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Use of dietary nitrate to increase productivity and reduce methane production of defaunated and faunated lambs consuming protein-deficient chaff

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Abstract. The effects of dietary nitrate supplementation and defaunation on methane (CH_4) emission, microbial protein outflow, digesta kinetics and average daily gain were studied in lambs fed chaff containing 4.1% crude protein in dry matter. Twenty ewe lambs were randomly allocated in a 2 × 2 factorial experiment (0% or 3.1% calcium nitrate supplementation and defaunated or faunated protozoal state). Nitrate supplementation increased blood methaemoglobin concentration ($P < 0.05$), rumen volatile fatty acids, ammonia concentration, dry matter intake, microbial protein outflow, average daily gain, dry matter digestibility, clean wool growth and wool fibre diameter ($P < 0.01$). Nitrate increased CH_4 production (g/day) due to greater dry matter intake, but did not affect CH_4 yield (g/kg dry matter intake). Nitrate-supplemented lambs had a shorter total mean retention time of digesta in the gut ($P < 0.05$). Defaunation reduced CH_4 production and CH_4 yield by 43% and 47%, but did not cause changes in dry matter intake, microbial protein outflow, average daily gain or clean wool growth. Defaunation decreased total volatile fatty acids and the molar percentage of propionate, but increased the molar percentage of acetate ($P < 0.05$). Interactions were observed such that combined treatments of defaunation and nitrate supplementation increased blood methaemoglobin ($P = 0.04$), and decreased CH_4 yield ($P = 0.01$).

Additional keywords: methanogensis, protozoa, sheep.

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Introduction

Residues of crops and agricultural by-products are the major feed sources for livestock in tropical and subtropical regions, but they are often of low to moderate digestibility with low levels of protein and minerals (Preston 1995). Ruminant production from these low-quality feeds is limited by the deficiency of absorbed amino acids and energy, resulting from decreased feed intake and digestibility due to low growth and fermentation of rumen microbes (Leng 1990).

Removal of protozoa from the rumen (defaunation) increases average daily gain (ADG) by 11% (Eugène *et al*. 2004) due to increased bacterial biomass and increased availability of protein at the duodenum (Bird and Leng 1978; Jouany 1996). Defaunated cattle grew at a 43% greater rate than faunated cattle on the same intake of a low-protein molasses-based diet (Bird and Leng 1978) whereas defaunated lambs showed increased growth rate and wool growth on a low-protein diet (Bird *et al*. 1979). Defaunation also decreases enteric methane (CH_4) production (MP; Kreuzer *et al*. 1986; Hegarty 1999; Morgavi *et al*. 2008) by eliminating methanogens that exist as endo- and ecto-symbionts with ciliate protozoa (Finlay *et al*. 1994) and by changing the molar proportions of volatile fatty acids (VFA) to a greater proportion of propionate and less proportion of butyrate (Eugène *et al*. 2004). In contrast, studies of isolated lambs raised without protozoa from birth and defaunated ewes had been shown that absence of rumen protozoa did not reduce MP (Bird *et al*. 2008; Hegarty *et al*. 2008). Therefore, there is a lack of certainty whether CH_4 emissions are decreased by defaunation.

Dietary nitrate has shown potential to decrease CH_4 emissions from ruminants due to its consistent and persistent efficacy (Guo *et al*. 2009; Nolan *et al*. 2010; van Zijderveld *et al*. 2010, 2011; Lee and Beauchemin 2014). This is because hydrogen (H_2) is used by microbes to reduce carbon dioxide (CO_2) to CH_4 (Nolan 1999), but when nitrate is present in the rumen, ~2 mol of H_2 will be needed to convert nitrate to nitrite and 6 mol H_2 will be required in order to reduce nitrite to ammonia (NH_3; Allison and Reddy 1984). A review by Leng and Preston (2010) concluded that the use of nitrate as a H_2 sink could theoretically reduce MP by 16–50%, depending on diet and the inclusion rate of nitrate, but there is little data on productivity of nitrate-supplemented ruminants on protein-deficient roughage. Previous studies have largely focussed on a comparison of nitrate and urea as non-protein nitrogen (NPN) sources for ruminant diets and on reducing MP (Nolan *et al*. 2010; Li *et al*. 2012; de Raphélis-Soissan *et al*. 2014). Despite these potential benefits of nitrate

supplements, little is known about the effects of nitrate on microbial fermentation and growth in the rumen without protozoa, especially in animals offered unbalanced N and energy diets. This experiment aimed to quantify the effects of nitrate as a source of NPN and the interaction with defaunation on MP and productivity of lambs offered a protein-deficient chaff diet.

Materials and methods

Animals and feeding

All protocols for care and treatment of the sheep were approved by the University of New England Animal Ethics Committee (AEC 14–083). Merino ewe lambs (n = 20; 38 ± 1.9 kg; 13 months of age) were selected and acclimated to a diet of oaten chaff (OC). Lambs were allocated to a dietary N level by stratified randomisation based on liveweight (LW). The experiment was a 2 × 2 factorial design (calcium nitrate supplementation at 0% or 3.1%; protozoa status either defaunated or faunated). The diet of 3.1% calcium nitrate [$5Ca(NO_3)_2.NH_4NO_3.10H_2O$, Bolifor CNF, Yara, Oslo, Norway] was prepared by sprinkling a dilute solution of the nitrate onto OC while the chaff was tossed in a rotary feed mixer (+NO_3; Table 1). Another diet (Control) was only OC (–NO_3; Table 1).

The experiment lasted for 93 days. Lambs were gradually adapted to nitrate-supplemented OC from Day 0 to Day 15 from initial inclusion of calcium nitrate of 1% up to 3.1% with the dose of calcium nitrate increased every 2 days. After this period of nitrate adaptation, lambs were given *ad libitum* access to nitrate-supplemented OC with 3.1% added calcium nitrate from Day 16 to Day 40. Lambs were placed on restricted intake (80% individual *ad libitum* intake) 5 days before entering respiration chambers on Day 45 to Day 50 and continued receiving restricted intake from Day 50 to Day 59 for digesta kinetics and total collection and from Day 59 to Day 64 for repeated-measure of CH_4 emissions in respiration chambers. Lambs resumed *ad libitum* intake on Day 64 until the end of the experiment. Lambs were fed twice daily in two equal portions at 0930 hours and 1500 hours. Water was available at all times.

Table 1. Chemical composition of the oaten chaff and nitrate-supplemented chaff (% in DM)
Bolifor CNF: $5Ca(NO_3)_2.NH_4NO_3.10H_2O$ (63.12% nitrate in Bolifor CNF)

Component	Oaten chaff (–NO_3)	Oaten chaff (+NO_3) (3.1% Bolifor)
Dry matter (% as fed)	90.2	89.6
Dry matter digestibility	71	70
Digestible organic matter	67	66
Inorganic ash	6.4	7.3
Organic matter	93.6	92.7
Neutral detergent fibre	49	48
Acid detergent fibre	26	25
Crude protein	4.1	7.1
Metabolisable energy (MJ/kg)	10.6	10.4
Nitrate-nitrogen (mg/kg)	60.3	4300
Nitrate	0.03	1.9

Feed sampling and chemical analyses

Samples of OC (100 g) were collected before and after each mix of feed and stored in −20°C. All samples were pooled and subsamples were taken to analyse chemical composition (Table 1). Feed samples were analysed by the NSW DPI Feed Quality Service, Wagga Wagga Agriculture Institute. Feed DM, acid detergent fibre, neutral detergent fibre and inorganic ash were determined by wet chemistry. Feed DM digestibility and digestible organic matter were determined by near-infrared spectroscopy. Feed crude protein (CP) was determined by DUCMAS combustion method (AOAC 990.03). The calculation of metabolisable energy (ME) was based on AFIA method (2–2R). Nitrate-N was determined by FIA SPAC10.

Defaunation of lambs

Ten lambs were offered lucerne cereal mix supplemented with coconut oil distillate with initial inclusion of 3% to 5% of coconut oil distillate over 7 days to suppress rumen protozoa. After 7 days feeding coconut oil distillate, lambs were fasted for 24 h and orally dosed with sodium 1-(2-sulfonatooxyethoxy) dodecane (Empicol ESB/70AV, Allright and Wilson Australia Ltd, Melbourne, Vic., Australia) administered at 10 g/day in a 10% v/v solution for three consecutive days to remove protozoa. Feed was withheld during this period. Animals required 12 days to recover to their pretreatment voluntary intake and the 3-day dosing with Empicol was then repeated. Defaunated lambs were offered lucerne cereal mix during the second drenching period and a further 14 days after the second drenching program to recover from defaunation treatment. Fourteen days after second drenching, defaunated and faunated lambs were given *ad libitum* access to OC for 7 days before Day 0. During the defaunation period, the 10 faunated lambs were restricted fed at their maintenance requirement calculated according to the equations used in SheepExplorer (2003) to prevent divergence in LW while the defaunated group was being prepared.

Blood methaemoglobin (MetHb)

Blood was sampled between 2.5 and 3 h after morning feeding on Days 0, 15, 50 and 85. A sample of 8 mL was taken from a jugular vein, using lithium heparinised vacutainers (BD Franklin Lakes, NJ, USA). Whole blood MetHb concentration was determined within 30 min using a blood gas analyser (ABL 800 Flex, Radiometer, Brønshøj, Denmark).

Methane production

Methane production (g CH_4/day) of each lamb was measured in open-circuit respiration chambers over 2 × 22-h consecutive periods (Bird *et al*. 2008). Lambs were placed in individual respiration chambers by 1100 hours, with their feed and water available inside the chambers. The chambers were opened to collect feed refusals, clean faecal trays, and supply fresh feed and water at 0900 hours the following day and then were resealed at 1100 hours.

Subsamples of air within each chamber and of the ambient air were collected every 13 min into Tedlar gas sampling bags (Supelco, Bellefonte, PA, USA) continuously over the 22 h of confinement for analysis. Methane concentration was measured by a photoacoustic gas analyser (Innova Model 1312, AirTech

Instruments, Ballerup, Denmark). Recovery of CH_4 through the chambers was determined by injection of a known volume of CH_4 and measurement of CH_4 concentration every 2 min for 20 min, with recovery of the dose being calculated by integrating the area under the concentration curve over time.

Digestibility, digesta kinetics and microbial protein outflow

Lambs were placed in metabolism cages and a 6-day collection of faecal and urinary output was conducted. All faecal output over the 6 days was collected and weighed with feed DM intake (DMI) and faecal DM output used to determine DM digestibility (DMD). Concurrent with determining DMD, the mean retention time (MRT) of digesta was estimated in all lambs over 6 days by reference to faecal excretion of a dosed particle-phase marker (5 g per lamb of Cr-mordanted neutral detergent fibre from OC) and liquid-phase marker (5 g per lamb of cobalt-ethylenediamine tetraacetic acid) from AVA Chemicals (Mumbai, India) in 45 mL of Milli-Q water). These non-digestible kinetic markers were prepared in accordance with Udén *et al*. (1980) and administered via intubation directly into the rumen as a single dose at 1000 hours on Day 53. Faecal samples were collected every 2 h for the first 24 h, starting 8 h after marker administration, then every 4 h for the next 48 h, every 8 h for the next 24 h, and every 12 h for the next 48 h.

Dry matter content of feed and faeces were determined by drying samples at 80°C in a fan-forced oven to a constant weight. Samples were ground through a 1-mm sieve before analysis of chromium and cobalt concentrations by portable X-ray fluorescence spectroscopy (Bruker Tracer III-V pXRF, Bruker Corp., Billerica, MA, USA) using calibration curves (Barnett *et al*. 2013). Analysis of digesta kinetics was undertaken using non-linear curve fitting algorithms of WinSAAM (Aharoni *et al*. 1999).

The concentration of allantoin in the urine was determined using the colourimetric methods (IAEA 1997), using a UV-1201 spectrophotometer (Shimadzu, Japan) reading at 522 nm. The yield of total microbial N from the rumen was calculated using equations of Chen *et al*. (1992).

Rumen fluid sampling, NH_3, VFA concentrations, and protozoal enumeration

Samples of rumen fluid (20 mL) were collected from each lamb before feeding using oesophageal intubation for protozoal enumeration, VFA and NH_3 analyses on Day 0, 25, 39, 65 and 92. Rumen pH was measured immediately using a portable pH meter (Orion 230 Aplus, Thermo Scientific, Beverly, MA, USA). A 15-mL subsample was placed in wide-neck McCartney bottle acidified with 0.25 mL of 18 M sulfuric acid and stored at −20°C for VFA and NH_3 analyses. A 4-mL subsample was placed in wide-neck McCartney bottle containing 16 mL of formaldehyde-saline (4% formalin v/v) for protozoa enumeration. Protozoa were counted using a Fuchs–Rosenthal optic counting chamber (0.0625 mm^2 and 0.2 mm of depth) using a staining technique adapted from the procedure described by Dehority (1984). The protozoa were differentiated into large (>100 μm) and small (<100 μm) holotrichs and entodiniomorphs. The VFA concentrations were determined by gas chromatograph (Nolan *et al*. 2010) using a SMARTGAS Varian CP 3800 gas chromatograph instrument (Varian Inc., Palo Alto, CA, USA) and ammonia (NH_3-N) analysis was determined based on a modified Berthelot reaction using a continuous flow analyser (San++, Skalar, Breda, The Netherlands).

Liveweight and clean wool growth

Lambs were weighed in the morning before feeding on Days 0, 15, 21, 30, 65 and 93 to monitor LW and determine ADG over the experiment. Clean wool growth (CWG) rate, wool yield, and fibre diameter were determined on the mid-side of the sheep from Day 25 to Day 92 by clipping a patch ~10 × 10 cm (Oster Golden A5 clippers, blade size 30 model, Cryogen X, USA). After the wool from the patch was clipped, four sides and one diagonal were measured and the area of the patch was calculated using Heron's formula (De Barbieri *et al*. 2014). Wool samples were sent to New England Fibre Testing to determine yield and fibre diameter.

Statistical analyses

Data was statistically analysed using SAS 9.0 (SAS Institute, Cary, NC, USA). Data for rumen fermentation characteristics, digesta kinetics, wool growth, microbial protein outflow and MP were subject to ANOVA in PROC GLM; factors being protozoa, nitrate (NO_3) and protozoa × NO_3 interaction. For analysis of final LW and ADG, the model used the initial LW as a covariate. For parameters which had more than one measures, all measures were averaged. Homogeneity of variance and normal distribution were tested using PROC UNIVARIATE before statistical analysis. Data on MetHb and protozoa count were log-transformed before statistical analysis. For all analyses, means were analysed using the least-squares means (LSMEANS) procedure and a probability of <5% was considered to be statistically significant.

Results

Blood MetHb concentration

The averaged, blood MetHb concentration over the whole experiment was significantly increased by defaunation ($P = 0.03$) and by supplementation of nitrate ($P < 0.01$) and there was a significant interaction between defaunation and nitrate supplementation on MetHb ($P = 0.04$; Table 2). In defaunated lambs on nitrate, two lambs were observed having MetHb values of 18.1% and 18.3% on Day 50 and one lamb observed with MetHb value of 19.1% on Day 85. In faunated lambs on nitrate, the highest MetHb was one lamb found with 6.2% of MetHb on Day 50.

Performances and digestion

Productivity of lambs was significantly increased by nitrate supplementation, but was not affected by defaunation. Supplementation of OC with nitrate significantly increased DMI from 622 to 895 g/day and DMD from 57.8% to 64.5% ($P < 0.001$; Table 3). Nitrate supplementation significantly increased microbial protein outflow, ADG, CWG and wool fibre diameter ($P < 0.01$). The intakes of ME and CP were significantly increased by nitrate supplementation from 3.8 to 6.1 MJ/day and from 14.9 to 41.7 g/day, respectively. Defaunation did not change DMI, ADG, microbial protein outflow or CWG, but decreased DMD ($P = 0.05$). There were

Table 2. Rumen fermentation characteristics, concentration of methaemoglobin (MetHb) and protozoal population of defaunated and faunated lambs fed diets of oaten chaff with or without nitrate supplementation

–P (defaunated) and +P (faunated)

Parameter	Treatment				Pooled s.e.	*P*-values		
	–P		+P			P	NO_3	P × NO_3
	–NO_3 (n = 5)	+NO_3 (n = 5)	–NO_3 (n = 5)	+NO_3 (n = 5)				
Rumen pH	6.72	6.61	6.69	6.57	0.03	0.16	<0.001	0.98
Total volatile fatty acids (mM)	32.71	37.71	35.17	49.03	3.10	0.04	0.01	0.17
Acetate (mol %)	72.89	72.62	68.15	71.02	0.95	<0.01	0.18	0.11
Propionate (mol %)	17.15	15.42	20.63	17.79	1.18	0.02	0.06	0.63
Butyrate (mol %)	8.00	9.58	9.51	9.42	0.75	0.38	0.34	0.28
Acetate : propionate ratio	4.37	4.76	3.34	4.07	0.30	0.01	0.08	0.59
NH_3-N (mg/L)	6.04	21.51	11.21	30.70	2.72	0.02	<0.001	0.46
MetHb (%)	0.91	5.48	0.87	1.55	0.94	0.03	<0.01	0.04
Total protozoa ($\times 10^5$/mL)	0	0	4.78	6.51	0.61	–	0.20	–
Small entodiniomorph	0	0	4.08	5.54	0.53	–	0.19	–
Large entodiniomorph	0	0	0.18	0.13	0.08	–	0.51	–
Small holotrich	0	0	0.50	0.79	0.19	–	0.33	–
Large holotrich	0	0	0.02	0.05	0.02	–	0.42	–

Table 3. Intake, performances, methane (CH_4) emissions and digesta kinetics of defaunated and faunated lambs fed diets of oaten chaff with or without nitrate supplementation

–P (defaunated) and +P (faunated). (n = 4) during measures of CH_4 emissions and total collection. DMI, dry matter intake; ADG, average daily gain; CWG, clean wool growth; MRT, mean retention time

Parameter	Treatment				Pooled s.e.	*P*-values		
	–P		+P			P	NO_3	P × NO_3
	–NO_3 (n = 5)	+NO_3 (n = 5)	–NO_3 (n = 5)	+NO_3 (n = 5)				
DMI (g/day)	620.71	849.32	624.61	941.72	53.79	0.37	<0.001	0.41
ME intake (MJ/day)	3.61	5.75	4.08	6.48	0.45	0.20	<0.001	0.77
Crude protein intake (g/day)	13.97	39.23	15.77	44.21	2.75	0.24	<0.001	0.57
Final LW(kg)	35.42	40.31	32.62	39.84	1.30	0.14	<0.001	0.37
ADG (g/day)	–0.95	54.55	–32.81	49.26	14.77	0.21	<0.001	0.39
CWG (μg/cm^2.day)	468	662	482	704	53.5	0.61	<0.01	0.79
Wool fibre diameter (μm)	18.85	22.87	19.58	21.96	0.82	0.96	<0.01	0.35
Methane production (g CH_4/day)	3.49	7.65	7.13	12.29	0.85	<0.001	<0.001	0.57
Methane yield (g CH_4/kg DMI[A])	7.30	10.14	18.88	14.20	1.31	<0.001	0.50	0.01
DM digestibility (%)	54.88	63.77	60.81	65.25	1.72	0.05	<0.01	0.22
Microbial N outflow (g/day)	3.17	8.55	3.14	8.29	1.45	0.89	<0.01	0.96
Rumen particulate MRT (h)	40.20	32.83	29.10	21.68	1.74	<0.001	<0.01	0.98
Hindgut particulate MRT (h)	15.05	20.53	21.97	14.70	2.33	0.82	0.71	0.02
Total particulate MRT (h)	55.25	53.35	50.82	36.37	3.66	0.01	0.05	0.11
Rumen solute MRT (h)	25.37	19.20	20.75	14.10	1.90	0.03	0.01	0.90
Hindgut solute MRT (h)	12.25	12.65	15.20	10.28	1.52	0.85	0.16	0.11
Total solute MRT (h)	37.63	31.85	35.95	24.38	3.22	0.18	0.02	0.38

[A]DMI was calculated during restricted intake period.

no significant interactions between defaunation and nitrate supplementation for productivity parameters.

Digesta kinetics as characterised by MRT of both rumen solute and particulate fractions were significantly affected by defaunation and nitrate supplementation. Defaunation significantly increased rumen MRT of solute and particulate fractions ($P < 0.05$) whereas nitrate supplementation significantly decreased rumen MRT of these fractions ($P < 0.05$). There was a negative correlation between MRT and DMI across all data (particulate MRT = 70.8 – 0.028 DMI, $r^2 = –0.52$, $P = 0.038$ and solute MRT = 52.2 – 0.028 DMI, $r^2 = –0.67$, $P = 0.004$), such that greater DMI was associated with shorter MRT in the rumen. Despite the slowing effect of defaunation and accelerating effect of nitrate on rumen MRT, there were no significant effects of defaunation or nitrate on MRT of solute and particulate fractions in the hindgut. There were no interactions between defaunation and nitrate in other gut segment, except hindgut particulate MRT ($P = 0.02$).

Rumen fermentation and methane emissions

Defaunated lambs remained protozoa-free throughout the experiment. In faunated lambs, nitrate supplementation did not affect protozoal population ($P > 0.05$; Table 2). Small entodiniomorphs in both nitrate and non-nitrate supplemented lambs accounted for 85% of total protozoa.

Nitrate supplementation significantly increased concentration of NH_3-N and total VFA ($P < 0.05$; Table 2). There was a tendency towards a lower molar percentage of propionate ($P = 0.06$) and higher molar ratio of acetate to propionate ($P = 0.08$) in nitrate-supplemented lambs. Defaunation, contrastingly, decreased total VFA, NH_3-N concentration and molar percentage of propionate. Defaunation increased the molar percentage of acetate and molar ratio of acetate to propionate ($P < 0.05$). Rumen pH was not affected by defaunation, but was decreased by nitrate supplementation (Table 2). No interactions between defaunation and nitrate were found for any rumen fermentation parameter.

Methane production was significantly decreased by defaunation, but was increased by nitrate supplementation ($P < 0.001$; Table 3) with no interaction between defaunation and nitrate for MP. There was a positive correlation between DMI and MP (MP = 0.014 DMI – 3.38; $r^2 = 0.75$, $P = 0.001$), such that MP was significantly increased by higher DMI. Defaunation significantly decreased methane yield (MY, g CH_4/kg DMI) but nitrate did not change MY. However, there was a significant interaction between defaunation and nitrate ($P = 0.01$) in MY such that in defaunated lambs, nitrate did not change MY, but in faunated lambs, nitrate decreased MY by 25%. Nitrate-supplemented defaunated lambs had lower MY than nitrate-supplemented faunated lambs ($P < 0.001$; 10.14 versus 14.20 g/kg DMI).

Discussion

Effects of nitrate on blood MetHb concentration and protozoa population

Nitrate toxicity remains a major constraint to commercial nitrate feeding because excessive nitrate in the rumen may accumulate nitrite concentrations in the rumen and then blood. Nitrite in blood reduces the ferric ion of haemoglobin and transforms the molecule to MetHb (Lundberg *et al*. 2008), which is unable to transport oxygen to tissues. Methaemoglobinaemia is diagnosed if more than 30% of haemoglobin is present on MetHb (Bruning-Fann and Kaneene 1993). In this study, feeding faunated lambs with 3.1% calcium nitrate (1.9% nitrate) maintained low MetHb concentration in agreement with previous studies when nitrate was supplemented at levels up to 2.6% by gradually introducing nitrate to allow adaptation of rumen microbes (Nolan *et al*. 2010; van Zijderveld *et al*. 2010; Li *et al*. 2012). This is because rumen microbes are capable of reducing nitrate or nitrite to NH_3 as nitrate is introduced as reviewed by Leng and Preston (2010).

Defaunated lambs in the present study showed increased MetHb concentration after 85 days of feeding nitrate, suggesting that protozoa may have an important role in the reduction of nitrate or nitrite in the rumen and consequently formation of MetHb in the blood of sheep. Lin *et al*. (2011) incubated different microbial fractions of whole rumen fluid, protozoa, bacteria, and fungi to assess their ability to reduce nitrate. The authors found that nitrate disappearance rate was similar in whole rumen fluid and protozoal fractions. Nakamura and Yoshida (1991) also reported that nitrate and nitrite disappearance rates in the rumen of faunated sheep were faster than in defaunated sheep and lower MetHb was observed in faunated sheep, potentially indicating active involvement of protozoa in the reduction of nitrate and nitrite. However, there were no clinical signs of nitrate toxicity in either defaunated and faunated lambs when 3.1% calcium nitrate was supplemented in these *ad libitum*-fed lambs.

The protozoal population was not affected by nitrate supplementation in this study; this agreed with previous studies (Nolan *et al*. 2010; van Zijderveld *et al*. 2010; Li *et al*. 2012).

Effects of nitrate supplementation and defaunation on performances and digestion

Oaten chaff used in this study as a basal diet for lambs was characterised by a low protein content (41 g per kg DM) providing only 4 g of CP per MJ of ME. Consequently, this diet was inadequate to support maintenance of growing lambs (CSIRO 2007), resulting in losing weight in lambs without nitrate supplementation. Similarly, the average value of 7 g of CP per MJ of ME in the nitrate-supplemented diet was below the microbial protein yield required for growing sheep (CSIRO 2007). The averaged concentrations of NH_3-N in the rumen increased from 8.6 to 26 mg/L rumen fluid with nitrate and were associated with an increased microbial growth efficiency (3.2–8.4 g/day). However, it was suggested by Satter and Slyter (1974) that 20–50 mg NH_3-N/L rumen fluid is required to maintain growth of rumen bacteria with forage diets, so the amount of NH_3-N even in nitrate-supplemented lambs in this study was suboptimal, although it still increased microbial growth and activity as indicated by greater total VFA compared with unsupplemented lambs. Lambs supplemented with nitrate had an ADG of 52 g/day and grew 683 μg/cm^2.day in CWG, suggesting high efficiency of nutrient utilisation by lambs on nitrate supplementation. Lambs without nitrate lost 25 g LW/day and CWG grew 475 μg/cm^2.day, suggesting that wool growth was utilising amino acids as a priority over body growth.

Defaunation resulted in a significant reduction of rumen NH_3-N concentration as less digestion of engulfed feed-protein and bacteria occurs in the absence of protozoa; this agreed with previous assessments (Jouany *et al*. 1988; Eugène *et al*. 2004; Santra *et al*. 2007; Morgavi *et al*. 2012). Defaunated lambs in this study had 13.8 mg NH_3-N/L rumen fluid, which was below the requirement for the maximum growth of microbes (Satter and Slyter 1974) and thus inadequate NH_3-N availability inhibited ruminal fermentation (Leng 1990). This resulted in lower total VFA, DMD and longer MRT, but no changes in microbial protein outflow, ADG or CWG by defaunated lambs. This contrasts with previous results where defaunation increased rumen bacterial outflow and increased the availability of protein at the duodenum (Bird and Leng 1978; Jouany 1996).

The 30% increase in DMI by nitrate-supplemented lambs was probably due to increased NH_3-N and fermentation. The negative correlation between DMI and particulate and soluble MRT (R^2 = –0.52 and –0.67; $P < 0.05$) may reflect that. The shorter MRT in nitrate-fed lambs allowed these animals to consume more feed

due to a reduced rumen fill constraint, faster passage and greater fermentation. In addition, higher DMD through the whole tract would have increased CP and ME intake as Leng (1990) suggested the availability of nutrients from low-quality forages can be improved by ruminants if microbes in the rumen grow efficiently. This is in keeping with the finding that lambs supplemented with nitrate in this study had higher ADG and CWG than unsupplemented lambs. In contrast, nitrate did not increase DMI, DMD and ADG in previous studies where protein was above ruminal requirement (van Zijderveld *et al*. 2010; Li *et al*. 2012; de Raphélis-Soissan *et al*. 2014). However, because these studies aimed to replace urea to nitrate in N-adequate diets, the authors were unlikely to observe positive effects of nitrate on fermentation and productivity of animals as reported here in the protein-deficient diet.

Effects of nitrate supplementation and defaunation on methane emissions and rumen fermentation

The higher MP in nitrate-supplemented lambs contrasts with results from protein adequate diets and was a consequence of higher DMI and increased ruminal fermentation as evidenced by higher total VFA concentration and DMD, leading to greater ruminal H_2 availability. In faunated lambs, the 24.8% reduction in MY by nitrate supplementation agrees with previous studies, which have shown CH_4 reduction range between 23% and 35% when 1.9% to 2.6% nitrate were supplemented (Nolan *et al*. 2010; van Zijderveld *et al*. 2010; Li *et al*. 2012). A review by Leng and Preston (2010) showed that CH_4 can be reduced by 16% to 50% depending on diets and the inclusion rate of nitrate. As the same amount of H_2 is used to reduce 1 mol of nitrate to NH_3 as 1 mol of CO_2 to CH_4 (Nolan *et al*. 2010), the faunated lambs in this study were given 3.1% calcium nitrate (14.3 g nitrate per day during the restricted intake period), which theoretically reduces 0.23 mol or 4.92 g CH_4/kg DMI. In this study, a reduction of 4.68 g CH_4/kg DMI was measured, which is 95% of the expected reduction, showing that most of the calcium nitrate was reduced to NH_3-N. The 63.5% greater in NH_3-N by nitrate-supplemented lambs could be contributed by an efficacy of nitrate reduction. Nitrate caused changes in rumen fermentation shifting to increased acetate and decreased propionate as high affinity H_2 of nitrate is more favourable in nitrate reduction than in formation of propionate or CH_4 (Ungerfeld and Kohn 2006). The present study showed a tendency of lower propionate and higher molar ratio of acetate to propionate, which was consistent with previous observation by Nolan *et al*. (2010). The reduced MP by nitrate supplementation may also be a consequence of inhibiting methanogens (van Zijderveld *et al*. 2010) as the reduction of nitrate to nitrite and then to NH_3-N resulted in a metabolic H_2 sink, which decreased H_2 availability for methanogens (van Zijderveld *et al*. 2011).

The reduced MP after defaunation is consistent with Eugène *et al*. (2004) and can be explained by fermentation shifting to a greater proportion of propionate and decreasing the proportion of butyrate. However, results from this study showed MP was reduced with decreased total VFA and propionate proportion, but an increased acetate proportion. A higher proportion of acetate and lower proportion of propionate in defaunated animals fed low-quality diets was also reported by Bird (1982). The lower MP indefaunated lambs could be due to restricted growth of microbes in the rumen, evidenced by the lower fermentation, NH_3-N concentration and DMD of defaunated lambs. Alternatively, by removing the endo-symbiotic and ecto-symbiotic methanogens associated with protozoa, H_2 may have accumulated, stimulating reductive acetogenesis (Ungerfeld 2013). Fonty *et al*. (2007) also reported that reductive acetogens established in the rumen lacking methanogens and can replace methanogens as a sink for H_2 in the rumen, thus reductive acetogens can be potentially important to reduce enteric CH_4 emissions (Joblin 1999). Because reductive acetogenesis involves the reduction of CO_2 by H_2 to acetate (Ungerfeld 2013), this might explain the low CH_4 emissions, but high acetate concentration in defaunated lambs. However, the reduction of MP was a direct consequence of the loss of rumen protozoa associated with methanogens was unknown in this study.

Interaction of defaunation and nitrate supplementation

In the present study, significant interactions of protozoa and nitrate were occurred on MetHb and MY. Concerning the role of protozoa on nitrate/nitrite reduction and MetHb, the present study confirmed the *in vitro* study by Lin *et al*. (2011) and showed that blood MetHb became greater in defaunated lambs after 85 days of feeding nitrate, which was in agreement with a study by Nakamura and Yoshida (1991) who reported that nitrate disappearance rates and blood MetHb were rapidly decreased in faunated animals compared with defaunated animals. Feeding nitrate to defaunated and faunated lambs in this study was hypothesised that dietary nitrate in combination with defaunation treatment additively decreased methanogens and MP. Methanogens use H_2 availability as a substrate for their metabolism and the use of H_2 by bacteria to reduce nitrate to nitrite and then NH_3 caused lower numbers of methanogens (van Zijderveld *et al*. 2010, 2011). The combined treatment of nitrate and defaunation could be expected to greater effects on reducing methanogens, although methanogens were not measured in this study. Significant interaction between protozoa and nitrate supplementation on MY occurred suggesting that nitrate supplemented to defaunated lambs would be positively additive in lowering MY compared with faunated lambs with or without nitrate supplementation.

Conclusion

Nitrate was an effective NPN source for rumen microbes, especially in the protein-deficient diet. From the point view of greenhouse gas mitigation strategy, nitrate was an effective strategy to reduced enteric CH_4 emissions, provided it was supplemented with appropriate levels. Defaunation reduced fermentation and digestion with no changes in microbial protein outflow or ADG. However, on the protein-deficient basal diet, defaunation reduced CH_4 emissions. Moreover, fermentation and digestion of defaunated lambs were increased by nitrate supplementation and the combined treatments of defaunation and nitrate were additive in reducing CH_4 emissions. This needs further investigation as combined two CH_4 mitigation strategies may be an effective approach.

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