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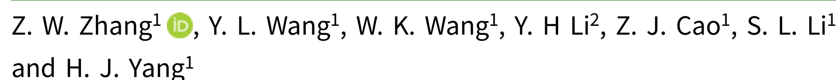
The inhibitory action mode of nitrocompounds on *in vitro* rumen methanogenesis: a comparison of nitroethane, 2-nitroethanol and 2-nitro-1-propanol



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Abstract

Nitroethane (NE), 2-nitroethanol (NEOH) and 2-nitro-1-propanol (NPOH) were investigated in order to determine their inhibitory effects on *in vitro* ruminal fermentation and methane (CH_4) production of a hay-rich substrate (alfalfa hay: maize meal = 4:1, w/w). The rumen liquor collected from cannulated Holstein dairy cows was incubated at 39 °C for 72 h. The addition of NE, NEOH and NPOH slowed down the fermentation process and notably decreased molar CH_4 proportion by 96.8, 96.4 and 35.0%, respectively. The abundance of total methanogen and methanogens from the order *Methanobacteriales* were all decreased with NE, NEOH and NPOH supplementation. Meanwhile, the nitrocompound addition reduced *mcr*A gene expression, coenzyme F_{420} and F_{430} contents. The correlation analysis showed that CH_4 production was correlated positively with the population abundance of total methanogens, *Methanobacteriales*, *mcr*A gene expression, coenzyme contents of F_{420} and F_{430}. The nitrocompound addition decreased acetate concentration and increased propionate and butyrate concentrations in the culture fluid. In summary, both NE and NEOH addition presented nearly the same inhibitory effectiveness on *in vitro* CH_4 production; they were more effective than NPOH. The results of the current study provide evidence that NE, NEOH and NPOH can dramatically decrease methanogen population, *mcr*A gene expression and the coenzyme content of F_{420} and F_{430} in ruminal methanogenesis.

Introduction

Methane (CH_4) production by ruminants, a major source of greenhouse gas emissions, is generally recognized as a fermentative inefficiency resulting in 2–12% loss of the gross energy consumed by the host (Johnson and Johnson, 1995; Thauer *et al*., 2010). During the past few decades, researchers have put a great deal of effort into mitigating CH_4 emission in ruminant animals (Patra, 2012; Vyas *et al*., 2018; Ochoa-García *et al*., 2019). Among these mitigating strategies, nitroethane (NE), 2-nitroethanol (NEOH), 2-nitro-1-propanol (NPOH) and 3-nitro-1-propionic acid (NPA) have been found to inhibit ruminal methanogenesis by as much as 90% *in vitro* through inhibiting biochemical conversions involved in methanogenesis (Anderson *et al*., 2008; Smith and Anderson, 2013; Latham *et al*., 2016; Correa *et al*., 2017; Ochoa-García *et al*., 2019), and NE and NPOH have been shown to reduce CH_4-producing activity by more than 69% *in vivo* (Anderson *et al*., 2006; Gutierrez-Bañuelos *et al*., 2007; Latham *et al*., 2016). However, it is not clear if the aforementioned inhibition can also be achieved directly by decreasing the population and/or carbon dioxide (CO_2)-reducing activity of methanogens. It is well known that methylcoenzyme M reductase (MCR) catalyses the final step in methanogenesis, converting the coenzyme M-bound methyl group to CH_4 (Thauer, 1998), and the prosthetic group of the MCR is coenzyme F_{430}, which is a nickel porphinoid (Ankel-Fuchs *et al*., 1984). Additionally, coenzyme F_{420} shows strong fluorescence in the oxidized form and was believed to exist only in methanogenic bacteria (Schulze *et al*., 1988). However, it is unknown how nitrocompounds could affect these coenzymes' activities during inhibition of ruminal methanogenesis.

Zhang and Yang (2011) noted that the optimal combination of 15 mM NE, 10 mM NEOH, 5 mM NPOH, 0.07 mM pyromellitic diimide and 0.01 mM 2-bromoethanesulphonate in cultures with an orthogonal experiment led to >95% CH_4 inhibition of a hay-rich substrate, and the combination of these inhibitors shifted ruminal fermentation from acetate towards propionate production. However, it was not clear what difference existed among NE, NEOH and NPOH at same dosage in terms of CH_4 inhibition as well as the shift of the methanogen community.

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In the present study, methyl-coenzyme M reductase activity as well as related coenzymes (e.g. F_{420} and F_{430}) was determined, and the objective was to explore the inhibition action mode of nitrocompound on *in vitro* rumen methanogenesis through a comparison of NE, NEOH and NOPH under a fixed dosage of 10 mM. The obtained outcomes were expected to provide a scientific reference for future *in vivo* investigation of these nitrocompounds in reducing methane emission in ruminant animals.

Materials and methods

Nitrocompound chemicals

The nitrocompound products were purchased commercially from Sigma Aldrich (St. Louis, MO, USA) and stored at 4 °C. Among these nitrocompounds, the NE product is a colourless oily liquid and almost insoluble in water. Both NEOH and NPOH products are light yellow liquids with a low boiling point. Their analytical grades were 99, 90 and 98%, respectively.

In vitro *batch cultures and sampling*

Alfalfa hay, harvested at the early bloom stage, was chopped into 2–5 mm strips with a paper cutter and oven dried at 65 °C for 48 h. The dried hay samples were then ground in a Wiley mill to pass through a 2.0 mm sieve, and then mixed with maize meal (4:1, w/w) to prepare a hay-rich substrate for subsequent *in vitro* batch culture experiments.

Five rumen-cannulated lactating Holstein dairy cows, fed in a free stall, served as donor animals for rumen fluids. The cows had free access to water and were fed a total mixed ration of 18.0 kg maize silage, 4.0 kg alfalfa hay and 14.5 kg concentrate daily. On the day before starting *in vitro* batch cultures, the animals were driven away from the lactating herds: rumen fluid was collected from each animal through rumen fistula 3 h after the morning feed and kept in pre-warmed vacuum flasks.

Glass bottles (volume capacity of 120 ml) with Hungate stoppers and screw caps were used as incubators. A completely randomized design was applied to three runs of *in vitro* batch cultures, and 0.5 g hay-rich substrate was weighed into 80 bottles/run with 20 bottles for each treatment. The treatment included a nitrocompound-free control, 10 mM of NE, 10 mM of NEOH and 10 mM of NPOH, respectively.

Following the experimental design, 80 bottles in each run were incubated anaerobically with 25 ml of rumen fluids strained through four layers of cheesecloth and 50 ml of 39 °C pre-warmed media buffer (pH 6.85; Menke and Steingass, 1988). In addition, four fermentations without substrate and nitrocompounds were used as blanks. The batch cultures were carried out at 39 °C in both automated and manual systems. In the automated system, cumulative gas production (GP) was recorded continuously by connecting treated bottles (five bottles/treatment) to the gas inlets of an automated gas recording system and incubating continuously for 72 h. In the manual system, fermentation gas samples were collected from the treated bottles (three bottles/treatment/incubation time) by connecting them to pre-emptied air bags which were then removed at 6, 12, 24, 48 and 72 h of incubation. A 1.0 ml gas sample was taken from the airbags, and CH_4, CO_2 and H_2 contents in fermentation gas samples were determined using a gas chromatographic method (Zhang and Yang, 2011).

The biomass content of each bottle was filtered through a nylon bag (8 × 12 cm, 42 μm pore size) to determine *in vitro* dry matter disappearance (IVDMD) at 6, 12, 24, 48 and 72 h. Then the filtered culture fluid (5 × 1.0 ml) was sampled into DNase-free polypropylene tubes and stored at −80 °C for the analysis of volatile fatty acid (VFA), methanogen population, *mcr*A gene expression, coenzyme contents of F_{420} and F_{430}.

Determination of in vitro *dry matter digestibility, volatile fatty acids and coenzyme content*

The difference between initially incubated dry matter (DM) and the residual DM in nylon bags (corrected using the blanks, after incubation) was calculated to determine the IVDMD. The culture fluid samples (1.0 ml) were treated with 0.3 ml metaphosphoric acid solution (25 mg/ml) and centrifuged at 15 000 ***g*** for 10 min at 4 °C. The concentrations of acetate, propionate, butyrate and branch-chained VFAs of iso-butyrate and iso-valerate in the supernatants were measured by a gas chromatography (GC522, Wufeng Instruments, Shanghai, China). Coenzyme F_{420} content was determined as previously described by Reuter *et al.* (1986) and expressed as fluorescence intensity of the coenzyme. Following the method of Ellefson *et al.* (1982), coenzyme F_{430} content was determined via the ultraviolet/visible spectrum by measuring the loss of absorbance and expressed as the relative absorbance of coenzyme F_{430} at 430 nm.

Expression analysis of mcrA gene

Total genomic RNA was extracted from a 1 ml aliquot of culture fluid samples using a RNeasy Mini kit (Tiangen® Biotech, Beijing, China) with an RNase-Free DNase Set (Qiagen) following the manufacturer's instructions. The cDNA was synthesized with a FastKing RT cDNA Kit (Tiangen® Biotech). The enumeration of cDNA of *mcr*A gene was measured on a Bio-Rad Multicolor Real-Time Polymerase Chain Reaction (PCR) Detection System (Bio-Rad Company, California, USA) using the RealMasterMix SYBR® Green (Tiangen® Biotech). The $2^{-\Delta\Delta Ct}$ method was used for expression analysis of the *mcr*A gene with 16S rRNA set as the reference gene (Livak and Schmittgen, 2001). The specific primer set for 16S rRNA gene and *mcr*A gene (Supplementary Material Table S1) were applied as described by Denman and McSweeney (2006) and Denman *et al.* (2007), respectively.

Determination of methanogenic population with real-time polymerase chain reaction

A bead-beating method, described by Denman and McSweeney (2006) and the FastDNA kit and FastPrep instrument (Tiangen® Biotech) were used for total DNA extraction. Total genomic DNA was isolated from a 1 ml aliquot of cultural fluid samples. Following the real-time PCR method as described by Denman and McSweeney (2006) and Denman *et al.* (2007), the enumeration of total methanogens (a primer applied as described by Zhou *et al.*, 2009), *Methanobacteriales*, *Methanococcales* and *Methanomicrobiales* (primers applied as described by Yu *et al.*, 2005) was measured on a Bio-Rad Multicolor Real-Time PCR Detection System (Bio-Rad Company) using the RealMasterMix SYBR® Green (Tiangen® Biotech). Their microbial abundances are expressed as a proportion of total estimated rumen bacterial 16S rDNA (Denman and McSweeney, 2006) according to the equation: relative quantification = $2^{-(CT\ target-CT\ total\ bacteria)}$, where CT represents the threshold cycle (Guo *et al.*, 2008).

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Table 1. Effect of nitroethane (NE), 2-nitroethanol (NEOH) and 2-nitro-1-propanol (NPOH) addition (10 mM) in culture fluids on kinetic gas production and fermentation gas composition during 72 h incubation

Parameter	Treatment				S.E.M.	*P* value		
	Control	NE	NEOH	NPOH		Treatment	Time	Interaction
$IVDMD_{72}$ (g/kg)	758	801	766	765	26.0	0.472	–	–
GP_{72} (ml/g DM)	139	122	122	136	1.3	<0.001	–	–
Kinetic gas production								
A (ml/g DM)	140	123	122	136	1.3	<0.001	–	–
c (/h)	0.15	0.15	0.15	0.13	0.004	0.026	–	–
$T_{1/2}$ (h)	2.58	2.62	2.57	2.75	0.030	0.024	–	–
AGPR (ml/h)	15.3	12.9	13.4	12.5	0.41	0.012	–	–
Fermentation gas composition (mol/100 mol)								
H_2	0.3	5.2	5.0	1.4	0.09	<0.001	<0.001	<0.001
CO_2	84.6	94.2	94.4	88.8	0.24	<0.001	<0.001	<0.001
CH_4	15.0	0.4	0.5	9.7	0.17	<0.001	<0.001	<0.001

NE, nitroethane; NEOH, 2-nitroethanol; NPOH, 2-nitro-1-propanol; $IVDMD_{72}$, *in vitro* dry matter disappearance of 72 h; GP_{72}, cumulative gas production at 72 h; *A*, the asymptotic gas production (ml/g DM); *c*, the fractional gas production rate (/h); $T_{1/2}$, the time when half of *A* occurred (h); AGPR, the average gas production rate (ml/h) between the start of the incubation and the time when half of *A* occurred; H_2, hydrogen gas; CO_2, carbon dioxide; CH_4, methane.

Calculations

The Microsoft Excel data of the cumulative gas production against the different incubation time (GP_t, ml/g DM) were imported into an SAS data set and fitted with the non-linear (NLIN) procedure of SAS 9.4 (Statistical Analysis for Windows, SAS Institute Inc., Cary, NC, USA) according to the France *et al.* (2000) model using Eqn (1):

$$GP_t = A \times [1 - e^{-c\times(t-L)}] \quad (1)$$

where GP_t is the cumulative gas production at time *t* (h); *A* is the estimated asymptotic gas production (ml/g DM); *c* is the fractional gas production rate (/h), and *L* is the lag time phase before GP commenced.

Following the method of García-Martínez *et al.* (2005), the average gas production rate (AGPR, ml/h) was calculated using Eqn (2):

$$AGPR = \frac{A \times c}{2 \times (\mathrm{Ln}2 + c \times L)} \quad (2)$$

The time when half of *A* occurred ($T_{1/2}$) was calculated using Eqn (3):

$$T_{1/2} = \log\left(\frac{1}{c}\right) + L \quad (3)$$

Following Demeyer and Graeve (1991), hydrogen recovery ($2Hrec$) was calculated using Eqn (4):

$$2Hrec = (2 \times \text{propionate} + 2 \times \text{butyrate} + 4 \times CH_4 + H_2) / (2 \times \text{acetate} + \text{propionate} + 4 \times \text{butyrate}) \quad (4)$$

where acetate, propionate and butyrate are given as their molar percentages in total VFA production, and CH_4 and H_2 as their molar percentages in the total gas production.

Statistical analysis

Data were analysed by analysis of variance using the general linear model procedure of SAS 9.4 (Statistical Analysis for Windows, SAS Institute Inc.). The model was applied as:

$$Y_{ij} = m + N_i + T_j + (N \times T)_{ij} + e_{ij} \quad (5)$$

where Y_{ij} is the dependent variable under examination; μ is the overall mean; N_i is the fixed effect of nitrocompound treatment (i = control, NE, NEOH and NPOH); T_j is the fixed effect of incubation time (6, 12, 24, 48 and 72 h); $N \times T$ is the interaction effect between nitrocompound treatment and incubation time. Least square means (LSMEANS) and standard errors of the means (S.E.M.) across 6, 12, 24, 48 and 72 h were calculated using the LSMEANS statement of SAS and tabulated in Tables 1 and 2. Overall differences among nitrocompound treatments were determined by Tukey's test. Pearson correlation analyses between variables under examination were performed using the correlation (CORR) procedure of SAS 9.4. Significance was declared at $P < 0.05$ unless otherwise noted.

Results

In vitro dry matter disappearance and kinetic gas production

After 72 h incubation, $IVDMD_{72}$ did not differ among different treatments (Table 1 and Supplementary Material Fig. S1, $P = 0.472$). Asymptotic gas production (*A*) and GP_{72} were decreased in both NE and NEOH in comparison with the control ($P < 0.001$), but no difference occurred between NPOH and the control. As shown in Table 1 and Fig. S1, neither NE nor NEOH addition altered *c* and $T_{1/2}$, but NPOH decreased *c* ($P = 0.026$) and

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Table 2. Effect of nitroethane (NE), 2-nitroethanol (NEOH) and 2-nitro-1-propanol (NPOH) addition (10 mM) on the relative abundance of methanogenic populations, coenzyme content, *mcr*A gene expression and volatile fatty acid production in fermentation fluids across different incubation times of 6, 12, 24, 48 and 72 h

Parameter	Treatments				S.E.M	P value		
	Control	NE	NEOH	NPOH		Treatments	Time	Interaction
Total methanogen	0.42	0.14	0.13	0.19	0.063	0.007	<0.001	<0.001
Methanobacteriales	0.16	0.11	0.11	0.08	0.033	<0.001	<0.001	<0.001
Methanomicrobiales ($\times 10^{-2}$)	0.49	0.17	0.23	0.20	0.042	0.045	<0.001	<0.001
Methanococcales ($\times 10^{-2}$)	0.26	0.07	0.05	0.09	0.012	0.007	<0.001	<0.001
Coenzyme content								
F_{420}	16.7	12.6	11.6	13.3	0.46	<0.001	<0.001	<0.001
F_{430}	0.63	0.56	0.54	0.51	0.030	<0.001	<0.001	0.063
*mcr*A expression	1	0.16	0.20	0.46	0.025	<0.001	<0.001	<0.001
Total VFA (mmol/l)	99	100	99	103	2.2	0.087	<0.01	0.074
Acetate (mmol/l)	62	58	57	61	1.6	<0.001	<0.001	0.062
Propionate (mmol/l)	22.4	24.1	24.9	24.1	0.60	<0.001	<0.001	0.188
Butyrate (mmol/l)	10.5	11.8	11.6	11.8	0.35	<0.001	<0.001	0.126
BCVFA (mmol/l)	5.1	5.6	5.2	5.9	0.33	0.004	<0.001	0.217
2Hrec	0.68	0.42	0.44	0.58	0.009	<0.001	<0.001	<0.001

F_{420}, coenzyme content expressed in fluorescence intensity; F_{430}, coenzyme content expressed in ultraviolet absorbance; VFA, volatile fatty acids; BCVFA, branch-chained VFAs including iso-butyrate and iso-valerate; 2Hrec, hydrogen recovery.

increased $T_{1/2}$ ($P = 0.024$). Consequently, AGPR was decreased by the addition of NE, NEOH and NPOH ($P = 0.012$).

Fermentation gas composition

The accumulation of H_2 in fermentation gases was far greater in nitrocompound treatments than the control (Table 1, $P < 0.001$). The addition of NE, NEOH and NPOH increased the molar CO_2 proportion by 11.3, 11.6 and 5.0%, respectively, in comparison with the control. Meanwhile, the addition of NE, NEOH and NPOH in comparison with the control notably decreased the molar CH_4 proportion, by 97.3, 96.7 and 35.3%, respectively. Interaction did occur between the nitrocompound addition and incubation time for the fermentation gas composition as shown in Fig. 1 ($P < 0.001$).

As the incubation time increased, the molar CH_4 proportion increased continuously in the control and NPOH group though it was lower with NPOH than the control ($P < 0.001$, Fig. 1(*a*)). The molar CH_4 proportion in NE and NEOH group was continuously far lower than that of control. In contrast, H_2 accumulation in fermentation gases continuously increased in NE and NEOH group against the increase of incubation time, and the accumulation was far greater than the control and NPOH ($P < 0.001$, Fig. 1(*b*)). As the incubation time increased, molar CO_2 proportion continuously decreased in all groups, and it was greater in nitrocompound groups than in the control ($P < 0.001$, Fig. 1(*c*)).

Methanogen populations

The relative abundance of total methanogens, *Methanobacteriales*, *Methanomicrobiales* and *Methanococcales* across different incubation time was decreased with the addition of NE, NEOH and NPOH (Table 2, $P < 0.001$). Interaction did occur between nitrocompounds addition and incubation time for all of the relative abundance of methanogen populations ($P < 0.001$). Briefly, total methanogens (Fig. 2(*a*)) and methanogen from *Methanobacteriales* (Fig. 2(*b*)) in nitrocompound treatments in comparison with the control presented less difference under first 24 h incubation time than subsequent incubation time. In contrast, a limited abundance of *Methanomicrobiales* (Fig. 2(*c*)) and *Methanococcales* (Fig. 2(*d*)) were detected during first 12 h incubation time, but all of them decreased almost to zero in subsequent incubation times.

Expression of the mcrA gene, coenzyme F_{420} and coenzyme F_{430} contents in cultures

The coenzyme contents of F_{420} and F_{430} in culture fluids across different incubation time were decreased with the addition of NE, NEOH and NPOH in comparison with the control (Table 2, $P < 0.001$). The *mcr*A gene expression relative to the control decreased remarkably (by 83.1, 79.7 and 53.5%, respectively) with the addition of NE, NEOH and NPOH ($P < 0.001$). Interaction did occur between nitrocompounds addition and incubation time ($P < 0.001$) for *mcr*A gene expression, coenzyme F_{420} and F_{430} contents ($P < 0.01$). Briefly, F_{420} content in NE group and F_{430} in NEOH continuously decreased to the lowest levels against the incubation time (Figs 3(*a*) and (*b*)). After 36 h incubation, F_{420} content ranked: control > NPOH > NEOH > NE and less decline of F_{430} content was observed for NE and NPOH in comparison with the control (Figs 3(*a*) and (*b*)). The *mcr*A gene expression in nitrocompound groups relative to the control peaked at 24 h, and thereafter it was greater in NPOH than NE and NEOH group (Fig. 3(*c*)).

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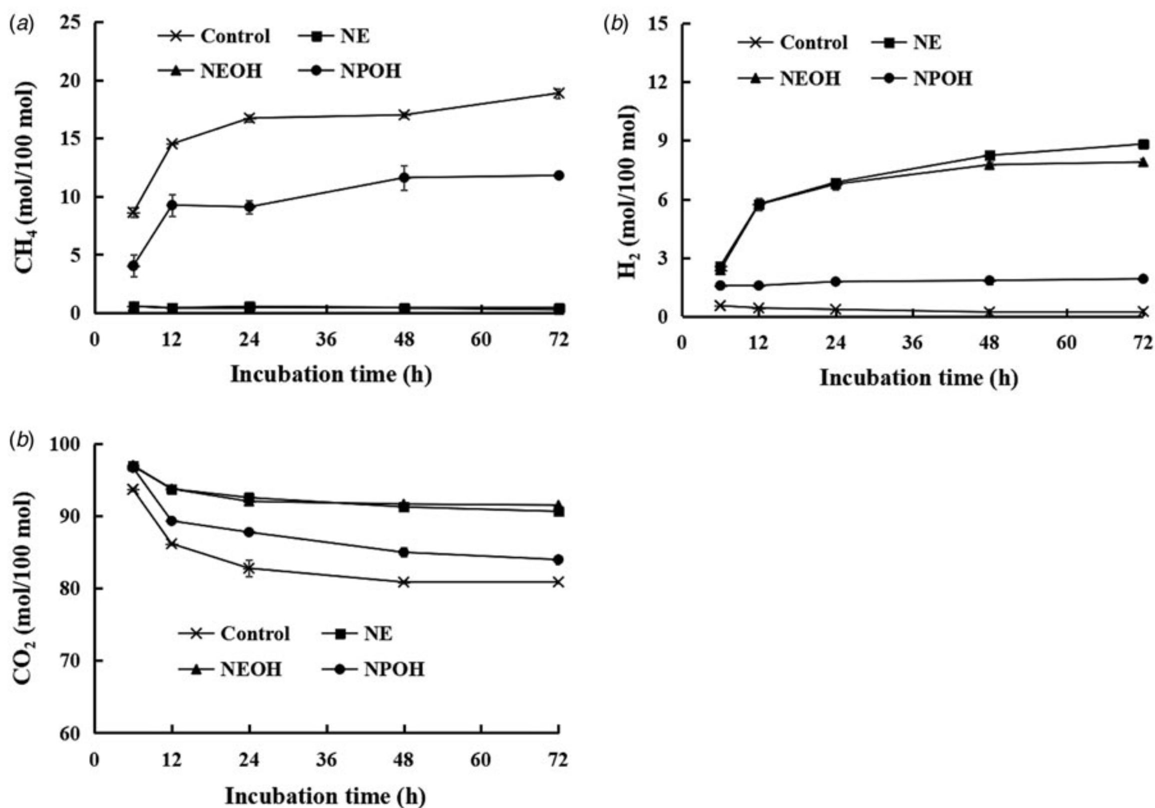

Fig. 1. Molar proportion of (*a*) methane (CH_4), (*b*) hydrogen gas (H_2) and (*c*) carbon dioxide (CO_2) of a hay-rich feed incubated with rumen fluids in the presence of nitroethane (NE), 2-nitroethanol (NEOH) and 2-nitro-1-propanol (NPOH). Effect of nitrocompounds was significant at $P < 0.001$.

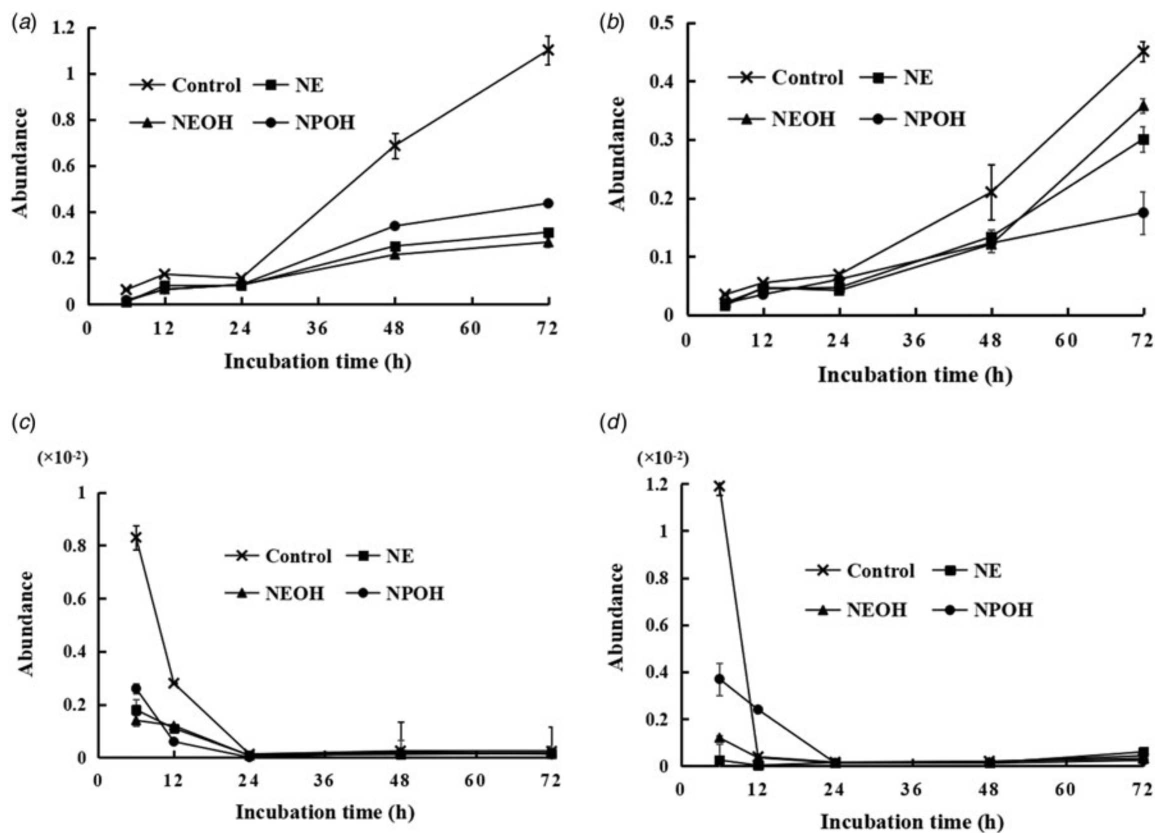

Fig. 2. Relative abundance changes of (*a*) total methanogens, (*b*) *Methanobacteriales*, (*c*) *Methanomicrobiales* and (*d*) *Methanococcales* of a hay-rich feed incubated with rumen fluids in the presence of nitroethane (NE), 2-nitroethanol (NEOH) and 2-nitro-1-propanol (NPOH). Effect of nitrocompounds was significant at $P < 0.001$.

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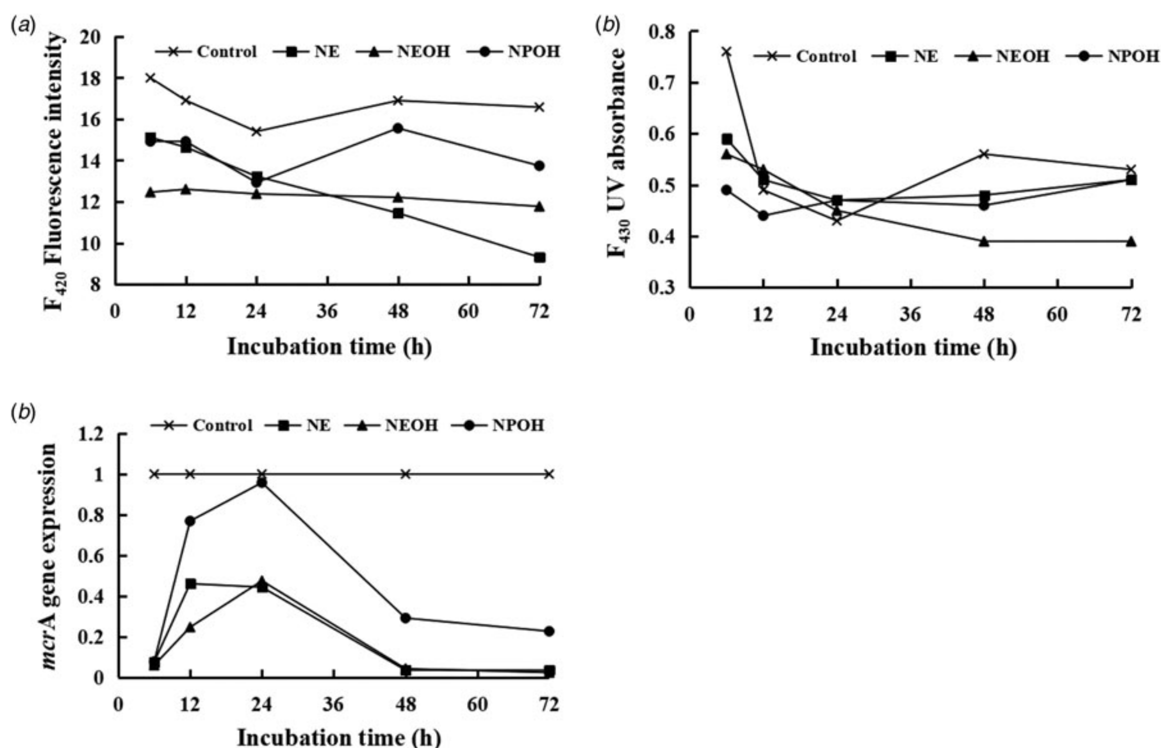

Fig. 3. Relative expression of (*a*) mcrA gene, (*b*) content of coenzyme F_{420} and (*c*) F_{430} of a hay-rich feed incubated with rumen fluids in the presence of nitroethane (NE), 2-nitroethanol (NEOH) and 2-nitro-1-propanol (NPOH). Effect of nitrocompounds was significant at $P < 0.001$.

Table 3. Pearson correlation coefficients between methane production, abundance of methanogenic populations, *mcr*A gene expression, coenzyme F_{420} content and coenzyme F_{430} content regardless of the type of nitrocompound

Parameter	Total methanogen	*Methanobacteriales*	*Methanomicrobiales*	*Methanococcales*	*mcrA*	F_{420}	F_{430}
Methane production	0.61 (<0.01)	0.35 (0.02)	0.24 (0.42)	0.12 (0.78)	0.74 (<0.01)	0.56 (<0.01)	0.31 (<0.01)
Total methanogen		0.79 (<0.01)	0.55 (<0.01)	0.17 (0.66)	0.26 (0.05)	0.24 (0.09)	0.52 (<0.01)
Methanobacteriales			0.62 (<0.01)	−0.25 (<0.05)	0.08 (0.58)	−0.10 (0.99)	0.35 (0.02)
Methanomicrobiales				0.34 (<0.01)	0.66 (0.02)	0.73 (0.01)	0.92 (<0.01)
Methanococcales					0.66 (0.05)	0.47 (0.03)	0.42 (<0.01)
mcrA						0.69 (<0.01)	0.44 (<0.01)
F_{420}							0.67 (<0.01)

P values for the Pearson correlation coefficient are noted in parenthesis.

Fermentation characteristics in culture fluids

No significant difference occurred for total VFA though its numerically highest concentration was reached in the NPOH group (Table 2 and Supplementary Material Fig. S2). However, the nitrocompound addition decreased acetate and increased the concentrations of propionate, butyrate and BCVFA ($P < 0.01$), in comparison with the control.

The response of methane production to mcrA gene expression and coenzyme contents

As shown in Table 3, CH_4 production during the 72 h incubation period was correlated positively with the abundance of the total methanogen population ($r = 0.61$), *Methanobacteriales* population ($r = 0.35$), *mcr*A gene expression ($r = 0.74$), coenzyme contents of F_{420} ($r = 0.56$) and F_{430} ($r = 0.31$). The total methanogen population was correlated positively with the *Methanobacteriales* population ($r = 0.79$), *Methanomicrobiales* population ($r = 0.55$), *mcr*A gene expression ($r = 0.26$) and coenzyme F_{430} content ($r = 0.52$). In addition, *mcr*A gene expression was correlated positively not only with coenzyme F_{420} content ($r = 0.69$) but also the coenzyme F_{430} content ($r = 0.44$). Meanwhile, a positive correlation was observed between coenzyme F_{420} content and coenzyme F_{430} content ($r = 0.44$).

Discussion

The inhibition of CH_4 production sometimes results in a depression of rumen fermentative parameters associated with digestive

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efficiency, including gas production (Zhang and Yang, 2012). Similarly, the nitrocompound additions in the present study slowed down AGPR, and NE and NEOH decreased cumulative gas production notably, when compared with NPOH and control. Latham *et al.* (2016) noted that most ruminal microbes tolerate relatively high concentrations of nitrocompounds, with only 0.10–0.20 of the population being inhibited by concentrations likely to be present in the rumen of animals exposed to nitrocompounds. The gas production profile differences presented in the current study suggested that rumen microbes were more sensitive to NE and NEOH than NPOH under the same inclusion level (10 mM).

Total amounts of VFA produced in present incubations were not significantly lower within the nitrocompound-supplemented cultures, in agreement with Anderson *et al.* (2003). The results of the current study indicate that the inclusive dose level of nitrocompounds (10 mM) may have no adverse effect on fermentative bacterial population. To compensate for the disruption of electron flow in the production of CH_4, the rumen microbial ecosystem often disposes of excess reducing equivalents by increasing the production of more reduced VFA (e.g. propionate, butyrate), which results in decreased production of acetate (Van Nevel and Demeyer, 1996). This phenomenon also occurred in the present study, and a portion of the reduced equivalents spared from CH_4 production appeared to have been used for the production of more reduced VFA under the conditions of the current experiment.

The greatest CH_4 inhibition occurred with the addition of NEOH and NE (96.8 *v.*96.4%) in comparison with NPOH (35.0%). These results are consistent with earlier studies by Anderson *et al.* (2003, 2006), who reported that NE and NEOH were almost equally effective in inhibiting ruminal CH_4 production *in vitro* and that NE inhibited CH_4 production more effectively than NPOH in the ovine rumen. Regarding CH_4 inhibition in the rumen, these nitrocompounds generally serve as alternative electron acceptors by competitively consuming reducing equivalents and inhibiting H_2 and formate oxidation (Zhang and Yang, 2012; Zhang *et al.*, 2018). A notable accumulation of H_2 occurred in the present study, which might indicate an inhibition of H_2 oxidation by NE, NEOH and NPOH (Božic *et al.*, 2009; Latham *et al.*, 2016; Ochoa-García *et al.*, 2019). Hydrogen is usually present at approximately 1 μM (0.1 kPa) in the unperturbed rumen (Thauer *et al.*, 1977); however, H_2 concentration often increases to levels that inhibit hydrogenase activity (1 kPa) when ruminal CH_4 production is inhibited due to decreased H_2 consumption by methanogens (Van Nevel and Demeyer, 1996). Due to the remarkable inhibition of CH_4 production by the nitrocompounds, H_2 accumulation in cultures in the current study might have been great enough to inhibit hydrogenase activity in NE-, NEOH- and NPOH-supplemented incubations.

The addition of NE, NEOH and NPOH decreased 2*Hrec*; however, the accumulation of H_2 did not have an adverse effect on IVDMD and the synthesis of total VFA in the present study. This could be explained by the fact that nitrocompounds have high electron-accepting characteristics (Latham *et al.*, 2016). However, the significant accumulation of H_2 within the incubations supplemented with NE, NEOH and NPOH implies that microbial interspecies-hydrogen transfer might not have been completely optimized. In addition, the reduction of 2*Hrec* by supplementation of NE, NEOH and NPOH suggested that the efficiency of H_2 utilization for the synthesis of VFA and CH_4 was reduced, consequently resulting in the increase of H_2 accumulation. Therefore, accumulation of H_2 in the current study confirmed the inhibitory effect of nitrocompounds on the H_2 oxidation and thereafter inhibited the ruminal methanogenesis. The molar H_2 proportion in total fermentation gas production was 5.2, 5.0 and 1.4% in NE, NEOH and NPOH groups, respectively. However, the extent to which 2*Hrec* decreased was far lower than that of CH_4 production. The fate of the remaining H_2 was not known with certainty, but possible sinks include use for anabolic processes, such as microbial cell growth, reduced products of nitrocompounds metabolism, or synthesis of intracellular polyhydroxyalkanoate or extracellular polysaccharides (Wachenheim and Patterson, 1992; Russell, 1998).

The inhibition of ruminal methanogenesis can also be achieved directly, by diminishing the numbers and/or activity of methanogens (Cieslak *et al.*, 2013). In the current study, methanogen populations were quantified using real-time PCR and the results showed that total methanogens and methanogens from the order *Methanobacteriales* were all decreased significantly with the addition of NE, NEOH and NPOH. Thus, it was possible that the nitrocompounds exerted a direct inhibitory effect on rumen methanogens, with NE and NEOH showing a greater reduction of methanogens than NPOH. The greater capacities of NE and NEOH to inhibit methanogen populations compared to NPOH may explain why NE and NEOH inhibited CH_4 production more effectively than NPOH in the present study.

In the rumen, most methanogens are hydrogenotrophic obligate anaerobes, involved in the reduction of CO_2 to CH_4 with formate or H_2 as the electron donor (Saminathan *et al.*, 2016). Coenzyme F_{420} acts as a cofactor for formate dehydrogenase and hydrogenase, which is important for ruminal methanogenesis and believed to be present in almost all methanogens (Hendrickson and Leigh, 2008). Dolfing and Willem (1985) reported that coenzyme F_{420} was an indicator of methanogenic activity. In addition, the formation of CH_4 from methyl-CoM is also a key step in ruminal methanogenesis from H_2 and CO_2. Methyl-CoM reductase (MCR) is one of the components involved in the catalysis of this reaction (Thauer, 1998). The *Mcr*A gene, encoding the α subunit of MCR and determining *mcr*A gene expression, has been well-accepted as a means of measuring MCR activity (Guo *et al.*, 2008). In the present study, a significant positive correlation between the content of these enzymes and CH_4 production was observed, suggesting that MCR, coenzyme F_{420} and F_{430} indeed play an important role in the process of CH_4 production. Furthermore, the addition of NE, NEOH and NPOH decreased *mcr*A gene expression and coenzyme F_{420} content to different degrees, suggesting that nitrocompounds exerted a direct effect on the activity of the methanogens via deactivation of the aforementioned enzymes. In the present study, NE and NEOH presented greater inhibitory efficiency on *mcr*A gene expression, coenzyme contents of F_{420} and F_{430} than that of NPOH and corresponded a greater decrease in CH_4 production by NE and NEOH treatment than that of NPOH.

Conclusion

Both NE and NEOH presented much greater capacities to inhibit CH_4 production compared to NPOH, resulting in a dramatic increase of H_2 accumulation during the *in vitro* rumen fermentation of a hay-rich feed. Although the addition of nitrocompounds did not decrease feed digestion and total VFA production, rumen fermentation shifted towards increasing propionate and decreasing acetate production. The CH_4 inhibition response to the

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nitrocompounds was associated with the direct inhibition of methanogen and a substantial depression of not only *mcrA* gene expression, but also the coenzyme contents of F_{420} and F_{430} in rumen methanogenesis. The findings in the present study ultimately provide a scientific, concrete reference for the practical use of these nitrocompounds with the aim of reducing CH_4 emissions in ruminant animals.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0021859619000868.

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Conflict of interest. The authors declare no conflict of interest.

Ethical standards. The experiment was conducted at China Agricultural University. All animal care and experimental operations were complied with the Guidelines of the Beijing Municipal Council on Animal Care.

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