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Quantification of ruminal microbiota and production of methane and carbonic dioxide from diets with inclusion of glycerin

[*Quantificação da microbiota ruminal e produção de metano e gás carbônico de dietas com inclusão de glicerina*]

V.C. *Santos*[1], J.M.B. *Ezequiel*[1], A.C. *Homem Junior*[1], R.S.B. *Pinheiro*[2]

[1]Universidade Estadual Paulista "Júlio de Mesquita Filho"– FCAV-UNESP – Jaboticabal, SP
[2]Universidade Estadual Paulista "Júlio de Mesquita Filho"– FEIS-UNESP – Ilha Solteira, SP

ABSTRACT

The aim of this study was to quantify the liquid-associated bacteria and protozoa of the rumen and to evaluate the potential of *in vitro* production of gases CH_4 and CO_2 of diets with inclusion of glycerin for sheep. Two diets were formulated with a roughage:concentrate ratio of 20:80, according to the presence or absence of glycerin: G0 – control, without glycerin; and G10 – 10% glycerin as part of the concentrate. To quantify the liquid-associated bacteria and protozoa from the rumen and production of CH_4 and CO_2 gases, a completely randomized design was used. The diets had no effect (P>0.05) on production and composition of liquid-associated protozoa and bacteria from the rumen of lambs. Likewise, a (P>0.05) diet effect was not observed on production of CH_4 and CO_2 *in vitro*, which presented average concentrations of 24.55mL/g MS and 51.52mL/g MS, respectively. The use of 10% glycerin did not alter production or composition of ruminal microflora, and moreover, did not negatively impact the production of CH_4 and CO_2 gases *in vitro*.

Keywords: sheep, bacteria, gases, nitrogen, protozoa

RESUMO

Objetivou-se com este estudo quantificar as bactérias e protozoários líquido-associados do rúmen e avaliar o potencial de produção dos gases CH_4 e CO_2 in vitro *de dietas com inclusão de glicerina para ovinos. Duas dietas foram formuladas, na proporção volumoso:concentrado de 20:80, conforme a presença ou ausência de glicerina: G0 – controle sem glicerina, G10 – 10% glicerina como parte do concentrado. Para a quantificação das bactérias e protozoários líquido-associados do rúmen e produção dos gases CH_4 e CO_2, utilizou-se o delineamento inteiramente ao acaso. Não foi observado efeito de dieta (P>0,05) sobre a produção e composição de protozoários e bactérias líquido-associados do rúmen de cordeiros. Da mesma forma, não houve efeito de dieta (P>0,05) sobre a produção de CH_4 e CO_2* in vitro, *os quais apresentaram concentrações médias de 24,55mL/g MS e 51,52mL/g MS, respectivamente. O uso de 10% glicerina não altera a produção ou composição da microflora ruminal, e também não impacta negativamente a produção dos gases CH_4 e CO_2* in vitro.

Palavras-chave: ovino, bactéria, gases, nitrogênio

INTRODUCTION

The use of glycerin in diets for ruminants is not recent; however, this interest has been renewed due to its increased availability and favorable price. Although the effects of glycerin in the feeding of ruminants either as an energy supplement or as a substitute of corn (Parsons *et al*., 2009) on animal performance are well-documented, little is known about its effects on the fermentation pattern and on rumen microorganisms. Evaluating the quantity and quality of ruminal microorganisms is extremely important for animal nutrition, as growth and milk-yield prediction models require estimates of the microbial protein synthesis.

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E-mail: vivianecorreasantos@gmail.com

Along with the maximization of the efficiency of microbial protein synthesis, the optimization of rumen fermentation has been the target of many scientists, as well as utilization of energy by the rumen, maximization of nitrogen use by the rumen bacteria and the reduction of methane and ammonia losses, which are some of the goals of nutritionists to efficiently improve animal performance (Eugène *et al*., 2004). For this purpose, it is necessary to study the microorganisms' populations present in the ruminal environment and the factors that result from modifications in their quality and functionality.

The CH4 emission by ruminants depends on several factors such as animal species, composition and amount of concentrate in the diet, level of intake, type of carbohydrate in the diet, feed processing, addition of lipids or ionophores in the diet and change in the ruminal microflora (Morgado *et al*., 2013).

The differences in the nutritional composition of feedstuffs may result in different fermentation patterns in ruminants, which cause the end products of microbial fermentation - especially acetic, propionic and butyric fatty acids - to be at different proportions; these products represent an important source of energy for the ruminant metabolism. Along with these fermentation processes there is production of CH_4 and CO_2; the production of CH_4 is influenced mainly by the carbohydrates in the diet. Production of CH_4 represents loss of dietary energy consumed by ruminants, in addition to contributing to global warming. Strategies such as increasing the level of grains in the diet, including lipids and supplementing the diet with ionophores are highly likely to reduce emissions of rumen CH_4, in addition to improving production efficiency (Beauchemin *et al*., 2008). According to (Pereira *et al*., 2006) little is known about the emissive behavior of gaseous pollutants from sheep, since the emphasis has been given to differences between emissive components of the diet. Thus, the aim of this study was to quantify the liquid-associated bacteria and protozoa of the rumen and to evaluate the potential of *in vitro* production of gases CH_4 and CO_2 of diets with inclusion of glycerin.

MATERIAL AND METHODS

The experiment was carried out at the Animal Unit for Digestive and Metabolic Research of the Department of Animal Science of the Faculty of Agricultural and Veterinary Sciences of UNESP, Campus Jaboticabal-São Paulo, Brazil.

Two diets (Table 1) were formulated (NRC, 2006) with a roughage:concentrate ratio of 20:80, using chopped *Cynodon* spp. as a source of roughage. The concentrate feed was composed by ground corn grain, soybean meal, limestone, mineral supplement, and inclusion or not of glycerin according to the diets: G0 – control, without glycerin; and G10 – 10% glycerin as part of the concentrate. The glycerin was extracted from soybean oil, which contains 83% glycerol, 11% water, 6% salts (of which 99% is NaCl) and 0.01% methanol (Caramuru Alimentos Ltda.).

Table 1. Chemical composition of experimental diets, with inclusion (G10) or not (G0) of glycerin, for sheep

	Diets (% of DM)	
Ingredients	G0[1]	G10[2]
Tifton hay	20.00	20.00
Corn grain	55.40	45.40
Glycerin	-	10.00
Soybean meal	23.00	23.00
Limestone	0.60	0.60
Mineral supplement*	1.00	1.00
Total	100.00	100.00
	Composition (% of DM)	
Dry matter	89.59	87.81
Ash	5.05	5.27
Organic matter	84.54	82.54
Ether extract	3.19	3.15
Crude protein	20.70	20.00
Metabolizable energy (Mcal/kg DM)**	3.00	3.00

[1]G0: control without glycerin; [2]G10: formulated with 10% glycerin.
*Commercial mineral supplement for sheep (P=60g; Ca=110g; Na=195g; Cl=300g; Mg=10g; S=25mg; Zn=4,000mg; Cu=600mg; Mn=600mg; Fe=1,200mg; Co=100mg; I=180mg; Se=12mg; Fl (maximum)= 0.60mg).
** EM = 12,71 – 0,0108 (ADF) + 0,0262 (EE).
** de Boever *et al*. (1999), values obtained in MJ/kg DM and transformed to Mcal/kg DM

For quantification of ruminal microbiota 40 uncastrated male Santa Inês × Dorper weaned lambs with an average age of 80 days and weight

of 21±2.3kg were utilized. Animals were identified and distributed in a completely randomized design with 20 replications.

Diets were fed twice daily (08h00 and 16h00) and animals had free access to water during the entire experimental period.

When animals reached approximately 33kg of weight, they were weighed and fed only water for 16 hours. The animals were stunned by a bolt pistol without penetration. Animals were bled through their jugular vein and carotid arteries immediately after being stunned.

After the lambs were slaughtered, their gastrointestinal tract was emptied and approximately 200g of samples of the ruminal content were collected manually. To separate the liquid phase from the solid phase the ruminal content was initially filtrated in a nylon filter with a pore size of 100µm for subsequent separation and quantification of liquid-associated bacteria and protozoa according to the methodology of Martin *et al*. (1994).

To identify the liquid phase, the rumen fluid (700 mL) was diluted in an equal volume of saline solution (Coleman, 1978) preheated at 39ºC. This final solution was incubated from 30 to 60 minutes at 39ºC. Glucose was added (1g/L) five minutes before the end of incubation so as to separate the protozoa from the rest of the rumen content and to optimize flocculation.

The protozoan pellet was recovered by centrifuging the clarified fluid at 1,000 x *g* for 10 minutes, at room temperature. This pellet was later washed with a saline solution in a nylon filter with pore size of 20µm (1 liter, at 39^{o}C), to minimize contamination by bacteria and residue of feeds. Liquid-associated bacteria (LAB) were obtained by centrifuging the protozoa-free supernatant fluid at 15,000 x *g* for 20 minutes at 4ºC. Subsequently, samples were lyophilized and analyzed for the contents of nitrogen (N), dry matter (DM) and mineral matter (MM), and the organic matter (OM) content was estimated.

For production of gases CH_4 and CO_2 four Santa Inês x Dorper sheep with mean body weight of 47.3kg, cannulated in the rumen were used, and measurements of production of CH_4 and CO_2 gases were done using Incubater SHAKER SL 222 consisting of three stages: 1 – Preparation of the sample: prior to feeding four rumen-cannulated sheep, we collected approximately 500mL of the rumen content, filtered the material through a nylon fabric (100µm) and homogenized it. In erlenmeyers with a capacity of 250mL, 150mL of ruminal fluid were placed added of 1.56g DM of the diets studied ground to 1mm, in order were previously added so as to keep the ratio of 1g sample:8mL rumen fluid. 2 – Production and storage of gases: the erlenmeyers with samples and rumen fluid were closed with stoppers and kept in a water bath for 12 hours at 39ºC in a dark room, and the gases produced were led by capillary system of silicone and stored in a proper plastic container with internal volume of 600mL. Such collectors were immersed in water enabling measurement of total gases by water displacement therein. 3 – Quantitative and qualitative analysis of the gas produced: an aliquot was directly collected from the erlenmeyers with the aid of a 1mL syringe, then immediately injected in a gas chromatograph (Trace GC Ultra, Thermo Scientific©) metanador and equipped with a flame ionization detector, using argon as carrier gas with a flow of 25 mL per minute, and the oven temperature was set to 70ºC. The calibration was performed with a mixture of gases CH_4 and CO_2. The peak areas were integrated with software Chromquest 5.0. The total gas produced was measured by displacement of collectors, immersed in water after 12 hours of fermentation.

To quantify the liquid-associated bacteria and protozoa from the rumen production of methane and carbonic gases we used a completely randomized design with 20 replications per diet for quantification of ruminal microbiota and 15 replications per diet for production of gases CH_4 and CO_2. Data were analyzed utilizing the PROC GLM procedure on the Statistical Analysis System (SAS, 2001).

RESULTS AND DISCUSSION

The diets had no effect (P>0.05) on production and composition of liquid-associated protozoa from the rumen of lambs fed diets with/without glycerin. Although there were no statistical differences, dry matter, organic matter and nitrogen productions were respectively 21.20%,

23.14% and 27.54% lower than the average of the diet with inclusion of glycerin (Table 2).

The average chemical composition of protozoa obtained by Martin *et al*. (1994) in organic matter and nitrogen were respectively 89.5 and 5.1% and Ezequiel *et al*. (2002) found 75.2 and 4.9% organic matter and nitrogen, respectively. In the present study, protozoa showed on average 49.7% organic matter and 9.7% nitrogen. The organic matter was lower than the values obtained in the aforementioned studies and the nitrogen values were above them; this can be explained by the differences between the diets evaluated and the presence of saline solution, which resulted in a higher mineral matter content in the samples. This hypothesis was also mentioned by Mendes *et al*. (2006) as the cause of the large variation in the microbial organic matter values found in the literature.

No effect of diets (P>0.05) was observed on production and composition of liquid-associated bacteria from the rumen of lambs fed diets with/without glycerin. Although they showed no statistical differences, productions of dry matter, organic matter and nitrogen were respectively 8.00, 8.58 and 13.68% below the average of the diet with inclusion of glycerin (Table 2).

Table 2. Production and composition of liquid-associated protozoa and bacteria in the rumen of lambs fed with glycerin

	Diets			
Variables	G0[1]	G10[2]	CV (%)	P value
Protozoa				
Dry matter (mg/L)	576.10	731.00	57.12	0.3763
Organic matter (%)	50.62	48.75	16.69	0.6822
Organic matter (mg/L)	294.70	383.40	74.89	0.4547
Nitrogen (% MO)	9.25	10.10	22.23	0.3964
Nitrogen (mg/L)	52.71	72.74	55.39	0.2228
Bacteria				
Dry matter (mg/L)	1069.70	1162.80	47.87	0.7015
Organic matter (%)	73.24	72.72	4.83	0.7462
Organic matter (mg/L)	785.30	859.00	50.66	0.6971
Nitrogen (% MO)	10.38	10.87	14.44	0.4834
Nitrogen (mg/L)	112.10	129.86	54.88	0.5571

[1]G0: control without glycerin; [2]G10: formulated with 10% glycerin. OM: organic matter. CV = coefficient of variation. P = probability.

The average organic matter (%DM) and nitrogen (%OM) contents of the bacterial fractions associated with the rumen fluid obtained in this study were 72.98 and 10.63%, respectively. Homem Júnior *et al*. (2010) found mean organic matter values of 81.1% and 11.3% nitrogen in bacteria from the rumen fluid and Ezequiel *et al*. (2002) reported organic matter and nitrogen contents of 81.6% and 6.7% for the liquid-associated bacteria. The organic matter content found by these authors was higher than those obtained in our experiment. However, Martin *et al*. (1994) reported organic matter contents of bacteria from the liquid phase within the range from 60.3 to 64.7% and Mendes *et al*. (2006) obtained an average 57.0% organic matter, and these numbers are lower than those found in our study.

The absence of a significant effect on nitrogen contents should be taken into account, since most of the nitrogen that the animal uses is of microbial origin, and if the contents decreased with inclusion of glycerin, this decrease would probably cause problems because of the low nitrogen availability. Ezequiel *et al*. (2002) reported nitrogen contents of 6.7% in the liquid-associated fraction, which is different from the present study, and may be linked to the fact that the protein content of the diets in our study was higher.

Preliminary research indicates that glycerin affects the growth of some rumen bacteria. Abo El-Nor *et al*. (2010) evaluated the effect of replacing corn with glycerin at concentrations of 0, 36, 72 and 108g/kg DM in a diet for Holstein

cows on rumen fermentation and observed a decrease in the concentration of DNA of bacteria *Butyrivibrio fibrosolvens* and *Selenomonas ruminantium* as the glycerin content in the diet was elevated. However, this mechanism still isn't elucidated, because the sensitivity of microorganisms may vary according to the level of glycerin inclusion in the diet. Moreover, glycerin can also interfere with the bacterial adhesion to food particles.

In the literature there are descriptions of large variations in the chemical composition of rumen microorganisms (Homem Júnior *et al*., 2010). Variations depend on the methodology of isolation adopted and on the several diets that can be formulated and which modify the supply of fermentable substrate and consequently microbial growth and its composition.

Diets did not differ (P>0.05) as to the production of CH_4 and CO_2 produced *in vitro* (Table 3). This demonstrates that inclusion of 10% glycerin does not change the production of CH_4 and CO_2, which disagrees with the results of Van Cleef *et al*. (2012), who evaluated the *in vitro* production of greenhouse gases using different concentrations of crude glycerin (0, 7.5, 15, 22.5 and 30%) in diets for Nellore cattle and observed that regardless of the concentration, crude glycerin caused reduction in CH_4 and CO_2 during 12 hours of incubation.

The mean value obtained for methane production is higher than those reported by Avila-Stagno *et al*. (2013) from 9.6 to 12.3mL/g DM intake, using the methodology of respirometric chambers to determine the methane production of lambs fed diets with increasing levels of 0, 7, 14 and 21% glycerol. The highest methane production observed in this study may be due to high concentrate (80%), which correlates positively with the total gas production (Lee *et al*., 2011), which may also explain the lack of effect the addition of glycerin has on the reduction of methane. According to Lee *et al*. (2011) methane production of in vitro alfalfa was 17.9, while corn was 29.6mL/g DM incubated, the latter being very close to the value obtained in this study, in which a high proportion of used concentrate was used.

According to some authors (Abo El-Nor *et al*., 2010; Ávila *et al*., 2011), glycerin has a ruminal fermentation characteristic of formation of propionic acid and reduction in acetate: propionate ratio, which would lead to reduction in the production of methane removal of H_2 from the middle, but it is possible that on a diet with high concentrate this effect is not as pronounced as on a diet with a high proportion of roughage feed (Lee *et al*., 2011).

Evaluating the *in vitro* production of diets containing 12% crude glycerin in replacement of barley, Ávila *et al*. (2011) did not observe any effect on methane production. Also, incubating a few ingredients with and without glycerin *in vitro* for up to 48 hours, Lee *et al*. (2011) did not observe alterations in methane production in their study.

Table 3. *In vitro* production of methane (CH_4) and carbon dioxide (CO_2) of diets including glycerin

	Diets			
Variable	G0[1]	G10[2]	CV (%)	P
CH_4 (mL/g MS)	24,93	24,16	6,76	0,3114
CO_2 (mL/g MS)	52,65	50,39	17,69	0,5876

[1]G0: control without glycerin; [2]G10: formulated with 10% glycerin.
CV = coefficient of variation
P = probability

CONCLUSIONS

The use of glycerin did not affect production or composition of liquid-associated bacteria and protozoa from the rumen. The *in* vitro production of gases CH_4 and CO_2 does not change with the inclusion of 10% crude glycerin in the diet.

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