

## Article

# Crude Glycerol Increases Neutral Detergent Fiber Degradability and Modulates Rumen Fermentative Dynamics of Kikuyu Grass in Non-Lactating Holstein Cows Raised in Tropical Conditions

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**Abstract:** This study evaluated the effects of increasing levels of crude glycerol (CG) on the effective degradability of neutral detergent fiber (EDNDF) in *Pennisetum clandestinum Hochst. Ex Chiov* (kikuyu forage) and ruminal fermentation parameters in grazing dairy cows. Four non-lactating cannulated Holstein cows were used in a 4 × 4 Latin square design. Treatments consisted of CG infusion in the rumen at the following levels: 0 (G0), 500 (G500), 1000 (G1000), and 1500 (G1500) g/animal/day. Two kikuyu forages harvested (D) at 35 (DR35) and 45 (DR45) days of regrowth were incubated in the rumen for 72 h. The infusion of CG into the rumen increased ( $p < 0.05$ ) EDNDF in both incubated forages. Total volatile fatty acids (VFAs) and pH values in the ruminal fluid were unaffected ( $p > 0.05$ ) by the infusion of CG. However, propionate and butyrate molar proportions increased ( $p < 0.05$ ) at the expense of acetate at all CG levels. In addition, the NH<sub>3</sub>-N levels decreased ( $p < 0.05$ ) by approximately 20% with the infusion of 1000 and 1500 g of CG. In conclusion, supplementation with CG increases ruminal EDNDF, improving rumen fermentation dynamics in cows grazing kikuyu forage under tropical conditions. This greater EDNDF was achieved for both harvesting times.

**Keywords:** biofuel; fermentative dynamics; sugar alcohol; tropical pasture



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## 1. Introduction

Crude glycerol (CG) is a plentiful by-product derived from biodiesel production, with approximately ~1 kg of glycerol produced for every 10 kg of biodiesel [1]. The global expansion of the biodiesel industry is leading an increase in CG production, which could enhance its potential use in the pharmaceutical, cosmetics, and food industries [2–4]. Therefore, exploring alternative strategies for utilizing this by-product is highly desirable. One successful option is its use as a feedstuff in ruminant nutrition, which improves the energy metabolism, enhances productivity, and prevents the onset of pathologies in dairy cows [5,6].

Crude glycerol is composed predominantly of glycerol, which is a sugar-alcohol known for its gluconeogenic characteristics [7,8], making it desirable for ruminant nutrition [5]. Supplementation with energy sources such as sugars or sugar-alcohols could improve fiber degradability in the rumen [9], especially when there is adequate availability of ruminal degradable protein (RDP) [10]. However, it is also recognized that an excess of sugar alcohols in the rumen could cause a drop in ruminal pH, affecting fiber degradability [11–13].

Glycerol in the diet is fermented in the rumen, producing propionate as the main fermentation product [14,15]. Additionally, part of the glycerol can be directly absorbed by the rumen mucosa and can be transformed into glucose by the liver, thereby constituting an energy source for cellular metabolism [16]. According to Mertens et al. [17], one of the main factors affecting the dry matter intake of forage (DMI<sub>f</sub>) is the neutral detergent fiber (NDF) content; therefore, increasing the degradation of this fraction is key to increasing DMI<sub>f</sub> and milk production from dairy animals fed primarily with roughage [18]. However, the effects of glycerol on NDF degradability, and the degradability of other nutrient fractions (e.g., dry matter (DM), organic matter (OM), crude protein (CP)), as well as fermentation parameters in dairy cattle, are still inconclusive. Literature reports have shown a lack of response [19–21], an increase [22,23], or a reduction in NDF degradability [24,25] when glycerol is included in ruminants' diets.

One of the most used forages for dairy cow nutrition in the high tropics of Colombia is *Pennisetum clandestinum* Hochst. Ex Chiov (kikuyu forage), which has significant levels of NDF, CP, and RDP and low contents of non-fiber carbohydrates (NFCs) [26,27]. Consequently, combining kikuyu grass with CG could offer a suitable feeding strategy for improving the production of grazing dairy cows in the tropics. Nevertheless, information regarding the effects of CG supplementation on rumen fermentation parameters and ruminal degradability of NDF in dairy cows grazing kikuyu forage is scarce. Hence, the aim of this study was to explore the effects of increasing inclusion levels of CG derived from biodiesel production on in situ ruminal NDF degradability and fermentative parameters in Holstein cows grazing kikuyu forage. This study hypothesized that the infusion of CG in the rumen of cows grazing kikuyu forage would modulate the rumen fermentation parameters and enhance the degradability of neutral detergent fiber.

## 2. Materials and Methods

### 2.1. Location

This experiment was conducted at the Paysandú experimental farm of the Universidad Nacional de Colombia, Medellín Campus, located in Santa Elena, Department of Antioquia, Colombia. The experimental farm is located at an average elevation of 2579 m.a.s.l., and it has an annual mean temperature of 14 °C.

### 2.2. Cows, Treatments, and Experimental Design

Four non-lactating Holstein cows (8 years old, 600 ± 33 kg BW) provided with a ruminal cannula (No. 1 C 4", Bar Diamond, Inc<sup>®</sup>, Parma, ID, USA) were used in this study in a 4 × 4 Latin square design. All cows had ad libitum access to water and feed and were grazing a Kikuyu pasture that had undergone 40 days of regrowth. In the morning at 06:00 h, cows were supplemented with a protein-energetic concentrate at a rate of 500 g per cow per day (Table 1).

Cows received each of the four treatments in a 4 × 4 Latin square design without replication. Treatments comprised the daily infusion of CG into the rumen at the following levels: 0 (G0), 500 (G500), 1000 (G1000), and 1500 (G1500) g of crude glycerol per cow. The CG was infused once at 6:00 h through the rumen cannula and manually mixed with the ruminal content from the dorsal and central regions of the rumen. In addition, kikuyu forage harvested at 35 (DR35) and 45 (DR45) days of regrowth (Table 2) placed in nylon bags was incubated in the rumen through the rumen cannula for in situ ruminal NDF degradability determination. The experimental period included four 16-day periods (13 days for adaptation to the diet and 3 days for measurements), for a total of 64 days.

Crude glycerol was obtained from OLEOFLORES<sup>®</sup>, a biodiesel production company based in Codazzi, Department of Cesar, Colombia, using African palm oil (*Elaeis guineensis*) as the feedstock. The crude glycerol had the following composition: glycerol (88%), water (8.0%), ash (4.0%), and methanol (0.8%). The glycerol was infused into the rumen of each cow daily at 06:00 h via the rumen cannula.

**Table 1.** Feedstuffs and chemical compositions of forage and concentrate.

	Kikuyu <sup>4</sup>	Supplement
<i>Feedstuffs (g/kg DM)</i>		
Wheat bran	-	404.2
Ground corn	-	313.8
Soybean meal	-	150.1
Cottonseed	-	65.4
Sugarcane molasses	-	49.7
Fish meal	-	9.8
Vitamin and mineral premix <sup>1</sup>	-	2.5
Calcium bicarbonate	-	2.5
Magnesium oxide	-	2.0
<i>Chemical composition (g/kg DM)</i>		
Dry matter	174.7 ± 1.4	903.1 ± 1.1
Crude protein	219.1 ± 1.6	235.6 ± 0.5
Neutral detergent fiber	622.4 ± 1.6	416.4 ± 1.1
Acid detergent fiber	274.0 ± 1.7	153.8 ± 1.1
Lignin	41.4 ± 0.6	22.0 ± 0.3
Ether extract	25.8 ± 0.5	36.0 ± 0.2
Ash	88.0 ± 1.4	71.2 ± 0.7
Non-fiber carbohydrates (NFCs) <sup>2</sup>	44.7 ± 1.2	240.8 ± 1.0
In vitro dry matter digestibility (IVDMD) <sup>3</sup>	601.0 ± 2.9	645.0 ± 1.3

<sup>1</sup> Vitamin–mineral premix supplied vitamin A: 5000 IU; vitamin D3: 1500 IU; vitamin E: 4 IU; Cu: 5 mg; Fe: 25 mg; Zn: 20 mg; Mn: 20 mg; Co: 0.05 mg; I: 0.3 mg; BHT antioxidant: 60 mg; excipient C.S.P.: 2.0 g. <sup>2</sup> NFC (g/kg DM) = 1000 – (CP + NDF + Ash + EE). <sup>3</sup> IVDMD at 48 h when using an Ankom Daisy II<sup>®</sup> incubator. <sup>4</sup> *Pennisetum clandestinum* Hochst. Ex Chiov after 40 ± 4 days of regrowth.

**Table 2.** Chemical compositions of incubated kikuyu forage harvested (g/kg DM).

Item	DR35	DR45
Dry matter	170.3	178.6
Crude protein	239.2	209.7
Neutral detergent fiber	542.7	599.0
Acid detergent fiber	227.0	249.6
Lignin	36.8	39.2
Ether extract	26.1	19.2
Ash	116.0	114.0
Non-fiber carbohydrates (NFCs) <sup>1</sup>	76.0	58.1
In vitro dry matter digestibility (IVDMD) <sup>2</sup>	712.0	601.0

<sup>1</sup> NFC (g/kg of DM) = 1000 – (CP + NDF + Ash + EE). <sup>2</sup> IVDMD at 48 h using an Ankom Daisy II<sup>®</sup> incubator.

### 2.3. In Situ Ruminant NDF Degradation

Determination of in situ ruminal NDF degradability was conducted using the nylon bag technique [28], while forage samples were collected using the “Hand Plucking” method [29]. Approximately 4 g of each kikuyu forage harvested [30], ground to 2 mm, was weighted into nylon bags (10 × 5 cm Ankom<sup>®</sup> Dacron/polyester with 50 ± 3 µm pores, Ankom Technologies, Fairport, NY, USA).

After the adaptation period of each experimental period and for all treatments, the bags containing each forage-type material were incubated in duplicate. Before incubation, all the bags were submerged in clean tap water at room temperature for 30 s. This step aimed to pre-hydrate the bags and establish a correction factor for particle escape at the initial 0 h time point [30]. All the bags were fastened to a galvanized iron chain and placed simultaneously into the rumen, and then they were removed at 0, 3, 6, 9, 12, 24, 48, and 72 h. The bags at 0 h time were left hanging in the rumen for 20 min. After the designated incubation times were completed, the bags were taken out of the rumen; to ensure consistent and uniform washing, bags were thoroughly washed in a washer for 10 min. Subsequently, the bags were dried at 60 °C for 72 h in a forced air oven. Once dried,

the remaining residue of the incubated substrate was further analyzed to obtain the DM and the fraction of remanent neutral detergent fiber (NDF).

#### 2.4. Rumen Fermentation Parameters

Collection of rumen fluid samples was performed on day 14 of each experimental period, specifically 4 h after the supplementation of crude glycerol. Approximately 50 mL of rumen fluid was taken from different regions of the rumen. The fluid was filtered through cheesecloth (four layers) to remove any solid particles. Subsequently, the pH of the rumen fluid was measured using a digital pH meter (Metrohm 704 pH meter, Metrohm, Herisau, Switzerland). To determine the concentration of rumen  $\text{NH}_3\text{-N}$ , a 20 mL aliquot of the ruminal fluid was preserved by adding 0.5 M HCl. Thereafter, ruminal fluid ammonia nitrogen ( $\text{NH}_3\text{-N}$ ) levels were measured using a selective ion electrode, as described by Valencia et al. [31].

Another subsample was stored at  $-4\text{ }^\circ\text{C}$  for further analysis of VFA concentrations. Before analysis, the samples were centrifuged at  $4000\times g$  for 4 min at  $-4\text{ }^\circ\text{C}$ . Then, the supernatant (800  $\mu\text{L}$ ) was mixed with an acidifying and deproteinizing solution (500  $\mu\text{L}$ ) composed of metaphosphoric (100 g/L) and crotonic (0.6 g/L) acids. The mixture was immediately subjected to centrifugation for 10 min at  $13,000\times g$  and  $-4\text{ }^\circ\text{C}$ . Finally, the resulting supernatant (1 mL) was analyzed for VFA analysis using gas chromatography coupled to a flame ionization detector (GC-FID; GC-2014, Shimadzu Corporation, Kyoto, Japan). The GC-FID was performed using an Agilent HP-FFAP capillary column (0.32 mm ID  $\times$  25 m length, Agilent Technologies, Frederick, CO, USA). The split injection and detection port temperatures remained at  $260\text{ }^\circ\text{C}$  and  $280\text{ }^\circ\text{C}$ , respectively. Helium (He) gas was used as the carrier gas, and the flow rate was 42 cm/s.

#### 2.5. Chemical Analyses of the Feedstuffs and Diet

Chemical composition analyses were performed on feedstuff and the remaining residue of the incubated forage samples. Ash, DM, and nitrogen contents were determined according to the procedures outlined by the AOAC [32]. Crude protein (CP) was calculated as nitrogen  $\times$  6.25. The contents of acid detergent fiber (ADF), NDF, and lignin were obtained in accordance with Van Soest et al.'s [33] suggestions. The ether extract (EE) contents were determined using the Soxhlet method with a fat analyzer (ANKOM XT15, Ankom Technologies, Fairport, NY, USA) according to the AOAC [34]. The IVDMD was determined after 48 h using an Ankom Daisy II<sup>®</sup> incubator (Ankom Technologies, Fairport, NY, USA) following the methodology described by Goering [35]. The NFC contents were calculated according to the following [36]:

$$\text{NFC (g/kg)} = 1000 - (\text{CP} + \text{NDF} + \text{Ash} + \text{EE}) \quad (1)$$

where CP, NDF, and EE are the crude protein, neutral detergent fiber, and ether extract, respectively.

#### 2.6. Calculations

To calculate the in situ ruminal degradability parameters of NDF of both kikuyu forages incubated (DR35 and DR45, respectively), an exponential model was used through a Marquardt algorithm iterative process using nonlinear regression via the PROC NLIN of SAS (Statistical Analysis System V9.4; SAS Inst. Inc., Cary, NC, USA).

To estimate each ruminal degradability parameter of the NDF, the exponentials were fitted into a model as follows:

$$y = b [1 - e^{-c(t-L)}] \quad (2)$$

where “y” represents the degradation of NDF in the nylon bag at each incubation time t (in hours: h), b is the potential degradation of NDF, c is the degradation rate of fraction b ( $\text{h}^{-1}$ ), and L is the pre-fermentative period or lag phase (h), according to the procedures

described by Giraldo et al. [37]. To estimate the effective degradability of NDF (EDNDF) in each substrate, the following model was used [38]:

$$\text{EDNDF} = [(b \times c)/(c + kp)] \quad (3)$$

This model considers a rumen outflow rate of 0.050 kp/h, which was estimated for grazing Holstein cows fed kikuyu grass [39].

### 2.7. Statistical Analysis

Data from the in situ ruminal degradability of NDF were analyzed under a split-plot arrangement using a randomized 4 × 4 Latin square experimental design with CG levels in the rumen as the factor assigned to the main plots and days of regrowth of forage as the factor attributed to the subplots. Interactions between glycerol levels in the rumen and days of regrowth of forage were analyzed using the PROC MIXED of SAS (SAS Inst. Inc., Cary, NC, USA). Data on pH, NH<sub>3</sub>-N, and VFA were analyzed under a 4 × 4 Latin square design using the PROC MIXED of SAS. The glycerol level was considered as a fixed effect, while the animal and period were defined as random effects. When a significant effect ( $p < 0.05$ ) was detected via ANOVA for all variables, a comparison of means was conducted using the Tukey adjusted multiple PDIFF comparison test.

### 3. Results

The in situ ruminal degradability parameters of NDF revealed an interaction effect between CG × incubated days of regrowth of forage (DR) on the “b” fraction and “c” rates ( $p = 0.001$ ; Table 3). The fraction “b” of DR45 increased by approximately 6.7% with increasing levels of CG supplementation in the rumen. For DR35, fraction “b” was unaffected by the levels of CG infusion in the rumen. A similar result was observed in the “c” rate for both DR and all levels of CG infusion in the rumen (Table 3).

**Table 3.** Effect of infusion of crude glycerol on in situ ruminal degradation parameters of NDF in incubated kikuyu forage harvested at 35 (DR35) and 45 (DR45) days of regrowth.

		Crude Glycerol Level in Rumens (g/animal/day)				p-Value			
		G0	G500	G1000	G1500	SEM <sup>2</sup>	CG <sup>3</sup>	DR <sup>4</sup>	CG × DR
In situ ruminal degradation parameters of NDF <sup>1</sup>									
b	DR35	0.954	0.904	0.926	0.932	0.017	0.715	<0.001	0.001
	DR45	0.848 <sup>b</sup>	0.933 <sup>a</sup>	0.903 <sup>a</sup>	0.879 <sup>ab</sup>				
c, h <sup>-1</sup>	DR35	0.035 <sup>b</sup>	0.051 <sup>a</sup>	0.051 <sup>a</sup>	0.046 <sup>a</sup>	0.003	0.083	<0.001	0.006
	DR45	0.033	0.033	0.037	0.034				
Lag, h	DR35	1.20	1.10	1.30	1.30	0.122	0.896	0.340	0.238
	DR45	1.30	1.40	1.30	1.30				
EDNDF	DR35	0.393	0.449	0.462	0.446	0.015	0.030	<0.001	0.294
	DR45	0.333	0.364	0.384	0.352				

<sup>1</sup> b: insoluble but potentially degradable fraction; c: degradation rate of fraction b; Lag: lag period; EDNDF: effective degradability of NDF considering a rumen outflow rate (kp h<sup>-1</sup>) of 0.050. <sup>2</sup> SEM: standard error of the mean. <sup>3</sup> CG: crude glycerol level infusion effect in the rumen. <sup>4</sup> DR: rumen incubated days of regrowth of forage effect. <sup>a,b</sup> Values followed by different letters in the same row are significantly different based on the CG × DR interaction effect ( $p < 0.05$ ).

The lag period of the incubated DR was not influenced by the level of CG in the rumen when compared with that of the G0 group ( $p = 0.238$ ). Conversely, there was a CG × DR interaction effect on the effective degradability of NDF (EDNDF). The EDNDF of both DR increased approximately by 13.0 ± 0.72% and 11.0 ± 0.74% for DR35 and DR45, respectively, when compared with that of the diets of animals that were not supplemented with CG ( $p = 0.030$ ; Table 3).

Ruminal pH and the total VFA concentration were unaffected by the CG infusion level (Table 4). However, the molar acetate concentration and the acetate: propionate ratio decreased with increasing CG in the rumen; thus, G1500 showed the lowest molar acetate concentration and acetate: propionate ratio values among the CG levels. Additionally, the variables for G500 and G1000 were statistically similar but quantitatively lower than those for G0 ( $p < 0.001$ ). In contrast, the molar concentration of propionate increased with increasing CG levels in the rumen. Furthermore, the molar propionate concentration values were similar between G1000 and G500 when compared with G0 ( $p < 0.001$ ). The butyrate ruminal concentration increased when CG was added to the rumen, irrespective of the infusion level ( $p < 0.001$ ). The ruminal  $\text{NH}_3\text{-N}$  concentration decreased at CG supplementation levels of 1000 (G1000) and 1500 (G1500) g/cow/day, respectively ( $p = 0.03$ ).

**Table 4.** Effects of the infusion levels of crude glycerol on ruminal fermentation parameters in grazing dairy cows.

	Crude Glycerol Level in the Rumen (g/animal/day) <sup>1</sup>					SEM <sup>3</sup>	<i>p</i> -Value <sup>4</sup>
	G0	G500	G1000	G1500			
<i>Mol/100 mol</i>							
Acetate (A)	65.4 <sup>a</sup>	51.1 <sup>b</sup>	52.3 <sup>b</sup>	47.9 <sup>c</sup>	0.74	<0.001	
Propionate (P)	20.8 <sup>c</sup>	25.9 <sup>b</sup>	24.2 <sup>b</sup>	29.1 <sup>a</sup>	0.63	<0.001	
Butyrate (B)	12.0 <sup>b</sup>	21.2 <sup>a</sup>	20.6 <sup>a</sup>	21.5 <sup>a</sup>	0.69	<0.001	
Other VFA <sup>2</sup>	1.7	1.6	1.8	1.6	0.33	0.155	
A:P (mol:mol)	3.2 <sup>a</sup>	2.0 <sup>b</sup>	2.1 <sup>b</sup>	1.7 <sup>c</sup>	0.06	<0.001	
Total VFA (mM)	104.4	109.4	108.7	99.4	4.75	0.237	
pH	6.4	6.3	6.3	6.2	0.06	0.363	
N-NH <sub>3</sub> (mg/dl)	21.5 <sup>a</sup>	18.9 <sup>ab</sup>	17.1 <sup>b</sup>	17.0 <sup>b</sup>	1.32	0.030	

<sup>1</sup> G0 (without crude glycerol infusion); G500, G1000 and G1500: 500, 1000 and 1500 g/cow/day of crude glycerol in the rumen, respectively. <sup>2</sup> Sum of valerate and isovalerate. <sup>3</sup> Standard error of the mean,  $n = 4$  cows/treatment. <sup>4</sup> *p*-values of treatment. <sup>a-c</sup> Values with different letters on the same row represent significant differences ( $p < 0.05$ ).

#### 4. Discussion

The infusion of CG in the rumen induced an increase in the EDNDF of the kikuyu grass samples harvested at 35 (DR35) and 45 (DR45) days of regrowth. This was associated with an increase in both the “c” degradation rate and the potential degradability of the “b” fraction at both kikuyu maturity stages. Ørskov and McDonald [38] suggested that an increase in the effective degradability of nutrient fractions, such as the NDF, is effectively described through the increase in different parameters (a, b, c, and kp). Changes in the effective degradability of nutrient fractions are influenced by the chemical composition of the forage, as well as by the growth and ability of fibrolytic microorganisms to degrade it [25].

As expected, the harvesting time of kikuyu grass affected its chemical composition, resulting in a higher fiber fraction content (NDF, ADF, and lignin) and lower CP content, as well as its degradability, as indicated by the lower IVDMD with the increasing days of regrowth. However, the infusion of CG positively influences the ruminal degradability of kikuyu grass regardless of the harvesting time, suggesting that CG supplementation can improve the utilization of this forage at different maturity stages. Specifically, the increase in the degradation rate “c” of the NDF fraction with all levels of CG infusion in the rumen indicates that CG has a positive effect on substrate degradation. Furthermore, these results suggest that the infusion of CG in the rumen of grazing dairy cows modifies the environment of the rumen and the activity of its microorganisms, thus degrading the “b” fraction into a less digestible substrate. Van Milgen et al. [40] indicated that except for fraction “a”, which disappears quickly in a rumen environment and is defined as an intrinsic factor to the substrate, other fractions are not always inherent to the incubated substrate, and this was observed in the current study.

The influence of glycerol on ruminal fermentation is directly associated with its dose and disappearance rate in the rumen [41]. The rate of glycerol disappearance is high because of adaptative microbial mechanisms to the glycerol presence in the rumen [42]. Schröder and Südekum [43] indicated that in diets with a high forage content vs. high concentrates, glycerol presents greater opportunities to improve the microbial activity in the rumen and induce a higher degradation of the structural carbohydrates of the diet, as was observed in this study. Similarly, Wang et al. [22] revealed that feeding steers with 100, 200, and 300 g of glycerol/day increased the ED of corn stover (the principal fibrous component of the diet), improving its degradation rate and the potentially degradable fraction. At the same time, other studies observed an increased NDF digestibility of diets in lambs supplemented with CG [3].

Several studies also suggest that a decrease in NDF digestibility is a possible consequence of a reduction in the molar proportion of acetate and the acetate: propionate ratio and a decrease in ruminal fibrolytic bacteria involved in NDF digestibility [44,45]. Hence, the results obtained for the degradability of NDF in kikuyu grass at 35 and 45 days of regrowth indicate that ruminal microorganism activity caused by feeding with glycerol depends largely on the continuous exposure to this sugar-alcohol; additionally, continuous glycerol exposure may increase ruminal degradability, specifically in the NDF fraction of incubated forages.

Ruminal pH was unaffected by the infusion of CG even at 1500 g/animal/day. These results agree with those of Boyd et al. [46] and Ariko et al. [47], who reported that ruminal pH was not influenced by CG incorporation into the total mixed ration of dairy cows. Conversely, Kijora et al. [48] revealed that when diets based on hay and silage forage with a high nonstructural carbohydrate content are mixed with 200 g of glycerol infused into the rumen, a decrease in the ruminal pH is observed. This suggests that the administration method (direct infusion vs. mixed in the ration) is a key factor in modulating ruminal environment changes when glycerol is used in dairy cow diets with high starch contents. However, our results indicate that when high-forage diets are used in combination with glycerol, there is a different response of fermentative parameters compared to high-starch diets. High-forage diets stimulate chewing and saliva production, which helps buffer the rumen [49]. This agrees with several already published studies exploring the effects of glycerol inclusion under different dietary conditions [43,50].

Even though the total VFA content was unaffected by glycerol infusion into the rumen, which aligns with the lack of glycerol effects on ruminal pH, the molar concentration of propionate and butyrate increased at the expense of acetate reduction, resulting in a decline in the acetate: propionate ratio. These results make biological sense because the inclusion of sugar-alcohols, such as glycerol, induces propionate synthesis due to its gluconeogenic properties [31,51]. Additionally, Ciriaco et al. [14] found that increasing supplementation doses of 50:50 (as-fed) molasses and CG mixed with Tifton 85 bermudagrass hay increases the propionate and butyrate concentrations with a concomitant decrease in acetate and the acetate: propionate ratio. From a biochemical standpoint, Avila-Stagno et al. [50] suggested that the increase in the molar concentration of propionate and butyrate may be due to those VFAs being the main products during glycerol fermentation in the rumen. Furthermore, the lower acetate: propionate ratio resulting from glycerol infusion may be associated with the increased EDNDF of forage observed in the in situ assay, supporting the hypothesis that the infusion of CG in the rumen of grazing cows can modulate the ruminal environment and enhance the fibrolytic activity of its microbiota. This modulation potentially leads to a better overall performance in dairy cows by optimizing the utilization of kikuyu forage. The decrease in the acetate: propionate ratio may be beneficial for ruminant performance, as this tendency may increase the total DM intake, as well as forage DM and NDF disappearance in beef cattle [52]. In a performance trial, Valencia et al. [53] reported that supplementing grazing dairy cows with 1500 g of GL per day increased the milk yield by 14.2%. Thus, incorporating CG into supplements could offer a suitable nutritional strategy to optimize the utilization of kikuyu forage and enhance the performance of dairy cows.

Our data revealed that the infusion of 1000 and 1500 g of CG reduced the  $\text{NH}_3\text{-N}$  levels in the rumen by approximately 20%. Paiva et al. [23] and Van Cleef et al. [15] suggested that the effects of dietary inclusion of CG on ruminal  $\text{NH}_3\text{-N}$  may be attributed to several factors, such as diet composition, the quality of the protein in the diet, and the glycerol feeding level. However, the results of different studies are still inconclusive regarding the effects of glycerol on ruminal  $\text{NH}_3\text{-N}$  concentrations. Ariko et al. [8] revealed an increase in the concentrations of ruminal  $\text{NH}_3\text{-N}$  when barley is replaced with CG up to 156 g/kg in high-starch diets; conversely, in vitro experiments conducted by Valencia et al. [31] observed a decrease in ruminal  $\text{NH}_3\text{-N}$  concentration when glycerol was included in forage diets with high CP contents. According to Wang and Zhiliang [54] and Abbasi et al. [55], cellulolytic bacteria are significant users of ruminal N, primarily in the chemical form of  $\text{NH}_3\text{-N}$ , which they utilize as a source of nitrogen for their growth and energy metabolism. This may explain the increase in NDF degradability of both kikuyu forages with high CP contents observed in this study. The inclusion of glycerol provides an additional energy source, improving the efficiency of nitrogen utilization by ruminal microbes and enhancing fiber degradation.

## 5. Conclusions

This study indicates that the infusion of CG into the rumen of non-lactating Holstein cows grazing kikuyu grass effectively modulates rumen fermentation parameters and enhances the degradability of NDF. The results show that infusion of CG at levels of 500, 1000, and 1500 g/cow/day increases the in situ ruminal NDF degradability of kikuyu grass at both 35 and 45 days of regrowth. Moreover, a CG rumen infusion at 1000 and 1500 g/cow/day decreases rumen acetate and ammonia nitrogen levels while increasing propionate and butyrate concentrations. These changes in rumen fermentation dynamics suggest that CG has significant potential to improve the efficiency of fiber utilization and the overall performance of dairy cows grazing kikuyu forage under tropical conditions. Thus, incorporating CG into the diet of grazing dairy cows could offer a viable supplementation strategy for optimizing forage utilization and enhancing dairy production in high-tropical regions. Further research is needed to explore the effects of CG supplementation on lactating cows' health and milk yield, as well as its economic viability and environmental impact.

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