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Effect of crude glycerol on *in-vitro* ruminal fermentation kinetics

Efeito do glicerol sobre a cinética da fermentação in vitro

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SUMMARY

The interest in using crude glycerol in animal feeding has reemerged due to its increasing availability and favorable price resulting from the expansion of biofuel industry. The objective of the present study was to evaluate the effects of substituting corn for crude glycerol at different levels in the diet on ruminal fermentation using *in-vitro* true digestibility parameters. The experimental treatments consisted of substituting corn for liquid crude glycerol (0; 4; 8 and 12%) in dry matter basis. Diets consisted of 60% alfalfa hay and 40% corn and glycerol substituted the corn in the diet. In addition to the 48 hours traditionally applied in digestibility assays, different *in-vitro* digestibility times were used (0; 4; 8; 16; 48, 72 and 96 hours) in order to study digestion kinetics. The dietary corn substitution for increasing crude glycerol levels did not affect ammonia nitrogen content, metabolizable energy content, *in-vitro* digestibility of organic matter and neutral detergent fiber, nor ruminal degradation parameters. However this by-product of biodiesel production may be tested *in-vivo* as an alternative energy feedstuff in ruminant diets.

Keywords: co-product of biodiesel, degradation parameters, effective degradability, *in-vitro* neutral detergent fiber digestibility, ruminant

RESUMO

O interesse na utilização da glicerina bruta na alimentação animal ressurge, devido ao aumento na disponibilidade e preço favorável, como conseqüência da expansão das indústrias de biocombustíveis. O objetivo deste trabalho foi avaliar os efeitos da substituição do milho por diferentes níveis de glicerina bruta na dieta sobre a fermentação ruminal através da digestibilidade *in vitro* verdadeira. As dietas experimentais consistiram na substituição do milho por glicerol (0; 4; 8 e 12%) na matéria seca da dieta (MS). As dietas eram compostas por 60% de feno de alfafa e 40% de grão de milho e o glicerol foi adicionado, substituindo o milho nas dietas. Além de trabalhar com as 48 horas tradicionais, foram utilizados diferentes horários de digestibilidade *in vitro* (0; 4; 8; 16; 48; 72 e 96 horas), com a finalidade de estudar a cinética da digestão. A substituição do milho por níveis crescente de glicerina bruta na dieta não afetou a concentração de N-NH_3, o teor de energia metabolizável, as digestibilidades *in vitro* da matéria orgânica da fibra em detergente neutro, bem como os parâmetros da degradação ruminal. Portanto, esse subproduto da produção do biodiesel deve ser testado *in vivo* como uma alternativa energética na formulação de dietas para ruminantes.

Palavras-chave: co-produto do biodiesel, degradabilidade efetiva, digestibilidade *in vitro* da fibra em detergente neutro, parâmetros de degradação, ruminantes

INTRODUCTION

As ruminant production systems have become increasingly intensified, economic assessment related to feeding became critical, as feed accounts for 30 to 70% of total production costs depending on activity and type of operation (RESTLE et al., 2007; RODRIGUES & RONDINA, 2013) reducing the profit margin for producers (GOES et al., 2008). Energy is the most expensive component of ruminant diets and its price has been influenced due to use of corn, soybean, and other grains for ethanol and biodiesel production. Also, oil price has risen due to growth of global population and income in countries whose economies grew at a faster rate. In this context, there has been an increasing number on the use of renewable energy sources due to rise in oil prices caused by the possible exhaustion of fossil energy reserves couples with concerns on global climate changes.

Among the renewable energy sources, biodiesel production has received much attention. Brazil has a great potential for the production of biofuels. In addition to planting several oil seeds that can be used for biodiesel production, it has cutting-edge technology and industrial capacity to develop it (OLIVEIRA et al., 2008).

Crude glycerol is the main by-product generated from biodiesel production: approximately 100 milliliters of crude glycerol are produced from each liter of biodiesel (THOMPSON & HE, 2006). There are several industrial applications of purified crude glycerol, such as in cosmetics, pharmaceuticals and food industries. However, the degree of purity required for these applications demands complex and expensive processes, as crude glycerol contains impurities, such as water, oil, catalyzer, reagent residues, ethanol or methanol, propanediol and minerals (SOARES et al., 2010).

On the other hand, crude glycerol could be used as an alternative energy source for ruminant feeding, due to its increasing availability and favorable price as a result of the expansion of the biofuel industry, as well as increase in grain prices. Nevertheless, adequate inclusion levels, the impact and level of contaminants and the nutritional value of crude glycerol need to be determined in order to prevent intoxication or reduction of the efficiency of utilization of other dietary components.

The objective of this study was to evaluate *in vitro* digestibility and ruminal fermentation kinetics of substituting corn for different levels of crude glycerol in diets consisting of alfalfa hay and ground corn.

MATERIALS AND METHODS

The experiment was carried in the ruminant sector of Laboratory of Animal Science and the chemical analyses were performed at the Animal Nutrition Laboratory, both belonging to the Department of Animal Science of the School of Agronomy of the Federal University of Rio Grande do Sul.

Two Texel sheep, with 40kg average body weight ruminally cannulated, were used as inoculum donor. The animals were kept in a 120m^2 paddock with a shelter during the entire experiment. Alfalfa hay (88.51% dry matter, 88.32% organic matter, 19.20% crude protein, 54.45% neutral detergent fiber, and 30.64% acid detergent fiber) was fed at 3% body weight twice daily (8:00 AM and 5:00 PM). Mineral salt (13.2% calcium, 8.0% phosphorus, 1.8% sulphur, 14.7% sodium, 0.13% manganese; 0.27%

zinc; 0.0044% cobalt; 0.0088% iodine; 0.0018% selenium and 0.0800% fluorine) and water were supplied *ad libitum*. Before the experiment started, the animals were subjected to a 10 day adaptation period to the above described diet. The experimental protocol followed the guidelines of the Ethics Committee on the Use of Animals in Research as Number 18.442 in compliance with Law 11.794.

The experimental treatments consisted of substituting corn for liquid crude glycerol (0, 4, 8 and 12%) in dry matter basis. Alfalfa hay *(Medicago sativa)* was used as roughage and comprised 60% of the diet. Table 1 shows the nutritional composition of the ingredients of the experimental diets.

Table 1. Dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), crude energy (CE) and glycerol contents of the ingredients in the experimental diets

Ingredients	DM	OM	CP	NDF	ADF	CE	Glycerol
	(%)	% DM				(MJ/kg)	(%)
Alfalfa hay	88.51	88.32	19.20	54.45	30.64	18.67	-
Ground corn	88.89	99.11	8.53	16.06	3.46	18.77	-
Crude glycerol*	81.44	-	0.11	-	-	14.53	80.00

*Methanol content lower than 45 mg/L.

In-vitro true digestibility was determined according to Goering & Van Soest (1970); however, in addition to the 48 hours traditionally used, digestibility was also determined at different times (0; 4; 8; 16; 48; 72 and 96 hours) aiming at studying the digestion kinetics of the different treatments.

One day before the incubation, in each 120 ml fermentation flask, 0.5 grams of a sample consisting of the experimental treatments was placed, and the flasks were then kept in an oven at 39ºC. On incubation day, two hours after the animals received the morning meal, ruminal fluid was collected and kept in a thermos bottle at 39ºC, filtered through four gauze layers, and kept in a water bath to maintain temperature close to 39ºC. The ruminal fluid was mixed with artificial saliva (McDOUGALL, 1948), which was kept in water bath at 39ºC and saturated with CO_2, at a ratio of 1 part of ruminal fluid to 4 parts of artificial saliva. The mixture was homogenized in water bath and saturated with CO_2.

Subsequently, 50 ml of the mixture containing the culture medium and the ruminal fluid were added to each of fermentation flasks containing the different treatments, saturated with CO_2 for about 30 seconds, rapidly closed with a rubber stopper with Bunsen valve and place in the incubator. The incubator was opened to agitate the flasks, three times per day, at 08:00, 12:00 and 17:00 hours. This procedure was carried out as quickly as possible to avoid drop of temperature.

Eight flasks (4 treatments and 2 replicate) were removed from the incubator at 0; 4; 8; 16; 48; 72 and 96 hours of incubation and were placed in iced water to interrupt microorganism activity. Flasks were then immediately centrifuged at 10.000rpm for 10 min, the supernatant was removed, and 100ml of neutral detergent solution

were then added, following the method of Van Soest & Robertson (1985). Flasks were sealed with aluminum foil and placed in a forced-circulation oven at 90ºC for 16 hours, according to the technique proposed by Chai & Udén (1998). The flask content was then filtered in sinterized glass crucible with coarse pore diameter. The crucibles with the residue were placed in an oven at 105ºC for 12 hours, and weighed in order to obtain moisture-free residue weight, and later placed in the oven at 450ºC for 5 hours.

The *in-vitro* organic matter true digestibility (IVOMTD) was calculated as the difference between the incubated organic matter (OM) and the non-digested OM, considered as the residue remaining in the crucibles. *In-vitro* neutral detergent fiber digestibility (IVNDFD) was calculated as the difference between incubated NDF and non-digested NDF, considered as the residue remaining in the crucibles after filtration and drying in the oven at 105ºC.

After the flasks were removed from the incubator and centrifuged, and before the neutral detergent solution was added, 20 ml samples of the supernatant were removed for analysis of ammonia nitrogen (NH_3-N). The concentration of NH_3-N was determined by distillation with magnesium oxide (PRATES, 2007).

In order to study the kinetics of ruminal degradability, IVOMTD results obtained at the different times were subjected to the model of McDonald (1981), as follows: $Y_t = a + b(1-e^{-c(t-to)})$, where Y_t = degradation after t hours; a = substrate solubilized immediately; b = insoluble, but potentially degradable material; a+b = potential degradability; c = degradation rate of b; to = lag time. The same model was used to obtain NDF degradation parameters, but the "a" parameter was excluded due to the absence of NDF solubilized immediately. Effective degradability (ED) was calculated using the equation proposed by McDonald (1981): $ED = a + [(b \times c)/(c + k)]e^{-(c+k)t}$, where a, b, and c follow the previous definitions, and k = feed passage rate of 2 or 5%/h. The same model was used to calculate ED of NDF but the parameter "a" was excluded from the model.

The experiment was replicated in three runs with two duplicates within runs (56 treatment flasks corresponding to seven different times, 4 level of glycerol substitution and 2 duplicates plus 4 blanks flasks in each run).

Samples of the incubated feedstuffs (alfalfa hay and ground corn) were ground and analyzed for dry matter, organic matter and crude protein (PRATES, 2007). Neutral detergent fiber and acid detergent fiber were determined using a fiber analyzer (ANKOM's Fiber Analyzer Ankom®) as described by Prates (2007). The enzyme alpha-amylase was used to determine NDF. Crude energy expressed in MJ/kg dry matter, were determined in duplicate using an isoperibol calorimeter bomb (IKA® calorimeter C 2000). The analyses aforementioned were performed in triplicate. The nutritional composition of crude glycerol and its contamination with methanol were evaluated by a specialized laboratory (CBO Análises Laboratoriais, Campinas, SP).

A metabolizable energy (ME), expressed in MJ/kg dry matter, was estimated considering the organic matter degradability (OMDeg) values obtained by the above mentioned technique and applied to the equation proposed by Menke & Steingass (1988), where ME = 1.15 + (0.16*OMDeg).

The effect of the dietary inclusion of increasing crude glycerol levels on *in-*

vitro digestibility with 48 hours of incubation, degradation rate, effective degradability at passage rate of 2 or 5%/h, lag time and average N-NH_3, at all times, were analyzed as a completely randomized design using the PROC MIXED (STATISTICAL ANALYSIS SYSTEM, 2012) according with the following mathematical model: $yij = \mu + \alpha i + \beta j + (\alpha\beta)ij + \varepsilon ij$, where yij is the observation at run j given treatment i; μ is the overall mean; αi is the fixed effect of treatment i (0, 4, 8 and 12 % glycerol); βj is the aleatory effect of run j (1, 2, 3) and $(\alpha\beta)ij$ is the interaction of treatment i by run j. Means were compared by the PROC MIXED (STATISTICAL ANALYSIS SYSTEM, 2012) considering linear or quadratic effect of the glycerol level inclusion. Statistical significance was declared at $P \leq 0.05$.

RESULTS AND DISCUSSION

Substituting corn for crude glycerol had no effect on *in-vitro* organic matter true digestibility (IVOMTD) or on *in-vitro* neutral detergent fiber digestibility (IVNDFD) after 48 hours of incubation, with average values of 81.89 % and 60.04 %, respectively (Tables 2 and 3). These results are consistent with the previous research which shows that feeding levels of glycerol up to 20% of the total ration does not have any effect on nutrient digestibility or animal performance (DONKIN 2008; KRUEGER et al., 2010). On the other hand, Wang et al. (2009) and Paggi et al. (2004) observed that glycerol decreased the *in-vitro* organic matter digestibility and *in-vitro* neutral detergent fiber digestibility in diets.

These results suggest that glycerol can modulate the digestion in a dose-dependent manner. In this study, one can concluded that the IVOMTD and IVNDFD were not affected by crude glycerol due to its low level in the diet, allowing optimum rumen fermentation, as growth, adhesion and cellulolytic activity were inhibited when glycerol was included in cultures at high concentration but not at low concentration (ROGER et al., 1992; PAGGI et al. 2004).

Table 2. Effect of crude glycerol inclusion level on the mean values of *in-vitro* organic matter true digestibility (IVOMTD, % - with 48 hours of incubation), organic matter degradation parameters (a, b, c, and lag time) and effective degradability of organic matter at passage rates of 2 and 5 %/h

Variables	Crude glycerol inclusion levels (%)				SEM	P value	
	0	4	8	12		Linear	Quadratic
IVOMTD, %	81.99	81.72	81.88	81.95	0.18	0.8519	0.8793
a, %	66.05	64.89	65.49	65.20	0.24	0.2463	0.3174
b, %	16.54	17.44	16.67	17.09	0.33	0.2927	0.3141
c, %/h	7.14	6.88	6.87	7.14	0.06	0.9189	0.9718
Lag time, h	4.70	3.96	4.33	4.86	0.86	0.7701	0.8328
ED 2%/h	77.70	77.30	77.23	77.23	0.11	0.8037	0.8975
ED 5%/h	73.66	73.13	73.16	73.03	0.09	0.7138	0.7989

Table 3. Effect of crude glycerol inclusion level on the mean values of *in-vitro* neutral detergent fiber digestibility (IVNDFD, % - with 48 hours of incubation), and neutral detergent fiber degradation parameters (b, c, and lag time) and effective degradability of neutral detergent fiber at passage rates of 2 and 5%/h

Variables	Crude glycerol inclusion levels (%)				SEM	P value	
	0	4	8	12		Linear	Quadratic
IVNDFD, %	59.77	59.78	59.89	60.70	0.45	0.9786	0.9621
b, %	39.32	39.87	38.26	40.40	0.73	0.5096	0.4068
c, %/h	8.88	5.64	7.73	6.95	0.00	0.0861	0.1211
Lag-time, h	5.93	3.83	5.73	4.60	0.86	0.1798	0.1924
ED 2%/h	50.10	49.03	49.40	50.26	0.26	0.5107	0.6716
ED 5%/h	40.23	39.23	39.73	40.30	0.27	0.5843	0.6823

Mean degradation rates of OM and NDF insoluble but potentially degradable fractions (c), expressed in %/h, for each crude glycerol level were 7.01%/h for OM and 7.30%/h for NDF, and were not influenced by the dietary crude glycerol levels.

According to Caton & Dhuyvetter (1997), effects of energy supplementation on substrate degradation rate are rarely observed, as detected in the present study. However, Wang et al. (2009), evaluating the *in-situ* digestion kinetics of corn stover, obtained a quadratic effect on the degradation rate of insoluble, but potentially degradable, fractions of DM and NDF with increasing crude glycerol supplementation levels in the diet of steers. These results are different from those obtained in the present study, where the substitution of up to 12% corn for crude glycerol in dry matter basis had no influence on the degradation of that fraction, possibly due to the better nutritional value of the substrate used and the lower level of crude glycerol in the diet, in agreement with the results observed by Hess et al. (2009).

No effects of the increasing substituting levels of corn for crude glycerol was observed on mean OM and NDF lag time, expressed in hours, with values of 4.46h for OM and 5.02h for NDF (Tables 2 and 3). These results are consistent with Krueger et al. (2010) that evaluated the *in-vitro* incubation of alfalfa hay and demonstrated that increasing dietary crude glycerol levels did not affect substrate colonization rate (2.40h). However, this rate was lower than that found in the present study (5.02h), possibly due to the presence of corn, which may have increased the colonization time of the fiber fraction, and due to the lower level of crude glycerol in the diet, because according with Hess et al. (2009), the NDF lag time tended to decrease linearly (P = 0.07) as crude glycerol increased to 30% of the in vitro substrate.

Effective degradability (ED) is a measure that integrates ruminal degradation parameters, such as losses due to washing in time zero (a), insoluble but potentially degradable material (b) and its degradation rate (c), with the passage rate of the solid fraction through the gastrointestinal tract. The results obtained for passage rates of 2%/h and 5%/h were not influenced by substituting corn for crude glycerol levels. Mean values of ED at 2%/h were 77.36% for OM and 49.70% for NDF, whereas the results relative to 5%/h were 73.25% for OM and 39.87% for NDF (Tables 2 and 3).

In the present study, we worked with effective degradability (ED) values with solid fraction passage rates of 2 and 5%/h, because, whereas a low-quality forages present passage rates of approximately 2%/h, most concentrates mixed with forages have passage rates of about 5%/h. However, passage rate is closely related to intake, and therefore, it would be more correct to say that the results with passage rates corresponding to low and high intake were analyzed (SHAVER et al., 1986). Passage rates were not influenced with crude glycerol substitution up to 12%. However, Wang et al. (2009), evaluating the *in-situ* digestion kinetics of corn stover, observed a quadratic effect on ED with a fraction solid passage rate of 2%/h for DM and for NDF with increasing crude glycerol supplementation levels in the diet of steers. This was possibly due to the lower nutritional value of corn stover as compared to alfalfa hay, which was used as substrate in the present experiment.

Mean NH_3-N results, in mg/dl, and ME, in MJ/kg DM, are presented in Table 4 for each crude glycerol dietary level. Neither NH_3-N nor ME were influenced by corn substitution for crude glycerol, with mean values of 15.69mg/dl and 11.95MJ/kg DM, respectively.

Table 4. Effect of crude glycerol inclusion level on the mean ammonia nitrogen values (NH_3-N, mg/dL) and metabolizable energy content (ME, MJ/kg DM)

Variables	Crude glycerol inclusion levels (%)				SEM	P value	
	0	4	8	12		Linear	Quadratic
NH_3-N, mg/dL	16.63	15.69	14.82	15.64	0.33	0.9385	0.8818
ME, MJ/kg DM	11.97	11.93	11.95	11.96	0.02	0.8677	0.8970

In *in-vitro* media, NH_3-N concentration works as an indicator of protein degradability, because there is no nitrogen absorption or recycling as in the rumen media *in-vivo* (DETMANN et al., 2011). Because most cellulolytic bacteria require ammonia for growth, low NH_3-N concentrations may limit microbial activity and thereby, reduce the rate and degree of cell wall digestion. The mean NH_3-N results obtained in the present study of 15.69mg/dl were within the optimal ruminal NH_3-N range (12 to 17mg/dl, MAPATO et al., 2010; LUNSIN et al., 2012) for rumen ecology, fermentation and optimal microbial growth (ANANTASOOK & WANAPAT, 2012). These results are consistent with the previous work that shows that feeding glycerol substituting corn or barley grain in the diet does not have any effect on NH_3-N concentration (ABO EL-NOR et al.; 2010; AVILA et al., 2011).

The energy value of glycerol is approximately equal to the energy contained in corn starch. However, the energy value of glycerol is variable due to the difference between the levels studied, unknown interactions with other dietary components and the proportion of corn and starch in the diet (DONKIN, 2008). Mach et al. (2009) estimated for Holstein young bulls, a metabolizable energy content of crude glycerol (86% glycerol) of 14.52 MJ/kg DM, higher than the value observed in this study. But the lack of differences in ME in the present study suggests that

corn can be substituted for glycerol without adjustments for the energy content in the diet.
The dietary corn substitution for increasing crude glycerol levels did not affect ammonia nitrogen content, metabolizable energy content, *in-vitro* digestibility of organic matter and neutral detergent fiber, nor ruminal degradation parameters. However this by-product of biodiesel production may be tested *in-vivo* as an alternative energy feedstuff in ruminant diets.

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