Research article

In vitro reduction of methane with the cyanogenic glucoside Linamarin

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ABSTRACT

Objective. To assess the effect of rising doses of the cyanogenic glucoside Linamarin on the reduction of *in vitro* rumen methane. **Materials and methods.** Rumen fluid from two fistulated Merino Precoz sheep, inoculated with a fermentation substrate comprising alfalfa hay (*Medicago sativa*) and ground oat grain (*Avena sativa L.*), and added with buffer solution and Linamarin (purity ≥98%) in rising doses, was incubated for eight hours *in vitro*. Methane was measured each hour with an infrared gas monitor. **Results.** According Linamarin doses were increased (0, 6, 13, 20 and 26 mg/L), the methane concentration fell in a linear manner ($p \leq 0.05$) by (9.7, 9.2, 18.1 and 29.4%), respectively. A significant reduction of methane was seen whit the highest dose of Linamarin. **Conclusions.** Linamarin, in pure state, was effective to reduce methane during *in vitro* ruminal fermentation. Therefore, this study constitutes a basis for future experiments including vegetable sources of Linamarin as well as other rumen variables, leading to find a strategy for reducing greenhouse gases.

Keywords: Food additive, methane production, rumen fermentation (*Source: Thesaurus of the National Library of Agriculture*).

RESUMEN

Objetivo. Evaluar el efecto de dosis crecientes del glucósido cianogénico Linamarina, en la reducción de metano ruminal *in vitro.* **Materiales y Métodos.** Se empleó líquido ruminal de dos ovejas fistuladas de la raza Merino Precoz, con el que se inoculó un sustrato fermentativo constituido por heno de alfalfa (*Medicago sativa*) y grano de avena molido (*Avena sativa L.*), se adicionó solución buffer y Linamarina (pureza de ≥98%) en dosis crecientes, lo que se llevó a incubación por ocho horas *in vitro*. El metano se midió cada hora, con un monitor de gases infrarrojo. **Resultados.** De acuerdo con el incremento de las dosis de Linamarina (0, 6, 13, 20 y 26 mg/L), la concentración de metano disminuyó de forma lineal ($p \leq 0.05$) en (9.7, 9.2, 18.1 y 29.4%) respectivamente. Se observó una reducción significativa de metano con la dosis más alta de Linamarina. **Conclusiones.** La Linamarina, en su estado puro, fue eficaz en la reducción de metano durante la fermentación ruminal *in vitro*. Por lo tanto, este estudio constituye una base para futuros experimentos que incluyan fuentes vegetales de Linamarina y otras variables ruminales, lo que puede conducir a encontrar estrategias para reducir los gases de efecto invernadero.

Palabras clave: Aditivo alimentario, fermentación ruminal, producción de metano (*Fuente: Tesauro de la biblioteca nacional de agricultura*).

INTRODUCTION

The rise in atmospheric greenhouse gas (GHG) concentrations (1) has accelerated climate change processes (2). The main GHG are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), which have increased by 40, 150 and 20%, respectively, since 1990 (3).

With regard to CH_4, it was estimated that total emissions from solely ruminant livestock in 2014 were around 97.1 million tons (1) which constituted 18 % of the total CH_4 released into the environment (4).

During the digestive process, ruminants generate CH_4 made up of methanogenic bacteria, (5) which is expelled through burping (6,7), which has an environmental impact and constitutes an energy loss for the animal in the order of 5 to 12% (6). However, CH_4 emissions could be reduced through sanitary improvement, herd management, diet or with the inclusion of plant secondary metabolites (PSM) (5).

There are different PSM, such as condensed tannins (CT), essential oils (EO) and cyanogenic glucosides (CG), with different mechanisms of action that enable them to reduce rumen CH_4 generation (8,9). These PSM are chemical compounds synthesized by the plant itself (5) and the primary mechanism related to the reduction of methanogenesis is the modification of antimicrobial activity (7).

The reducing action of the CH_4 production from CT and EO has been widely proven with favorable results. In this regard, when 20% of *Amaranthus spinosus* (plant containing CT) was replaced in an experimental diet in an *in vitro* fermentation trial, a 26 % reduction of CH_4 was obtained (10). Moreover, when clove (*Syzygium aromaticum*), white thyme (*Thymus mastichina*) and anise (*Pimpinella anisum*) EO were used, CH_4 was reduced by 37, 76 and 32%, respectively (8). Likewise, when clove EO 200 mg was used, CH_4 was reduced by 84.21, 69.49 and 80.34%, respectively (9), when the substrate was a mixed diet, concentrated and hay, respectively, demonstrating the effect of PSM as CH_4 reducers.

Linamarin, a kind of CG, is found mainly in cassava (*Manihot esculenta Cranz*), with a higher concentration in bitter varieties. It has been observed that using cassava under *in vitro* conditions has an anti-methanogenic effect (11). When analyzing the concentrations of Linamarin in cassava cortex, it was found 28.40±3.38 g/kg of dry weight and 7.71±0.97 g/kg in fresh weight, while in cassava parenchyma it was found 14.71±1.91 and 5.77±0.74 g/kg in dry and fresh

form respectively (12). The effect of cassava on the CH_4 reduction *in vitro* fermentation, was demonstrated whit the inclusion of 12 mg/DM of root and leaf cassava. The CH_4 production was 70.50±1.32 and 65.70±1.32 mL/g of digested cassava root or leaf DM respectively, while when cassava was not included, the CH_4 production was 74.2±1.32 mL/g of digested DM (13).

Linamarin, has a potential to reduce CH_4 production in ruminants, when suitable doses are used. However, the effect of the inclusion of different doses of pure Linamarin *in vitro* is little known. The objective of this study was to quantify the effects of including rising doses of Linamarin on the CH_4 concentration in a period of time during *in vitro* ruminal fermentation.

MATERIALS AND METHODS

Animal handling. Two four-years-old rumen fistulated Merino Precoz sheep were used, which were fistulated in 2014 in accordance with the protocol of the Bioethics and Animal Welfare Committee of the Faculty of Veterinary Sciences of the University of Chile. They were handled at the Dryland Young and Grazing Ruminants section of Germán Greve Silva Experiment Station, which belongs to the Faculty of Agricultural Sciences of the University of Chile, located in Maipú Commune, Metropolitan Region, Chile (Lat. 33º 28' S and Long. 70º 51' W; 470 m.a.s.l.). The experiment was conducted at the Laboratory of the Department of Animal Production of the Faculty of Agricultural Sciences, University of Chile.

Diet. During the experimental period, the sheep were fed 1.2 kg of alfalfa hay and 300 g of oat grain per animal per day, dispensed in two portions, one given in the morning and the other in the afternoon. Table 1 shows the nutritional composition of the diet offered and the supplies that were used as fermentation substrates (FS) for incubation.

Table 1. Bromatological composition of the diet supplied and components.

	DM (%)	NDF (%)	ADF (%)	CP (%)	Energy (Mcal/kg DM-)
Alfalfa Hay	92.84	49.33	38.09	16.66	2.21
Oat Grain	89.89	28.69	13.79	11.06	2.72
Diet	91.37	39.01	25.94	13.86	2.50

MD: Dry Matter, NDF: Neutral detergent fiber, ADF: Acid detergent fiber, CP: Crude Protein, (Mcal/kg DM) Mega Calories per kilogram of DM.

Ruminal fluid collection (RF). On each incubation day, the collection of RF was carried out in the morning, in preprandial conditions. Two liters were collected during the three incubation days. The RF was filtered with a double-layer cotton cloth and

kept in thermoses, which were kept in a container with water at 39ºC, with the purpose of maintaining a stable temperature in order to be taken to the laboratory and to carry out the inoculation.

Experimental design. This study was repeated measures with a single factor having five levels, where Linamarin (purity ≥98%; Sigma-Aldrich Chemical, Darmstadt, Germany, Cat. No. 68264-50 mg) was tested in five different dosages (0, 6.0, 13.0, 20.0, and 26.0 mg/L). Each dose represented a treatment. In each treatment, a RF sample was supplemented with the respective Linamarin dose and *in vitro* incubated in 5 test tubes (repetitions), measuring CH_4 concentration hourly, during 8 hours of incubation. This protocol was repeated in three consecutive days.

Inoculation. The *in vitro* technique described by Theodorou et al (14) was used. Twenty-five 100 mL test tubes with a rubber stopper and Bunsen valve were used. The FS, consisting of 0.5 g of alfalfa hay and 0.5 g of 1 mm-ground oat grain, was put into each tube in a Wiley mill. Each test tube was added with RF 30 mL and buffer solution 40 mL. The buffer solution composition was: 238 mL macromineral solution (5.7 g Na_2HPO_4 + 6.2 KH_2PO_4 + 0.6g $MgSO*7H_2O$ + distilled water 1 L) plus 238 mL phosphate buffer solution (35 g $NaHCO_3$ + 4g NH_4HCO_3 + distilled water 1 L, pH 7.0) in 1 L distilled water (15).

Incubation. The tubes were placed on test tube racks and put into a thermoregulated chamber (Memmert 854, Germany) at 39ºC for a period of eight consecutive hours. The temperature was constantly monitored, and the tubes underwent rotary motion.

CH_4 measurements. This procedure was carried out every hour during a period of eight consecutive hours, using an RKI Eagle 2 gas monitor (RKI instruments, California, USA). This monitor is a high-precision instrument, using a thermal conductivity (infrared) sensor, capable of detecting CH_4 concentrations within a range of 0 to 50.000 ppm.

Statistical analysis. Data were analyzed by means analysis of variance, using Statgraphics 5.0 software. These were normally distributed, no differences were obtained when compared data between days of incubation, then data from different incubation days were analyzed together. The data were analyzed by time, treatment and the interaction time *vs* treatment when it was relevant. The differences among groups were analyzed using the Tukey test. Differences were considered when p≤0.05. Data are presented as group average±SEM. Additionality polynomial

contrasts were performed to determine linear or quadratic effects.

RESULTS

Reduction of CH_4 concentration. Table 2 shows the general averages of CH_4 concentrations, according to each Linamarin dose, the linear and quadratic effect, as well as the significance of the treatments, time of fermentation and the interaction time *vs* treatment.

Table 2. Mean concentration values of CH_4 at increasing doses of Linamarin in ruminal fermentation *in vitro*.

	Treatment (LIN concentration mg/L)				
	0	6	13	20	26
CH4 (ppm)	2738[a]	2472[a]	2485[a]	2243[ab]	1932[b]

SEM[1]	P[2]			P[3]	
	T	H	T*H	L	Q
154.2	<0.001	<0.001	<0.001	0.002	0.015

[a, b] Means with different letters are different (p≤0.05). [1]SEM, Standard error of the mean; [2]Probability of differences between treatments (T), sampling schedule (H) and interaction (T*H);[3]Probability linear (L) or quadratic (Q) to concentration of LIN.

The CH_4 reductions in relation to the doses (6, 13, 20, 26 mg/L) were 9.7, 9.2, 18.1 and 29.4%, respectively. When the CH_4 concentration values were regressed in accordance with the treatments, a significant linear (p=0.002) and quadratic (p=0.015) effect was obtained in the reduction of CH_4 with increasing doses of Linamarin.

The analysis of the data by incubation time shows a reduction (p≤0.05) in CH_4 concentration (Figure 1), where hours 1, 2 and 3 were the higher (p≤0.05), but similar among them.
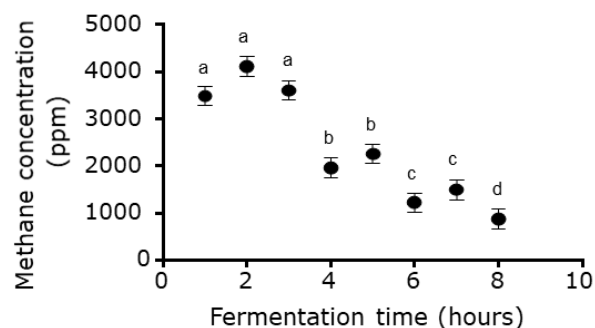

Figure 1. Reduction of methane concentration during eight hours of fermentation whit Linamarin.

A significant decrease begun at hour 4 of incubation, with a CH_4 concentration of 3.480±20 ppm; this value was similar to that of hour 5. Low concentrations of CH_4 were obtained at hours 6 and 7 of incubation, with values of 1.218±20 and

1.494±20 ppm, respectively. Hour 8 was the lowest in CH_4 concentration with 840±20 ppm. When the CH_4 concentration values were regressed as a function of incubation time, a significant decreasing linear ratio was obtained (R^2=0.843; p≤0.001).

An interaction between time of incubation and treatments was obtained (p≤0.005). The less value of CH_4 concentration was obtained with the higher dose of Linamarin at hour 1. At this time, CH_4 concentration increased while Linamarin dose decreased (Figure 2), showing a dose/response effect. However, after 2 hours of fermentation, a similar CH_4 reducing pattern throughout the rest of the fermentation time was observed.
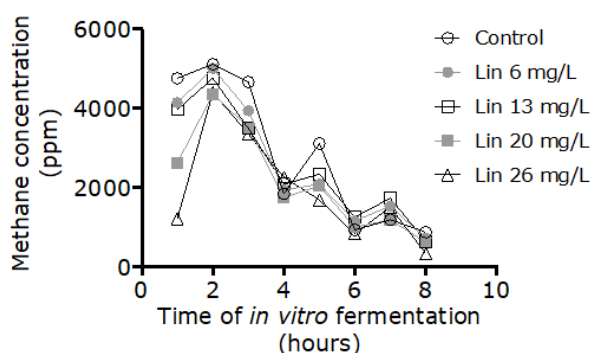
Figure 2. Effect of five doses of Linamarin on the production of CH_4, during eight hours of *in vitro* fermentation.

DISCUSSION

No studies regarding the use and dosage of pure Linamarin in the reduction of CH_4 were found in literature. As was previously mentioned, Linamarin is a PSM present in cassava. Concentrations of Linamarin in cassava fluctuate according to the variety, place, age of the plant, harvest time and how it is supplied in diets. In one reported analysis, 538.4±4.91 mg of Linamarin were found in 100g DM cassava, while 102.3±0.93 mg of Linamarin was obtained from 100 mL of aqueous mixture, therefore, it is important to consider the way in which cassava is offered, when a specific dose of Linamarin must be supplied (16).

The use of bitter varieties of cassava as a food additive has been demonstrated to reduce CH_4 concentrations. In this regard, Phuong et al. (11) obtained a 50 % CH_4 reduction in a 24 hours *in vitro* fermentation assay when included 2.16 g/DM of bitter cassava leaves. We have estimated the amount of Linamarin included in the previously described experiment, in accordance to Maherawati et al (16), although these authors did not report the used cassava variety for the analysis. Thus, the inclusion of bitter cassava had an equivalent to 11.62 mg Linamarin. Then, the results of Phoung et al (11) are similar to the results obtained in the present study, with the dose of 13 mg/L of pure Linamarin. Other results reported by Inthapaya and Preston (17), when they supplemented with 12 g/DM of cassava leaf flour, with an equivalent of 64 mg/Linamarin, obtaining the lowest CH_4 values, with a reduction of 35.5% CH_4 at 24 hours of *in vitro* fermentation. These percentage of reduction is higher than the maximal reduction obtained in our study. However, the inclusion of Linamarin used by Inthapaya and Preston (17) was about 2 folds than the maximal dose used in our experiment, confirming the dose/response effect of the Linamarin on CH_4 reduction in *in vitro* ruminal fermentation.

The findings of this research show that the four Linamarin doses had a linear response in the reduction of CH_4 concentrations. This result is primarily related to the way in which Linamarin was used, since having ≥98% purity makes it highly available for rumen microorganisms. In this regard, when Do et al (18) used cassava leaves as substrate, plus cassava root flour as an additive, equivalent to a 10.76 mg/Linamarin, they obtained CH_4 32.2 mL/g of substrate, while when they did not include the additive, CH_4 43.5 mL/g substrate was obtained. Furthermore, the addition of cassava root flour to the substrate lead to reduce total gas production from 237 mL to 197 mL.

The regression of the incubation time *vs* CH_4 concentration, show that the addition of Linamarin into the incubation with sheep RF, presents a trend to reduce CH_4 concentration. PSM have a period of action that could vary depending on the doses given, the diet of the animal (primarily) and the antimicrobial capacity, that leads to the reduction of protozoa and methanogenic bacteria, which as a result leads to a constant reduction of CH_4 in a certain period (19,20).

Previous studies have demonstrated that fermentation is influenced by the type of process in the substrates used as Linamarin source. In this regard, Inthapaya et al (21) assessed the effects using fresh, ensiled, sun- or oven-dried cassava leaves on *in vitro* CH_4 production, observing that the CH_4 percentage was lower for fresh and ensiled leaves compared to sun- and oven-dried leaves, with values of 12, 11, 14 and 15% of CH_4, respectively. The fresh leaves of cassava contain a greater amount of Linamarin, therefore the reduction of CH_4 is greater in comparison to dry leaves of cassava, which due to drying process, decreases the concentration of Linamarin (21). These results are in agreement with those of the

present study, in which higher doses of Linamarin results in less CH_4 concentration. However, Inthapaya et al (21), also observed that as the fermentation time elapses, the total gas production increased, as well as the percentage of CH_4 from 11 to 16% from 12 to 24 hours of fermentation. It has been observed that in long fermentation times, the CH_4 reducing activity tends to decrease, probably due to a habituation of the microorganisms to the additive inclusion and/or to a reduction of the additive effectiveness (22,23).

In conclusions this study demonstrates that the addition of Linamarin to the rumen fermentation substrate reduces CH_4 production in a dose-dependent manner. Also, the reducing action of the methanogenesis of Linamarin is dependent on the fermentation time. This study constitutes a basis for any future tests including vegetable sources of Linamarin as well as other rumen variables and longer *in vitro* fermentation times.

Conflict of interests

There is no conflict of interest.

Acknowledgements

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