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Influence of calcium propionate on *in vitro* fermentation of sorghum-based diets

Efecto del propionato de calcio en la fermentación *in vitro* de dietas a base de sorgo

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Abstract

The objective of the present study was to evaluate the effect of calcium propionate (CaPr) on *in vitro* ruminal fermentation using a factorial arrangement 2 x 2 evaluating CaPr (0 *vs.* 1%) and grain level (55 *vs.* 65%). There was a CaPr x Grain interaction in the volume of gas produced (V; p = 0.04). Addition of CaPr prolonged Lag time (1.4 *vs.* 1.04 h; P<0.01), and increasing the grain level also prolonged Lag time (1.56 *vs.* 0.89 h; p < 0.03) and gas production rate (0.046 *vs.* 0.041 h-1; P<0.04). However, there were no differences in CH_4, CO_2, acetate, propionate and butyrate concentrations. Therefore, the addition of calcium propionate in a diet with 55 or 66% of grain increased Lag phase but it is not affected fermentation pattern or methane losses.

Keywords
in vitro gas production • propionate • grain • fermentation

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Resumen

El objetivo de este estudio fue evaluar *in vitro* el efecto de la adición de propionato de Ca (PrCa). El diseño experimental fue completamente al azar con un arreglo factorial 2x2; PrCa (0 o 1%) y grano (55 o 65%). Se encontró diferencias (p>0,05) entre tratamientos para volumen de gas, pero la tasa de producción de gas no fue diferente (p<0,05), se observó un efecto por parte del grano (p<0,04). La adición de PrCa prolongó el tiempo Lag (1,4 *vs.* 1.04 h; P<0,01) y aumentando el nivel de grano también prolongó la fase Lag (1,56 *vs.* 0,89 h; P<0,03) y la tasa de producción de gas (0,046 *vs.* 0,041 h-1; P <0,04). Sin embargo, no hubo diferencias en la concentración de CH_4 y CO_2, ni de propionato, acetato y butirato. La adición de propionato de calcio a una dieta con 55 o 65% de grano prolonga la fase Lag pero no afecta el patrón de fermentación o las pérdidas de metano.

Palabras clave
gas *in vitro* • propionato • grano • fermentación

Introduction

In intensive production systems it is important to evaluate metabolic modifiers to maximize energy intake (7) and frequently uses different levels of grain. The fermentable carbohydrates of these diets produce volatile fatty acids (VFA) (12), as an end product of the fermentation, where 95% of these VFA are represented by acetate, propionate and butyrate (11). The inclusion of grains is desirable to promote more propionic in the fermentation because it is an important glucose precursor (2). This makes it a metabolic mediator (1). The increase in propionate production in diets that are rich in highly fermentable carbohydrates has been associated with an increase in animal performance (3).

In ruminants, the increase of glucose precursors, such as propionate, could optimize nutrient use and thus improve production (9). Furthermore, there is also an improvement in energy use when the end product is propionate (6). The additions of propylene glycol presented the lowest volume of gas production and the short lag time (7) therefore it was expected that inclusion of calcium propionate could reduce methane and the volume of gas production, and be metabolized rapidly reducing lag time in the diet fermentation. Therefore, the objective of the present study was to evaluate the *in vitro* fermentation of lambs with two levels of grain containing different amounts of calcium propionate.

Materials and methods

Fermentation substrates

The fermentation substrates were randomly assigned to one of four treatments (diets, table 1, page 187): (1) 650 g/kg grain (450 sorghum + 200 corn) and 0 g/kg Ca propionate (780 g/kg propionic acid); (2) 550 g/kg grain (350 sorghum + 200 corn) and 0 g/kg Ca-Pr; (3) 650 g/kg grain (450 sorghum + 200 corn) and 10 g kg Ca-Pr; (4) 550 g/kg grain (350 sorghum + 200 corn) and 10 g/kg Ca-Pr.

Table 1. Experimental diets and chemical composition
Tabla 1. Dieta experimental y composición química.

	0% Ca-Pr		1% Ca-Pr	
	65% grain	55% grain	65% grain	55% grain
Dry matter basis, g/kg				
Sorghum grain	450	350	450	350
Corn stover	200	20	20	20
Molasses	100	10	10	10
Soybean meal	70	85	70	85
Urea	10	10	10	10
Minerals[1]	10	10	10	10
Ca propionate[2]	0	0	10	10
Acid buff[3]	5	5	5	5
Chemical composition (g/kg DM)				
Dry matter	873	878	874	879
Crude protein	156	144	156	144
NDF	193	273	168	252
ADF	95	145	80	153
Starch	438	385	446	374

Ca-Pr: calcium propionate; DM: dry matter; NDF: neutral detergent fibre; ADF: acid detergent fibre.

[1] Ca 27%, P 3%, Mg 0.75%, Na 6.55%, Cl 10%, K 0.05, S 42 ppm, lasalocid 2000 ppm, Mn 2000 ppm, Fe 978 ppm, Fe 3000 ppm, Se 20 ppm, Co 15 ppm, vitamin A 35000 IU, vitamin D 150000 IU and vitamin E 150 IU.

[2] 78% propionic acid and 22% Ca.

[3] $CaCO_3$ 750 g and $MgCO_3$ 190 g.

Ca-Pr: propionato de calcio; DM: materia seca; NDF: fibra detergente neutro; ADF: fibra detergente ácido.

[1] Ca 27%, P 3%, Mg 0,75%, Na 6,55%, Cl 10%, K 0,05, S 42 ppm, lasalocida 2000 ppm, Mn 2000 ppm, Fe 978 ppm, Fe 3000 ppm, Se 20 ppm, Co 15 ppm, vitamina A 35000 UI, vitamina D 150000 UI y vitamina E 150 UI.

[2] 78% ácido propionico y 22% Ca.

[3] $CaCO_3$ 750 gy $MgCO_3$ 190 g.

The manufacturer of the calcium propionate was Alimentaria Mexicana Bekarem, S.A. de C.V. from México, D.F. For the experiments, 1 kg of each diet was prepared. Feed samples were dried at 55°C for 24 hours and ground into 1-mm-sized pieces.

Culture media

The culture media used were similar as the one described by Cobos and Yokoyama (1995). Briefly, they contained: mineral solution I (6 g of K_2HPO_4 in 1 L of distilled water), mineral solution II (6 g of K_2HPO_4; 6 g $(NH_4)_2SO_4$; 12 g NaCl; 2.45 g $MgSO_4$; and 1.6 g $CaClH_2O$ in 1 L of distilled water), 18% sodium carbonate buffer solution, reduced cysteine solution (2.5 g of L-cysteine in 15 ml of NaOH (2N)), 2.5 g of Na_2S and 0.1 ml of rezarsurin (1%).

Inoculum and inoculation

Ruminal fluid was obtained from two Suffolk wethers with an average weight of 45 kg, fed with a diet containing 50% corn silage and 50% corn grain, using a vacuum pump, the rumen fluid from both donors was mixed to homogenized before the incubation. Donors were not fed with calcium propionate. After the fluid was obtained it was filtered through gauze, and kept at 39°C until later use.

The ruminal fluid was added to the culture media at a final concentration of 10% (13). A total of 0.5 g of dry feed was added to a 120 ml vial with 90 ml of the culture medium plus 9 ml inoculum, and vials with only the 90 ml of inoculum were used as controls. The vials were incubated in water bath at 39°C.

Gas production

Gas production was measured at different times using a hypodermic needle connected to a 0–1 kg/cm^2 gauge inserted in the vial plug. The pressure was measured at 0.5, 1, 2, 4, 6, 9, 12, 16, 36, and 48 hours after incubation. The pressure (kg/cm^2) was converted to volume and accumulated gas production with the model proposed by Menke and Steingass (1988):

$$Y= v / (1 + \exp (2-4*s* (t – L)))$$

where:
Y= total gas produced
v= volume
s= gas production rate
t= time
L= lag phase; all these parameters were estimated.

Volatile fatty acids

Eight vials were incubated for each treatment for inoculum sampling. Samples were collected from eight repetitions of each treatment (inoculum, 2 ml per replication) after 24 hours of fermentation. The samples were acidified with metaphosphoric acid (25%) to stop microbial activity, and frozen until further analysis. The samples were centrifuged at 5000 rpm for 10 min, and the supernatant was used to measure concentrations of VFA, by gas chromatography (5).

Volatile fatty acid (propionate, acetate and butyrate) concentrations were determined by chromatography on a Supelco-29329, 30 m 0.53 mm 0.10-mm column (Sigma Aldrich Canada, Oakville, ON, Canada) installed in a gas chromatograph (Agilent 6890, Agilent United States, Santa Clara, CA, USA) by flame ionization detection. Volatile fatty acids from the ruminal liquid samples were identified by comparison with retention times of known standards (Sigma Aldrich Canada).

A temperature-programmed cycle from 90 to 130°C, rising by 4°C per minute, was used. The injection block was maintained at 265 °C to provide rapid vaporization of the injected fluid. Nitrogen was used as a carrier gas with a flow rate of 60 ml per minute.

The hydrogen flow rate to the detector was 50 ml. per minute, and the air flow was 380 ml per minute. Under these conditions, the analysis of a rumen fluid sample requires about 8 minutes from acetic to butyric acid. Concentrations of CO_2 and CH_4 were calculated as described by (Russell and Strobel, 1989).

Variables of gas production

The gas production technique was used to evaluate the fermentation. The samples of diet were placed in amber glass jars with a capacity of 125 mL: 2.5 g for the humid ones and 0.5 g for the dry ones, six teen samples for each treatment were

incubated; 90 mL of standardized ruminal liquid was added, with a continuous flow of CO_2, and the jars were sealed.

The variables of the gas production kinetics: maximum volume of gas produced (Vmax), lag phase (L) and gas production rate (S), were found with a logistical model. Gas volume data and incubation time were used to obtain the parameters of kinetics of gas production: maximum volume of gas produced (Vm), lag phase (L) and rate of gas production (S) using the logistic model Vo = Vm / (1 + exp (2-4 * s * (t L)).

Statistical analysis

The results were analyzed as a completely randomized design with a 2 x 2 factorial arrangement of the treatments (with amount of grain and propionate as main factors) using the PROC GLM statistical software (19).

The volume of gas produced, gas production rate and initial gas production were calculated using the PROC NLIN BEST method (19).

Results and discussion

The lag phase was prolonged with higher levels of grain (1.32 *vs.* 1.86) and with the addition of CaPr (0.76 *vs.* 1.03) (table 2). Mertens and Loften (1980) noted that lag time increased as starch was added to the substrate under constant pH. Oliveira *et al*. (2011) observed that the addition of propionic acid increased the initial gas production, which do not coincide with the results found in this experiment.

Table 2. *In vitro* cumulative gas production and estimated kinetic parameter model for feed with two levels of sorghum grain and calcium propionate.

Tabla 2. Producción de gas acumulado y parámetros de cinética *in vitro* para alimentos con dos niveles de grano de sorgo y propionato de calcio.

	0% CaPr		1% CaPr	
	65% grain	55% grain	65% grain	55% grain
Lag time (h)	1.32	0.76	1.80	1.03
Volume of gas produced (V) mL	421.02	396.73	414.32	420.01
Gas production rate (R), h^{-1}	0.044	0.040	0.048	0.043
CH_4 mol/100mmol VFA	34.20	34.34	33.65	34.00
CO_2 mol/100mmol VFA	56.59	56.51	55.87	55.78

	SEM	Significance		
		Grain	CaPr	Grain x CaPr
Lag time (h)	0.17	0.03	0.01	0.55
Volume of gas produced (V) mL	6.95	0.19	0.24	0.04
Gas production rate (R), h^{-1}	0.001	0.04	0.08	0.24
CH_4 mol/100mmol VFA	2.05	0.76	0.34	0.13
CO_2 mol/100mmol VFA	1.08	0.34	0.72	0.46

Ca-Pr: calcium propionate; SEM: standard error of the mean; *P*-value (P<0.05); Values of CH_4 and CO_2 were calculated as described by Wolin (1960).

Ca-Pr: propionato de calcio; SEM: error estandar de la media; Valor de *P* (P<0,05); Valores de CH_4 y CO_2 fueron calculados por ecuaciones descritas por Wolin (1960).

Differences may be because the dissociation of Ca propionate contributed to VFA in a minor proportion than propionic acid and the calcium released (17.4 mg/L of soluble Ca) may have other effects as a nutrient for *Fibrobacter succinogenes* (6, 8).

However, in this study, osmotic pressure could be increased affecting microbial growth and lag time, resulting in similar conditions like those when a grain of fats rate of starch digestion is increased in the diet (12).

The volume of gas produced showed an interaction CaPr and grain, maintaining the same volume when samples were incubated with CaPr. The CaPr did not affect gas production rate, which was higher with 65% than 55% of grain (0.045 *vs.* 0.044/h respectively) (table 2, page 189). Ferraro *et al*. (2009) found a long lag time and a low gas production using gluconeogenic precursors including propylene glycol. That partially coincides whit our results. Different substrates may result in varied fermentation kinetics, particularly in the gas production rate and its lag phase (7).

De Visser *et al*. (1998) found a longer lag phase by supplementing rumen-degradable starch, as it was observed in this study when adding higher amounts of grain (4). Mertens and Loften (1980) found a positive relationship between grain proportion and lag phase(r=0.9) (14).

The estimated production of CH_4 and CO_2 were not affected ($p > 0.05$) by the grain level, or by the addition of propionate. Regarding to fermentation patterns, the molar concentrations of acetic acid, butyric acid, propionic acid and total acids showed no significant differences ($p > 0.05$) (table 3).

Table 3. *In vitro* fermentation pattern for feed with two levels of sorghum grain and calcium propionate.

Tabla 3. Concentracion ruminal de ácidos grasos volátiles de fermentación *in vitro* para alimentos con dos niveles de grano de sorgo y propionato de calcio.

	0% CaPr		1% CaPr	
	65% grain	55% grain	65% grain	55% grain
VFA (mol/100 mol)				
Acetate	67.07	67.39	66.74	67.34
Propionate	21.06	20.87	21.90	21.33
Butyrate	11.86	11.73	11.34	11.21
VFA Total mmol/L	26.48	25.75	23.81	25.56

	SEM	Significance		
		Grain	CaPr	Grain x CaPr
VFA (mol/100 mol)				
Acetate	26.48	0.34	0.79	0.57
Propionate	26.48	0.15	0.65	0.20
Butyrate	26.48	0.45	0.52	0.84
VFA Total mmol/L	2.34	0.28	0.56	0.74

Ca-Pr: calcium propionate; SEM: standard error of the mean; Volatile Fatty Acids; *P*-value (P<0.05).

Ca-Pr: propionato de calcio; SEM: error estandar de la media; VFA: Ácidos grasos volátiles; Valor de *P* (P<0,05).

The calcium propionate added in the diet contributes only 0.003 mmol/L and quantified AGVt production was 0.25 mmol/L and that this could not be expected to have modified this variable.

Nevertheless, *in vitro* study showed that both Ca propionate and $CaCO_3$ increased total VFA in ruminal cultures but only Ca propionate increased ruminal propionate, butyrate and valerate (6) and one experiment with steers, supplemental Ca propionate resulted in greater proportions of propionate compared to $CaCO_3$ and also increased ruminal soluble calcium (9).

Although no methane was measured most of the experiments the VFA patterns allow to conclude on methane emission with the addition of calcium propionate. Lee *et al.* (2012) observed a reduction in the acetate by adding calcium propionate and this was also observed by Ferraro *et al.* (2009) with propionate precursors in *in vitro* studies where propionate was increased. It has been demonstrated that altering rumen fermentation pattern may affect CH_4 production (21).

The addition of organic acids (aspartate, fumarate and malate) in the diet increased the molar concentration of propionate and decreased CH_4 concentrations in rumen (11). Trabue *et al*. (2007) noted that the use of glucogenic precursors reduced acetate concentrations. Lee *et al*. (2012) observed the highest molar concentrations of propionate in sheep fed with high-grain diets plus calcium propionate as compared to the same diets without calcium propionate. Similarly, adding various doses of propionic acid *in vitro* increased its concentration which was not metabolized by microorganisms. Higher doses of propionic acid inhibited growth of some bacteria (7).

Conclusions

The addition of calcium propionate to a diet with 55 or 65% grain, under *in vitro* conditions, extended the Lag time of gas production. However, it does not affect the *in vitro* VFA concentrations and methane produced.

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