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Effects of the methane-inhibitors Nitrophenol, 5-Nitrobenzimidazol and two new synthetic nitrocompounds on *in vitro* ruminal fermentation

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ABSTRACT

The objective of this study was to examine the effects of four nitrocompounds (Nitrophenol, 5-Nitrobenzimidazol and two synthetic nitrocompounds ABLE 244 and ABLE 245) on methane production and fermentation characteristics using *in vitro* rumen batch culture. 0, 2, 8 or 12 μM of each nitrocompound were incubated. The higher concentrations of Nitrophenol and 5-Nitrobenzimidazol produced 60% less CH_4 ($P < 0.05$) compared to controls, while two synthetic nitrocompounds ABLE 244 and ABLE 245 had no effect on CH_4 production. Quantification of fermentation end-products indicated that fermentation efficiencies were not compromised by the nitro-treatments.

1. Introduction

Methane is a greenhouse gas that contributes to global warming (Lassey, 2007). After carbon dioxide; methane is considered the most potent greenhouse gas (IPCC et al., 2001), due to the higher efficiency (20–30 times) of long-wave radiation absorption relative to CO_2 and involvement of CH_4 in chemical reactions that give ozone as the final product (Crutzen, 1995). Due to the increased concentration of CH_4 in the atmosphere in the post-industrial era, several investigations have been involved to identify sources and sinks of methane and to estimate their effects (Bodelier Paul and Laanbroek, 2004; Hilary et al., 2012; Guangming et al., 2013).

In the livestock sector, ruminants contribute significantly to global greenhouse gas emissions (Yáñez-Ruiz and Martín-García, 2016). In terms of the environment, ruminal methanogenesis accounts for about 12–14% of total greenhouse gas emissions (Zervas and Tsiplakou, 2012). But methane production results in a loss of raw energy (4–12%) for cattle fed on forage and fodder (Zhenming et al., 2012). In the rumen, CH_4 is produced by methanogens catalyzing the transfer of hydrogen and carbon dioxide into methane. In addition to methane production, the low hydrogen partial pressure by methanogenesis has a great influence on other products of the non-methanogenic and fermentative microbial community (Wolin et al., 1997). In many cases, the reduction of CH_4 production in the rumen may thus affect digestive function and microbial cell yields due to altered fermentation efficiencies associated with microbial hydrogen transfer reactions (Miller, 1995; Van Nevel and Demeyer, 1996; Anderson et al., 2008)

Several methods have been developed by ruminant microbiologists to reduce the energy losses associated with the production of ruminal CH_4 (Anderson et al., 2008), and many chemical inhibitors reduce methanogenesis (eg monensin and lasalocide) (Russell and Strobel, 1989), plant extracts (tannins for example) (Hariadi and Santoso, 2010) or new synthetic compounds (Patra et al., 2017). These strategies involve supplementing ruminants with anti-methanogenic compounds that directly inhibit methanogens or inhibit the biochemical reactions involved in methane production (Bozic et al., 2009). Among these methods; is the change in electron acceptors that consume more efficiently the reducing equivalents produced during fermentation to redirect the electron flux from the reduction of carbon dioxide to CH_4 (Anderson and Rasmussen, 1998; Sar et al., 2005). Several nitrocompounds have the ability to reduce ruminal methane *in vitro* up to 90% (Anderson et al., 2003), such as nitroethane, 2-nitroethanol, 2-nitro-1-propanol and 3- nitro-1-propionic inhibit the rumen. CH_4 production (Anderson and Rasmussen, 1998; Anderson et al., 2003, 2008; Bozic et al., 2009; Gutierrez-Banuelos et al., 2008). In addition, nitroethane and 2-nitro-1-propanol reduce CH_4-producing activity *in vivo* (Anderson et al., 2006; Gutierrez-Banuelos et al., 2008; Zhang and Yang, 2011), as well as ethyl-3. -nitrooxy propionate and 3-

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nitrooxypropanol have shown potential for successful use as anti-methanogenic additives in ruminants (Martínez-Fernández et al., 2014). Short-chain nitro compounds have demonstrated potently inhibiting methanogenesis and can serve as terminal electron acceptors (Zhenming et al., 2011).

The use of nitrates in reducing methane has been limited because of the risk of potential nitrite accumulation and its ability to cause methemoglobinemic cattle (Zhenming et al., 2012). Recent work, however, suggests that the risk of ruminal nitrite accumulation maybe alleviated by co-supplementation with nitrite-reducing bacteria or decreasing the rapidity of nitrate reduction in the rumen by feeding nitrate with a slow-release coating (Raphélis-Soissan et al., 2017). Therefore, nitrate may be a potential inhibitor to attenuate methane in cattle.

Literature survey revealed that benzimidazole derivatives have considerable interest as an antimicrobial (Ates-Alagoz, 2016) and anticancer agents (Yadav et al., 2016). However, *in vitro* antitumor screening of benzimidazoles toward cancer cell lines demonstrated that these compounds are the most potent analogs toward all tested cell lines (El-Gohary and Shaaban, 2017).

Smith et al. (1988) experiment's on male rats exposed for 2 weeks to up to 2 mg Nitrophenol have demonstrated that no histopathological alterations in the esophagus, stomach, small intestine, colon, and cecum. On the other hand, No studies were located regarding the carcinogenic effects in humans or animals following inhalation exposure to Nitrophenol or Nitrophenol derivatives.

The objective of this study was to evaluate the effects of two newly synthesized nitrocompounds, 13- (4-nitrophenyl) -3,4-dihydro-2H-indazolo [1,2-b] phthalazine-1,6, 11 (2 H, 13 H) -trione (ABLE 244) and 16- (4-nitrophenyl) -1,16-dihydrophthalazino [2', 3′: 1,2] pyrazolo [4,3-a] carbazole-9,14 dione (ABLE 245), and two commercial nitrocompounds, Nitrophenol (NIP) and 5-Nitrobenzimidazol (5-NBZ) on the total *in vitro* production of volatile gas, methane and fatty acid in the rumen.

2. Materials and methods

2.1. Chemicals

Newly synthesized nitrocompounds (Fig. 1) were synthesized at the Crystallography Laboratory (University of Constantine, Algeria) as follows:

The 13-(4-nitrophenyl)-3,4-dihydro-2*H*-indazolo [1,2-b] phthalazine-1,6,11(2*H*, 13*H*)-trione (ABLE 244) was prepared according to the modified procedure (Sayyafi et al., 2008) *via* the multi component reaction of phthalhydrazide (1 mmol), 1,3-cyclohexadione (1.05 mmol) and 4-nitrobenzaldehyde (1.05 mmol) at reflux of acetic acid and in the presence of a catalytic amount of trifluoroacetic acid.

The 16-(4-nitrophenyl)-1,16-dihydrophthalazino [2′,3′:1,2] pyrazolo [4,3-a]carbazole-9,14 dione (ABLE 245)was obtained according to the previously described procedure (Lamera et al., 2017) from reaction of phthalhydrazide (1 mmol), 4-nitrobenzaldehyde (1 mmol), 1,3-cyclohexadione (1.05 mmol) and phenylhydrazine (1.7 mmol) using a sequential MCR/Fisher indolization strategy at reflux of acetic acid and in the presence of a catalytic amount of trifluoroacetic acid.

2.2. Experimental design and animal management

Animals were cared and handled in accordance with the Spanish guidelines for experimental animal protection (Royal Decree 53/2013 on the protection of animals used for experimentation or other scientific purposes) in line to corresponding European Directive (2010/63/EU). The corresponding experimental protocol was approved by the Ethics and Animal Welfare Committee of the Estación Experimental del Zaidín (Spanish National Research Council).

Rumen content from three canulated goats was collected before morning feeding, through two layers of sterilized cheesecloth under a steady stream of oxygen-free CO_2 and maintained at 40 °C in a water bath. Then, in an interval of time lasting 30 min, the rumen fluid was used as inoculums for *in vitro* batch incubations as follow.

2.3. In vitro incubation

Tests for effects of inhibitors on ruminal methane production were accomplished by batch culture (Theodorou et al., 1987). The culture medium consisted of an artificial saliva (Menke and Steingass, 1988) that was bubbled with CO_2 until saturated before being used (Krishnamoorthy et al., 1991) and the clarified rumen fluid in a 3:1 ratio in crimp-top Wheathon bottles (capacity 120 ml). Each *in vitro* culture tube contained 30 ml medium and 10 ml fresh rumen fluid collected from three canulated goats before morning feeding; containing 0.3 g ground oats hay, and 0, 2, 8 and 12 μM of NIP, 5-NBZ, ABLE 244 and ABLE 254.

Culture tubes were immediately closed with rubber stoppers to contain the respective gas phase, and incubated upright without agitation at 39 °C during 24 h. Gas pressure in headspace was released and quantified using a Pressure Meter (Wide Range 840065) after 2, 4,6,12 and 24 h.

2.4. Gas and methane measurement

After 24 h incubation, a gas sample (about 5 ml) was stored in an evacuated tube (Terumo Europe N.V., Leuven, Belgium) to determine CH_4 produced from 12 to 24 h by gas chromatography using a HP Hewlett 5890 Packard Series II gas chromatograph (Waldbronn, Germany) equipped with a flame ionization detector (FID) and an HPINNOWAX cross linked polyethylene glycol column

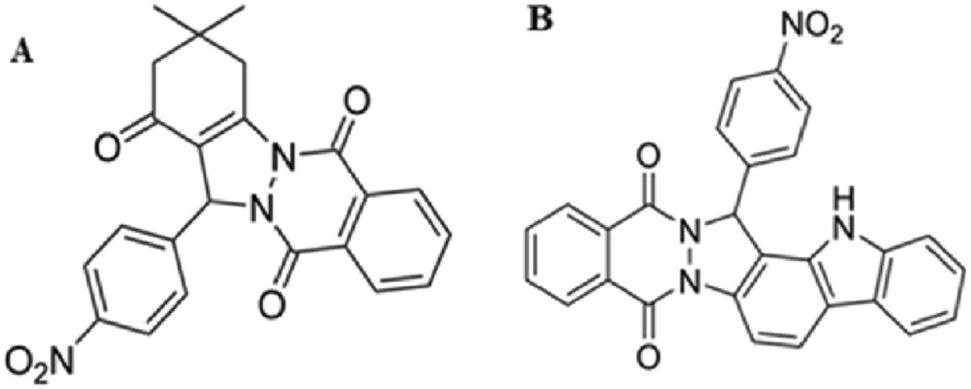

Fig. 1. Structure of ABLE 244 (A) (Sayyafi et al., 2008) and ABLE 245 (B) (Lamera et al., 2017).

(25 m × 0.2 mm × 0.2 μm; Teknokroma, Madrid, Spain). The carrier gas was $N_2$and peaks were identified and quantified by using with a standard curve made by injecting different volumes of 99.9% pure CH_4 pre and post the injection of samples. Samples of 0.5 ml of gas were injected using a 1 ml Sample-Lock® syringe (Hamilton, Reno, NV, USA). Bottles were then opened and a sample was taken (0.8 ml was added to 0.8 ml of deproteinising solution consisting in 20 g of metaphosphoric acid and 0.8 g of crotonic acid, as internal standard, per liter) for GC VFA determination following procedure described by Arco-Pérez et al. (2017).

2.5. Statistical analyses

The incubation run was performed using the rumen fluid from each of the three goats to inoculate each of the 3-replicates per treatment (per compound and dose). The total gas and CH_4 production, VFA and Protozoa counts data were analyzed using the SPSS software (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, New York, USA) by general linear model (GLM) repeated measures (dose), including the fixed effects of compound and dose × compound interaction, with the donor animal as the experimental unit. Polynomial response to incremental amounts of each compound was evaluated using orthogonal contrasts. When a significant effect was found, post hoc comparison of means by compound was made using the LSD test. Differences were declared significant at $P < 0.05$ and considered as tendencies towards significance at $P < 0.10$.

3. Results and discussion

3.1. Total gas and CH_4 production

Total gas production (GP) was decreased ($P < 0.05$) by near 30% with 12 μM Nitrophenol (NIP) compared to unsupplemented control cultures (Table 1). These results indicate that NIP at this level negatively affects the *in vitro* rumen fermentation. However, 5-NBZ, ABLE 244 and ABLE 245 did not affect gas production.

A number of *in vitro* studies have demonstrated the anti-methanogenic potential of a class of nitro-compounds such as nitroethane, 2-nitroethanol, 2-nitro-1-propanol and 3-nitro-1- propionic acid (Anderson and Rasmussen, 1998), consistent with similar studies using other commercially available compounds like nitroethane (Anderson et al., 2010) and with earlier *in vitro* studies with short chain nitro-compounds (Gutierrez-Banuelos et al., 2008; Saengkerdsub et al., 2006), that with the higher concentration being near that used in earlier studies (Anderson et al., 2003, 2008, 2010), NIP and 5-NBZ markedly inhibited CH_4 production ($P < 0.05$) with 12 μM NIP and 12 μM 5-NBZ by more than 60% when compared to control (Table 1).

These nitrocompounds, that reduce CH_4 and gas production, may have transformed by ruminal bacteria; Kulkarni and Chaudhari (2007) reported that most organisms contain redox enzymes (nitroreductase), which transform nitro-aromatics into corresponding amines, through the successive addition of electron pairs donated by co-substrates. Microorganisms of the rumen fluid biocatalyzed the reduction of nitro-compound substrates to yield the respective amines (Rodríguez et al., 2011).

Rhodococcus erythropolis, *Rhodococcus* sp., *Nocardioides* sp., *Nocardioides simplex* and *Rhodococcus* sp. are all able to grow using Nitrophenol as sole carbon and/or nitrogen and energy sources (Kou-San and Rebecca, 2010).

A main effect on total gas production was not observed ($P > 0.05$) with ABLE 244 and ABLE 245, probably because the biodegradability of long-chain products by bacteria is difficult (Tarayre, 2012). Likewise, the absence of the adaptation of bacteria to the nitrocompounds (due to insufficient contact time) makes its degradation difficult (Mazzeo et al., 2010).

Microorganisms are the only entities in the biosphere with the ability to exploit various organic/inorganic compounds for their growth. They are able to inhabit various ecological niches and to pursue unusual metabolic and physiological activities (Timmis et al., 1994). We propose that NIP and 5-NBZ be reduced under anaerobic conditions to corresponding amines (Nishino and Spain, 2002; Zhang and Bennett, 2005; Kulkarni, and Chaudhari, 2007).

3.2. Volatile fatty acid production

To compensate the accumulation of H_2, the rumen microbial ecosystem often disposes of excess reducing equivalents by increasing the production of more reduced volatile fatty acids (Van Nevel and Demeyer, 1996).

The results of the fermentation analyses indicate that the production of VFA in the *in vitro* ruminal cultures has been modified by the addition of nitrocompounds, and that these alterations have varied between the concentrations tested (Table 2). It is possible that organic nitrocompounds reduce methane production and redirect reducing equivalents to butyrate and propionate production (Zhenming et al., 2012).

Compared to the control cultures, total VFA was significantly increased by NIP at 2 μM, but not at 8 and 12 μM. However, the lack of effect on total VFA concentration at both tested doses suggests that fermentation was not compromised by majority of compounds (Martínez-Fernández et al., 2014). Acetate was reduced by 2, 8 and 12 μM 5-NBZ, but not affected by addition of others nitrocompounds.

Table 1
Effects of nitro-compounds on fermentation characteristics after 24h batch rumen microorganisms culture.

Item	Compounds	Dose (μM)				SEM	*p value*
		0	2	8	12		
Total gas production (ml/g DM)	**NIP**	321[a]	327[a]	275[ab]	226[b]	3.39	0.009
	5-NBZ	321	320	305	284	3.21	0.546
	ABLE244	321	327	321	321	3.25	0.995
	ABLE245	321	331	323	343	2.60	0.827
CH_4 (ml/ml)	**NIP**	0.181[a]	0.183[a]	0.122[ab]	0.062[b]	0.01	0.002
	5-NBZ	0.181[a]	0,158[a]	0.139[ab]	0.121[b]	0.01	0.031
	ABLE244	0.181	0.156	0.167	0.156	0.01	0.469
	ABLE245	0.181	0.158	0.162	0.177	0.01	0.954
CH_4 (ml/gDM)	**NIP**	58.1[a]	59.8[a]	33.5[ab]	14.0[b]	1.36	0.004
	5-NBZ	58.1[a]	50.5[a]	42.3[ab]	34.3[b]	1.21	0.050
	ABLE244	58.1	51.0	53.6	50.0	1.22	0.870
	ABLE245	58.1	52.2	52.3	60.7	1.25	0.822

NIP, Nitrophenol; 5-NBZ, 5-Nitrobenzimidazol; ABLE 244 and ABLE 245, synthesized nitrocompounds.
[a,b, ab]Values within columns with unlike superscripts differ ($P < 0.05$).

Table 2
Effects of nitrocompounds on volatile fatty acid (VFA) after 24 h batch rumen microorganisms' culture.

Item	Compounds	Dose (μM) 0	2	8	12	SEM	*p value*
Total VFA(mM)	NIP	102^{ab}	138^{a}	99.1^{ab}	83.4^{b}	5.53	0.087
	5-NBZ	102	119	105	96.3	4.70	0.461
	ABLE244	102	110	104	102	2.66	0.810
	ABLE245	102	113	104	102	2.85	0.729
Acetate (%)	NIP	62.3	62.0	59.9	60.5	0.66	0.253
	5-NBZ	62.3^{a}	61.3^{a}	58.8^{ab}	57.7^{b}	0.49	0.006
	ABLE244	62.3	61.5	61.8	61.1	0.36	0.352
	ABLE245	62.3	61.6	62.1	61.8	0.44	0.853
Propionate (%)	NIP	17.8^{a}	17.6^{a}	19.9^{ab}	21.1^{b}	0.52	0.030
	5-NBZ	17.8^{a}	18.6^{ab}	21.3^{ab}	22.8^{b}	0.43	0.002
	ABLE244	17.8	18.0	18.1	18.5	0.25	0.359
	ABLE245	17.8	18.2	18.0	18.1	0.26	0.722
Isobutyrate (%)	NIP	1.66^{ab}	1.76^{a}	1.56^{ab}	1.20^{b}	0.06	0.024
	5-NBZ	1.66	1.76	1.56	1.70	0.05	0.829
	ABLE244	1.66	1.70	1.70	1.80	0.06	0.478
	ABLE245	1.66	1.63	1.70	1.73	0.06	0.648
Butyrate (%)	NIP	13.1	13.3	13.9	13.8	0.30	0.326
	5-NBZ	13.1	13.2	13.6	13.2	0.32	0.839
	ABLE244	13.1	13.5	13.4	13.5	0.22	0.573
	ABLE245	13.1	13.4	13.4	13.3	0.22	0.802
Isovalerate (%)	NIP	3.20^{a}	3.36^{ab}	3.20^{a}	2.46^{b}	0.09	0.027
	5-NBZ	3.20	3.20	2.96	3.00	0.08	0.322
	ABLE244	3.20	3.40	3.20	3.30	0.10	0.921
	ABLE245	3.20	3.30	3.10	3.20	0.10	0.843
Valerate (%)	NIP	1.93^{a}	1.86^{ab}	1.36^{ab}	0.70^{b}	0.07	0.000
	5-NBZ	1.93^{a}	1.76^{ab}	1.66^{b}	1.56^{b}	0.02	0.001
	ABLE244	1.93^{a}	1.80^{ab}	1.73^{b}	1.70^{b}	0.02	0.015
	ABLE245	1.93^{a}	1.73^{a}	1.56^{b}	1.66^{ab}	0.03	0.018
Acetate: propionate	NIP	3.50	3.52	2.99	2.90	0.12	0.065
	5-NBZ	3.50^{a}	3.30^{ab}	3.03^{ab}	2.80^{b}	0.10	0.039
	ABLE244	3.50	3.41	3.42	3.30	0.06	0.356
	ABLE245	3.50	3.38	3.45	3.41	0.07	0.783

NIP, Nitrophenol; 5-NBZ, 5-Nitrobenzimidazol; ABLE 244 and ABLE 245, synthesized nitrocompounds.
$^{a,b,\ ab}$Values within columns with unlike superscripts differ ($P < 0.05$).

The decrease in acetate concentrations by treatment with 5-NBZ could indicate a decrease in fiber degradation, as some fibrolytic microorganisms are more sensitive to high hydrogen partial pressure (Morgavi et al., 2010). At all the four 5-NBZ concentrations examined, 5-NBZ increased propionate concentrations, with 12 μM reducing the most, also, 12 μM of NIP was increased propionate but it was not affected with others nitrocompounds. As such, 5-NBZ and NIP remarkably reduced acetate: propionate ratio (Table 2). This explanation corroborates the finding of a previous *in vitro* study; increases in pH typically result in a shift of fermentation towards reduced VFAs (*e.g.* propionate and butyrate) in the rumen, leading to decreased acetate:propionate ratio (Zhenming et al., 2012). A reduction in the acetate: propionate ratio has been described as a common feature of several antimethanogens. This indicates a concurrent decrease of methane formation and redirection of hydrogen from methane to more propionic metabolic pathways (Martínez-Fernández et al., 2014). This discrepancy might be attributable to other factors, such as selective consumption of individual VFAs by nitrate-respirating bacteria. Future studies using defined mixtures of VFAs as the sole substrates can help to verify this surmise.

Analysis of butyrate at the end of the incubation in this study revealed no effect ($P > 0.05$) of nitro-treatment on his concentration. This is agreement to a previous study where butyrate was not affected by level of organic nitrocompound (eg. Nitroethane) (Brown et al., 2011), suggesting that electrons spared from CH_4 production were not redirected to production of butyrate (Gutierrez-Banuelos et al., 2008; Bozic et al., 2009).

Isobutyrate concentrations were reduced by 8 and 12 μM of Nitrophenol, while not affected by other nitrocompounds. NIP reduced isovalerate at 12 μM ($P < 0.05$), but not at the other concentrations. On the contrary, valerate was markedly affected by all of the four treatments (NIP, 5-NBZ, ABLE244 and ABLE 245), with 12 μM of 5-NBZ reducing the most ($P = 0.001$), ABLE 244 and ABLE 245 reduced only valerate. These observations were contrary to observed by Anderson et al. (2010); using nitroethane, dimethyl-2-nitroglutarate and 2-nitromethyl-propionate with 2.97 or 11.88 μM.

Indeed, of the four compounds tested, NIP and 5-NBZ reduced methane production by a large margin, and the first appeared to be the most potent. In the case of NIP treatment, reductions in methane production were accompanied by lower production of total VFAs. These results suggest that reduced fermentation activities are among the possible reasons for the reduced methane production in the rumen cultures, a finding that corroborates previous studies using different inhibitors (Beauchemin and McGinn, 2006; Holtshausen et al., 2009). The effect of antimethanogenic compounds on total and individual VFAs can be affected by other factors, such as the presence or absence of fermentable sugars, and the VFAs detected may be those present in the original ruminal fluid. So, the effects of antimethanogenic compounds on fermentation and ruminal digestion should be interpreted taking into account the substrates present in the cultures (Zhenming et al., 2011). In this study, there might be little fermentation or VFA production in the blank bottles without ground oats hay; due to the lack of fermentable sugars, and the VFAs detected might be those present in the original rumen fluid.

3.3. Total protozoa

Protozoa are the greatest producers of hydrogen in the rumen ecosystem (Szumacher-Stabel and Cieslak, 2012). The microbial mechanisms under CH_4 production involve interspecies H_2 transfer between H_2-producers and methanogens (Wolin et al., 1997). The most studied example of this H_2 transfer is the symbiotic relationship between methanogens and protozoa (Newbold et al., 1995): methanogens are positioned on the protozoa to reduce the distance for diffusion of H_2 from the hydrogenosome. These methanogens associated with protozoa would be responsible for between 9% and 25% of methanogenesis in rumen fluid (Newbold et al., 1995).

The quantity of total protozoa affected by compounds is presented in Table 3. As observed with other parameters, the addition of ABLE 244 and ABLE 245 had no significant effects on the population of total protozoa. While 5-NBZ significantly ($P < 0.05$) suppressed the population of total protozoa. Previous studies have shown that the addition of nitrate in the ration of ruminants negatively affects the protozoa population (Guyader et al., 2015). The inhibition of methanogenesis is expected to increase the partial pressure of H_2, potentially leading to an inhibition of H_2-producing microorganisms such as *Ruminococci*, protozoa, and fungi (Martínez-Fernández et al., 2014).

Table 3
Effects of nitro-compounds on total protozoa counts (log10) after 24 h batch rumen microorganisms culture.

Compounds	Dose μM 0	2	8	12	SEM	*p value*
NIP	8.44^{a}	8.41^{a}	8.30^{ab}	8.18^{b}	0.06	0.179
5-NBZ	8.44^{a}	8.41^{a}	8.47^{a}	8.10^{b}	0.03	0.017
ABLE244	8.44	8.40	8.56	8.39	0.06	0.614
ABLE245	8.44	8.23	8.29	8.41	0.04	0.983

NIP, Nitrophenol; 5-NBZ, 5-Nitrobenzimidazol; ABLE 244 and ABLE 245, synthesized nitrocompounds.
$^{a,b,\ ab}$Values within columns with unlike superscripts differ ($P < 0.05$).

4. Conclusion

Results from the present study confirm the CH_4-inhibiting activity of Nitrophenol and 5-Nitrobenzimidazol. The reduction in CH_4 emissions observed in this trial using 5-NBZ did not cause a rumen dysfunction.

The two newly tested compounds, ABLE 244 or ABLE 245 did not have CH_4-inhibiting activity and we hypothesize that these long chain nitrocompounds are not easily degradable by the ruminal flora. However, those new synthetic compounds seem have no adverse effects on rumen microbial fermentation. Further studies on longer-term fermentation (over 24 h) are needed to elucidate the need for additional time for microorganism to adapt to these compounds.

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