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Effect of added dietary nitrate and elemental sulfur on wool growth and methane emission of Merino lambs

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Abstract. The effects of dietary nitrate (NO_3) and elemental sulfur (S) on nutrient utilisation, productivity, and methane emission of Merino lambs were investigated. Forty-four lambs were randomly allocated to four groups (n = 11) fed isonitrogenous and isoenergetic diets. The basal feed was supplemented with 1% urea + 0.18% S (T1), 1.88% NO_3 + 0% S (T2), 1.88% NO_3 + 0.18% S (T3), or 1.88% NO_3 + 0.40% S (T4). Retention of S was improved by increasing the content of elemental S in the NO_3-containing diet ($P < 0.001$), yet the N retention (g/day) by the animal, and the N and S content of wool (%), were not altered by S supplementation ($P > 0.05$). Dry matter intake, liveweight gain, and feed conversion ratio did not differ ($P > 0.05$) between treatments. Replacing urea with NO_3 improved the rate of clean wool growth by 37% ($P < 0.001$, T1 vs T3). Clean wool growth increased by 26% ($P < 0.001$) when the S content of the NO_3-containing diet was increased from 0 to 0.18% (T2 vs T3). Methane production (g/day) and methane yield (g/kg DM intake) were reduced ($P < 0.05$) by 24% when urea was replaced by NO_3 (T1 vs T3). The addition of 0.4% S to a diet containing 1.88% NO_3 also reduced methane production ($P = 0.021$) and methane yield ($P = 0.028$). In conclusion, the addition of 1.88% NO_3 and 0.18% elemental S to a total mixed diet increased clean wool production and reduced methane production. However, there was no evidence of inter-relationships between NO_3 and S.

Additional keywords: liveweight gain, feed conversion ratio, wool growth rate.

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Introduction

The impact of animal production systems on air quality has been under increasing public and political scrutiny. There is an urgent need to identify practical feeding strategies to reduce methane emissions from sheep and cattle in intensive and extensive systems. The potential for using nitrate (NO_3) supplementation as a methane mitigation technology has been established (Leng 2010; Nolan *et al*. 2010; van Zijderveld *et al*. 2010; Li *et al*. 2012), and methane emissions from ruminants are consistently reduced when nitrate (NO_3) is present in concentrate-based diets (van Zijderveld *et al*. 2010; van Zijderveld *et al*. 2011; Li *et al*. 2012; Hegarty *et al*. 2013).

On the other hand, there are strong indications that NO_3 inclusion in ruminant diets can reduce feed intake and liveweight gain (Weichenthal *et al*. 1963; Goodrich *et al*. 1964; Farra and Satter 1971; Hegarty *et al*. 2013). Farra and Satter (1971) suggested that the suppression of feed intake when NO_3 was included in ruminant diets was probably caused by poor palatability. Others have shown that excessive NO_3 intake can cause rumen metabolic imbalances (Marais 1988), vasodilation (Sakai *et al*. 2000; Pinder *et al*. 2009), reduced blood pressure (Whatman *et al*. 2013), and methaemoglobinaemia. It appears that reduced feed intake may be a protective mechanism that animals use to avoid metabolic post-ingestive effects of excessive NO_3 (Forbes and Mayes 2002).

It has been suggested that the increased capillary-bed blood flow would enhance nutrient delivery to the wool follicle and, hence, wool growth (Sokolowski *et al*. 1969; Hales and Fawcett 1993). In theory, the cardiovascular effects of nitric oxide (NO) formed from the reduction of absorbed NO_2 (Sakai *et al*. 2000; Pinder *et al*. 2009) would increase capillary-bed blood flow and nutrient supply to the wool follicle, which should stimulate wool growth. In a study of growing, white-faced south-western lambs, which appears to be the only study of its type, wool growth tended to increase when NO_3 replaced urea in a concentrate-based diet (Sokolowski *et al*. 1969).

In the same study by Sokolowski *et al*. (1969), addition of inorganic sulfur (S) to the NO_3-containing diet improved the utilisation of NO_3-N by the lambs and facilitated NO_3 and NO_2 reduction to NH_3 in the rumen, thereby reducing the likelihood of methaemoglobinaemia (Leng 2010). van Zijderveld *et al*. (2010) showed that the addition of sulfate (SO_4)-S (0.85% total S in dry matter (DM)) to a diet containing NO_3 (2.6% NO_3 in DM) reduced methane production by ~21% compared with a diet containing NO_3 with no added sulfate. Despite these diets differing in their ingredients and also in their total NO_3 and S content, it is still reasonable to hypothesise that differences in dietary NO_3 : S ratio could alter nutrient utilisation, wool production, animal growth performance, and methane emissions. The current study was undertaken primarily to investigate

whether wool growth of fine-wool Merino sheep would be increased by the addition of NO_3 to the diet. The other aims of the study were to assess the efficacy of NO_3 for mitigating methane production, to quantify the production responses of lambs to the replacement of dietary urea with NO_3, and to assess whether any response to NO_3 is affected by the level of S in the diet.

Materials and methods

Animals and diets

All protocols for the care of the animals used in this experiment were approved by the University of New England Animal Ethics Committee (AEC 12/127).

Forty-four weaned wether lambs (~3.5 months old, fine-wool Merino, liveweight 22.7 ± 0.17 kg) were selected from a local commercial farm (Warrane Station, Armidale, NSW). Upon entry to the Animal House, lambs were vaccinated (2 mL/animal of Cydectin Weaner Guard 6 in 1 vaccine and wormer for sheep; Virbac Animal Health, Australia, Regents Park, NSW) and treated for intestinal parasites (3 mL/animal of First Drench (levamisole 37.5 g/L and praziquantel 18.8 g/L); Virbac Animal Health, Australia). Lambs were monitored daily for clinical symptoms of methaemoglobinaemia.

Lambs were allocated to four dietary treatment groups (T1–T4, Table 1) by stratified randomisation within weight ranges and were penned individually. The four barley (64.5%) and wheat chaff (21.5%) based complete diets were formulated to be isonitrogenous and isoenergetic (15.5% crude protein on a DM basis and 11.6 MJ metabolisable energy (ME)/kg DM) (Table 2).

The experiment lasted for 69 days and consisted of two periods: an initial 65-day *ad libitum* feeding period in which each lamb was offered feed at a level of 3 × maintenance ME, followed by a 4-day restricted feeding period in which each lamb was placed in a respiration chamber to determine methane output. In this period, each lamb was offered 80% of its average voluntary intake during the preceding 7-day period to facilitate comparison of the effects of dietary treatments on methane production without the potential confounding effect of animals refusing feed while in the respiration chamber.

During the first 14 days, the diet of all lambs was progressively changed from 100% chopped lucerne–oaten chaff on day 1 to 100% treatment pellets on day 14. However, soon after sheep were fed the 100% treatment diets, diarrhoea became prevalent (on average 14% lambs had diarrhoea); hence, from day 21 onwards, all lambs were offered 91% treatment pellets and 9% chaff. Once the diarrhoea prevalence declined, lamb growth performance was monitored (days 27–65). Daily feed intake for each lamb was recorded. Animals had access to fresh water at all times.

Table 1. Supplementary levels of nitrate (provided as $5Ca(NO_3)_2.NH_4NO_3.10H_2O$), urea, and elemental sulfur inclusion (%, DM basis) in pelleted barley grain and chaff based diets

Diet	Nitrate	Urea	Elemental S
T1	0	1.00	0.18
T2	1.88	0	0.00
T3	1.88	0	0.18
T4	1.88	0	0.40

Table 2. Ingredients and nutrient composition of experimental diets (% DM basis) fed to lambs
All feed ingredients were pelleted using a 1-cm die

Ingredients	T1 1% urea + 0.18% S	T2 1.88% NO_3+ nil S	T3 1.88% NO_3 + 0.18% S	T4 1.88% NO_3 + 0.4% S
Wheat chaff	21.41	21.50	21.49	21.48
Molasses	2.60	2.54	2.41	2.24
Cotton seed meal	7.20	7.23	7.23	7.23
Barley	64.26	64.54	64.51	64.48
Mitavite Performa Oil[A]	0.56	0.56	0.56	0.56
Mineral Pac[B]	0.56	0.56	0.56	0.56
Limestone	1.68	0.00	0.00	0.00
Urea	1.00	0.00	0.00	0.00
Powdered sulfur (S)	0.18	0.00	0.18	0.40
NaCl	0.56	0.56	0.56	0.56
Calcium nitrate[C]	0.00	2.49	2.49	2.49
Total	100	100	100	100
	Calculated nutrient composition			
Nitrogen (N), %	2.48	2.48	2.48	2.48
S, %	0.33	0.15	0.33	0.56
N : S ratio	7.5	16.5	7.5	4.4
Nitrate (NO_3), %	0.0	1.89	1.88	1.88
NO_3 : S ratio	0.0	12.6	5.7	3.4

[A]Mitavite Performa 3: Omega 3 fatty acids, Vitavite, NSW, Australia.
[B]Mineral Pac: Mega Min, Agsolutions, Qld, Australia.
[C]$5Ca(NO_3)_2.NH_4NO_3.10H_2O$ (75% NO_3 in DM).

Methaemoglobin (MetHb) concentration in blood

Blood was sampled 1 h before morning feeding on days 7, 22, 35, and 65. Blood samples (~8 mL) were taken from the jugular vein, using lithium-heparin vacutainers (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). Concentration of MetHb in blood was determined within 30 min of blood collection as described by Hegesh *et al*. (1970).

Dry matter, nitrogen, and sulfur utilisation

On day 43, five lambs were randomly selected from each group, i.e. 20 lambs in total, moved to metabolism cages, and offered *ad libitum* feed in preparation for the nutrient balance measurement. These 20 lambs were re-employed for methane study at a later stage (days 66–69). Total collections of faeces, urine, and refusals were made on days 47–53 to determine DM, N, and S digestibility. The total daily faecal output was well mixed then subsampled (10% w/w), and subsamples were pooled over 7 days and stored at −20°C. Urine was collected into buckets containing ~500 mL of 1.8 M H_2SO_4 as a preservative (IAEA 1997). The daily urine output was recorded and then diluted with tap water to a constant final weight of 3 kg. Representative samples of diluted urine (3% w/w) were taken, pooled over 7 days, and stored at −20°C. Feed offered and any feed refused were subsampled daily (25% w/w), pooled, and stored at −20°C until analysis. The daily water intake was recorded.

Dry matter concentration of experimental diets, feed refusals, and faeces were determined by drying a subsample at 65°C in a forced-draught oven to a constant weight. Total N in feed, faecal, and urine samples was determined using an automated Organic Nitrogen Determinator (FP-2000; LECO Corp., St Joseph, MI, USA). Total S in the feed, faecal, and urine samples was determined using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Vista MPX Radial; Varian Inc., Mulgrave, Vic.) after a modified sealed chamber digestion (Anderson and Henderson 1986).

The concentration of allantoin in the urine was determined using the colourimetric method of IAEA (1997), and the yield of total microbial N from the rumen was then calculated using the prediction equations of Chen *et al*. (1992).

Methane production

Total methane production (L/day at standard temperature and pressure (STP)) was measured for 22 h on days 66–69 of the trial in eight open-circuit respiration chambers as described by Bird *et al*. (2008). Six lambs, of which four were used in the nutrient balance study, were selected from each of the four treatments for methane production measurement. Of the 24 lambs, eight lambs per day (2 lambs per treatment × 4 treatments) were randomly allocated for placement in a respiration chambers on days 66, 67 and 68.

Lambs were offered the daily ration at 1100 hours in the chamber. Chambers were ~1200 L in volume and the external air was drawn through the chamber at ~180 L/min at STP; thus, the mean retention time of air in the chambers was ~7 min. Methane concentration was measured using a 1312 Photoacoustic Multi-gas analyser with inbuilt moisture correction (Innova Airtech Instruments, Ballerup, Denmark). Methane production was calculated as airflow (L/day) × methane concentration in air immediately after leaving the chamber (ppmv) and was adjusted for methane concentration in the incoming air and for the temperature and atmospheric pressure in the chamber.

Clean wool growth rate and skin surface temperature

Clean wool growth (CWG) of each sheep was measured between day 24 and day 60 by clipping a 10 cm by 10 cm mid-side patch on the left and right side. On day 60, wool produced from both patches was removed as close to the skin surface as possible and stored separately in plastic sealable bags. Two shearers operated on day 24 and day 60 and sheep were randomly allocated to each shearer. The greasy wool samples were washed and assessed by a third-party laboratory (New England Fibre Testing Pty Ltd, Walcha, NSW). The washing yield (%) was calculated as clean wool weight (g)/greasy wool weight (g) × 100. Total N concentration in the clean wool samples was determined using an automated Organic Nitrogen Determinator (FP-2000, LECO Corp.). Total S concentration in the wool samples was determined using ICP-OES as for feed and excreta.

Sheep were completely shorn on day 70 and skin surface (mid-side flank area) temperature was measured using an infrared thermometer (MS6530; Wiltronics Research Pty Ltd, Ballarat, Vic.) twice daily at 0830 and 1630 hours on days 71, 72, and 73.

Statistical analyses

All results were assessed by analysis of variance (ANOVA), using Genstat 12th edition (VSN International Ltd, Hemel Hempstead, UK). Dry matter intake (DMI), liveweight gain (LWG), and nutrient utilisation were analysed using a one-way ANOVA for treatments. Data for CWG were analysed using a split-plot ANOVA with initial liveweight as the covariate; treatment, location of patch, and shearer as the treatment factors; animal as the experimental unit; and patch-side as the subplot. The effects of treatment and measurement day on methane production and yield were assessed using an unbalanced ANOVA with the average DMI over the previous 2 days as the covariate. When differences between means existed, a least-significant differences test was used to compare means from within a fixed factor. Unless stated otherwise, the results are expressed as the mean ± standard error of the difference of the mean (s.e.d.). The significance level was set at $P = 0.05$.

Results

Many of the lambs developed diarrhoea by day 14 (a mean rumen pH of 5.8 was observed for lambs experiencing diarrhoea). Thiamine (vitamin B1) deficiency was diagnosed in one lamb (in T2) in the final week, and it was removed from the trial.

Dry matter intake, liveweight gain, and clean wool growth

Dry matter intake, LWG, and feed conversion ratio (FCR) did not differ ($P > 0.05$) between treatments (Table 3). The average CWG was increased ($P < 0.001$) by ~30% when 1% urea was replaced by 1.88% NO_3 in the diet (T3 vs T1). The rate of CWG was improved ($P < 0.001$) when the content of elemental S in the NO_3-containing diet was increased from 0 (T2) to 0.18% (T3);

Table 3. Average dry matter intake (DMI), daily liveweight gain (LWG), feed conversion ratio (FCR), clean wool growth (CWG), and skin surface temperature of wether lambs fed diets with and without nitrate and/or elemental sulfur over a 38-day measurement period (days 27–65)
Data were based on 11 lambs per treatment. For CWG, means followed by the same letter are not significantly different at $P = 0.05$

Variable	T1 1% urea + 0.18% S	T2[A] 1.88% NO_3 + nil S[A]	T3 1.88% NO_3 + 0.18% S	T4 1.88% NO_3 + 0.4% S	s.e.d.	*P*-value
Initial LW (kg)	22.4	22.5	21.9	22.2	0.54	0.72
DMI (g/day)	761	687	813	741	103.9	0.682
LWG (g/day)	114	91	153	122	30.7	0.264
FCR (g DMI/g LWG)	7.58	7.62	5.49	8.53	1.680	0.28
CWG (μg/cm^2.day)	486a	530a	668b	738b	52.0	<0.001
Skin surface temp. (°C)[B]	29.4	30.0	29.8	30.2	0.29	0.07

[A]One lamb diagnosed with thiamine deficiency was removed.
[B]Measured on days 71, 72, and 73 after sheep were shorn.

Table 4. Main effects of treatment (means ± s.e.d., $n = 5$) on dry matter digestibility (DMD), and nitrogen and sulfur utilisation of sheep fed *ad-libitum* diets with and without nitrate and/or elemental sulfur over a 7-day period of total collection
Within rows, means followed by the same letter are not significantly different at $P = 0.05$

Variable	T1 1% urea + 0.18% S	T2 1.88% nitrate + nil S	T3[A] 1.88% nitrate + 0.18% S	T4 1.88% nitrate + 0.4% S	s.e.d.	*P*-value
DM intake (g/day)	981	956	891	960	69.60	0.61
WG (g/day)	175	135	160	122	29.8	0.32
DMD (%)	69ab	73c	67a	71bc	1.30	0.01
Microbial N outflow (g/day)	10.2	9.76	9.41	10.1	1.818	0.97
N intake (g/day)	21.43	21.34	20.48	21.99	1.57	0.81
N retention (g/day)	6.41	8.02	6.81	8.52	1.30	0.36
$N_{retention}/N_{intake}$ (%)	29.2	37.9	32.8	40.3	5.13	0.17
S intake (g/day)	3.51a	1.86b	3.19a	4.95c	0.40	<0.001
S retention (g/day)	1.35a	0.67b	1.23a	2.29c	0.16	<0.001
$S_{retention}/S_{intake}$ (%)	38.2a	36.6a	38.4a	47.1b	2.95	0.01
Wool N (%)	15.3	15.3	15.3	15.3	0.10	0.87
Wool S (%)	3.72	3.55	3.61	3.51	0.130	0.46
Wool N : S ratio	4.13	4.31	4.24	4.37	0.162	0.50

[A]One sheep removed from the analysis due to severe diarrhoea.

however, additional elemental S supplementation (to 0.4%) did not further increase CWG in animals fed a diet containing NO_3 (T4 vs T3). Sheep offered diets containing added NO_3 instead of urea also tended ($P = 0.07$) to have higher skin surface temperatures.

Nitrogen and sulfur utilisation

Throughout the 7-day total collection, DMI and LWG were not affected by diet ($P > 0.05$). Nitrogen intake, microbial N outflow, and N retention did not differ between treatments ($P > 0.05$). Whereas the addition of 0.4% elemental S to a 1.88% NO_3 diet increased S intake and retention ($P < 0.001$), addition of elemental S to the diet did not affect N retention of sheep receiving NO_3-containing diets ($P > 0.05$). Lambs offered the T2 diet ingested less S ($P < 0.001$) and retained less ingested S ($P < 0.001$) than those in the other treatments. The N and S content of wool were not affected by any of the four treatments.

Enteric methane production

There was a substantial increase in daily DMI as the experiment progressed; however, there was no effect of treatment on DMI over days 27–65 (Table 3), during the digestibility study (Table 4), or during the measurement of methane emission in the final week of the study (Table 5). Methane production (g/day) and methane yield (g/kg DMI) were reduced ($P < 0.05$) by ~24% when urea was replaced by NO_3 in the diet (T1 vs T3). The addition of 0.4% elemental S to the 1.88% NO_3 diet also reduced methane production ($P = 0.021$) and methane yield ($P = 0.028$).

Methaemoglobin concentration in blood

There was no treatment difference for the blood MetHb concentration of lambs ($P > 0.05$), which averaged 0.3% of total haemoglobin across all animals. The blood MetHb concentrations in lambs receiving 1.88% NO_3 did not change over the entire MetHb-monitoring period, averaging ~0.38%. None of the sheep had blood MetHb concentrations >2.5% during the MetHb-monitoring period.

Discussion

Lambs in this study were newly weaned at commencement of this trial. Weaning stress and the high cereal content of the diets may have contributed to the development of diarrhoea in many of

Table 5. Least square means for dry matter intake (DMI), methane production and methane yield of wether lambs (n = 6) fed diets with and without supplementary nitrate and/or elemental sulfur

Within rows, means followed by the same letter are not significantly different at P = 0.05

Variable	T1 1% urea + 0.18% S	T2 1.88% NO_3 + nil S	T3 1.88% NO_3 + 0.18% S	T4 1.88% NO_3 + 0.4% S	Av. s.e.d.	P-value
DMI (g/day)[A]	789	771	878	904	74.8	0.331
Methane production (g/day)	14.3a	10.0b	10.7b	7.41c	1.875	0.021
Methane yield (g/kg DMI)	17.2a	13.2b	13.1b	8.22c	1.872	0.028

[A]DMI on the day that methane production was measured.

the lambs by day 14. One lamb (T2) experienced thiamine deficiency in the final week. Young growing lambs fed high-grain diets are very susceptible to thiamine deficiency, as high grain diet encourages the growth of certain thiaminase-producing bacteria in the rumen, and their presence might induce thiamine deficiency (Rammell and Hill 1986). Perhaps a longer adaptation period, i.e. 28 days, allowing the newly weaned animals to be introduced to the high-grain diet gradually, would have reduced the likelihood of problems associated with excessive lactic acid production in the rumen. The low MetHb concentration and absence of visible symptoms of NO_3 toxicity in lambs was in accord with findings for animals fed similar diets (Sapiro *et al*. 1949; Barnett and Bowman 1957; Butler 1959; Li *et al*. 2012).

Effect of added dietary nitrate and elemental sulfur on nutrient utilisation

All lambs were in positive balance for N and S, indicating that all rations contained sufficient N and S to meet the growth requirements of the lambs. Replacing 1% urea with 1.88% NO_3 in the diet containing 0.18% elemental S (T1 vs T3, both diets containing ~2.48% total N and 0.33% total S) did not affect apparent digestibility of DM, N, or S, and microbial N outflow did not differ between diets. In contrast, Sokolowski *et al*. (1969) reported that addition of 1.96% NO_3 to a basal diet containing 1.38% N and 0.5% S increased N retention and N digestibility. The lack of an NO_3 effect on DM and N utilisation are in agreement with data reported by Nolan *et al*. (2010) and Li *et al*. (2012). When the lower N and S utilisation levels are considered in conjunction with the increased CWG observed in this study, a clarification of the increased CWG is apparent, as explained below.

Effect of added dietary nitrate and elemental sulfur on dry matter intake, liveweight gain, and wool growth

Variable effects of NO_3 on DMI and LWG in sheep have been reported. Hoar *et al*. (1968), Sokolowski *et al*. (1969), and Li *et al*. (2012) observed that DMI and LWG were reduced when 14–21 g NO_3/kg DM was added to a concentrate diet fed to growing lambs. In contrast, van Zijderveld *et al*. (2010) reported that feeding 26 g NO_3/kg DM to crossbred Texel lambs had no adverse effects on DMI or LWG.

The N : S ratio affects rate of fermentation and microbial synthesis in the rumen, and a total N : S ratio of 12.5 is recommended for sheep (Freer *et al*. 2007), but Leng (2010) reviewed the literature at length and proposed that ruminants fed NO_3-containing diets may benefit from lower N : S ratios. The wool growth results obtained from our study appear to support the suggestion by Leng (2010). In this study, the addition of elemental S in T3 (N : S = 7.5 : 1) increased CWG when with T2 (N : S = 16.5 : 1).

There is little information on the effect of dietary NO_3 on wool growth in Merino sheep. Sokolowski *et al*. (1969) reported that in sheep not selected for wool growth, there was a tendency for CWG to increase when NO_3 was added to a total mixed diet. This finding was supported by the current study, which clearly showed that feeding a diet containing NO_3 produced higher CWG than the same diet with isonitrogenous amounts of urea. The major limitation to wool growth is the supply of amino acids, especially the S-containing amino acids, to the wool follicle (Hales and Fawcett 1993; Hynd and Masters 2002). It has been suggested that additional dietary S could increase microbial S-amino acid supply to the sheep by increasing the concentration of S-amino acids in the microorganisms flowing out of the rumen, which could increase wool growth (Murray *et al*. 1990). However, there were no differences in digestible DMI and net microbial growth from the rumen to provide a ready explanation for greater nutrient delivery to the wool follicles of sheep given diets supplemented with NO_3. One possibility is the cardiovascular effects of NO formed from the reduction of absorbed NO_2 and its conversion to NO following absorption, which would induce arteriolar relaxation and hence an increase in capillary-bed blood flow (Pinder *et al*. 2009). This may have increased blood flow to the skin and skin temperature, which tended to be 0.6°C higher in sheep ingesting diets containing NO_3 in our study. The increased capillary-bed blood flow would enhance nutrient delivery to the wool follicle and, hence wool growth (Sokolowski *et al*. 1969; Hales and Fawcett 1993). The potential importance of such an effect warrants further study.

Effect of added dietary nitrate and elemental sulfur on methane production

The reported effect of NO_3 on methane production varies from 16 to 50% reduction depending on the inclusion rate of NO_3 and the type of diet and animal used in various studies (Leng 2010). Stoichiometrically, 1 mol NO_3 fed per sheep per day should reduce rumen methane production by 1 mol or 22.4 L per day in NO_3-supplemented sheep, provided fermentation pathways within the rumen are unaffected and hydrogen or electron production is not increased. In our study, sheep fed the NO_3 treatments consumed ~0.26 mol NO_3/day, which with the above proviso should theoretically reduce rumen methane production by ~4.2 g/day (T3 [1.88% NO_3 + 0.18% S] vs T1 [1% urea +

0.18% S]). The observed reduction in methane production was 3.6 g/day, which is 86% of the expected stoichiometric potential. This is in general agreement with the conclusions of Nolan *et al*. (2010), van Zijderveld *et al*. (2010), and Li *et al*. (2012), and suggests that most, but not all, NO_3-N ingested by animals is reduced to ammonia by the rumen microorganisms. Mitigation of methanogenesis below stoichiometrically predicted levels could result from NO_3 effects on volatile fatty acid proportions and microbial cell yield that changed the balance of other H_2-using and -producing reactions in the rumen (Nolan *et al*. 2010), or from absorption of NO_3 and NO_2 from the rumen. Nevertheless, the current study confirms that NO_3 can be used to mitigate enteric methane release in sheep fed concentrate-based diets.

It has been proposed that many anaerobic organisms could utilise S (SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, and elemental S) as an additional electron (or H) sink (Hedderich *et al*. 1998; Kung *et al*. 1998; Hinsley and Berks 2002). van Zijderveld *et al*. (2010) showed that the addition of sulfate (SO_4)-S (0.85% total S in DM, more than four times higher than the recommend level of 0.2% S for growing sheep) to a diet containing NO_3 (2.6% NO_3 in DM) reduced methane production by ~21% compared with diet containing NO_3 but without S. Elemental S is a much less expensive source of S than any form of sulfate salts, although the EU has banned the use of elemental S in the animal feed industry for occupational health and safety reasons (H. Perdock, pers. comm.). Our study shows that when the appropriate level of elemental S is added to a NO_3-containing diet, methane emissions could be further reduced.

Cappenberg (1975) observed that S-reducing bacterial end products, i.e. H_2S and sulfide ions, inhibited methanogenesis *in vitro*. Therefore, it is possible that when S reduction and its end product, H_2S, reaches a critical level over a certain period of time in the rumen, methanogenic activity is further decreased, which means that the decreased methane production would persist for some time after feeding, as observed by van Zijderveld *et al*. (2010). Adding 0.18% S to the 1.88% NO_3 diet did not reduce daily methane production, and methane yield may reflect the direct uptake of S into S-containing products such as thiamine (which was deficient in the NO_3 diet with no S supplement; NRC 1996) by ruminal microbes rather than complete reduction to H_2S. The fact that supplementation with the highest level of S reduced daily methane production and methane yield may indicate that high levels of dietary S do inhibit methanogenesis, although the mechanism responsible for this is unknown. Further study is required to elucidate how NO_3 interacts with S to inhibit methane production.

Conclusion

This study demonstrates that nitrate provides a safe means of providing a ruminally degradable N source and mitigating enteric methane emissions in sheep. The addition of 1.88% NO_3 and 0.18% elemental S to a total mixed diet increased clean wool production. However, the inter-relationships between $NO_3$and S are still not fully understood and more research on Merino sheep under grazing conditions is needed.

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