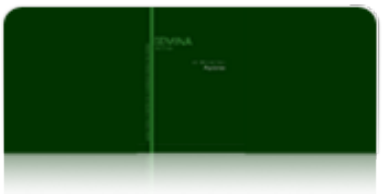

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Crude glycerin as an alternative to corn as a supplement for beef cattle grazing in pasture during the dry season

Glicerina bruta como alternativa ao milho no suplemento de bovinos de corte em pastagem durante a estação seca

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Abstract

Two experiments were conducted to evaluate the effects of inclusion of crude glycerin (CG) in the supplement (0, 70, 140, 210, and 280 g kg^{-1} dry matter (DM) of supplement) of Nellore cattle grazing tropical grasses during dry season. In Experiment 1, intake, digestibility, ruminal fermentation, and the rumen microbial profile were evaluated in two simultaneous 5 × 5 Latin squares, using 10 ruminally cannulated Nellore steers (408.8 ± 38.5 kg of body weight (BW)). In Experiment 2, cattle growth performance was evaluated in 50 young Nellore bulls (279.52 ± 16.3 kg of BW) distributed in a randomized complete block design. The increasing inclusion of CG did not affect intake (P=0.813), diet digestibility (P=0.895), however linearly increased pH (P=0.001), butyrate concentration ($P < 0.001$) and *Fibrobacter succinogenes* (P=0.003) population. CG inclusion linearly decreased total ruminal volatile fatty acids (VFA) ($P < 0.001$), acetate concentration ($P < 0.001$) and quadratically affected (P=0.009) acetate: propionate ratio. In experiment 2, the inclusion of CG quadratically affected DM intake (DMI) (P=0.005), DM total-tract apparent digestibility ($P < 0.001$), linearly increased additional gain ($P > 0.001$), average daily gain ($P > 0.001$) and feed efficiency ($P > 0.001$). CG in the supplement of Nellore steers grazing tropical grass during dry season doesn't affect intake and digestibility but alters ruminal fermentation, without negative effect on relative proportion of cellulolytic bacteria population. The increasing replacement of corn grain by CG in the supplement of pasture-raised growing Nellore bulls (up to 280g/kg DM) improved BW gain and consequently feed efficiency.

Key words: Body weight. Forage. Glycerol. Nellore. Rumen Bacteria.

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Resumo

Foram realizados dois experimentos para avaliar os efeitos da inclusão de glicerina bruta (GB) no suplemento (0, 70, 140, 210 e 280 g kg^{-1} de suplemento) de bovinos Nelore pastejando gramíneas tropicais durante a estação seca. No experimento 1, o consumo, digestibilidade, fermentação ruminal e o perfil microbiano do rúmen foram avaliados em dois quadrados latinos 5x5 simultâneos, usando 10 novilhos Nelore canulados no rúmen (408,8 ± 38,5 kg de PC). No Experimento 2, o desempenho do crescimento do gado foi avaliado em 50 novilhos Nelore (279,52 ± 16,3 kg de PC) distribuídos em delineamento em blocos casualizados. A inclusão crescente de GB não afetou o consumo (P = 0,813), a digestibilidade da dieta (P = 0,895), mas aumentou linearmente o pH (P = 0,001), a concentração de butirato (P < 0,001) e a população de *Fibrobacter succinogenes* (P = 0,003). A inclusão de GB diminuiu linearmente os ácidos graxos voláteis totais (AGVs) no rúmen (P < 0,001), a concentração de acetato (P < 0,001) e afetou quadraticamente a relação acetato: propionato (p = 0,009). No experimento 2, a inclusão de GB modificou quadraticamente o consumo de matéria seca (CMS) (P = 0,005), a digestibilidade aparente total (P < 0,001), aumentou linearmente o ganho adicional (P > 0,001), o ganho médio diário (P > 0,001) e a eficiência alimentar (P > 0,001). GB no suplemento de novilhos Nelore pastejando gramíneas tropicais durante a estação seca não afeta a ingestão e digestibilidade, mas altera a fermentação ruminal, sem efeito negativo sobre a proporção relativa de bactérias celulolíticas. A crescente substituição do grão de milho por GB no suplemento touros Nelore em crescimento criados em pastagem (até 280g / kg de DM) melhorou o ganho de peso e consequentemente a eficiência alimentar**.**

Palavras-chave: Peso corporal. Forragem. Glicerol. Nelore. Bactérias ruminais**.**

Introduction

Supplementation of grass-fed beef cattle during the growth phase may be an attractive way to improve the efficiency of beef production. Recent studies have shown that improved performance because of concentrate supplementation during the growth phase in grazing systems is maintained during the finishing phase (DUCKETT et al., 2014). Furthermore, growth in the biofuel industries has resulted in volatile future markets and increased the cost of the grains traditionally used in cattle supplements. Thus, the use of alternative feedstuffs may be economically interesting to maintain or increase profitability.

Crude glycerin (CG), a by-product of the biodiesel industry, has become an attractive ingredient to replace grains in ruminant diets as a viable energy source (PARSONS et al., 2009; HALES et al., 2013), with the potential for continuous growth in its production over the next several years (QUISPE et al., 2013). In the rumen, CG is converted to propionate and acts as a precursor for hepatic glucose synthesis (CHUNG et al., 2007), providing energy for cellular metabolism (GOFF; HORST, 2001). Thus, it is expected that an addition to the glucose supply would increase lipogenesis and weight gain in cattle supplemented with CG.

In feedlot diets, CG decreased dry matter (DM) intake but increased feed efficiency (PARSONS et al., 2009; HALES et al., 2013), average daily gain (PARSONS et al., 2009), and duodenal flow of unsaturated fatty acids (GRANJA-SALCEDO et al., 2017a). Nevertheless, the addition of CG may improve the efficiency of ruminants fed forage more than that of those fed concentrate diets (DROUILLARD, 2008) by enhancing the digestibility of forage diets (AVILA et al., 2014). CG is a viable feed additive for ruminants consuming roughage-based diets (HESS et al., 2008) and was recently studied in grazing pasture finished cattle (FACURI et al., 2014; SAN VITO et al., 2015).

During warm-season grass feeding, the addition of CG at up to 4% of DM reduced *in situ* fiber degradation but did not affect forage intake or total

apparent digestibility in beef cattle (SAN VITO et al., 2016). However, the literature on the feeding values and ruminal fermentation parameters of CG remains inconclusive, in particular, for animals grazing tropical grasses during the dry season. In tropical conditions, the dry season is the most critical phase of the cattle grazing system (REIS et al., 2009). Thus, further studies are warranted to elucidate the mechanism of CG metabolism in the digestive tract (WANG et al., 2009). Based on this, we hypothesized that the addition of CG to the supplement of growing cattle grazing tropical grasses during the dry season could change their rumen fermentation, without negatively affecting digestibility, leading to an improvement in animal performance. This study aimed to evaluate the effects of increasing concentrations of CG (replacing corn as an energy source) in the supplement of growing Nellore cattle grazing tropical grasses during the dry season on growth performance, total tract digestibility, ruminal fermentation, and the ruminal microbiological profile.

Materials and Methods

This study was carried out in strict accordance with the recommendations in the Brazilian College of Animal Experimentation (COBEA - Colégio Brasileiro de Experimentação Animal) guidelines and was approved by the Ethics, Bioethics, and Animal Welfare Committee (CEBEA - Comissão de Ética e Bem Estar Animal) of the Faculdade de Ciências Agrárias e Veterinárias, UNESP - Univ Estadual Paulista - Jaboticabal campus (protocol number 021119/11).

Animals and management

Two experiments were simultaneously conducted during the dry season (June to November 2011) at the São Paulo State University (Julio de Mesquita Filho" - UNESP, in Jaboticabal, São Paulo State, Brazil (21°15′22″ S latitude, 48°18′58″ 77 W longitude, and 595 m elevation). The typical climate is classified as humid subtropical, with mild, dry winters and hot, wet summers; during the experimental period, the average monthly precipitation was 19.4 mm, with an average maximum monthly temperature of 34.1 °C and average minimum monthly temperature of 8.1 °C. The pasture used as the site of this study was planted in 2009 (*Brachiaria brizantha* 'Xaraes') and was divided into 10 paddocks of 1.8 ha each by electric fencing. Each paddock was served by an automatic metal water trough with a capacity of 1000 L and collective covered plastic feeders to provide the supplement.

The treatments included CG at 0, 70, 140, 210, and 280 g kg^{-1} dry matter (DM) in the supplement. Crude glycerin (870.98 g kg^{-1} DM; 50.72 g kg^{-1} mineral matter; 10.15 g kg^{-1} crude protein (CP); 10.81 g kg^{-1} ether extract (EE); 800.34 g kg^{-1} glycerol; and 0.3 g kg^{-1} methanol) was acquired from a soybean-oil-based biodiesel production company (ADM, Rondonópolis, Brazil). Supplements were formulated according to Valadares Filho et al. (2010) for steers of body weight (BW) of 300 kg and 750 g d^{-1} of average daily gain, containing approximately 75.98 g kg^{-1} DM of total digestible nutrients (TDN). Supplemental concentrations of corn gluten meal were increased with increasing CG to maintain a similar concentration of CP in the DM. Ingredients were sampled every 15 d to determine the proportion of ingredients and chemical composition of the supplements (Table 1). Supplements were not stored for more than three days after mixing. In both experiments, animals were group-supplemented at the rate of 700 g 100 kg^{-1} BW, daily at 10h00, in collective feed bunks arranged in each paddock. The BW of individual animals was recorded at the beginning of each period, without fasting, in order to adjust supplementation supply in both experiments.

Table 1. Ingredients and chemical composition of supplement and pasture of *Brachiaria brizantha* cv. Xaraés.

	Crude glycerin in the supplement (g kg^{-1} DM)					
Item	0	70	140	210	280	Forage*
Supplement composition, g kg^{-1}						
Corn grain	490	395	317	239	160	-
Crude glycerin	-	70	140	210	280	-
Soybean meal	460	460	460	460	460	-
Urea/ammonium sulfate	10	10	10	10	10	-
Commercial premix†	40	40	40	40	40	-
Chemical composition						
Dry matter	929	926	924	921	919	914 ± 3.4
Ash, g kg^{-1} DM	74	77	80	84	87	53 ± 6.8
Crude protein, g kg^{-1} DM	343	352	350	348	347	64 ± 6.7
Neutral detergent fiber‡, g kg^{-1} DM	197	182	168	155	142	708±21.5
Ether extract, g kg^{-1} DM	29	27	25	23	21	7.3 ± 1.2

* = Average and standard deviation of the mean of samples obtained by technique of simulated grazing in five periods. †Composition = Ca: 210g kg^{-1}; P: 20g kg^{-1}; S: 37g kg$^{-1;}$ Na: 80g kg^{-1}; Cu: 490mg kg^{-1}; Mn: 1.424mg kg$^{-1;}$ Zc: 1.83mg kg$^{-1;}$ I: 36mg kg^{-1}; Co: 29mg kg$^{-1;}$ Se: 9mg kg^{-1}. ‡NDF assayed with a heat stable α-amylase and expressed exclusive of residual ash.

Forage characteristics

The grazing method used was the continuous grazing system (ALLEN et al., 2011). Forage height were randomly measured weekly at 80 points ("hits") per paddock by using a graduated stick (BARTHRAM, 1985) to estimate the average paddock height. In order to estimate the herbage mass, 5 samples per paddock (average spot heights) were collected from a 0.25 m^2 area (5 cm residual height) every 28 days (June to November 2011). Clipping samples were then dried at 55 ± 5 °C to constant weight under forced air to estimate the DM availability per hectare. The paddocks had an average forage mass of 10048.8, 9059.7, 9652.1, 9484.7, and 9365.8 kg DM ha^{-1} and an average sward height of 30.2, 25.6, 22.2, 21.8, and 23.2 cm, respectively for treatments with 0, 70, 140, 210, and 280 g kg^{-1} DM of CG in the supplement. To estimate forage nutritive value, samples were hand-plucked every 28 d at the same time as herbage mass sampling (20 average spot heights in each paddock; according to Johnson (1978), dried at 55 ± 5 °C to constant weight under forced air and then ground through a 1 mm screen in a shear mill (Thomas-Wiley Laboratory Mill Model 4, H. Thomas Co.) for further analysis.

Ruminal fermentation study (Exp. 1)

Two 5 x 5 Latin square trials, balanced for residual effects, using ten ruminally cannulated Nellore steers (BW 408.8 ± 38.5 kg and 18 months of age) were used to assess the effect of different concentrations of CG in the supplement on intake, nutrient digestibility, ruminal pH, ammonia-N (NH_3-N) concentration, volatile fatty acids (VFA), and ruminal microbial profile over five 14 d periods. Each period consisted of 10 days for adaptation to the treatments and 4 days for sampling. Initially, the animals were weighed, identified, and treated against endo- and ectoparasites by the administration of ivermectin (Ivomec, Merial, Paulínea, BR),

and allocated (1 animal/paddock) into the same paddocks as animals from the performance study. Intake and digestibility estimation was performed in all periods.

Ruminal pH, NH_3-N, and VFA were measured on day 11 of each period. Aliquots of 50 mL of ruminal contents were obtained at 0, 3, 6, 12, and 18 h after supplementation. Ruminal fluid was obtained from several sites within the rumen and was subsequently strained through two layers of cheesecloth. Ruminal pH was immediately measured using an electric pH meter (Nova Técnica, PHM, Piracicaba, SP). After pH measurement, the samples were acidified by the addition of 1 mL of 9 M H_2SO_4 and then stored at -20 °C for further analysis of NH_3-N and VFA concentration. Ruminal fluid NH_3-N was analyzed by distillation with 2 M KOH in a micro-Kjeldahl system, according to the procedures of Fenner (1965). The samples collected for analysis of VFA were centrifuged at 13,000 x *g* (4 °C) for 30 min and quantified by gas chromatography (GC2014, Shimatzu Corporation, Kyoto, Japan) with an HP-INNOWax capillary column (30 m x 0.32 mm; 0.50 µm film thickness; Agilent Technologies, Colorado, USA) at an initial temperature of 80 °C and a final temperature of 240 °C.

Ruminal microbial (bacteria, archaea, and protozoa) samples were collected on d 11, 3h after supplementation. For protozoa, population cell counts were determined from aliquots of rumen content, obtained by taking a handful of ruminal contents from mid-point of the rumen, that were fixed in equal volume of 18.5% formalin, according to D'Agosto and Carneiro (1999). Each sample was homogenized, and 1 mL of ruminal content was pipetted and transferred to vials with lugol, after 15 min, 9 mL of glycerin at 30% was added to the vials. To quantify the protozoa, 1 mL of content from each vial was pipetted to fill the Sedgewick-Rafter chamber, according to Dehority (1984).

For ruminal cellulolytic bacteria (*Ruminococcus albus, Fibrobacter succinogenes, Ruminococcus flavefaciens*) and total rumen methanogens (archaea) only samples from treatments with 0 and 280 g kg^{-1} DM of CG were analyzed. Fifty grams of the ruminal content was obtained by taking a handful of rumen contents from mid-point of the rumen placed in a container. A second handful of rumen contents was squeezed to obtain liquid, which was added to the same container. The samples were weighed and immediately processed to obtain a bacterial pellet as describe by Granja-Salcedo et al. (2017b).

DNA extraction was conducted in 250 mg of bacterial pellet using the extraction kit FastDNA® SPIN Kit for Soil (MP Biomedical, LLC). The integrity and quantity of DNA was verified on a 0.8% agarose gel stained with ethidium bromide (5 mg mL^{-1}); size was estimated by comparison with 1-kb plus DNA ladder marker (Invitrogen, Waltham, MA, USA), and the yield and quality of DNA were evaluated by spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA, USA) and by fluorometry (Qubit 3.0, Life Technology, Waltham, MA, USA). All forward and reverse primers used in this study (Table 2) were tested in different concentrations to determine the minimum concentration of primer with the lowest threshold cycle (Ct), and reduce nonspecific amplifications. The validation of the selected-primers concentrations was performed with different concentrations of DNA. The value of "slope" was determined and efficiency primers were calculated.

Table 2. Primers used for specific quantification of cellulolytic bacteria and Methanogens by q-PCR.

Primer	Sequence (5` to 3`)	bp*	Efficiency (%)
Total bactéria†,§	F: CGGCAACGACAACCC R: CCATTGTAGCACCTGTGTAGCC	130	98
Fibrobacter succinogenes†	F: GGTATGGGATGAGCTTGC R: GCCTGCCCCTGAACTATC	121	95
Ruminococcus flavefaciens†	F: GGACGATAATGACGGTACTT R: GCAATC(CT)GAACTGGGACAAT	132	94
Ruminococcus albus†	F: CCCTAAAAGCAGTCTTAGTTCG R: CCTCCTTGCGGTTAGAACA	175	96
Methanogens‡	F: TTCGGTGGATCDCARAGRGC R: GBARGTCGWAWCCGTAGAATCC	140	95

F = "forward"; R = "reverse". * = Amplicon size in base pairs. † = Primer set from Denman and McSweeney (2006). ‡ = Primer set from Denman et al. (2007). § = Primers used for the normalization of qPCR.

The amplifications were performed in triplicate and negative controls were run in the assay, omitting the DNA. The reactions were conducted in the 7500 Real Time PCR System (Applied Biosystems, Foster City, California 94404, USA). Rox was used as a passive reference dye. The qPCR reaction was carried out using 120 ng of total DNA in a reaction containing: 6.25 µl of SYBR® Green PCR Master Mix (Bio-Rad, Hercules, California, USA), 10 pmol of primer pair, and H_2O to a final volume of 12.5 µl. Cycling conditions were 50°C for 2 min; 95°C for 10 minutes; and 40 cycles with denaturation of 95°C for 15 seconds, pairing 60°C for 1 minute, and extension 78°C for 30 seconds. After amplification cycles, a step was added in which temperature was increased from 60 to 95°C to obtain dissociation curve of the reaction products, used for analyzing the specificity of amplification. Relative quantification was used to determine species proportion. The results were expressed as a 16S rDNA ratio of general bacteria, following the equation:

$$\text{Relative quantification} = 2^{-(\text{Ct target} - \text{Ct total bacteria})};$$

where C_t is defined as the number of cycles required for the fluorescent signal to cross the threshold.

Performance study (Exp. 2)

Fifty Nellore bulls (BW 279.52 ± 16.31 kg) with an average age of 12 ± 2 months were used. The experimental period lasted 136 d, divided into five periods of 28 d. Initially, the animals were weighed, identified, treated against endo- and ectoparasites by the administration of ivermectin (Ivomec, Merial, Paulínea, BR), and distributed in a randomized complete block design with two replicates (paddocks) per treatment and five animals per paddock. Intake and digestibility were evaluated in the last 28 d of the study. Every 28 d the animals were weighed without fasting, to adjust supplement supply. Individual bull BW was recorded at day 0 and day 136 of study after a 16-h fasting period from feed and water. Average daily gain (ADG) was calculated by dividing BW gain (final BW - initial BW) by the number of days in the study. Feed efficiency was calculated as the ratio between ADG and DM intake (g of BW gain g^{-1} of DM intake).

Intake and apparent digestibility were estimated for all animals using three markers. In Exp. 1 the evaluation was performed in all periods, and in Exp. 2, the evaluation was performed in the last 28 d of study. Lignin isolated, purified, and enriched from *Eucalyptus grandis* (LIPE®), titanium dioxide

(TiO_2), and indigestible neutral detergent fiber (iNDF) were used to estimate the excretion of fecal matter (as dry weight), supplement intake, and forage intake, respectively.

The LIPE was provided daily for 8 days by cannula infusion (Exp. 1) or oral administration (Exp. 2) of a 500-mg bolus, with five days to stabilize fecal excretion of the marker, and in the last three days for sample collection (SANTOS et al., 2011). Fecal samples were collected on days 12, 13, and 14 of each period, directly from the rectum, at 11h00 and 16h00, 9h00 and 15h00, and 7h00 and 14h00, during the first, second, and third day of collection, respectively. Fecal samples were dried at 55±5°C for 72 h, pooled in animal basis, ground to pass a 1-mm screen in a Wiley mill (Thomas-Wiley Laboratory Mill Model 4, H. Thomas Co.). Approximately 10 g of each composed sample of feces was sent to the Federal University of Minas Gerais (Belo Horizonte, MG, Brazil) to analyze the lignin marker concentration using the infrared spectroscopy method (SALIBA et al., 2013).

To estimate DMI of supplement, titanium dioxide (TiO_2) was added as external marker to the supplement at a rate of 10 g d^{-1} of TiO_2 per animal (10 g d^{-1} × no. of animal/paddock) for 9 days, with six days to stabilize fecal excretion of the marker, and in the last three days for sample collection (TITGEMEYER et al., 2001). Feces samples were taken during the same time of the fecal excretion procedures. Feces were dried at 55±5°C for 72 h, to constant weight, pooled in animal basis, ground and digested using sulfuric acid. A standard curve was prepared adding 0, 2, 4, 6, 8 and 10 mg of TiO_2, through spectrophotometry read at 410 nm as described by Myers et al. (2004). Individual supplement intake was estimated using the following equation:

$$\text{Supplement DMI} = \frac{[\text{g of } TiO_2/\text{g of feces}] \times \text{fecal excretion}}{[\text{g TiO2/g of supplement}]}$$

The forage DMI was estimated using as internal marker undigested neutral detergent fiber (NDF), determined by ruminal incubation for 288 h (VALENTE et al., 2011). Forage DMI was estimated from the fecal output of the internal marker corrected for the supplement contribution as follows:

$$\text{Herbage DMI} = \frac{\text{FE} \times [i\text{MF}] - \text{DMIS} \times [i\text{MS}]}{[i\text{MH}]}$$

where FE is the fecal excretion, DMIS is the DMI of supplement, [*i*MF], [*i*MS] and [*i*MH] are the concentrations of the internal marker in feces, supplement and herbage, respectively. Total DMI was obtained by addition of herbage and supplement DMI.

For proximate analysis, samples of ingredients of supplements, forage and feces were dried at 55ºC for 72 h. Samples were then ground in a Wiley mill (Thomas-Wiley Laboratory Mill Model 4, H. Thomas Co.) to pass through a 1-mm screen. All samples were analyzed for DM (934.01), organic matter (OM) (942.05), and EE (920.85) according to the Association of Official Analytical Chemists (AOAC, 1990). Concentrations of N in each sample were determined by rapid combustion (Leco model FP-528; LECO Corporation, St. Joseph, MI). Neutral detergent fiber (NDF) was determined with a heat stable α-amylase and expressed exclusive of residual ash without the addition of sodium sulfite according to Van Soest et al. (1991), adapted to an Ankom^{200} Fiber Analyzer (Ankom Technology, Fairport, NY) and TDN was calculated according NRC (2001).

Statistical analyses: ruminal fermentation (Exp. 1)

The experimental design used for the evaluations of feed intake, digestibility, protozoa and ruminal fermentation parameters were analyzed in two simultaneous 5 x 5 Latin squares with 5 supplement sources, 10 animals and 5 experimental periods. Ciliated protozoa data were transformed to log10, plus a drive to meet the requirements of the SAS analysis. Statistical model included the fixed effect

of treatment and random effects of Latin square, period with square, animal with square, and the error. The interaction between treatments and Latin squares was included in the model and removed when $P>0.05$. Statistical evaluations of pH, ammonia N and VFA were summarized by sampling time and then analyzed using compound symmetry. The structure of errors was fitted with the Bayesian information criterion (BIC). Linear, quadratic, cubic and quartic effects of CG levels were tested with orthogonal contrasts using the contrast option of the MIXED procedures of SAS (SAS Inst. Inc., Cary, NC, USA). Cubic and quartic effects were not significant. Significance was declared when $P<0.05$.

Comparison of ruminal bacteria proportion and archaea between treatment 0 and 280 g/kg DM of CG in the supplement were compared using Wilcoxon test (n=10, $P<0.05$).

Statistical analyses: performance study (Exp.2)

Data were analyzed as a complete randomized experimental design using the GLM procedure of SAS (SAS Inst. Inc., Cary, NC, USA). The paddock was the experimental unit, and the model effects included treatment. The initial body weight was used as a covariate for the statistical analysis of the ADG. Homogeneity of the data was verified using the UNIVARIATE procedure of SAS. Residuals were plotted against the predicted values using the plot procedure to analyze data for outliers. The LSMEANS statement of the mixed procedure of SAS was used to calculate mean values. Orthogonal contrasts were used to estimate the linear and quadratic effects of glycerin treatment, with significance considered at $P<0.05$.

Results

Ruminal fermentation study (Exp.1)

Inclusion of CG (0 to 280 g kg^{-1} DM) in the supplements of Nellore steers grazing tropical grass did not affect intake of DM ($P = 0.813$), supplement ($P = 0.072$), forage ($P = 0.843$), OM ($P = 0.741$), CP ($P = 0.122$), EE ($P = 0.083$), ($P = 0.299$), digestible OM ($P = 0.830$), or TDN ($P = 0.869$) (Table 3). Inclusion of CG (0 to 280 g kg^{-1} DM) did not affect digestibility of DM ($P = 0.895$), OM ($P = 0.828$), CP ($P = 0.558$), NDF ($P = 0.395$), or TDN ($P = 0.963$; Table 3).

Table 3. Effect of crude glycerin (CG) inclusion in the supplements on intake and total tract apparent digestibility of young Nellore steers grazing tropical grass during dry season (Exp. 1)

	CG in the supplement (g kg^{-1} DM)						P-value*	
Item	0	70	140	210	280	SEM‡	L	Q
Intake, kg d^{-1}								
Dry matter	7.86	7.96	6.50	7.95	7.69	0.788	0.813	0.240
DM, g kg^{-1} body weight	1.92	1.96	1.61	1.91	1.83	0.210	0.534	0.363
Supplement	2.66	2.65	2.63	2.64	2.62	0.133	0.072	0.603
Pasture	5.19	5.31	3.87	5.28	5.06	0.816	0.843	0.252
Organic matter	7.32	7.39	6.03	7.44	7.09	0.692	0.741	0.227
Crude protein	1.20	1.28	1.20	1.31	1.25	0.084	0.122	0.301
Ether Extract	0.11	0.11	0.09	0.09	0.10	0.012	0.083	0.123
NDF†	4.25	4.21	3.17	4.05	3.82	0.460	0.299	0.200
Digestible OM intake, kg d^{-1}	3.85	4.04	2.84	4.29	3.87	0.701	0.830	0.353
Total digestible nutrient	3.97	4.16	3.01	4.36	3.97	0.711	0.869	0.335

continue

continuation

Digestibility, g kg^{-1} DM								
Dry matter	452.9	474.2	402.9	461.0	452.6	5.398	0.895	0.312
Organic matter	511.6	539.5	462.6	546.6	517.3	4.670	0.828	0.648
Neutral detergent fiber	462.2	475.7	371.8	454.1	416.0	6.145	0.395	0.393
Total digestible nutrient	491.2	516.1	451.2	515.3	489.5	4.224	0.963	0.645

† = Neutral detergent fiber with heat stable α-amylase and expressed exclusive of residual ash. ‡ = standard error of mean. * = Probability of a linear or quadratic effect of inclusion of CG in the supplement, contrast = linear (L) or quadratic (Q) (P<0.05).

Inclusion of CG in the supplement did not affect ruminal NH_3-N concentration ($P = 0.070$), but linearly increased pH ($P = 0.001$) and linearly decreased the total ruminal VFA concentration ($P < 0.001$). Inclusion of CG did not affect propionate ($P = 0.860$), isobutyrate ($P = 0.083$), valerate ($P = 0.146$), or isovalerate concentration ($P = 0.077$). However, butyrate concentration increased linearly ($P < 0.001$), whereas acetate decreased linearly ($P < 0.001$) with increasing CG in the supplement. Consequently, the inclusion of CG quadratically affected ($P = 0.009$) the ratio of acetate to propionate (A:P) in the rumen, with the lowest value observed with 280 g kg^{-1} of GC inclusion in the supplement (Table 4).

Table 4. Effect of crude glycerin (CG) inclusion in the supplements on pH, NH_3-N and volatile fatty acids (VFA) concentrations of young Nellore steers grazing tropical grass during dry season (Exp. 1).

	CG in the supplement (g kg^{-1} DM)						*P*-Value*	
Item	0	70	140	210	280	SEM ‡	L	Q
pH	6.39	6.35	6.44	6.43	6.61	0.078	0.001	0.081
NH_3-N mg dL^{-1}	17.21	18.13	16.88	16.37	15.02	1.580	0.070	0.339
VFA, mM								
Total VFA	114.0	111.1	105.5	104.4	97.3	3.279	<.001	0.704
Acetate	80.23	74.87	68.73	65.96	60.40	1.892	<.001	0.637
Propionate	19.07	20.27	20.09	20.46	18.74	0.929	0.860	0.090
Butyrate	10.46	11.78	12.87	13.77	14.23	0.840	<.001	0.462
Isobutyrate	1.19	1.14	1.11	1.12	1.08	0.071	0.083	0.729
Valerate	1.42	1.44	1.42	1.64	1.69	0.168	0.146	0.635
Isovalerate	2.25	2.24	1.89	1.96	2.02	0.163	0.077	0.266
A:P ratio†	4.22	3.85	3.49	3.45	3.39	0.122	<.001	0.009

‡ = standard error of mean. †A: P ratio = acetate: propionate ratio. * = Probability of a linear or quadratic effect of inclusion of CG in the supplement, contrast = linear (L) or quadratic (Q) (P<0.05).

Inclusion of CG in the supplement did not affect the number of rumen protozoa genres (remaining at 7) ($P > 0.05$; Table 5) or the rumen relative proportion of *R. albus* ($P = 0.236$), *R. flavefaciens* ($P = 0.129$), or archaea ($P = 0.150$), but increased that of *Fibrobacter succinogenes* ($P = 0.003$; Table 6).

Table 5. Effect of level of crude glycerin (CG) inclusion in the supplements on rumen fluid protozoa numbers of Nellore steers grazing tropical grass during dry season (Exp. 1).

	CG in the supplement (g kg^{-1} DM)					SEM‡	*P*-value*	
Protozoa †	0	70	140	210	280		L	Q
Entodinium	6.70	6.65	6.72	6.74	5.83	0.329	0.129	0.161
Dasytricha	4.31	4.67	4.76	4.38	4.19	0.565	0.723	0.387
Isotricha	2.51	3.70	2.84	3.20	2.89	0.683	0.891	0.426
Eremoplastron	3.19	2.94	3.30	2.82	2.53	0.636	0.497	0.710
Eudiplodinium	2.51	1.74	1.35	1.73	1.35	0.509	0.145	0.371
Elytroplastron	2.05	2.13	1.70	2.12	2.81	0.650	0.500	0.438
Polyplastron	1.84	1.35	1.70	2.50	1.00	0.602	0.688	0.355

† = Log^{10} of number of protozoa mL^{-1} ‡ = standard error of mean. * = Probability of a linear or quadratic effect of inclusion of CG in the supplement, contrast = linear (L) or quadratic (Q) (P<0.05).

Table 6. Effect of crude glycerin (CG) inclusion in the supplements on relative proportion of cellulolytic bacteria and methanogens of young Nellore steers grazing tropical grass during dry season (Exp. 1)

	CG in the supplement (g kg^{-1} DM)			
Cellulolytic Bacteria †	0	280	SEM ‡	*P*-value*
Ruminoccocus albus	0.0038	0.0054	0.001	0.236
Ruminoccocus flavefasciens	0.0050	0.0039	0.002	0.129
Fibrobacter succinogenes	0.0033	0.0296	0.017	0.003
Methanogens	0.0149	0.0190	0.072	0.150

† = measured based on the proportion of the specific 16S rRNA associated with total bacteria. ‡ = standard error of mean. * = Differences were considered significant at P<0.05 using the Wilcoxon test.

Performance study (Exp.2)

Inclusion of CG in the supplements quadratically affected DM intake ($P < 0.05$), forage intake ($P = 0.004$), NDF intake ($P = 0.002$), digestibility of DM ($P > 0.001$), and digestible OM intake (P = 0.002), which had the lowest values at 140 g/kg of CG inclusion in the supplement. However, inclusion of CG in the supplement did not affect intake of supplement ($P = 0.214$) or CP ($P = 0.399$; Table 7).

Table 7. Effect of crude glycerin (CG) inclusion in the supplements on performance of young Nellore bulls in tropical grass during dry season (Exp. 2).

	CG in the supplement (g kg^{-1} DM)					SEM‡	*P*-Value†	
Item*	0	70	140	210	280		L	Q
Dry matter intake, kg d^{-1}	9.75	8.99	7.51	7.93	7.77	0.99	<.001	0.005
Dry matter intake, g kg^{-1} BW	2.46	2.27	1.89	2.03	1.97	0.30	<.001	0.023
Forage intake, kg DM d^{-1}	7.20	6.38	4.54	5.29	5.12	1.09	<.001	0.004
Supplement intake, kg DM d^{-1}	2.55	2.61	2.60	2.64	2.64	0.16	0.214	0.777
Crude protein intake, g d^{-1}	1.18	1.21	1.13	1.16	1.16	0.09	0.399	0.496
NDF intake, g d^{-1}	5.96	5.28	4.24	4.52	4.32	0.71	<.001	0.002
DM digestibility, % DM	66.51	64.03	55.53	60.68	59.52	3.71	<.001	<.001

continue

continuation

Digestible OM intake, kg d^{-1}	6.52	5.75	4.13	4.86	4.74	0,91	<.001	0.002
Initial BW, kg	279.0	283.7	276.5	280.7	277.7	16.6	0.740	0.841
Final BW, kg	374.7	382.8	377.9	391.5	395.7	25.0	0.060	0.732
Additional gain, kg	95.7	99.1	101.4	110.8	118.0	15.1	<.001	0.430
Average daily gain, kg	0.703	0.728	0.745	0.814	0.867	0.11	<.001	0.432
Feed efficiency	0.072	0.083	0.106	0.101	0.112	0.01	<.001	0.205

*BW = body weight; NDF = neutral detergent fiber with heat stable α-amylase corrected for ash and nitrogen; feed efficiency = additional body weight gain per dry matter intake. † = Probability of a linear or quadratic effect of inclusion of CG in the supplement, contrast = linear (L) or quadratic (Q) (P<0.05). ‡ = standard error of mean.

Inclusion of CG in the supplement did not affect final BW ($P = 0.060$), but linearly increased additional gain ($P > 0.001$), average daily gain ($P > 0.001$), and feed efficiency ($P > 0.001$; Table 7).

Discussion

We observed that steers supplemented with increasing concentrations of CG showed similar DMI, increased *F. succinogenes* population, increased pH and butyrate concentration, and increased additional gain, average daily gain, and feed efficiency. Thus, the hypothesis that addition of CG in the supplement of growing cattle grazing tropical grasses during the dry season could change the rumen fermentation, without negatively affecting digestibility, with an improvement in animal performance was accepted.

Ruminal fermentation study

There are limited studies on the effects of including CG in the supplement for cattle grazing tropical pastures. Most studies have reported the effect of glycerin use in high-grain diets, or diets with a high proportion of forage, but always in feedlot conditions. Previously, only *in vitro* experiments on the effects of glycerin inclusion on the digestibility and fermentation of similar diets to animals raised in pastures in the dry season have been reported. In our study, the inclusion of CG at the level of 280 g kg^{-1} DM in the supplement (95 g kg^{-1} of diet DM) did not affect intake, digestibility, or rumen microbes proportion in cannulated steers grazing low quality tropical grass, although the concentration of rumen fermentation products were changed.

Previous studies that investigated the effects of CG inclusion in the supplementation of grazing heifers on ingestive behavior are in agreement with our study (FARIAS et al., 2012; FACURI et al., 2014) reported no negative effects on feed intake when CG was included at up to 150 g kg^{-1} diet DM. It could also be suggested that CG can be used as an energetic ingredient that can effectively substitute cereals in the diets of grazing cattle.

Apparent total digestibility of DM was not affected by inclusion of CG in the supplement. This corroborates the studies of Hess et al. (2008), which showed that CG could be added at up to 150 g kg^{-1} of diet DM for forage-based ruminant diets without negatively affecting the DM or fiber digestibility, indeed, CG may enhance digestibility in forage diets (SCHRÖDER; SÜDEKUM, 1999; AVILA et al., 2014). Based on this evidence, we can suppose that inclusion of CG does not cause negative effects on intake or digestibility when included in low-energy forage-based diets.

The trend towards reduction in the ruminal NH_3-N concentration could be due to the improved growth of ruminal microbial populations that would increase the NH_3-N consumption with CG supplementation, especially the fiber-degrading populations (WANG et al., 2009). According to

Russell et al. (1992), cellulolytic bacteria derive their nitrogen (N) exclusively from NH_3-N. The reduction in ruminal NH_3-N concentration could be explained by the increased relative proportion of *Fibrobacter succinogenes* due to the accretion of CG in the supplement. A similar result was found by Shin et al. (2012), who reported that use of dietary N by ruminal microbes increased as CG concentration increased in the diet; Shin et al. (2012) also observed a reduction in NH_3-N of ruminal fluid across sources of roughage including cottonseed hulls and corn silage by feeding with CG in lactating cow diets.

The increase in ruminal pH value with increased concentrations of CG in the supplement is consistent with the reduction in total VFA across the treatments. Ruminal pH was greater than 6.0, within the optimum range for cellulolytic bacteria activity, which may have also contributed to the increased proportion of *F. succinogenes* in the diets with CG. Values below that level generally inhibit the growth of ruminal cellulolytic bacteria, and in turn, reduce the cellulolytic activity in the rumen (RUSSELL; DOMBROWSKI, 1980; GRANJA-SALCEDO et al., 2016).

Feeding with CG has been reported to shift VFA production in favor of propionate at the expense of acetate, mainly in *in vitro* conditions using glycerol in forage substrates (AVILA et al., 2011; KRUEGER et al., 2010; AVILA et al., 2014). In part, this is in agreement with the results of the present study. However, the propiogenic properties of CG were not confirmed, because of the reduction in the total VFA concentration and the acetate:propionate ratio, derived at the expense of acetate and increased concentration of butyrate and valerate. No changes in propionate concentration were observed. Thus, our results do not confirm the propiogenic properties of CG or the hypothesis that CG is likely to be entirely converted to propionate in the rumen (PARSONS et al., 2009), but support a possible suppressive effect of CG on acetate formation in the rumen, as found by Trabue et al. (2007).

The increased butyrate and valerate in the current study may have resulted from an increased production of lactate by the fermentation of CG (TRABUE et al., 2007), which provided a substrate for *Megasphaera elsdenii* (KLIEVE et al., 2003). These results are in agreement with those of Rémond et al. (1993) and Shin et al. (2012), who found a decrease in the proportion of acetic acid and an increase in the proportions of butyrate and valerate when CG was supplemented at increasing levels. Contrasting with these results, previous studies (DeFRAIN et al., 2004; TRABUE et al., 2007; WANG et al., 2009) have reported that animals supplemented with CG had greater total rumen VFA, greater rumen molar proportions of propionate, and a decreased ratio of acetate to propionate. The discrepancies among studies on the effects of CG on rumen fermentation may be the result of the amounts of CG administered (RÉMOND et al., 1993) and the interaction between CG and the substrate used in the diets (SAN VITO et al., 2014).

Currently, it is still unknown how CG feeding affects protozoa populations, but it is known that butyrate concentrations in ruminal fluid increase when the numbers of ciliated protozoa increase (WHITELAW et al., 1972). However, we did not detect changes in the total number of ciliated protozoa, despite the changes that occurred in the rumen fermentation and the increased concentration of butyrate.

The lack of negative effects on the intake and digestibility of the diet of CG inclusion at up to 280 g kg^{-1} DM in the supplement provided to low-quality forage grazing animals, may have resulted from lack of negative effect of CG inclusion on rumen microbes. It was expected that higher substitution replacement levels of corn by CG might adversely affect rumen fermentation through reducing fiber digestion and bacterial populations (ABO EL-NOR et al., 2010); possibly due to the greater inhibition of growth and cellulolytic activity of rumen bacteria and fungi (ROGER et al., 1992; PAGGI et al., 2004).

Interestingly, we observed an increase in the relative proportion of *F. succinogenes* with CG supplementation, with no negative effects of CG inclusion on *R. albus* and *R flavefaciens*. Sensitivity of rumen microbes to CG may also depend on the level of CG inclusion (ABO EL-NOR et al., 2010). For example, Roger et al. (1992) showed increased growth of *F. succinogenes* with the inclusion of glycerol in the growth medium at a low level (0.02 v v^{-1}) but the growth was inhibited when the concentration of glycerol reached the level of 0.5 v v^{-1}. Differing sensibilities to the effects CG between cellulolytic bacteria species were also reported by Abo El-Nor et al. (2010), corroborating the results of Wang et al. (2009), which suggested that CG supplementation in corn stover-based diets fed to steers particularly improved microbial activity of gram-negative bacteria. Thus, it is still possible that CG has a different effect on cellulolytic bacteria by modifying cell membrane permeability and therefore affecting bacterial cellulolytic activities. This observation may have contributed to the increased proportion of *F. succinogenes* (gram-negative) compared to *R. albus* and *R. flavefaciens* (gram-positive) throughout the inclusion of CG.

Addition of crude glycerin also did not affect the *Archaean* population, which is consistent with previous reports (AVILA et al., 2014; DANIELSSON et al., 2014). The consistent numbers of ciliated protozoa, which live in the rumen in a symbiotic relationship with archaea (USHIDA et al., 1997), may have contributed to the lack of effects on the proportions of methanogens.

Performance study

Based on the intake of the animals in this study, the inclusion of 280 g kg^{-1} DM of CG in the supplement corresponded to approximately 95 g kg^{-1} of diet DM. This level of inclusion of CG decreased DM intake, and increased the average daily gain (ADG) and feed efficiency of the young bulls raised in pasture.

Compared with the control supplement, cattle receiving 280 g CG kg^{-1} DM supplement increased ADG to 23.3%. Similarly, Gunn et al. (2011), observed an increase in the ADG when feeding calves with up to 150 g kg^{-1} of diet DM; however, Pyatt et al. (2007) and Parsons et al. (2009) reported an ADG increase in finishing steers and heifers when CG was added to the diet. Crude glycerin has a high energy content, which is approximately the same as that of corn starch (DONKIN et al., 2009), and can potentially be used for animal feeding. Glycerin has a faster fermentation rate than starch (SHIN et al., 2012) and this may increase the efficiency of use of dietary energy in the ruminant diet (LEE et al., 2011), contributing to better performance.

Unlike the result of Exp. 1, CG inclusion resulted in a decrease of 20.3% in DM intake and 27.3% in digestible OM intake, due to a 28% decrease in the forage intake. The difference between Exp. 1 and 2 may be due to the fact that bulls have a higher intake compared to steers (BURNHAM et al., 2000), and the higher intake potentiated the observation of a negative effect of glycerol on DM intake. The use of glycerin in the substitution of corn results in rapid ruminal fermentation (TRABUE et al., 2007), which can cause alterations in ingestive behavior. Facuri et al. (2014) showed that the addition of CG at a level of 150 g kg^{-1} of diet DM in substitution of corn in supplements for animals raised on pastures does not influence feed intake but reduces the grazing time. This fact may have contributed to the decreased forage intake in the present study, indicating that glycerol acts as a gluconeogenic precursor, which increases satiety, thus affecting feed intake.

The reduction in DM intake and increase in ADG contributed to increasing the enhanced feed efficiency by 55.5% when CG was included in the supplement in the present study (comparing the 0 to the 280 g kg^{-1} levels of supplement). Parsons et al. (2009) reported an improvement in feed efficiency when glycerin was included to up to 12% of the diet whereas Pyatt et al. (2007) showed a 21.9% improvement in feed efficiency when

glycerin replaced 100 g kg^{-1} of the dry-rolled corn in the diet, leading to a 10.1% reduction in DM intake. Furthermore, the ingestion of supplement changes the ingestive behavior of grazing ruminants (MARQUES et al., 2005) and when part of the nutrient requirement is met by CG intake, dietary energy usage efficiency may improve due to improved conditions for the activity of the ruminal microbiota. An example of this was observed in Exp.1, with a decrease in NH_3-N concentration and an increase in ruminal pH and the relative proportion of *F. succinogenes* when CG was included.

Our study shows that the response of glycerin in studies is dependent on what glycerin replaces in experimental diets, whether grain or roughage (HALES et al., 2013). Thus, we suggest that glycerin can be used as an energetic ingredient that can effectively substitutes cereals in the supplement of growing bulls raised in low and medium-quality pastures.

Inclusion of crude glycerin in the supplement of Nellore steers grazing tropical grass during the dry season does not affect intake and apparent total tract digestibility. However, it alters rumen fermentation, increasing butyrate and valerate while reducing acetate and total VFA, and showing no negative effect on the relative proportions of *R. albus, R. flavefaciens,* methanogenic, and protozoa populations. Nevertheless, including glycerin at concentrations of up to 280 g kg^{-1} DM supplement (95 g kg^{-1} DM diet) to growing Nellore bulls grazing tropical grass improves BW gain and feed efficiency and can be used as a profitable alternative to replace corn in the supplements of Nellore cattle grazing pasture.

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