisms was not affected, while the concentration of particle-associated bacteria fraction was decreased by the addition of crude glycerin in the diets. The inclusion of up to 30% of crude glycerin in diets for beef cattle decreased ruminal concentration of total VFA and acetate, increased propionate, reduced concentration of particle-associated bacteria, and decreased production of methane.

1. Introduction

The increase in biodiesel production worldwide has been welcomed as a much needed alternative energy source for the challenges facing the world. However, it also results in price increases of several agricultural commodities that are used for biofuel production, including corn (Anderson et al., 2008). Besides being a staple human food, corn is an important feed grain for livestock, but its market price is driving farmers to search for alternative feed sources. As the biodiesel industry produces a large surplus of crude glycerin, this by-product is increasingly being considered an attractive source of energy to replace corn. The economic and humanitarian gains of such strategy may be auspicious as it could increase the availability of this crop for the needs of human nutrition.

Promising results regarding productivity and feed efficiency have been reported for the inclusion of up to 20% crude glycerin in diets for ruminants (Gunn et al., 2010). Because the amount of glycerin fed to the animals may affect fermentation and absorption in the rumen, studies examining greater inclusions of crude glycerin in the diet are warranted. Indeed, previous studies have shown a negative impact of glycerin on the growth of cellulolytic bacteria (Roger et al., 1992), resulting in reductions in digestibility of fiber (Donkin et al., 2009; Shin et al., 2012; Van Cleef et al., 2014), and probably decreasing the production of methane (Holter and Young, 1992). Thus, it becomes imperative to investigate whether replacing corn with a greater percentage of glycerin in cattle diets can maintain adequate ruminal conditions reducing enteric methane emissions. This is an important question because while the global demand for food availability stimulates livestock production, it also leads to increasing emissions of greenhouse gases.

The objective of this study was to evaluate how the inclusion of up to 30% crude glycerin in Nellore cattle diets affects *in vivo*

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ruminal parameters such as pH, ammonia, volatile fatty acids, and microbial concentrations as well as *in vitro* greenhouse gas production.

2. Materials and methods

The trials were conducted at Animal Science Department of São Paulo State University (UNESP), Jaboticabal Campus. The cannulation procedures and the use of the ruminally cannulated animals in these experiments were approved by the Animal Welfare and Ethics Commission from São Paulo State University (Protocol 010707).

2.1. Animals and experimental design

Five ruminally cannulated Nellore steers averaging 24 months of age and 400 kg BW were assigned to a 5 × 5 Latin square arrangement of treatments. The animals were housed in individual semi-roofed, concrete-surfaced pens (12 m^2), with feed bunks and waterers, and received the experimental diets for 21-d periods, composed of 14 d of adaptation and 7 d of data collection.

The diets contained similar protein (122 g CP/kg DM) and energy (2.5 Mcal EM/kg DM) concentrations and were formulated to supply the requirements of Nellore steers in feedlot according to NRC–National Research Council (1996), and fed to the animals in a roughage:concentrate ratio of 30:70 (Table 1). The following treatments were evaluated: G0 – (control), no crude glycerin (CGL) inclusion in the diet; G7.5–75 g CGL/kg of dietary dry matter (DM); G15–150 g CGL/kg dietary DM; G22–220 g CGL/kg dietary DM; and G30–300 g CGL/kg dietary DM. The CGL inclusion concentrations are described on a DM basis.

The CGL tested contained 860 g glycerol/kg of DM, 950 g of DM/kg, 1.1 g CP/kg of DM, 60 g salts/kg of DM, and less than 0.01% methanol. The roughage used was corn silage and the concentrate was composed of ground corn grain, soybean hulls, sunflower meal, salt, limestone, and dicalcium phosphate. The animals were fed twice daily at 0800 and 1800 h, about 8 kg (DM basis), and fresh water was available throughout the experiment. The feed delivery was controlled by observing bunk scores between 0 and 1 (Pritchard, 1993).

Table 1
Percentage of feed ingredients and nutrient composition of experimental diets.

Item	Treatments[a]				
	G0	G7.5	G15	G22.5	G30
Ingredients (g/kg DM)					
Corn silage	300.0	300.0	300.0	300.0	300.0
Corn grain	350.0	255.0	180.0	125.0	50.0
Soybean hulls	192.0	180.5	145.5	89.0	54.5
Sunflower meal	146.0	178.0	213.0	249.0	284.0
Crude glycerin	–	75.0	150.0	225.0	300.0
Salt (NaCl)	5.0	5.0	5.0	5.0	5.0
Limestone	7.0	6.5	5.5	7.0	6.5
Dicalcium phosphate	–	1.0	1.0	–	–
Calculated chemical composition					
CP (g/kg DM)	122.0	122.1	122.2	122.0	122.1
ME (Mcal/kg DM)	2.5	2.5	2.5	2.5	2.5
EE (g/kg DM)	29.0	25.8	23.1	20.9	18.1
NDF (g/kg DM)	408.3	400.7	381.1	350.1	330.8
ADF (g/kg DM)	254.1	255.6	247.1	228.9	220.6
HEM (g/kg DM)	154.2	145.1	134.1	121.2	110.2
Ca (g/kg DM)	5.6	5.6	5.5	5.7	5.5
P (g/kg DM)	3.2	3.2	3.5	3.4	3.4

[a] G0=Without crude glycerin; G7.5=75 g crude glycerin/kg DM; G15=150 g crude glycerin/kg DM; G22.5=225 g crude glycerin/kg DM; G30=300 g crude glycerin/kg DM

2.2. Rumen pH, ammonia, and VFA profiles

This trial was conducted at Laboratory of Ingredients and Pollutant Gases of the Animal Unit of Digestive and Metabolic Studies from São Paulo State University–Jaboticabal Campus.

Rumen fluid samples were collected on d 15 of each experimental period, at −1, 0, 2, 4, 6, and 8 h after feeding. Approximately 500 g of ruminal content of each animal adapted to each experimental diet were collected from the dorsal and ventral rumen, and strained through four layers of cheesecloth to separate liquid and solid phases. Five milliliters of the filtrate was preserved by storing at −20 °C in a freezer for later analysis of VFA profiles. The pH (n=1) was determined immediately after rumen fluid sampling by using a digital pH meter, and ammonia concentrations (n=2) was determined using a micro-Kjeldhal device, using 5 mL of KOH 2 N, and a distillation flux of 2 mL/min. The distilled sample was dropped in 10 mL boric acid solution (2%), and then titrated with HCl 0.005 N.

In due course, the rumen fluid samples reserved previously were thawed and VFA profiles were evaluated (n=2). For sample preparation, 1.6 mL of rumen fluid was centrifuged (15,000*g*; 15 min; 4 °C; Sorvall Superspeed RC2-B, Newton, CT, USA) with 0.4 mL of 3:1 metaphosphoric acid 25% (Vetec Química Fina Ltd) and formic acid 98–100% (Merck KGaA), and 0.2 mL of 2-ethilbutyric acid 100 mM (internal standard; PM=116.16; CAS 88-09-5; Sigma Chemie Gmbh). After centrifugation, approximately 1.2 mL of supernatant was transferred to chromatographic vials. The concentration of VFA was determined by injecting 1 μL of sample in a gas chromatograph (CG HP 7890 A; Injector HP 7683B, Agilent Technologies, Palo Alto, CA, USA) equipped with a HP-FFAP capillary column (19091 F-112; 25 m; 0.320 mm; 0.50 μm; J&W Agilent Technologies Inc.; Palo Alto, CA, USA). The calibration curve was made using chromatographic standards (Chem Service, West Chester, PA, USA) of acetic acid (99.5%; CAS 64-19-97), propionic acid (99%; CAS 79-09-4), isobutyric acid (99%; CAS 79-31-2), butyric acid (98.7%; CAS 107-92-6), isovaleric acid (99%; CAS 503-74-2), and valeric acid (99%; CAS 109-52-4).

2.3. Gas measurements

The incubation took place between d 16 and d 18 of each experimental period. The methodology adopted in this trial for determination of *in vitro* gas production was adapted from Pereira et al. (2006), and consists of three main steps:

Sample preparation: Approximately 4 kg of ruminal content of each adapted animal to each diet were manually collected, and strained through a nylon filter (100 μm); 125 mL of the filtrate was then transferred to 250 mL plastic Erlenmeyers containing 1.7 g of each diet used (n=3), in order to maintain a 1:8 ratio of sample and ruminal liquid. Two Erlenmeyers with only rumen fluid were used as blanks for each treatment in each period.

Gas production and storage: The Erlenmeyers were purged with anaerobic gas, capped with stoppers, and attached to a gasometer made of PCV pipe, and 500-mL PET bottles. The device was incubated in a water-bath at 39 °C for a 12-h period.

Gas sampling and qualitative analysis: The total gas production was measured using a graduated ruler and correlated with a standard curve previously made to convert height to volume, according to the bottle area. The gas was sampled with a 1-mL syringe and 0.5 mL was immediately injected into a gas chromatograph (Trace GC Ultra™, Thermo Scientific). The GC was equipped with a Porapak column and molecular sieve. The oven temperature was set to 70 °C, and the injector temperature used was 110 °C. The carrier gas used was argon, with 25 mL/min flow.

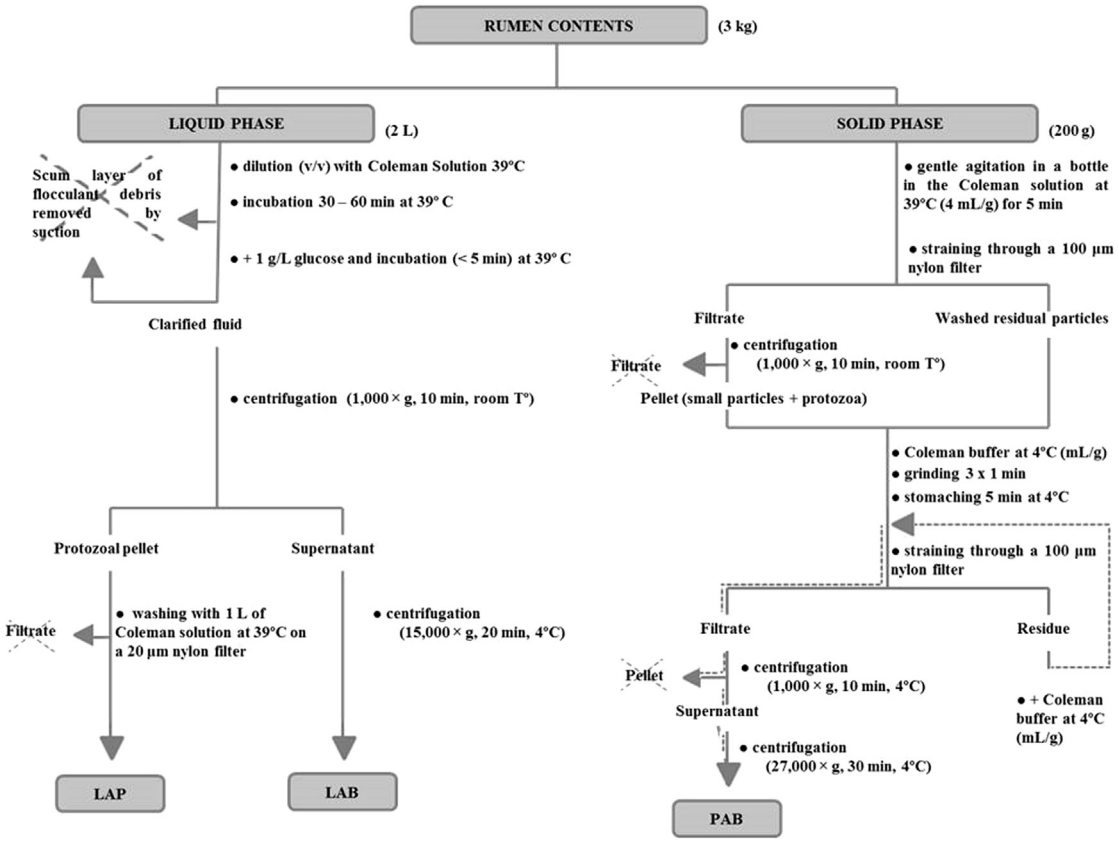

Fig. 1. Procedure to isolate liquid-associated protozoa (LAP) and bacteria (LAB) and particle-associated bacteria (PAB).

2.4. Protozoal and bacterial fractions

This trial used methodology adapted from Martin et al. (1994), illustrated in Fig. 1. Approximately 3 kg of ruminal content was sampled from the dorsal and ventral rumen from each adapted animal in each period, 2, 4, and 12 h after morning feeding. To avoid any interference in ruminal function only one sample of ruminal content was taken per day, between d 19 and d 21 of each experimental period.

The ruminal content was strained through a nylon filter (100 μm) in order to separate liquid and solid phases. Two hundred grams of the solid content was weighed, of which 30 g was used for DM analyses, and 170 g was used for isolation of particle-associated bacteria (PAB). The solid material was initially washed by manual shaking with a pre-warmed (39 °C) Coleman saline solution (0.63% K_2HPO_4, 0.5% KH_2PO_4, 0.065% $NaCl \cdot 6H_2O$, 0.09% $MgSO_4 \cdot 7H_2O$, 0.5% cysteine hydrochloride), using a ratio of 1 g of solid to 4 g of saline solution. The content was filtered through a nylon filter (100 μm). The filtrate was then centrifuged at room temperature (1000*g*, for 10 min). The pellet of small particles obtained was added to the content retained on the filter. This combined material was suspended in pre-cooled Coleman solution (4 °C), and homogenized at 200 rpm for 5 min using a Stomacher device (Seward and Co., London). The material was filtered again and the solid retained was discarded. The filtrate was centrifuged (1000*g*, for 10 min, at 4 °C) and the supernatant was centrifuged again (27,000*g*, for 30 min, at 4 °C). The resulting pellet was considered as the PAB. The bacterial material was transferred to 100-mL plastic flasks and dried at 55 °C for 72 h. Subsequently, the flasks with dried PAB were weighed and reserved for further analyses of DM, mineral matter, and N (AOAC, 1990).

From the liquid phase, separated at the beginning from the 3 kg of ruminal content, 700 mL was diluted with pre-warmed (39 °C) Coleman saline solution (1:1) and incubated in water bath at 39 °C for 30 min, until the flocculation was complete. After 25 min of incubation, 1 g/L glucose was added in order to separate the protozoa from the rest of the ruminal liquid. After that, 400 mL of clarified fluid was centrifuged (1000*g*, for 10 min, at room temperature) and the pellet of liquid-associated protozoa (LAP) was recovered. The pellet was washed with a pre-warmed Coleman saline solution (39 °C), and filtered through a 20-μm nylon filter.

The liquid-associated bacteria (LAB) was recovered by centrifuging the protozoa-free supernatant (15,000*g*, for 20 min, at 4 °C). The pellets of LAP and LAB were transferred to 100-mL plastic flasks and dried at 55 °C for 72 h. Subsequently, the flasks with dried microorganisms were weighed and reserved for further analyses of DM, mineral matter, and N (AOAC, 1990).

2.5. Statistical analysis

The variables NH_3–N, pH, VFA, and microbial fractions (LAB, PAB, and LAP) were analyzed as a 5 × 5 Latin square with repeated measures, using the MIXED procedure of SAS, version 9.1. The model included fixed effects of treatment, time, and their interaction, as well as random effects of animal and period. Several covariance structures were tested and the best one was chosen for each variables, based on Akaike information criterion. The degrees of freedom and tests were adjusted using the option KR. The linear, quadratic and cubic effects of crude glycerin were tested, as well as the contrast control treatment × crude glycerin treatments. Data from the gas production study were analyzed as a 5 × 5 Latin square and the same effects were evaluated. The significance was declared as $P < 0.05$, and tendency was declared as $P \leq 0.10$.

Table 2
Effects of crude glycerin on ruminal volatile fatty acids, pH, and ammonia nitrogen in Nellore steers.

Item	Treatment (% Crude glycerin)[a]					SE	Contrast, *P*-value[b]			
	0	7.5	15	22.5	30		L	Q	C	0 × Gly
VFA, mM/L										
Total VFA	136.1	122.2	118.7	115.1	120.2	6.6	0.03	0.42	0.71	0.03
Acetic acid	86.6	73.4	59.9	54.1	49.3	4.9	< 0.0001	0.39	0.68	0.0001
Propionic acid	24.5	22.3	24.1	29.9	33.1	1.56	0.007	0.008	0.97	0.54
Isobutyric acid	1.29	1.26	1.28	1.18	1.27	0.09	0.25	0.53	0.48	0.47
Butyric acid	19.3	21.0	28.8	24.6	30.5	2.7	0.04	0.25	0.11	0.07
Isovaleric acid	2.16	2.10	1.94	2.01	2.10	0.25	0.50	0.73	0.76	0.52
Valeric acid	2.02	2.11	2.61	3.41	4.00	0.23	0.0001	0.13	0.91	0.01
Acetate:propionate	3.65	6.56	2.82	1.92	1.53	0.16	< 0.0001	0.50	0.41	< 0.0001
pH	6.39	6.11	6.20	6.27	6.23	0.11	0.48	0.04	0.32	0.05
NH_3-N, mg/dL	20.0	20.5	15.0	15.9	18.3	3.09	0.10	0.93	0.24	0.30

[a] G0 = Without crude glycerin; G7.5 = 75 g crude glycerin/kg DM; G15 = 150 g crude glycerin/kg DM; G22.5 = 225 g crude glycerin/kg DM; G30 = 300 g crude glycerin/kg DM.
[b] L = Linear; Q = Quadratic; C = Cubic; 0 × Gly = Treatment G0 × Glycerin treatments

3. Results

3.1. Rumen pH, ammonia, and VFA profiles

Table 2 shows the effects of the inclusion of increasing concentrations of crude glycerin in the Nellore cattle diet on ruminal parameters. The total VFA concentration linearly decreased ($P=0.03$) with the inclusion of increasing concentrations of crude glycerin. There was a linear reduction in the concentration of acetic acid ($P<0.0001$), and a quadratic effect in the concentration of propionic acid ($P=0.008$), leading to a linear decrease in the acetate:propionate ratio ($P<0.0001$). Glycerin supplementation also increased butyric acid (linear, $P=0.04$) and valeric acid (linear, $P=0.0001$) concentration, however no changes were observed for isobutyric and isovaleric acids. Additionally, a treatment × sampling time interaction was observed for butyric acid ($P=0.03$), with the greatest average values observed 4 h post-feeding (32.08 mM/L) and the least, 1 h before feeding (17.16 mM/L).

No changes were observed in ruminal NH_3–N concentrations when crude glycerin was added up to 30% in Nellore cattle diets, however pH values were lesser in animals fed glycerin when compared with controls (P = 0.05), and presented a quadratic effect with the least value being observed for treatment with 7.5% crude glycerin (pH = 6.11, SE = 0.11). Time of sampling had an effect on pH ($P<0.01$). The values observed for the treatment without crude glycerin were close to 5.7 between 2 and 4 h post-feeding, tending ($P=0.06$) to be lesser when compared the other treatments.

3.2. Protozoal and bacterial fractions

The inclusion of crude glycerin in the diet, regardless of the concentration, decreased the concentration of PAB ($P=0.003$), and tended to promote a quadratic effect on this variable ($P=0.05$, Table 3). Although the concentration of OM of PAB did not change with the inclusion of crude glycerin, the concentration of PAB (mg

Table 3
Effects of crude glycerin on production and composition of protozoal and bacterial fractions of Nellore steers.

Item[a]	Treatment (% Crude glycerin)[b]					SE	Contrast, *P*-value[c]			
	0	7.5	15	22.5	30		L	Q	C	0 × Gly
PAB										
mg DM/kg	4046.5	3722.2	3809.8	3779.0	3471.2	128	0.03	0.05	0.11	0.003
%OM	72.2	71.9	71.5	72.1	71.5	0.86	0.86	0.51	0.71	0.68
mg OM/kg	2919.9	2679.3	2723.6	2727.1	2483.5	84	0.02	0.02	0.15	0.001
mg N/kg DM	239.8	225.7	235.7	233.8	215	26.3	0.72	0.26	0.13	0.19
%N/OM	9.25	9.53	9.82	9.69	9.76	0.27	0.03	0.19	0.51	0.02
LAB										
mg DM/L	2416.2	2268.3	2401.7	2165.1	1915.2	155	0.26	0.71	0.24	0.33
%OM	62.1	62.1	62.2	61.5	61.1	1.17	0.62	0.64	0.82	0.87
mg OM/L	1502	1410.5	1501	1333	1170.2	90.8	0.25	0.63	0.22	0.34
mg N/L	152.3	120.6	162.1	107.7	106.1	13.9	0.04	0.22	0.0006	0.06
%N/OM	9.70	8.40	10.39	7.90	8.97	0.72	0.22	0.34	0.01	0.26
LAP										
mg DM/L	2265.3	2083.4	2007.1	2051.5	1956.3	156	0.22	0.38	0.97	0.16
%OM	59.7	59.8	59.1	58.9	55.8	0.71	0.14	0.68	0.47	0.45
mg OM/L	1252.5	1240.3	1185.1	1208.2	1084.3	94	0.17	0.39	0.95	0.13
mg N/L	87	86.9	71.1	84.5	73.1	8.7	0.90	0.16	0.73	0.19
%N/OM	6.28	5.34	5.93	6.73	6.65	0.56	0.44	0.14	0.61	0.67

[a] PAB = Particle-associated bacteria; LAB = Liquid-associated bacteria; LAP = Liquid-associated protozoa.
[b] G0 = Without crude glycerin; G7.5 = 75 g crude glycerin/kg DM; G15 = 150 g crude glycerin/kg DM; G22.5 = 225 g crude glycerin/kg DM; G30 = 300 g crude glycerin/kg DM.
[c] L = Linear; Q = Quadratic; C = Cubic; 0 × Gly = Treatment G0 × Glycerin treatments.

OM/kg), was lesser in treatments with this co-product, when compared with control treatment ($P=0.001$), and showed a quadratic effect of treatments ($P=0.02$). The concentration of nitrogen in PAB (mg N/kg), did not change among treatments, however the concentration of N (%N/OM) increased ($P=0.02$) with glycerin supplementation, and showed a linear effect with the treatments ($P=0.03$).

There was interaction of glycerin treatments versus sampling time for the concentration of PAB (mg DM/kg, $P=0.02$ and mg OM/kg, $P=0.01$). At 4 h post-feeding with the glycerin treatments, we observed the least concentration of PAB.

The results presented in Table 3 show that the glycerin treatments did not affect the concentration of LAB (mg DM/L and mg OM/L). We also observed cubic effects of glycerin treatments on N concentration of LAB (P= 0.0006), and in the percentage of N of this bacterial fraction ($P=0.01$). Comparing glycerin treatments with controls, a tendency of decreasing concentration of N (mg N/L) in LAB was observed ($P=0.06$). The average values observed for LAP were 2072.7 mg DM/L, 58.7% OM, 1194.1 mg OM/L, 80.5 mg N/L, and 6.2%N/OM (Table 3). There was interaction between treatment and post-feeding time ($P=0.02$). Treatments with 15% crude glycerin and control diets increased the percentage of OM of LAP at 2 h post-feeding, when compared with the other treatments. Furthermore, the inclusion of 30% crude glycerin exhibited the least values at 4 and 12 h post-feeding.

3.3. Greenhouse gases measurements

The greater the concentration of crude glycerin added to the diets, the lesser was the production of methane *in vitro* after 12 h of incubation ($P<0.0001$). Indeed, regardless of the concentration of glycerin added, methane production was always lesser than in the controls ($P=0.0001$). The inclusion of crude glycerin also affected *in vitro* production of ruminal CO_2 ($P=0.02$), with a cubic effect observed for the treatments ($P<0.0001$), in which the least values were observed for those containing 7.5 and 30% of crude glycerin (Table 4).

4. Discussion

This study appears to be the first study using such elevated concentrations of glycerin. This is also important in the field of human nutrition, once it could mitigate the competition for feed ingredients between humans and animals. We report that the inclusion of 30% crude glycerin in beef cattle diet led to the withdrawal of 86% of corn from the animal rations and reduced emission of enteric methane. However, further studies are necessary to elucidate the effects on digestibility of dietary fiber and starch, identification of microorganism species associated with animal performance results.

4.1. Ruminal parameters

Increasing addition of crude glycerin to Nellore diets led to an unexpected linear decrease in total VFA because the pronounced reduction in acetic acid (as much as 43.1%) was not matched by the increase in propionic acid (as much as 35.1%). Indeed in the literature (Boyd et al., 2013; Chanjula et al., 2014), total VFA is not affected by glycerin diets, even when the proportions of acetic and propionic acid vary. For instance, when Ramos and Kerley (2012) and Chanjula et al. (2014) replaced dietary corn with up to 20% glycerin, they observed no changes in total VFA although the production of acetate linearly decreased and propionic acid increased. Previous studies such as the one published by Boyd et al. (2013) have shown that even adding small concentrations of glycerin to the diets (up to 4%), it is possible to detect the same effect (acetic acid reduction and propionic acid increase), albeit with less intensity. The observed reduction in acetic acid may be explained by the deleterious selection of fibrolytic microorganisms, which are known to be sensitive to glycerin (Abo El Nor et al., 2010).

The inclusion of crude glycerin leads to a reduction in the amount of non-fiber carbohydrate in the diet. However, crude glycerin has similar fermentative characteristics, generating mostly propionic, and less butyric and valeric acids. Once ingested and in the animal rumen, glycerol mostly disappears in the first 24 h (Trabue et al., 2007).

Previous studies have reported the deleterious effect of glycerin on fiber digestibility (Donkin et al., 2009; Shin et al., 2012). Glycerin inclusion reduces the number of microorganisms involved in fiber digestion (Roger et al., 1992; Abo El-Nor et al., 2010); leading to the reduction of acetic acid, which is a by-product of fiber digestibility, and consequently reduces methane production, as we observed herein.

The linear increase observed in the production of butyric and valeric acids was also reported by Remond et al. (1993), Wang et al. (2009), and Shin et al. (2012). Glycerin is largely metabolized by specific groups of bacteria such as *Megasphaera elsdenii* and *Selenomonas ruminantium* (Stewart et al., 1997; Krehbiel, 2008). *Megasphaera elsdenii* produces butyric acid as one of its end products from the fermentation of lactic acid (Klieve et al., 2003), which is increased with inclusion of glycerin. On the other hand, *Selenomonas ruminantium* ferments glycerin and converts the succinate produced in the rumen by other bacteria to propionic acid (Wolin et al., 1997). Thus, the addition of crude glycerin in cattle diets may increase *Megasphaera elsdenii* and *Selenomonas ruminantium* populations resulting in changes in VFA profiles, increasing concentration of propionate and butyrate, as showed herein. The increased concentration of butyric acid plays an important role in rumen health, since it provides the rumen epithelium most of its energy requirements (Schröder and Südekum, 1999).

Decreases in ruminal pH may be deleterious to the fermentative activity of ruminal microorganisms (Strobel, Russell 1986). Several studies reported that the addition of crude glycerin reduces the ruminal pH (Kijora et al., 1998; Mach et al., 2009; Ramos

Table 4
Effects of crude glycerin on *in vitro* enteric greenhouse gas concentrations in Nellore steers

Item (mL/g DM)	Treatment (% Crude glycerin)[a]					SE	Contrast, *P*-value[b]			
	0	7.5	15	22.5	30		L	Q	C	0 × Gly
CH_4	12.03	10.97	7.64	6.99	6.43	0.39	< 0.0001	0.12	0.18	< 0.0001
CO_2	55.82	54.11	55.34	57.80	50.56	0.47	0.0006	0.0001	< 0.0001	0.02

[a] G0=Without crude glycerin; G7.5=75 g crude glycerin/kg DM; G15=150 g crude glycerin/kg DM; G22.5=225 g crude glycerin/kg DM; G30=300 g crude glycerin/kg DM.

[b] L=Linear; Q=Quadratic; C=Cubic; 0 × Gly=Treatment G0 × Glycerin treatments.

and Kerley, 2012), just as we observed herein for intermediate concentrations of glycerin. However, all values obtained in this study remained within the range considered as optimal for the fermentative activity of bacteria, between 6.0 and 6.4 (Van Soest, 1994).

The protein source used in present study derived from sunflower meal, which is greatly soluble in the rumen (over 93%, Van Cleef, 2012). This may be the reason underlying the similar values of ruminal ammonia nitrogen observed among treatments. The average values obtained for this variable ranged from 15 to 20.5 mg NH_3-N/dL, considered adequate for ruminal fermentative activities. Leng (1990) reported that ruminal ammonia nitrogen value must be greater than 10 mg NH_3-N/dL to maximize ruminal digestion of DM and greater than 20 mg NH_3-N/dL to increase ruminant DM intake.

4.2. Protozoal and bacterial fractions

The reduction in the concentration of PAB caused by the inclusion of crude glycerin in the diets is consistent with other reports that revealed reduction of the bacteria groups *Butyrivibrio fibrisolvens* and *Ruminococcus flavefaciens* (Abo El-Nor et al., 2010; Roger et al., 1992). These groups of bacteria are responsible for the digestion of the fibrous portion of feedstuff and are some of the microorganisms composing the PAB fraction (Michalet-Doreau et al., 2001). Because glycerin is liquid, its inclusion removes particulate ingredients from the diet, thereby reducing PAB.

The reason underlying the decrease in the concentration of nitrogen of LAB, which occurred without a decrease in the concentration of this fraction, is not clear. A possibility is that the inclusion of crude glycerin in the diets changes the osmolarity of the rumen fluid thus affecting the permeability of the bacterial membrane and changing the bacterial nutrient concentration. Although Mach et al. (2009) reported no effect on ruminal fluid osmolarity when they included up to 12% crude glycerin in diets, the explanation just given may still stand because in the present study the concentrations of glycerin added were greater.

Crude glycerin addition to diets did not affect the concentration of protozoa. This result contrasts to that reported by Fávaro et al. (2014), who showed a decrease in the LAP with inclusion of up to 20% crude glycerin in cattle diets. Here we found that LAB were also not affected, suggesting that both microorganism fractions associated with the liquid phase of rumen content was not affected by crude glycerin inclusion.

Taken together, our data suggest that the deleterious effect observed in PAB (Table 3), which is associated to the solid phase of the rumen content, is mostly related to the adherence of bacteria to feed particles.

4.3. Greenhouse gases

Recent studies have shown contrasting results of gas production, specifically methane, probably due to different methodologies adopted. Meale et al. (2013) and Avila-Stagno et al. (2013) found no effect of addition of glycerin on those parameters using maximal concentrations of 12 and 21% of this by-product, respectively. However, the decrease observed in methane concentration with the addition of glycerin, as also demonstrated in prior studies (Lee et al., 2011), could be a result of the decreased rumen fermentation of diets, evidenced by total VFA decrease, reduced acetate:propionate ratio generated by the fermentation of these diets, as well as the effect of decreasing concentration of NDF in treatments with greater concentrations of crude glycerin. Thus, the amount of H_2 in the rumen decreases, and hence its availability for methanogen microorganisms, which use it to reduce CO_2 to CH_4 (Van Soest, 1994; Janssen, 2010). The decrease in the production of CO_2 was lesser than that observed for CH_4 (as much as 46.6 and 9.4%, respectively). The disproportionate reduction of these gases may indicate a reduction in the population of methanogens, considering that much of the rumen methane comes from CO_2 (McAllister and Newbold, 2008) and that methane production decreased dramatically, even with substrate CO_2 available in the environment. Besides being indicators of energy loss by the animals (estimated between 6 and 18% of the diet gross energy), these gases are important factors in global warming (Pedreira and Primavesi, 2006). Thus, the inclusion of crude glycerin in the diet of ruminants becomes an interesting alternative for mitigating methane emission by livestock.

5. Conclusions

The inclusion of up to 30% of crude glycerin in diets for beef cattle decreased ruminal concentration of total VFA and acetate, increased propionate, reduced production of particle-associated bacteria, and decreased production of methane.

Further studies using greater concentrations of crude glycerin in the diets of beef cattle are needed to evaluate the optimal concentration able to maintain productive and economic efficiency of beef production.

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