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SHORT COMMUNICATION

High concentrations of crude glycerin on ruminal parameters, microbial yield, and in vitro greenhouse gases production in dairy cows

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Abstract: Holstein cows were used to evaluate the inclusion of elevated concentrations of crude glycerin on ruminal parameters, production of greenhouse gases, and microbial yield. The use of up to 300 g kg^{-1} reduces ruminal bacteria yield and NH_3-N, without affecting the pH. Moreover, 300 g kg^{-1} glycerin increases methane production.

Key words: bacteria, glycerol, dairy cow, methane, microorganism.

Résumé : Les vaches holsteins ont été utilisées pour évaluer l'ajout de concentrations élevées de glycérine brute sur les paramètres du rumen, la production des gaz à effet de serre et le rendement microbien. L'utilisation d'une quantité jusqu'à 300 g kg^{-1} réduit le rendement bactérien du rumen ainsi que le NH_3-N, sans effet sur le pH. De plus, 300 g kg^{-1} de glycérine augmente la production de méthane. [Traduit par la Rédaction]

Mots-clés : bactéries, glycérol, vache laitière, méthane, microorganisme.

The advent of biodiesel due to the change in world's energetic matrix generates new residues and by-products, such as crude glycerin. Approximately 10% of total biodiesel produced become crude glycerin. This by-product has been intensively tested as energy source in diets for livestock. The glycerol (main constituent of crude glycerin) can be converted to glucose by the liver and kidneys to provide energy for cellular metabolism. In ruminants, the glycerol is fermented in the rumen into short chain fatty acids, mainly to propionic and butyric. In general, the inclusion of crude glycerin involves the concomitant removal of energy ingredients from diets, such as corn grain. Thus, in addition to increasing the availability of corn for human consumption, it could also interfere with the emission of greenhouse gases, particularly methane. As glycerol seems to be fermented mainly into propionic and butyric acids and not into acetic acid (Rémond et al. 1993), which is the main precursor of ruminal methane, a mitigation of the production of this pollutant is also expected. However, the impact of crude glycerin on rumen parameters and microbial mass is not completely elucidated, especially when the addition is greater than 150 g kg^{-1}, in dry matter of diets. Thus, the objectives of this study were to evaluate the effects of high levels of crude glycerin as a partial replacement of corn, in medium-yield dairy cows' diets on ruminal parameters, microbial yield, and in vitro greenhouse gases.

The Unesp/Jaboticabal Institutional Animal Care and Use Committee approved all the procedures adopted in this study (protocol No. 1892108). The research was conducted at Animal Unit of Digestive and Metabolic Studies and at the Greenhouse and Ingredients Laboratory from the Animal Science Department of São Paulo State University, Jaboticabal, Brazil.

Six multiparous ruminally cannulated lactating Holstein cows (587 ± 39 kg BW, 114 ± 29 DIM, and milk production of 20 ± 1.5 kg d^{-1}) were used in this study, in a replicated 3 × 3 Latin square design. Cows were paired according to initial weight, parity, and milk production.

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Abbreviations: **ADF**, acid detergent fiber; **BW**, body weight; **CP**, crude protein; **DIM**, days in milk; **DM**, dry matter; **DMI**, dry matter intake; **EE**, ether extract; **LAB**, Liquid-associated bacteria; **LAP**, liquid-associated protozoa; **ME**, metabolizable energy; **NDF**, neutral detergent fiber; **NFC**, nonfiber carbohydrate; **OM**, organic matter; **PAB**, particle-associated bacteria; **RDP**, rumen degradable protein; **RUP**, rumen undegradable protein.

Table 1. Ingredient and chemical composition of experimental diets containing increasing concentrations of crude glycerine.

	Treatments[a]		
Item	G0	G150	G300
Ingredients (g kg^{-1} DM)			
Corn silage	450	450	450
Corn grain	365	193	28
Sunflower meal	107	114	113
Corn gluten meal	51	66	81
Urea	5	5	6
Crude glycerin	0	150	300
Mineral–vitamin premix[b]	23	23	23
Chemical composition			
CP (g kg^{-1} DM)	144	143	141
RDP (g kg^{-1} DM)	98	97	95
RUP (g kg^{-1} DM)	46	46	46
NDF (g kg^{-1} DM)	348	333	315
ADF (g kg^{-1} DM)	190	189	186
NFC[c] (g kg^{-1} DM)	453	333	215
EE (g kg^{-1} DM)	32	26	21
ME (Mcal kg^{-1} DM)	2.8	2.8	2.8

[a]G0: control treatment; G150: addition of 150 g kg^{-1} crude glycerin; G300: addition of 300 g kg^{-1} crude glycerin.

[b]Composition of mineral–vitamin premix (g kg^{-1}) = vitamin A: 200 kIU; vitamin D: 60 kIU; vitamin E: 60 IU; Ca: 190 g; P: 73 g; Na: 62 g; Mg: 44 g; Cl: 90 g; S: 30 g; Zn: 1350 mg; Mn: 940 mg; Co: 3 mg; Cu: 340 mg; I: 16 mg; Se: 16 mg; Fe: 1064 mg; F: 730 mg.

[c]NFC = 100 – (% CP + % NDF + % ash + % EE).

After lactation peak, each pair was randomly assigned to one of the three experimental diets and progressed through the three 21 d periods until everyone had received all treatments. Cows were housed in individual tie stall (1.5 m × 1.9 m) equipped with individual feed bunks and waterers.

The experimental diets were composed of corn silage, cracked corn grain, sunflower meal, corn gluten, urea, mineral premix, and 0 (G0), 150 (G150), or 300 g kg^{-1} (G300) crude glycerin (Table 1). Corn silage was top-dressed with crude glycerin and then mixed with the concentrate at the time of feed delivery. The crude glycerin used in this experiment was soybean based and composed of 83 g kg^{-1} glycerol, 89 g kg^{-1} DM, 6 g kg^{-1} NaCl, and less than 0.01 g kg^{-1} methanol.

The methodology of ruminal microbial isolation used in this trial was described by Martin et al. (1994). Approximately 3 kg of ruminal content was sampled from the rumen, between the cranial and ventral coronary pillars at 0, 4, 9, and 15 h after morning feeding. An additional sample of 100 g of ruminal content was collected to determine pH values and ammonia nitrogen concentrations. To avoid any interference in ruminal function, only one sample was collected per day, between d 18 and d 21 of each experimental period.

The ruminal content was strained through four layers of cheesecloth to separate liquid and solid phases. From solid phase, 200 g was weighed and 30 g was used for DM analysis, and 170 g was used for isolation of particle-associated bacteria (PAB). The solid material was initially manually washed with a prewarmed (39 °C) Coleman solution (0.63 g kg^{-1} K_2HPO_4, 0.5 g kg^{-1} KH_2PO_4, 0.065 g kg^{-1} $NaCl{\cdot}6H_2O$, 0.09 g kg^{-1} $MgSO_4{\cdot}7H_2O$, and 0.5 g kg^{-1} cysteine hydrochloride), using a ratio of 1 g solid to 4 g saline solution. The content was filtered through a nylon filter (100 μm), and the filtrate was then centrifuged at room temperature (1000*g*, for 10 min, at room temperature). The pellet of small particles obtained was added to the content retained on the filter. This combined material was suspended in a precooled Coleman solution (4 °C) and homogenized at 200 rpm for 5 min using a Stomacher device (Seward and Co., London, UK). Material was filtered and the solids retained were discarded. Filtered was centrifuged (1000*g*, for 10 min, at 4 °C) and the supernatant was centrifuged (27 000 *g*, for 30 min, at 4 °C). Resulting pellet was considered as the PAB. Bacterial material was transferred to 100 mL plastic flasks and dried at 55 °C for 72 h. Flasks with the dried PAB were weighed and reserved for analysis of DM, ash, and N (Association of Official Analytical Chemists 1990).

From the liquid phase, 700 mL was diluted with a prewarmed (39 °C) Coleman solution (1:1) and incubated in a water bath at 39 °C for 30 min, until the flocculation was completed. After 25 min of incubation, 1 g L^{-1} glucose was added to separate the protozoa from the rest of ruminal liquid. After the separation, 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. Pellets were washed with a prewarmed saline solution (39 °C) and filtered through a 20 μm nylon filter.

Liquid-associated bacteria (LAB) were recovered by centrifuging the protozoa-free supernatant (15 000 *g*, for 20 min, at 4 °C). Pellets of LAP and LAB were transferred to 100 mL plastic flasks and dried at 55 °C for 72 h. Flasks with dried microorganisms were weighed and reserved for analysis of DM, ash, and N.

For the evaluation of ruminal parameter, 100 g of ruminal content was strained through four layers of cheesecloth to separate liquid and solid phases. Ammonia concentrations and pH values were immediately measured after rumen fluid sampling and separation. The pH was determined using a digital pH meter and the ammonia using a microkjeldhal device, using 5 mL of 2 M KOH, and distillation flux of 2 mL min^{-1}. The distilled was dropped into a 10 mL boric acid solution (20 g kg^{-1}) and then titrated with 0.005 M HCl.

For in vitro gas production trial, approximately 4 kg of ruminal content was manually collected and strained through four layers of cheesecloth. Strained rumen fluid mixed with McDougall's buffer in 2:1 ratio (120 mL in

Table 2. Dry matter intake, ruminal parameters, protozoal, and bacterial fractions and in vitro gas production in lactating Holstein cows fed diets supplemented with different amounts of crude glycerine.

	Treatments[a]			Contrast, *P*-value		
Item	G0	G15	G30	G0 × G[b]	G15 × G30[c]	SE
DMI[d] (kg d^{-1})	17.8	17.2	15.0	0.02	0.01	1.48
Ruminal parameters						
pH	6.0	5.9	5.9	0.47	0.91	0.05
NH_3-N (mg dL^{-1})	28.1	20.3	20.9	0.04	0.65	1.80
Microorganisms						
LAB[e] (mg L^{-1})						
DM	1575.9	1155.1	1235.9	<0.0001	0.24	56.79
OM	1165.9	815.3	852.3	<0.0001	0.56	46.19
N	151.6	114.5	117.9	<0.0001	0.65	5.27
PAB[f] (mg kg^{-1})						
DM	5442.5	4957.9	5837.8	0.15	0.004	454.78
OM	4894.6	4025.3	4786.3	0.18	0.008	390.40
N	573.6	486.5	555.7	0.31	0.06	41.29
LAP[g] (mg L^{-1})						
DM	1800.6	1785.8	1910.9	0.17	0.54	62.34
OM	765.4	854.3	812.1	0.37	0.10	29.76
N	113.3	121.0	114.7	0.23	0.32	4.31
Gas production						
Total (mL g^{-1} DM)	30.7	22.5	28.4	0.25	0.27	7.90
CH_4 (mL g^{-1} DM)	5.1	4.4	7.2	0.52	0.04	2.08
CO_2 (mL g^{-1} DM)	18.9	15.8	17.5	0.52	0.66	6.30
CO_2/CH_4	3.7	3.6	2.6	0.67	0.03	0.96

[a]G0: control treatment; G150: addition of 150 g kg^{-1} crude glycerin; G300: addition of 300 g kg^{-1} crude glycerin.
[b]Control treatment vs. glycerin treatments.
[c]150 vs. 300 g kg^{-1} crude glycerin.
[d]Ezequiel et al. (2015).
[e]Liquid-associated bacteria.
[f]Particle-associated bacteria.
[g]Liquid-associated protozoa.

total volume) was transferred to 250 mL plastic erlenmeyers containing 1.7 g (DM basis) of each experimental diet, to keep a 1:8 ratio of sample and inoculum. The erlenmeyers were purged with CO_2, capped with silicon stoppers, and attached to gasometers made of plastic pipe and 500 mL plastic bottles. The erlenmeyers were incubated in a water bath at 39 °C for a 12 h period. The total gas production was measured using a graduated ruler and correlated with a standard curve previously built to convert height in 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) to evaluate the concentrations of CO_2 and CH_4. 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 at 110 °C. The carrier gas used was argon, with a 25 mL min^{-1} flow.

Data were analyzed as a replicated 3 × 3 Latin square design using PROC MIXED for repeated measures of SAS (version 9.1; SAS Institute, Inc., Cary, NC, USA). Orthogonal contrasts were used to determine the effects of crude glycerin and the effect of 150 vs. 300 g kg^{-1} crude glycerin. For the analysis of repeatedly measured variables, sampling time and time × treatment interaction terms and a repeated-measures statement with the unstructured covariance structure, based upon its smallest Akaike's information criterion value, were used. Significance was defined as $P < 0.05$ and trends as $0.05 \leq P \leq 0.10$. Values are reported as least-squares means and associated standard errors.

There was no interaction of treatments and sampling time for all variables evaluated in this experiment. No differences were observed among treatments for ruminal pH (average 6.93). Ruminal ammonia decreased ($P = 0.04$), when control treatment was compared with treatments with glycerin (Table 2), but the increase from 150 to 300 g kg^{-1} crude glycerin in the diet did not change ruminal ammonia concentrations (average 20.6 mg dL^{-1}). The pH values observed in the present trial

agree with previous studies in which glycerin was supplemented to dairy cows (Boyd et al. 2013). The average of 5.93 indicates that even with 300 g kg^{-1} crude glycerin supplementation, the pH ranges from 5.8 to 6.2, which is considered normal for lactating dairy cows. The lower concentration of ruminal ammonia in G150 and G300 can be attributed to the lower DMI observed in those treatments (Table 2), as reported by Ezequiel et al. (2015). Ruminal ammonia is the main source of nitrogen of several cellulolytic bacteria, and it could be the reason for lower production of PAB in G150. Previous studies report similar effects of glycerin on ruminal ammonia concentrations. Shin et al. (2012) fed lactating cows with 100 g kg^{-1} glycerin, in corn silage or cottonseed hulls-based diets, and observed reduction of ruminal ammonia in animals fed the glycerin treatment, in both conditions.

The addition of crude glycerin, regardless the concentration, decreased ($P < 0.0001$) the DM, OM yield, and N contents of LAB (Table 2); however, no changes were observed between G150 and G300. The amount of PAB was similar when control treatment was compared with glycerin treatments together; however, G150 promoted less PAB than G300 ($P = 0.004$). The nitrogen content of PAB tended to be lower ($P = 0.06$) in treatments with 150 g kg^{-1} glycerin compared with G300, and no difference was observed between control treatment and glycerin treatments. There were no differences among treatments in the amount of LAP, showing 1832.4 mg DM L^{-1} and 116.3 mg N L^{-1}, on average. Manipulation of dietary factors may alter chemical composition of bacteria, bacterial attachment on rumen feed particles, and the relative proportions of liquid and PAB. The mechanism of action of crude glycerin on the populations of bacteria is still unclear and may be due to the formation of an unfavorable ruminal environment in terms of osmolality, encapsulation of feed particles avoiding microorganism to adhere, and competition of bacteria or substrate preference.

The negative effect of crude glycerin on solid-associated bacteria, observed in G150 from present study, is very important because bacterial colonization of ruminal particles is an important prerequisite for fiber degradation. On the other hand, the negative effect of crude glycerin inclusion on LAB yield was probably due to the reduction in DM intake and changes in ruminal osmolality. Moreover, considering that approximately 43% of glycerol is absorbed through the ruminal wall (Krehbiel 2008), it probably contributes to the reduction of subtract available to LAB. The lack of effect of crude glycerin on liquid-associated protozoa disagrees with the results presented by Fávaro et al. (2014) who observed linear decrease of LAP, when feeding crossbred steers with 0, 50, 100, 150, or 200 g kg^{-1} crude glycerin. In current study, it expected a reduction in LAP yield due to the reduction of LAB, which is engulfed by protozoa as its main protein source.

The production of methane (Table 2) was not affected by glycerin supplementation ($P = 0.52$), though G300 showed higher production than G150 ($P = 0.04$). Nonetheless, the production of CO_2 was similar among treatments. These results caused the lower CO_2 to CH_4 ratio for G300 compared with the other treatments ($P = 0.03$). The similar CO_2 production among treatments was associated with the increased production of CH_4 in treatment G300 compared with treatment G150, decreasing the CO_2/CH_4 ratio in treatment G300. From the total gas produced, 62%, 70%, and 62% corresponded to CO_2, and 17%, 20%, and 25% to CH_4, respectively for G0, G150, and G300. This means that after 12 h of ruminal incubation, the G300 treatment provided gas production proportionally richer in methane than the other treatments. The hypothesis is that the presence of glycerol in the rumen favored other pathways of action of methanogenic bacteria, with elevated production of methane, which was demonstrated in this study. Justifying this statement, Jarvis et al. (1997) mentioned that there is formation of formate and ethanol from glycerol molecule in the rumen. In turn, the formate produced turns into methane. In addition, large amounts of ethanol are oxidized to acetate in the rumen (Pradhan and Hemken 1970), a process that releases reducing equivalents used for the production of CH_4.

The use of high concentrations of crude glycerin (150 or 300 g kg^{-1}) in diets for lactating dairy cows reduces liquid- and solid-associated bacteria yield and ammonia nitrogen without affecting ruminal pH. Nonetheless, 300 g kg^{-1} crude glycerin increases in vitro methane production.

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