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Degradability and gas production of diets enriched with additives in cattle or sheep inoculum[1]

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***Abstract*—** *The study of ruminal kinetics of feedstuffs and the influence of feed additives on degradability and gas production can contribute to the formulation of more efficient diets. This study proposes to examine cumulative gas production from rumen fermentation and the in vitro degradability of diets containing maize and cottonseed cake enriched with amylolytic enzyme, protected lysine, lysophospholipids or protected methionine. In the degradability trial, the samples were incubated in anaerobic medium for 0, 3, 6, 12, 24 and 48 h, at 39 °C. In vitro gas production was determined at the incubation times of 3, 6, 9, 12, 16, 24, 32, 48 and 72 h, and the use of cattle and sheep ruminal fluid was compared. The inclusion of lysophospholipid increased ($P<0.05$) the degradability of dry matter in the diet, whereas the addition of protected methionine reduced this variable. Gas production was greater in sheep inoculum up to 48 h of fermentation, and no differences were detected at 72 h. The amylolytic enzyme increased the gas production only up to 24 h of fermentation. After this time, none of the tested additives increased gas production.*

[1] Part of the first author's Master's dissertation.

I. INTRODUCTION

Ruminant finishing systems in Brazil range from extensive to intensive production types. The former require more time to produce the same unit of animal product, as a greater portion of the feed provided is used for the maintenance of vital activities in the animal. Thus, extensive farming is less efficient in terms of preserving the energy contained in the diet in the final product, be it meat, milk, or others. In these systems, grasses usually form the basis of the diet. In intensive finishing systems, on the other hand, the diet is more grain-based, which translates into superior conversion of the dietary energy into animal product.

Much of the energy lost during ruminal fermentation result from the elimination of gases (Lana *et al*., 1998). Approximately 99% of these gases are carbon dioxide and methane, with nitrous oxide corresponding to a very small portion (Kozloski, 2002).

Diets with higher proportions of concentrate generate larger volumes of cumulative gas per time unit, as they contain larger amounts of non-fibrous carbohydrates, which are rapidly digested in the rumen (Mertens, 1987).

Substrates with higher acetate production capacity (i.e., higher fiber content) produce proportionally larger amounts of gas, when compared with high-starch substrates. The latter, in turn, provide greater propionate production and less gas production per unit of fermented glucose (Blümmel *et al*., 1997). This can be demonstrated mainly by relating the amount of gas produced per unit of animal gain.

The study of ruminal kinetics of feedstuffs and the influence of dietary additives on the digestibility, digestion rate and potential of ruminal digestion, among others, can significantly contribute to the formulation of more economical and efficient diets that more adequately meet the requirements of ruminal microorganisms and compatibility between feed ingredients.

This study was conducted to examine cumulative gas production from rumen fermentation and the *in vitro* degradability of diets containing maize and cottonseed cake enriched with amylolytic enzyme, protected lysine, lysophospholipids or protected methionine, in cattle and sheep inocula.

II. MATERIAL AND METHODS

All procedures involving animals were approved by the Animal Use Ethics Committee of the Federal University of Goiás (CEUA-UFG) (approval no. 37288).

The evaluated diets were composed of ground maize, cottonseed cake and a vitamin-mineral premix (Table 1). The following treatments were tested: control (CON), amylolytic enzyme (ENZ), protected lysine (LYS), lysophospholipids (LIP) and protected methionine (MET).

Table 1. Percentage composition of ingredients (as-fed basis) and chemical composition of diets (dry-matter basis)

Ingredient	Treatment				
	CON	ENZ	LYS	LIP	MET
Cottonseed cake (%)	55.00	55.0	55.0	55.0	55.0
Grain maize (%)	42.0	42.0	41.7	42.0	41.9
Mineral-vitamin premix[a] (%)	3.0	3.0	3.0	3.0	3.0
Enzyme (%)	-	0.076	-	-	-
Lysine (%)	0.0845	0.0845	0.1183	0.0845	0.0845
Lysophospholipid (%)	-	-	-	0.0380	-
Methionine (%)	0.0425	0.0425	0.0425	0.0425	0.1555
Chemical composition					
Dry matter (%)	90.5	90.5	90.5	90.5	90.5
CP (% DM)	20.11	20.11	20.11	20.11	20.11
EE (% DM)	7.41	7.41	7.41	7.41	7.41
NDF (% DM)	34.23	34.23	34.23	34.23	34.23
ADF (% DM)	21.50	21.50	21.50	21.50	21.50
MM (% DM)	6.52	6.52	6.52	6.52	6.52

[a]Nutrient/kg of premix: calcium = 262 g, phosphorus = 60 g, sulfur = 50 g, magnesium = 40 g, sodium = 30 g, iron = 3000 mg, zinc = 1000 mg, manganese = 900 mg, fluorine = 600 mg, copper = 221 mg, iodine = 15 mg, selenium = 10 mg and cobalt = 5 mg.

The amylolytic enzyme was produced from the fungus *Aspergillus awamori* and used in freeze-dried form at a dose of 16.9 U/kg diet (as-fed basis). Enzyme production, characterization and evaluation were carried out at the Laboratories of Enzymology and Digestion Physiology of the Institute of Biological Sciences II, at the Federal University of Goiás. Amylase activity was determined by the saccharification method, which is based on the quantification of the reducing sugars produced by the enzymatic reaction (Miller, 1959). The protected methionine used in the experiment was supplied from the MetiPEARL® product (55.3% methionine, Kemin) and protected lysine from LysiPEARL® (48.5% L-lysine hydrochloride, Kemin). The lysophospholipid was supplied from the Lysoforte Booster Dry® product (Kemin).

In the *in vitro* degradability trial, approximately 2.0 L of rumen fluid were collected from a 24-month-old uncastrated bull that was previously acclimated to each diet for seven days. All materials (thermos, funnel, beakers and blender) involved in the handling of the collected fluid were previously heated to 39 ºC. The rumen fluid was mixed in the blender at high speed for 30 s to release part of the microorganisms adhered to the suspended material in it. Afterwards, the fluid was filtered through cotton fabric.

Four 0.4-L aliquots of filtered rumen fluid were separated and added to 1.6 L of Kansas buffer solution, resulting in four 2.0-L volumes. Each volume was placed in incubation jars (TECNAL), where the ANKOM® F57 incubation bags were placed. The four jars were then placed in the DAISY II TE-150 (TECNAL) *in vitro* incubator. All handling procedures involving rumen fluid occurred under constant CO_2 infusion.

Twelve ANKOM® F57 bags containing 0.5 g of sample were added to each jar. The samples were incubated in anaerobic medium for 0, 3, 6, 12, 24 and 48 h at 39 °C. Two bags were removed per jar at each incubation time, with an average value calculated for each jar. The average of the two bags from each jar at each time constituted a replicate; accordingly, there were four replicates for each removal time. Once removed from the incubator, the ANKOM® bags were placed in cold water to stop microbial activity and subsequently washed in running water until it was clear. After the excess water was removed, the bags were washed with acetone for five minutes and completely dried in a forced-air oven at 105 °C for 12 h. The bags were then placed in a desiccator for 30 min and their weight was recorded. The entire procedure was performed five times, with one treatment being incubated at a time. In this way, it was possible to avoid applying two treatments in the same jar and prevent the interaction of two additives in the same sample.

The *in vitro* fermentation of dry matter (DM) was achieved by the artificial-fermenter methodology described by Holden (1999). Dry matter degradability (DMD) was calculated using the formula described by Tilley & Terry (1963):

$$DMD\ (\%) = (A - (B - Br) \times 100)/A,$$

where A = weight of the initial DM of the bag plus the sample; B = weight of the residual DM of the bag plus the digested sample; and Br = weight of the bag without sample (termed 'blank').

Degradability data were adjusted using the Orskov & Mcdonald (1979) model, according to the following equation:

$$p = a + b\ (1 - e^{-c.t}),$$

where p = rate of degradation over time; a = rapidly degradable fraction; b = potentially degradable fraction; c = hourly rate of degradation of the potentially degradable fraction; e = natural logarithm; and t = incubation time. The sum of a and b must be less than or equal to 100%.

The values of a, b and c were used to calculate potential degradability (a + b), which represents the feed solubilized or degraded in the rumen when time is not the limiting factor, and effective degradability, by the following equation proposed by Orskov *et al*. (1980):

$$p = a + (b.c)/(c + Kp),$$

where p represents the rate of effective degradability and Kp is the estimated rate of passage of particles through the rumen per hour.

The *in vitro* gas production trial was carried out according to Theodorou *et al*. (1994) with modifications by Maurício *et al*. (1999). Samples of 1.0 g of the substrates to be evaluated were weighed and sealed in ANKOM® F57 degradability bags. For the fermentation of the samples, glass bottles with a volume of 160 mL were used, which were filled with CO_2. The bags containing the samples were placed in these bottles together with 90 mL of buffer medium and 10 mL of sheep or cattle rumen inoculum. Then, the bottles were filled again with CO_2 and sealed with rubber stoppers.

One bull and two rams (adult, castrated, rumen-fistulated) were used as donors of rumen fluid. The bull was kept in brachiaria pasture (*Brachiaria brizantha*), whereas the sheep was kept in a stall receiving fresh and chopped bermuda grass (*Cynodon dactylon*) in the trough. Mineral mixture and water were freely available to all animals, but the rams also received 150 g/day of concentrate supplementation per animal.

A total of 128 bottles were incubated, eight of which contained only rumen fluid and the buffer medium as control (blanks), which were used to determine the production of gas from the rumen content for a later correction of net gas production. The remaining 120 bottles corresponded to twelve repetitions per inoculum (rams and bull) for each treatment (CON, ENZ, LYS, LIP and MET), with nine replicates, corresponding to the post-incubation times of 3, 6, 9, 12, 16, 24, 32, 48 and 72 h.

Pressure readings were taken 3, 6, 9, 12, 16, 24, 32, 48 and 72 h after incubation, using a pressure transducer (model Press Data). The transducer is connected to a three-outlet valve, one outlet being connected to the transducer, another to a 25 mm × 0.7 mm needle and the third free to remove the gas after the reading.

The pressure data (obtained in PSI) were converted to volume of gas produced using the equation found by Guimarães Júnior *et al*. (2008), for the temperature and atmospheric pressure conditions of Planaltina - DF, Brazil:

$$\text{Volume (mL)} = 4.50231 \times \text{pressure (PSI)} + 0.05164 \times \text{pressure}^2 \ (R^2 = 0.996).$$

The kinetics of gas production in each treatment was determined by the equation from the model described by France *et al*. (1993):

$$Y = A\ \{1 - \exp[-b(t - L) - c(\sqrt{t} - \sqrt{L})]\},$$

where Y = cumulative gas production (mL); A = maximum gas production potential (mL); L = colonization time or lag time (h); b (h^{-1}) and c ($h^{-0.5}$) = constant fractional rates; and t = time (h).

In vitro degradability was analyzed in a randomized complete-block design in which each jar constituted a replicate. In statistical analysis, the *in vitro* degradability fractions were compared by the F test at 5% significance and the obtained curves were analyzed by the model identity test (Regazzi, 2003), using R statistical software (R, Development Core Team, 2012).

The gas production trial was laid out in a completely randomized design with a 5 × 2 factorial arrangement, where the factors were represented by the substrates (CON, ENZ, LYS, LIP and MET) and the inocula (sheep and cattle). Cumulative gas production data were subjected to analysis of variance and means were compared by Tukey's test at 5% significance using R statistical software (R Development Core Team, 2012).

III. RESULTS AND DISCUSSION

The results and parameters used to calculate the *in vitro* DM degradability (IVDMD) are shown in Table 2. Fraction a, which represents the fraction of rapid ruminal degradation, was lower ($P<0.05$) in MET than in the CON and LIP treatments. The highest result for fraction b ($P<0.05$), which represents the fraction potentially degradable in the rumen, was found in LYS, followed by LIP, MET and CON. The ENZ treatment obtained the lowest value for this fraction, which not differ from CON. The hourly rate of degradation (c) of fraction b did not differ ($P>0.05$) between the treatments. Each degradation curve estimated from the Orskov & Mcdonald (1979) equation is a model. The model identity test described by Regazzi (2003) allows for a comparison of the parameters and regressions of this model using the F test, which makes it possible to determine whether or not there is similarity in the regression profile. The comparisons between parameters a, b and c of the models was pairwise (Table 2).

Table 2. In vitro dry matter degradability of experimental diets and model identity test of fractions and in vitro degradability curves.

Parameter	Treatment				
	Control	Methionine	Enzyme	Lysine	Lysophospholipid
Fraction a (%)	14.05^{a1}	7.67^{b}	12.66^{ab}	13.33^{ab}	15.96^{a}
Fraction b (%)	45.50^{bc}	48.01^{b}	37.50^{c}	71.14^{a}	56.68^{b}
Fraction c	0.0281^{a}	0.0489^{a}	0.0479^{a}	0.0233^{a}	0.0264^{a}
PD (%)	59.54	55.68	50.16	84.49	72.64
ED (kp=2%)	40.62	41.74	39.12	51.59	48.22
ED (kp=5%)	30.41	31.41	31.01	35.93	35.56
ED (kp=8%)	25.87	25.88	26.71	29.37	30.03
Lag time (h)	3.24	2.69	2.10	2.36	0.03

Comparison	Model-identity test			Model identity
	Fraction			
	a	b	c	
CON × ENZ	ns[2]	ns	ns	ns
CON × LYS	ns	0.022	ns	<0.001
CON × LIP	ns	ns	ns	<0.001
CON × MET	0.022	ns	ns	0.017
ENZ × LYS	ns	0.007	ns	<0.001
ENZ × LIP	ns	0.006	ns	<0.001
ENZ × MET	ns	0.029	ns	0.014
LYS × LIP	ns	ns	ns	ns
LYS × MET	ns	0.011	ns	0.001
LIP × MET	0.004	ns	ns	0.001

[1]Means followed by common letters in the rows do not differ by the F test (P<0.05)

[2]Comparisons between means and models not significant in the F test (P<0.05)

The fact that a fraction or all fractions are similar between treatments does not necessarily imply equal models. Small differences in the values of the fractions between treatments may not be noticeable in statistical tests for comparison of means. However, they can substantially modify the graphic behavior of the model, since the small numerical differences between the fractions in the treatments can add to their effects. Therefore, model identity tests are necessary to demonstrate the differences between treatments, when analyzing the whole set. The comparisons of CON × ENZ and LYS × LIP revealed similar results. Figure 1 shows the visual similarity between the curves drawn from these treatments.

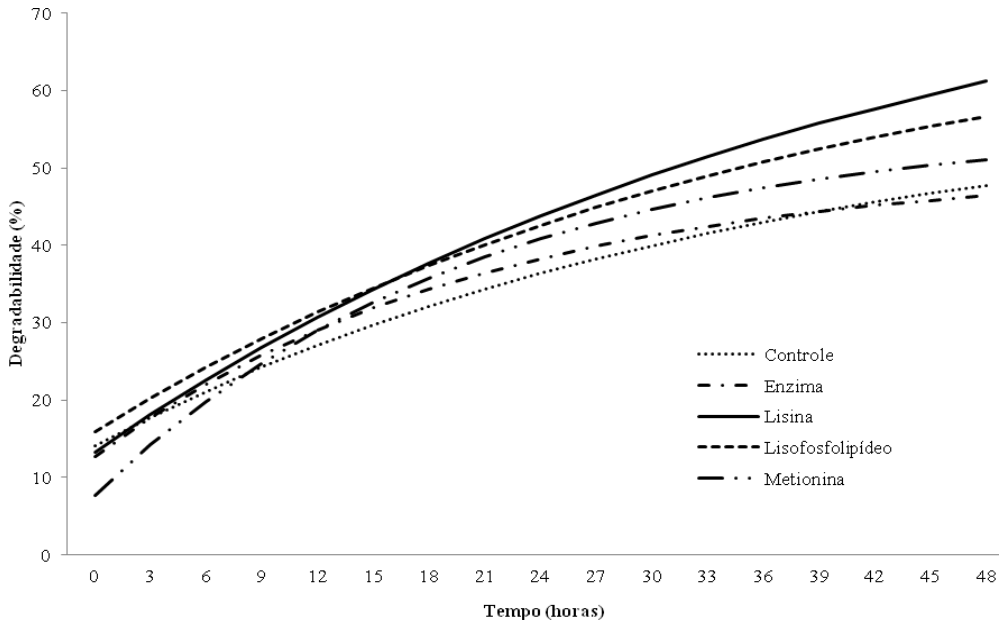

Fig.1: DM degradation curve of the experimental diets according to the adjusted parameters of Orskov & Mcdonald (1979).

The MET and LYS provided showed higher (P<0.05) IVDMD than CON. Obeidat *et al*. (2008) and Acosta *et al*. (2012), however, found no differences in the *in vivo* digestibility of sheep diets enriched with MET, which had already been described by other researchers (Oke *et al*., 1986; Antongiovanni *et al*., 2002). Han *et al*. (1996) also observed no differences in the *in vitro* degradability of lamb diets enriched with LYS and MET analogues, and the same was reported by Sun *et al*. (2007).

Sun *et al*. (2007) observed an increase in the activity of endo-1,4-β-D-glucanase and β-glucosidase after supplementing LYS and MET. These enzymes are responsible for degrading the dietary fiber (Bowman & Firkins, 1993). It is not known how these amino acids affect the activity of these enzymes. Fiber degradability was not evaluated, but the increase observed in the IVDMD of the LYS and MET treatments may be due to greater degradation of the fibrous portion.

There was no difference in IVDMD between ENZ and CON. However, Crosby *et al*. (2006) observed an 8.9% increase in the *in vivo* digestibility of lamb diets enriched with different doses of *Bacillus licheniformis* amylases.

There was an increase in IVDMD with the addition of LIP, in comparison to CON. This result agrees with the descriptions of the Cong *et al*. (2009), who observed an increase in IVDMD using three different surfactants. Hristov *et al*. (2007) also reported increased *in situ* degradability of starch and DM with the addition of a surfactant to the diet.

The parameters estimated through the gas production model developed by France *et al*. (1993) are shown in Table 3 and the cumulative gases production means of the CON, ENZ, LYS, LIP and MET treatments incubated with cattle or sheep inoculum at different times are described in Table 4.

Table 3. Gas production potential (A), constant fractional rates (b and c) and lag time (L) calculated for different substrates in rumen fluid of sheep and cattle.

Treatment	A (mL/g DM)		b (h^{-1})		c ($h^{-0.5}$)		L (h)	
	Sheep	Cattle	Sheep	Cattle	Sheep	Cattle	Sheep	Cattle
CON	212.8	192.0	0.030	0.028	-0.077	-0.080	1.6461	2.0372
ENZ	194.6	258.2	0.029	0.016	-0.074	-0.035	1.6132	1.1655
LYS	217.1	248.7	0.032	0.018	-0.115	-0.055	3.2115	2.2665
LIP	173.7	184.2	0.043	0.032	-0.150	-0.112	3.0947	3.4837
MET	150.7	162.0	0.045	0.044	-0.150	-0.180	2.7931	4.2357

Table 4. Mean cumulative gas production values (mL/g DM) at 24, 48 and 72 h of fermentation in rumen filtrate of cattle and sheep.

Treatment	24 h		48 h		72 h	
	Sheep	Cattle	Sheep	Cattle	Sheep	Cattle
CON	69.21^{aA}	53.96^{bA}	131.06^{aA}	$108,42^{bA}$	$168,87^{aA}$	$144,81^{aA}$
ENZ	61.79^{aAB}	53.77^{bA}	116.60^{aAB}	$106,22^{aA}$	$152,27^{aAB}$	$152,10^{aA}$
LYS	57.86^{aB}	45.59^{bB}	124.77^{aAB}	$102,15^{bA}$	$165,76^{aAB}$	$146,20^{aA}$
LIP	57.09^{aB}	46.89^{bAB}	119.69^{aAB}	$103,63^{bA}$	$147,37^{aAB}$	$139,08^{aA}$
MET	56.00^{aB}	46.23^{bAB}	110.99^{aB}	$106,53^{aA}$	$131,29^{aB}$	$134,34^{aA}$
Mean	60.39^{a}	49.29^{b}	120.62^{a}	$105,39^{b}$	15311^{a}	$143,31^{a}$
CV	12.16		14.43		15.85	
P1*	<0.001		<0.001		0.1125	
P2**	<0.001		0.2577		0.0751	
P3***	0.4128		0.3098		0.5604	

Means followed by distinct lowercase letters in the rows or uppercase letters in the columns differ from each other by Tukey's test (P<0.05). Probability values of analysis of variance for inocula (*), treatments (**) and their interaction (***).

At the incubation times of 24 and 48 h, there were significant differences in gas volumes between the inocula, with a larger amount produced in the sheep inoculum. However, there was no significant difference at 72 h. As rumen fluid donors, the rams received a diet with a higher non-fibrous carbohydrate content than the bull. Thus, it is possible that the microbiota of the rams was more able to digest non-fibrous carbohydrates than that of the bull, which explains the higher initial gas production. Nonetheless, over time, the microbiota present in the cattle rumen fluid may have adapted to the substrate, or the very existing microbiota managed to digest the substrate that had not yet been fermented, resulting in a similar final production in both inocula.

Bueno *et al*. (2005) compared the use of cattle and sheep inoculum in the production of gases from various substrates and found higher values in cattle inoculum (345.9 mL) than in sheep inoculum (323.8 mL) per gram of organic matter, after 96 h of incubation.

By 24 h of fermentation, CON produced more gas than LYS, LIP and MET, but was similar to ENZ in the sheep inoculum. In the cattle inoculum, however, CON and ENZ showed higher production ($P<0.05$) than LIS and similar results to other treatments. After 48 h and 72 h of fermentation, CON produced more gas than MET, but was similar to the other treatments in the sheep inoculum, with no differences occurring between the treatments in cattle inoculum for these times. When only the treatments were analyzed regardless of inoculum source, differences were solely present at 24 h, when CON and ENZ were superior to the other treatments.

Wang et al. (2004) observed no differences in the volume of gas produced from cattle diets enriched with a nonionic surfactant. For the same product, there was a decrease in the cumulative gas production from barley grains (*Hordeum vulgare*) after 36 h of incubation, when added at the dose of 0.10%. In the proportion of 0.05%, there was no difference in relation to control (Lee & Ha, 2003). However, in the same study, no difference in gas production was observed after 96 h of incubation for orchard grass hay (*Dactylis glomerata* L.). Cong et al. (2009), on the other hand, observed an increase in gas production following the addition of three different surfactants.

Gas production curves are illustrated in Figure 2.

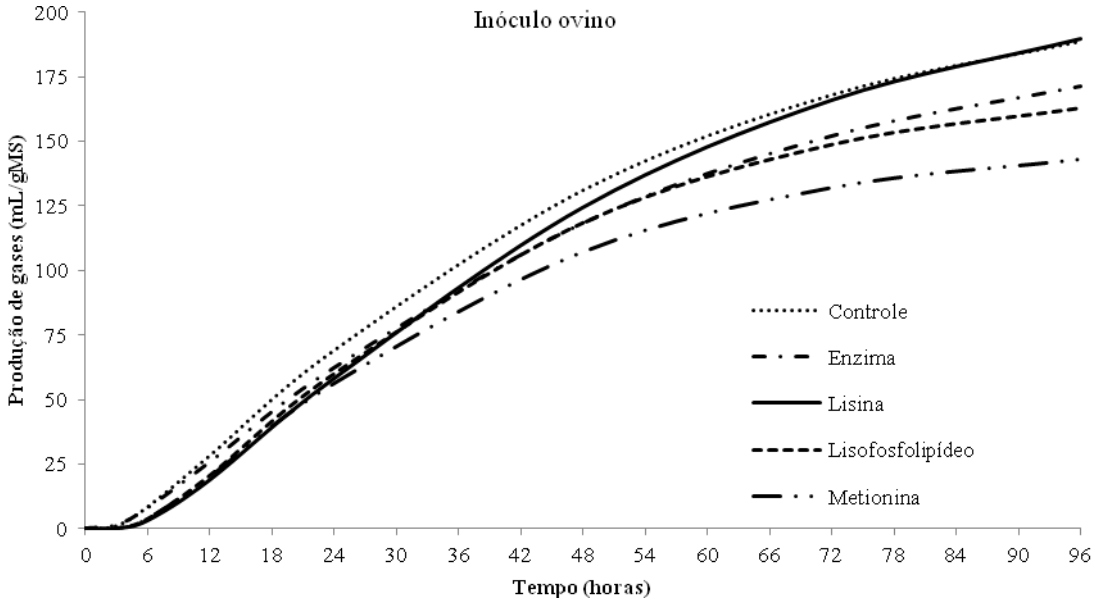

Fig.2: Cumulative gas production during 96 h of fermentation process in cattle or sheep inoculum adjusted to the model of France et al. (1993).

The treatments showed different responses in terms of gas production in each inoculum. However, in both inocula, MET and LIP represented the curves with the lowest gas production values visually.

IV. CONCLUSIONS

The inclusion of lysophospholipid increases the *in vitro* dry matter degradability of high-concentrate diets for finishing lambs, whereas the addition of protected methionine reduces this variable. Gas production is greater in sheep inoculum up to 48 h of fermentation. The addition of amylolytic enzyme increases gas production up to 24 h of fermentation.

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