# Reticular Outflow, Rumen Dynamics, and Ingestive Behavior in Buffaloes (*Bubalus bubalis*) Fed Diets with Different Sources of Energy

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**Abstract:** The objective of this study was to evaluate the effects of two energy sources, associated or not (crude glycerin and castor oil) in buffaloes' diets on intake, digestibility, rumen kinetics, feeding behavior, nitrogen balance, microbial protein synthesis, rumen fermentation, and blood metabolites. Four ruminally-cannulated Murrah buffaloes [526 ± 29 kg of initial BW] were randomly assigned according to a 4 x 4 Latin square design in which the animals were randomly allocated to each treatment: CONT = control with soybean meal associated with ground corn; GLY = crude glycerin, dietary inclusion of 90 g/kg; CAO = castor oil, dietary inclusion of 50 g/kg; GLYCAO = crude glycerin associated with castor oil, dietary inclusions of 50 g/kg GLY and 50 g/kg CAO. A higher ruminal renewal rate of DM and NDF and DM passage rate was observed for animals fed CON and GLY than the other diets ($P < 0.05$), the same fact was observed for rumination efficiency grams DM/hour ($P < 0.001$). Among the feed sources, crude glycerin can partially replace ground corn in the buffalo's diet without compromising intake, nutrient metabolism, and rumen dynamics.

**Keywords:** Animal nutrition, biodiesel, by-products, ethology, fatty acids, nitrogen utilization, passage rate.

## INTRODUCTION

The search for alternative feeds in animal nutrition requires a holistic view global. Among the energy possibilities are biofuels, considered low-carbon substitutes for fossil fuels, which can help reduce greenhouse gas emissions and the effects of climate change. On the other hand, the increase in demand for vegetable raw materials (corn, wheat, and oilseeds) for biodiesel production impacts an increase in the prices of foods commonly used in animal nutrition.

The production of conventional feed energy concentrates, such as starchy ones like ground grain corn, is a very expensive option that can be seen as directly competing with human food security [1]. Thus, the increase in the price of this cereal, due to its high utilization in human food and ethanol production, in addition to the high demand for feed for non-ruminants, is causing a growing interest in the use of alternative and lower-cost feeds for ruminants.

Several other raw materials are potential substrates for the synthesis of biofuels, generating products that can be used in animal feed and that do not compete with human food. Among the products generated is crude glycerin (GLY), a by-product obtained from the chemical process based on the transesterification of vegetable oils with alcohol through base catalysis for biodiesel production. Glycerol makes up the majority of GLY, along with other substances like lipids, alcohol, and minerals. As glycerol has an energy content comparable to corn grain, using it in ruminant diets produces satisfactory results and reduces production costs.

Glycerol can be absorbed directly into the rumen epithelium of a ruminant animal after ingestion, where it is subsequently transformed into glucose in the liver. In addition, it can serve as a substrate for the rumen microbial population to produce volatile fatty acids (VFAs), mainly propionate, a gluconeogenic substance crucial for ruminants.

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ISSN: 1927-5196 / E-ISSN: 1927-520X/25

Castor oil (CAO), a non-starchy energy feed, is another food alternative capable of replacing corn in the diet of ruminants. Due to its vast industrial use, castor bean (*Ricinus communis L.)* is widely cultivated worldwide, especially in developing countries. Castor oil contains about 89 to 92% ricinoleic acid (12-hydroxycis-9-18:1) [2], which gives the oil unique characteristics, such as its ability to modulate rumen fermentation through its antimicrobial properties. The oil obtained from pressing castor bean seeds has a longer shelf life when compared to other vegetable oils, which is justified by its excellent oxidative stability. In addition, it is an oil inedible by humans, reducing competition for food between humans and animals.

Like divalent ionophores, ricinoleic acid acts on the structure of bacterial cell walls, causing protein denaturation and coagulation, especially in Gram-positive bacteria [3]. Research has been done to evaluate the impacts of crude glycerin on cattle diets [4]. In addition, the effects of the inclusion of castor oil were evaluated in diets for bulls [5].

Like current research, Brant *et al.* [6] examined the effects of replacing soybean meal and ground corn with cottonseed cake and crude glycerin on Murrah buffalo diets, respectively. Despite this, there is a lack of research evaluating the effects of replacing conventional energy sources compared to diets containing castor oil or crude glycerin, alone or in combination, and their impacts on buffalo diets managed in tropical conditions. In this sense, the current research was carried out to test the following hypotheses: (a) GLY and CAO could be included as alternative energy sources in buffalo diets without compromising nutrient intake or digestibility; (b) the use of GLY and CAO could improve nitrogen balance and increase microbial protein synthesis; c) the inclusion of GLY and CAO alters the parameters and kinetics of rumen fermentation, without changes in the metabolic profile and feeding behavior.

This study evaluated the effects of two associated or non-associated energy sources (crude glycerin and castor oil) in buffalo diets on intake and digestibility, rumen kinetics, feeding behavior, nitrogen balance, microbial protein synthesis, rumen fermentation, and blood metabolites.

## MATERIAL AND METHODS

### Animals, Experimental Design and Treatments

Four buffaloes (*Bubalus bubalis*) of the Murrah breed, castrated, with an initial average body weight of 526 kg ± 29 kg and aged 30 months, were used. The animals were cannulated in the rumen with latex cannulas (Kehl®, São Carlos, Brazil). The design used in the experiment was a 4 x 4 Latin square in which the animals were randomly allocated. Each experimental period lasted 21 days, with 13 days of adaptation to the experimental treatments and eight days for collecting samples and information.

The experimental treatments were: 1) Soybean meal in association with ground corn (CONT); 2) Crude glycerin (GLY), dietary inclusion of GLY up to 90 g/kg in the total dry matter (DM); 3) Castor oil (CAO), dietary inclusion of CAO up to 50 g/kg in the total DM; and 4) Crude glycerin associated with Castor oil (GLYCAO), dietary inclusions of GLY at 50 g/kg of glycerin and CAO at 50 g/kg in the total DM (Table **1**).

The diets tested in this experiment were calculated according to the recommendations of Paul and Lal [7], being isoproteic. Thus, the current study used the same level of protein to isolate the effect of energy sources.

Roughage represented 70% of the diet, using sorghum silage in particles measuring approximately 5 cm. The concentrate (30% of the diet) consisted of ground corn, soybean meal, crude glycerin, castor oil, urea, salt, and mineral premix (Table **1**).

### Intake, Apparent Digestibility of Nutrients, Body Condition Score, and Body Weight

The experimental diets were offered at 08:00 and 15:00 h, in identical quantities, as a total mixed ration (TMR), allowing leftovers of up to 10%. Feed consumption per animal was recorded daily by measuring each animal's amount of feed offered and leftovers after 24 hours. Sorghum silage and concentrate ingredient samples were collected at the beginning of each experimental period. During the data collection period (14-21st day of each period), the remains of each animal were collected. At the end of the experimental period, a composite sample of the remains of each animal was taken. The samples of sorghum silage, concentrate ingredients, and collected leftovers were identified and stored at -20ºC for analysis after the end of the experiment.

Feces were collected 24 hours on the 18th day of each experimental period to determine digestibility. Feces were collected directly from the floor of the stalls instantly after spontaneous defecation and stored in 200 l containers. The floor was constantly cleaned

**Table 1: Proportion of Ingredients and Chemical Composition of the Experimental Diets**

| Item | Experimental diets (g/kg DM)[1] | | | |
|---|---|---|---|---|
| | CONT | GLY | CAO | GLYCAO |
| Proportion of ingredients (g/kg DM) | | | | |
| Sorghum silage | 700 | 700 | 700 | 700 |
| Ground corn | 182 | 89.0 | 129 | 79.0 |
| Soybean meal | 70.0 | 70.0 | 70.0 | 70.0 |
| Crude glycerin | - | 90.0 | - | 50.0 |
| Castor oil | - | - | 50.0 | 50.0 |
| Urea | 12.0 | 15.0 | 15.0 | 15.0 |
| Salt | 6.00 | 6.00 | 6.00 | 6.00 |
| Mineral premix[2] | 30.0 | 30.0 | 30.0 | 30.0 |
| Chemical composition (g/kg DM) | | | | |
| Dry matter (g/kg as fed) | 460 | 455 | 452 | 471 |
| Organic matter | 921 | 931 | 922 | 920 |
| Crude protein | 133 | 132 | 136 | 132 |
| Ether extract | 32 | 79 | 79 | 80 |
| Neutral detergent fiber (NDF) | 488 | 463 | 492 | 470 |
| NDF corrected for ash and protein | 430 | 417 | 423 | 415 |
| Indigestible NDF | 134 | 133 | 134 | 133 |
| Potentially digestible NDF | 350 | 332 | 339 | 333 |
| Acid detergent fiber | 295 | 290 | 252 | 300 |
| Lignin | 41.0 | 37.0 | 39.0 | 37.0 |
| Neutral detergent insoluble protein (g/kg TN) | 212 | 295 | 205 | 208 |
| Acid detergent insoluble protein (g/kg TN) | 105 | 147 | 100 | 102 |
| Non-fibrous carbohydrates[3] | 326 | 354 | 284 | 296 |
| Liquid energy (MJ/kg)[4] | 5.00 | 5.00 | 5.90 | 5.90 |

[1]CONT, soybean meal associated with ground corn; GLY, crude glycerin; CAO, castor oil; GLYCAO, glycerin in association with castor oil.
[2]Assurance levels (per kilogram of active elements): 128 g Calcium; 44 g Phosphorus; 178 g Sodium; 12 g Sulfur; 5 g Magnesium; 107 mg Cobalt; 50 mg Copper; 50 mg Iodine; 750 mg Manganese; 12 mg Selenium; 3.7 g Zinc; 1.4 g Iron; 440 mg Fluoride.
[3]According to Hall [16]: NFC = 100 − [(CP – CP from urea + urea)] + NDF + EE + ash.
[4]Calculated according to NRC [17] equations.

during collection to avoid contamination of the samples. After total collection, the collected feces were weighed and homogenized, and a representative sample of 10% was removed and stored at -20ºC.

The body condition score (BCS) and body weight (BW) of the buffaloes were measured at the beginning of the study and on the last day of each experimental period after the days of adaptation to the diets, according to the methodology of Anitha *et al*. [8].

### Ruminal Kinetics and Feeding Behavior

To determine ruminal kinetics, rumen contents were emptied on the 20th and 21st day of each period, according to Harvatine and Allen [9]. Representative samples of 10% of the digesta were taken during the evacuation. The collected samples were separated into solid and liquid fractions using filters with a pore size of 1.000 µm and frozen for subsequent analyses.

According to the methodology of Krizsan *et al*. [10], to determine rumen flow, reticular digesta samples were collected every 9 hours between days 14 and 16 of each experimental period. At the end of the experimental period, a pool of digesta samples from each animal was prepared, then weighed and divided into two fractions using a 100-µm nylon filter. The two fractions were the filtrate (liquid phase and small particles) and residue (large particles) [9]. The samples were then preserved for later evaluations.

Feeding behavior was analyzed on the 17th day of each period through visual assessments by previously trained individuals. The analysis was conducted over 24 hours, every 5 minutes, to determine the buffaloes'

time spent feeding, ruminating, and idle time. In three 2-hour periods throughout the day (morning, afternoon, and night), assessments were made to determine the number of chews per ruminated bolus and the time taken to ruminate this bolus using stopwatches. Artificial lights were used at night, to which the animals were previously adapted. The determination of eating behavior variables was calculated using the equations proposed by Bürger *et al.* [11].

**Chemical Analysis**

Samples of sorghum silage, concentrate ingredients, leftovers, feces, ruminal digesta, and reticular digesta phases were dried at 55ºC for 72 hours and then ground on a 2 and 1-mm sieve.

According to Casali *et al*. [12] methodology, the 2 mm samples were analyzed for Indigestible neutral detergent fiber ($_{i}$NDF) content. The potentially digestible NDF ($_{pd}$NDF) was calculated by subtraction between neutral detergent fiber (NDF) and $_{i}$NDF.

Samples ground to 1 mm of concentrate ingredients, sorghum silage, and feces were analyzed for dry matter content (DM) (ID:920.15); ash (MM) (ID: 942.05), ether extract (EE) (ID: 920.39), crude protein (CP) (ID: 948.13) and lignin (ID: 973.18) using the AOAC methods [13].

In samples of the ingredients used in the diet, neutral and acid detergent fiber (NDF and ADF) contents were determined according to Mertens [14], neutral detergent insoluble protein and acid detergent insoluble protein by Licitra *et al.* [15], in addition to measuring non-fibrous carbohydrates (NFC) and net energy according to Hall [16] and NRC [17], respectively.

In samples 1 mm from the ruminal digesta and reticular digesta fractions, DM, MM, and NDF were determined according to the abovementioned methodologies.

**Nitrogen (N) Balance and Microbial Protein Synthesis**

Total urine collections were carried out over 24 hours for three days to determine nitrogen balance and microbial protein synthesis. For urine collection, funnels were attached to the animals, and a hose was attached to buckets with 100 ml of 20% sulfuric acid to prevent the volatilization of ammonia.

Urinary volume was measured every 24 hours, and representative samples were collected. These samples were filtered and stored at -20ºC for subsequent analyses.

Determining nitrogen in urine was carried out using method nº 984.13 proposed by AOAC [13]. Microbial protein synthesis was calculated according to Chen and Gomes [18] using the concentration of purine derivatives (allantoin, uric acid, and xanthine-hypoxanthine). Uric acid was analyzed by colorimetric methodology using a commercial kit (K139 Bioclin®, São Paulo, Brazil), and the other purine derivatives were estimated using the method of Chen and Gomes [18].

**Ruminal Fermentation and Blood Metabolites**

Representative samples from five locations in the rumen were collected through the ruminal cannula on the 13th day of each collection period every 2 hours, starting at 8 a.m. (before feeding) and ending at 8 p.m.

The collected samples were filtered, and then the pH of the ruminal liquid was immediately determined using a digital pH meter (ORP 8651, AZ Instrument Corp., Tanzi District, Taichung City, Taiwan). Subsequently, the ruminal fluid samples were stored at -20ºC for later analysis.

At the end of the experiment, the samples were thawed and centrifuged at 2,000 x for 15 minutes using a centrifuge (Centribio 80-2B Centribio, São Paulo, SP, Brazil) at room temperature. One milliliter of the supernatant was collected and placed in tubes with 25% metaphosphoric acid, then analyzed for the concentration of short-chain fatty acids (acetic acid, propionic acid, and butyric acid) using the method proposed by Mathew *et al.* [19].

Ammonia nitrogen ($NH_3$-N) was determined using one milliliter of 10% (w/v) sodium tungstate solution mixed with one-milliliter aliquot samples of rumen fluid. According to Broderick and Kang [20], samples were immediately mixed and stored at −20°C for further analysis [20]. The equation to determine methane emission by animals ($CH_4$) was used as proposed by Moss *et al.* [21].

On the 16th day of each period, 10 mL of blood was collected at 8 a.m. (before feeding) through jugular venipuncture in sterile Vacutainers®. Afterward, the samples were refrigerated and centrifuged (1800 x g for 15 min at 5°C) (Centribio, São Paulo, SP, Brazil). Then, the serum was collected and stored at -20ºC.

Blood concentration analyses were performed using commercial kits (Doles Reagents Ltd., Goiânia, GO, Brazil) for total protein, urea, creatinine, albumin, glucose, cholesterol, and triglycerides.

### Statistical Analysis

Dietary effects were statistically analyzed according to a 4 x 4 Latin square using the PROC MIXED procedure of the Statistical Analysis System (SAS) Software, Institute, Inc. Cary, North Carolina, USA (version 9.4), according to the following model:

$$Y_{ijk} = \mu + A_i + P_j + D_k + e_{ijk},$$

Where $Y_{ijk}$ = observed values of animal $_i$, period $_j$, and diet $_k$; $\mu$ = overall mean of the experiment for the variable; $A_i$ = random effect of animal (i = 1-4); $P_j$ = random effect of the period ($_j$ = 1-4); $D_k$ = fixed effect of diet ($_k$ = 1-4); $e_{ijk}$ = random error caused by the animal, period, and diet.

Data of rumen fermentation were analyzed as repeated measures over time using to the following model:

$$Y_{ijkl} = \mu + A_i + P_j + D_k + \gamma_{ijk} + T_l + (T * D)_{lk} + \varepsilon_{ijkl},$$

Where: $Y_{ijkl}$ = observation of animal "i" in period j, on diet k, at time l; μ = overall mean; $A_i$ = random effect of animal (i = 1-4); $P_j$ = random effect of the period ($_j$ = 1-4); $D_k$ = fixed effect of diet ($_k$ = 1-4); $\gamma_{ijk}$ = random error to test diet effect; $T_l$ = fixed effect of time (l = 0, 2, 4, 6, 8, 10, 12, and 14 h after feeding); $T*D_{lk}$ = fixed effect of the interaction between diet k and time l, and $\varepsilon_{ijkl}$ = random error to test time effect and their interactions.

The time effect of the variables was evaluated by orthogonal polynomial trend test (POLYANOVA), the coefficient for equally spaced polynomial Linear (-7 -5 -3 -1 1 3 5 7), Quadratic (7 1 -3 -5 -5 -3 1 7) and Cubic (-7 5 7 3 -3 -7 -5 7) was applied on PROC MIXED. For those variables in which a significant interaction effect was detected, the interaction effect was decomposed (PROC MIXED – SAS), with the diet effect evaluated each time and vice versa.

The Akaike information criterion (AIC) was used to determine the structured covariance matrix. Degrees of freedom were defined according to Kenward and Roger [22]. All means were estimated by the LSMEANS procedure and compared by the Tukey test, considering 0.05 as the probability of type-I error.

## RESULTS

### Intake, Apparent Digestibility of Nutrients, Body Condition Score, and Body Weight

Higher intakes of DM, OM, NDF, iNDF, and NFC were verified in buffaloes fed CONT and GLY than those fed CAO and GLYCAO (P < 0.050) (Table **2**). Intake EE was bigger in buffaloes fed with CAO and GLYCAO than in those animals that received the GLY diet (P < 0.001), while higher TDN intake was checked in animals fed with CONT and GLY than in buffaloes fed with GLYCAO (P < 0.001) (Table **2**).

Buffaloes that received the CONT diet consumed more CP than those that received CAO and GLYCAO diets (P < 0.001). Only a difference in EE digestibility (P = 0.013) was observed between the diets evaluated (Table **2**). Thus, buffaloes fed CAO and GLYCAO showed greater EE digestibility than those fed GLY.

Diets had no effects on buffaloes' body condition score (P = 0.778) and body weight (P = 0.691) (Table **2**).

### Ruminal Kinetics and Feeding Behavior

Animals fed the GLYCAO diet had a more natural matter ruminal pool than buffaloes who received the CONT and GLY diets (Table **3**). However, buffaloes fed CONT and GLY diets had a higher rate of rumen renewal (%/h) of DM (P < 0.001), NDF (P < 0.001), and iNDF (P = 0.002), and a higher rate of DM passage (P = 0.001) than those who received CAO and GLYCAO diets.

A significant difference was observed in the rate of ruminal renewal of $_{pd}$NDF (P = 0.014) and the rate of DM digestion (P = 0.010), with higher rates for animals that received the CONT diet compared to CAO and GLYCAO (Table **3**). Furthermore, there was a higher NDF passage rate in buffaloes fed with GLY than those fed with CAO and GLYCAO (P = 0.005). Buffaloes fed CAO, and GLYCAO spent more time in rumination than animals fed CONT and GLY (P = 0.002) (Table **4**). Besides, buffaloes that received the CONT diet had longer idle time than those that received the CAO and GLYCAO diets (P = 0.011). In addition, more time spent chewing was observed in buffaloes fed CAO and GLYCAO than animals fed CONT (P = 0.011) (Table **4**).

The greater dietary efficiency of DM was observed in animals that received the CONT diet compared to

**Table 2: Nutrient Intake, Apparent Digestibility, Body Weight (BW), and Body Condition Score (BCS) in Buffaloes Fed Diets Composed of Soybean Meal in Association with Ground Corn (CONT), Crude Glycerin (GLY), Castor Oil (CAO), and Crude Glycerin in Association with Castor Oil (GLYCAO)**

| Item | Experimental diets[1] | | | | SEM[2] | p-value |
|---|---|---|---|---|---|---|
| | CONT | GLY | CAO | GLYCAO | | |
| Intake (kg/day) | | | | | | |
| Dry matter | 10.5[a] | 10.0[a] | 8.17[b] | 7.74[b] | 0.06 | <0.001 |
| Organic matter | 9.70[a] | 9.28[a] | 7.62[b] | 7.16[b] | 0.30 | <0.001 |
| Crude protein | 1.45[a] | 1.30[ab] | 1.12[bc] | 1.01[c] | 0.05 | <0.001 |
| Ether extract | 0.32[bc] | 0.25[c] | 0.58[a] | 0.53[ab] | 0.04 | <0.001 |
| Neutral detergent fiber | 5.10[a] | 4.73[a] | 4.00[b] | 3.72[b] | 0.16 | <0.001 |
| $_{i}$NDF[3] | 1.42[a] | 1.38[a] | 1.09[b] | 1.09[b] | 0.05 | <0.001 |
| $_{pd}$NDF[4] | 3.68[a] | 3.35[ab] | 2.91[bc] | 2.64[c] | 0.11 | <0.001 |
| Non-fibrous carbohydrates | 2.89[a] | 2.99[a] | 1.69[b] | 1.84[b] | 0.17 | <0.001 |
| Total digestible nutrients | 6.24[a] | 6.11[a] | 5.43[ab] | 5.03[b] | 0.44 | <0.001 |
| Digestibility coefficient (%) | | | | | | |
| Dry matter | 67.0 | 67.0 | 61.1 | 61.0 | 1.30 | 0.153 |
| Organic matter | 68.0 | 67.7 | 64.4 | 62.3 | 1.17 | 0.280 |
| Crude protein | 74.3 | 73.3 | 74.0 | 73.2 | 0.86 | 0.970 |
| Ether extract | 56.6 [ab] | 52.3[b] | 75.7[a] | 73.1[a] | 3.35 | 0.013 |
| Neutral detergent fiber | 59.7 | 54.6 | 48.1 | 49.2 | 1.97 | 0.118 |
| Non-fibrous carbohydrates | 80.0 | 86.2 | 83.0 | 78.2 | 1.22 | 0.192 |
| Total digestible nutrients | 65.3 | 64.6 | 63.1 | 62.1 | 1.15 | 0.761 |
| Performance | | | | | | |
| BCS (unity) | 3.95 | 3.80 | 3.95 | 3.75 | 0.14 | 0.788 |
| BW (kg) | 539 | 517 | 517 | 530 | 7.26 | 0.691 |

[a,b]Values in the same row with different superscript letters differ significantly by Tukey's test (P < 0.05).
[1]CONT, soybean meal associated with ground corn; GLY, crude glycerin; CAO, castor oil; GLYCAO, glycerin in association with castor oil.
[2]SEM, standard error of the means.
[3]$_{i}$NDF, Indigestible neutral detergent fiber.
[4]$_{pd}$NDF Potentially digestible neutral detergent fiber.

**Table 3: Ruminal Kinetics and Post-Ruminal Flow of Buffaloes Fed Diets Composed of Soybean Meal in Association with Ground Corn (CONT), Crude Glycerin (GLY), Castor Oil (CAO), and Crude Glycerin in Association with Castor Oil (GLYCAO)**

| Item | Experimental diets[1] | | | | SEM[2] | p-value |
|---|---|---|---|---|---|---|
| | CONT | GLY | CAO | GLYCAO | | |
| Rumen digesta, kg | | | | | | |
| Fresh matter | 44.9[bc] | 43.6[c] | 51.7[ab] | 52.4[a] | 1.41 | 0.036 |
| Dry matter | 5.36 | 5.15 | 6.00 | 6.36 | 0.19 | 0.054 |
| NDF[3] | 2.40 | 2.39 | 2.67 | 2.81 | 0.09 | 0.299 |
| $_{i}$NDF[4] | 1.27 | 1.09 | 1.26 | 1.35 | 0.05 | 0.334 |
| Ruminal removal rate, %/h | | | | | | |
| Dry matter | 8.19[a] | 8.22[a] | 5.68[b] | 5.10[b] | 0.39 | <0.001 |
| NDF[3] | 8.84[a] | 8.39[a] | 6.33[b] | 5.59[b] | 0.40 | <0.001 |
| $_{pd}$NDF[5] | 14.0[a] | 11.0[ab] | 9.10[b] | 7.78[b] | 0.80 | 0.014 |
| $_{i}$NDF[4] | 4.69[a] | 5.32[a] | 3.57[b] | 3.45[b] | 0.24 | 0.002 |
| Digestion rate, %/h | | | | | | |
| Dry matter | 3.15[a] | 2.66[ab] | 1.73[c] | 1.82[bc] | 0.19 | 0.010 |
| NDF[3] | 4.18 | 3.38 | 2.66 | 2.56 | 0.24 | 0.053 |

**(Table 3). Continued.**

| Item | Experimental diets[1] | | | | SEM[2] | p-value |
|---|---|---|---|---|---|---|
| | CONT | GLY | CAO | GLYCAO | | |
| Passage rate, %/h | | | | | | |
| Dry matter | 5.03[a] | 5.56[a] | 3.94[b] | 3.28[b] | 0.27 | 0.001 |
| NDF[3] | 4.65[ab] | 5.01[a] | 3.67[bc] | 3.03[c] | 0.25 | 0.005 |
| Ruminal digestibility | | | | | | |
| Absolute, kg/d, $_{pd}$NDF[5] | 1.28 | 1.42 | 1.26 | 0.94 | 0.08 | 0.287 |
| Relative, g/kg, $_{pd}$NDF[5] | 649 | 576 | 565 | 637 | 26.1 | 0.621 |
| Relative, g/kg, DM[6] | 443 | 323 | 301 | 358 | 26.6 | 0.256 |

[a,b]Values in the same row with different superscript letters differ significantly by Tukey's test (P < 0.05).
[1]CONT, soybean meal associated with ground corn; GLY, crude glycerin; CAO, castor oil; GLYCAO, glycerin in association with castor oil.
[2]SEM, standard error of the means.
[3]NDF, Neutral detergent fiber.
[4]$_{i}$NDF, Indigestible neutral detergent fiber.
[5]$_{pd}$NDF, Potentially digestible neutral detergent fiber.
[6]DM, Dry matter.

**Table 4: Feeding Behavior in Buffaloes Fed Diets Composed of Soybean Meal in Association with Ground Corn (CONT), Crude Glycerin (GLY), Castor Oil (CAO), and Crude Glycerin in Association with Castor Oil (GLYCAO)**

| Item | Experimental diets[1] | | | | SEM[2] | p-value |
|---|---|---|---|---|---|---|
| | CONT | GLY | CAO | GLYCAO | | |
| Time spent (minutes/day) | | | | | | |
| Feeding | 184 | 195 | 203 | 178 | 8.56 | 0.453 |
| Rumination | 493[b] | 541[b] | 615[a] | 633[a] | 16.9 | 0.002 |
| Idling | 764[a] | 704[ab] | 623[b] | 630[b] | 18.1 | 0.011 |
| Chewing | | | | | | |
| Number/bolus | 52.4 | 55.7 | 59.5 | 55.0 | 0.90 | 0.083 |
| Hour/day | 11.3[b] | 12.3[ab] | 13.6[a] | 13.5[a] | 0.30 | 0.011 |
| Feeding behavior (number of periods/day) | | | | | | |
| Feeding | 16.0 | 18.8 | 16.0 | 16.0 | 0.85 | 0.578 |
| Rumination | 19.8 | 21.0 | 22.3 | 23.8 | 0.87 | 0.150 |
| Idling | 31.5 | 33.3 | 30.8 | 31.5 | 0.78 | 0.753 |
| Feeding efficiency | | | | | | |
| grams DM[3]/hour | 3489[a] | 3104[ab] | 2567[b] | 2710[ab] | 160 | 0.027 |
| grams NDF[4]/hour | 1687 | 1463 | 1254 | 1312 | 78.0 | 0.067 |
| Rumination efficiency | | | | | | |
| grams DM[3]/hour | 1296[a] | 1113[a] | 797[b] | 738[b] | 64.3 | <0.001 |
| grams NDF[4]/hour | 627[a] | 525[b] | 390[c] | 356[c] | 30.5 | <0.001 |

[a,b]Values in the same row with different superscript letters differ significantly by Tukey's test (P < 0.05).
[1]CONT, soybean meal associated with ground corn; GLY, crude glycerin; CAO, castor oil; GLYCAO, glycerin in association with castor oil.
[2]SEM, standard error of the means.
[3]DM, Dry matter.
[4]NDF, Neutral detergent fiber.

the CAO diet (P = 0.027). Furthermore, it was found that buffaloes fed CONT and GLY diets had higher DM and NDF rumination efficiencies (P < 0.001) (Table **4**).

### Nitrogen (N) Balance and Microbial Protein Synthesis

Buffaloes that received the CONT diet had higher N intake than animals that contained castor oil in the diet (CAO and GLYCAO)(P < 0.001) (Table **5**). Furthermore, greater N retention (P = 0.045) and N excretion in feces (P = 0.030) were found in buffaloes fed the CONT diet compared to those fed the GLYCAO diet.

### Ruminal Fermentation Parameters and Blood Metabolites

Buffaloes fed CONT had a lower rumen pH than those fed CAO and GLYCAO (P = 0.029) (Table **6**).

**Table 5: Nitrogen (N) Utilization, Microbial Protein Synthesis in Buffaloes Fed Diets Composed of Soybean Meal in Association with Ground Corn (CONT), Crude Glycerin (GLY), Castor Oil (CAO), and Crude Glycerin in Association with Castor Oil (GLYCAO)**

| Item | Experimental diets[1] | | | | SEM[2] | p-value |
|---|---|---|---|---|---|---|
| | CONT | GLY | CAO | GLYCAO | | |
| Nitrogen utilization (g/day) | | | | | | |
| N-intake | 232[a] | 208[ab] | 185[bc] | 162[c] | 7.89 | <0.001 |
| Fecal-N | 37.2[a] | 33.7[ab] | 29.7[ab] | 26.8[b] | 1.43 | 0.030 |
| Urinary-N | 41.7 | 37.2 | 31.6 | 32.6 | 3.45 | 0.758 |
| Retained-N | 153[a] | 137[ab] | 124[ab] | 102[b] | 6.90 | 0.045 |
| Microbial protein | | | | | | |
| Efficiency[3] | 77.1 | 82.6 | 69.0 | 69.9 | 10.4 | 0.970 |
| Synthesis (g/day) | 568 | 556 | 398 | 355 | 74.5 | 0.706 |

[a,b]Values in the same row with different superscript letters differ significantly by Tukey's test (P < 0.05).
[1]CONT, soybean meal associated with ground corn; GLY, crude glycerin; CAO, castor oil; GLYCAO, glycerin in association with castor oil.
[2]SEM, standard error of the means.
[3]Grams of microbial protein/kg of TDN.

**Table 6: Ruminal Fermentation in Buffaloes Fed Diets Composed of Soybean Meal in Association with Ground Corn (CONT), Crude Glycerin (GLY), Castor Oil (CAO), and Crude Glycerin in Association with Castor Oil (GLYCAO)**

| Item | Experimental diets[1] | | | | SEM[2] | p-value | | | p-value[3] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CONT | GLY | CAO | GLYCAO | | Diet | Time | Diet x Time | Linear | Quadratic | Cubic |
| pH | 6.45[b] | 6.59[a] [b] | 6.67[a] | 6.64[a] | 0.06 | 0.029 | 0.029 | 0.834 | <0.001 | <0.001 | 0.308 |
| N-$NH_3$ (mg/dL)[4] | 17.2[a] | 13.1[b] | 8.88[c] | 8.52[c] | 0.72 | <0.001 | <0.001 | <0.012 | 0.002 | 0.051 | 0.218 |
| Total SCFA(mMoL)[5] | 42.2[a] | 40.6[a] | 35.2[b] | 35.0[b] | 1.53 | <0.001 | <0.001 | 0.468 | <0.001 | 0.025 | 0.187 |
| Acetate (C2) (mMol) | 29.0[a] | 26.8[a] | 23.5[b] | 23.2[b] | 1.01 | <0.001 | <0.001 | 0.401 | <0.001 | 0.013 | 0.158 |
| Propionate (C3) (mMol) | 9.81[a] | 9.68[a] | 8.13[b] | 8.05[b] | 0.39 | <0.001 | <0.001 | 0.568 | <0.001 | 0.056 | 0.143 |
| Butyrate (C4) (mMol) | 4.35[a] | 4.14[ab] | 3.54[bc] | 3.69[c] | 0.17 | 0.005 | 0.005 | 0.553 | <0.001 | 0.217 | 0.827 |
| C2:C3 ratio (MoL/100 MoL) | 2.95 | 2.82 | 2.89 | 2.90 | 0.05 | 0.308 | 0.308 | 0.146 | 0.535 | 0.154 | 0.879 |
| $CH_4$ (MoL/100 MoL)[6] | 12.1[a] | 11.1[a] | 9.72[b] | 9.75[b] | 0.41 | <0.001 | <0.001 | 0.372 | <0.001 | 0.015 | 0.234 |
| **Regression equations** | | | | | | | | | | | |
| $\hat{Y}_{pH} = 7.03 – 0.118*T + 0.007*T2$ ($R^2 = 84.62$) | | | | | | | | | | | |
| $\hat{Y}_{N\text{-}NH3\ within\ CON} = 19.38 - 0.297*T$ ($R^2$=21.55) | | | | | | | | | | | |
| $\hat{Y}_{N\text{-}NH3\ within\ GLYCAO} = 13.57 – 1.588*T + 0.092*T2$ ($R^2 = 75.33$) | | | | | | | | | | | |
| $\hat{Y}_{SCFA} = 27.34 + 1.979*T - 0.089*T2$; ($R^2 = 89.96$) | | | | | | | | | | | |
| $\hat{Y}_{SCFA} = 27.34 + 1.979*T - 0.089*T2$; ($R^2 = 89.96$) | | | | | | | | | | | |
| $\hat{Y}_{Acetate} = 17.97 + 1.40*T - 0.064*T2$; ($R^2 = 91.20$) | | | | | | | | | | | |
| $\hat{Y}_{Propionate} = 6.90 + 0.165*T$ ($R^2 = 69.16$) | | | | | | | | | | | |
| $\hat{Y}_{Butyrate} = 3.13 + 0.076*T$ ($R^2 = 72.11$) | | | | | | | | | | | |
| $\hat{Y}_{CH4} = 7.53 + 0.572*T - 0.026*T2$ ($R^2 = 92.65$) | | | | | | | | | | | |

[a,b]Values in the same row with different superscript letters differ significantly by Tukey's test (P < 0.05).
[1]CONT, soybean meal associated with ground corn; GLY, crude glycerin; CAO, castor oil; GLYCAO, glycerin in association with castor oil.
[2]SEM, standard error of the means.
[3]Probability values for the effect of hours (time) relative to fed.
[4]N-$NH_3$, Ruminal ammonia nitrogen.
[5]SCFA, Short chain fatty acids.
[6]Enteric methane production estimated according equation to Moss *et al.* [21]: $CH_4$ (grams/day) = 0.45 acetate (mmol/L) − 0.275 propionate (mmol/L) + 0.40 butyrate (mmol/L).

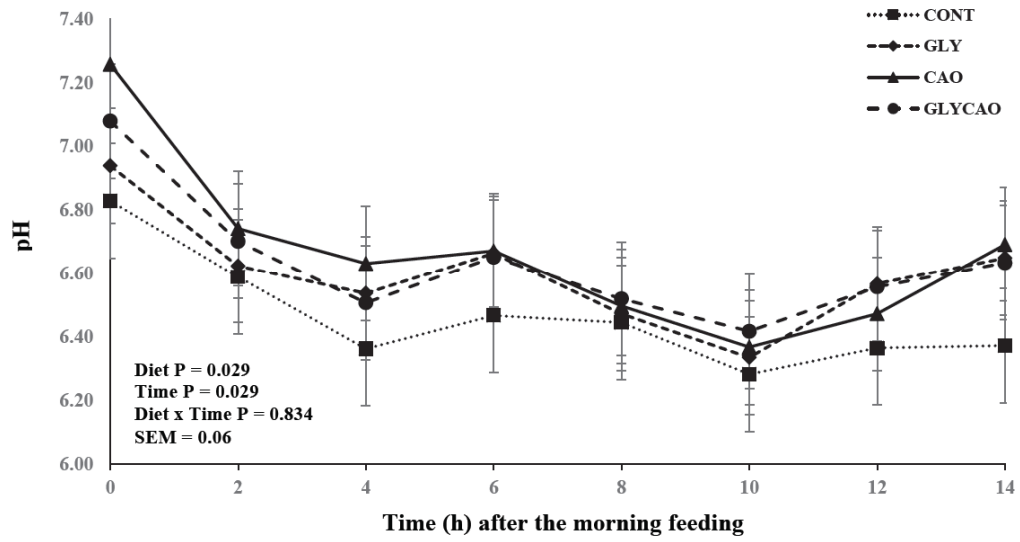


**Figure 1:** Ruminal pH of buffaloes fed diets composed of soybean meal in association with ground corn (CONT), crude glycerin (GLY), castor oil (CAO), and crude glycerin in association with castor oil (GLYCAO). Standard error of the mean (SEM).

Besides, there was a time effect on rumen pH, which was quadratically influenced in a negative manner by diets ($P < 0.05$; Figure **1**). The highest rumen pH of 6.54 was observed in buffaloes nine hours after the morning feeding.

There was a higher concentration of rumen ammonia nitrogen in buffaloes that received the CONT and GLY diets ($P < 0.05$; Figure **2**). Furthermore, an interaction was verified between the experimental diets and time for the variable ammonia nitrogen.

Buffaloes fed CONT and GLY showed a higher concentration of total short-chain fatty acids ($P < 0.001$) and acetate ($P < 0.001$) than those fed CAO and GLYCAO (Table **6**). Besides, the effect of time was verified for total SCFA ($P < 0.001$) and acetate concentration ($P < 0.001$), observing a positive quadratic effect ($P < 0.050$). Thus, the highest total SCFA and acetate concentrations of 38.34 mMol and 25.58 mMol approximately were observed in buffaloes at 12 and 11 hours after morning feeding, respectively.

Buffaloes fed CONT and GLY diets showed a higher concentration of propionate than those fed CAO and GLYCAO ($P < 0.001$) (Table **6**). Furthermore, the effect of time on propionate concentration was verified, and the effect was linearly increased by the diets.

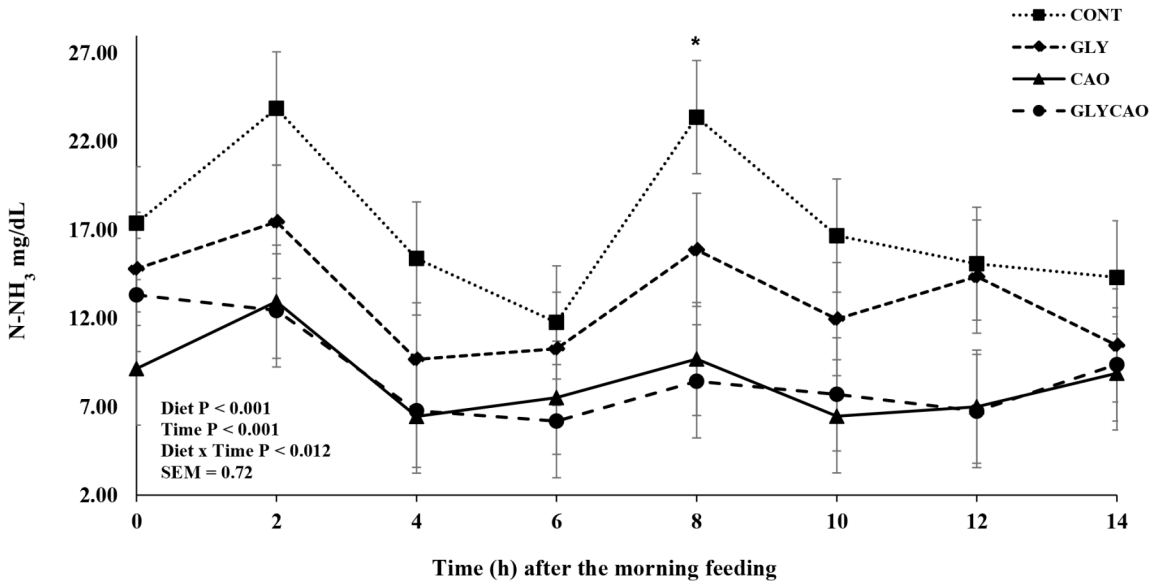


**Figure 2:** Concentration of ruminal ammonia nitrogen ($NH_3$-N, mg/dL) of buffaloes fed diets composed of soybean meal in association with ground corn (CONT), crude glycerin (GLY), castor oil (CAO), and crude glycerin in association with castor oil (GLYCAO). Standard error of the mean (SEM).

**Table 7: Serum Blood Metabolites of Buffaloes Fed Diets Composed of Soybean Meal in Association with Ground Corn (CONT), Crude Glycerin (GLY), Castor Oil (CAO), and Crude Glycerin in Association with Castor Oil (GLYCAO)**

| Item | Experimental diets[1] | | | | SEM[2] | p-value |
|---|---|---|---|---|---|---|
| | CONT | GLY | CAO | GLYCAO | | |
| Total protein (mg/dL) | 13.7 | 13.2 | 12.4 | 12.8 | 0.39 | 0.730 |
| Urea (mg/dL) | 31.0 | 34.0 | 32.0 | 35.8 | 1.24 | 0.582 |
| Creatinine (mg/dL) | 0.22 | 0.27 | 0.19 | 0.26 | 0.02 | 0.535 |
| Albumin (g/L) | 2.44 | 2.53 | 2.30 | 2.53 | 0.10 | 0.860 |
| Glucose (mg/dL) | 127[a] | 117[ab] | 107[b] | 112[ab] | 2.62 | 0.012 |
| Cholesterol (mg/dL) | 78.8 | 67.8 | 83.8 | 77.3 | 2.75 | 0.219 |
| Triglycerides (mg/dL) | 12.8 | 11.0 | 16.8 | 13.0 | 1.48 | 0.616 |

[a,b]Values in the same row with different superscript letters differ significantly by Tukey's test (P < 0.05).
[1]CONT, soybean meal associated with ground corn; GLY, crude glycerin; CAO, castor oil; GLYCAO, glycerin in association with castor oil.
[2]SEM, standard error of the means.

A higher butyrate concentration was observed in buffaloes fed CONT than those fed CAO and GLYCAO (P = 0.005). Besides, animals fed GLY showed a higher butyrate concentration than animals fed GLYCAO. In the same way, there was a time effect on butyrate concentration, which was increased linearly by diets (P < 0.001).

Buffaloes fed CONT and GLY showed a higher enteric methane production than those fed CAO and GLYCAO (P < 0.001) (Table **6**). Furthermore, there was a time effect on methane production, which was influenced in a positive quadratically manner by diets (P = 0.015). Thus, it was estimated that the maximum value was 10.67 Mol /100 Mol 11 hours after morning feeding.

Regarding the serum blood metabolites, a higher glucose concentration has been checked in buffaloes fed CONT than those fed CAO (P = 0.012) (Table **7**).

## DISCUSSION

### Intake, Apparent Digestibility of Nutrients, Ruminal Kinetics, and Feeding Behavior

Animals fed CAO and GLYCAO had lower DM, TDN, and nutritional component consumption. This result is probably associated with the unsaturated fatty acids present in glycerin and castor oil, which can inhibit the growth of some bacterial populations in the ruminal environment [23], mainly fibrolytic bacteria, including *Ruminococcus flavefaciens* and *Butyrivibrio fibrisolvens*. Additionally, such fatty acids can also reduce the adhesion of bacteria to dietary fiber particles, increasing the amount of ruminal digesta, and, consequently, such associated effects may have led the animal to physical filling, resulting in a reduction in consumption [24].

Although the total and ruminal digestibility of NDF showed no difference between the diets evaluated, it is essential to highlight that the amount of food in the rumen of animals submitted to the CAO and GLYCAO diets was more significant, which implies filling physical, due to the effect of the oil on bacteria and the adhesion of food particles, causing a lower digestion rate, renewal rate and digesta passage rate [25]. In addition to the effect of oils on ruminal microbiota and particle adhesion, lipid ingestion may have stimulated the release of cholecystokinin, a hormone responsible for inhibiting gastric leakage [26]. Thus, it is possible to infer that diets containing high levels of lipids increase plasma concentrations of cholecystokinin and, consequently, decrease the digestion passage rate, leaving the animal complete for longer and causing a reduction in food consumption.

Considering the deleterious effects of lipids on the fibrous fraction, it would be natural for there to be a reduction in the total digestion of DM and NDF. This effect was not observed. However, the reduction in the passage rate implies a longer stay of digesta in the ruminal environment, which increases rumination and chewing time [25], thus being able to compensate for the deleterious effects of added fatty acids. Fatty foods, as increasing chewing time facilitates fermentation, increases saliva production, and exposes a greater surface area of the food to ruminal microbes [27].

It is also noteworthy that when buffaloes were fed GLY and CAO, the feed efficiencies of DM and

rumination of DM and NDF were decreased. This was likely caused by the longer ruminal retention time of digesta and the impact of lipid inclusion in the adhesion of bacteria to dietary fiber particles, as was previously discussed. However, these activities remained rhythmic due to the high level of sorghum silage in the diet.

Diet composition affects the time an animal spends ruminating, the physical form of dietary ingredients, and NDF concentration [28]. Feeding behavior activities are mutually exclusive; therefore, as the diets showed no differences in the amount of time spent eating, idle time was the opposite of time spent ruminating.

**Nitrogen Balance and Microbial Protein Synthesis**

The higher DM intake can explain higher ingested nitrogen in buffaloes fed CONT than those fed with CAO or GLYCAO. The GLYCAO reduced the N losses in the feces, but this decreased excretion was insufficient to prevent the reduction of N retained by the animals.

The N balance results suggest that neither GLY nor CAO was influential, but the lower N intake caused decreased N retention. The similar urinary N excretion in buffaloes fed CONT, and alternative energy sources is probably a consequence of the similarity among the diets. Although the effect of diets on the intake of CP was observed, it was not sufficient to promote differences in CP's digestibility and urinary nitrogen excretion.

The lack of effect of diets on CP digestibility may be associated with the protein source of the diet, which was the same for all treatments, as well as its inclusion proportion, which was the same in all diets.

**Ruminal Fermentation and Blood Metabolites**

The lower rumen pH of buffaloes fed CONT compared to the CAO and GLYCAO diets may be associated with the breakdown of carbohydrates, mainly starch, and subsequent lactic acid production. As a result, this promotes a decline in pH values after feeding the animals. Despite this, the rumen pH values in this study were above the critical threshold that could affect cellulolytic bacteria's growth [24].

Rumen pH decline occurs immediately after feeding and persists for up to 4 to 8 hours, depending on the nature of the diet and the animals' feeding behavior [29]. The energy sources evaluated have properties that do not cause a rapid pH decline compared to corn, whose main carbohydrate is starch, which is highly fermentable. The current experiment observed the CAO and GLYCAO effects on ruminal fermentation and SCFA production.

When ruminant animals are fed diets that include vegetable oils as a source of unsaturated fatty acids, there are often negative effects on DMI and rumen fermentation [15]. As observed in the present study and already mentioned, unsaturated fatty acids can reduce the adhesion of bacteria to dietary fiber particles and inhibit the growth of ruminal microorganisms, especially fibrotic bacteria, which consequently reduces the total digestibility of NDF and the production of acetate and butyrate.

The increase of propionate concentrations by feeding with glycerol has been reported when crude glycerin replaced roughage or rapidly fermentable starch in the concentrates. However, no increase in propionate concentrations was observed in glycerinated diets, which may result from the low glycerol content of the glycerin used.

In the current experiment, animal intake and protein degradation can justify the effect of diets on ruminal ammonia nitrogen concentration as a function of hours concerning morning feeding. According to Wanapat and Pimpa [30], the rumen's N-$NH_3$ concentration of 14 mg/100 mL is considered optimal for buffaloes. This study shows that diets with GLY, CAO, and GLYCAO influenced the N-$NH_3$ concentration, lowering it to the optimum level mentioned previously. Despite this, no effect was noted in the digestibility of fibrous fractions and nutrients.

Changes in microbial protein synthesis or protein degradation can cause ruminal ammonia variations caused by lipid supplementation. This may also be related to UFAs in the ruminal environment from castor oil and glycerin, which can be associated with a decrease in the permeability of the outer membranes in gram-positive bacteria [31], which are primarily responsible for protein degradation. Thus, their inhibition may decrease the rumen N-$NH_3$ concentration.

Higher enteric methane production was noticed in this study in buffaloes fed CON and GLY than those fed CAO and GLYCAO, which is already expected. This result is likely related to the fact that CAO has ricinoleic acid as the main component in its composition. This component exhibited antimicrobial activity, especially the fatty acids (approximately 90%

ricinoleic acid) in castor oil, which has been described as an inhibitor of biohydrogenation and methane production, according to Morales *et al.* [32]. In addition, it also has action and impacts on some Gram-positive bacteria, as highlighted by Novak *et al.* [33].

In the present study, the buffaloes fed with the CONT, GLY, and GLYCAO diets had the highest serum glucose concentration, which may be related to fermentation modulators. This result is probably related to the animals' intake of CAO, which decreased the propionate concentration in the rumen [34]. Compared to previous studies, the glucose concentration was higher than those conducted by Dias *et al.* [35], with healthy Murrah buffaloes of different physiological categories managed in tropical conditions, averaging between 90.8 and 92.6 mg/dl.

Glycerol administration promotes serum glucose production [5] in the livers of animals, which agrees with the current study that hepatic gluconeogenesis produces more than 90% of the total glucose. This result strongly correlates with the amount of digestible energy consumed, propionate absorption, and glucose synthesis in the developing ruminant liver. The majority of dietary glycerol is absorbed directly by the rumen epithelium of the small intestine and transported to the liver, where it is converted to glycerol-3-phosphate, which is used to fuel gluconeogenesis by the enzyme glycerol kinase.

## CONCLUSION

Among food sources, crude glycerin can partially replace ground corn in buffaloes' diets without compromising intake, nutrient metabolism, or ruminal dynamics.

## ETHICS APPROVAL

The experimental procedures, housing facility, and routine care of the buffaloes were in accordance with the Ethics Committee on Animal Experimentation of the State University of Southwestern Bahia (UESB) [Protocol number: 114/2015] and performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

The authors acknowledge the Federal University of Bahia (UFBA) for providing the animals and the State University of Southwest Bahia (UESB) for the physical structure laboratories for the development of the experiment.

## FINANCIAL SUPPORT STATEMENT

This work was financially supported by the Brazilian research funding institutions CNPq (National Research Council of Brazil), and FAPESB (Bahia State Agency for Research Funding)(PRONEX Funding Call, Grant number PNX6446_2009).

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Received on 13-12-2024 Accepted on 15-02-2025 Published on 30-03-2025

https://doi.org/10.6000/1927-520X.2025.14.04