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Response of Ruminal Fermentation, Methane Production and Dry Matter Digestibility to Microbial Source and Nitrate Addition Level in an *in vitro* Incubation with Rumen Microbes Obtained from Wethers

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Abstract: Researchers evaluated the response of ruminal fermentation, methane production and dry matter digestibility to microbial source and nitrate addition level in an *in vitro* incubation using rumen microbes from wethers fed a diet with or without supplementary nitrate. Potassium nitrate was added to the fermentation substrates *in vitro* to form three different levels of nitrate-N (4, 8 and 16 g kg^{-1} DM). Compared with microbial source from urea diet-fed wethers, ruminal microbes from nitrate diet-fed animals had increased total gas production, CH_4 and CO_2 production, acetate molar proportion and DM digestibility but had decreased ruminal nitrite accumulation, NH_3-N concentration, total VFA production and molar proportion of propionate and butyrate. There was no effect of interaction between microbial source and nitrate-N addition level on ruminal fermentation characteristics, methane production and dry matter digestibility. Along with the increase of nitrate-N addition level, production of CH_4 and CO_2 and molar proportion of propionate were reduced whereas residual concentrations of both nitrate and nitrite in the incubation medium were increased. Nevertheless, total VFA production and DM digestibility was not affected by nitrate-N addition level *in vitro*. In conclusion although, nitrate addition has ability to inhibit ruminal methane production, the inhibition ability would gradually vanish after the microbes acclimate to the nitrate diet. The results imply that nitrate has a potential value as a dietary nitrogen supplement used by ruminant animals.

Key words: Urea diet, nitrate diet, potassium nitrate, rumen fermentation, methane, digestibility *in vitro*

INTRODUCTION

Methane (CH_4) is an important greenhouse gas that contributes to global warming. It is >21 times more effective in trapping heat in the atmosphere than carbon dioxide (CO_2) (EPA, 1997). Ruminants produce about 80 million metric tons of CH_4 annually, accounting for about 28% of global CH_4 emissions from human-related activities. Meanwhile, CH_4 emission represents a loss of 2-15% gross energy intake of ruminants (Johnson and Johnson, 1995). Some reports demonstrated that nitrate could inhibit methane production in the rumen (Anderson and Rasmussen, 1998; Sar *et al*., 2002; Zhou *et al*., 2012).

However, when a relatively large quantity of nitrate is abruptly introduced into the rumen of ruminants that are not adapted to the nitrate diet, nitrite, an intermediate metabolite product can accumulate, resulting in toxic effects to the animal (Lewis, 1951). Therefore, increasing nitrate reduction to ammonia in the rumen could prevent the undesirable toxicity caused by the accumulation of nitrite thus, enhancing the safe use of nitrate in feeds. Some studies demonstrated that ruminants are able to adapt to a high-nitrate diet by reducing nitrate and nitrite more rapidly through an increase in the number of nitrate-metabolizing microbes in the rumen (Allison and Reddy, 1984; Alaboudi and Jones, 1985; Marais *et al*., 1988). This would suggest that nitrate could be used as a methanogenesis inhibitor and nitrogen source for ruminants provided the animal has adapted to the nitrate diet.

However, earlier studies have mainly focused on nitrate toxicity and on the effect of nitrate on methane production in ruminal microorganisms which were not-adapted to nitrate and little is known on the methanogenesis inhibitory effect of nitrate and on the changes in ruminal fermentation in animals which are gradually adapted to the nitrate diet. The objective of this study was to assess the response of ruminal fermentation,

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methane production and dry matter digestibility to microbial source and nitrate addition level in an *in vitro* incubation with rumen microbes from wethers fed a nitrate or a urea-based diet.

MATERIALS AND METHODS

Diet of donor animals: Six adult Inner Mongolia wethers (approx. 35 kg body weight) with permanent rumen-cannulae were used as rumen microbial donor animals. All procedures involving animals were approved by the China Agricultural University Institutional Animal Care and Use Committee. Wethers were housed in individual cages and fed twice daily at 7:00 and 19:00. Drinking water was freely accessible to the animals. Six wethers were randomly assigned to a Urea-based died group (UR; 3 wethers) or a Nitrate-based diet group (NI; 3 wethers). Each of the wethers in the UR group was fed a diet consisting of 70% roughage (*Leymus chinensis*) and 30% mixed concentrate (89.7 corn grain, 4.5 soybean meal, 2.5 urea, 0.8 salt, 1.5 limestone and 1.0% vitamin and trace mineral premix on dry matter). Wethers in the NI group were fed a similar diet with the exception of an administration of potassium nitrate instead of urea. KNO_3 was added to the NI diet in a stepwise mode with an initial concentration of 0.39% during the 1st week and a final concentration of 2.27% at the end of week 4. The final concentration was maintained constantly throughout the period of rumen content collection.

Substrates for *in vitro* incubation: Three substrates were formulated using 400 g kg^{-1} DM soluble starch (S4251, Sigma-Aldrich), 600 g kg^{-1} DM CM-Cellulose (C4021, Sigma-Aldrich) and three different dosages of KNO_3 (P6083, Sigma-Aldrich) in order to maintain different nitrate-N concentrations of 4, 8 and 16 g kg^{-1} DM. Actual concentrations of nitrate-N were determined to be 3.84, 8.26 and 16.02 g kg^{-1} DM, accounting for 2.40, 5.16 and 10.01% of total crude protein in the substrate diet (DM basis).

***In vitro* culture procedure and gas production:** Triplicates of rumen content were collected 1 h before morning feeding from each of the wethers fed nitrate-based or urea-based diet. The contents from same treatment diet-fed animals were pooled, strained through four layers of cheesecloth and then added to the anaerobic buffer solution at a ratio of rumen fluid to buffer of 1:2 (Menke *et al*., 1979) under a constant flow of O_2-free CO_2. Then, 60 mL of inoculated culture medium were delivered with a dispenser into each of prewarmed (39°C) glass syringes (HFT000025, Haberle Maschinenfabrik GmbH, Germany). The syringes earlier purged of O_2 by anaerobic N_2 were then sealed and incubated in a shaking water bath at 39°C (Lin *et al*., 2011). Three blank syringes which contained only inoculated culture medium with no any substrate were simultaneously incubated. Each treatment had three replicates. The incubations were carried out at 39°C for 24 h.

Sampling and analysis: At 24 h incubation, gas production of each syringe was measured by reading the position of the syringe piston and then all syringes were immediately immerged into cold water to terminate incubation. Gas samples were taken to pre-emptied gasbags for analyzing gas composition by gas chromatography (TP-2060T, Beifen Ruili Analytical Equipment Co., Beijing, China) equipped with a thermal conductivity detector and a stainless steel column (1 m ×3 mm ×2 mm) packed with TDX-01. The carrier gas was argon with a flow rate of 30 mL min^{-1}. Temperatures of the injectors, column and detector were 120, 100 and 160°C (Guo *et al*., 2009; Lin *et al*., 2011).

Fermentation content was collected from each syringe at 24 h incubation for determination of pH values with a pH meter (Model PHS-3C, Shanghai Leici Scientific Instrument Co., Ltd., China). The samples were then centrifuged at 2500×g for 15 min at 4°C. The supernatants were divided into four parts for determination of Ammonia-Nitrogen (NH_3-N), Nitrate-Nitrogen (NO_3-N), Nitrite-Nitrogen (NO_2-N) and Volatile Fatty Acid (VFA) concentrations. Ammonia-N concentration was measured by the procedure of Broderick and Kang (1980) using a spectrophotometer (UV-VIS 8500, Shanghai Tianmei Scientific Instrument Co., Ltd., China).

Concentration of NO_3-N and NO_2-N were analyzed by the procedure of Lin *et al*. (2011) by using an ion chromatograph (Model Dionex-2500, Dionex Co., Ltd., USA) with an Ionpac AS11HC 2-mm analytical column. For sample preparation of VFA determination, 1 mL supernatant was mixed with 25% metaphosphoric acid and frozen at -20°C for 2 h. After the acidified supernatants thawed they were centrifuged at 10,000×g at 4°C for 15 min and analyzed for VFA using an Agilent 6890N gas chromatograph with a 30 m ×0.32 mm ×0.5 μm capillary column packed with HP-INNOWax with N_2 as the carrier gas and 2-ethylbutyric acid as the internal standard (Supelco Inc., Bellefonte, PA, USA). The injector port temperature was 220, Flame Ionization Detector (FID) detector temperature was 250°C and the oven temperature was held at 180°C. As to dry matter digestibility determination after centrifugation and removal of supernatants, all the fermentation contents were subjected to further washing with 50 mL of H_2O and dried at 60°C for 48 h to determine the *In Vitro* Dry Matter Digestibility (IVDMD) calculated by the following equation:

$$IVDMD\ (\%) = \left[\frac{\text{Initial dry substrate weight-(Sample residue weight-Control weight)}}{\text{Initial dry substrate weigh}}\right] \times 100$$

Statistical analyses: Data were analyzed using a complete random design with a 2×3 factorial arrangement of treatments using the GLM procedure of SAS (2008) except for the data of nitrate disappearance and nitrite accumulation. The treatments sums of squares were partitioned into rumen microbial source, nitrate-N addition level and their interaction. Nitrate disappearance and nitrite accumulation were analyzed using a complete random design with a 3×3 factorial arrangement of treatments using the GLM procedure of SAS (2008). The treatments sums of squares were partitioned into rumen microbial source, nitrate-N addition level, time and their interaction. Differences between rumen microbial source and between nitrate-N addition levels were compared using Duncan's multiple range test. The Least Square Means (LSMEANS) of the interaction of rumen microbial source and nitrate-N addition level were calculated using the LSMEANS statement. Main treatment effects and interactions of main treatment effects are declared significant at $p<0.05$ and trends are declared at $p<0.10$.

RESULTS AND DISCUSSION

Total gas and CH_4 production: The total and individual gas production and the relative proportions of hydrogen (H_2), methane (CH_4), carbon dioxide (CO_2) are shown in Table 1. Total gas production was significantly influenced by both microbial source ($p = 0.013$) and nitrate-N addition level ($p = 0.032$) whereas there was no interaction ($p = 0.718$) between microbial source and nitrate-N addition level. Compared with the rumen microbes from UR diet-fed wethers, the microbes from NI diet-fed animals had higher ($p = 0.013$) total gas production whilst the total gas production was reduced ($p = 0.032$) as nitrate-N addition level increased. With the exception of the volume of H_2 ($p = 1.000$), the volume of CH_4 ($p = 0.002$) and CO_2 ($p = 0.021$) was considerably higher for the microbes from NI diet-fed wethers than that from UR diet-fed animals. Similarly, the percentage of CH_4 was also higher ($p = 0.028$) for NI microbes than those for UR microbes whereas the percentage of CO_2 was lower ($p = 0.003$) for NI microbes than those for UR microbes. Along with increasing nitrate-N addition level in the substrate both production of CH_4 ($p = 0.053$) and CO_2 ($p = 0.030$) dropped. However, there were no effects of interactions of microbial source and nitrate-N addition level on the volume and percentage of H_2 ($p>0.05$), CH_4 ($p>0.05$) and CO_2 ($p>0.05$).

Nitrate disappearance and nitrite accumulation: Figure 1 shows nitrate disappearance >0-24 h *in vitro* incubation. Compared with UR microbes, NI microbes had faster rate of nitrate degradation ($p<0.0001$) with zero nitrate residues

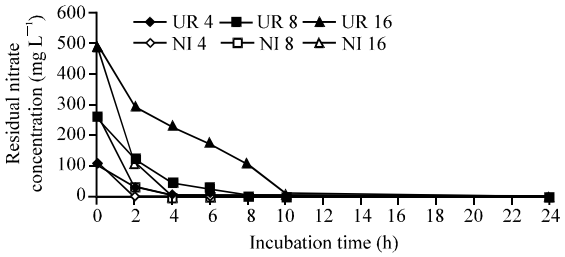

Fig. 1: Residual concentrations of nitrate in the medium of 0-24 h *in vitro* incubation. UR 4 (◆), 4 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with Urea (UR) diet; UR 8 (■), 8 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with UR diet; UR 16 (▲), 16 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with UR diet; NI 4 (◆), 4 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with nitrate (NI) diet; NI 8 (□), 8 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with NI diet; NI 16 (▲), 16 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with NI diet; microbial source effect ($p<0.0001$), nitrate-N addition level effect ($p<0.0001$), Microbial source x Nitrate-N addition level interaction ($p<0.0001$; SEM = 0.20)

Table 1: Effects of Microbial Source (MS) and Nitrate-N addition Level (NL) on total Gas Production (GP) and compositions in the *in vitro* incubation

	Microbial source			Nitrate-N addition level (g kg^{-1} DM)‡				Interaction		
Parameters	UR	NI	SEM	4	8	16	SEM	MS	NL	MS×NL
Total GP* (mL g^{-1} DM)	108.9^{b}	129.2^{a}	5.800	53.05^{a}	45.60^{b}	43.70^{b}	2.300	0.013	0.032	0.718
Gas volume (mL)										
H_2	0.010^{a}	0.010^{a}	0.001	0.010^{a}	0.010^{a}	0.010^{a}	0.001	1.000	0.735	0.571
CH_4	14.20^{b}	19.40^{a}	1.130	19.20^{a}	16.60^{b}	14.60^{c}	1.340	0.002	0.053	0.304
CO_2	94.70^{b}	109.8^{a}	4.760	114.6^{a}	97.40^{b}	94.60^{b}	4.620	0.022	0.030	0.775
Gas proportion (%)										
H_2	0.010^{a}	0.010^{a}	0.002	0.010^{a}	0.010^{a}	0.010^{a}	0.001	0.666	0.809	0.471
CH_4	12.81^{b}	15.03^{a}	0.490	14.31^{a}	14.39^{a}	13.06^{a}	0.610	0.028	0.160	0.087
CO_2	87.18^{a}	84.96^{b}	0.490	85.68^{a}	85.60^{a}	86.94^{a}	0.610	0.003	0.160	0.087

*GP = Gas production; UR = Microbes from Urea diet; NI = Microbes from Nitrate diet; MS = Microbial Source; NL = Nitrate-N addition Level; SEM = Standard Error of Means. $^{a-c}$Means with different superscript letters in the same row significantly differ ($p<0.05$), ‡Nitrate-N concentrations in the substrates are 4, 8 and 16 g kg^{-1} DM, respectively

Table 2: Effects of Microbial Source (MS) and nitrate-N addition Level (NL) on ruminal pH, NH_3-N concentration, total VFA production and individual VFA molar proportion in the *in vitro* incubation

	Microbial source			Nitrate-N addition level (g kg^{-1}DM)‡				p-values		
Factors	UR*	NI	SEM	4	8	16	SEM	MS	NL	MS×NL
pH	6.590	6.600	0.02	6.570	6.600	6.620	0.02	0.8360	0.170	0.1070
NH_3-N (mg/100 mL)	10.830	7.360	0.34	8.890^b	8.960^b	9.440^a	0.72	<0.0001	0.013	<0.0001
Total VFA (mmol L^{-1})	59.340	42.150	2.87	53.100	45.920	53.21	4.13	0.0040	0.862	0.3010
VFA molar proportion (M%)										
Acetate	68.250	71.560	0.94	69.560	69.430	70.72	1.12	0.0470	0.743	0.9430
Propionate	15.440	12.770	0.21	14.400^a	14.330^a	13.60^b	0.52	<0.0001	0.045	0.4620
Isobutyrate	1.220	1.420	0.05	1.220^b	1.330^{ab}	1.390^a	0.06	0.0004	0.018	0.0020
Butyrate	11.980	11.010	0.25	11.740	11.510	11.23	0.30	0.0250	0.567	0.7680
Isovalerate	1.800	1.770	0.05	1.650^b	1.880^a	1.820^{ab}	0.04	0.5880	0.031	0.5450
Valerate	1.300	1.550	0.10	1.430	1.520	1.340	0.11	0.1160	0.605	0.3260

*UR = Microbes from Urea diet; NI = Microbes from Nitrate diet; MS = Microbial Source; NL = Nitrate-N addition Level; SEM = Standard Error of Means. $^{a-c}$Means with different superscript letters in the same row significantly differ ($p<0.05$). ‡Nitrate-N concentrations in the substrates are 4.00, 8.00 and 16.00 g kg^{-1} DM, respectively

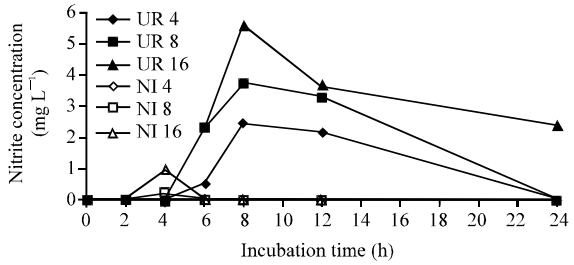

Fig. 2: Residual concentrations of nitrite in the medium of 0-24 h *in vitro* incubation UR 4 (◆), 4 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with Urea (UR) diet; UR 8 (■), 8 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with UR diet; UR 16 (▲), 16 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with UR diet; NI 4 (◇), 4 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with Nitrate (NI) diet; NI 8 (□), 8 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with NI diet; NI 16 (△), 16 g kg^{-1} DM nitrate-N in substrate DM *in vitro* with NI diet; microbial source effect ($p<0.0001$), nitrate-N addition level effect ($p<0.0001$), Microbial source x Nitrate-N addition level interaction ($p = 0.004$; SEM = 0.01)

in the medium during 2-4 h incubation. In contrast, UR microbes always maintained a slower rate of nitrate disappearance than NI microbes at each incubation time point with zero nitrate residues occurring >4-12 h incubation. Figure 2 shows a dynamic accumulation of nitrite during 0-24 h *in vitro* incubation. Residual nitrite concentrations were much lower for NI microbes than those for UR microbes ($p<0.0001$) at each incubation time point. The time at which the peak value of nitrite concentration appeared differed between microbial sources. Nitrite concentration reached the peak value at 8 h incubation for UR microbes while nitrite concentration peaked at 4 h incubation for NI microbes. Moreover, nitrite accumulation increased with increasing dietary nitrate-N level ($p<0.0001$). Effects of interactions were observed between microbial source and nitrate-N level on both nitrate disappearance ($p<0.0001$) and nitrite accumulation ($p = 0.004$).

The pH value and VFA production: The pH value, NH_3-N concentration, total VFA production and VFA molar proportion are shown in Table 2. The pH value was not affected by microbial source ($p = 0.836$), nitrate-N addition level ($p = 0.170$) and the interactions between microbial source and nitrate-N addition level ($p = 0.107$). The concentration of NH_3-N was lower for microbes from NI diet-fed wethers than those from UR diet-fed animals ($p<0.0001$). With increasing nitrate-N level, the concentration of NH_3-N increased ($p = 0.013$). At the same time, the concentration of NH_3-N was influenced by the interaction between microbial source and nitrate-N addition level ($p<0.0001$). Compared with UR microbes, NI microbes had increased molar proportions of acetate ($p = 0.047$) but had decreased total VFA production ($p = 0.004$) and reduced molar proportions of propionate ($p<0.0001$) and butyrate ($p = 0.025$). Nitrate-N addition level did not influence total VFA production ($p = 0.862$) and molar proportions of acetate ($p = 0.743$). When nitrate-N addition level was increased from 8-16 g kg^{-1} DM, molar proportions of propionate decreased ($p = 0.045$) while the molar proportions of Isobutyrate ($p = 0.018$) and isovalerate ($p = 0.031$) increased. Total VFA production and molar proportions were little influenced by the interaction between microbial source and nitrate-N addition level ($p>0.05$).

Dry matter digestibility: Figure 3 shows the results of *in vitro* Dry Matter (DM) digestibility. The DM digestibility was higher for NI microbes than UR microbes ($p = 0.0002$). However, there was no difference in DM

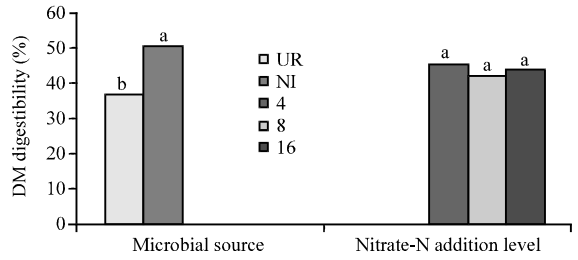

Fig. 3: Effects of Microbial Source (MS) and Nitrate-N addition Level (NL) on DM digestibility in the *in vitro* incubation microbial source effect (p = 0.0002, SEM 1.74%), nitrate-N addition level (p = 0.669, SEM 3.03%) interaction of microbial source and nitrate-N addition level (p = 0.467, SEM 2.25%); UR = urea diet; NI = nitrate diet; 4 = 4 g kg^{-1} DM nitrate-N in substrate DM *in vitro*, 8 = 8 g kg^{-1} DM nitrate-N in substrate DM *in vitro*, 16 = 16 g kg^{-1} DM nitrate-N in substrate DM *in vitro*. Means with different superscript letters in the same row significantly differ (p<0.05)

digestibility among three nitrate-N addition levels (p = 0.669). In addition interaction between microbial source and nitrate-N addition level did not affect DM digestibility *in vitro* (p = 0.467). Generally it is recognized that dietary addition dose of nitrate-N at <0.12% (DM basis) is generally safe to ruminant animals (Meng *et al*., 2011). Nitrate-N addition level at 0.12-0.23% would cause chronic nitrate toxicosis, e.g., depressed appetites (Farra and Satter, 1971), reduced weight gains and milk production (Jainudeen *et al*., 1964) and increased susceptibility to infections in ruminant animals.

Nitrate-N addition level >0.23% (DM basis) usually causes acute nitrate poisoning for ruminant animals. When acute poisoning occurs, animals die before any visibly toxic signs appear (Jainudeen *et al*., 1964). In the study when nitrate-N was added to the NI diet in a stepwise fashion with an initial concentration of 0.05% at week 1 and a final concentration reaching 0.31% at the end of week 4, the donor wethers showed a depressed appetite and reduced weight gain only during the 1st 2 weeks and recovered in week 4. The concentration of blood methemoglobin in donor wethers fed NI diet only was 0.45-0.93% of total hemoglobin indicating acclimation of donor animals to the nitrate diet before the beginning of the present experiment.

Effects of microbial source on ruminal fermentation, methane production and dry matter digestibility: Total gas production was higher for microbes from NI diet-fed wethers than those from UR diet-fed animals, suggesting higher ability of ruminal microorganisms from NI diet-fed wethers to use nitrate-N as N supply for their growth *in vitro*. Farra and Satter (1971) indicated that feed intake was reduced and methemoglobinemia appeared when dietary nitrate level was added >4% of dietary DM. In the present study, no matter what level of nitrate-N was added to the substrate, microbes from NI diet-fed wethers had higher gas production than the microbes from UR diet-fed animals indicating ruminal microbial activity unaffected by the NI addition to the diet of wethers. The unaffected microbial activity due to feeding nitrate included diet to wethers may be attributed to the adaptation of ruminal microorganisms to added nitrate by the donor wethers fed KNO_3 in a stepwise fashion for 4 weeks.

Similarly, researchers observed that there were no poisonous signs when mature sheep were adapted to a diet containing 4% KNO_3 (Caver and Pfander, 1974). Allison and Reddy (1984) demonstrated that ruminants could adapt to nitrate diet by an increase in the nitrate-metabolizing bacteria in the rumen. Alaboudi and Jones (1985) showed that ruminants could increase their tolerance to nitrate 3-5 times and not display toxic symptoms by gradually adapting to nitrate. The present study further confirmed the results of these earlier reports. Methane represents both the loss of feed energy and a major greenhouse gas. When nitrate is added to the ruminant diet, the reduction of CH_4 emission is expected. At least two mechanisms are included in this explanation for nitrate inhibition of CH_4 production: nitrate can replace carbon dioxide as an electron acceptor, thereby inhibiting the reduction of CO_2-CH_4 (Allison and Reddy, 1984) and methanogens are more sensitive to nitrite and the inhibition of methanogenesis by nitrite may be attributed to its toxic effect on growth of methanogens (Iwamoto *et al*., 2002; Sar *et al*., 2005).

However, in the present study, CH_4 production increased when the nitrate-N was incubated with rumen microbes from NI diet-fed wethers (Table 1). This indicates that the ability of inhibition of CH_4 production by nitrate may gradually vanish after rumen microorganisms acclimate to the nitrate diet. Lewis (1951) indicated that nitrate can be reduced to nitrite and subsequently ammonia in the rumen of sheep. When ruminants consume high-nitrate diets, the nitrate reduction often causes nitrite accumulation in the rumen. The accumulated nitrite would inhibit ruminal fermentation (Sar *et al*., 2005) and is readily absorbed into blood leading to high concentration of blood methaemoglobin (Lewis, 1951).

However, ruminants have the ability to adapt to nitrate included diet (Allison and Reddy, 1984). In the

study, the rate of nitrate degradation was rather faster in the NI diet than the UR diet (Fig. 1). With comparison of residual nitrate concentration (Fig. 1) with nitrite concentration (Fig. 2) *in vitro* even if high amount of nitrate-N (16.02 g kg^{-1} DM) was added to the incubation substrate, the peak value of residual nitrite concentration only appeared when 0.99 mg L^{-1} was added to the NI diet at 4 h incubation, suggesting nitrite from nitrate reduction would be quickly reduced to ammonia by ruminal microorganisms. Lewis (1951) indicated that the administration of nitrite in the diet of sheep at <3.34% (w/v) solution had little effect on the concentration of blood methaemoglobin.

The present study further demonstrated that nitrite accumulation can be prevented by acclimation of animals to the nitrate included diet.

Effects of nitrate-N addition level on ruminal fermentation, methane production and dry matter digestibility: In this study, pH values were about 6.57-6.62 which was the normal fermentation range. The concentration of NH_3-N was markedly lower in the incubation with microbes from NI diet-fed wethers than those from UR diet-fed animals which is in line with the former observation of higher gas production in incubation with ruminal microbes from NI diet-fed wethers than with the microbes from UR diet-fed animals (Table 1). A lower total VFA concentration was observed for the microbes from NI diet-fed wethers which is similar to the result of Guo *et al.* (2009). In addition, the pattern of VFA production was characterized by enriched acetate and diminished propionate and butyrate with the microbes from NI diet-fed wethers.

These results are consistent with earlier reports (Farra and Satter, 1971; Takahashi *et al*., 1989; Guo *et al*., 2009; Lin *et al*., 2011) and could reflect the electron sink of nitrate in rumen fermentation. In the experiment, DM digestibility was higher for incubation with the microbes from NI diet-fed wethers than those from UR diet-fed animals. The magnitude of the effect of nitrate on rumen fermentation depends on the degree to which nitrite is accumulated in the rumen and on the ability of ruminal microbes in particular to change towards a population containing increased proportions of microbial species capable of tolerating nitrite (Marais *et al*., 1988). In the present study, nitrite accumulations were rather lower in the incubation with rumen microbes from NI diet-fed wethers (Fig. 2).

Researchers have reasons to extrapolate that the population of nitrate-metabolizing bacteria is likely higher for incubation with rumen microbes from NI diet-fed wethers than those from UR diet-fed animals. For this reason, higher DM digestibility for the incubation with rumen microbes from NI diet-fed wethers should be expected. This result supported the hypothesis that nitrate can be used as a nitrogen source for ruminants if they well acclimate to the nitrate diet. In this study, nitrate-N addition level showed a negative effect on total gas and CO_2 production. The CH_4 production however, tended to be reduced along with the increase of nitrate-N addition level. These results are in agreement with earlier reports (Jones, 1972; Anderson and Rasmussen, 1998).

Nitrate concentration and nitrite accumulation increased with increasing nitrate-N levels. This result is consistent with Dai *et al*. (2010) in which *in vitro* ruminal concentrations of nitrate and nitrite were increased with increasing nitrate level in the *in vitro* incubation diet. Ammonia is an important intermediate of nitrogenous compounds such as nitrate substances fermented in the rumen and is further incorporated into rumen microbial proteins. The concentration of NH_3-N declined with increasing nitrate-N addition level. This observation in combination with the result of reduced gas production (Table 1) suggests that increasing nitrate-N levels may have certain inhibition impacts on rumen microbial growth. With increasing nitrate-N level there was a reduced molar proportion of propionate. This result is in agreement with the observation of Farra and Satter (1971) who noted a decreased molar proportion of propionate owing to nitrate addition. The alterations in VFA profile observed in this study would be indicative of greater reducing equivalent release by the fermentative population due to nitrate reduction as described by Russell and Wallace (1997).

Marais *et al*. (1988) reported that nitrate or its reduced product temporarily present in the digesta would decrease ruminal DM digestibility. Dai *et al*. (2010) showed that ruminal *in vitro* DM digestibility changed in a quadratic fashion with the rise of nitrate-N addition level. The present result however did not exhibit any positive effect of increasing nitrate-N addition levels on ruminal DM digestibility. The difference between the different experiments may be related to the different rumen microbial source used for the *in vitro* incubation. In the present study, ruminal microbes from wethers fed nitrate diets in a stepwise fashion may cover up the effect of increase in nitrate-N levels to some extent.

CONCLUSION

The incubation with rumen microbes from NI diet-fed wethers had higher total gas production, CH_4 production, DM digestibility and lower nitrite accumulation in comparison with incubation with microbes from UR diet-fed animals. Although, nitrate addition has the ability to inhibit ruminal methane production, the inhibition ability would be gradually reduced after the microbes acclimate

to the nitrate diet. Increasing nitrate levels resulted in decreasing total gas production, propionate molar proportion and individual production of methane and CO_2 increasing NH_3-N concentration and nitrite concentrations but no change in individual proportions of hydrogen, methane and CO_2, totel VFA production and dry matter digestibility. The results imply that nitrate has a potential value as a dietary nitrogen supplement used by ruminant animals. Further study is needed to provide more information about safe usage of nitrate in the ruminant diet in practice.

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