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ANIMAL PRODUCTION

Metabolic profile and milk production of cows grazing on ryegrass with crude glycerin supplementation

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ABSTRACT: To assess the effect of crude glycerin inclusion on the metabolic profile of lactating cows grazing on ryegrass, an experiment was conducted with eighteen Holstein cows, divided between two treatments: 1) a basal diet without crude glycerin supplementation, 2) a basal diet supplemented with crude glycerin at a rate of 0.3% of the animals' live weight. A double crossover design was used over a 56-day experiment, divided into four 14-day periods, with the initial period used only to standardize the diet. Data were analyzed blocking for lactation period, including results for milk production (liters per day and corrected to 4% fat content), blood and urine urea and glucose levels, and milk urea nitrogen levels. The inclusion of crude glycerin at 3% of live weight in the diet of lactating Holstein cows grazing on ryegrass was found to reduce urea excretion in milk and increase blood glucose levels, without affecting milk production. Therefore, its inclusion in their diet is recommended.
Key words: dairy cattle, glycerol, glucose, urea nitrogen.

Perfil metabólico e produção de leite de vacas em pastejo com azevém e inclusão de glicerina bruta na dieta

RESUMO: Com o objetivo de avaliar o efeito da inclusão de glicerina bruta sobre o perfil metabólico de vacas em lactação, em pastejo de azevém, foi conduzido um experimento com dezoito animais, da raça holandesa, divididos entre os tratamentos: 1) Dieta basal sem adição de glicerina bruta, 2) Dieta basal suplementada com glicerina bruta na proporção de 0,3% do peso vivo dos animais. O delineamento utilizado foi o duplo reverso, em um período experimental de duração de 56 dias, dividido em quatro períodos de 14 dias, sendo o primeiro período utilizado apenas para padronização da dieta. Foram analisados, bloqueando por período de lactação, resultados referentes à produção de leite (litros dia^{-1} e corrigida para produção de 4% de gordura), nível de ureia e glicose no sangue e urina, nível de nitrogênio ureico do leite. Verificou-se que a inclusão de 0,3% de glicerina bruta sobre o peso vivo de vacas holandesas, em lactação, em pastejo de azevém, reduz a eliminação de ureia via leite e aumenta os níveis sanguíneos de glicose, não tendo efeito sobre a produção de leite, sendo recomendada sua utilização na dieta.
Palavras-chave: bovinos leiteiros, glicerol, glicose, nitrogênio ureico.

INTRODUCTION

In Brazil's subtropical regions, dairy cattle diets during the winter season rely on cultivated forage plants, primarily ryegrass (*Lolium multiflorum* Lam.), which is characterized by its high crude protein content. However, excessive protein intake in dairy cow diets can compromise animal health and milk production (BUTLER, 1998). Consequently, there is a growing interest in alternative energy sources that can help balance cattle diets.

This study is a pioneering investigation into the inclusion of crude glycerin in the diet of dairy cows grazing on ryegrass, representing an innovation in the use of agro-industrial by-products for animal nutrition. Using this by-product as an energy source not only contributes to the nutritional balance of protein-rich diets but also shows significant potential to improve milk production and reduce feeding costs, thereby providing economic and environmental benefits to the agricultural sector.

Crude glycerin is readily assimilated by ruminal microorganisms and extensively metabolized in the liver (ABO EL-NOR et al., 2010). It has the potential to reduce the biohydrogenation of fatty acids in the rumen (KRUEGER et al., 2010) and increase energy supply by raising propionate production (LEE et al., 2011). The increase in propionate enhances glucose synthesis in the liver through precursor molecules in the gluconeogenesis pathway. Glucose is vital for maintaining and improving milk production (BERCHIELLI et al., 2006)

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To assess the effect of crude glycerin supplementation in the diet of dairy cows grazing on ryegrass, data from the animals' metabolic profile variables (blood and urine urea and glucose levels, as well as milk urea nitrogen levels) can be used and correlated. Despite evidence of the energetic potential of crude glycerin, few studies have directly correlated its effects on the metabolic profile of dairy cows grazing on ryegrass. This study aims to fill that gap by assessing the effect of including crude glycerin at 0.3% of live weight on the metabolic profile and milk production of lactating Holstein cows grazing on ryegrass.

MATERIALS AND METHODS

The experimental trial was carried out in the municipality of Augusto Pestana, in Rio Grande do Sul state (RS), Brazil (28°31'2" South, 53°59'39" West), from July to August 2015.

Eighteen lactating Holstein cows (600 ± 50 kg body weight) were assigned to two treatments:1) a basal diet without crude glycerin supplementation, 2) a basal diet with crude glycerin supplementation at 0.3% of live weight. The experimental period lasted 56 days and was divided into four 14-day periods. The cows were randomly paired and, during the initial experimental period, all received the same standard diet, based on ryegrass pasture, corn silage, and commercial feed (referred to as the adaptation period). The animals were blocked according to days in lactation (DIL) into three categories: 60-120 days, 121-200 days, and 201-320 days, and by similar milk production levels, in a sequence of three periods (1, 2, and 3) using a simple crossover design.

The cows were maintained on a ryegrass (*Lolium multiflorum* Lam.) pasture, the tetraploid cultivar Bar HQ®, under rotational grazing, as a single herd in the experimental area and supplied with a minimum dry matter allowance (DMA) of 25 kg/animal/day.

The DMA (kg of DM/animal/day) was calculated using bromatological analyses, as described by RODRIGUES (2010). The available biomass at paddock entry and exit was calculated based on the following steps. First, forage samples were collected before the animals entered the paddock, by cutting the forage at ground level within randomly distributed quadrats of known area (0.25 m²). Next, forage mass was estimated by immediately weighing the collected samples to determine green biomass (fresh mass). These samples were then dried in an oven at 65 ºC for 48 hours to determine dry matter content and the available biomass per hectare. Thus, the DMA was adjusted according to the paddock area and the number of animals present.

All animals were managed under the same feeding and grazing conditions, namely, rotational grazing on ryegrass between milking sessions, 17% crude protein concentrate (adjusted according to milk production) and 10 kg of corn silage dry matter per day in troughs. The only difference between treatments was the inclusion or exclusion of crude glycerin. Each animal was weighed at the beginning of every experimental period to determine daily crude glycerin intake, based on a dry matter consumption of 3% of live weight. Corn silage was placed in individual troughs, followed by the concentrate on top, with the glycerin mixed into the concentrate.

The crude glycerin used was in liquid form, with an oily texture and dark yellow color. It was produced from 100% soybean feedstock and had the following composition: 80% glycerol, 12% water, 1% methanol, 2% mineral matter, 3% organic matter, and 2% sodium chloride.

Forage samples were collected at the entrance of each paddock during the experiment using a simulated grazing method. A composite sample was prepared for each experimental period. Similarly, corn silage and concentrate samples were collected and analyzed for each period. Forage samples were kept frozen until they were sent to the laboratory for bromatological analysis. The analyses followed standardized chemical methods using protocols described by RODRIGUES (2010) in the Embrapa Clima Temperado manual. The following procedures were used:

Dry Matter (DM) was determined using the gravimetric method in an oven at 105 ºC until a constant weight was reached, following the Weende system. Pre-drying was conducted at 65 ºC in a forced-air oven to prevent the volatilization of nitrogen compounds.

Mineral Matter (MM) was determined in a muffle furnace at 550-600 ºC using the simple incineration method to obtain ash weight.

Crude Protein (CP) was calculated based on nitrogen content, determined by the Kjeldahl method. This involved block digestion at 400 ºC, followed by distillation and titration with 0.05 N H_2SO_4. A factor of 6.25 was used to calculate CP.

Neutral Detergent Fiber (NDF) and Acid Detergent Fiber (ADF) were analyzed using the method described by VAN SOEST et al. (1991), using specific detergent solutions to remove soluble components and retain fibrous fractions.

Ether Extract (EE) was determined using the Soxhlet method, with petroleum ether as the solvent for lipid extraction.

These methods were carried out at the Bromatology Laboratory of the Universidade Regional do Noroeste do Estado do Rio Grande do Sul (UNIJUÍ), following the safety and standardized procedures described by RODRIGUES (2010). The bromatological analysis results of the feeds are presented in table 1. Diet analyses during the different experimental periods, with and without crude glycerin, are detailed in table 2.

During the final two days of each experimental period, milk samples (approximately 40 mL per animal) were collected from both morning and afternoon milkings (60% from the morning and 40% from the afternoon, proportional to total milk production). Samples were stored in vials containing the preservative Bronopol (2-bromo-2-nitro-1,3-propanediol) and sent to an official laboratory for Milk Urea Nitrogen (MUN) analysis using infrared spectroscopy.

Milk production (liters/day) was recorded over the final six days of each experimental period by measuring milk yield from morning and afternoon milkings using a semi-automated milking system. The average production per animal per period was then calculated. Milk production was corrected to 4% fat content using the formula: [(0.4 × milk yield) + 15 × (milk yield × % fat / 100)] (NRC, 2001).

Blood samples were collected from all animals via coccygeal vein puncture on the final day of each experimental period, always in the morning after feeding, using 10 mL vacuum tubes containing a clot activator. After centrifugation, the serum was separated, frozen, and sent to the Clinical Analysis Laboratory at UNIJUÍ for glucose and urea analysis. Urea concentration was determined using the urease-colorimetric method with the commercial Urea CE® kit (Labtest Diagnóstica S.A., Lagoa Santa, Minas Gerais state, Brazil). This method

Table 1 - Nutritional composition of ryegrass pasture, corn silage, and concentrate in the 3 experimental periods.

Period	Items	----------Foods----------		
		Ryegrass Pasture	Corn Silage	Concentrate**
1	DM%	9.24	23.43	83.50
	MM% in DM	15.54	4.62	10.83
	OM% in DM	84.46	95.38	89.17
	CP% in DM	31.81	7.49	17.05
	NDF% in DM	50.87	59.98	-
	ADF% in DM	43.93	51.84	-
	EE% in DM	5.45	4.21	4.16
2	DM%	13.70	23.43	83.50
	MM% in DM	13.25	4.62	10.83
	OM% in DM	86.75	95.38	89.17
	CP% in DM	21.95	7.49	17.05
	NDF% in DM	58.45	59.98	-
	ADF% in DM	38.51	51.84	-
	EE% in DM	3.46	4.21	4.16
3	DM%	10.27	23.43	83.50
	MM% in DM	14.34	4.62	10.83
	OM% in DM	85.66	95.38	89.17
	CP% in DM	28.88	7.49	17.05
	NDF% in DM	54.00	59.98	-
	ADF% in DM	43.28	51.84	-
	EE% in DM	3.83	4.21	4.16

*DM = Dry Matter, MM = Mineral Matter, OM = Organic Matter, CP = Crude Protein, NDF = Neutral Detergent Fiber, ADF = Acid Detergent Fiber, EE = Ether Extract.

**Concentrate Composition: Antioxidant additive (BHA and BHT), Palatability additive, Sodium bicarbonate, Biotin, Calcitic limestone, Potassium chloride, Sodium chloride (common salt), Ventilated sulfur (flower of sulfur), Rice bran, Soybean meal (GMO from *Agrobacterium* sp.), Calcium iodate, Ground whole corn (GMO from *Bacillus thuringiensis*, *Streptomyces* sp.), Sodium monensin, Magnesium oxide, Zinc oxide, Saccharomyces cerevisiae, Sodium selenite, Cobalt sulfate, Copper sulfate, Iron sulfate, Manganese sulfate, Vitamin A, Vitamin D3, and Vitamin E.

Table 2 - Nutritional composition of the diet in the 3 experimental periods.

Period	Items'	------------------------------------Diet------------------------------------	
		With Crude Glycerin	Without Crude Glycerin
1	DM%	18.40	17.00
	NFC% in DM	35.70	29.40
	CHO Ferm.% in DM	43.70	41.70
	PB% in DM	20.44	22.71
	FDN% in DM	18.10	20.50
	EE% in DM	4.60	5.10
2	DM%	24.80	23.00
	CNF% in DM	35.70	29.40
	Ferm CHO% in DM	45.50	43.70
	CP% in DM	16.56	18.41
	NDF% in DM	19.90	22.60
	EE% in DM	3.80	4.20
3	DM%	20.00	18.50
	NFC% in DM	35.70	29.40
	Ferm. CHO% in DM	44.40	42.50
	CP% in DM	19.28	21.43
	NDF% in DM	18.80	21.30
	EE% in DM	4.00	4.40

*DM = Dry Matter, NFC = Non-Fibrous Carbohydrates (NFC), Ferm. CHO = Fermentable Carbohydrates, CP = Crude Protein, NDF = Neutral Detergent Fiber, EE = Ether Extract.

involves the enzymatic hydrolysis of urea by urease, followed by a reaction with Berthelot's reagent, measuring absorbance at 570 nm using a spectrophotometer. Glucose was oxidized by the enzyme glucose oxidase, producing hydrogen peroxide, which reacted with specific chromogens to form a colored complex read by spectrophotometer at 505 nm. The enzymatic GOD/POD method was performed using the Glucose Liquiform® Kit (Bioclin/Quibasa Química Básica Ltda, Belo Horizonte, Minas Gerais, Brazil).

Urine samples (100 mL) were collected from each cow after morning and afternoon milkings via perineal or vulvar massage. Samples were centrifuged at 3,000 rpm for 10 minutes and filtered through gauze. A 10 mL aliquot was then diluted with 1 mL of 20% sulfuric acid to maintain a pH below 3.0, preventing bacterial growth and volatilization. The sample was homogenized and pH was determined using test strips, then stored at -20 ºC. From each collection, 5 mL were taken from the morning and afternoon samples over seven days, resulting in a 70 mL composite sample per cow per period. These samples were submitted to the Clinical Analysis Laboratory at UNIJUÍ for urea and glucose quantification. Urine urea concentration was determined using the enzymatic urease method, with the commercial Ureia CE kit (Labtest Diagnóstica), and urine glucose using the enzymatic glucose oxidase/peroxidase (GOD/POD) method, with the Glucose Liquiform Kit (Bioclin/Quibasa).

For analytical purposes, crude glycerin supplementation in the diet was considered a fixed effect, and the block (blocked lactation days) a random effect. To assess the effect of crude glycerin inclusion on the metabolic profile, the following variables were evaluated: blood and urine urea levels, milk urea nitrogen, blood and urine glucose levels, and milk production (liters/day and 4% fat-corrected milk yield). The results for metabolic variables (urine, blood, and milk), milk production, and feed intake were analyzed using the MIXED procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Data were submitted to analysis of variance (ANOVA), and treatment effects were assessed using Tukey's test for paired data. Differences were considered statistically significant when $P < 0.05$. Pearson's correlation coefficient was used to analyze the relationship between blood, urine and milk urea levels; blood glucose and urine levels; and milk production. Significance was set at $P < 0.05$.

RESULTS

Table 3 presents the mean values for urea (blood, milk, and urine), glucose (blood, urine), and milk production at different stages of lactation for Holstein cows fed a diet with or without crude glycerin supplementation. In this study, the inclusion of 0.3% crude glycerin based on live weight reduced MUN excretion. The group treated with crude glycerin maintained MUN levels approximately two percentage points lower than the control group. A significant increase in blood glucose levels was observed when crude glycerin was included in the diet, but there was no effect on milk production (both in liters and fat-corrected to 4%) or glucose levels in urine.

With respect to correlation analysis, a positive linear correlation was observed between blood urea, urinary urea, and MUN in both the group supplemented with crude glycerin and the control group. Additionally, increases in urea levels (milk, blood, and urine) were associated with a decrease in milk production, showing a negative correlation.

DISCUSSION

The blood urea levels (Table 4) were within the range typically found for lactating cows, which is between 42.8 and 64.2 mg/dL (KANEKO et al., 1997). As expected, urea levels in urine were higher than in blood, given the kidneys substantial capacity

Table 3 - Average values of urea (in blood, milk, and urine), glucose (in blood and urine), milk production, and feed leftovers in the trough, across different lactation periods of Holstein cows, fed a diet supplemented or not with 10% crude glycerin in the total dry matter.

Lactation days	With Crude Glycerin	Without Crude Glycerin	P-value	CV (%)
----------Blood Urea (mg dL^{-1})----------				
60 to 120	49	52		
121 to 200	51	56		
201 to 320	53	58		
Mean and Standard Deviation	51 ± 14	55 ± 12	0.3648	23.95
----------Urine Urea (mg dL^{-1})----------				
60 to 120	157	166		
121 to 200	166	186		
201 to 320	167	223		
Mean and Standard Deviation	163 ± 88	192 ± 94	0.3716	52.03
----------Milk Urea Nitrogen (mg dL^{-1})----------				
60 to 120	20.28	21.79		
121 to 200	21.47	23.51		
201 to 320	22.64	23.43		
Mean and Standard Deviation	21^{a} ± 5	23^{b} ± 6	0.0452	23.91
----------Blood Glucose (mg dL^{-1})----------				
60 to 120	71	64		
121 to 200	69	63		
201 to 320	64	58		
Mean and Standard Deviation	68^{a}± 5	62^{b}± 9	0.0283	11.47
----------Urine Glucose (mg dL^{-1})----------				
60 to 120	24	12		
121 to 200	18	10		
201 a 320	15	9		
Mean and Standard Deviation	19 ± 24	10 ± 22	0.2692	172.82
----------Milk Production (liters day^{-1})----------				
60 to 120	36	36		
121 to 200	28	27		
201 to 320	27	25		
Mean and Standard Deviation	30 ± 6	29 ± 7	0.6187	15.62
----------Milk production (4% fat corrected)----------				
60 to 120	30	29		
121 to 200	25	24		
201 to 320	24	23		
Mean and Standard Deviation	26 ± 8	25 ± 9	0.7051	17.26

Different letters in the same row indicate significant differences for $P < 0.05$ due to the inclusion of crude glycerin, according to Tukey's test.

Table 4 - Correlations among blood urea (mg/dL), urine urea (mg/dL), milk urea nitrogen (mg/dL), blood glucose (mg/dL), urine glucose (mg/dL), and milk production (liters/day) of Holstein cows, and feed leftovers in the trough (g/day) under diets with and without crude glycerin inclusion.

-----With Crude Glycerin-----						
Variables	Blood Urea	Urine Urea	Milk Urea Nitrogen	Blood Glucose	Urine Glucose	Milk production
Blood urea	-	-	-	-	-	-
Urine urea	0.9078	-	-	-	-	-
Milk urea nitrogen	1.0000	0.9099	-	-	-	-
Blood glucose	-0.9707	-0.7805	-0.9695	-	-	-
Urine glucose	-0.9820	-0.9707	-0.9829	0.9078	-	-
Milk production	-0.9122	-0.9999	-0.9142	0.7871	0.9732	-
-----Without Crude Glycerin-----						
Variables	Blood Urea	Urine Urea	Milk Urea Nitrogen	Blood Glucose	Urine Glucose	Milk production
Blood urea	-	-	-	-	-	-
Urine urea	0.9357	-	-	-	-	-
Milk urea nitrogen	0.9306	0.7416	-	-	-	-
Blood glucose	-0.8486	-0.9807	-0.5960	-	-	-
Urine glucose	-1.0000	-0.9357	-0.9306	0.8486	-	-
Milk production	-0.9869	-0.8665	-0.9775	0.7521	0.9869	-

to excrete urea and maintain higher concentrations in urine compared to plasma levels (ORTOLANI, 2002).

With respect to MUN, there are some differences in the literature. According to JOHNSON and YOUNG (2003), values range from 10 to 15 mg/dL, while FERGUSON et al. (1993), JONKER et al. (1998), and BUTLER et al. (1996) consider values above 19 mg/dL as high, associating them with reduced milk production and reproductive losses. ALMEIDA (2012) reports that the average concentration should be between 12 to 16 mg/dL. In general, the values for MUN in this study were above those described in the literature, likely due to the high CP content in the diet which allowed for the assessment of this effect (Table 2).

Despite the occurrence of glucosuria, blood glucose levels remained close to the physiological parameters for the species (Table 3). For cattle, blood glucose concentrations range from 45 to 75 mg/dL according to KANEKO et al. (1997) and 50 to 80 mg/dL according to FERNANDES et al. (2012).

As reported by BUTLER (2001), excessive intake of degradable protein in the rumen results in elevated plasma and tissue levels of ammonia, urea, and other nitrogenous compounds. This is because most of the ammonia absorbed in the digestive tract is converted to urea by the liver, resulting in increased circulating blood levels and excretion of urea in urine or milk. The reduction in MUN observed in the present study is related to the effect of crude glycerin, which directly increases the availability of metabolizable energy to the animals and, indirectly, enhances ruminal microbial growth and fiber digestibility.

Crude glycerin incorporated into the animals' diet is fermented in the rumen, where its glycerol is converted primarily into propionic acid (PAIVA et al., 2016). Once absorbed into the bloodstream, propionate is metabolized in the liver, where it is used to form glucose through the gluconeogenesis pathway (VIVENZA, 2012). Glycerol absorbed by the ruminal epithelium is also converted into glucose in the liver (KREHBIEL, 2008). Glycerol also positively affects amino acid or nitrogen retention (CERRATE et al., 2006), by inhibiting the activity of the enzymes phosphoenolpyruvate carboxykinase and glutamate dehydrogenase. This conservation of gluconeogenic amino acids increases the amount of ammonia available in the ruminal fluid for microbial development.

Thus, in the present study, the ammonia released in the ruminal fluid from the degradation of nitrogenous compounds from ryegrass, combined with an adequate energy supply via crude glycerin, was used to synthesize microbial protein, resulting in lower urea excretion in the milk.

ÁVILA-STAGNO et al. (2014), studying the inclusion of up to 15% crude glycerin in the DM of forage-based diets, observed an increase in propionate followed by a rise in butyrate in the rumen. The use of crude glycerin can provide a significant amount of gluconeogenic precursors due to its higher conversion to propionate. This increases the availability of carbon that can be used for fatty acid synthesis (VIVENZA, 2012), potentially leading to changes in milk production, observed in our study.

According to GONZALEZ et al. (2000), increased energy intake can inversely influence ruminal ammonia concentration due to enhanced microbial protein synthesis, which also reduces circulating and excreted urea levels. Additionally, SILVA (2017) underscores that urea nitrogen levels in milk are strongly correlated with plasma urea concentration. Due to its low molecular weight, urea easily diffuses through organic fluids, including the milk in the mammary gland (FERGUON & CHALUPA, 1989). Studies confirm that urine nitrogen excretion has a linear, positive correlation with blood and milk levels (KAUFFMAN & ST-PIERRE, 2001).

MUN is as an indicator of adequate or excess ruminal ammonia in relation to the energy available for microbial growth in the rumen. High levels of ruminally available (degradable/soluble) protein relative to the amount of available carbohydrates result in high MUN levels. When crude glycerin was not included in the animals' diet, no significant correlation was found between milk urea and blood glucose levels. This highlights the potential of crude glycerin inclusion in the diet to increase blood glucose and reduce MUN. Moreover, the increase in blood glucose is often associated with an increase in milk production.

Although total dry matter intake (DMI) was not directly measured in this study, some inferences can be made based on the nutritional composition of the diet, the inclusion of glycerin, and the metabolic results obtained. Including glycerin in ruminant diets can alter voluntary DMI due to its rapid ruminal fermentation and efficient conversion into propionate (BORGES, 2014). This metabolic mechanism may result in a reduction in the consumption of fibrous forages. Thus, it is likely that, even without direct measurement, DMI was influenced by the palatability of glycerin and its effect on the digestibility of other components in the diet. The metabolic results suggest that including glycerin may have influenced fibrous forage intake and, consequently, affected urea levels in the blood, urine, and milk.

Glycerin can also affect crude protein (CP) and neutral detergent fiber (NDF) intake, since including easily fermentable energy sources can reduce ruminal retention time and fiber degradation (BOYD et al., 2013). As a result, in diets containing high glycerin levels, lower effective fiber consumption can be expected, potentially affecting blood urea concentration, as observed in the data presented here.

Although these hypotheses are consistent with the observed metabolic effects, we suggest that future experiments be designed to include direct measurement of DM and nutrient intake, allowing for a more accurate analysis of the relationship between intake and metabolic outcomes. This improvement could clarify whether the observed effects are due to a possible modification in intake or the inclusion of glycerin itself.

CONCLUSION

The inclusion of 0.3% crude glycerin based on live weight in the diet of lactating Holstein cows, grazing on ryegrass and supplemented with corn silage and concentrate, reduces the excretion of urea via milk and increases blood glucose levels, without affecting milk production or feed intake. Its inclusion in the diet is therefore recommended.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHORS' CONTRIBUTIONS

All authors equally contributed to the development of this manuscript.

BIOETHICS AND BIOSECURITY COMMITTEE APPROVAL

The experiment was conducted in accordance with the regulations of the animal welfare ethics committee at the Universidade Regional do Noroeste do Estado do Rio Grande do Sul State, Brazil, under protocol number 018/2014.

DATA AVAILABILITY STATEMENT

The raw data may be obtained with authors at request.

DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE

No use of AI was made in the present paper.

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