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EFFECT OF NITRO COMPOUNDS ON *in vitro* RUMINAL METHANE, CARBON DIOXIDE, HYDROGEN AND DRY MATTER DEGRADABILITY

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ABSTRACT

This study determined the *in vitro* effects of ethyl nitroacetate (ENA), ethyl 2-nitropropionate (E-2-NPP) and 3-nitropropionic acid (NPA) on ruminal production of CH_4, H_2, volatile fatty acids (VFAs) and *in vitro* dry matter disappearance of ground alfalfa. *In vitro* incubations were carried out over 24-h succeeding days (3 batches). Total gas and methane production decreased with all nitro compounds ($P < 0.05$) in all the batches. Total production of VFAs was reduced by ENA and E-2-NPP ($P < 0.05$), but not by NPA. DM disappearance was similar between treatments but decreased across batches. Further studies should be aimed at understanding the impact of NPA on ruminal metabolism, as well as its effect on *in vivo* models.

Keywords: Cattle, CH_4, rumen fermentation, hydrogen, nitro compounds.

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INTRODUCTION

Ruminal methanogenesis is a biological activity that keeps a low partial pressure of hydrogen (H_2) in the rumen. Conversely, methane (CH_4) production is deemed as energy waste, which can reach 10% of the gross energy consumed by the ruminant (Clodagh et al., 2022) and greatly contributes to the human-caused greenhouse gas emissions. The U.S. Environmental Protection Agency (EPA, 2021) estimates that ruminant animals produce 20% of the U.S. total emissions of CH_4. Due to the environmental and economic implications of methanogenesis, their reduction is a global goal. In this regard, several approaches have been developed. These approaches include feed additives, microbiome manipulation, chemical intervention, genetic selection, and forage management (Króliczewska et al., 2023).

The most frequently used strategy is chemical intervention and some nitro compounds have also received attention for their potential to mitigate CH_4 emissions from the livestock industry. Some of these compounds have been previously evaluated: 3-nitropropionate (Anderson and Rasmussen, 1998); Nitroethane, 2-nitro-1-propanol (Anderson et al., 2003); dimethyl-2-nitroglutarate, 2-nitro-methyl-propionate (Anderson et al., 2010); 2-nitroethanol (Zhang et al., 2020); ethyl-nitroacetate (Anderson et al., 2011); 3-nitrooxypropanol (Martínez-Fernández et al., 2014); and 3-nitro-1-propionic acid (Ochoa-García et al., 2019). All these compounds reduced rumen CH_4 production by as much as 97%. Of all of them, the 3-nitrooxypropanol is the only compound that is currently being used as a commercial additive and no adverse effects have been reported (Alemu et al., 2023).

This nitro compound differs structurally from all the remaining nitro compounds, and its mode of action includes the capability to dock into the active site of the Methyl-Coenzyme M reductase and interferes in the last step of CH_4 production by ruminal archaea (Attwood and McSweeney, 2008). On the other hand, the mode of action of the remaining nitro compounds is through substrate competition, specifically for H_2, which is used to reduce them to amines (Anderson et al., 1993). Nevertheless, some of these nitro compounds are not likely to be used as feed additives in the short term because these are not naturally occurring compounds and there is limited research about food safety issues. In addition, the end products of their reduction (aminoethane, ethanolamine and aminopropanol, mainly) have little or non-nutritional value for ruminants. However, the exception is the 3-nitropropionic acid, which is produced by some plants such as those from the *Astragallus* genus and the fungus *Aspergillus flavus* (Doxtader and Alexander, 1966) and *Penicillium atrovenetum* (Shaw and Wang, 1964). Moreover, in most cases, the resulting reduced product has some toxicity or little or no nutritional value. Some exceptions are 3-nitro-1-propionic acid, which is reduced to β-alanine (Anderson et al., 1993), and ethyl-nitroacetate, which may be reduced to glycine, although this has not yet been proven. In both cases, the reduced products can be used as nutrients by the host. However, further research is required to determine if these or other nitro compounds have effects on dry matter degradability and if their final products are positive for rumen fermentation parameters. Accordingly, this study determined the effect of ethyl nitroacetate, ethyl 2-nitropropionate, and 3-nitropropionic acid on *in vitro* ruminal production of CH_4, H_2, VFA's, and dry matter degradability of alfalfa.

MATERIALS AND METHODS

Chemicals

All the chemicals were purchased from Sigma-Aldrich Chemicals Inc. (St. Louis, MO). The purity of the chemicals was 97, 97, and 96% for ethyl nitroacetate, ethyl 2-nitropropionate and 3-nitropropionic acid, respectively.

In vitro incubations

All treatments were tested in two separate incubation runs with three replicates (tubes; analytical replicates) in each run for each treatment. In each incubation run, three tubes with inoculum but without feed (blanks) were also included to establish a baseline for total gas production (TG). Animal donors for rumen fluid were maintained according to the procedures approved by the University of Chihuahua Animal Care and Use guidelines and the Mexican Government guidelines for the use of research animals (NOM-062-ZOO-1999)

In vitro incubations of 24-h were conducted as consecutive batch culture in 18 ×150 mm crimp-top tubes preloaded with 9 mL of basal medium as described by Theodorou et al. (1994), 0.2 g of finely ground (2 mm length) alfalfa hay plus the nitro compound (treatment). The treatments were designed to achieve 12 μmol/mL of each nitro compound in the incubation fluid (Anderson et al., 2011). Treatments and control were cultured in triplicate and replicated two times (runs).

Rumen contents were obtained at 8:00 before the morning meal from two rumen fistulated crossbreed Hereford × Angus heifers fed a maintenance ration based on oat straw and corn silage (1:1). Five hundred mL from each heifer were

placed in a 500 mL container, which was sealed hermetically and transported to the laboratory. The fresh ruminal fluid was filtered through five-cheese cloth layers and mixed under CO_2 flushing. A basal medium was prepared and distributed to the tubes according to the methods of Gutierrez-Bañuelos et al. (2008). The treatments were added to each tube by supplementing 0.3 mL of stock solution of each nitro compound or distilled water for the control. All the stock solutions of each nitro compound were prepared as sodium salt solution (Gutierrez-Bañuelos et al., 2007).

The initial culture (batch A) was inoculated with 1 mL of fresh rumen fluid. After 24-h incubation at 39 ° C, 1 mL from each culture was used to inoculate the next batch of tubes. A total of three batches (A, B, and C) were conducted in this study by sequential transfer of inoculum from the previous batch of tubes to new tubes preloaded with fresh medium and feed substrate (replicated two times). Total gas, CH_4, H_2, VFAs, and dry matter disappearance were measured at the end of the 24-hour incubation period of each batch. Total VFA production was calculated by deducting the concentration at the initial time (0 h) from the concentration at the ending time (24 h) for each batch. The average of the two replicates was used for the statistical analysis.

Fermentation parameters

Total gas production was determined by measuring volume displacement in a 30-cc glass syringe in each experimental unit after 24 h of incubation. Gas composition was determined by gas chromatography according to Allison *et al*. (1992) using a GOW-MAC Series 580 chromatograph, fitted with a Carbosphere® 80/100 packed column. Nitrogen gas was used as a carrier at a flow of 20 mL min^{-1}. VFA's production was determined by gas chromatography following the methodology described by Galyean and May (1989), where 5 mL of the centrifuged sample was added to a mixture of 1 mL of 25% metaphosphoric acid, an internal standard of 2-ethylbutyric acid and subjected to an ice bath for 30 min followed by centrifugation at 10,000 × g. The standards were treated in the same way. The VFA quantification was carried out using a Clarus 400 Perkin Elmer Chromatograph (PerkinElmer, Waltham, MA), using a stainless-steel capillary column of Poropak-Q of 30 m length; helium was used as a carrier gas (20 mL min^{-1}). *In vitro* dry matter degradability (IVDMD) was determined by drying the total solid content of each tube (Tilley and Terry, 1963).

Statistical analysis

Data (TG, IVDMD, gas composition (CH_4 and H_2), and VFA's) were analyzed using a completely randomized design, and batch effects were determined using Tukey-adjusted least-squares-mean multiple comparisons to assess treatment differences using SAS (Statistical Analysis for Windows, SAS Institute Inc., Cary, NC, USA).

RESULTS AND DISCUSSION

Total gas production and gas composition

Total gas was reduced by all nitro compounds ($P < 0.05$; Table 1) and across batches just the control was reduced ($P < 0.05$), which agrees with the results of Anderson et al. (2010), who reported less production of TG when three different nitro compounds were supplemented in two doses to *in vitro* cultures. Even though decreased TG production was observed for all nitro compounds, the effect was stronger with ethyl nitroacetate and ethyl-2-nitropropionate than with 3-nitropropionic acid. This could be related to the reduced microbial diversity caused by the consecutive transferring, considering that the gas volume logged is partially generated from bacterial fermentation (Amanzougarene and Fondevila, 2020). It has been determined that the growth of protozoa decreases under *in vitro* conditions, including that of hydrogen-producing protozoa (Solomon et al., 2021). Thus, it is possible that the loss of H_2 from that protozoa could contribute to TG reductions since H_2 is part of the gaseous fraction in the rumen. Further studies should consider analyzing the rumen microbiome to confirm the data from the present study.

Methane production was lowered ($P < 0.05$) for all nitro compounds (Table 1). Ethyl nitroacetate reduced CH_4 production by as much as 99 % in batches A and B and inhibited it in batch C. These results agree with Anderson et al. (2011), who found a reduction of >93% when ENA was supplemented in a 12-mM dose. Ethyl 2-nitropropionate and 3-nitropropionic acid inhibited CH_4 production in all batches (Table 1). As noted by other authors (Anderson and Rasmussen, 1998; Anderson et al., 2010), reduction of CH_4 production from ruminal microbes could be due to the lack of electron-donating substrates available for methanogenesis such as hydrogen (Lan and Yang, 2019). It is suspected that some nitro compounds could inhibit the activity of formate dehydrogenase/formate hydrogen lyase leading to low production of H_2 (Anderson et al., 2008). Additionally, it is known that the reduced end products of some of these nitro compounds are amino acids or other amines that can be used as nutrients for the host (Anderson et al., 1993).

Hydrogen produced in the supplemented cultures in batch A was slightly higher compared

Table 1. Gas composition and volatile fatty acids produced by rumen microbes under different nitrocompound supplementation.

Item	Treatment	Batch A*	Batch B*	Batch C*
Total gas (mL/200 mg MS)	Control	22.4±0.49a(a)	15.8±1.8a(b)	15.9±1.46a(b)
	NPA	17.4±0.96b(a)	14.6±0.93a(a)	13.4±2.96ab(a)
	ENA	11.1±0.49c(a)	6.78±0.32b(a)	9.15±3.42b(a)
	E-2-NPP	7.86±3.18c(a)	8.07±2.26b(a)	9.36±0.64b(a)
CH_4 (umo/mL)	Control	9.96±0.61a(a)	2.49±1.33a(b)	3.03±1.36a(b)
	NPA	ND	ND	ND
	ENA	0.09±0.13b(a)	0.02±0.04b(a)	ND
	E-2-NPP	ND	ND	ND
H_2 (umo/mL)	Control	ND	0.19±0.05a(ab)	0.30±0.14a(b)
	NPA	0.21±0.10ac(a)	0.26±0.07a(a)	1.0±0.36a(b)
	ENA	0.71±0.05b(a)	0.33±0.06a(a)	0.46±0.45a(a)
	E-2-NPP	0.29±0.14c(a)	0.54±0.06a(a)	0.34±0.08a(a)
Acetate (umo/mL)	Control	42.54±4.97a(a)	59.7±4.0a(b)	49.5±4.85a(ab)
	NPA	47.22±1.95a(a)	57.2±0.2a(b)	53.2±4.38a(ab)
	ENA	28.16±2.16b(a)	41.8±10.3b(a)	43.4±4.44a(a)
	E-2-NPP	23.96±5.53b(a)	45.00±3.63ab(b)	49.10±4.55a(b)
Propionate (umo/mL)	Control	17.40±1.21a(a)	24.2±4.99a(a)	20.90±3.03a(a)
	NPA	21.89±0.53b(a)	21.4±0.57a(a)	18.3±2.91a()a
	ENA	15.23±0.94ac(a)	9.13±1.89b(b)	16.90±1.81a(a)
	E-2-NPP	12.22±2.10c(a)	17.2±2.75a(a)	15.90±0.32a(a)
Butyrate(umo/mL)	Control	4.58±0.15a(a)	7.42±0.45a(b)	6.42±0.75a(b)
	NPA	2.40±0.20b(a)	6.47±1.18a(b)	6.76±0.51a(b)
	ENA	4.70±0.32a(a)	5.40±1.33a(a)	5.17±0.22b(a)
	E-2-NPP	1.95±0.43b(a)	6.07±0.33a(b)	5.36±0.57b(b)
In vitro dry matter disappearance	Control	71.2±2.31a(a)	57.1±7.33a(b)	56.5±1.03a(b)
	NPA	67.8±4.82a(a)	50.2±4.62a(b)	57.9±1.48a(b)
	ENA	62.2±8.48a(a)	56.3±6.58a(a)	63.2±4.86a(a)
	E-2-NPP	65.8±7.26a(a)	53.1±3.07a(a)	58.7±8.19a(a)

*Different letters denotes difference between treatments. Letters in parenthesis denote differences between batches. ND. Not detected. (Detection threshold for CH_4 and H_2 was 0.015 and 0.017 mg mL^{-1}, respectively)

with non-supplemented tubes (Table 1). In the batches, increases were observed just for control and NPA. However, the increase was not enough to exceed the level at which the hydrogenase activity is inhibited (Miller, 1995; Van Nevel and Demeyer, 1996). The H_2 production from non-supplemented tubes showed a tendency to increase through batch because of the inhibition of H_2 oxidation combined with reduced CH_4 production (Anderson et al., 2008) and low disposal of H_2 to other pathways. Jenssen (2010) indicated that the energy metabolism of ruminants is highly influenced by the flow of metabolic H_2, which could be driving different fermentative pathways. As above-mentioned, the supplemented tubes produced less CH_4 and more H_2, but levels of propionate and butyrate were also lower than those in non-supplemented tubes, confirming a shift in the fermentative pathways (Martinez-Fernandez et al., 2016).

Volatile fatty acid production

The production of VFAs in batch A was reduced for all the nitro compounds ($P < 0.05$), except for NPA. During the subsecuent batches B nad C, this effect was maintained for ENA and E-2-NPP and ENA, respectively. For VFA production in the presence of added nitro compounds, different results with inconsistencies have been reported. Our results agree with Anderson et al. (2003), who reported a decrease in propionic acid when 2-nitropropanol was supplemented. Conversely,

these results differ from the report of Brown et al. (2011), who reported no appreciable effects of nitroethane on VFA production. However, the effect of nitro compounds in VFA production tends to disappear over time. In this sense, Gutierrez-Bañuelos et al. (2008) found that levels of acetate and propionate did not differ from the control after incubation series, being in agreement with our study. These results suggest that an adaptation of ruminal microbes to NPA could be a consequence of an increase in bacterial populations specialized in nitro compound reduction (Zhang et al., 2018).

However, the specific mechanisms are still unclear. The VFA production on NPA supplementation was similar to that of the control and the production of propionate was higher than the other nitro compounds, probably due to the partial conversion of NPA in propionate at the ruminal level (Ungerfeld, 2015). There is evidence that reductive cleavage of the nitro moiety can occur with some of the short-chain nitro compounds in rumen incubations, but the amount is small, being less than 5% of the amount of nitro compounds metabolized (Anderson et al., 1993; Zhang et al., 2020).

Dry matter disappearance

Dry matter disappearance was similar between nitro compounds (Table 1). Previous studies have also reported no differences in ruminal fermentations and digestibility of dry matter when nitro compounds are used as anti-methanogenic supplementation (Zhang et al., 2019; Romero-Perez et al., 2015; Martínez-Fernandez et al., 2014). It is well known that the accumulation of H_2 because of methanogenesis reduction has an impact on microbial fermentation, principally on metabolic pathways that include cofactors such as NADH and NADPH (Leng, 2014). Nevertheless, nitro compounds could allow the shift from H_2 to formate, reducing the ruminal pressure to normal levels for microbial populations (Leng, 2014). Moreover, the excess of H_2 could end up being used by microbial organisms such as non-fermentative, anaerobic *Denitrobacterium detoxificans*, thereby decreasing the pressure to which the rest of the microbial ecosystem is exposed (Anderson et al., 2010).

An effect of the consecutive inoculation was observed for the control tubes and those supplemented with NPA, where DMD decreases from batch A to C, following the same pattern as TG, showing the lowest digestibility for all tubes during the B series. These results differ from those of McDermott et al. (2020), who indicated that digestibility of dry matter on ruminal cultures tends to increase with consecutive culturing, due to a microbial adaptation under *in vitro* conditions, associated with reduced diversity of the microbiome. Nevertheless, Lin et al. (2019) demonstrated that adaptation to *in vitro* conditions may take a long time, and that the transfer-to-transfer process has an impact on fermentative parameters, including gas produced, pH, and short-chain fatty acids produced, which may cause variability in dry matter degradability of our experiment.

CONCLUSIONS

The use of nitro compounds such as ethyl nitroacetate and ethyl 2-nitropropionate showed great efficiency in reducing methane production under *in vitro* conditions, without changes in the digestibility of dry matter. However, these nitro compounds had a strong negative effect on the production of volatile fatty acids. The obtained results indicate that NPA is the most promissory nitro compound because it decreased CH_4 emission, redirecting the H electrons to a more efficient pathway as the acetogenic pathway. Further studies should be aimed at understanding the impact of 3-nitropropionic acid on ruminal metabolism for a long period and its effect on *in vivo* models.

Conflicts of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Authors' contributions

Pedro Antonio Ochoa-García, Robin C. Anderson and Agustín Corral-Luna: Planning and design of the study; Pedro Antonio Ochoa-García, Martha María Arevalos-Sanchez: Conducting, sampling and samples analysis; Felipe Alonso Rodríguez-Almeida and Adrián Omar Maynez-Pérez: Statistical analysis; Martha María Arévalos-Sánchez, Monserrath Félix-Portillo, Alberto Muro-Reyes, Aleksandar K. Božić: Manuscript review: All the authors

contribute equally to editing and finalizing the manuscript: Agustín Corral-Luna and Einar Vargas Bello-Pérez: Final version approval.

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