Short Communication

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Partial replacement of corn with glycerin: digestibility and ruminal fermentation kinetics by *in vitro* gas production¤

Sustitución parcial de maíz con glicerina: digestibilidad y cinética de la fermentación ruminal mediante producción de gas in vitro

Substituição parcial do milho pela glicerina: digestibilidade e cinética da fermentação ruminal através da produção de gases in vitro

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Summary

Background: glycerin, a co-product of biodiesel production, could be included in animal feeds. **Objective:** to evaluate the effects of partial replacement of corn with glycerin on digestibility and ruminal fermentation kinetics, estimated by the *in vitro* gas production technique. **Methods:** dietary treatments consisted of corn substitution with crude glycerin (0, 4, 8, and 12% on a dry matter basis). *In vitro* digestibility of neutral detergent fiber and organic matter were calculated as the difference between the amount of incubated and undigested substrate. Cumulative gas pressure was measured *in vitro* using automatic equipment. Gas production kinetics was analyzed using a dual-pool logistic model. **Results:** increasing levels of crude glycerin to replace corn did not affect *in vitro* digestibility of neutral detergent fiber, organic matter, ammonia nitrogen content, or degradation rates. A negative linear effect on the partitioning factor and a linear increase in the rapidly degradable fraction were observed with the inclusion of crude glycerin. **Conclusions:** dietary inclusion of up to 12% crude glycerin (dry matter basis) replacing corn did not affect diet digestibility. A greater volume of gas was observed with the highest inclusion level of glycerin, indicating that alfalfa hay, corn and crude glycerin combination could affect fermentation, suggesting the occurrence of associative effects.

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Keywords*: bicompartimental logistic model, biodiesel by-product, energy, neutral detergent fiber, partitioning factor.*

Resumen

Antecedentes: una alternativa al uso de la glicerina, generada como residuo de la producción de biodiesel, es su utilización en la alimentación animal. **Objetivo:** evaluar los efectos de la sustitución parcial de maíz con glicerina sobre la digestibilidad de la dieta y la cinética de la fermentación ruminal, usando la técnica de producción de gas *in vitro*. **Métodos:** los tratamientos consistieron en la substitución de maíz por glicerina cruda (0, 4, 8 y 12% en base seca). La digestibilidad *in vitro* de la fibra detergente neutra y la materia orgánica fue calculada por la diferencia entre la cantidad del sustrato incubado y el no digerido. La presión acumulativa de gas *in vitro* fue medida por un equipo automático. La cinética de la producción de gas fue analizada empleando un modelo logístico bicompartimental. **Resultados:** la inclusión de niveles crecientes de glicerina en substitución del maíz no afectó la digestibilidad *in vitro* de la fibra detergente neutra, la materia orgánica, la tasa de nitrógeno amoniacal o las tasas de degradación. Se observó un efecto lineal negativo en el factor de partición y un aumento lineal en la fracción de rápida degradación por la inclusión de glicerina. **Conclusión:** la inclusión dietaria de hasta 12% de glicerina (base seca) para reemplazar al maíz no afectó la digestibilidad de la misma. Se observó una mayor produccion de gas con el mayor nivel de inclusión de glicerina, lo que indica que la combinación de heno de alfalfa, maíz y glicerina podría alterar la fermentación, lo que sugiere la existencia de efectos asociativos.

Palabras clave: *co-producto del biodiesel, energía, factor de partición, fibra detergente neutra, modelo logístico bicompartimental.*

Resumo

Antecedentes: um uso alternativo da glicerina gerado como um coproduto da produção de biodiesel pode ser a sua inclusão na alimentação animal. **Objetivo:** avaliar o efeito da substituição parcial do milho pela glicerina sobre a digestibilidade da dieta e a cinética de fermentação através da técnica *in vitro* de produção de gás. **Métodos:** os tratamentos consistiram na substituição do milho por glicerina bruta (0, 4, 8 e 12%) com base na matéria seca. A digestibilidade da fibra em detergente neutro e da matéria orgânica foi calculada como a diferença entre a quantidade de substrato incubado e o não digerido. A pressão acumulativa de gás foi mensurada *in vitro* utilizando um equipamento automático de medição de gás. A cinéticas da produção de gás foi analisada utilizando o modelo logístico bicompartimental. **Resultados:** o aumento dos níveis de inclusão da glicerina bruta para substituir o milho na dieta não afetou a digestibilidade *in vitro* da fibra em detergente neutro, a digestibilidade *in vitro* da matéria orgânica, teor de nitrogênio amoniacal e a taxa de degradação. Foram observados efeito linear negativo no fator de partição e aumento linear na fração rapidmente degradável com a inclusão da glicerina bruta na dieta. **Conclusão:** a inclusão dietética de até 12% da glicerina bruta (na matéria seca) para substituir o milho não afetou a digestibilidade da dieta. O maior volume de gás produzido foi observado para o maior nível de inclusão de glicerina indicando que a combinação de feno de alfafa, milho e glicerina bruta poderia alterar a fermentação, sugerindo a ocorrência de efeitos associativos.

Palavras chave: *coproduto do biodiesel, energia, fator de partição, fibra em detergente neutro, modelo logístico bicompartimental.*

Introduction

Biodiesel production has increased worldwide, as well as the volume of co-products generated, particularly crude glycerin. Brazilian production of biodiesel was approximately 3.4 billion L in 2014, which generated about 311 million L of crude glycerin (ANP, 2014). There is no feasible alternative to absorb this volume of crude glycerin in the existing Brazilian National Policy for the Production and Use of Biodiesel.

Utilizing glycerin as a feed ingredient in ruminant diets could potentially inhibit fat degradation by bacteria and may promote ruminal passage of total lipid content, thereby providing higher proportions of beneficial unsaturated fat for incorporation into beef products (Krueger *et al*., 2010). Glycerin retention within the rumen is directly associated with its absorption. It can be absorbed from the rumen in significant amounts (Krueger *et al.,* 2010; Omazic *et al*., 2015). Omazic *et al*. (2015) reported that glycerin

also disappeared via microbial digestion and outflow from the rumen through the omasal orifice. Absorbed glycerin is metabolized in the liver and requires glycerin kinase (Hagopian *et al*., 2008), an enzyme responsible for channeling glycerin into the triose phosphate step of glycolysis/gluconeogenesis.

Gross energy of glycerin ranges from 3 to 6 Mcal/Kg, depending upon its composition (Mendoza *et al*., 2010, Meurer *et al*., 2012; Françozo *et al*., 2013; Eiras *et al*., 2014). Since glycerin has high-energy content — similar to cornstarch (Yang *et al.,* 2012) — it could potentially be used for animal feed. Therefore, it is necessary to evaluate crude glycerin as an alternative energy feedstuff to replace corn grain in ruminant diets.

The objective of this study was to evaluate the effect of increasing levels of crude glycerin replacing corn in corn-alfalfa hay diets on digestibility, cumulative gas production and ruminal fermentation kinetics using *in vitro* techniques for true digestibility and cumulative gas production.

Materials and methods

Ethical considerations

Animal care procedures throughout the study followed protocols approved by the Ethics Committee for Animal Use (CEUA) at the University of Rio Grande do Sul, Brazil (number 18442/2010).

Experimental design

The dietary treatments consisted in the inclusion of 0, 4, 8, and 12% of crude glycerin replacing ground corn (dry matter basis; DM basis). The experimental diets were composed of 60% alfalfa hay and 40% concentrate (DM basis). One gram (dry matter) of composite sample was added to 250 mL fermentation bottles. Four replicates were used per treatment. Four blank fermentation bottles were also incubated (i.e. no substrate), totaling twenty bottles for the experiment. Bottles were kept in an oven at 39 ºC. One hundred mL of a mixture containing culture medium (Goering and Van Soest, 1970), also maintained at 39 ºC, and saturated with CO_2 were added to the bottles. The bottles were saturated with CO_2, closed and kept in the incubator until 25 mL of inoculum was added to each bottle. The bottles were then placed in an incubator at 39 ºC for 48 hours. The nutritional composition of ingredients and the chemical composition of experimental diets are described in Tables 1 and 2, respectively.

Samples of the incubated feedstuffs (alfalfa hay and ground corn) were analyzed for dry matter (DM; AOAC, 1990; method 934.1 and 930.15), organic

Table 1. Nutritional composition of ingredients.

Ingredients	DM (fresh material)	OM	CP	NDF % DM	ADF	ADL	Energy (MJ/Kg)	Glycerin (g/Kg)
Alfalfa hay	88.5	88.3	19.2	54.4	30.6	9.7	18.6	-
Ground corn	88.8	99.1	8.5	16.1	3.3	0.3	18.7	-
Crude glycerin*	81.4	-	0.1	-	-	-	14.5	80.0

*Methanol content lower than 45 mg/L. Dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF), gross energy (GE), acid detergent lignin (ADL) and glycerin contents.

Table 2. Chemical composition of the experimental diets.

Chemical fraction	Corn substitution with crude glycerin (% DM)			
	0	4	8	12
DM (fresh material)	88.5	88.3	88.2	88.4
OM (% DM)	92.4	92.2	92.0	91.7
CP (%DM)	16.5	16.3	16.2	16.0
NDF (% DM)	33.8	33.5	32.9	32.6
EE (%DM)	3.1	3.0	2.9	2.8
Energy (MJ/Kg)	18.6	18.5	18.3	18.0

Dry matter (DM), organic matter (OM), crude protein (CP), neutral detergent fiber (NDF), ether extract (EE), gross energy (GE) contents.

matter (OM, AOAC, 1975; method 7.010), and crude protein (CP, AOAC, 1990; method 988.05). Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined according to Van Soest *et al*. (1991). A specialized laboratory evaluated the nutritional composition of crude glycerin (B.E.T. Laboratories do Brasil S/C Ltda). All analyses were performed in triplicates.

Two Texel sheep (60 ± 1.2 Kg average body weight) fitted with a rumen fistula were used as inoculum donors. Two hours after the animals received the morning meal, ruminal material was collected (1 part of liquid to 1 part of solid). One hundred mL of buffer solution plus 25 mL of rumen fluid were incubated.

In vitro cumulative gas pressure was automatically measured every 10 minutes for 48 hours, using an automatic gas-measuring equipment (ANKOM® Gas Production System, Macedon, NY, USA), equipped with a pressure transducer attached to each bottle that transmits by radiofrequency the cumulative gas pressure values to a computer. The bottles were automatically vented every 10 minutes. Four runs were carried out with two replicates per treatment. Gas production values were corrected for the blanks. Gas production data were fitted to the dual-pool logistic model (Schofield *et al*., 1994). The model parameters (A, B, C, D, E, and F) were estimated by the iterative method of Marquardt using the NLIN procedure of SAS® (SAS Institute, Cary, NC, USA; 2002). The partitioning factor (PF) was determined according to Makkar (2004), where: PF = mg of TDOM (truly degraded organic matter)/mL gas produced.

After 48 hours of incubation, fermentation was stopped by putting the bottles in ice. The bottles were then centrifuged, the supernatant was removed, and 50 mL of neutral detergent solution (Goering and Van Soest, 1970) was added to the bottles, which were maintained at 90 ºC in an oven during 16 hours to extract the neutral detergent soluble fraction (Chai and Udén, 1998). The residue was filtered in sintered glass crucibles with coarse pore diameter (100 to 160 microns) and placed in an oven at 105 ºC for 12 hours, weighed, and burnt in a muffle at 450 ºC for 5 hours.

The *in vitro* organic matter true digestibility (IVOMTD) and *in vitro* neutral detergent fiber digestibility (IVNDFD) were calculated as the difference between the incubated OM or NDF and the non-digested OM or NDF residue remaining in the crucibles (Goering and Van Soest, 1970).

After removing from the incubator and centrifuging the bottles, and before adding the neutral detergent solution, 20 mL of the supernatant was removed and acidified for ammonia nitrogen (NH_3-N) analysis. NH_3-N concentration was determined by distillation with magnesium oxide (AOAC, 1990; method 990.03).

Statistical analysis

Maximum gas production from the rapidly and slowly degradable fractions of organic matter (Schofield *et al*., 1994), their respective degradation rates and lag time were estimated by the NLIN procedure. The effects of increasing crude glycerin inclusion levels in the diet on the estimated parameter, *in vitro* digestibility with 48 hours of incubation, ammonia nitrogen and partitioning factor were analyzed using the GLM procedure. Means were compared by the REG procedure. *In vitro* cumulative gas production was evaluated using the GLM procedure as repeated measures. All statistical procedures were performed using the SAS package (Statistical Analysis System, version 9.3, SAS Institute, Cary, NC, USA; 2002).

Results

As there was no significant effect ($p>0.05$) on the parameters evaluated, only the significant effects of glycerin levels ($p<0.05$) will be shown. Increasing crude glycerin levels to replace corn did not influence *in vitro* neutral detergent fiber digestibility (IVNDFD) nor *in vitro* true organic matter digestibility (IVTOMD) after 48 hours of incubation. Mean values for those parameters were 57.8 and 80.4%, respectively (Table 3).

Total cumulative gas production ranged between 82.1 (0% glycerin in the diet) and 123.1 (12% glycerin in the diet) mL/g of incubated OM (Figure 1). There was no effect of glycerin inclusion on cumulative

Table 3. Effects of four inclusion levels of crude glycerin on the mean values of several parameters.

Parameters	Crude glycerin inclusion levels (%)				Mean	P-values		
	0	4	8	12		Linear	Quadratic	Cubic
IVNDFD (%)	57.4	57.1	58.7	58.1	57.8	0.4133	0.8596	0.3879
IVTOMD (%)	80.2	80.3	80.8	80.5	80.4	0.5227	0.6568	0.5134
A (mL)[1]	46.9	71.1	95.2	111.5	81.2	0.0020[1]	0.7381	0.8828
B (%/h)	0.028	0.016	0.012	0.010	0.016	0.0574	0.4058	0.8095
C (h)	6.7	23.4	20.0	14.4	16.1	0.5591	0.1576	0.5980
D (mL)	37.5	38.4	24.9	14.9	28.9	0.3326[2]	0.4648	0.5980
E (%/h)	0.022	0.011	0.008	0.019	0.015	0.5955	0.0592	0.7848
F (h)[2]	40.1	54.9	76.3	84.7	64.0	0.0146[3]	0.0542	0.2867
PF (mg/mL)[3]	9.7	8.8	7.5	7.2	8.3	<0.0001[4]	0.2161	0.2314
NH3-N(mg/dL)	16.6	15.6	14.8	15.6	15.6	0.5379	0.5303	0.7977

[1]Ŷ = 48.53 + 5.44x; [2]Ŷ = 41.13 – 2.03x; [3]Ŷ = 40.75 + 3.87x; [4]Ŷ = 9.66 – 0.22x. *In vitro* neutral detergent fiber digestibility (IVNDFD, %), *in vitro* true organic matter digestibility (IVTOMD, %), maximum gas production of the rapidly (A, mL) and slowly (D, mL) degradable fractions of organic matter and their respective degradation rates (B and E, %/hours), lag times (C and F, hours), partitioning factor (PF - mg TDOM/mL gas, 24 hours of incubation), and ammonia nitrogen values (NH3-N, mg/dL).

gas production during the first hours of incubation. However, after 14 h of incubation, the inclusion of 12% glycerin yielded higher cumulative gas production (79.9 mL/g OM) than the treatment with no glycerin (54.8 mL/g OM; p = 0.0613), and this pattern was observed until 30 hours of incubation. After 30 hours of incubation, the 12 and 8% crude glycerin levels produced more gas (118.2 and 107.7 mL/g OM, respectively) compared with the no-glycerin treatment (79.3 mL/g OM; p<0.01), and this trend was maintained until the end of 48 hours of incubation. The standard deviations of gas production values at 16, 30, and 48 hours of incubation were 15.03, 18.83, and 19.65, respectively.

The partitioning factor, which integrates total cumulative gas production with substrate disappearance, is an indicator of fermentative efficiency. The crude glycerin levels used in the present study significantly decreased the PF (Table 3).

Maximum gas production of the rapidly (A) and slowly (D) degradable fractions of organic matter were affected by increasing levels of crude glycerin in replacement of corn (Table 3).

Mean degradation rates of the rapidly (B) and slowly (E) degradable fractions were 0.016 and 0.015 %/h, respectively, and were not affected by crude glycerin inclusion levels. The mean NH3-N result obtained in the present study was 15.6 mg/dL (Table 3).

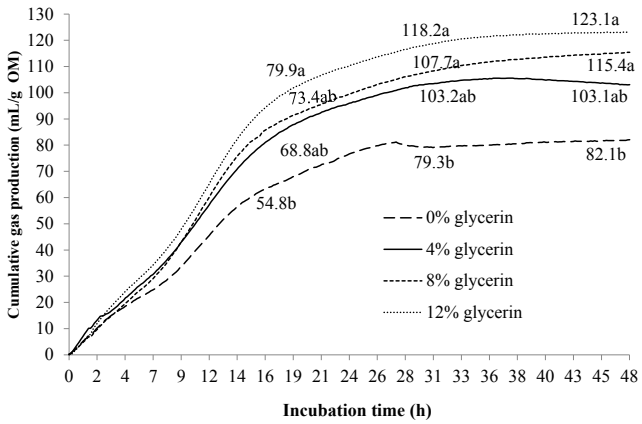

Figure 1. Effect of crude glycerin inclusion level to replace the corn in the diet on the mean values of cumulative gas production (mL/g of OM incubated) during 48 hours of incubation.

Discussion

The digestibility results are consistent with previous studies, which showed that feeding glycerin levels up to 12% of the total ration does not have an effect on nutrient digestibility or animal performance (Krueger *et al*., 2010; Meral *et al*., 2015). On the other hand, Abo El-Nor *et al*. (2010) observed that glycerin decreased the *in vitro* organic matter digestibility and *in vitro* neutral detergent fiber digestibility in diets. These results suggest that glycerin can modulate digestion in a dose-dependent manner. Drouillard (2012) describes that the deleterious effects of glycerin on fiber digestion are evident because the

inhibitory effects of higher crude glycerin levels on cellulolytic bacteria and fungi activity are clearly evident and may provide a plausible explanation for reduced fiber digestion. In this study, IVOMTD and IVNDFD were not affected by crude glycerin due to its low level in the diet, allowing optimum rumen fermentation, as growth, adhesion and cellulolytic activity were inhibited when glycerin was included at high concentration but not at low concentration (Benedeti *et al*., 2016).

Total cumulative gas production values are low, but according to Lee *et al.* (2011), adding glycerin to alfalfa or corn tended to decrease the *in vitro* gas production compared to alfalfa or corn substrates when they were incubated without glycerin. Krueger *et al.* (2010) also reported a linear increase in gas production when glycerin was added to alfalfa hay at 10, 20 and 40% of the DM *in vitro*. The differences in the gas production profiles were not expected as corn and glycerin produce the same amount of gas (Lee *et al.,* 2011), and the replacement of corn for glycerin should not cause modification. However, we can hypothesize that alfalfa hay, corn and glycerin combination could affect fermentation, suggesting the occurrence of associative effects. Corn grain has a hydrophobic protein matrix, which interferes negatively on its ruminal degradation rate (Larson and Hoffman, 2008). However, alfalfa hay has a higher percentage of soluble fraction and lower NDF, thus more nonstructural carbohydrate content. These carbohydrates are associated with glycerin, which is rapidly metabolized (between 4 and 6 h), providing greater synchronism with the sources of nitrogen of rapid degradation, and consequently producing more gas.

Partitioning factors indicate fermentative efficiency, and therefore, higher PF values mean greater incorporation of degraded organic matter to the microbial mass, thereby increasing the efficiency of microbial protein synthesis. According to Makkar (2004), optimal PF values range between 2.74 and 4.41 mg TDOM/mL gas produced. The high PF values obtained in the present study indicate that fermentation conditions were good, stimulating production of microbial protein and resulting in high partitioning values.

The higher gas production of the rapidly degradable fraction relative to the slowly degradable one (81.2 vs 28.9 mL/g OM) indicates that glycerin increased the soluble fraction of the diet. This result is consistent with the findings by Getachew *et al*. (2004) and Krueger *et al*. (2010), who reported that maximum gas production of the rapidly degradable fraction (A) increased linearly as the amount of glycerin in the diet increased.

The B and E degradation rates may be considered as intrinsic characteristics of the feed, and depend on the chemical composition of the roughage, the maturation stage of the plant, and the cell wall structure. The degradation rate can influence the efficiency of microbial synthesis, thereby affecting gas production of each fraction of the incubated feed. According to Caton and Dhuyvetter (1997), there are usually no effects of dietary energy supplementation on substrate degradation rate, as observed in the results obtained in the present study, probably due to efficiency of energy use.

To be degraded in the rumen, the substrate must undergo an attachment process by rumen bacteria, known as colonization time or lag time, which allows enzymes to reach the substrate. In the present study, the inclusion of increasing glycerin levels did not affect the colonization time of bacteria to the rapidly degradable fraction (C, h) of feed. However, the colonization time to the slowly degradable fraction (F, h) of feed was showing direction of the effect, probably due to a direct relationship between the presence of highly fermentable glycerin and the colonization time.

In this study, the kinetics of gas production from glycerin fermentation were similar to results reported by Lee *et al.* (2011) and Krueger *et al*. (2010) who reported long colonization times. This indicates that kinetics of *in vitro* fermentation of glycerin seem to depend on the colonization of rumen microbes. In addition, fermentation of glycerin may be altered by the presence of other feed ingredients (Lee *et al*., 2011).

Fermentation kinetics of feedstuffs can be determined by the gas production and by buffering of volatile short-chain fatty acids (SCFAs) and depends on the relative ratio of soluble, insoluble but degradable, and non-degradable particles of the feed. In *in vitro* media, NH_3-N concentration is an indicator

of protein degradability because there is no nitrogen absorption or recycling, compared with the *in vivo* rumen media (NRC, 1985).

The mean NH_3-N results obtained in the present study were above 5 mL/dl, which is, according to Satter and Slyter (1974), the concentration that maximizes microbial protein synthesis. These results are consistent with previous works showing that feeding glycerin in substitution for corn or barley grain does not affect NH_3-N concentration (Abo El-Nor *et al.,*2010; Avila *et al*., 2011).

In conclusion, inclusion of up to 12% crude glycerin (on dry matter basis) in replacement of corn did not affect diet digestibility. The greater volume of gas produced was observed at the highest inclusion level of glycerin, indicating that alfalfa hay, corn and glycerin combination could change fermentation, suggesting the occurrence of associative effects.

References

Abo El-Nor S, Abughazaleh AA, Potua RB, Hastings D, Khattab MSA. Effects of differing levels of glycerol on rumen fermentation and bacteria. Anim Feed Sci Tech 2010; 162:99-105.

ANP. Agência Nacional do Petróleo 2013. Available from: http://www.anp.gov.br

AOAC. Official Methods of Analysis. 12th ed., Association of Official Analytical Chemists, Washington, DC; 1975.

AOAC. Official Methods of Analysis. 15th ed., Association of Official Analytical Chemists, Washington, D.C;1990.

Avila JS, Chaves AV, Hernandes-Calva M, Beauchemin KA, McGinn SM, Wang Y, Harstad OM, McAllister TA. Effects of replacing barley grain in feedlot diets with increasing levels of glycerol on *in vitro* fermentation and methane production. Anim Feed Sci Tech 2011; 166-167:265-268.

Benedeti PDB, Paulino PVR, Marcondes MI, Maciel IFS, Silva MC, Faciola AP. Partial replacement of ground corn with glycerol in beef cattle diets: intake, digestibility, performance, and carcass characteristics. PLOS.One 2016; 28:1-16.

Caton JS, Dhuyvetter DV. Influence of energy supplementation on grazing ruminants: requirements and responses. J. Anim. Sci. 1997; 75:533-542.

Chai W, Udén P. An alternative oven method combined with different detergent strengths in the analysis of neutral detergent fiber. Anim Feed Sci Tech 1998; 74:281-288.

Drouillard JS. Utilization of crude glycerine in beef cattle. In: MAKKAR, H. P. S. Biofuel co-products as livestock feed – opportunities and challenges. Rome: FAO; 2012. p. 155-162.

Eiras CE, Barbosa LP, Marques JA, Araújo FL, Lima BS, Zawadzki F, Perotto D, Prado IN. Glycerine levels in the diets of crossbred bulls finished in feedlot: Apparent digestibility, feed intake and animal performance. Anim Feed Sci Tech 2014; 197:222-226.

Françozo MC, Prado IN, Cecato U, Valero MV, Zawadzki F, Ribeiro OL, Prado RM, Visentainer JV. Growth performance, carcass characteristics and meat quality of finishing bulls fed crude glycerin-supplemented diets. Braz Arch Biol Technol 2013; 56:327-336.

Getachew G, Robinson PH, Depeters EJ, Taylor SJ. Relationships between chemical composition, dry matter degradation and *in vitro* gas production of several ruminant feeds. Anim Feed Sci Tech 2004; 111:57-71.

Goering HK, Van Soest PJ. Forage fiber analyses (apparatus, regents, procedures, and some aplications). Agriculture Handbook 379. U.S. Government Printing Office, Washington (DC); 1970.

Hagopian K, Ramsey JJ, Weindruch, R. Enzymes of glycerol and glyceraldehyde metabolism in mouse liver: effects of caloric restriction and age on activities. Bioscience Rep 2008; 28:107-115.

Krueger NA, Anderson RC, Tedeschi LO, Callaway TL, Edrington TS, Nisbet DJ. Evaluation of feeding glycerol on free-fatty acid production and fermentation kinetics of mixed ruminal microbes *in vitro*. Bioresour. Technol 2010; 101:8469-8472.

Larson J, Hoffman PC. Technical note: A method to quantify prolamin proteins in corn that are negatively related to starch digestibility in ruminants. J Dairy Sci 2008; 91:4834-4839.

Lee SY, Lee SM, Cho YB, Kam DK, Lee SC, Kim CY, Seo S. Glycerol as feed supplement for ruminants: *In vitro* fermentation characteristics and methane production. Anim Feed Sci Tech 2011; 166-167:269-274.

Makkar HPS. Recent advances in the *in vitro* gas method for evaluation of nutritional quality of feed resources. In: Assessing Quality and Safety of the Animal Feeds. FAO Animal Production and Health Series 160. FAO: Roma; 2004. p.55-88.

Mendoza O, Ellis M, McKeith F, Gaines A. Metabolizable energy content of refined glycerin and its effects on growth performance and carcass and pork quality characteristics of finishing pigs. J. Anim. Sci. 2010; 88:3887-3895.

Meral Y, Kara Ç, Biricik H. Influence of glycerol supplementation to dairy and feedlot cattle diets on performance and health: a review. J Biol Environ Sci 2015; 9:109-117.

Meurer F, Franzen A, Piovesan P, Rossato KA, Santos LD. Apparent energy digestibility of glycerol from biodiesel production for Nile tilapia (*Oreochromis niloticus, Linnaeus* 1758). Aquac Res 2012; 43:1734-1737.

NRC. Ruminant Nitrogen Usage. Washington (DC): National Academic Press; 1985.

Omazic AW, Kronqvist C, Zhongyan L, Martens H, Holtenius, K. The fate of glycerol entering the rumen of dairy cows and sheep. J Anim Physiol a Anim Nutr 2015; 99:258-264.

SAS, 2002. SAS/STAT package. Version 9.3. SAS Inst. Inc., Cary, NC, USA.

Satter LD, Slyter LL. Effect of ammonia concentration on rumen microbial protein production *in vitro.* Br J Nutr 1974; 32:199-208.

Schofield P, Pitt RE, Pell AN. Kinetics of fiber digestion from *in vitro* gas production. J. Anim. Sci. 1994; 72: 2980-2991.

Van Soest PJ, Robertson JB, Lewis BA. Methods for dietary fiber, neutral detergent fiber and non-starch polysaccharides in relation to animal nutrition. J Dairy Sci 1991; 74:3583-3597.

Yang F, Hanna MA, Sun R. Value-added uses for crude glycerol–a byproduct of biodiesel production. Biotechnol Biofuels 2012; 5:1-10.