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RESEARCH ARTICLE

Linamarin-eugenol and its combination as food additive on methanogenic bacteria, fermentation parameters and methane production in vitro

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ABSTRACT

Secondary plant metabolites (SPMs) can influence the reduction of enteric methane (CH_4) emissions in ruminants. This study aimed to evaluate the effects of supplementing diet with cyanogenic glucoside linamarin (LIN), essential oil eugenol (EU), and their combination (LIN+EU) on methanogenic microorganisms, CH_4 production, and rumen fermentation parameters in vitro. The basal diet (BD) (alfalfa [*Medicago sativa* L.] hay and oat [*Avena sativa* L.] grain in a 3:1 ratio) was supplemented with LIN, EU, and LIN+EU mixtures and placed in amber bottles. The experimental treatments were: T1, BD (control); T2, BD-LIN (20 mg L^{-1}); T3, BD-LIN (40 mg L^{-1}); T4, BD-EU (400 mg L^{-1}); T5, BD-LIN+EU (20 mg L^{-1} + 400 mg L^{-1}); and T6, BD-LIN+EU (40 mg L^{-1} + 400 mg L^{-1}). The treatments were inoculated with rumen fluid and incubated for 6, 12, and 24 h to assess abundance of total bacteria (TB), methanogenic archaea, and ruminal fermentation parameters. The CH_4 production was significantly reduced ($p < 0.001$) with inclusion of LIN, EU, and LIN+EU, particularly at 12 and 24 h incubation. However, at these same time points, in vitro DM disappearance (IVDMD) was also reduced ($p < 0.001$), except when LIN was included alone, highlighting its advantage in this variable. Rumen pH remained relatively stable at 6 and 12 h but decreased ($p < 0.001$) at 24 h with LIN. The pH values ranged between 6.65 (minimum) and 6.85 (maximum), which are optimal for microbial activity. Total bacterial abundance (TB) was not affected ($p > 0.05$) by treatments, but methanogenic archaea abundance was significantly reduced ($p < 0.001$) at 24 h with the LIN+EU mixtures (T5 and T6), coinciding with the highest CH_4 reductions of 36.9% and 56.7%, respectively. At 24 h, IVDMD with LIN alone was 51.83%, whereas EU alone resulted in 46.48%. In conclusion, LIN+EU mixtures T5 and T6 had a synergistic effect, effectively reducing CH_4 emissions, demonstrating the combined impact of different SPMs mechanisms of action.

Key words: Archaea, methanogenesis, mitigation, ruminal metabolism, secondary metabolites.

INTRODUCTION

Livestock production faces a major challenge: Producing meat and milk for a rapidly growing population, but at the same time reducing its environmental impact. Ruminants produce CH_4, a greenhouse gas, which is a product of ruminal fermentation, representing an energy loss that could be used to produce more meat or milk (Eckard and Clark, 2018; Kleppel, 2020). Several studies have demonstrated CH_4 reduction through dietary manipulation of ruminal fermentation via inclusion of plant secondary metabolites (PSMs), including essential oils (EOs), tannins, N compounds, flavonoids, and saponins. These compounds employ distinct mechanisms of action, representing viable alternatives for CH_4 mitigation (Eckard and Clark, 2018; Ku-Vera et al., 2020).

Furthermore, less-studied metabolites such as cyanogenic glycosides show promising potential for reducing ruminal CH_4 production (Phuong et al., 2015; Zavaleta et al., 2019).

Essential oils, such as eugenol (EU), oregano (*Origanum vulgare* L.), rosemary (*Rosmarinus officinalis* L.), cinnamon (*Cinnamomum verum* J. Presl), garlic (*Allium sativum* L.), reduce methanogenesis by reducing methanogenic archaeal populations, and influence fermentation and ruminal parameters, due to their antimicrobial activity (Patra and Yu, 2015; Cobellis et al., 2016a; Garcia et al., 2020). The EO could have effect in the microorganism's Gram-negative type; the aromatic hydrocarbons destroy the external membrane (Ugbogu et al., 2019). The challenge in using these metabolites lies in defining the optimal doses to reduce methanogenic Archaea, without affecting ruminal parameters. Benetel et al. (2022) reported that the EO from oregano and white thyme (*Thymus vulgaris* L.) reduced net CH_4 production at 250 mg L^{-1} without negative effects on feed digestibility. In addition, Garcia et al. (2020) evaluate increasing doses (0, 0.3, 3, 30, and 300 µL L^{-1}) of EO of *Lippia turbinata* Griseb or *Tagetes minuta* L., finding that the intermediate dose (30 µL L^{-1}) inhibited CH_4 with a slight reduction in substrate digestibility.

Cassava (*Manihot esculenta* Crantz) is an important food crop for human and animals, cassava leaves and peel have high levels of PSM, especially better varieties, such as, cyanogenic glycosides. Linamarin (LIN) is the predominant form, accounting for 93% (Ndubuisi and Chidiebere, 2018; Boukhers et al., 2022). Cyanogenic compounds inhibit cytochromes present in methanogens (Ortiz et al., 2014). Several studies have investigated cassava, few have focused specifically on LIN. Inthapanya and Preston (2014) included cassava leaf meal at level of 6 g crude protein per 100 g substrate in vitro, CH_4 production was reduced by 2.8% at 24 h. On the other hand, Zavaleta et al. (2019) showed that with increasing doses of LIN (6, 13, 26, and 30 mg L^{-1}), CH_4 was proportionally reduced by 9.7%, 9.2%, 18.1%, and 29.4%, respectively.

The present study aimed to demonstrate the potential of PSMs of different origin and action mechanisms could imply a synergy between them, reducing CH_4 production, without negatively affecting digestibility or ruminal fermentation. The effect of LIN combined with other PSMs, is unknown. Therefore, the objective of this research was to evaluate the effect of including LIN, EU, and their combinations (LIN+EU), on methanogenic microorganisms, ruminal fermentation parameters and methanogens in vitro.

MATERIALS AND METHODS

The study was conducted at the Nutrition Laboratory of the Department of Animal Production, School of Agricultural Sciences, Universidad de Chile, and the Livestock Systems and Nutrition Laboratory, Universidad de Concepción (UdeC), Chillán, Chile. The experiment followed protocols approved by the UdeC Animal Welfare and Ethics Committee (CBE-13-2019).

Incubation was performed according to Avila et al. (2011). Continuous CO_2 infusion was maintained in all bottles throughout the incubation period. Fermentation substrates were alfalfa hay (*Medicago sativa* L.) and oat grain (*Avena sativa* L.), ground to 2 mm in a mill (grain mill, Breuer, Temuco, Chile), which was used to prepare the base diet (BD) in a 3:1 ratio (Table 1). Substrates and BD were determined at the UdeC Animal Nutrition Laboratory. Dry matter (DM; #934.01), ash (CT; #942.05), crude protein (CP; #945.01) and acid detergent fiber (ADF; #973.18) were determined according to AOAC (1995) methodology and neutral detergent fiber (NDF) as described by Mertens (2002).

For the design of the treatments, linamarin (LIN) (purity ≥ 98%; Sigma-Aldrich Chemical, Darmstadt, Germany), eugenol (EU) (≥ 98% purity; Sigma-Aldrich Chemical, Chile), and LIN+EU were added to BD prepared in bottles, generating six treatments: BD without additives (control) (T1); BD + 20 mg LIN L^{-1} (T2); BD + 40 mg LIN L^{-1} (T3); BD + 400 mg EU L^{-1} (T4); BD + 20 mg LIN L^{-1} + 400 mg EU L^{-1} (T5); and BD + 40 mg LIN L^{-1}+ 400 mg EU L^{-1} (T6). The doses of 20 and 40 mg L^{-1} LIN were selected based on the study by Zavaleta et al. (2019), as these concentrations were found to reduce CH_4 production. The doses of 400 mg L^{-1} EU were chosen based on previous research suggesting that this concentration is effective in reducing methanogenesis without negatively affecting ruminal fermentation (Ortiz et al., 2014; Cobellis et al., 2016b).

Table 1. Bromatological analysis of the substrates and diet. DM: Dry matter; NDF: neutral detergent fiber; ADF: acid detergent fiber; TA: total ash; CP: crude protein. [1]Basal diet 750 g alfalfa hay kg^{-1} DM + 250 g oat grain kg^{-1} DM.

Components	Ingredients		Basal diet (%)[1]
	Alfalfa hay	Oat grain	
DM	92.84	89.89	91.36
NDF	49.33	28.69	39.01
ADF	38.09	13.79	25.94
TA	12.29	1.93	10.36
CP	16.66	11.06	13.96

Inoculation and incubation of the in vitro culture

The ruminal liquid (RL) was obtained from two cannulated Aberdeen Angus cows, fed *ad libitum* on a diet based on alfalfa hay, oat grain, and a vitamin-mineral mixture in a (70:25:5) ratio. The RL was extracted post-prandially, in a thermos at 39 °C, and immediately taken to the laboratory where it was filtered using a four layers cotton cloth. The RL was immediately poured into a glass flask at 39 °C, containing Menke buffered solution in a 1:3 ratio.

The BD (0.5 g) was placed in pre-weighed ANKOM F57 bags (Macedon, New York, USA) heat-sealed, and allocated to 50 mL amber bottles, according to treatment groups prior to the application of LIN, EU and LIN+EU were applied. Afterwards, bottles were inoculated with 25 mL inoculum for in vitro fermentation. Three replicates for each treatment and two blank replicates (no treatment) were incubated during 6, 12 or 24 h in three consecutive days to evaluate net gas production (NGP) and in vitro DM disappearance (IVDMD). Incubation temperature was maintained at 39 °C (Form Series II 3110 WaterJacketed CO_2, Thermo Fisher Scientific, Waltham, Massachusetts, USA), with an orbital shaker (Unimax, Heidolph, Schwabach, Germany) at 90 oscillations min^{-1} and constant CO_2 supplementation in the atmosphere (Avila et al., 2011). A total of 180 bottles were incubated.

Determination of total gas and CH_4 by gas chromatography

At each incubation time, bottles were removed from the incubator to measure NGP by water displacement (Avila et al., 2011). Prior to each measurement, a 15 mL sample of gas was removed and placed in a vacuum exetainer tube (Labco Ltd., High Wycombe, UK). The CH_4 was measured in a gas chromatograph (Agilent 7890B, Agilent Technologies, Santa Clara, California, USA), equipped with a thermal conductivity detector and 30 m column (GS-CarbonPLOT, Agilent Technologies). The column oven, injector and detector temperature were set to 35, 185 and 150 °C, respectively. A 2 mL gas subsample was manually injected into the gas chromatograph (GC) from each Exetainer vial. The CH_4 gas used to prepare the standards was of analytical quality (99.5%; Linde Group, Santiago, Chile). The stock CH_4 gas was diluted with N gas (Linde Group) at room temperature (≈ 22-24 °C) to obtain standards of 15.0%, 10.0%, 7.5%, 5.0%, 2.5% and 1.0% CH_4.

Determination of pH and in vitro DM disappearance (IVDMD)

After measuring the gas and sampling for CH_4, the pH of each bottle, treatment and incubation time was measured with a portable pH meter (Orion Star A121 pH, portable meter kit, RK-58825-08, Thermo Scientific, Toronto, Ontario Canada). Subsequently, each bag containing residual BD was extracted from the corresponding bottle, washed with distilled water, and dried for a period of 24 h, at 60 °C, in an oven (Memmert UN 55, Deutschland, Germany), for IVDMD determination.

Determination of ammoniacal N (NH_3-N) concentration

Once the bottles were opened, a sample of 1.5 mL was taken from each treatment and incubation period and transferred in 2 mL cryovials containing 150 µL 10% trichloroacetic acid. The samples were stored at -20 °C until determination of NH_3-N content. The concentration of NH_3-N was measured a UV-VIS spectrophotometer (Spectroquant Pharo 300, Merck KGaA, Darmstadt, Germany).

Extraction and quantification of bacterial DNA

Samples (2 mL) were taken from each treatment bottle and stored in Eppendorf tubes at -80 °C until processing. Prior to analysis, the samples were freeze-dried (Christ Alpha, model 1-4 LSC, Martin Christ, Osterode am Harz, Germany) at 57 °C. Subsequently, DNA extraction was performed following the protocol described by Yu and Morrison (2004). The quality and quantity of DNA obtained was determined in a micro-spectrophotometer (Nano-400, Hangzhou, China), at a wavelength of 260 nm. The DNA concentration was estimated in ng μL^{-1} and DNA purity was estimated by the ratio of absorbance at 260/280 nm, with a range of 1.64 and 2.90.

Amplification and quantification of bacterial populations by qPCR

The extracted DNA was diluted 1:10 before qPCR analyses on a thermal cycler (LightCycler Nano, Roche Life Science, Indianapolis, Indiana, USA). Previously validated primers targeting bacterial 16S ribosomal coding DNA were used. The primer sequences are described in Table 2. The following conditions were used for PCR: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, then a denaturation curve. Quantification was performed using the thermal cycler (LightCicler real-time PCR system, Roche Diagnostics, Indianapolis, Indiana, USA) using 10 µL Power SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK), 0.9 µL each primer, 2 µL DNA sample and 6.20 µL MilliQ water to a final volume of 20 µL.

Table 2. Sequence of primers for quantitative PCR. bp: Base pairs. Both primers were obtained from IDT& Fermelo-Chile Portal Group. Integrated DNA Technologies (IDT) Fermelo Biotec, Santiago, Chile.

Target species	Sequence	Size bp	Reference
Total bacteria	f-CGGCAACGAGCGCGAACCC r-CCATTGTAGCACGTGTGTAGCC	130	Denman and McSweeney (2006)
Methanogenic archaea	f-TTCGGTGGATCDCARAGRGC r-GBARGTCGWAWCCGTAGAATCC	140	Denman et al. (2007)

Quantification of total bacteria and methanogenic archaea

To quantify bacterial DNA, a standard curve was generated with serial dilutions of known DNA concentrations (1×10^{1} to 1×10^{5} ng μL^{-1}). The standard curve was generated using a series of known concentrations of bacterial DNA; each dilution was measured by a spectrophotometer. The resulting standard curve allows the quantification of bacterial DNA in unknown samples. Fluorescence values obtained from qPCR of the samples were recorded after each extension step and quantification cycle (qc). This value was related to the logarithm (base 10) of the standard concentrations to calculate the amount of bacterial DNA in each sample. The efficiency (€) of the PCR was calculated with the formula $€ = [10^{(-1/slope)} - 1] \times 100\%$ (Saro et al., 2014).

Statistical analysis

The treatments were distributed in a factorial design (3×2) with randomized blocks, where the factors are represented by the doses of LIN (0, 20 and 40 mg L^{-1}), EU (0 and 400 mg L^{-1}) and their interaction (LIN×EU). The blocks were each day of the test run. The variables were analyzed using a linear mixed model with six treatments, considered as the fixed effects (n = T1 - T6) and random effect were the incubation run (n = 3 d) and the fermentation time (6, 12 and 24 h). The experimental unit was represented by each bottle (n = 3). The data were analyzed using the R platform within the InfoStat statistical software (Grupo InfoStat, FCA, Universidad Nacional de Córdoba, Cordoba, Argentina). Significant differences were considered when $p \leq 0.05$. Data are showed as average ± SEM.

RESULTS

Response of total bacteria (TB) and methanogenic archaea

The results showed that, at 24 h fermentation, methanogenic archaea were reduced ($p < 0.001$) with treatments 5 and 6 corresponding to LIN 20 or 40 mg + EU 400 mg (Figure 1). However, TB was not reduced ($p > 0.05$).

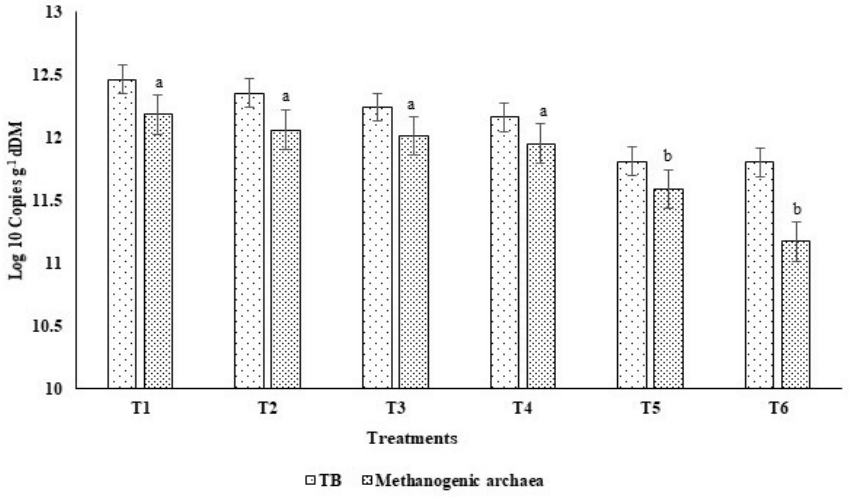

Figure 1. Effect of treatments on total bacteria (TB) and methanogenic archaea at 24 h in vitro fermentation. BD: Base diet; LIN: linamarin; EU: eugenol; T1: (control); T2: BD + 20 mg LIN L^{-1}; T3: BD + 40 mg LIN L^{-1}; T4: BD + 400 mg EU L^{-1}; T5: BD + 20 mg LIN L^{-1}+ 400 mg EU L^{-1}; T6: BD + 40 mg LIN L^{-1} + 400 mg EU L^{-1}. Different letters indicate differences ($p \leq 0.05$) of the same variable in the different treatments.

Relationship between Archaea and CH_4

Figure 2 shows the response of methanogenic archaea to treatments at 6 h (Figure 2a), 12 h (Figure 2b) and 24 h fermentation (Figure 2c). At 6 h, there was no effect of LIN, EU and LIN+EU treatments on CH_4 production ($p \geq 0.05$). However, at 12 and 24 h fermentation (Figures 2b and 2c) a decrease of CH_4 was present as response to the addition of these PSMs ($p < 0.001$), meanwhile, methanogenic archaea showed a decrease at 24 h fermentation in vitro in presence of mixtures of LIN+EU ($p < 0.001$).

Net gas production (NGP) and ruminal fermentation parameters

The inclusion of LIN, EU and its combinations did not affect ($p \geq 0.05$) NGP (mL) at 6, 12 and 24 h in vitro fermentation (Table 3). Regarding ruminal fermentation parameters, at 6 h in vitro fermentation, the inclusion of LIN, EU and its combinations had no effect ($p \geq 0.05$) on IVDMD, pH and NH_3-N. However, at 12 h fermentation, the inclusion of EU and LIN+EU, decreased IVDMD ($p \leq 0.05$), while pH and NH_3-N were not affected at this time with the treatments. At 24 h fermentation, IVDMD also decreased ($p < 0.001$), in the same treatments as at 12 h, while pH increased with the inclusion of EU and LIN+EU mixtures ($p < 0.001$). The NH_3-N showed no differences between treatments ($p \geq 0.05$).

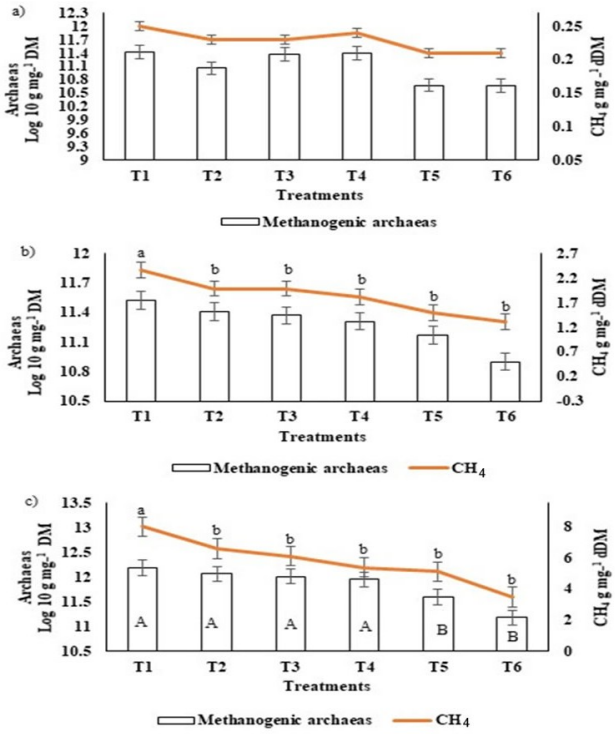

Figure 2. Effect of treatments on methanogenic archaea and CH_4 production at 6 h (a), 12 h (b), and 24 h (c) of ruminal fermentation. BD: Base diet; LIN: linamarin; EU: eugenol; T1: (control); T2: BD + 20 mg LIN L^{-1}; T3: BD + 40 mg LIN L^{-1}; T4: BD + 400 mg EU L^{-1}; T5: BD + 20 mg LIN L^{-1} + 400 mg EU L^{-1}; T6: BD + 40 mg LIN L^{-1} + 400 mg EU L^{-1}. Means with different letters indicate differences in CH_4 production per digested DM (dDM). Means with different capitalized literals indicate differences in methanogenic archaea ($p \leq 0.05$).

Table 3. Effect of linamarin (LIN), eugenol (EU) and their mixture on net gas production (NGP), in vitro DM disappearance (IVDMD), pH and NH_3-N at three fermentation times in vitro. Means with different letters in the same row indicate differences ($p \leq 0.05$). T1 = Base diet (BD) control; T2 = BD + 20 mg LIN L^{-1}; T3 = BD + 40 mg LIN L^{-1}; T4 = BD + 400 mg EU L^{-1}; T5 = BD + 20 mg LIN L^{-1} + 400 mg EU L^{-1}; T6 = 40 mg LIN L^{-1} + 400 mg EU L^{-1}. SEM: Standard error of the mean.[1]Probability of significant difference between: Treatments (T), LIN, EU and interaction LIN×EU.

	Treatments							*p*-value[1]			
Item	T1	T2	T3	T4	T5	T6	SEM	T	LIN	EU	LIN×EU
0-6 h											
NGP, mL	68.24	66.06	62.23	59.37	56.61	56.71	7.75	0.15	0.49	0.11	0.84
IVDMD, %	42.50	42.22	42.32	41.71	41.21	41.44	1.40	0.15	0.65	0.14	0.96
pH	6.85	6.86	6.86	6.86	6.88	6.86	0.04	0.74	0.46	0.56	0.68
NH_3-N, mg dL^{-1}	6.38	5.87	6.10	7.17	6.24	6.29	1.73	0.95	0.86	0.74	0.81
0-12 h											
NGP, mL	124.93	123.12	119.61	116.70	116.04	114.90	10.97	0.96	0.93	0.38	0.97
IVDMD, %	45.33^a	45.30^a	45.37^a	43.63^b	44.41^b	44.33^b	1.16	< 0.001	0.338	< 0.001	0.31
pH	6.75	6.85	6.74	6.74	6.73	6.74	0.05	0.44	0.55	0.60	0.19
NH_3-N, mg dL^{-1}	6.95	6.62	6.41	6.24	6.41	6.26	1.54	0.81	0.82	0.32	0.76
0-24 h											
NGP, mL	185.87	181.33	177.26	174.40	164.16	161.02	15.50	0.08	0.72	0.22	0.98
IVDMD, %	52.70^a	52.62^a	51.83^a	46.48^b	46.87^b	46.34^b	1.01	< 0.001	0.37	< 0.001	0.72
pH	6.62^b	6.62^b	6.65^b	6.70^a	6.71^a	6.71^a	0.03	< 0.001	0.26	< 0.001	0.62
NH_3-N, mg dL^{-1}	8.93	8.86	8.37	8.30	8.09	8.06	0.09	0.74	0.72	0.18	0.90

DISCUSSION

Total bacteria and methanogenic archaea

In the scientific literature, exist several reports describing experiments with supplementation of essential oil (EO) mixtures (Castro-Montoya et al., 2015; Günal et al., 2017) for CH_4 reduction in ruminants, but no trials with mixtures of cyanogenic glycosides with EO have been reported. In the present study, the reducing methanogenic archaea plant secondary metabolites (PSMs) (Günal et al., 2017; Zavaleta et al., 2019), eugenol (EU) and linamarin (LIN), and its combinations were probed as alternative supplementation strategy for ruminal CH_4 reduction.

Results indicate that 0.5 mg LIN + 10 mg EU and 1.0 mg LIN + 10 mg EU reduced CH_4 by 36.9% and 56.7%, respectively, at 24 h in vitro fermentation. These results concur with the major reduction of methanogenic archaea by 4.8% and 8.3%, respectively. The effect of PSMs on various ruminal microorganisms depends on the chemical composition of the metabolite, dose and base diet, among other factors (Patra et al., 2017). One pathway for CH_4 reduction is the decrease in methanogenic bacteria among other microorganisms through PSMs (Zhou et al., 2020). Our study is the first using LIN+EU, confirming that its mixtures can decreases methanogenic archaeal populations and in turn reducing CH_4.

Previous research evaluated the effect of different EO on the abundance of total bacteria and archaea methanogenic bacteria (Patra and Yu, 2012) by qPCR, finding a linear reduction with increasing doses of EO. The highest dose of 1 g L^{-1} for EU oil and oregano (*Origanum vulgare*) resulted in the lowest concentration of bacteria 38.4% and 12.4%, regarding control.

Net gas (NGP) and CH_4 production

No effect of treatments on NGP were observed in any of the three in vitro fermentation times. Regarding the production of CH_4, the inclusion of 20 mg L^{-1} LIN had a 17.6% reduction effect at 24 h, a value similar to those obtained in a previous experiment of our group (Zavaleta et al., 2019) with the same dose, confirming the effect of LIN on CH_4 reduction. Inthapanya and Preston (2014) showed that by adding cassava leaf meal (64 mg LIN L^{-1}); they obtained a 35.5% reduction of CH_4 at 24 h fermentation. However, this percentage was lower than that reported in the same study, at the same fermentation time, with a dosage of 40 mg L^{-1}. As it is evident, the doses of LIN used by Inthapanya and Preston (2014) were higher than that used in our study. According to Zagrobelny et al. (2004), LIN can be toxic in high doses and could be detrimental in the IVDMV. Therefore, the PSMs mixture can be an alternative that complies with CH_4 reduction; however, these compounds should be administered in small doses to minimize adverse effects on ruminal fermentation parameters.

In vitro DM disappearance (IVDMD)

The IVDMD was not affected with the inclusion of 20 and 40 mg L^{-1} LIN, with values of 52.6% and 51.8% at 24 h, respectively. These values could increase with longer incubation time. However, Soto et al. (2009) mention that a digestibility above 55% is considered acceptable, values which are very close to that observed in the LIN treatments. In the present study, IVDMD was significantly reduced with 400 mg L^{-1} EU (considered an intermediate dose), obtaining 43.6% and 46.5% at 12 and 24 h, respectively, which was concomitantly associated to a CH_4 reduction. It has been described for Patra and Yu (2012) that increasing doses of EO can linearly reduce IVDMD, since bacterial populations are affected. In a trial that included an EU 500 mg L^{-1} (Günal et al., 2017), a higher dose than that used in our research, a digestibility of 50.7% and a 30.0% reduction of CH_4 were obtained. This reduction, which is lower, compared with that of our study (32.7%), in the same in vitro fermentation time. This result could be due to the fact that in our trial we used EU with a purity of 98%, while in the aforementioned work clove oil (*Syzygium aromatic*), the spice from which EU is obtained, was used. The 500 mg L^{-1} dose of EU used by Günal et al. (2017) showed no negative effect on pH and volatile fatty acids, so the doses could be comparable.

pH and NH_3-N

The inclusion of LIN, EU and its mixture did not affect pH or NH_3-N, in any of the three in vitro fermentation times, being both found in ranges considered optimal, pH 6.2-6.7 (Agarwal et al., 2015; Marte-Pereira et al., 2025), NH_3-N 5-25 mg dL^{-1} (Cheeke, 2004). By including 20 and 40 mg L^{-1} LIN, pH was 6.86 for both at 6 h in

vitro fermentation, remained at 6.85 and 6.74, respectively, at 12 h and finally reduced to 6.62 and 6.65 at 24 h. Even with the obtained reduction, the pH is within the optimal values. At 24 h in vitro fermentation, the treatments with EU and LIN+EU had pH values of 6.70 and 6.71, respectively, which were higher, as compared to the two doses of LIN at the same time. Regarding the inclusion of LIN and in accordance with Sepulveda et al. (2011), this response could be influenced by the numerical increase of NH_3 indicating that the higher the ruminal pH, the higher the non-ionized or free proportion of NH_3. Baraka and Abdl-Rahman (2012) obtained lower pH value (6.3) with the same EU dose as in the current trial. It should be considered that pH values below 6.0 could be detrimental to cellulolytic and amylolytic bacteria by reducing IVDMD (Agarwal et al., 2015).

The NH_3-N concentration was not affected by the addition of EU, LIN and its combinations. The average values among treatments were 6.3, 6.4 and 8.4 mg dL^{-1} at 6, 12 and 24 h in vitro fermentation, respectively. These values allow for microbiota to remain stable and a proper synthesis of microbial protein (Lu et al., 2019). According to the observed NH_3-N values, the doses of LIN and EU used in our trial did not affect the proteolytic bacteria. Baraka and Abdl-Rahman (2012) used a dose of 400 mg L^{-1} EU and obtained similar NH_3-N values (8.7 mg dL^{-1} at 24 h). However, they added 0.5 mg L^{-1} fumaric acid. According to Newbold and Rode (2006), fumaric acid is a metabolic precursor of propionate, acting as a sink for the H^+ remaining available in ruminal metabolism, also reducing CH_4 concentration.

Considering the results obtained in this study, LIN is a glycoside that should be further investigated at different doses, either alone or in combination with PSMs, to determine which types of bacteria or other microorganisms may be affected. Additionally, given the presence of LIN in bitter cassava varieties, it could serve as a low-cost mitigation strategy for producers with access to this crop.

It is interesting to note that in countries such as Colombia and Mexico, it is common for producers to offer cassava to livestock, either the foliage or the root, without taking into account the amount of LIN that may be in the diet provided (Wanapat and Kang, 2015), so it is of interest to continue this line of research.

CONCLUSIONS

The inclusion of the cyanogenic glucoside linamarin (LIN), the essential oil eugenol (EU) and its combinations in diets for ruminal in vitro fermentation, showed to be effective for the reduction of ruminal methanogenesis at 12 and 24 h in vitro fermentation, without causing significant changes in other variables of ruminal metabolism, such as pH and NH_3-N, that could influence animal health and productivity. The use of EU decreased in vitro DM disappearance, in contrast to LIN, which did not affect this parameter. The LIN+EU combinations synergize in the reduction of methanogenic bacteria and CH_4 at 24 h in vitro fermentation. These results open an important possibility for the study of the inclusion of natural sources of plant secondary metabolites in ruminant diets as antimethanogenic additives, contributing to the reduction of greenhouse gases.

Author contribution

Conceptualization: C.Z-C. Methodology: N.V-A., V.B-V. Software: D.J-A. Validation: J.A-S. Formal analysis: D.J-A. Investigation: N.V-A. Resources: C.Z-C. Data curation: E.E.S-R. Writing-original draft: C.Z-C. Writing-review & editing: N.V-A. Visualization: N.V-A. Supervision: J.A-S. Project administration: V.H.P-G. Funding acquisition: C.Z-C. All co-authors reviewed the final version and approved the manuscript before submission.

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