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In vitro degradation and gas production of glycerin generated in the biodiesel production chain

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ABSTRACT. This study was realized to evaluate the production of gas and volatile fatty acids when glycerin was used to replace four forage species through the use of a semi-automated technique *in vitro*. The experimental design included 4 treatments and 4 replications. The treatments consisted of increasing levels of glycerin (0, 30, 50 and 70%), which was used to replace the forage plants Elephant grass, sugar cane silage, corn silage and Brachiaria grass. Replacement of the Brachiaria and Elephant grasses with glycerin linearly reduced the production of methane and carbon dioxide and ammonia nitrogen and had a positive effect on forage quality ($p < 0.05$). With the addition of glycerin, the values for acetate concentration decreased ($p < 0.05$); however, when added glycerin there was increased in propionic and butyric acid concentration ($p < 0.05$). Substitution of forage species for glycerin by tests for semi-automatic technique *in vitro* reduce the production of greenhouse gases (CH_4 and CO_2) which reduction in energy loss, and increase volatile fatty acids propionic and butyric.

Keywords: acetate, butyrate, N-NH_3, propionate, VFAs.

Degradação e produção de gases *in vitro* de glicerina da cadeia produtiva do biodiesel

RESUMO. Este estudo foi realizado para avaliar a produção de gás e ácidos graxos voláteis da glicerina em substituição à quatro espécies forrageiras pela técnica semi-automática *in vitro*. O delineamento experimental incluiu 4 tratamentos e 4 repetições. Os tratamentos consistiram em níveis crescentes de glicerina (0, 30, 50 e 70%), em substituição às forrageiras capim Elefante, cana-de-açúcar, silagem de milho e Braquiária. A substituição das gramíneas forrageiras capim Brachiaria e capim Elefante pela glicerina afetou a produção de metano, dióxido de carbono e nitrogênio amoniacal e teve um efeito positivo sobre a qualidade da forragem ($p < 0,05$). Com a adição de glicerina, os valores da concentração de acetato reduziram ($p < 0,05$). Todavia, a adição de glicerina aumentou as concentrações de ácido propiônico e butírico ($p < 0,05$). A substituição de espécies forrageira pela glicerina através da técnica semiautomática *in vitro* reduz a produção de gases de efeito estufa (CH_4 e CO_2) que diminuirá a perda de energia e eleva os ácidos graxos voláteis propiônico e butírico.

Palavras-chave: acetato, butirato, N-NH_3, propionato, AGVs.

Introduction

Byproducts originating from the biodiesel production chain have been studied as potential ingredients for ruminant diets to reduce greenhouse gas (GHG) production and increases animal production (Eiras et al., 2014; Françozo et al., 2013). Studies aimed at characterizing the ruminal metabolism of these byproducts are needed to identify potential byproducts that could be used efficiently in the diet of ruminants to replace conventional ingredients (Cruz et al., 2014; Hulshof et al., 2012; Moreira et al., 2014; Silva et al., 2014). Among these byproducts, glycerin, which is obtained in the transesterification process from oil to biodiesel in the crude form, is an alternative (Cruz et al., 2014; Farias et al., 2012; Martínez-Fernández et al., 2014).

The main component of glycerin, glycerol, is highly energetic, is generated by animal metabolism. The inclusion of glycerol in the diet has been reported (Gunn et al., 2010; Mach et al., 2009) and different fates have been reported for glycerol when entering the rumen: passage to the lower gut, absorption through the rumen wall and conversion to glucose in the liver, or fermentation to propionate resulting in increases in blood glucose in cattle (Krehbiel, 2008).

Rumen fermentation breaks down glycerol, and the main product is propionic acid, which is a precursor of fatty acids and glucose metabolites in ruminants. The use of glycerin may reduce the inclusion of high amounts of concentrate in the diet, which could adversely affect animal performance (Farias et al., 2012). And this process in the rumen, the production of gases occurs. The production of carbon dioxide and methane by the rumen and intestinal bacteria (*Methano brevibacter* spp. and *Methano microbium mobile*) can cause emissions that vary from 2 to 12% of the gross energy of the food ingested, with an average loss of 6% (Hristov et al., 2013). Emission of methane and carbon dioxide has led to the identification of livestock activity as a major source of GHG production. Thus, the objective was examined the amount of methane, carbon dioxide, VFAs, and N-NH_3 produced when using glycerin to replace a portion forage species in semi-automated technique *in vitro*.

Material and methods

This research was conducted at the Experimental Station of Coronel Pacheco, MG (owned by Embrapa Gado de Leite – CNPGL), located in the Mata of Minas Gerais State, Brazil.

In this experiment, four forage species were used: Elephant grass (*Pennisetum purpureum*) with 60 days of age, corn silage (*Zea may*) after 50 days, Brachiaria grass (*Brachiaria decumbens*) with 40 days of age and sugar cane silage after 365 days (*Sacharum* ssp.) from the experimental field of Embrapa in Coronel Pacheco in Minas Gerais Stage. An approximately 300 g sample of a glycerin byproduct, derived from the processing and/or extraction of vegetable oil to produce fuel for the biodiesel industry, was collected. Subsequently, samples were taken to the laboratory, packaged and stored at 10ºC prior to analysis. At the start of the analysis, the samples were thawed, pre-dried using forced ventilation at 55°C for 72 hours, ground in a Wiley mill equipped with 5 mm sieve, packaged in plastic bags, identified and sent for laboratory analysis at Embrapa Dairy Cattle, Juiz de Fora, Minas Gerais State.

In the laboratory, the samples belonging to each fodder and the glycerin were mixed and homogenized to form a single sample. From this sample, a portion (100 g) was used for chemical analysis, and the remainder of the material was used to formulate the test diets. Each diet was formulated to evaluate various levels of glycerin in the diet, with ratios of 100/0, 70/30, 50/50 and 30/70% (roughage/glycerin), as fed basis.

The substrates consisting of forage materials and the byproduct were pre-dried in forced air ovens at 60°C for 48 hours and then ground in a Willey mill equipped with a 1.0 mm sieve to determine dry matter (DM) (Method 967.03 - AOAC (2005), ash (Method 942.05 - AOAC (2005), crude protein (CP) (Method 981.10 - AOAC (2005), and ether extraction (EE) (Method 920.29 - AOAC (2005). To determine the NDF and ADF contents, the methodology of Van Soest et al. (1991) was used with the modifications that were proposed in the Ankon device manual (Ankon Technology Corporation, Macedon, New York, US). Acid detergent lignin (ADL) was determined according to method 973.18 (AOAC, 2005), in which the ADF residue was treated with 72% sulfuric acid. The proportion of ingredients and chemical composition are shown in Table 1. The total carbohydrate (TC) values were determined using the equation described by Sniffen et al. (1992): TC= 100 - (% CP + % EE + % Ash).

Glycerine was produced in a soy-diesel facility and its chemical composition was determined at the Embrapa Dairy Cattle (Embrapa Dairy Cattle, Juiz de Fora, Minas Gerais State, Brazil), as follows: ashes – 57.1 g kg^{-1}; glycerol, 811.1 g kg^{-1}; sodium, 26.4 g kg^{-1}; calcium, 60.0 mg kg^{-1}; phosphorus, 32.9 mg kg^{-1}.

The assay for in vitro digestibility of dry matter (IVDMD) was performed according to the methodology of Tilley and Terry (1963). Three fistulated steers were used for this *in vitro* test. The ruminal contents were collected in the morning, transferred to preheated thermo-flasks (39ºC) and transported immediately to the laboratory. In the laboratory, the rumen fluid was filtered with cheesecloth into three layers. Mineral buffer solution was later added to the rumen fluid in a water bath maintained at 39ºC with continuous CO_2 injection. The samples were held in an incubator, in a state of rotation, for 72h. Peptides were added to the incubators within 48h to act as an intermediate compound for the action of the microorganisms.

The substrates used for *in vitro* incubations were glycerin, which was used to replace the byproduct fodder in ratios of 100/0, 70/30, 50/50 and 30/70% (roughage/glycerin). The feed ingredients were dried at 55ºC for 24h and then ground to pass a 1 mm screen and incubate *in vitro* (Meale et al., 2012). The entire incubation procedure was repeated twice (*i.e.*, two incubation runs x three replicates per treatment, resulting in a total of six replicate vials per treatment).

The *inoculum* for the *in vitro* incubation was obtained from the three ruminally fistulated cows

grazing on beard grass supplemented with 2 kg of concentrate (220 g kg^{-1} CP and 126 g kg^{-1} NDF in DM). Rumen fluid was collected 200h before morning milking from 4 distinct sites in the rumen, filtered through 4 layers of cheesecloth, combined in equal portions from each animal and immediately transported in a pre-warmed Thermos® flask to the laboratory. *Inocula* were prepared by mixing rumen fluid and a mineral buffer with 0.5 mL of cysteine sulfide solution in a ratio of 1:5 (Vitti et al., 1999). *Inoculum* (30 mL) was then transferred into pre-loaded, pre-warmed (39ºC) vials under a stream of O_2-free N gas. The vials were sealed and placed on an orbital shaker rack set at 120 oscillations per min. in an incubator set at 39ºC.

To Determination of total gas, methane concentration and *in vitro* dry matter disappearance (IVDMD), the net gas production of each vial was measured at 6, 12, 24 and 48h of incubation with a water displacement apparatus (Fedorah & Hrudey, 1983). At 6 and 12h prior to gas measurement, the headspace gas was sampled from each vial with a 20 mL syringe and immediately transferred into a 5.9 mL evacuated Exetainer® (Labco Ltd., High Wycombe, Buckinghamshire, UK), which was analyzed to determine the CH_4 concentration using gas chromatography. Methane was expressed as mg of CH_4 g^{-1} of DM disappeared, and total net gas production was expressed as mL g^{-1} of incubated DM. After the gas was sampled for CH_4 and total gas production was measured at 48h of incubation, the fermentation vials were opened, and the pH of the culture was measured using a pH meter (Orion Model 260A, Fisher Scientific, Toronto, ON, Canada). The ANKOM® bags with the residues were then removed from the bottles, rinsed thoroughly with distilled water and dried at 55ºC for 48h to a constant weight to estimate IVDMD.

To determination of N-NH_3 and VFAs, a subsample (1.6 mL) of the culture media from each vial was transferred to a 2 mL micro-centrifuge tube and centrifuged at 14,000 *x g* for 10 min. at 4ºC (Spectrafuse 16M, National Labnet Co., Edison, NJ, USA) to precipitate particulate matter and protein. The supernatant was transferred into 2 mL micro-centrifuge tubes and analyzed for ammonia-N. In addition, a subsample (1.5 mL) of each vial was collected, acidified with 300 μL of metaphosphoric acid (0.25; w/v), and centrifuged as previously described for the ammonia-N analysis. The supernatant was frozen at -20ºC until analysis for VFA concentrations. The 0f samples were also analyzed for ammonia-N and VFA to calculate net ammonia-N and net total VFA production (Holtshausen et al., 2009).

The experimental design used a randomized 4 x 4 factorial arrangement (glycerin levels x forages). When the variable was independent of the main factors of the byproducts, we applied the mean test, and replacement levels determined the most representative regression model. Those variables where the effect of the main factors was dependent, the regression model that best represented the data were applied to the byproducts according to the level of substitution of sugar cane silage. Additionally, the effect of the glycerin at each level of substitution was subjected to the mean test. When choosing the regression models that best represented the behavior of the data, we considered significant at a P-value of ≤ 0.05 using the Tukey's test (SAS, 2004).

Results

An analysis of the chemical composition (Table 1) verified that the Elephant grass and Brachiaria had higher levels ($p < 0.05$) of CP, which is positive in terms of forage quality.

However, the levels of ADF (351.1 g kg^{-1}) and lignin (144.4 g kg^{-1}) were the highest compared with the other products studied, directly influencing the digestibility of this grass in the rumen.

The diet compositions had different characteristics then the corresponding fodder. The sugar cane silage had the lowest levels of DM (271.4 g kg^{-1}), CP (22.5 g kg^{-1}) and EE (11. 9 g kg^{-1}), making more difficult to achieve the requirements of cattle, and had the lowest level of DDM 554.6 g kg^{-1}. Corn silage had lower ($p < 0.05$) levels of NDF (469.3 g kg^{-1}) and ADF (314.5 g kg^{-1}), and compared with the forage grass Brachiaria, corn silage was less fibrous and had higher levels of (EE) (32.2 g kg^{-1}), ash (6.1 g kg^{-1}) and IVDMD (652.0 g kg^{-1}), enabling the material to have a positive effect.

The results (Table 2) show the average production of methane (CH_4) for fodders with different levels of glycerin. All of the fodder and levels differed, and within the types of fodder, Elephant grass ($p < 0.0001$), sugar cane silage ($p = 0.0452$), corn silage ($p = 0.0662$) and Brachiaria grass ($p = 0.0003$) had a linear decrease regression effect.

The Elephant grass (12.77 mL g^{-1} DM) with 70% glycerin had the lowest methane content ($p < 0.05$), while the Brachiaria (27.70 mL g^{-1} DM) with 70% glycerin had the highest methane content. The replace of forages by glycerin mitigated methane, reducing the values at each level; 64% Elephant grass, 69% sugar cane silage, 46% corn silage, 72% Brachiaria grass.

Table 1. Ingredient and chemical composition (DM basis) (g kg^{-1}) of the ingredients fed.

Forages	DM[1]	CP	NDF	ADF	ADL	EE	ASH	TC	IVDMD
Elephant grass	117.7	126.1	555.0	351.1	144.4	14.2	25.4	834.3	591.4
Sugar cane silage	271.4	22.5	518.2	362.3	40.4	11.9	48.5	917.1	554.6
Corn silage	157.3	62.5	469.3	314.5	29.7	22.2	51.7	863.6	607.0
Brachiaria grass	129.2	122.2	556.2	277.7	34.7	32.2	86.1	759.5	652.0
Glycerin	875.9	95.8	-	-	-	-	57.1	-	929.0

Abbreviations: DM, Dry Matter; CP, Crude Protein; NDF and ADF, Neutral and Acid Detergent Fiber; ADL, Acid Detergent Lignin; EE, Ether Extract; ASH, Ashes; TC, Total Carbohydrates; IVDMD, In Vitro Dry Matter Degradability. [1]Organic Matter base.

Table 2. Mean values, probability (*P*-value) and regression equations illustrating the effects on production of CH_4 (mL g^{-1} DM), CO_2 (mL g^{-1} DM) and N-NH_3 (mL g^{-1} DM) when different levels of glycerin.

Forages	Levels of substitution for Glycerin				Regression Equations	R^2	*P*-value
	0%	30%	50%	70%			
Methane production (CH_4, mL g^{-1} DM)							
Elephant grass	19.92[b]	19.27[b]	15.93[b]	12.77[b]	$\hat{Y}$ = 23.17-2.479x	0.93	< 0.0001
Sugar cane silage	8.94[d]	9.28[c]	8.44[d]	6.18[c]	$\hat{Y}$ = 10.49-0.912x	0.71	0.0452
Corn silage	19.42[b]	13.27[c]	11.91[c]	9.04[c]	$\hat{Y}$ = 21.53-3.25x	0.91	0.0662
Brachiaria grass	27.70[a]	25.20[a]	23.53[a]	20.10[a]	$\hat{Y}$ = 30.25-2.447x	0.98	0.0003
Carbon Dioxide (CO_2, mL g^{-1} DM)							
Elephant grass	120.92[a]	111.42[a]	103.94[a]	99.70[a]	$\hat{Y}$ = 126.7-7.114x	0.97	< 0.0001
Sugar cane silage	71.25[b]	65.61[b]	56.04[b]	32.24[b]	$\hat{Y}$ = 87.93-12.66x	0.90	0.0193
Corn silage	72.25[b]	58.92[b]	46.83[c]	39.55[b]	$\hat{Y}$ = 81.93-11.01x	0.98	0.0064
Brachiaria grass	7.36[c]	5.23[c]	5.23[d]	4.86[c]	$\hat{Y}$ = 7.545-0.75x	0.72	0.0018
Ammonia Nitrogen (N-NH_3, mL g^{-1} DM)							
Elephant grass	21.46[a]	17.73[a]	11.90[a]	8.40[a]	$\hat{Y}$ = 22.112+0.192x	0.92	< 0.0001
Sugar cane silage	1.86[c]	2.56[c]	3.50[c]	3.26[c]	$\hat{Y}$ = 0.553-0.047x	0.80	< 0.0001
Corn silage	6.53[b]	2.63[c]	1.40[d]	0.70[d]	$\hat{Y}$ = 5.963+0.083x	0.88	< 0.0001
Brachiaria grass	6.36[b]	6.03[b]	6.73[b]	5.50[b]	$\hat{Y}$ = 6.453+0.007x	0.06	0.4587

*Means in the same column with different letters differ statistically by the Tukey test at 5% probability.

The results for the production of and carbon dioxide (CO_2) *in vitro* showed that there was a decrease linear relationship ($p < 0.05$) for almost all forages analyzed. Analyzing the substitution levels of glycerin for fodder individually, we observed that the Elephant grass produced the highest ($p < 0.0001$) final volumes of gas with 120.92 mL g^{-1} of DM (incubated), while Brachiaria grass had the 66% lowest volumes, 7.36 mL g^{-1} DM to 4.86 mL g^{-1} DM at 70% glycerin replace, respectively. The sugar cane silage and corn silage showed a gradual decrease in CO_2 production from 0% (control) to 70% glycerin, presented reduced of 54% in corn silage and 45% in sugar cane silage.

The production of ammonia nitrogen (N-NH_3) in the different forages with different levels of glycerin varied ($p < 0.05$) and fit a linear regression. The Elephant grass (21.46 mL g^{-1} DM) at the 0% level had the highest ammonia nitrogen content, while the sugar cane (1.86 mL g^{-1} DM) at the 0% level had the lowest ammonia nitrogen content ($p < 0.05$). The values for corn silage and Elephant grass decreased with each level; however, at a level of 0%, the N-NH_3 content of the Elephant grass was relatively high compared with the other forages (21.46 mL g^{-1} DM).

The production of NH_3 with the sugar cane silage increased with increasing levels of glycerin, with values of 0.06 mL g^{-1} DM at the 0% level to 2.56 mL g^{-1} DM at the 30% level. The values for corn silage decreased with increasing levels of glycerin, from 6.53 mL g^{-1} DM at the 0 % level to 2.63 mL g^{-1} DM at the 30% level.

The results for the concentration of acetic acid *in vitro* showed that, for sugar cane silage ($p = 0.0206$) and corn silages ($p = 0.0001$) there was a linear decreased relationship, and Brachiaria grass a linear increased relationship ($p = 0.0013$). Analyses of each level showed significant differences within different fodders. At level 0% (control) the highest production occurred with corn silage (37.72 μmol mL^{-1}), while the lowest production was the Brachiaria grass (10.26 μmol mL^{-1}) (Table 3).

Analyzing the substitution levels of glycerin for fodder individually, it was observed that at the level at 0% corn silage and 70% Brachiaria grass replacement presented the highest concentration ($p < 0.05$) of acetic acid (37.72 μmol mL^{-1}), Brachiaria showed the lowest (10.26 μmol mL^{-1}) volume ($p < 0.05$). The Elephant grass showed no significant differences ($p = 0.8073$) between the levels of substitution studied.

The results for the concentration of propionic acid in vitro showed that in almost all forages analyzed ($p < 0.05$), there was a linear relationship, except for Brachiaria, where there was no difference ($p = 0.8279$) between replacement levels. Analyzing each level, there were differences ($p < 0.05$) between different forages. The 0% level (control) differed between Elephant grass and Brachiaria, with values of 11.76 μmol mL^{-1} and 35.37 μmol mL^{-1}, respectively (Table 3).

Table 3. Mean values, probability (*P*-value) and regression equations illustrating the effects on production of volatile fatty acids (VFAs) when different levels of glycerin.

Forages	Levels of Glycerin				Regression Equations	R^2	*P*-value
	0%	30%	50%	70%			
Concentration of Acetate (μmol mL^{-1})							
Elephant grass	26.51^b	26.60^b	28.60^a	24.69^b	$\hat{Y}$ = 27.084+0.012x	0.01	0.8073
Sugar cane silage	24.89^b	22.26^b	23.00^b	21.37^b	$\hat{Y}$ = 24.543+0.044x	0.43	0.0206
Corn silage	37.72^a	30.27^a	25.76^b	22.11^b	$\hat{Y}$ = 37.399+0.224x	0.90	< 0.0001
Brachiaria grass	10.26^c	12.60^c	15.10^c	19.93^a	$\hat{Y}$ = 6.595+3.151x	0.96	0.0013
Concentration of Propionate (μmol mL^{-1})							
Elephant grass	11.76^d	22.13^b	27.79^a	18.56^b	$\hat{Y}$ = 11.255-0.675x-0.007x^2	0.48	0.0474
Sugar cane silage	22.27^b	25.62^a	25.14^b	27.77^a	$\hat{Y}$ = 22.546-0.070x	0.45	0.0175
Corn silage	19.63^c	24.22^a	33.17^a	36.42^a	$\hat{Y}$ = 18.825-0.254x	0.79	0.0001
Brachiaria grass	35.37^a	25.44^a	23.34^c	39.24^a	$\hat{Y}$ = 30.149-0.018x	0.01	0.8279
Concentration of Butyrate (μmol mL^{-1})							
Elephant grass	3.343^a	5.42^a	5.80^a	4.16^a	$\hat{Y}$ = 3.298-0.125x-0.001x^2	0.52	0.0198
Sugar cane silage	10.07^b	11.61^b	11.27^c	10.95^c	$\hat{Y}$ = 10.535-0.011x	0.05	0.4869
Corn silage	12.41^a	9.95^b	12.72^b	13.52^b	$\hat{Y}$ = 11.335-0.021x	0.11	0.2914
Brachiaria grass	16.61^c	22.24^c	28.10^d	32.83^d	$\hat{Y}$ = 16.139-0.234x	0.94	< 0.0001

*Means in the same column with different letters differ statistically by the Tukey test at 5% probability.

The average concentration of propionic acid in sugar cane silage, elephant grass and corn silage increased as the glycerin concentration increased. Specifically, as glycerin increased from 0 to 30%, propionic acid increased from 22.27 to 25.62 μmol mL^{-1}, 11.76 to 22.13 μmol mL^{-1} and 19.63 to 24.22 μmol mL^{-1} in sugar cane silage, elephant grass and corn silage, respectively.

The results for the in vitro production of butyric acid showed a linear relationship to Brachiaria grass (p = 0.0001) and quadratic relationship to elephant grass (p = 0.0198) was observed. There was effect of the substitution levels to sugar cane silage (p = 0.4869) and corn silage (p = 0.2914). The 0% level (control) differed between Brachiaria grass and Elephant grass, with values of 16.61 μmol mL^{-1} and 3.34 μmol mL^{-1}, respectively.

At the levels of 30, 50 and 70% replacement, the Elephant grass produced the lowest final volumes of butyric acid (5.42, 5.80 and 4.16 μmol mL^{-1}, respectively), while the Brachiaria grass showed the highest volumes (22.24, 28.10 and 32.83 μmol mL^{-1}, respectively).

The sugar cane silage and corn silage showed an oscillation between the levels of 0% (control) and 30% intake of the substrate, ranging in maize silage from 12.41 to 9.95 μmol mL^{-1}, respectively, and in sugar cane silage from 10.07 to 11.61 μmol mL^{-1}, respectively.

Discussion

Ruminants have evolved a pre-gastric fermentation structure with four spacious compartments and have a symbiotic relationship with microbes that can break down complex structural polysaccharides (cellulose and hemicellulose) into compounds that can be absorbed by the animal. This relationship allows the use of different fodder as food sources. However, this fermentation process, which occurs mainly in the rumen-reticulum portion of the gastrointestinal tract of ruminants, results in the production of gasses, which reduce the efficiency of this process and harm the environment. According to these results, the amount of methane gas produced in this experiment similar greatly between the different types of fodder, and the addition of glycerin to elephant grass, sugar cane silage and corn silage decreased its production (Krehbiel, 2008).

This decrease was enhanced by glycerol, which had a greater effect on methane production, keeping hydrogen concentrations low, thereby facilitating the growth of other bacterial species and a more efficient fermentation process (Hristov et al., 2013).

Ruminant livestock can produce 250 to 500 L of methane per day. This level of production results in estimates of the contribution by cattle to global warming that may occur in the next 50 to 100 yr to be a little less than 2%. Many factors influence methane emissions from cattle and include the following: level of feed intake, type of carbohydrate in the diet, feed processing, addition of lipids or ionophores to the diet, and alterations in the ruminal microflora. Manipulation of these factors can reduce methane emissions from cattle (Hammond et al., 2015). All forages caused a decrease in the levels of methane as the levels of added glycerin increased. Morgado et al. (2013) stated that the potential to produce CH_4 and CO_2 was related to the chemical composition of the feeds; rapidly fermentable ingredients tended to produce minor amounts of CH_4 g^{-1} dry mass in comparison with fermented ingredients with higher fiber.

The proportion of gas is dependent on the ecology of the rumen and the fermentation balance. Typically, the amount of CO_2 produced in the reticulum rumen is approximately two to three

times the amount of methane, as demonstrated by previous research. In this study, glycerin in the forages elephant grass, sugar cane silage and corn silage had a large impact on the production of carbon dioxide.

The production of methane and carbon dioxide are directly related because methane production is mainly modulated by the presence of free carbon and hydrogen dioxide in the rumen; free hydrogen from the reduction of carbon dioxide of methanogenic microorganisms aids in forming methane (Cieslak et al., 2013). In this study, combining glycerin with forages decreased the percentage of methane and carbon dioxide produced slightly, which was a positive result.

The ammonia nitrogen production differed for all forages and determines the concentration of ammonia allows evaluation of the balance of power with the protein diet because high ammonia concentrations are related to excess rumen-degraded protein and/or low concentrations of carbohydrates in the rumen-degraded protein (Ítavo et al., 2002).

The glycerin, which is a carbohydrate, enhanced different fodder characteristics. For example, the relatively high level of ammonia in elephant grass was reduced with the introduction of glycerin. Sugar cane silage had only 0.06 mL g^{-1} ammonia, and with the addition of glycerin, this concentration increased to 2.56 mL g^{-1}.

Corn silage and Brachiaria grass had similar concentrations of ammonia nitrogen. The addition of glycerin produced different results; for example, the concentration of ammonia nitrogen in the corn silage was reduced considerably with increasing levels of glycerin. The reduction in the concentration of ammonia nitrogen with increasing levels of glycerin can be explained by the increase in ruminal availability of energy, allowing the greater use of ammonia for microbial growth (McMurphy et al., 2014).

Five mg of N 100 mL^{-1} would be the ideal minimum and 19-23 mg of N 100 mL^{-1} would be the ideal maximum in rumen fluid during fermentation activity. Based on these percentages, sugar cane silage was the only fodder that, even with the introduction of glycerin, did not reach the minimum level for maximum occurrence of microbial fermentation in the rumen. The ammonia content in corn silage with added glycerin decreased to a percentage lower than the ideal minimum, reducing forage quality. In the elephant and brachiaria grasses, the introduction of glycerin had a positive effect on the production of ammonia, which was within the optimal levels for fermentation quality (Ítavo et al., 2002).

The main pathway for methanogenesis requires CO_2 and H_2, and methanogenesis occurs at higher production rate of H_2. During this fermentation, the production of acetic and butyric acids increases (Williams et al., 2014). In general, acetate production values decreased with the addition of glycerol. The reduction of acetate in the study of glycerin with feedstuffs is a result of the chemical composition of glycerin (Table 1), which does not include fibrous compounds used by cellulolytic bacteria for effective production of volatile fatty acids and, especially, acetic acid, which is directly related to the production of acetate, as the formation of pyruvate is degraded to CO_2 (Manatbay et al., 2014).

The addition of glycerin resulted in an increase in the levels of propionate and butyric because when glycerol undergoes fermentation, the main product is propionic acid, a precursor of fatty acids and glucose metabolites in ruminants. These increased amounts of propionic and butyric acid may occur when a diet is rich in cereals, which can lead to an increase in blood flow in the ruminal epithelium. In turn, this increase stimulates the growth of buds. When the diet of the ruminants is changed significantly, this transition must be made gradually to allow the rumen papillae to increase in size as well to adapt to a microbial concentrate; an adjustment period of between three and four weeks is required for efficient growth of the papillae to reduce the risk of ruminal acidosis (Romero-Huelva & Molina-Alcaide, 2013).

However, the ideal level of glycerin to add to the feed of ruminants would depend on several factors, including tolerance and adaptation of the animal and handling of the glycerol (due to its high density and viscous nature). An in vivo assessment would lead to better interpretation of the integrated data in this study.

Conclusion

Substitution of forage species for glycerin by tests for semi-automatic technique *in vitro* reduce the production of greenhouse gases (CH_4 and CO_2) which reduction in energy loss, and increase volatile fatty acids propionic and butyric. To confirm these findings in the *in vitro* phase, studies must be conducted *in vivo*.

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