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RESEARCH ARTICLE

# Effects of linseed and glycerol inclusion in concentrate ruminant diets on methane production using a Rusitec semicontinuous system

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## ABSTRACT

Improving the quality of ruminant diets can be an effective strategy for mitigating enteric methane ($CH_4$) production. The aim of this study was to assess the effects of increasing concentrations of glycerol in concentrate diets supplemented with linseed on $CH_4$ production and rumen fermentation parameters. Experimental diets were: Control (61%:25%:14% corn, mixed hay, and soy); positive control (51%:25%:9%:15% corn, mixed hay, soy, and linseed); Gly 5% (46%:25%:9%:15:5% corn, mixed hay, soy, linseed, and glycerol); and Gly 10% (40%:25%:10%:15%:10% corn, mixed hay, soy, linseed, and glycerol). Diets were incubated in a completely randomized design in a Rusitec apparatus (10 d adaptation, 5 d sampling). Differences were analyzed using Tukey's test, contrasts, and principal component analysis. Results showed that linseed supplementation reduced $CH_4$ ($P \leq 0.004$) and ammonia N ($NH_3$-N; $P = 0.012$) production. However, while the use of glycerol increases $CH_4$ production ($P \leq 0.002$) in linseed-supplemented diets, it simultaneously reduces $NH_3$-N production ($P = 0.004$). Linseed supplementation decreased the degradability of DM, crude protein, and non-fibrous carbohydrates but increased ether extract (EE) degradability ($P \leq 0.013$). Similarly, glycerol enhanced EE degradability and increases DM degradability in linseed-supplemented diets ($P \leq 0.006$). Both linseed and glycerol shift the fermentation profile by decreasing acetate proportions ($P \leq 0.002$) and increasing propionate proportions ($P < 0.001$), which reduces the acetate-to-propionate (A:P) ratio ($P < 0.001$). Overall, the combination of linseed and glycerol in concentrate diets reduces $NH_3$-N production without lowering $CH_4$ emissions and may even increase them. This combination also decreases the A:P ratio and enhances EE and DM degradability in linseed-added diets.

Key words: Biodiesel, corn, methanogenesis, mitigation, oilseed, rumen.

## INTRODUCTION

Livestock production plays a key role in the global food system supporting over 8 billion people (Shinkai et al., 2024). Ruminant production, in particular, faces the challenge of providing quality food to the growing population while being scrutinized for its contribution to greenhouse gases (GHG) emissions (Herrero et al., 2016). Carbon dioxide ($CO_2$), methane ($CH_4$), and nitrous oxide ($N_2O$) are GHGs that absorb infrared radiation, thereby contributing to global warming (Castañeda-Rodríguez et al., 2023). Methane contributes with 16% of the total anthropogenic GHG emissions, and 35%-40% of total $CH_4$ emissions arise from enteric fermentation in domesticated ruminants, which makes it a promising source of mitigation (Moss et al., 2000; Herrero et al., 2016). Additionally, $CH_4$ production represents a gross energy loss for the animal, ranging from 2%-12%.

Therefore, mitigating enteric $CH_4$ emissions without compromising animal productivity is desirable both to reduce the environmental impact of livestock and to improve ruminant production (Martin et al., 2016).

Improving the quality of ruminant diets can be an effective strategy for mitigating enteric $CH_4$ production (Castañeda-Rodríguez et al., 2023). Concentrate feeds such as corn, have shown lowering $CH_4$ emissions per unit of animal product, by providing high yields of DM that are readily digestible, increasing animal intake and performance, and improving efficiency of post-ruminal digestion in terms of energy (O'Mara et al., 1998). Besides, a concentrate-based diet leads to a higher proportion of propionate than a forage-based diet (Shinkai et al., 2024). Propionate is the main glucogenic precursor in ruminants (Benedetti et al., 2015) and acts as a hydrogen ($H_2$) sink in the rumen, resulting in lower $CH_4$ production compared to forage-based diets (Shinkai et al., 2024). A concentrate-based diet may additionally lower rumen pH (Benchaar et al., 2015), which in turn decreases ruminal $CH_4$ production, as rumen methanogens are highly sensitive to low pH (Shinkai et al., 2024). However, the use of corn as the primary energy source in cattle feed supplements competes with human consumption, making its use unsustainable (Benedetti et al., 2015; Almeida et al., 2021). The expanding biofuel industry has driven up demand for corn, impacting both prices and the livestock industry, therefore, alternative feed sources are needed (Paiva et al., 2016), and incorporating agro-industrial by-products into cattle diets offers a sustainable solution (Almeida et al., 2021).

Among biofuels, biodiesel production volume is expected to reach 60 billion liters by 2025, with approximately 6 billion liters of glycerol co-produced (Pandit et al., 2023). Indeed, glycerol is also a natural component of animal metabolism, produced through the lipolysis of adipose tissue or blood lipoproteins (Syahniar et al., 2016). As ruminant feedstock, glycerol, has been successfully used as diet ingredient in beef (Benedetti et al., 2015), sheep (Avila-Stagno et al., 2013) and dairy cattle (Karlsson et al., 2019). In the rumen, glycerol shifts rumen fermentation patterns towards propionate in in vitro (Avila-Stagno et al., 2014; Benedetti et al., 2015), and in vivo conditions (Ariko et al., 2015). However, the use of glycerol on $CH_4$ production have been variable, with slight reductions (Lee et al., 2011), no effects (Avila-Stagno et al., 2013) or increase in concentrate diets (van Cleef et al., 2016). Glycerol has also been shown to improve fatty acid profiles (Avila-Stagno et al., 2013), while glycerine, a commercial product with glycerol as its main component, has been shown to reduce total saturated fatty acids (SFA) and increase monounsaturated and polyunsaturated fatty acids (PUFA), resulting in a higher PUFA:SFA ratio (Eiras et al., 2014).

On the other hand, linseed, an n-3 rich oilseed, is considered one of the most effective dietary lipid sources for mitigating enteric $CH_4$ emissions (van Gastelen et al., 2017). This seed has effectively reduced $CH_4$ production (Benchaar et al., 2015; Martin et al., 2016), whereas its use has resulted in improvements of fatty acid profiles in beef and dairy products, due its n-3 rich fatty acid profile (Caroprese et al., 2010; He et al., 2012). However, diet digestibility may be adversely affected by the addition of dietary lipid (Vargas et al., 2020).

The use of concentrated feeds has demonstrated positive effects on GHG emissions. Replacing corn with glycerol in concentrate diets addresses the need for alternative feed sources. Both glycerol and n-3 lipids are known to have beneficial properties concerning lipid profiles; however, the methanogenic properties of glycerol may be detrimental to climate change efforts. Therefore, we hypothesized that incorporating increasing concentrations of glycerol, as a replacement for corn in concentrate diets supplemented with linseed, would reduce $CH_4$ production due to lipid supplementation. The objective of this study was to assess the effects of the inclusion of increasing concentrations of glycerol in concentrate diets supplemented with linseed, on rumen fermentation parameters and $CH_4$ production using a Rumen Simulation Technique (Rusitec) fermentation system.

## MATERIALS AND METHODS

This experiment was conducted at the Nutrition and Livestock Systems Laboratory, Universidad de Concepción, Chillán, Chile. The inoculum donor heifers were cared for in accordance with the guidelines of the Universidad de Concepción Bioethics Committee.

### Experimental design and treatments

The experiment was a complete randomized design, with four dietary treatments replicated in a Rusitec apparatus (Gutiérrez-Gómez et al., 2020). The experimental treatments included corn (*Zea mays* L.), mixed hay,

soy (*Glycine max* (L.) Merr.), linseed (*Linum usitatissimum* L.) and glycerol. The main substrates (corn grain and mixed hay of *Trifolium repens* L. and *Lolium perenne* L. in a 25:75 ratio) simulate typical feeding practices for finishing beef cattle supplemented with soy. Linseed concentrations were selected according to previous reports designed to improve fatty acid profiles in beef cattle (He et al., 2012) and glycerol concentrations according to practical inclusions to replace corn grain (Gutiérrez-Gómez et al., 2020). All dietary ingredients, except glycerol were ground through a 4 mm screen (Breuer, Temuco, Chile). Afterwards, all ingredients were thoroughly mixed, weighed into pre-labeled nylon bags (100 × 200 mm; pore size = 50 µm) and stored at -4 °C until placed in the fermenters. Experimental diets were: Control (6.1 g corn grain, 2.5 g mixed hay, 1.4 g soybean meal) (CTL); positive control (5.1 g corn grain, 2.5 g mixed hay, 0.9 g soybean meal, 1.5 g linseed) (+CTL); 5% glycerol DM (4.6 g corn grain, 2.5 g mixed hay, 0.9 g soybean meal, 1.5 g linseed, and 0.5 g glycerol) (Gly 5%); 10% glycerol DM (4.0 g corn grain, 2.5 g mixed hay, 1.0 g linseed, 1.5 g linseed, and 1.0 g glycerol) (Gly 10%). Diet compositions are described in Table 1.

Table 1. Ingredients and chemical composition of the experimental diets. CTL: Control diet; +CTL: positive control (CTL plus 1.5 g linseed); Gly 5%: glycerol 5% (CTL plus 1.5 g linseed and 0.5 g glycerol); Gly 10%: glycerol 5% (CTL plus 1.5 g linseed and 1.0 g glycerol); DM: dry matter; FW: fresh weight; aNDFom: neutral detergent fiber; ADFom: acid detergent fiber; CP: crude protein; EE: ethereal extract; NFC: non-fibrous carbohydrates, calculated as 100 – (crude protein + neutral detergent fiber + ethereal extract + ash).

| | Diets | | | |
|---|---|---|---|---|
| Item | CTL | +CTL | Gly 5% | Gly 10% |
| Ingredients (g 100 $g^{-1}$ diet) | | | | |
| Corn grain | 61 | 51 | 46 | 40 |
| Mixed hay | 25 | 25 | 25 | 25 |
| Soybean meal | 14 | 9 | 9 | 10 |
| Linseed | 0 | 15 | 15 | 15 |
| Glycerol | 0 | 0 | 5 | 10 |
| Chemical composition (% DM) | | | | |
| DM (% FW) | 88.1 | 89.1 | 89.3 | 88.2 |
| aNDFom | 25.1 | 25.2 | 24.4 | 23.9 |
| ADFom | 17.6 | 18.3 | 17.7 | 17.2 |
| Ash | 5.38 | 5.54 | 5.33 | 5.18 |
| CP | 15.2 | 15.5 | 14.7 | 14.4 |
| EE | 2.53 | 9.61 | 9.49 | 9.39 |
| NFC | 51.8 | 44.2 | 46.1 | 47.1 |

## Incubations and Rusitec apparatus

Rumen fluid and solid digesta were obtained 2 h after feeding two Aberdeen Angus heifers fitted with rumen cannulas. The heifers were fed with a mixed diet consisting of commercial concentrate 14% crude protein (CP; Copeval, Los Angeles, Chile), and a hay mixture of *T. repens* and *L. perenne* in a 25:75 ratio, plus a vitamin/mineral block (Veterquímica, Santiago, Chile). The animals had unrestricted access to fresh water. Rumen fluid (800 mL per fermenter) was collected, pooled and filtered through four layers of cheesecloth into a thermos flask and immediately transported to the laboratory. In addition, 400 g solid digesta were collected for initial inoculation of the vessels.

The Rusitec apparatus was equipped with eight 1 L volume fermentation vessels, immersed in a thermos table stainless steel water tank at 39 °C. Each vessel had an inlet for buffer (McDougall, 1948), and an upper outlet for fermentation effluent. The effluent was collected in a 1-L Erlenmeyer flask connected to a 2 L gas collection bag. The four dietary treatments were randomly assigned to duplicate vessels. The experiment was carried out in two runs (four replicates per treatment), each lasting for 15 d, with 10 d adaptation, followed by 5 d for measurements and sample collection.

To begin the experiment (day 1), each fermentation vessel was filled with 200 mL McDougall buffer at 39 °C modified with ammonium sulfate (1.0 g $L^{-1}$) plus 800 mL rumen fluid and flushed with $CO_2$ (Avila-Stagno et al., 2014). Two Dacron bags were added, one bag contained 40 g rumen digesta, while the other contained the experimental diet corresponding to the fermenter. After 24 h fermentation, the bag with rumen digesta was replaced by another bag with the corresponding experimental diet. Similarly, the bag containing the experimental diet was replaced with a new bag containing the specific treatment after 48 h. Following this, each diet bag was removed after 48 h fermentation, squeezed moderately, and replaced by a new bag containing the specific treatment. Artificial saliva was infused continuously into the fermenters at a 3% $h^{-1}$ dilution rate according to flow rates for forage diets (Avila-Stagno et al., 2014). During the bag change, each fermentation vessel was gassed with $CO_2$ to maintain anaerobic conditions. The effluent accumulation was measured daily during feed bag exchange and collected in 1 L flask with 5 mL sodium azide (1 g $L^{-1}$) to stop fermentation.

### Substrate disappearance

Dry matter disappearance was determined at 48 h, from days 11 to 15. Feed bags were removed from each fermenter, washed in cold, running distilled water until water was clear, and dried at 55 °C (UN 55, Memmert, Schwabach, Germany) for 48 h before weighing. To ensure that there was enough sample for analysis, food residues from the bags of each experimental diet were pooled after fermentation. Samples were ground through a 1 mm screen prior to chemical analysis.

### Fermentation metabolites

Gas fermentation was collected in 2 L vinyl collecting bags attached to each fermenter. Just before exchanging the feed bags, total daily gas production of each fermenter was determined by water displacement (Soliva and Hess, 2007). From day 11 to 15, just before total gas determination, samples of 15 mL were collected from the septum collection bags with a 21 gauge needle and then transferred into evacuated Exetainers (Labco Ltd., Ceredigion, UK) for $CH_4$ analysis.

At the daily feed bag exchange, each Erlenmeyer flask was disconnected from its corresponding fermenter, and the pH of the effluent in each flask was immediately measured using a portable pH meter (Orion Star A121; Thermo Fisher Scientific, Waltham, Massachusetts, USA). To determine the concentration of volatile fatty acids (VFA), effluent subsamples (1.5 mL) were collected directly from each flask, placed in screw-capped vials with 200 µL 10% sulfuric acid and immediately frozen at -20 °C until analysis. Additionally, sub-samples with 1.5 mL effluent were placed in vials with 150 µL trichloroacetic acid until analysis of ammonia N ($NH_3$-N) concentration.

### Analysis

Subsamples of each treatment (feed and fermentation residues) were used for chemical analysis. The neutral detergent fiber (aNDFom) was analyzed according to the method described by Mertens (2002), utilizing heat-stable alpha-amylase. The following analyses were carried out according to the methods described by the AOAC (1997): Method #930.15 for the DM content, method #923.05 for total ash determination, and method #973.18 for the determination of acid detergent fiber (ADF). The ether extract (EE) was determined using method #920.39 with a Goldfish Fat Extractor (Labconco Corp., Kansas City, Missouri, USA). Total N was assessed using method #991.20 with a macro Kjeldahl distiller (VAPODEST; Gerhardt GmbH & Co. KG, Königswinter, Germany), and the CP content was determined using a conversion factor of 6.25. Whereas non-fibrous carbohydrates (NFC) were calculated as 100 – (CP + aNDFom + EE + ash).

The concentration of $CH_4$ in the gas samples was determined using gas chromatography (GC; Agilent 7890B, Agilent Technologies, Santa Clara, California, USA) equipped with GS-CarbonPLOT 30 m × 0.32 mm × 3 µm column and a thermal conductivity detector. Oven temperature was 35 °C (isothermal). The carrier gas was helium (27 cm $s^{-1}$), the injector temperature was 185 °C (1:30 split, 250 µL injector volume) and the thermal conductivity detector was 150 °C.

The concentrations of VFA and $NH_3$-N in the liquid effluent were analyzed using GC with an Agilent 7890B system (Agilent Technologies) and a UV-VIS spectrophotometer (Spectroquant Pharo 300; Merck, Darmstadt, Germany), respectively. Additionally, the concentrations of VFA (mmol $L^{-1}$) and $NH_3$-N (mg $dL^{-1}$) were multiplied by the outflow rate of fluid of the vessels (L $d^{-1}$) to calculate their respective productions.

### Statistical analysis

The normality and homogeneity of data were assessed by the Shapiro-Wilk's and Levene's tests, respectively, before the statistical analysis. Data were analyzed using the MIXED procedure of SAS (SAS Institute, Cary, North Carolina, USA). The model included the fixed effects of treatment, Day and Treatment × Day interactions with the day of sampling from each fermenter treated as a repeated measure. The individual fermenter was considered as the experimental unit for statistical analysis. The minimum values of Akaike's information criterion were used to select the covariance structure among compound symmetry, heterogeneous compound symmetry, autoregressive, heterogeneous autoregressive, Toeplitz, unstructured and banded for each parameter. Orthogonal polynomial contrasts were performed to test for linseed effects (all diets containing linseed compared to control) and the linear and quadratic effects of glycerol concentration within the linseed-added diets. Significance was declared at $P < 0.05$ and trends were discussed when $0.05 \leq P < 0.10$. The relationship between variables was visualized using principal component analysis (PCA) biplot.

## RESULTS

### Total gas and methane production

Total gas production (mL $d^{-1}$) was higher in the Gly 10% diet compared to the +CTL diet ($P = 0.028$), showing a linear increase ($P = 0.006$) with the addition of glycerol (Table 2). The percentage of $CH_4$ in gas was lower in the +CTL diet compared to both the CTL and Gly 10% diets ($P = 0.001$), showing a decrease with the supplementation of linseed ($P < 0.001$) but a linear increase ($P = 0.002$) with the addition of glycerol. Similarly, net $CH_4$ production (mg $d^{-1}$), $CH_4$ per gram DM incubated (mg $g^{-1}$ DMi), and $CH_4$ per gram of DM disappeared (mg $g^{-1}$ DMd) were all lower in the +CTL diet ($P < 0.001$), showing decreases with linseed ($P \leq 0.004$) and linear increases ($P < 0.001$) with glycerol.

### Rumen fermentation parameters

Incubation pH was unaffected by the treatments ($P = 0.138$), but tended to increase linearly ($P = 0.054$) with the addition of glycerol (Table 2). The $NH_3$-N production (mg $d^{-1}$) was lower in the Gly 10% diet compared to both the CTL and +CTL diets ($P = 0.004$), decreasing with linseed supplementation ($P = 0.012$) and showing a linear decrease ($P = 0.004$) with the addition of glycerol. Total VFA production (mmol $d^{-1}$) was unaffected by the treatments ($P = 0.284$), but showed a trend toward a linear increase ($P = 0.090$) with the addition of glycerol. The molar proportion of acetate (mmol 100 $mmol^{-1}$) was lowest in the Gly 10% diet compared to all other diets, while in the Gly 5% diet was lower compared to both the CTL and +CTL diets ($P < 0.001$), decreasing with linseed ($P = 0.002$) and showing a linear decrease ($P < 0.001$) with glycerol addition. Conversely, propionate molar proportion (mmol 100 $mmol^{-1}$) was highest in the Gly 10% diet compared to all diets, and in the Gly 5% diet was higher compared to both the CTL and +CTL diets ($P < 0.001$), increasing with linseed ($P < 0.001$) and glycerol, both linearly ($P < 0.001$) and quadratically ($P = 0.013$). The butyrate molar proportion (mmol 100 $mmol^{-1}$) was lower in the Gly 10% diet compared to the CTL diet ($P = 0.006$), showing a decrease with linseed ($P < 0.001$) and a trend toward a linear decrease ($P = 0.065$) with the addition of glycerol. Conversely, iso-butyrate molar proportion (mmol 100 $mmol^{-1}$) was unaffected by the treatments ($P = 0.190$) but increased linearly ($P = 0.035$) with the addition of glycerol. The molar proportion of valerate (mmol 100 $mmol^{-1}$) was higher in the Gly 10% diet compared to the CTL diet ($P = 0.049$), increasing with linseed ($P < 0.001$). The caproate molar proportion (mmol 100 $mmol^{-1}$) in both the Gly 5% and Gly 10% diets was lower compared to the CTL and +CTL diets ($P < 0.001$), with the addition of glycerol showing a linear decrease ($P = 0.004$) and a trend toward a quadratic effect ($P = 0.054$). The acetate-to-propionate (A:P) ratio was lowest in the Gly 10% diet compared to all other diets, while in the Gly 5% diet was lower compared to both the CTL and +CTL diets ($P < 0.001$), decreasing with linseed ($P < 0.001$), and with glycerol, both linearly ($P < 0.001$) and quadratically ($P < 0.001$).

Table 2. Effects of dietary treatments on fermentation characteristics. Mean values within the same row sharing no common superscript are significantly different ($P < 0.05$). CTL: Control diet (no additive); +CTL: positive control (CTL plus 1.5 g linseed); Gly 5%: glycerol 5% (CTL plus 1.5 g linseed and 0.5 g glycerol); Gly 10%: glycerol 10% (CTL plus 1.5 g linseed and 1.0 g glycerol); SEM: standard error of mean; DM: dry matter; VFA: volatile fatty acids; A:P ratio: acetate-to-propionate ratio; aNDFom: neutral detergent fiber; CP: crude protein; EE: ethereal extract; NFC: non-fibrous carbohydrates. The *P*-values are presented for the overall diet effects and for contrasts: Lin *vs*. CTL: all diets containing linseed compared to control (linseed effects); Gly L: linear effect by glycerol concentration within the linseed-added diets; Gly Q: quadratic effect by glycerol concentration within the linseed-added diets.

| | Diets | | | | | *P*-value | | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | CTL | +CTL | Gly 5% | Gly 10% | SEM | Diet | Lin *vs.* CTL | Gly L | Gly Q |
| Total gas, mL $d^{-1}$ | $1904^{AB}$ | $1634^{B}$ | $1800^{AB}$ | $1933^{A}$ | 85.70 | 0.028 | 0.104 | 0.006 | 0.808 |
| $CH_4$, % of gas | $5.67^{A}$ | $3.55^{B}$ | $4.56^{AB}$ | $5.30^{A}$ | 0.397 | 0.001 | < 0.001 | 0.002 | 0.784 |
| Net $CH_4$, mg $d^{-1}$ | $69.40^{A}$ | $39.00^{B}$ | $59.50^{A}$ | $78.20^{A}$ | 6.42 | < 0.001 | 0.003 | < 0.001 | 0.884 |
| $CH_4$, mg $g^{-1}$ DM incubated | $7.41^{A}$ | $4.11^{B}$ | $6.34^{A}$ | $8.43^{A}$ | 0.684 | < 0.001 | 0.003 | < 0.001 | 0.911 |
| $CH_4$, mg $g^{-1}$ DM disappeared | $9.47^{A}$ | $5.35^{B}$ | $8.18^{A}$ | $10.70^{A}$ | 0.845 | < 0.001 | 0.004 | < 0.001 | 0.836 |
| pH | 6.85 | 6.87 | 6.88 | 6.97 | 0.044 | 0.138 | 0.600 | 0.054 | 0.194 |
| $NH_3$-N, mg $d^{-1}$ | $56.60^{A}$ | $55.90^{A}$ | $52.70^{AB}$ | $49.80^{B}$ | 1.44 | 0.004 | 0.012 | 0.004 | 0.950 |
| Total VFA, mmol $d^{-1}$ | 51.30 | 49.20 | 49.40 | 52.30 | 1.38 | 0.284 | 0.246 | 0.090 | 0.381 |
| VFA, mmol 100 $mmol^{-1}$ | | | | | | | | | |
| Acetate | $56.90^{A}$ | $57.50^{A}$ | $52.00^{B}$ | $47.90^{C}$ | 0.47 | < 0.001 | 0.002 | < 0.001 | 0.300 |
| Propionate | $23.30^{C}$ | $23.70^{C}$ | $30.70^{B}$ | $34.70^{A}$ | 0.68 | < 0.001 | < 0.001 | < 0.001 | 0.013 |
| Butyrate | $14.60^{A}$ | $13.60^{AB}$ | $13.00^{AB}$ | $12.30^{B}$ | 0.46 | 0.006 | < 0.001 | 0.065 | 0.833 |
| Iso-butyrate | 0.73 | 0.71 | 0.75 | 0.77 | 0.021 | 0.190 | 0.805 | 0.035 | 0.479 |
| Valerate | $2.09^{B}$ | $2.28^{AB}$ | $2.22^{AB}$ | $2.42^{A}$ | 0.098 | 0.049 | < 0.001 | 0.300 | 0.334 |
| Caproate | $1.38^{A}$ | $1.23^{A}$ | $0.87^{B}$ | $0.84^{B}$ | 0.107 | < 0.001 | 0.258 | < 0.001 | 0.054 |
| A:P ratio | $2.51^{A}$ | $2.50^{A}$ | $1.70^{B}$ | $1.39^{C}$ | 0.056 | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| Disappearance, g 100 $g^{-1}$ | | | | | | | | | |
| DM | $79.80^{A}$ | $75.90^{B}$ | $77.60^{AB}$ | $79.30^{A}$ | 0.90 | 0.010 | 0.013 | 0.006 | 0.998 |
| aNDFom | 33.80 | 35.30 | 34.90 | 36.90 | 3.02 | 0.839 | 0.901 | 0.591 | 0.649 |
| CP | $84.40^{A}$ | $80.70^{B}$ | $81.90^{AB}$ | $82.40^{AB}$ | 0.79 | 0.011 | 0.001 | 0.123 | 0.663 |
| EE | $77.90^{C}$ | $79.80^{BC}$ | $83.20^{AB}$ | $84.10^{A}$ | 0.94 | < 0.001 | 0.006 | 0.001 | 0.244 |
| NFC | $89.50^{A}$ | $86.90^{B}$ | $88.70^{AB}$ | $88.60^{AB}$ | 0.61 | 0.033 | < 0.001 | 0.062 | 0.198 |

## Nutrient disappearance

The DM disappearance (g 100 $g^{-1}$) was lower in the +CTL diet compared to both the CTL and Gly 10% diets ($P$ = 0.010) and decreased with linseed ($P$ = 0.013), while a linear increase ($P$ = 0.006) was observed with the addition of glycerol (Table 2). The CP disappearance (g 100 $g^{-1}$) was lower in the +CTL diet compared to the CTL diet ($P$ = 0.011), decreasing with linseed supplementation ($P$ = 0.001). Similarly, NFC disappearance (g 100 $g^{-1}$) was also lower in the +CTL diet compared to the CTL diet ($P$ = 0.033), decreasing by the supplementation of linseed ($P$ = 0.001), and showing a trend toward a linear effect ($P$ = 0.062) with glycerol addition. However, EE disappearance (g 100 $g^{-1}$) was higher in the Gly 10% diet compared to both the CTL and +CTL diets, and in the Gly 5% diet compared to the CTL diet ($P < 0.001$), increasing with linseed ($P$ = 0.006) and linearly with glycerol ($P < 0.001$). The aNDFom disappearance (g 100 $g^{-1}$) was unaffected ($P$ = 0.839) by the treatments.

## Principal component analysis

The PCA biplot (Figure 1) shows that 93.9% of the variance was explained by the first two principal components (PC-1 and PC-2). The PC-1, with an eigenvalue of 10.6, accounted for 61.4% of the variance, while the PC-2, with an eigenvalue of 5.35, explained 31.5%. In the PC-1 score plot, two main groups were clearly discriminated based on glycerol addition: Diets without glycerol (CTL and +CTL) appeared mostly in the negative range, while diets with glycerol (Gly 5 and 10%) were in the positive range. Conversely, the PC-2 score plot discriminated +CTL and Gly 5% (negative range) from CTL and Gly 10% (positive range). Regardless of components, CTL was

oriented oppositely to Gly 5%, and +CTL was oriented oppositely to Gly 10%. The magnitude of vectors was similar across variables, except for total VFA, though directions varied. The vectors indicated that $CH_4$ production (mg $g^{-1}$ DMd) was clustered and positively associated with total gas and DM disappearance, in a direction opposite to acetate.

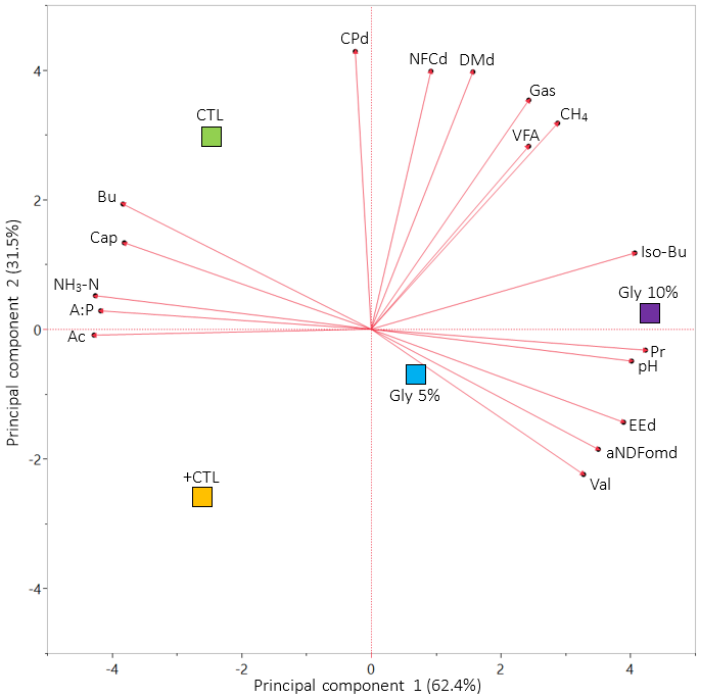


**Figure 1.** Principal component analysis biplot of fermentation characteristics. A:P ratio: Acetate-to-propionate ratio; Ac: acetate; aNDFomd: neutral detergent fiber disappearance; Bu: butyrate; Cap: caproate; $CH_4$: methane production (mg $g^{-1}$ DMd); CPd: crude protein disappearance; CTL: control diet (no additive); +CTL: positive control (CTL plus 1.5 g linseed); DMd: dry matter disappearance; EEd: ethereal extract disappearance; Gly 5%: glycerol 5% (CTL plus 1.5 g linseed and 0.5 g glycerol); Gly 10%: glycerol 10% (CTL plus 1.5 g linseed and 1.0 g glycerol); Iso-Bu: Iso-butyrate; NFCd: non-fibrous carbohydrates disappearance; $NH_3$-N: ammonia N; Pr: propionate; Val: valerate.

## DISCUSSION

Improving concentrate diets is an effective strategy to reduce enteric $CH_4$ production. We hypothesized that including increasing concentrations of glycerol, as a replacement for corn in concentrate diets supplemented with linseed, would reduce $CH_4$ production due to lipid supplementation. However, this study demonstrated that while linseed supplementation successfully reduces $CH_4$ production, the combination with glycerol in concentrate diets does not mitigate $CH_4$ emissions and may even increase them due to the methanogenic properties of glycerol.

### Total gas and methane production

The increase in gas production with glycerol aligns with previous findings by Avila-Stagno et al. (2014), Syahniar et al. (2016), and is also supported by Castañeda-Rodríguez et al. (2023), who indicate the presence of non-fibrous carbohydrates (NFC) associated with glycerin, which are rapidly fermented, consequently increasing gas production. The decreased $CH_4$ production in our study is attributable to the addition of linseed, as shown in the PCA biplot, where positive control (+CTL) is oriented in the opposite direction to $CH_4$. The linseed effect has

been observed in previous in vitro studies using linseed oil (Szumacher-Strabel et al., 2004; Vargas et al., 2020) and in vivo studies with both linseed oil (Benchaar et al., 2015; van Gastelen et al., 2017) and extruded linseed (Martin et al., 2016). This effect may be attributed to the lipid contents of linseed which shifts fermentation to propionate and inhibit protozoa (Szumacher-Strabel et al., 2004; Martin et al., 2016), several bacterial genera (van Gastelen et al., 2017), and methanogenic archaea (Vargas et al., 2020). However, in our study, linseed did not offset the increase in $CH_4$ production caused by glycerol addition as shown the PCA biplot, where the diets with glycerol (Gly 5% and Gly 10%) were positioned increasingly closer to $CH_4$, with Gly 10% being the closest. The increase in $CH_4$ output by glycerol addition is consistent with findings in both forage (Avila-Stagno et al., 2014) and concentrate (van Cleef et al., 2016) diets, suggesting that most of the glycerol was fermented (Karlsson et al., 2019). During diet fermentation by rumen microorganism, $H_2$ is released, and its transfer to different acceptors ensures the continuity of fermentation by re-oxidizing reduced co-factors. Both $CH_4$ and propionate production act as $H_2$ sinks in the rumen, with a strong negative correlation between them (Shinkai et al., 2024). However, in our study both $CH_4$ and propionate production increased with the glycerol addition. Avila-Stagno et al. (2014) indicated that glycerol must release two pairs of $H_2$ before converting to pyruvate and metabolizing subsequently towards propionate, acetate or butyrate, so that in itself the fermentation of glycerol to propionate does not act as a $H_2$ sink. Van Cleef et al. (2016) propose that the presence of glycerol in the rumen favored other pathways of action of methanogenic bacteria. The inability of linseed to fully mitigate the $CH_4$ produced by the glycerol addition suggests a more complex interaction between these dietary components and the rumen microbiota. Moreover, Gutiérrez-Gómez et al. (2020), in a forage-based diets with the same glycerol and linseed concentrations as in our study, found a decrease in $CH_4$ production with the use of the linseed, but with no effect from glycerol. This indicates that the basal diet determines the interaction between linseed and glycerol, and that as opposed as fibrous diets, in starch-rich diets as in our study, with propionate-oriented fermentation, the effects of linseed do not suffice to offset the methanogenic effects of glycerol. A microbiological analysis is necessary to estimate the differences between microbial populations to clarify this issue.

### Rumen fermentation parameters

The addition of linseed and glycerol did not affect pH, which is consistent with findings in forage diets supplemented with linseed and increasing glycerol concentrations (Gutiérrez-Gómez et al., 2020), barley-based feedlot diets with glycerol supplementation (Avila et al., 2011), and diets with a 70:30 forage-to-concentrate (F:C) ratio that included linseed oil (van Gastelen et al., 2017). The addition of linseed and glycerol decreased $NH_3$-N concentration, as shown in the PCA biplot, where $NH_3$-N concentration was positioned closer to the CTL diet, consistent with previous findings for linseed (Vargas et al., 2020) and glycerol (Avila-Stagno et al., 2014). However, unlike Gutiérrez-Gómez et al. (2020) that used glycerol and linseed in the same concentrations but in forage-based diets, the diets of this experiment are isoproteic; this may imply relative stability in the N supply in the different experimental diets. Concentration of $NH_3$-N is affected by ruminal protein degradability and its use to synthesize microbial protein. In an in vitro environment, there is no N absorption or recycling, unlike the rumen in vivo (Castañeda-Rodríguez et al., 2023). Additionally, the decrease in crude protein (CP) disappearance with linseed addition suggest a reduction in protein degradability, but the linear reduction of $NH_3$-N with glycerol suggests it may have stimulated microbial protein synthesis (Syahniar et al., 2016). However, further studies with labeled N are necessary to validate these findings.

Although no changes were observed in total volatile fatty acids (VFA), aligning with findings from linseed (Vargas et al., 2020), linseed oil (Ueda et al., 2003) and glycerol (Avila et al., 2011; Meale et al., 2013) supplementation, some individual VFAs showed distinct effects. The acetate proportion decreased, while the propionate proportion increased with the addition of linseed and glycerol, reducing the A:P ratio, as shown in the PCA biplot, where propionate was oriented in the opposite direction to the A:P ratio. The shift in fermentation patterns is consistent with previous studies on linseed oil (van Gastelen et al., 2017), linseed (Gutiérrez-Gómez et al., 2020), and glycerol (Avila et al., 2011; Gutiérrez-Gómez et al., 2020). The effect of linseed is also consistent with the previously discussed reduction in $CH_4$ production, highlighting the crucial role of re-oxidizing reduced co-factors in rumen fermentation (Shinkai et al., 2024). In contrast, the effect of glycerol confirms its propionogenic properties (Avila et al., 2011; Avila-Stagno et al., 2014). Butyrate decreased with linseed addition, aligning with previous studies using linseed oil (Szumacher-Strabel et al., 2004; Vargas et al.,

2020). In contrast, valerate increased with linseed addition, consistent with findings by Benchaar et al. (2015), who observed a valerate increase in cows when linseed oil was supplemented to a corn silage-based diet, but not to a red clover silage-based diet. These changes in the VFA profile may be attributed to bacterial sensitivity to polyunsaturated fatty acids (Huang et al., 2021). On the other hand, the decrease in caproate with glycerol contrasts with the findings of Trabue et al. (2007), where 25 mM glycerol increased caproate proportion over incubation time. Similarly, Avila-Stagno et al. (2014) reported a linear increase in caproate with 0, 0.5, and 1.0 g glycerol in a forage diet, but also noted a quadratic decrease with 1.5 g. The varying effects of glycerol on caproate proportion may be attributed to differences in concentration and diet composition.

### Nutrient disappearance

The DM disappearance decreased with linseed, which could imply a direct effect of linseed on ruminal microbial populations. With the inclusion of linseed in dairy cows' forage-based diets, Yang et al. (2009) reported lower cellulolytic bacteria and protozoa counts, which may explain the lower DM disappearance. Also, when feeding linseed oil in a high-concentrate diet, Ueda et al. (2003) reported a decrease ruminal fiber digestibility. On the contrary, DM disappearance increased with glycerol, aligning with previous studies on barley grain diets (Avila et al., 2011), wheat (Meale et al., 2013), brome hay, and maize silage (Avila-Stagno et al., 2014). Glycerol can increase DM disappearance, as it is a fully digestible compound that replaced corn, which is not fully digestible. Although, the DM disappearance increase may also reflect glycerol passing through the nylon bags, rather than indicating its digestion (Meale et al., 2013). Similarly, glycerol addition increased ethereal extract (EE) disappearance, consistent with previous findings (Paiva et al., 2016; Almeida et al., 2021). This effect is likely because glycerol fat predominantly consists of highly digestible fatty acids, in contrast to the vegetable waxes and terpenes found in corn grain fat (Almeida et al., 2021). Linseed also increased EE disappearance, aligning with the results of Benchaar et al. (2015) and further supported by van Gastelen et al. (2017), who suggested that unsaturated fatty acids from linseed may have a higher digestibility than saturated fatty acids. In contrast, CP and NFC disappearance decreased with linseed supplementation. This reduction in CP disappearance aligns with the findings of Petit (2003), who reported that linseed has lower CP digestibility compared to sunflower seed and soy bean meal, likely due to the intrinsic properties of linseed. On the other hand, the reduction in NFC disappearance, which was calculated, may be related to changes in EE and CP disappearances. The increase in EE disappearance likely could not compensate for the decrease in CP disappearance. The disappearance of neutral detergent fiber (aNDFom) in the present study was unaffected by the supplementations, consistent with findings from van Gastelen et al. (2017) regarding linseed, as well as from Avila-Stagno et al. (2013) and Gutiérrez-Gómez et al. (2020) regarding glycerol additions.

## CONCLUSIONS

The use of linseed reduces $CH_4$ production, but this effect is offset by the inclusion of glycerol in concentrate diets. Concentrations of $NH_3$-N are slightly reduced by linseed inclusion in the diets but linearly reduced by glycerol. Combining linseed and glycerol shifts the volatile fatty acids profile by increasing propionate, decreasing acetate, and reducing the A:P ratio. Additionally, it enhances nutrient degradability, particularly improving the degradability of ethereal extract and DM, although crude protein and non-fibrous carbohydrates degradability may decrease with the addition of linseed.

### Author contribution

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

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