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Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep

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Abstract. The effects of dietary nitrate on DM digestion, rumen volatile fatty acid concentrations, microbial protein outflow, rumen water kinetics, and methane production were studied. Eight rumen-cannulated sheep were acclimated to a diet consisting of chaffed oaten hay supplemented with either 4% KNO_3 or 0% KNO_3 but made iso-nitrogenous by the addition of urea. Nitrate supplementation did not affect blood methaemoglobin concentration, DM intake, whole tract or ruminal DM digestibility and the sheep appeared healthy at all times throughout the acclimation and experimental periods. Nitrate did cause changes in rumen fermentation consistent with its acting as a high-affinity hydrogen acceptor, i.e. there was a tendency towards a lower molar percentage of propionate in the rumen volatile fatty acids, and higher molar ratio of acetate to propionate. Methane yield (MY, L methane/kg DM intake) was reduced by 23% in KNO_3-supplemented sheep ($P < 0.05$) and these sheep tended to have a shorter mean fluid retention time in the rumen (MRT). There was a significant association between MRT and MY, such that a shorter MRT was associated with a lower MY. The results confirmed that the presence of nitrate in the diet lowers enteric methane production even though there was considerable between-animal variation in gut kinetics and methane production.

Introduction

In Australia, ~16% of total greenhouse gas (GHG) emissions arise from agricultural activities, but enteric methane from ruminants (11% of total) is the most significant source of agricultural GHG emissions (NGGI 2009). Practical ways of reducing GHG emissions from ruminants are required.

Microbial fermentative digestion of organic matter by rumen microorganisms depends on a supply of NAD^+, which is converted to NADH. H_2 and its electrons from NADH are used by Archaea to reduce CO_2 to methane, allowing NAD^+ to be regenerated so that fermentation of feed materials can continue. Although CO_2 reduction to methane is usually the major 'sink' for H_2 and its associated electrons, other oxidised inorganic compounds can remove H_2. Nitrate salts, for example, are potent inhibitors of methanogenesis in many anaerobic systems, including the rumen (Allison and Reddy 1984) and other secondary fermentation systems ranging from anaerobic biodigestors to sediments (Hungate 1966; Allison *et al*. 1981; Akunna *et al*. 1994). Nitrate has a greater affinity for H_2 than does CO_2 and so, when nitrate is present in the rumen, nitrite and ammonia formation are favoured over methane production (Ungerfeld and Kohn 2006).

After comprehensively reviewing the literature, Leng (2008) concluded that the inclusion of nitrate in feed supplements appeared to be entirely feasible as a means of reducing enteric methane emissions from ruminant livestock. While there is a risk of nitrite toxicity, nitrate supplementation also has potential advantages in addition to inhibiting methane emissions, viz. (1) the end product of nitrate reduction is ammonia, which for ruminants on low digestibility diets, is a major source of N for microbial growth and (2) nitrate reduction should theoretically be more efficient energetically than methanogenesis and so microbial growth should be increased when methanogenesis is inhibited by the presence of nitrate. This theory has been confirmed *in vitro* by Guo *et al*. (2009). Nitrate supplements could therefore potentially replace urea supplementation, which is widely accepted in the grazing and feedlot industries. Despite these possibilities, little is known about the effects of nitrate on microbial fermentation and growth in the rumen.

The aim of this study was to examine the digestive characteristics and microbial growth in the rumen of sheep given a diet of chaffed oat hay supplemented with iso-nitrogenous amounts of KNO_3 or urea.

Materials and methods

Animals and feeding

The study was approved by the University of New England Animal Care Committee (AEC 09/084). Eight Merino wethers (38.6 s.e. 2.4 kg; aged 3 years with long-established rumen fistulas) were housed in metabolism cages in rooms with continuous lighting and temperatures of 15–20°C and

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allocated randomly within weight ranges to two treatment groups. Two iso-nitrogenous diets of chaffed oaten hay (OC) were prepared. A diet with 4% added KNO_3 was prepared by sprinkling a solution of KNO_3 onto oaten hay while the hay was tossed in a rotary feed mixer. Another diet (Control) was similarly prepared using a solution containing urea so that 5.54 g N was added per kg hay for both diets. Both diets were then dried in warmed, ventilated rooms. The sheep were gradually acclimated to the nitrate-containing diet over 18 days. The daily ration (1 kg/day air-dry feed) was delivered to the sheep in equal portions each hour by automatic feeders during the digestibility trial, and every 2 h when the sheep were in respiration chambers. Feed intake and refusals were recorded daily.

Faeces and urine collection

After 18 days during which the four sheep in each of the treatment groups were acclimated to their diets, faeces and urine outputs from the sheep were collected in three consecutive 24-h collections. Faeces and urine were separated below the metabolism cages, faeces into a plastic bag and urine into a 10-L bucket acidified daily with 100 mL of 10 mM HCl to ensure pH remained below 3. The day's urine from each sheep was made up to 3 L and a 30-mL aliquot was bulked into a separate container and frozen. DM content of subsamples of the diets and faecal samples were determined by drying at 60°C for 4 days.

Microbial N outflow from the rumen

Samples of bulked urine from each sheep were thawed and allantoin concentration, daily excretion rate and microbial N outflow from the rumen were determined as described by FAO/IAEA (2003).

Methane production

Methane production was measured in four open-circuit respiration chambers as described by Bird *et al*. (2008). Each chamber had a total volume of 1200 L and the mean retention time of air was ~7 min. While sheep were in the chambers for 22 h, air was sampled every 13 min and methane concentration was measured by a photoacoustic infrared multigas analyser (Innova Model 1312, Innova Airtech Instruments, Denmark). A continuous subsample of chamber gas (3 ml/min) was also continuously pumped (peristaltic pump) into a gas collection bag over the 22-h period after which methane was determined using a CP4900 PRO Micro-GC (Varian, Palo Alto, CA, USA) fitted with M5A and PPQ capillary columns. Methane production of sheep on the control and 4% nitrate diets (n = 4/diet) was measured on successive days. One animal in the control group had relatively high emissions when first measured, so methane emissions from these four sheep were determined again 3 days later and mean values for each sheep in this group were used during the statistical analysis.

Feed digestion in situ

Oaten hay without additives (2 g) was ground to pass through a 2-mm sieve and placed, together with a glass marble, in porous dacron bags (pore size = 40 μm). Eight bags were inserted into the rumen of each sheep via the cannula at different intervals over a period of 36 h, but were removed from the rumen together. Four bags were similarly prepared at the same time but never placed in the rumen. All bags were washed in a bucket under a running tap until the water was clear, then squeezed loosely to remove the majority of the water and placed on trays in a fan-forced oven to dry at 60°C for 2 days. Washout loss (WL) was the decrease in weight of feed after washing for bags not incubated in the rumen.

The data for percentage removal of chaffed hay DM from bags placed for various times in each sheep were fitted using the non-linear curve fitting algorithms in WinSAAM (Stefanovski *et al*. 2003) to the model suggested by Ørskov and McDonald (1979), i.e.

$$P_t = a + b(1 - \exp^{-ct}) \quad (1)$$

where P_t indicates the percentage of the DM originally placed in each bag that was removed from the bag at time t; a is the intercept of the fitted curve at time zero; b is the percentage of the relatively insoluble DM that was potentially degradable and c is the rate of degradation of fraction b.

A lag time was estimated by fitting the model in Eqn 2 (McDonald 1981):

$$P = WL \quad \text{for} \quad t = t_0,$$
$$P = a + b(1 - e^{-ct}) \quad \text{for} \quad t > t_0 \quad (2)$$

The lag time (*Lag*) was given by:

$$Lag = 1/c \; log_e[b/(a + b - WL)] \quad (3)$$

The effective degradability (*ED*) of oat chaff DM was determined according to Eqn 4 below:

$$ED = a + (bc)/(c + k_p) \quad (4)$$

where t is time (h), k_p is the rate constant for particle flow out of the rumen, assumed to be 0.05/h. The undegradable DM fraction was given by 1– (a + b).

Volatile fatty acid concentrations, pH and ammonia

Four samples of rumen fluid (×15 mL) were taken over the feeding period at ~6-h intervals and each acidified with 0.1 mL 18 M H_2SO_4 then frozen. Samples were subsequently thawed and volatile fatty acid (VFA) concentrations determined by gas chromatography (GC) using a packed steel column (1.8 m by 2 mm i.d.) with 15% neopentyl glycol adipate and 2% phosphoric acid liquid phase on chromosorb W2W (80/100 mesh). The column was operated isothermally at 130°C in a Varian 3800 GC with a column flow of 30 mL/min N_2 and Varian Star integration software. Iso-caproic acid was added to all samples as an internal standard. On four occasions, the pH of rumen fluid was measured immediately after samples were obtained via the rumen cannula using a portable pH meter (Orion 230 Aplus, Thermo Scientific, Beverly, MA, USA). Rumen ammonia was measured by a modified Berthelot reaction using a continuous flow analyser (Skalar San++, Breda, The Netherlands) and a factory-made manifold. A sample of rumen fluid was taken from each sheep at ~0900 hours and analysed for ammonia concentration.

Rumen fluid kinetics

Rumen fluid volume, fluid outflow rate and the rate constant (k) were estimated from the decline in concentration of Cr-EDTA after its injection (~67 mg Cr) into the rumen (Downes and McDonald 1964). Mean retention time of rumen water (MRT) was calculated as 1/k (Faichney 1975). Rumen fluid samples ($n = 7$) were collected over a 6-h period after Cr-EDTA injection, acidified and stored frozen. After thawing and centrifugation, these samples were analysed for concentration of Cr using an atomic absorption spectrometer. Standards were prepared by dilution of a purchased standard (Spectrosol, BDH, London; 1007 mg Cr/L) in rumen fluid that had also been acidified, frozen and centrifuged.

Counting protozoa

Ciliate protozoa were enumerated in samples of rumen fluid (4 mL) collected at 0900 hours and mixed with 16 mL of formaldehyde saline (4 g formalin, 13 g NaCl/L). Preserved samples were stored at room temperature and counted using a Hawksley Cristalite B.S. 748 counting chamber (Sussex, UK). The protozoa were differentiated into large (>100 μm) and small (<100 μm) holotrich and entodiniomorph groupings.

Methaemoglobin determination

Concentration in blood was determined within 30 min of blood collection (10 mL) by the method of Hegesh *et al*. (1970).

Statistical analyses

One-way ANOVA tests for each parameter were conducted using Minitab (version 15; State College, PA, USA) to test for effects of nitrate.

Results

The sheep appeared healthy throughout the 18-day period of acclimation to the nitrate and urea-containing diets and afterwards during the experimental periods. There was no treatment difference in the rumen ammonia concentration of sheep ($P > 0.05$). Blood methaemoglobin concentrations in nitrate-supplemented sheep were not significantly different from those of control sheep and never exceeded 2.8% (Table 1).

There was no main effect of nitrate supplementation on whole-tract DM digestibility or rate of DM loss or potential degradability *in situ*, but effective degradability of DM in the rumen determined by the *in situ* technique was slightly reduced ($P < 0.05$) by dietary nitrate, and the lag period was longer ($P < 0.05$) in nitrate-supplemented sheep than in controls. The rate of degradation of the *b*-fraction did not differ ($P > 0.1$)

Table 1. Physiological and fermentation characteristics of sheep fed iso-nitrogenous diets of oaten chaff supplemented with 5.54 g N/kg feed as urea (control) or as KNO_3 (4% of air dry)

c, rate constant (see Eqn 1); WL, washout loss. n.s., not significant at $P < 0.05$

Measure	0% KNO_3	4% KNO_3	Pooled s.d.	*P*-value
DM intake (g/day)	863	870	34.0	n.s.
DM digestibility (%)	59.4	56.8	4.89	n.s.
Methane yield (L/kg DM)	29.8	22.9	3.71	0.04
Rumen ammonia (mg N/L)	102	115	17.9	n.s.
Microbial protein outflow (g/day)	58.4	73.9	16.2	n.s.
Total volatile fatty acid in rumen fluid (mM)	82.8	97.8	6.79	0.02
Acetate (mol %)	68.0	73.4	3.71	0.09
Propionate (mol %)	21.7	17.5	2.89	0.09
Butryate (mol %)	8.7	7.7	0.70	0.09
Acetate : propionate ratio	3.22	4.28	0.78	0.10
Rumen pH	6.37	6.45	0.135	n.s.
Blood methaemoglobin (%)	0.48	0.62	0.156	n.s.
Rumen fluid volume (L)	7.15	6.7	0.83	n.s.
Fluid rate constant (/h)	2.23	2.28	0.756	n.s.
Mean fluid retention time (min)	704	669	195	0.06
Protozoal numbers ($\times 10^{-4}$/mL)				
Total protozoa	32	22	10.7	n.s.
Small entodiniomorphs	31.3	20.7	10.9	n.s.
Large entodiniomorphs	0.3	0.6	0.16	0.03
Holotrichs	0.4	0.7	0.04	n.s.
In situ results				
Bag washing loss (WL, %)	30.8	30.8	–	–
Lag period (min)	127	173	26.8	0.05
Intercept (a, %)	23.8	21.2	1.67	0.07
Potential DM loss (WL, %)	46.0	47.7	1.5	0.15
DM loss rate (c, %/h)	4.62	4.68	0.211	0.77
Undegradable DM (%)	30.2	31.1	0.81	0.18
Potential degradability (%)	69.8	68.2	0.81	0.18
Effective degradability	52.0	50.9	0.59	0.04

between sheep given the nitrate-supplemented or control treatments.

Total VFA concentration in rumen fluid was higher ($P < 0.05$) in the nitrate-supplemented sheep than in control sheep. The nitrate-supplemented animals also tended ($P < 0.10$) to have a higher molar proportion of acetate in rumen fluid and lower proportion of propionate, as well as a higher molar ratio of acetate to propionate. There was no difference in rumen ammonia concentration between control and urea-supplemented sheep (102 versus 115 mg N/L, $P > 0.05$).

Methane yield (MY; L methane/kg DM intake) was lower ($P < 0.05$) by 23% or 6.9 L/day when 4% KNO_3 was added to the oaten hay diet. There was evidence of a repeated pattern of methane concentrations in the respiration chambers that matched the timing of the 2-hourly meals, i.e. methane concentration fell in the first hour after feeding then increased during the second hour. This pattern was more obvious for the nitrate-supplemented sheep than for the control sheep (Fig. 1).

Rumen fluid volume and rumen outflow did not differ ($P > 0.05$) between treatments. There was a positive association between the MRT of rumen liquid and MY (L/kg DMI = 13.3 + 0.0191 MRT, $r^2 = 0.39$, $P = 0.058$), such that MY increased with longer liquid retention times in the rumen. There were no significant relationships between MRT of rumen fluid and factors such as propionate proportion in the rumen fluid or microbial crude protein outflow from the rumen.

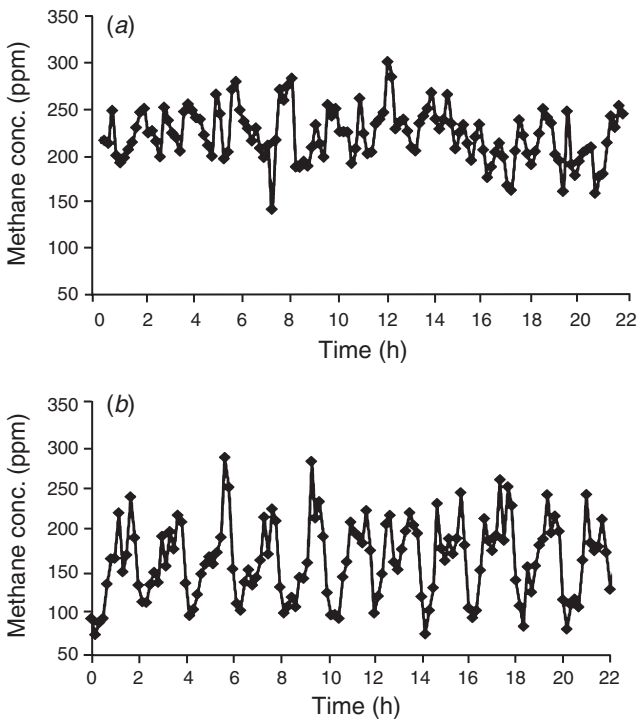

Fig. 1. Time-course of methane concentration in respiration chambers associated with 2-hourly feeding of iso-nitrogenous diets; (*a*) control diet with urea but no added KNO_3 and (*b*) diet with 4% added KNO_3.

Microbial crude protein outflow, total protozoal population and rumen pH were not affected significantly by the inclusion of nitrate in the diet.

Discussion

A major reason for undertaking this study was to determine the magnitude of the effect on methane output of sheep when nitrate is present in the diet. Stoichiometry shows that reduction of 1 mol nitrate to ammonia uses the same amount of H_2 and high potential electrons as does the reduction of 1 mol CO_2 to methane. Inclusion of 4% KNO_3 (0.396 mol nitrate fed/sheep per day) could be expected to reduce methane production by 0.396 mol or 8.87 L/day in supplemented sheep. The 6.9 L reduction achieved was 78% of that expected. The less-than-predicted reduction may have been a consequence of a greater ruminal fermentation rate, which increased H_2 availability in nitrate-supplemented sheep, as evidenced by a tendency for a higher total VFA concentration and a higher acetate percentage in the VFA. However, there was no increase in effective degradability of feed in the rumen as measured *in situ*. Another possibility is that a small fraction the nitrate and nitrite was absorbed into the blood, which would have decreased the H_2 available for nitrate reduction in the rumen. Considerable animal-to-animal variation in rumen parameters such as microbial protein outflow may have prevented the large differences in means for these traits being significantly different, making further explanations of the impact of nitrate on underlying fermentation responses uncertain.

The tendency for nitrate-supplemented sheep to have a shorter MRT is consistent with the lower MY achieved. Shorter MRT has been consistently observed to be associated with reduced methane production in sheep, and also in a range of species including humans (Hegarty 2004). From the present study, it is not clear whether nitrate reduced MRT by supporting faster fermentation and so faster rumen clearance. Raised VFA concentrations in supplemented sheep suggest this did occur, but the lack of effect on *in situ* DM loss is not consistent with there being a faster rate of ruminal fermentation.

As VFA production and concentration are related (Leng 1970), the higher total VFA concentrations in rumen fluid in sheep supplemented with nitrate rather than urea could be indicative of higher VFA production and higher digestible DM intake; however, VFA concentrations are a consequence of both production and removal rates and the latter can be affected by factors such as pH, rumen volume, rate of water outflow from the rumen and osmotic pressure (López *et al*. 1994). Because the control and nitrate-supplemented diets were iso-nitrogenous, it is unlikely that faster fermentation occurred as nitrate helped correct a ruminal N deficit.

More direct measures of production rate would be needed to establish whether VFA production rate is indeed increased when nitrate rather than urea is a major source of N for the rumen microbial population. The tendency for reduction in the molar proportion of propionate and increase in the molar proportion of acetate is consistent with result of Farra and Satter (1971). Nitrate has a higher affinity for H_2 than CO_2 and the reactions than generate propionate (Ungerfeld and Kohn 2006) so that

methane and propionate production will be suppressed by nitrate. Whenever H_2 is more effectively removed, NADH concentration in cells will be lowered and NAD^+ concentration increased and these events favour acetate formation, inhibit propionate formation and promote a more rapid fermentation of carbohydrate (Sutherland 1977).

In addition to nitrate lowering daily methane production, the patterns in methane chamber methane concentration corresponding to the intervals between 2-hourly meals of nitrate-containing feed (Fig. 1) showed that the nitrate released at the start of each meal resulted in a rapid and marked reduction in rumen methane production. This is confirmation of the higher affinity of nitrate for H_2 relative to that of CO_2 and other electron acceptors. It is also notable that the early reduction in methane output after any meal was not sustained over the 2 h between meals. Thus, a 'slow release' form of nitrate might enhance methane mitigation and, in addition, reduce nitrate and nitrite absorption from the rumen and thereby reduce the potential for nitrite toxicity.

In situ effective degradability of oaten hay DM in the rumen of sheep on the three diets did not differ significantly, even though the lag time before net oaten hay DM removal was ~0.7 h longer for nitrate-supplemented sheep than for urea-supplemented sheep.

The rate constant for degradation of 'insoluble DM' was apparently unaffected by the presence of dietary nitrate. This suggests that the small but significant effect of dietary nitrate on effective degradability of DM was probably attributable to a slower rate of microbial attachment to feed materials (longer lag time) when bags were placed in sheep offered the nitrate-supplemented diet. It seems unlikely, however, that the lag difference would be important production in practice, because Trinh *et al*. (2009) showed there were no differences in growth rate and N retention in goats when they offered iso-nitrogenous supplements of KNO_3 or urea with a diet of rice straw, molasses and foliage of *Sesbania grandiflora*.

Although nitrate in the diet can predispose animals to nitrite toxicity (Davidson *et al*. 1941) there were no signs of toxicity in the sheep used in this study, as judged by negligible blood methaemoglobin concentrations even at the 4% level of KNO_3 supplementation. Moreover, the sheep in this study appeared healthy at all times throughout the acclimation and experimental periods. Trinh *et al*. (2009) also reported that goats given supplements of urea or nitrate at similar levels to those used in this study remained healthy throughout a 22-week experimental period and grew as well as their counterparts given urea supplements.

We conclude that, as also shown by other workers (Carver and Pfander 1974; Trinh *et al*. 2009) nitrate can replace urea as a source of non-protein N for ruminant livestock. In the context of GHG reduction, there is the important additional benefit of reduced enteric methane emissions.

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