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Inclusion of glycerol in forage diets increases methane production in a rumen simulation technique system

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Abstract

We hypothesised that the inclusion of glycerol in the forage diets of ruminants would increase the proportion of propionate produced and thereby decrease *in vitro* CH_4 production. This hypothesis was examined in the present study using a semi-continuous fermentation system (rumen simulation technique) fed a brome hay (8·5 g) and maize silage (1·5 g) diet with increasing concentrations (0, 50, 100 and 150 g/kg DM) of glycerol substituted for maize silage. Glycerol linearly increased total volatile fatty acids production ($P<0·001$). Acetate production was quadratically affected ($P=0·023$) and propionate and butyrate production was linearly increased ($P<0·001$). Glycerol linearly increased ($P=0·011$) DM disappearance from hay and silage. Crude protein disappearance from hay was not affected ($P=0·789$), but that from silage was linearly increased ($P<0·001$) with increasing glycerol concentrations. Neutral-detergent fibre ($P=0·040$) and acid-detergent fibre ($P=0·031$) disappearance from hay and silage was linearly increased by glycerol. Total gas production tended to increase linearly ($P=0·061$) and CH_4 concentration in gas was linearly increased ($P<0·001$) by glycerol, resulting in a linear increase ($P<0·001$) in mg CH_4/g DM digested. Our hypothesis was rejected as increasing concentrations of glycerol in a forage diet linearly increased CH_4 production in semi-continuous fermenters, despite the increases in the concentrations of propionate. In conclusion, this apparent discrepancy is due to the more reduced state of glycerol when compared with carbohydrates, which implies that there is no net incorporation of electrons when glycerol is metabolised to propionate.

Key words: ***In vitro* techniques: Biodiesel by-products: Hydrogen sink: Methane**

Glycerol has been used as a feed ingredient to replace grain in dairy[1] and finishing beef cattle diets[2,3] and in growing-finishing lamb diets[4]. Results suggest that glycerol at 50–100 g/kg DM does not affect weight gain and may improve feed conversion when substituted for barley or maize grain in beef cattle diets[3]. However, a greater concentration (>120 g/kg diet DM) of glycerol has been reported to result in reduced intakes in cattle[2,3] and sheep[4–6] fed high-grain diets.

Glycerol can increase blood glucose levels in cattle and sheep by being directly absorbed through the rumen wall and converted to glucose in the liver[7] or by being fermented in the rumen mainly to propionate, which in turn can be absorbed and converted to glucose in the liver[1,8]. The replacement of wheat starch[9] or barley grain[10] with glycerol has been reported to linearly increase propionic acid production and reduce the acetate:propionate ratio *in vitro*. Shifts towards propionate fermentation have been suggested as a means of reducing CH_4 emissions, since the metabolic pathways leading to propionate have been proposed as a hydrogen sink[11–13].

Previous studies have reported increases in propionate production with only a numerical decrease of CH_4 *in vitro*[10] (mg CH_4/g DM incubated and mg CH_4/g DM digested) and *in vivo*[4] (g CH_4/lamb per d, g CH_4/kg DM intake, g CH_4/kg DM digested, percentage of gross and digestible energy intake lost as CH_4) when glycerol replaced barley grain in finishing lamb diets. A possible cause for this lack of effect is that the shift to propionate fermentation may be of relatively low magnitude given the propiogenic

Abbreviations: ADF, acid-detergent fibre; NDF, neutral-detergent fibre; RUSITEC, rumen simulation technique; VFA, volatile fatty acid.

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properties of high-starch diets. The findings reported by Rémond *et al.*[7] support this concept, as propiogenesis has been shown to substantially increase when glycerol is added to high-fibre diets than when added to high-starch diets incubated *in vitro*. CH_4 production (ml CH_4/g DM incubated) in 24 h *in vitro* batch cultures has been reported to decrease when glycerol is added to alfalfa- or maize grain-based diets[14]. Since the acetate:propionate ratio is progressively reduced over a period of 3–4 d after glycerol supplementation in cattle[7,15], it is probable that enteric CH_4 production may decrease as rumen microbial populations adapt to the inclusion of glycerol in the diet.

As feeding of forage to breeding and growing herds in the beef cattle industry accounts for more than 80 % of greenhouse gas emissions and 55 % of CH_4 emissions[16], inclusion of glycerol in forage diets is likely to have a greater impact on the reduction of greenhouse gases emitted by beef cattle. We hypothesised that the inclusion of glycerol up to 150 g/kg in forage-based diets may decrease CH_4 production in a semi-continuous fermentation system (RUSITEC, rumen simulation technique). Thus, the objective of the present study was to evaluate the effects of adding glycerol to a forage diet in a semi-continuous fermentation apparatus (RUSITEC) on fermentation variables, including CH_4 production.

Experimental methods

The present experiment was conducted at the Agriculture and Agri-Food Canada Research Centre in Lethbridge, Alberta, Canada. Donor cows used in the experiment were cared for in accordance with the guidelines of the Canadian Council on Animal Care[17].

Experimental design and treatments

The experiment was a complete randomised design with four dietary treatments replicated in two RUSITEC apparatuses[18]. The duration of the experiment was 15 d. The first 8 d were used for adaptation, followed by 7 d of sampling (day 9 to day 15). The experimental treatments included brome hay, maize silage and glycerol in the following proportions: (1) 8·5 g hay + 1·5 g maize silage (control); (2) 8·5 g hay + 1·0 g maize silage + 0·5 g glycerol; (3) 8·5 g hay + 0·5 g maize silage + 1·0 g glycerol; (4) 8·5 g hay + 1·5 g glycerol. These amounts were selected to test inclusions of 50–150 g/kg based on previous *in vivo* results[4], which indicated DM intake reductions at glycerol concentrations exceeding 140 g/kg.

The ingredients and chemical composition of the substrates are reported in Table 1. Hay and silage were ground through a 4 mm screen (Arthur Thomas Company). Glycerol (99·5 % pure, Sigma–Aldrich) was thoroughly mixed with the hay portion of the diet for each treatment before filling the polyester bags (100 × 200 mm; pore size = 50 μm; B. & S.H. Thompson). Maize silage was incubated in separate bags (50 × 100 mm; pore size = 50 μm).

Table 1. Chemical composition of the substrates

	Brome hay (g/kg DM)	Maize silage (g/kg DM)
DM	953	982
Organic matter	909	930
Neutral-detergent fibre	683	459
Acid-detergent fibre	485	346
Crude protein	87·5	86·3
Crude fat	24	26
Ash	91	70
Non-fibrous carbohydrates*	115	359

* Calculated as 1000 − (crude protein + neutral-detergent fibre + crude fat + ash).

Experimental apparatuses and incubations

Each RUSITEC apparatus was equipped with eight 920 ml volume anaerobic fermenters. Each fermenter had an inlet for the infusion of buffer and an effluent output port. The fermenters were immersed in a water-bath maintained at 39°C. The four dietary treatments were randomly assigned to duplicate fermenters within each RUSITEC apparatus (four replications per treatment). The experiment was started by filling each fermentation vessel with 180 ml of warmed McDougall's buffer[19] modified to contain 1·0 g/l of $(NH_4)_2SO_4$, 720 ml of strained rumen fluid, one bag containing 20 g of wet solid rumen digesta and two additional bags containing the dietary ingredients as described above. After 24 h, the solid rumen digesta bag was replaced with two bags containing each feed. Thereafter, bags that had been incubated for 48 h were replaced daily. Artificial saliva was continuously infused into the fermenters at a dilution rate of 2·9 %/h. During nylon bag exchange, each fermentation vessel was flushed with O_2-free CO_2 to maintain anaerobic conditions. Effluent accumulation was measured daily during feed bag exchange and collected in a 2·0 litres container containing sodium azide (1 g/l) to arrest microbial growth.

Inoculum was obtained 2 h after feeding from two ruminally cannulated cows fed a forage diet containing barley silage, barley grain and a mineral vitamin supplement (71:25:4 DM basis). Rumen fluid was collected, pooled and filtered through four layers of cheesecloth into an insulated thermos and transported immediately to the laboratory. Approximately 400 g of ruminal solid digesta were also collected for the initial inoculation of the fermenters. Fermentation was initiated in the RUSITEC apparatuses on two consecutive days (two runs).

Sample collection

DM disappearance. DM disappearance at 48 h was determined daily from day 9 to day 15. Feed bags were removed from each fermenter, washed in cold, running distilled water until water was clear, and dried at 55°C for 48 h. To ensure that there was sufficient sample for analysis, silage and concentrate bag residues were pooled over 2 and 3 d, respectively. Samples were ground through a 1 mm screen in a Wiley mill (standard model 4; Arthur H. Thomas) before chemical analysis.

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Fermentation metabolites

Fermentation gas was collected into reusable 2-litre vinyl urine collection bags (Bard, Inc.) attached to each fermenter. Just before feed bag exchange, daily total gas production from each fermenter was determined by water displacement[20]. From day 9 to day 15, just before the determination of total gas, gas samples were collected from the septum of the collection bags using a twenty-six-gauge needle (Becton Dickinson). Samples (20 ml) were transferred to evacuated 6·8 ml exetainers (Labco Limited) for immediate analysis of CH_4. Fermenter pH was recorded (Orion model 260A, Fisher Scientific) daily at the time of feed bag exchange. To determine the concentration of volatile fatty acids (VFA), subsamples of fermenter liquid (4·0 ml) were collected directly from the fermentation vessels[19] at the time of feed bag exchange and placed in screw-capped vials preserved with 400 μl of 25 % (w/w) metaphosphoric acid and immediately frozen at −20°C until analysis. At the same time, 4·0 ml subsamples of fermenter fluid were also collected, placed in screw-capped vials and preserved with 400 μl of TCA until the determination of the concentration of NH_3-N. The concentrations of VFA and NH_3-N (mmol/l) were multiplied by the outflow rate of fluid infused to the vessels (litres/d) to determine VFA and NH_3-N production (mmol/d).

Chemical analysis

Subsamples of each treatment were used for chemical analysis. Feed and fermentation residues were analysed for DM content (method no. 930.15)[21] and ash (method no. 942.05)[21]. The concentration of neutral-detergent fibre (NDF) was determined and expressed inclusive of residual ash[22]. The concentration of acid-detergent fibre (ADF) was determined according to the method 973.18 (Association of Official Analytical Chemists)[21]. The concentration of total N (method no. 990.03)[21] was determined using a mass spectrometer (NA 1500, Carlo Erba Instruments)[23]. The concentration of crude fat was determined by diethyl ether extraction (Association of Official Analytical Chemists[21], method 920.39) using the Goldfisch Fat Extractor (Labconco Corporation). The concentrations of VFA and NH_3-N in the liquid effluent were determined by GC[23] and the modified Berthelot method[24], respectively. The concentration of CH_4 in the gas samples was determined using a Varian gas chromatograph equipped with GS-Carbon-PLOT 30 m × 0·32 mm × 3 μm column and thermal conductivity detector (Agilent Technologies Canada, Inc.). Oven temperature was 35°C (isothermal). The carrier gas was helium (27 cm/s), the injector temperature was 185°C (1:30 split, 250 μl injector volume), and the detector temperature was 150°C (thermal conductivity detector).

Statistical analysis

Data were analysed using the MIXED procedure of SAS (SAS, Inc., 2013; SAS Online Doc 9.1.3).

The model included the fixed effects of treatment (substrate), day and treatment × day interactions with the day of sampling from each fermenter treated as a repeated measure. Therefore, the individual fermenter was used as the experimental unit for statistical analysis. The minimum values of Akaike's information criterion were used to select the covariance structure among compound symmetry, heterogeneous compound symmetry, autoregressive, heterogeneous autoregressive, Toeplitz, unstructured and banded for each parameter. Orthogonal polynomial contrasts were carried out to test for linear, quadratic and cubic responses to increasing concentrations of glycerol (0, 50, 100 and 150 g/kg DM) in the substrate. Significance was declared at $P \leq 0·05$, and a trend was discussed when $0·05 < P < 0·10$.

Results

Effects of glycerol on nutrient disappearance

Increasing concentrations of glycerol resulted in a linear increase in DM disappearance from hay ($P = 0·001$) and maize silage ($P = 0·011$; Table 2). Crude protein disappearance from hay was not affected ($P = 0·788$), but that from silage was linearly increased ($P < 0·001$). Glycerol linearly increased NDF ($P = 0·040$) and ADF ($P = 0·031$) disappearance from hay and silage.

Effects of glycerol on fermentation

There were no interactions between treatments and sampling day for any of the fermentation variables. The inclusion of glycerol linearly decreased culture pH ($P = 0·035$) and increased total VFA production ($P < 0·001$; Table 3). A quadratic effect ($P = 0·023$) for acetate production was detected with increasing concentrations of glycerol, whereas propionate production was linearly increased ($P < 0·001$), resulting in a linear and quadratic decline ($P < 0·001$) in the acetate:propionate ratio. Increasing concentrations of glycerol also resulted in a linear increase in butyrate ($P < 0·001$) and valerate ($P < 0·001$) production. The concentration of NH_3 was linearly reduced by the addition of glycerol ($P < 0·001$), although the magnitude of the effect was small.

With increasing concentrations of glycerol in the substrate, 24 h cumulative gas production tended to increase linearly ($P = 0·061$; Table 4) and CH_4 concentration in gas was linearly increased ($P < 0·001$). This resulted in a linear increase in CH_4 production when expressed as total mg CH_4/d, mg CH_4/g total DM incubated ($P = 0·001$) and mg CH_4/g of hay DM disappeared ($P < 0·001$).

Discussion

The effects of glycerol on fibre digestion have been variable. The linear increase in DM, NDF and ADF loss from hay and silage is in agreement with the findings of the study of Wang *et al.*[25], who reported increased *in sacco* effective degradability of DM and NDF from forage as well as an improved digestibility of total tract nutrients, including NDF, when steers were fed increasing concentrations of glycerol (0, 11, 22 and 33 g/kg DM) in mixed diets (600 g/kg maize

Table 2. Effects of increasing concentrations of glycerol on the disappearance of DM, crude protein (CP), neutral-detergent fibre (NDF) and acid-detergent fibre (ADF) of brome hay and maize silage in the rumen simulation technique

(Mean values with their standard errors)

	Glycerol* (g/kg)					*P*		
Items	0	50	100	150	SEM	Linear	Quadratic	Cubic†
DM loss (mg/g)								
Hay	382	377	386	405	4·1	0·001	0·005	NS
Silage	510	523	562	–	13·7	0·011	NS	–
Total DM	402	424	461	501	3·7	< 0·001	0·008	NS
CP loss (mg/g)								
Hay	622	624	642	633	15·5	NS	NS	NS
Silage	633	674	706	–	9·7	< 0·001	NS	–
NDF loss (mg/g)								
Hay	213	224	221	245	10·7	0·040	NS	NS
Silage	83	119	157	–	14·4	< 0·001	NS	–
ADF loss (mg/g)								
Hay	67·8	79·3	79·1	113·0	9·62	0·031	NS	NS
Silage	26·2	23·5	67·4	–	3·26	< 0·001	< 0·001	–

* Experimental substrates: 0 = 8·5 g brome hay + 1·5 g maize silage; 50 = 8·5 g brome hay + 1·0 g maize silage + 0·5 g glycerol; 100 = 8·5 g hay + 0·5 g maize silage + 1·0 g glycerol; 150 = 8·5 g hay + 1·5 g glycerol.
† Cubic contrasts for silage disappearance cannot be calculated since there were only three levels of silage in diet DM.

stover and 400 g/kg concentrate). The results of the present study also concur with those of the study of Schröder and Südekum[26], who reported that fibre digestion was increased in low-starch diets when glycerol was included at a concentration of 150 g/kg DM. In another study, increasing concentrations of glycerol (0–400 g/kg diet DM) have been shown to not affect the *in vitro* degradability of NDF when added to lucerne hay[27]. However, reductions in fibre digestion when glycerol is added to starch-containing diets *in vivo*[26] and *in vitro*[28] have been reported. These results have been associated with the inhibition of hemicellulolytic and cellulolytic bacteria[28] and fungi[29]. Increased crude protein digestibility from silage is in contrast with the findings of previous studies, which have reported unaffected digestibility of total tract proteins in dairy cows fed glycerol[30] or decreased *in sacco* degradability of proteins in steers[25]. Discrepancies among studies on the effects of glycerol on fibre and protein digestibility are difficult to explain. It is possible that some proteolytic and fibrolytic species may have responded differently to glycerol in the present study, but this is difficult to ascertain as microbial populations were not determined. The quantification of organisms would be important to resolve contradictory results of the effects of glycerol on fibre digestion.

Previous studies have consistently reported a decreased molar proportion of acetate and increases in the proportion of propionate in *in vitro* conditions using glycerol in starch-rich[9,10] and forage substrates[10,27], as well as *in vivo* in finishing beef cattle fed concentrate diets[3,26] and in transition dairy cows[31]. This concurs with the results of the present study and confirms the propiogenic properties of glycerol. Shifts towards reduced acetate:propionate ratio derived from the increased concentrations of propionate and increases in the concentrations of butyrate have also been reported

Table 3. Effects of increasing concentrations of glycerol on the fermentation characteristics of a brome hay–maize silage diet in the rumen simulation technique

(Mean values with their standard errors)

	Glycerol* (g/kg)					*P*		
	0	50	100	150	SEM	Linear	Quadratic	Cubic
VFA production (mmol/d)								
Total	18·6	19·2	20·8	25·5	1·02	< 0·001	0·045	NS
Acetate	10·2	9·2	8·8	10·6	0·56	NS	0·023	NS
Propionate	4·2	4·9	6·3	8·4	0·57	< 0·001	NS	NS
Butyrate	2·7	3·1	3·4	4·1	0·21	< 0·001	NS	NS
Valerate	1·0	1·5	1·8	1·9	0·11	< 0·001	0·102	NS
Caproate	0·11	0·14	0·15	0·13	0·001	0·041	0·013	NS
Acetate:propionate ratio	2·6	1·9	1·4	1·3	0·05	< 0·001	< 0·001	NS
NH_3-N (mmol/d)	7·0	6·9	6·5	6·5	0·12	< 0·001	NS	NS
pH	7·11	7·10	7·09	7·07	0·011	0·035	NS	NS

VFA, volatile fatty acids.
* Experimental substrates: 0 = 8·5 g brome hay + 1·5 g maize silage; 50 = 8·5 g brome hay + 1·0 g maize silage + 0·5 g glycerol; 100 = 8·5 g hay + 0·5 g maize silage + 1·0 g glycerol; 150 = 8·5 g hay + 1·5 g glycerol.

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Table 4. Effects of increasing concentrations of glycerol on cumulative gas production and methane production in the rumen simulation technique

(Mean values with their standard errors)

	Glycerol* (g/kg)					*P*		
	0	50	100	150	SEM	Linear	Quadratic	Cubic
Gas volume (ml)	930	1015	1058	1056	57·1	0·061	NS	NS
CH_4 (%)	1·13	1·59	1·78	2·02	0·158	< 0·001	NS	NS
CH_4 (mg/d)	7·51	11·54	13·54	15·32	1·639	0·001	NS	NS
CH_4 (mg/g hay DMD)	2·62	3·64	4·46	4·89	0·586	0·004	NS	NS
CH_4 (mg/g substrate incubated)†	0·78	1·21	1·44	1·63	0·175	0·001	NS	NS
CH_4 (mg/g total DMD)	1·96	2·86	3·15	3·29	0·302	< 0·001	0·113	NS

DMD, DM disappeared.
* Experimental substrates: 0 = 8·5 g brome hay + 1·5 g maize silage; 50 = 8·5 g brome hay + 1·0 g maize silage + 0·5 g glycerol; 100 = 8·5 g hay + 0·5 g maize silage + 1·0 g glycerol; 150 = 8·5 g hay + 1·5 g glycerol.
† DM basis.

in vitro using starch substrates[9] and *in vivo* using starch- and forage-based diets[26].

The linear increase in CH_4 proportion in total gas and total CH_4 production as a function of total DM disappeared contradicts our hypothesis. The fermentation of carbohydrates to propionate has been described as a hydrogen sink, and feeding propiogenic substrates has been proposed as a CH_4 abatement strategy[11–13]. However, glycerol is a more reduced substrate than sugars and releases two electron pairs for each mole of glycerol converted to pyruvate[32], one in the oxidation of glycerol to dihydroxyacetone, which is then phosphorylated and enters glycolysis, and the other in glycolysis itself, in the oxidation of 3-phosphoglyceraldehyde to 3-phosphoglycerate[33]. This compensates for electron incorporation in the conversion of pyruvate or phosphoenolpyruvate to propionate. Thus, there is no net electron incorporation in the conversion of glycerol to propionate:

$$CH_2OHCHOHCH_2OH \rightarrow CH_3CH_2COOH + H_2O$$

.

Glycerol failed to decrease CH_4 production as hypothesised, but increased it. There was an increase in butyrate production as glycerol replaced maize silage. Butyrate production from both carbohydrates and glycerol would result in a release of reducing equivalents and contribute to increasing CH_4 production:

$$CH_2OHCHOHCH_2OH \rightarrow \tfrac{1}{2}CH_3CH_2CH_2COOH + CO_2 + 2[2H].$$

Less amounts of glycerol seem to be fermented to acetate[34]. Acetate production was quadratically affected by the substitution of maize silage with glycerol, but changes were of relatively low magnitude. Glycerol stimulated DM disappearance, but because glycerol replaced maize silage, the amounts of total forage digested DM were actually lower as there were less amounts of maize silage to be digested and less amounts of carbohydrates were fermented. Therefore, changes in acetate production seem to have resulted from a shift in carbohydrate fermentation towards acetate, which would also release reducing equivalents and contribute to the increase in CH_4 production, because the increase in propionate production from glycerol would not demand extra reducing equivalents. The formation of some butyrate and acetate from glycerol instead of from carbohydrates would further contribute to the enhancement of methanogenesis, again because being more reduced than carbohydrates, glycerol would result in a greater release of reducing equivalents per mol of acetate and butyrate produced compared with carbohydrates.

An alternative explanation for the increase in CH_4 production with glycerol is based on the equimolar conversion of glycerol to formate and ethanol by an isolate from deer rumen identified as *Klebsiella planticola*[35]. Formate is a precursor of CH_4[36], and large amounts of ethanol are oxidised to acetate in the rumen[37,38], a process that releases reducing equivalents that can be used for CH_4 production[39]. It has been shown that pure cultures of *Ruminococcus flavefaciens*[40], *R. albus*[41] and a ruminal fungus[42] decrease formate and ethanol production when co-cultured with methanogens, as CH_4 becomes the main electron sink in the co-cultures. Also, some microorganisms can convert glycerol to 1,2-propanediol[43], and in turn there is some recovery of 1-^{14}C-1, 2-propanediol incubated in ruminal continuous cultures as $^{14}CH_4$[44].

The adaptation of donor animals to diets containing glycerol seems to have affected fermentation when glycerol was included in *in vitro* batch culture incubations. Gas and CH_4 production was increased when 150 g/kg glycerol was included in the substrates (900 g/kg concentrate based on rolled maize, maize gluten feed and soyabean hulls) using inoculum obtained from glycerol-adapted animals[45], but changes in CH_4 production were negligible when the inoculum was obtained from unadapted animals, suggesting that microbial adaptation influences digestion and fermentation end products. This explains, at least partially, the differences between previous studies reporting no effect[10] or decreased CH_4 production[14] when incubating glycerol in *in vitro* batch cultures using inoculum obtained from unadapted animals as opposed to the results of the present study, where increased propionate and total VFA production and linear increase in DM loss were found to be associated with increased CH_4 production (mg CH_4/g DM digested) using glycerol-adapted fermenters. When increasing concentrations of glycerol were fed to adapted lambs, no effects on CH_4 emissions were observed[4]. In this case, absorption through the rumen wall or passage to the lower gut or both may have impeded fermentation of an important proportion of glycerol[7], thus reducing the release of hydrogen electrons in the rumen environment when compared with *in vitro* fermenters where absorption is precluded.

Conclusions

Increasing concentrations of glycerol in forage diets incubated in a RUSITEC apparatus improved DM, NDF and ADF disappearance from brome hay and maize silage and crude protein disappearance from maize silage. The acetate:propionate ratio was linearly decreased as a result of increased production of propionate. The concentrations of CH_4 in gas and total CH_4 production per unit of DM digested or incubated were increased, as the fermentation of glycerol to propionate does not act as a hydrogen sink.

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The authors' contributions were as follows: J. A.-S., A. V. C. and T. A. M. designed the study; J. A.-S. and G. d. O. R. conducted the experimental procedures and laboratory analyses; J. A.-S. and A. V. C. analysed and interpreted the data; J. A.-S. wrote the first draft of the manuscript; A. V. C., T. A. M., E. M. U. and G. d. O. R. critically revised the manuscript.

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