Communication

Effect of Ethyl Nitroacetate, Ethyl 2-Nitropropionate and 3-Nitropropionic Acid on Ruminal Fermentation Characteristics and Dry Matter Degradability under *In Vitro* Conditions

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Abstract: The objective of this study was to investigate the effects of ethyl nitroacetate, ethyl 2-nitropropionate and 3-nitropropionic acid on ruminal production of CH_4, H_2, volatile fatty acids (VFAs) and on dry matter digestibility of ground alfalfa under *in vitro* conditions. *In vitro* incubations were conducted as consecutive batch cultures. Total gas and amounts of methane produced by ruminal microbes were decreased by all nitrocompounds ($P < 0.05$). Total production of VFAs was significantly reduced for all the nitrocompounds ($P < 0.05$) except for NPA. DM disappearance was not affected ($P > 0.05$) by treatment.

Keywords: cattle; CH_4; rumen fermentation; hydrogen; nitrocompounds

1. Introduction

Ruminal methanogenesis is a biological process, which contributes to maintaining a low partial pressure of the hydrogen (H_2) in the rumen. However, methane (CH_4) production is considered a loss of energy, which can reach 12% of the gross energy consumed by the ruminant (Johnson and Johnson, 1995). Furthermore, CH_4 is a greenhouse gas that greatly contributes to the global warming and the Environmental Protection Agency (EPA, 2021) estimates that ruminant animals produce nearly 20% of the total U.S. emissions of CH_4.

Due to the environmental and economic implications, some alternatives can be used to reduce the cost associated with ruminal methanogenesis, for example ionophores such as monensin and lasalocid have been proven effective as CH_4 inhibitors and animal growth promotors (Johnson and Johnson, 1995). While feeding monensin, a typical reduction of 30% in CH_4 production has been reported (Russell and Strobel, 1989; Johnson and Johnson, 1995). However, its efficiency to decrease CH_4 production appears to be transient due to microbial adaptation (Tedeschi et al., 2003). This microbial adaptation has caused public health concerns due to speculation that ionophores may lead to the development of antimicrobial resistance, which could limit their use as feed additive.

Similarly, plant essential oils (EO's) have been demonstrated to possess the capacity to decrease CH_4 production under certain ruminal conditions (Patra and Yu, 2012, Castañeda-Correa et al 2019). The most promising EO has been extracted from *Origanum vulgare*, and it decreased CH_4 production as much as 87% (Patra and Yu, 2012). However, these compounds are not selective to methanogenic bacteria, and their effects can alter populations of beneficial microbes such as cellulolytic bacteria which could decrease apparent dry matter and neutral detergent fiber degradability. Due to these adverse effects, EO may not be a practical additives to mitigate CH_4 emission from ruminants. Recently, the use of seaweed and seaweed bioactives have been investigated as a potential feed additives to mitigate the enteric methane emission (Abbott et al., 2020). However, there are some

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concerns about the bromoform the main antimethanogenic compound founded in the seaweed. There is evidence of their carcinogen effect, and also this compound has a negative effect of ozone layer.

By the other hand, some short chain nitrocompounds have also received attention as a potential additive to mitigate CH_4 emissions from the livestock industry. Some of them like 3-nitropropionate were earlier reported by 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), and 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 of these compounds have reduced rumen CH_4 production as much as 97%. From this list, only the 3-nitrooxypropanol is currently being used as a commercial additive and no adverse effects have been reported (Alemu, et al 2023). This nitrocompound differ structurally from all the remaining nitrocompounds, and its mode of action include the interference of the Methyl-Coenzyme M in the last step of CH_4 reduction by ruminal archaea (Attwood and McSweeney, 2008). Nevertheless, some of these nitrocompounds are not candidates to be used as feed additive, since these are no natural occurring compounds except for 3-nitro-1-propionic 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). Additionally, in most cases, the resulting reduction product is a toxic product or has little or no nutritional value. Some exceptions of this are 3-nitro-1-propionic acid that is reduced to β-alanine (Anderson et al., 1993) and ethyl-nitroacetate may possibly 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 warranted with these as well as other nitrocompounds to investigate if these compounds have effects on dry matter degradability and if their final products are positive for rumen fermentation parameters. Accordingly, the objectives of this study were to investigate the effects of ethyl nitroacetate, ethyl 2-nitropropionate and 3-nitropropionic acid on ruminal production of CH_4, H_2, VFAs and dry matter digestibility of alfalfa under in vitro conditions.

2. Materials and Methods

2.1. Chemicals

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

2.2. In vitro incubations

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 (Theodorou et al., 1994), 0.2 g of finely ground alfalfa hay plus the nitrocompound. The supplemented treatments where designed to achieve 12 μmol/mL of incubation fluid of each nitrocompound (Anderson et al., 2011). Treatments and control were cultured in triplicate. The initial culture was inoculated with 1 mL of fresh rumen fluid. Rumen fluid was collected at 8:00 before the morning meal from two cannulated crossbreed HerefordXAngus heifers feed maintenance ration based on oat straw and corn silage. Five hundred mL from each heifer were placed in a 500 mL container which was sealed hermetically and transported to the lab. 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). Treatments were added to each tube by supplementing 0.3 mL of stock solution of each nitrocompound or distilled water for the control. All the stock solutions of each nitrocompound were prepared as sodium salt solution (Gutierrez-Bañuelos et al., 2007). After 24 h of incubation at 39 ° C, 1 mL from each culture was used to inoculate the next series of tubes. A total of three series (A, B and C) were conducted in this experiment, by sequential transfer of inoculum from the previous series of tubes to new tubes preloaded with fresh medium and feed substrate. Total gas, CH_4, H_2, VFAs and dry mater degradability were measured at the end of the 24h incubation period

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of each series. Total VFAs production was calculated subtracting the initial time (0 h) from the end time (24 h) for each series.

2.3. Fermentation parameters

Total gas (TG) production was determined by measuring volume displacement in a 30-cc glass syringe in each tube after 24 h of incubation. The composition of headspace gas was determined by gas chromatography according (Allison et al., 1992). *In vitro* dry mater disappearance (IVDMD) was determined by drying total content of each tube (Tilley and Terry, 1963). Volatile fatty acids were quantified by gas chromatography following the procedure described by Salanitro and Muirhead, (1975) where 5 mL of the centrifuged sample were 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 x g. The standards were treated in the same way. The VFAs 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

2.4. Statistical analysis

The data for total gas production, IVDMD, gas composition (CH_4 and H_2), and VFAs were subjected to analysis of variance with the MIXED model procedure of SAS (Statistical Analysis for Windows, SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Total gas production and gas composition

Total gas produced by ruminal microbes was decreased by all nitrocompounds ($P < 0.05$) across series, which agrees with results of previous experiments (Anderson et al., 2010) reporting less production of TG when three different short chain nitrocompounds were supplemented in two doses to *in vitro* cultures. Even though decreased TG production was observed for all nitrocompounds, the effect was stronger with ethyl nitroacetate and ethyl-2-nitropropionate than with 3-nitropropionic acid (Table 1). No effect of series of incubation was observed, except for the control tubes produced less TG ($P < 0.05$) from series A to B and C (Table 1). This reduction could be related to the reduced microbial diversity caused for the consecutive transferring, considering that the gas volume recorded is in part directly produced from bacterial fermentation (Amanzougarene and Fondevila, 2020). Additionally, there is possible the loss of H2 producing protozoa could contribute to these reductions in total gas (Solomon et al., 2021), since H2 is part of the gaseous fraction in the rumen.

Methane production was markedly reduced ($P < 0.05$) for all nitrocompounds (Table 1). Ethyl nitroacetate reduced CH_4 production as much as 99 % in series A and B, but inhibit it in series C. These results are consistent with previous data reported by 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 series (Table 1). As was noted by others (Anderson and Rasmussen, 1998; Anderson et al., 2010), this effect on 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). Is suspected that some short chain nitrocompounds had the capacity to inhibit the activity of formate dehydrogenase/formate hydrogen lyase leading a low production of H_2, (Anderson et al., 2008). Additionally, it is known that the reduced end products of some of these nitrocompounds are amino acids or other amines, which can be used as nutrients for the host (Anderson et al., 1993).

Hydrogen produced for the supplemented cultures was slightly higher compared with unsupplemented tubes (Table 1). 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 unsupplemented tubes showed a tendency to increase through series as a consequence of the inhibition of H_2 oxidation combined to the CH_4 production decrease (Anderson et al., 2008) and a low

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disposal of H_2 to other pathways. As was noted by Jenssen (2010), the energy metabolism of ruminants is highly influenced by the flow of metabolic H_2 which could be driving different fermentative pathways. As was mentioned above, the supplemented tubes produced less CH_4 and more H_2, but also these tubes produced more propionate and butyrate, compared to unsupplemented tubes, confirming a shift in the fermentative pathways (Martinez-Fernandez et al., 2016).

3.2. Volatile fatty acid production

The production of VFAs was significantly reduced for all the nitrocompounds ($P < 0.05$) except for NPA. For VFAs production in presence of added nitrocompounds, different results with inconsistencies have been reported. Our results are agreeing with Anderson et al., (2003) who reported a decrease in propionic production 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 VFAs production. Nonetheless, the effect of nitrocompounds in VFAs production tends to disappear over time, as reported by Gutierrez-Bañuelos et al., (2008) where acetate and propionate did not differ from control after incubation series. These results agreed with those reported in this study, where differences in VFAs production after consecutive culturing were not found. These results suggest an adaptation of ruminal microbes to NPA, could be a consequence of an increase in bacterial populations specialized in nitrocompound reduction (Zhang et al., 2018). However, the specific mechanisms are still unclear. The VFAs production on NPA supplementation showed no significant difference compared to the control and the production of propionate was higher than the other nitrocompounds, probably due to the partial conversion of NPA in propionic acid at ruminal level (Ungerfeld, 2015). There is evidence that reductive cleavage of the nitro moiety can occur with some of the short chain nitrocompounds in rumen incubations, but the amount is small, being less than 5% the amount of nitrocompound metabolized (Anderson et al., 1993; Zhang et al., 2020).

3.3. Dry matter digestibility

DM disappearance was not affected ($P > 0.05$) by the addition of nitrocompounds (Table 1). These results agree with others (Zhang et al., 2019; Romero-Perez et al., 2015; Martínez-Fernandez et al., 2014) who reported no differences in ruminal fermentations and digestibility of dry matter when nitrocompounds are used as antimethanogenic supplementation. It is well known that the accumulation of H_2 as a consequence of methanogenesis reduction has an impact on microbial fermentation, principally on metabolic pathways that include cofactors such as NADH and NADPH (Leng, 2014). Nevertheless, the nitrocompounds 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 be 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). On the other side, an effect of the consecutive inoculation was observed for the control tubes and those supplemented with NPA, where DMD decreases from series A to C, following the same tendency that TG, showing the lowest digestibility for all tubes during the B series. These results differ from others (McDermott et al., 2020), who indicate that the 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 microbial ecosystem. Nevertheless, Lin et al., (2019) demonstrate that the adaptation to *in vitro* conditions may take a long time, and the transfer-to-transfer process has an impact on fermentative parameters, including gas produced, pH, and short chain fatty acids SCFA produced, which may cause variability in dry matter degradability of our experiment.

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

Item	Treatment	Serie		
		A*	B*	C*
Total gas (ml/200 mg MS)	Control	22.4±0.49$^{a(a)}$	15.8±1.8$^{a(b)}$	15.9±1.46$^{a(b)}$
	NPA	17.4±0.96$^{b(a)}$	14.6±0.93$^{a(a)}$	13.4±2.96$^{ab(a)}$
	ENA	11.1±0.49$^{c(a)}$	6.78±0.32$^{b(a)}$	9.15±3.42$^{b(a)}$
	E-2-NPP	7.86±3.18$^{c(a)}$	8.07±2.26$^{b(a)}$	9.36±0.64$^{b(a)}$
CH_4 (umo/mL)	Control	9.96±0.61$^{a(a)}$	2.49±1.33$^{a(b)}$	3.03±1.36$^{a(b)}$
	NPA	0.00	0.00	0.00
	ENA	0.09±0.13$^{b(a)}$	0.02±0.04$^{b(a)}$	0.00$^{(a)}$
	E-2-NPP	0.00	0.00	0.00
H_2 (umo/mL)	Control	0.00±0.00$^{a(a)}$	0.19±0.05$^{a(ab)}$	0.30±0.14$^{a(b)}$
	NPA	0.21±0.10$^{ac(a)}$	0.26±0.07$^{a(a)}$	1.0±0.36$^{a(b)}$
	ENA	0.71±0.05$^{b(a)}$	0.33±0.06$^{a(a)}$	0.46±0.45$^{a(a)}$
	E-2-NPP	0.29±0.14$^{c(a)}$	0.54±0.06$^{a(a)}$	0.34±0.08$^{a(a)}$
Acetate (umo/mL)	Control	42.54±4.97$^{a(a)}$	59.7±4.0$^{a(b)}$	49.5±4.85$^{a(ab)}$
	NPA	47.22±1.95$^{a(a)}$	57.2±0.2$^{a(b)}$	53.2±4.38$^{a(ab)}$
	ENA	28.16±2.16$^{b(a)}$	41.8±10.3$^{b(a)}$	43.4±4.44$^{a(a)}$
	E-2-NPP	23.96±5.53$^{b(a)}$	45.00±3.63$^{ab(b)}$	49.10±4.55$^{a(b)}$
Propionate (umo/mL)	Control	17.40±1.21$^{a(a)}$	24.2±4.99$^{a(a)}$	20.90±3.03$^{a(a)}$
	NPA	21.89±0.53$^{b(a)}$	21.4±0.57$^{a(a)}$	18.3±2.91$^{a(a)}$
	ENA	15.23±0.94$^{ac(a)}$	9.13±1.89$^{b(b)}$	16.90±1.81$^{a(a)}$
	E-2-NPP	12.22±2.10$^{c(a)}$	17.2±2.75$^{a(a)}$	15.90±0.32$^{a(a)}$
Butyrate(umo/mL)	Control	4.58±0.15$^{a(a)}$	7.42±0.45$^{a(b)}$	6.42±0.75$^{a(b)}$
	NPA	2.40±0.20$^{b(a)}$	6.47±1.18$^{a(b)}$	6.76±0.51$^{a(b)}$
	ENA	4.70±0.32$^{a(a)}$	5.40±1.33$^{a(a)}$	5.17±0.22$^{b(a)}$
	E-2-NPP	1.95±0.43$^{b(a)}$	6.07±0.33$^{a(b)}$	5.36±0.57$^{b(b)}$
***In vitro* dry matter disappearance**	Control	71.2±2.31$^{a(a)}$	57.1±7.33$^{a(b)}$	56.5±1.03$^{a(b)}$
	NPA	67.8±4.82$^{a(a)}$	50.2±4.62$^{a(b)}$	57.9±1.48$^{a(b)}$
	ENA	62.2±8.48$^{a(a)}$	56.3±6.58$^{a(a)}$	63.2±4.86$^{a(a)}$
	E-2-NPP	65.8±7.26$^{a(a)}$	53.1±3.07$^{a(a)}$	58.7±8.19$^{a(a)}$

*Superscript with different letter denotes difference between treatments. Letters in parenthesis denotes differences between series.

The text continues here.

4. Conclusions

The use of short-chain nitrocompounds such as ethyl nitroacetate, ethyl 2-nitropropionate, and 3-nitropropionic acid showed great efficiency to reduce methane production under *in vitro* conditions without changes in the digestibility of dry matter. The impact of ENA and E-2-NPP on H_2 and VFAs composition, suggests a negative effect of these nitrocompounds on microbial populations, altering the fermentation products. Moreover, the comparison of control and NPA supplemented tubes suggests an adaptation after time of the ruminal ecosystem to this nitrocompound. Further studies should be aimed at understanding the impact of NPA on ruminal metabolism, as well as 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.

Author Contributions: RCA, MFP, AKB and ACL responsible for the general conception of the research, PAOG, AOMP, MMAS and ACL responsible for the experimental procedures and PAOG, RCA, FARA, AOMP, MFP, AMR, AKB, MMAS, EVBP and ACL, manuscript writing and reviewing.

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