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Novel Strategies for Enteric Methane Abatement

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Novel strategies for enteric methane abatement

ABSTRACT

The effectiveness of dietary nitrate inclusion and of elimination on rumen protozoa to achieve reduced enteric methane emissions from ruminants was assessed. Dietary nitrate is a highly effective abatement technology, reducing methane emissions in proportion to the dose added. It can safely be delivered in processed feeds and in lick-blocks at levels which deliver quantifiable emissions reduction. Ruminal nitrate reduction is extremely rapid and emission levels return to normal within 3h of nitrate feeding. Providing nitrate in pelleted feeds and in lick blocks both reduced methane production, warranting its development as a CFI methodology. Methane yield of cattle without rumen protozoa did not differ from that of untreated cattle, but protozoa-free cattle were 30kg heavier than untreated counterparts by completion of the study. Bioactive compounds from a range of chemical families were assessed for antiprotozoal action in the laboratory. The lead compound showed no efficacy in sheep.

EXECUTIVE SUMMARY

- ✓ Inclusion of nitrate in the diet of sheep reduces enteric methane production in a near-quantitative manner as shown by repeated open circuit calorimeter studies in this project and a growing international literature.
- ✓ While nitrate accumulation in the rumen can occur, 85-95% of nitrate consumed is rapidly converted to ammonia.
- ✓ Methane mitigation (as a result of hydrogen utilization in reducing nitrate to ammonia) is extremely fast, with methane returning to normal levels within 3 hours of animals consuming nitrate.
- ✓ Nitrate supplementation has actions beyond methane mitigation in the rumen, causing short term reductions in rumen fermentation, an increase in the acetate:propionate ratio and an increase in rumen ammonia concentration.
- ✓ No effects of nitrate on growth rate of sheep were apparent in pen or field studies but wool growth was significantly greater in nitrate supplemented than urea supplemented sheep.
- ✓ Supplementary nitrate could be expected to improve animal productivity of grazing animals on low quality forage (eg. Standing dry-season grass pastures in tropical and subtropical Australia) by increasing rumen ammonia concentration, increasing rate of fermentation and so increasing pasture intake.

- ✓ Dietary nitrate provided in complete (pelleted) rations reduced enteric emissions and this technology could be readily and safely adopted. A commercial feed manufacturer who participated in this study is very interested in pursuing this product.
- ✓ Dietary nitrate provided to sheep by commercial lick-blocks reduced methane production at the levels voluntarily consumed, being a measured 22% reduction in sheep offered the block in penned studies (at 55g/d block intake) and an estimated 8% in a flock of grazing sheep in a paddock (at 20g/d block intake).
- ✓ Calcium nitrate, while the cheapest and most freely available form of dietary nitrate, is not highly palatable, with increasing nitrate inclusion levels (from 0.8 to 4.2% nitrate-N by weight) leading to reduced block intake. In contrast intake of urea-containing blocks increases over this N inclusion range. This means that ingestion of potentially toxic levels of this form of nitrate through lick-blocks is less of a risk than is ingestion of too much urea, because the sheep self-regulates nitrate intake. Our results suggest intake will self-limit at approximately 1.6g N/d, that could be expected to reduce emission by 1.8g methane/d/sheep. A commercial collaborator who manufactured blocks for this study is particularly interested in pursuing developing nitrate containing lick-blocks for Australia.
- ✓ A screening program of chemical compounds considered likely to interfere with key metabolic processes in rumen protozoa was developed and operated over 2 years.
- ✓ Of all compounds tested, ronidazole showed greatest *in-vitro* efficacy against rumen ciliate protozoa.
- ✓ In vivo tests of ronidazole however showed no sustained impact on protozoal populations or on methane emission from sheep.
- ✓ Cattle from which protozoa had been eliminated did not differ in methane yield (g methane/kg DM intake) from cattle with a normal (entodiniomorph dominant) rumen protozoal population.
- ✓ Cattle from which protozoa had been eliminated were significantly heavier at completion of this project than were cattle with a normal protozoal population, indicating scope for productivity gains in cattle akin to those seen in lambs and sheep. These are the only protozoa-free cattle population in the world.

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Nitrate as a Novel Mitigation Technology

Introduction and Objectives

The NO_3 molecule, like the SO_4 and CO_2 molecules are all capable of being fully hydrogenated (reduced) by microbial enzymes in anaerobic ecosystems such as the rumen. Since nitrate and sulphate reduction have a greater energy yield than does reduction of carbon dioxide to methane, they occur preferentially and can competitively reduce methane production through reduced hydrogen availability. This means nitrate supplementation can be expected to reduce rumen methanogenesis and increase rumen ammonia concentration. It should not be expected to increase animal production directly as it does not redirect hydrogen into an end-product used by the animals (eg acetate or propionate), but only into ammonia. The nitrate related objectives of this study were:

> By 1 December, 2011 the potential for effective and safe nitrate supplementation strategies developed that will reduce rumen methanogenesis, delivering:
> . The chemistry of rumen and animal nitrate metabolism clearly defined
> . Dose response of methane inhibition for dietary nitrate available
> . Delivery systems for nitrate demonstrated in grazing ruminants.
> . Recommendations on acclimation, safe feeding levels and methane inhibition associated with dietary nitrate

The investigations addressing these objectives are described below, in the format of draft or submitted manuscripts in most cases:

The kinetics of nitrate reduction to nitrite and ammonia and incorporation into bacteria in the rumen of sheep given roughage diets containing nitrate and/or urea

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Note: The values in this experimental report are subject to pending results of analysis confirming the nitrogen concentration of the basal feed.

Abstract. A 3-pool model was used to describe the kinetics of ^{15}N-labelled nitrate (NO_3^-) and ^{15}N-labelled urea in the rumen of sheep given chaffed oaten hay supplemented with 2.5% or 1.2% or 0% NO_3^- or iso-nitrogenous amounts of urea. The model enabled tracer data to be closely fitted and was well-determined statistically. For all sheep on all diets, NO_3-N was rapidly reduced via NO_2^- to NH_3.in the rumen; 85-95% of the NO_3-N ingested was recovered in the rumen fluid NH_3 pool and only 5-15% left the rumen by outflow in fluid or by direct absorption across the rumen wall. There was no evidence of direct utilisation of NO_3-N for polymer formation by rumen bacteria. Other components of the model were generally in accord with known biology.

There was no evidence of NO_3-/NO_2- toxicity in sheep offered the highest NO_3^- containing diet (2.45% NO_3^-) and results of this study raise questions concerning the effectiveness of the strategy of adaption of animals to NO_3-N containing diets to reduce the likelihood of NO_3-/NO_2^- toxicity.

Introduction

In Australia, about 16% of total greenhouse gas (GHG) emissions arise from agricultural activities, but enteric methane (CH_4) from ruminants (11% of total) is the most significant source of agricultural GHG emissions (NGGI 2009). The amount of CH_4 emitted by ruminants per unit production is higher in animals subjected to poor nutrition. When animals consume diets consisting of predominantly dry mature roughage - as occurs annually in northern Australia, non-protein nitrogen (NPN) supplements such as urea and nitrate (NO_3^-) have been shown to improve production (Clark and Quin 1951). Nitrate salts could potentially replace urea as a NPN source with the added benefit that NO_3^- will reduce CH_4 emissions (Leng 2008 van Zijderveld et al., 2010JDS). Microbial fermentative digestion of organic matter by rumen microorganisms depends on a continuing supply of NAD^+ which is converted to NADH and H_2 in energy requiring reactions. NAD^+ has to be regenerated by the removal of electrons (H_2) to enable fermentation to continue. H_2 and its electrons are used by Archaea to reduce CO_2 to CH_4, allowing NAD^+ to be regenerated. Other inorganic compounds such as NO_3^- have a greater affinity for H_2 than does CO_2 (Ungerfeld and Kohn 2006). NO_3^- salts and are potent inhibitors of methanogenesis in many anaerobic systems, including the rumen (Allison and Reddy 1984) and, when nitrate is

present in the rumen, NO_2^- and NH_3 formation are favoured over CH_4 production. After comprehensively reviewing the literature, Leng (2008) concluded that the inclusion of $NO3^-$ in feed supplements appeared to be entirely feasible as a means of reducing enteric methane emissions from ruminant livestock. However, there is a risk of nitrite toxicity. Nitrate supplementation also has potential advantages in that it should theoretically be more energetically efficient than methanogenesis and so microbial growth should be enhanced. This hypothesis has been confirmed in vitro by Guo *et al.* (2009).

Nitrate supplements could potentially complement replace urea supplementation which is widely practiced in the grazing industries. Despite these possibilities, little is known about the kinetics of nitrate metabolism by microorganisms in the rumen. The aim of this study was to examine the kinetics of nitrate and ammonia in the rumen using ^{15}N-labelled NO_3^- and ^{15}N-labelled ammonium salts. Part of this investigation showing that the nitrate in these diets significantly reduced methane emissions has been published (Nolan *et al.* 2010).

Materials and Methods

Animals and Diets

Twelve Merino-cross ewes (2 year old; 38 ± SD 2 kg) with long-established rumen cannulas were housed indoors in individual metabolism cages in two adjacent rooms (6 sheep per room). The cages were placed in pairs about 1 m apart in each room. A single overhead feeder with two moving conveyor belts was mounted on each pair of cages to deliver equal portions of each sheep's daily ration each hour. Two batches of feed based on chaffed oaten hay (8 MJ ME, 75 g CP/kg DM) were prepared. The first was made by adding a solution containing urea and Na_2SO_4) to 240 kg chaffed oaten hay while it was tumbling in a feed mixer (Diet 1). After thorough mixing, the feed was removed from the feeder and dried on a concrete floor in a warm ventilated room. A second batch of feed with 4% KNO_3 was made by adding 25 L of KNO_3 solution (384 g/L) to 240 kg of the same oaten chaff in the same mixer and drying it in the same way. Diets 2 and 3, containing 2% (1.23% NO_3^-) and 4% KNO_3 (2.45% NO_3^-), respectively, were then prepared by mixing the two batches of oaten hay in appropriate proportions. The sheep were offered 1 kg air-dry feed each day from the overhead feeders.

Sheep were acclimated to the urea diet (Diet 1) and the two diets containing KNO_3 by mixing increasing amounts of diet 3 (4% NO3-) with diet 1 (urea) over a period of 7 d, so that by Day 7, sheep scheduled for Diet 2 were consuming 2% KNO_3 and those on Diet 3 were consuming 4% KNO_3.

Intraruminal injections of $K^{15}NO3$ and $^{15}NH_3$

On 7 July 2009, intraruminal injections of $^{15}NH_3$ (approx. 2.0 mmol in 120 mL) were given to Sheep 1, 2, 5 and 6 in Room A, and intraruminal injections of $K^{15}NO_3$ (approx. 2.5 mmol in 120 mL) were given to Sheep 9, 10, 11 and 12 in Room B (see Table 1).

Table 1. Diary of events.

Diet	Mon	Tues	Wed	Thurs	Friday
Control Room A Sheep 1 and 2		*NH3		*NO3	
Control Room B Sheep 3 and 4		*NO3		*NH3	
2% NO3 Room A Sheep 5 and 6			*NH3		*NO3
2% NO3 Room B Sheep 7 and 8			*NO3		*NH3
4% NO3 Room A Sheep 9 and 10		*NH3		NO3	
4% NO3 Room B Sheep 11 and 12		*NO3	Chamber **9, 10**	NH3	

- Intra-ruminal doses of $^{15}NH_3$ or $^{15}NO_3^-$
- Shaded boxes represent days when sheep were in respiration chambers

The ^{15}N-labelled salts were dissolved in deionised water and delivered using a 140 mL syringe to which was attached a stiff plastic tube (2 mm i.d. and 20 cm length). The solution was injected continuously over about 0.6 min while the tube was moved through the rumen contents to disperse the ^{15}N-labelled solutions. Samples of ruminal fluid were taken from each sheep, via samplers consisting of a wire cage wrapped in nylon gauze and placed in the dorsal sac, before the dose was administered, and afterwards at approximately 20, 40, 60, 90, 120, 180, 330, 425, 760 and 1270 min post-injection. The rumen samples (20 mL) were placed in labeled 50 mL centrifuge tubes in crushed ice and delivered to the laboratory within 20 min. In the laboratory, they were immediately placed in a high speed centrifuge (Beckman Model J2-21M) and centrifuged at 15,000 x g for 15 min. The supernatant solution was then removed to storage containers containing 0.15 mL 18 M H_2SO_4 before being stored in a freezer at -18°C to await further analysis. The upper bacterial layers from the residues in the centrifuge tubes were transferred to new tubes and mixed with 40 mL water to wash the bacteria. The tubes were then centrifuged at 15,000 x g for 15 min as before, and the new supernatant solution was discarded. The top bacterial layers in the residues were then transferred to a 10 mL Bijou bottle, frozen and freeze-dried. The freeze-dried bacteria were stored at room temperature to await total N and ^{15}N analysis.

Samples of rumen contents were also taken with a core sampler consisting of a Perspex tube (20 mm i.d.) that was inserted quickly into the rumen contents so that a core of mixed rumen

contents was forced up the tube. These contents were trapped by pulling a wire passing through the tube with a rubber stopper on the far end and removed and placed in a 25 mL plastic container in liquid N_2 so that the contents were quickly frozen (within 1.5 min) before storage at -18°C.

The above procedures were repeated on 8, 9 and 10 July 2009, so that all 12 sheep received intraruminal injections of both ^{15}N-KNO_3 and $^{15}NH_3$ about 2 d apart.

In addition to the procedures already described, on 9 July 2009, 5 samples of rumen fluid were collected at intervals over the day to enable pH to be determined (portable pH meter). Cr-EDTA was included with the intraruminal doses of ^{15}N-labelled salts to enable rumen volumes of the sheep to be determined (Downes and McDonald 1964).

Analysis of samples for enrichment with ^{15}N

Total N concentration in ammonia-N and bacterial samples was determined on an automatic N/carbon/sulphur analyser (Carlo Erber Instruments; Model NA1500) and the nitrogen oxides produced were converted to N_2 that was passed into an isotope ratio mass spectrometer to determine ^{15}N abundance.

Model solutions

Enrichment *v.* time data for ammonia-N and bacterial N after intraruminal injections of $K^{15}NO3$ on one day and $^{15}NH_4Cl$ two days later (or in the reverse order – see Table 1) were adjusted to the same mass of ^{15}N injected (2 mmol) by linear scaling and then fitted to a 3-compartment model (Figure 1) using the WinSAAM software (Boston and Berman 1982; Stephanowski *et al.* 2003). The 4 sets of enrichment *v.* time data for the sampled NH_3-N and bacterial N pools derived from each of the 12 sheep were fitted to the model shown in Figure 1 to solve for fluxes and pool sizes and estimate the standard error of the fit (FSD).

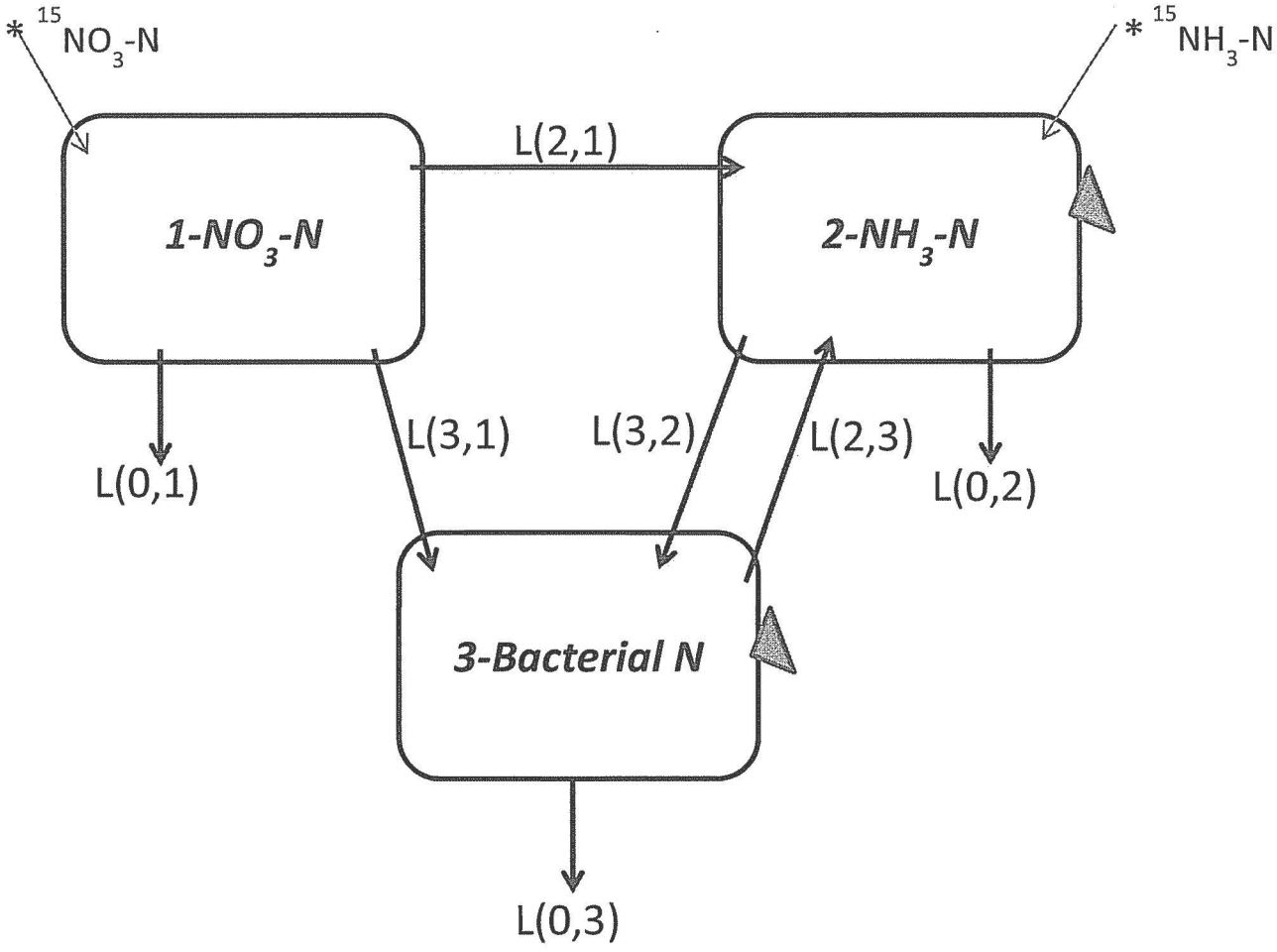

Fig. 1. Three-pool model of N kinetics in the rumen with fractional outflow rates from pool *i* to pool *j* described by L(*i,j*) as used in the WinSAAM software which was used to fit enrichment v. time results after administration of both $^{15}NH_4Cl$ and $K^{15}NO_3$ in experiments conducted 2 d apart. * Injection sites; ◣ Sampling sites.

The rate constants (L(i,j) are represented in WinSAAM by a system of differential equations. WinSAAM iteratively adjusts initial parameters (Bayesian information) entered by the user to minimise the objective function and optimise the fit to the experimental tracer data. The primary information to enable rate constants in the model (Fig. 1) to be determined was the 2 sets of enrichment v. time data (rumen fluid NH_3-N and bacterial N) for each of the 2 tracer administrations (intra-ruminal dosing with $^{15}NH_3$ or $^{15}NO_3^-$). This meant there were 4 sets of curves used simultaneously to give model solutions for each sheep. As well as the 4 sets of enrichment v. time results arising from the two injections of tracer, additional (Bayesian) information was used to help define the steady state pool sizes and tracee N flows.

Bayesian information

Intakes of NO_3^--N and urea-N by the sheep were calculated from DM intake of the relevant diet and the concentrations of KNO_3 or urea in that diet.

The entry of N into the 3 pools from outside the model system, U(1), U(2) and U(3), i.e. from feed and endogenous sources, was represented in WinSAAM by a G function, viz. G(20)=U(1)+U(2)+U(3); G(20) was set initially to be 13.7 (FSD=1) g/d of ruminally available N for a sheep eating all of its daily ration of 14.9 gN/d. Dietary NO_3^-, dietary urea and endogenous urea (the last assumed to be 2.5 gN/d) were considered to be totally available in the rumen, whereas ingested feed N (9.4 g/d) and endogenous Non-urea N (1.0 g/d) were assumed to be 60% available. U(1) represented the entry of NO_3-N from the feed for the 4% and 2% KNO_3 and control diet (5.54, 2.77 and 0 g /d, respectively). Another G function, G(21) = U(2)+U(3) was set initially to include the dietary urea (2 diets) and endogenous urea (2.5 gN/d) plus ruminally available feed N and endogenous N (5.54 gN/d).

Pool sizes in WinSAAM were represented by M(*i*) where i represents the i^{th} pool. The NO_3-N pool size for all sheep, M(1), was initially set to be 0.033 gN (±FSD=1), based on the mean water volume determined using Cr-EDTA and the mean NO_3-N concentration in rumen fluid in samples taken just before tracer injections. The initial NH_3-N pool size, M(2), was set in the same way. The pool size of the bacteria, (M(3), was set initially at 7 gN (FSD=2) based on values reported for sheep given low quality hay diets by Oosting *et al.* (1993). R(0,1) was given an initial value of 0.8 gN/d (FSD=1) based on the NO_3-N concentration in rumen fluid samples also collected just before tracers were administered and the rumen fluid outflow rate determined using CrEDTA. The irreversible loss of N from the bacterial pool, R(0,3) in WinSAAM, was also set initially to a value predicted according to AFRC (1993), i.e. 1.57 g microbial N/MJ fermentable ME intake or 10 gN/d) but also was not highly constrained (±FSD=2). The FSDs ascribed to the U and M values, and to R(0,3) were not highly constrained so that the values could change relatively freely during optimisation.

Statistical analysis

Models were fitted individually using the same initial conditions and parameter weightings for sheep within diets. The optimised pool sizes and flow rates were then presented as means (SE) for all sheep in each dietary treatment. The results for two sheep that did not ingest at least 95% of their ration over each of the 2 d periods after tracer administration were excluded from these analyses.

RESULTS

Visual appraisal of the curves generated by solving and optimising the pool sizes and flow rates in the 3-pool model (Fig 1) indicate the enrichment *v.* time data were well fitted by the model. The parameters were also well determined statistically. The enrichments of NH_3-N and bacterial N after administration of ^{15}N as either $^{15}NH_3$ or $^{15}NO_3^-$ were remarkably similar indicating that the rate of conversion of NO_3^- to NH_3 occurred quickly on all diets, *viz.* as was evident from a comparison of the enrichment *v.* time curves in rumen fluid NH_3-N and bacterial N after intraruminal injection of either tracer (Figs 2, 3 and 4). Moreover, in all sheep, simulated turnover of the NO_3-pool was extremely rapid (c.15/d) and the enrichment of NH_3-N after $^{15}NO_3^-$ injection - which must have been zero at the moment of tracer injection - had risen to its maximum value by the time the first or second sample of rumen NH_3-N was taken (Figs 2b, 3b and 4b).

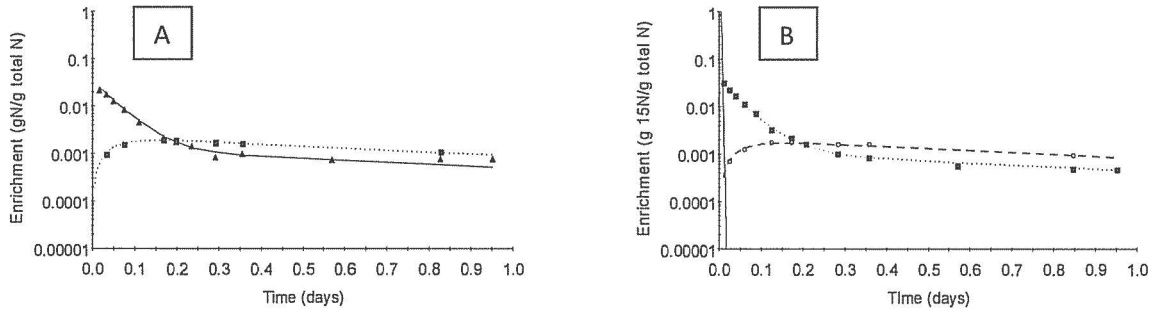

Fig. 2. Enrichment of NO_3-N (solid line, simulated by the model) and NH3-N (■) and bacterial N (o) in after intraruminal injection (a) of $^{15}NH_3$-N into the rumen and (b) of NH_3-N (■) and bacterial N (o) after injection of the same amount of ^{15}N in the form of $^{15}NO_3$-N into sheep 5 ingesting the diet containing 4% KNO_3.

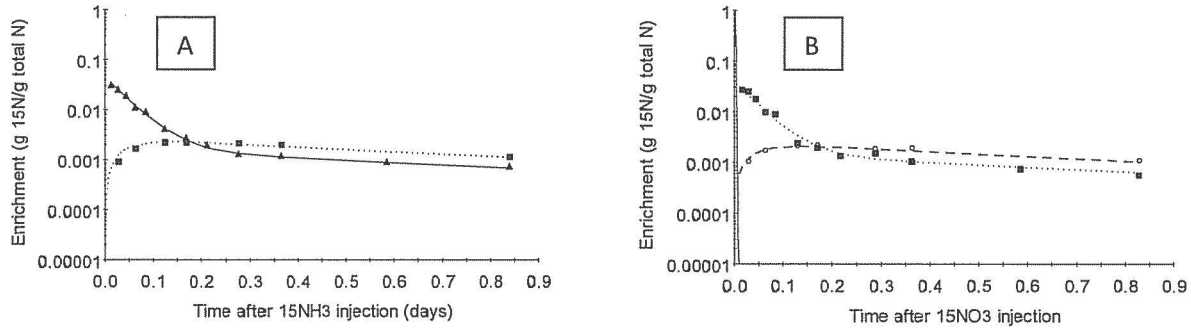

Fig. 3. Enrichment of NO_3-N (solid line, simulated by the model) and NH3-N (■) and bacterial N (o)in Sheep 6 after intraruminal injection of (a) $^{15}NH3$-N into the rumen and (b) of NH_3-N (■) and bacterial N (o) after injection of the same amount of ^{15}N in the form of $^{15}NO_3$-N into sheep 6 ingesting the 2% KNO_3 diet (iso-nitrogenous with control diet).

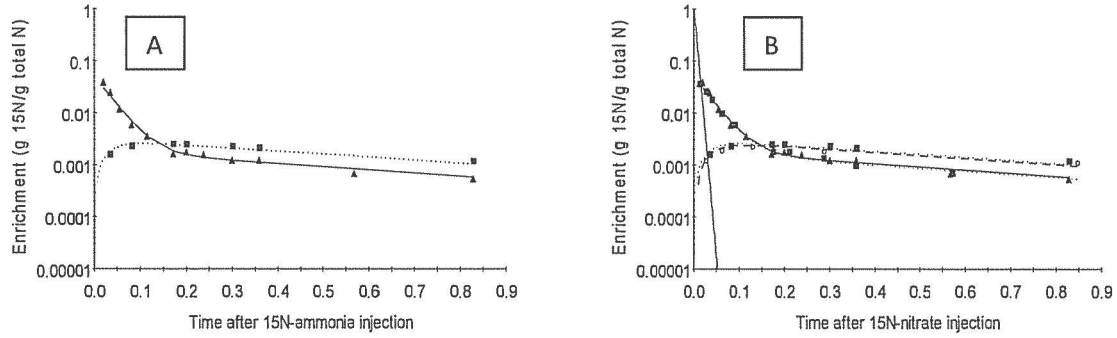

Fig. 4. Enrichment of NO_3-N (solid line, simulated by the model) and NH3-N (■) and bacterial N (o) after intraruminal injection of (a) $^{15}NH3$-N into the rumen (a) and (b) of NH_3-N (■) and bacterial N (o) after injection of the same amount of ^{15}N in the form of $^{15}NO_3$-N into a sheep 1 ingesting the 4% KNO_3 diet (iso-nitrogenous with control diet).

The pool sizes and N flow rates determined by solving the models for all sheep on the 3 diets are given in Figs 5, 6 and 7 and, in all cases, are close to the initial values used to begin the optimisation process.

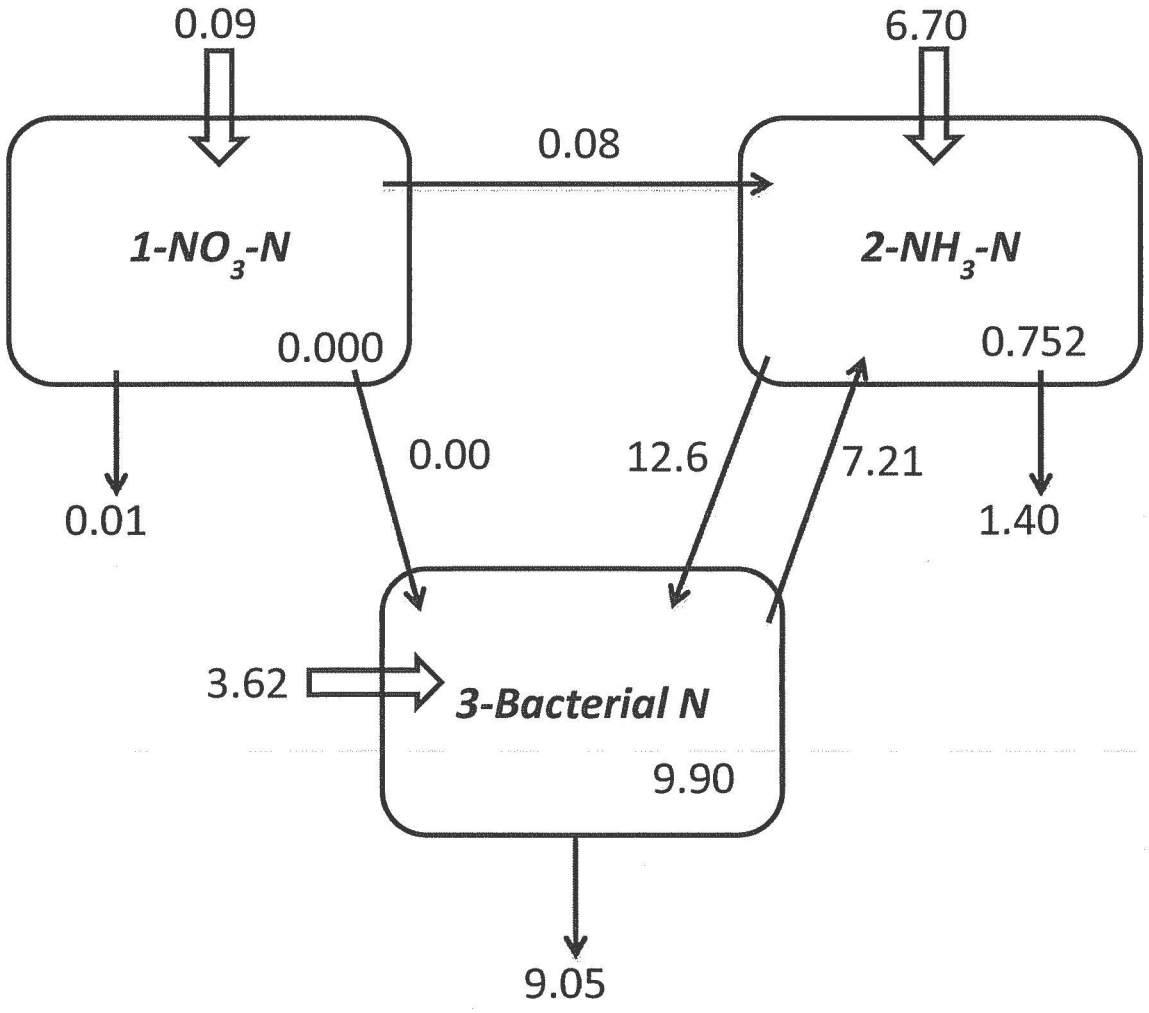

Fig. 5 Pool sizes (g N) and flow rates (g N/d) in the rumen of sheep 5 given a diet of chaffed oaten hay supplemented with urea (5.54 g N/d) based on the ^{15}N tracer data in Fig.2

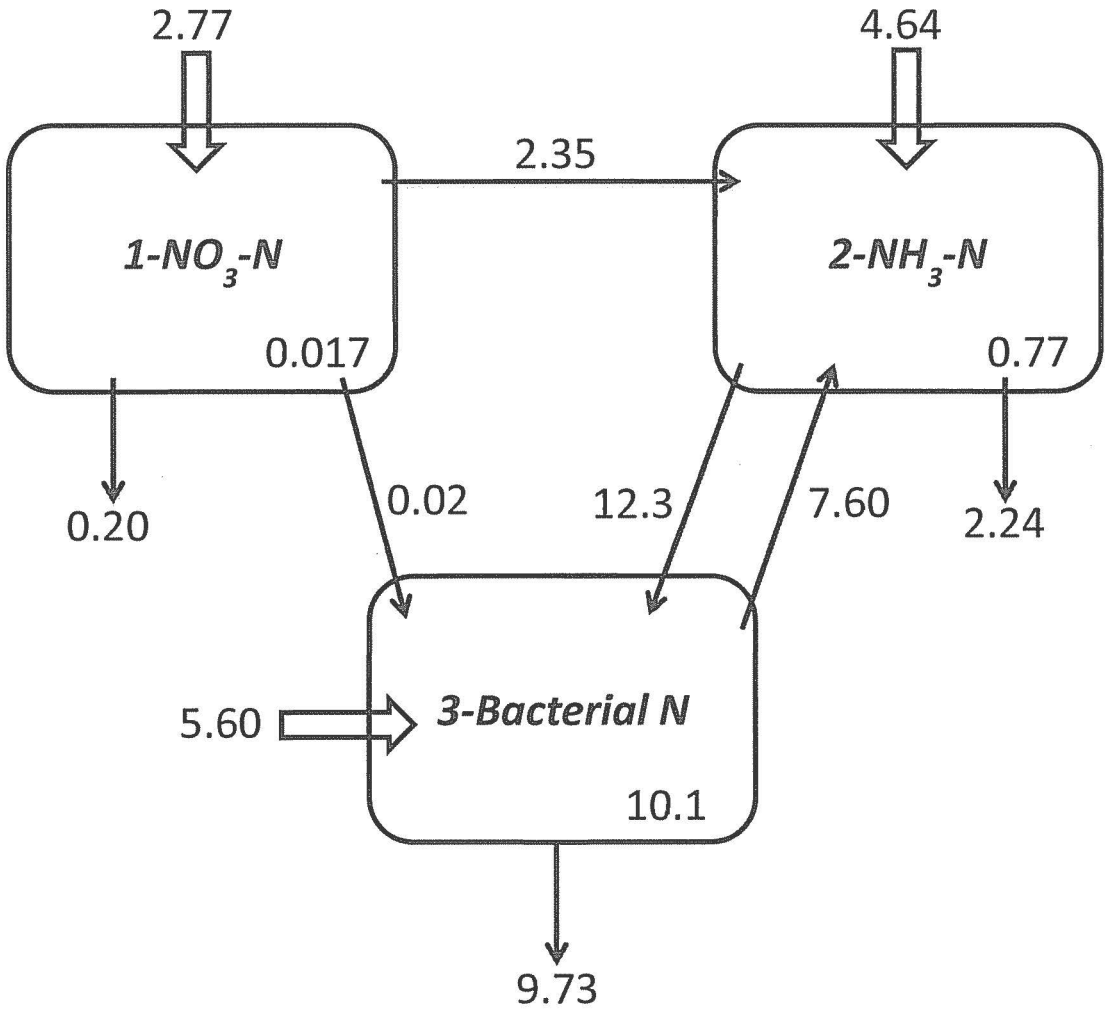

Fig. 6 Pool sizes (g N) and flow rates (g N/d) in the rumen of sheep 11 given a diet of chaffed oaten hay supplemented with urea (2.77 g N/d) and NO_3^- (2.77 g N/d) based on the ^{15}N tracer data in Fig.3.

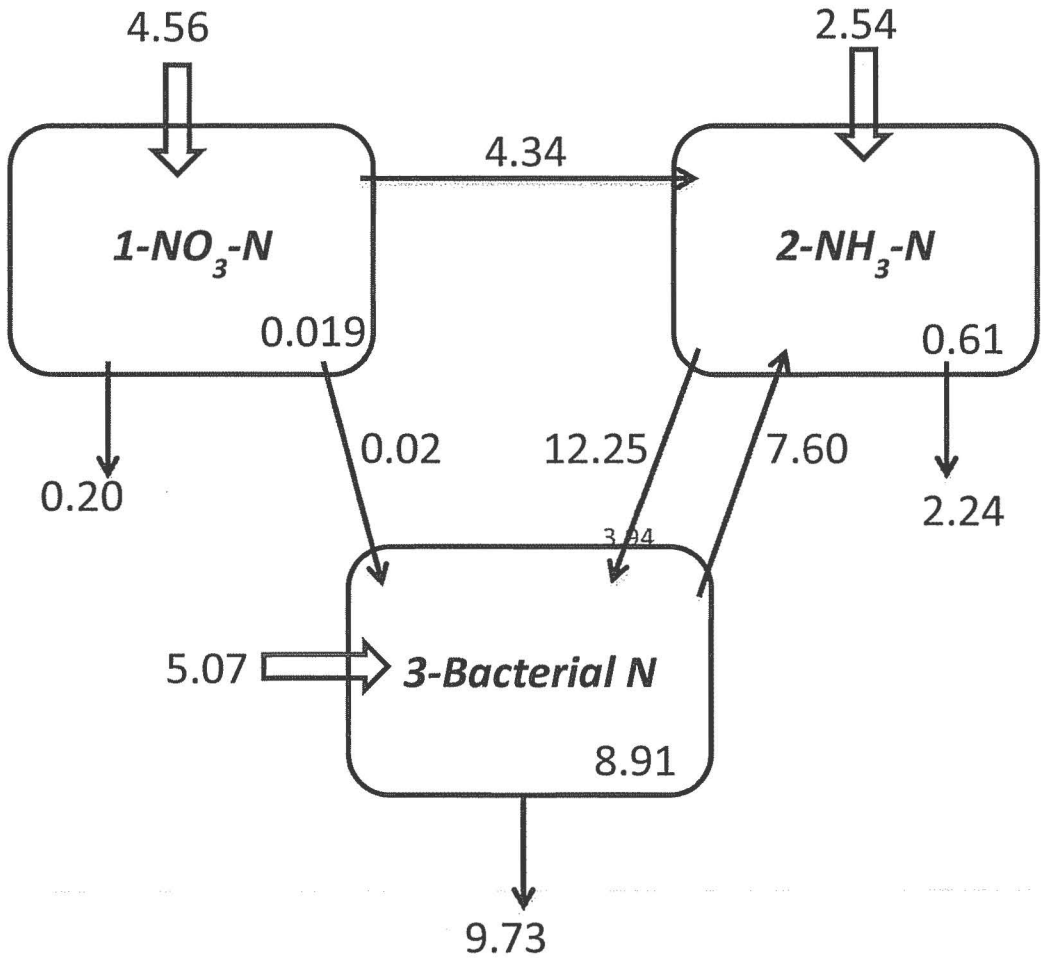

Fig. 7 Pool sizes (g N) and flow rates (g N/d) in the rumen of sheep 1 given a diet of chaffed oaten hay supplemented with NO_3^- (5.54 g N/d) based on the ^{15}N tracer data in Fig.4.

Models similar to those shown for Sheep 1, 5 and 11 (Figs 2-4) were solved individually and the means (±SE) accumulated.

The estimated pool sizes for rumen fluid NO_3-N and NH_3-N in the model solutions for each dietary treatment (Table 2) are close to the values derived in a concurrent study (Nolan et al. 2010) and used as the initial conditions to begin the model optimisation process. The optimised bacterial N pool size (8.9-10.1 gN) was about 30% higher than the initial values obtained from Oosting *et al.* (1993).

There was no evidence of direct uptake and utilisation of NO_3-N as a source of N for microbial polymer synthesis via R(3,1). More than 85% of the NO_3^- in the rumen was converted to NH_3-N via R(2,1), probably by reactions occurring outside bacteria. Bacteria obtained the majority of their N for polymer synthesis from NH_3 and the remaining 29-46% from NAN (probably mainly peptides and amino acids). The irreversible loss of bacterial N from the rumen (net synthesis)

was similar in sheep on each of the diets (9.0- 9.7 gN/d) and the total synthesis of bacterial crude protein exceeded the net synthesis by about 26%, so there was extensive recycling of NH_3-N between the bacterial N and NH_3-N pools. The flow of N via R(0,2) includes N in digesta flowing out of the rumen (about 1.1 g N/d) and the remainder would have been absorbed across the rumen wall as ammonia

Discussion

The same 3-pool model was used to fit all tracer results simultaneously and excellent fits were obtained by using this relatively simple representation of the rumen. The use of only one microbial pool with data obtained from analysing bacteria isolated from the fluid phase of digesta is a potential limitation but nevertheless this model appears to give statistically well determined fits to all sets of tracer data. A NO_2^- pool was not included because it was not possible to detect NO_2^- in rumen fluid or to determine its enrichment. There was also no explicit inclusion of urea or other labile N pools in tissues. Even with these limitations, results from the model supported previous findings. The net synthesis given by R(0,3) was similar to that predicted in the same sheep from excretion of purine derivatives in urine (Nolan et al. 2010) and from AFRC (1993) prediction equations using their ME intakes. The finding that there was appreciable intra-ruminal recycling of NH_3–N is also in accord with previous reports (Nolan and Leng 1972; Aharoni *et al.* 1991; Firkins et al. 1992; Koenig et al. 2000; Oldick *et al.* 1992).

The models provide new information concerning the metabolism of NO_3^- in the rumen. There was little evidence of direct incorporation of NO_3-N into microbial polymers. The model solutions indicate that, for the sheep in this study, label from $^{15}NO_3^-$ appeared rapidly in the NH_3 pool and therefore showed that NO_3^- was rapidly reduced in the rumen to NO_2^- and also NO_2^- was rapidly reduced to NH_3. NO_2-N was not detectable in rumen fluid of these sheep. This could have been because NO_2^- was rapidly absorbed across the rumen wall or because the rate of conversion of NO_2^- to NH_3 was at least as rapid as the conversion of NO_3^- to NO_2^-. As methaemoglobin formation in the blood of these sheep was minimal (Nolan *et al.* 2010), absorption of NO_2^- was probably also relatively low, although rapid removal of NO_2^- from the methaemoglobin in the blood could also explain the low methaemoglobin concentrations. Support for the conclusion that NO_2^- absorption was low was provided by the model solutions that showed that 85-95% of the NO_3-N in the rumen was recovered in the NH_3 pool in rumen fluid and only 5-15% left the rumen by outflow in fluid or by direct absorption.

Sudden intakes of NO_3^- by sheep can be fatal (Nicholls and Miles 1980). Sheep tolerated 224 mg NO_3^-/kg LW over 24 hours but the same amount was lethal as a single dose (Sinclair & Jones 1967). It has been argued that, to help avoid problems of NO_3^- toxicity, it is advantageous to adapt ruminants to NO_3^- by increasing the concentrations of NO_3^- in the diet over an extended period. Alaboudi and Jones (1985) adapted sheep to a high NO_3^- diet (1.5 g NO_3^-/kg W per day) over a period of 10 wk, after which the sheep subsequently exhibited no clinical signs of elevated methaemoglobin concentration in blood. In this study, sheep were adapted to their diets (containing 0.6 and 0.3 g NO_3^-/kg W) over a period of only 18 d but methaemoglobin concentrations were not elevated significantly during the subsequent experiments. In addition, when $^{15}NO_3^-$ was administered to the animals adapted to the control diet (containing negligible

NO_3^- but made iso-nitrogenous with the other diets by the addition of urea), these animals converted the tracer NO_3^- just as rapidly to NH_3 as did the sheep adapted to NO_3- on the other diets. Nitrate and NO_2^- reductase activity was therefore present in non-adapted sheep. It is the relative activities of these two enzymes that will be important and adaption will only be beneficial if it increases the rate of reduction of NO_2^- to NH_3 more than the rate of reduction of NO_3^- to NO_2.

We conclude that giving roughage diets (fed hourly) containing 0.6 g of NO_3^- /kg W, was sufficient to reduce CH_4 emissions (L/kg DM intake) in these sheep by 23% (Nolan et al. 2010), without causing NO_2^- toxicity. The reason is that they converted NO_3^- rapidly and almost completely to NH_3. There was no build-up in concentration of NO_2^- and therefore minimal absorption of NO_3^- from the rumen. The feeding or other conditions that pre-dispose animals to toxicity have not been fully clarified by this study but our results show that toxicity is not an automatic outcome when NO_3^- is included in diets for ruminants, even if they have not been adapted to those diets. To avoid toxicity, NO_2^- concentration needs to be kept low, either by limiting the rate of conversion of NO_3^- to NO_2^- or by increasing the rate of reduction of NO_2^- to NH_3.

References

Alaboudi AR Jones GA (1985) Effect of acclimation to high nitrate intakes on some rumen fermentation parameters in sheep. *Canadian Journal of Animal Science* **65**, 841-849.

Bird SH, Hegarty RS, Woodgate R (2008) Persistence of defaunation effects on digestion and methane production in ewes. *Australian Journal of Experimental Agriculture* 48, 152–155. doi: 10.1071/EA07298.

Boston RC, Grief PC, Bermen M (1982) The routine fitting of kinetic data to models: a mathematical formalism for digital computers. *Biophysical Journal* **2**, 275-287.

Clark, R. & Quin, J.I. 1951 Studies on the alimentary tract of the Merino sheep in South Africa. XXII. Effect of supplementing poor-quality grass hay with molasses and nitrogenous salts. *Onderstepoort Journal of Veterinary Research* **25**, 93–103.

Downes AM, McDonald IW (1964) The Chromium-51 complex of the ethylene tetra-acetic acid as soluble rumen marker. *British Journal of Nutrition* **26**, 153-162.

Firkins JL, Weiss WP, Piwonka EJ (1992) Quantification of intraruminal recycling of microbial nitrogen using nitrogen-15.Journal of Animal Science **70**, 3223-3233.

Koenig KM, Newbold CJ, McIntosh FM, Rode LM (2000) Effects of protozoa on bacterial nitrogen recycling in the rumen. *Journal of Animal Science* **78**, 2431-2445.

Nicholls TJ & Miles EJ (1980) Nitrate/nitrite poisoning of cattle on ryegrass pasture. *Australian Veterinary Journal* **56**, 95-96.

Leng RA (2008) The potential of feeding nitrate to reduce enteric methane production in ruminants. Report to Department of Climate Change, Commonwealth Government, Canberra. Available at: http//:penambulbooks.com/Downloads/Leng-final%20Modified%202017-9-2008.pdf [Verified 17 July 2010].

Nolan JV, Hegarty RS, HegartyJ, Godwin IR, Woodgate R (2010) Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. *Animal Production Science* **50**, 801-806.doi: 10.1071/AN09211.

Oldick BS, Firkins JL Kohn RA (2000) Compartmental modeling with nitrogen-15 to determine effects of degree of fat saturation on intraruminal N recycling. *Journal of Animal Science* **78**, 2421-2430.

Oosting SJ, Viets TC, Lammers-Wienhoven SCW, van Bruchem J (1993) Ammonia treatment of wheat straw. 2. Efficiency of microbial protein synthesis, rumen microbial protein pool size and turnover, and small intestinal protein digestion in sheep. *Wageningen Journal of Life Sciences* **41***(2) pp??*

Sinclair KB & Jones DIH (1967) Nitrate toxicity in sheep. *Research in Veterinary Science* **8,** 65.

Stefanovski D, Moate PJ, Boston RC (2003) WinSAAM: A windows-based compartmental modeling system. *Metabolism* **52**(9), 1153-1166.

The interactions between nitrate, sulphur and molybdenum in reducing nitrite accumulation and methane production from ruminants: an *in-vitro* incubation study oaten chaff as substrate

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Hypothesis

Supplementary sulphate in sulphur deficiency diet will reduce the nitrite accumulation in the rumen, hence methane production in a basal diet of oaten chaff in which the source of non-protein nitrogen (NPN) is nitrate.

Objectives

To study the effect of nitrate, sulphate and molybdenum on methane production in an *in vitro* system inoculated with rumen fluid using oaten chaff as the basal substrate.

Materials and methods

Duration

The experiment was conducted in the ruminant laboratory of UNE from 18th of August to 24th of August, 2011.

Experimental design

Two levels of sulphur, two levels of molybdenum and two levels of nitrate were investigated in an *in vitro* fermentation system. The design was a 2 × 2 × 2 factorial (8 treatments) arrangement with 2 replications. The levels of sulphur (as sodium hydrate sulphate, $NaHSO_4$) were 0 and 0.3% (in DM); the levels of molybdenum (as Sodium molybdate dihydrate, $Na_2MoO_4 \cdot 2H_2O$) were 0 and 6 mg Mo/kg DM; the levels of $Ca(NO_3)_2$ were 0 and 36 g N/kg DM (calcium nitrate was 3.8% in DM). The calculated ratio of N/S is 12:1. The individual treatments are listed in **Table 1**.

As the amount of each element used in each treatment is rather small, for instance, the amount of $Na_2MoO_4 \cdot 2H_2O$ required for the treatment T2, T4, T5 and T8 is 0.00000272 g each, so the only accurate way to dissolve 0.00136 g $Na_2MoO_4 \cdot 2H_2O$ in 1 L water (0.00000136 g/ mL), so 1 mL solution should provide the required amount of $Na_2MoO_4 \cdot 2H_2O$ for each treatment. The same technique was applied to $Ca(NO_3)_2$ and $NaHSO_4$.

Table 1. Ingredients in the substrate (g DM) If element not presents in the incubation medium using– symbol

Treatment No.	Treatment ID	Oaten chaff (g)	$Ca(NO_3)_2$ (g)	$NaHSO_4$ (g)	$Na_2MoO_4 \cdot 2H_2O$ (g)
T1	N – S – Mo –	178	-	-	-
T2	N – S + Mo +	178	-	0.002025	0.00000272
T3	N – S + Mo –	178	-	0.002025	-
T4	N – S – Mo +	178	-	-	0.00000272
T5	N + S – Mo +	178	0.037954	-	0.00000272
T6	N + S + Mo –	178	0.037954	0.002025	-
T7	N + S – Mo –	178	0.037954	-	-
T8	N + S + Mo +	178	0.037954	0.002025	0.00000272

***In vitro* incubation protocol for assessing bioactivity of compounds**

In-vitro incubations of rumen fluid were conducted in 60 mL plastic syringes.

Each syringe was fitted with a three-way luer-lock tap (BD-Connecta) and ground oaten chaff (500µm sieve size; 200 ± 10 mg chaff/syringe) was weighed directly into the syringe. Subsample of oaten chaff was dried at 105^{0}C for 24h to determine DM (DM 88.9%). Oaten chaff molybdenum concentration 0.8µg/g was determined using inductively coupled emission spectroscopy by a certified U.S. EPA method.

Rumen fluid was collected by rumen cannula from a 40 kg Merino wether (3.5 years old) approximately 45 minutes prior to the commencement of inoculations and maintained at 39ºC. The sheep were fed 800 g oaten chaff (DM 88.9%, Molybdenum 0.8 µg/g; S 0.139%) throughout the period during which the screening program was conducted. A vessel, fitted with one tube to allow withdrawal of incubation medium and another tube to allow constant bubbling of CO_2 through the incubation medium was placed in a 39ºC water bath and charged with a modified buffer solution (Tilly and Terry 1964) (e.g. 200 mL) (see Table 2 for the ingredients of buffer) while flushing with CO_2 (BOC anaerobic grade). Approximately 15 minutes prior to commencement of incubations, rumen fluid (e.g. 100 mL) was added, via syringe, to the vessel to provide a buffered rumen fluid inoculum for dispensing into incubation syringes. The volume of inoculum prepared varied with the number of syringes to be used in the runs (see Experimental Design) but the ratio of buffer to rumen fluid was always 2:1, while the total volume prepared was typically 60 to 70 mL in excess of that theoretically required for the inoculations. Bubbling with CO_2 through the rumen fluid/buffer mixture continued until all inoculations were completed.

Table 2. Ingredients of the buffer solution (modified from Tilly and Terry 1964)

Ingredients	$CaCl_2$	$NaHPO_4.12H_2O$	NaCl	KCl	$NaHCO_3$
(g/liter)	0.04	9.30	0.47	0.57	9.80

Prior to the commencement of incubations, the syringes were loaded with 3 mL of solutions (see Table 1 for treatment arrangements. i.e. T1 contains 3 mL of H2O and T2 contains 1 mL of H_2O, 1 mL of $NaHSO_4$ and 1 mL of $Na_2MoO_4 \cdot 2H_2O$). The buffered incubation medium (10 mL) was then drawn up into the syringe, the syringe shaken well, the initial volume of gas read from the scale on the syringe and the syringe returned to the shaking water bath for incubation. The duration of incubations was measured from the time of drawing up incubation solution. Incubations continued at 39°C for 18 h, with incubations commenced (and completed) in a staggered program using two minute intervals.

Measurements Post-Incubation

After 18 h, the syringe was removed from the shaking water bath and the final gas volume read. The tap was then removed from the syringe and the liquid expelled into a 25 mL plastic vial and a clean tap fitted to the syringe. The syringe was set aside for later measurement of the gas composition.

The pH of the liquid was measured (Eutech EcoScan 5/6) and then 3 mL 50% formalin was pipetted into the 25 mL plastic and stored at -20 °C for nitrite analysis. The nitrite concentration in the incubation medium was determined using xx method.

Analysis of Gas Composition

Gas samples were pumped directly from the incubation syringe, through two 17 mm syringe filters (Chromacol, PTFE, 0.45µm), into a Varian CP4900 Gas Chromatograph (GC), fitted with thermal conductivity detectors. Three channels consisting of a Molsieve 5Å (MS5A) column, with argon as carrier, a Molsieve 5Å column with helium as carrier and a CP-PoraPLOT U (PPU) column with helium as carrier were used to determine hydrogen and methane, methane and carbon dioxide respectively. In general, single measurements were conducted on each gas sample but in cases where the measured quantities appeared to be anomalous (usually typified by a very high nitrogen content) a duplicate was run if sufficient sample of gas remained.

Volatile Fatty Acid Analysis (Results are not available)

At the completion of each measurement period, an aliquot of the incubation medium (0.5 mL) was pipetted into a 1.5 mL Eppindorf microcentrifuge tube containing an internal standard solution (1 mL). The samples were centrifuged (13000g x 10 minutes) and the supernatant transferred to autosampler vials for analysis. The samples were chromatographed on a Varian CP-3800 Gas Chromatograph. Peak areas were normalised with respect to that of the internal standard and the concentrations of each VFA determined by reference to a commercial standard VFA mixture (Supelco). Samples were prepared and stored for future VFA analysis but VFA results were not available for this report.

Protozoal Counts

At the completion of a run, an aliquot of the incubation medium (0.5 mL) was pipetted into a 5 mL plastic vial containing a formaldehyde/sodium chloride solution (0.25 mL). One drop of Brilliant Green stain was added and after standing for 1-2 hours, the solution was diluted with 30% glycerol (2 mL) and protozoal numbers determined on a counting slide by visual enumeration.

Statistical analysis

One-way ANOVA tests for each parameter were conducted using GenStat 12.1 (VSN International Limited, UK). Unless stated otherwise, the results were expressed as mean ± standard error of difference of mean (s.e.d.). The significance level was set at $P < 0.05$, and trends were noted if $0.05 \leq P < 0.10$.

Results and Discussion

Nitrate inclusion significantly reduced total gas production and the production of methane and accumulation of hydrogen gas when expressed as either a volume or a percentage of total gas production (Table 3). Importantly, the decrease in total gas production (a measure of fermentation (Getachew et al. 1998), due to nitrate inclusion was far greater than the volume change attributable to less methane and hydrogen production alone, so CO_2 production or release from the media must also have been reduced by nitrate. This is consistent with nitrate incubations having a slightly but significantly higher pH (Table 4). The basal diet used in the incubation was not rich in either S or Mo but contained more of both minerals than the required concentration. However, methane production from the unsupplemented diet was slightly but significantly reduced by Mo supplementation. When nitrate was present, methane levels approached zero so no effect of Mo on methane production was evident. There was no apparent effect of sulphur inclusion on total gas production or any component gas productions

Table 3. Mean value for gas production, methane percentage in the gas and methane production per substrate fermented

Treatment	Total gas production (mL)	Methane (%)	Hydrogen (%)	Methane production (mL)	Hydrogen production (mL)
N – S – Mo –	14.3^{a}	13.52^{a}	0.0376^{a}	1.947^{b}	0.0054^{a}
N – S + Mo +	18.0^{b}	13.28^{b}	0.0348^{b}	2.387^{a}	0.00628^{a}
N – S + Mo –	16.7^{b}	13.38^{a}	0.0357^{a}	2.228^{b}	0.00594^{a}
N – S – Mo +	19.7^{b}	13.83^{a}	0.0336^{a}	2.717^{a}	0.00663^{a}
N + S – Mo +	7.7^{c}	0.631^{c}	0^{c}	0.049^{c}	0^{b}
N + S + Mo –	8.3^{c}	0.372^{c}	0.0001^{c}	0.031^{c}	0.00001^{b}
N + S – Mo –	7.7^{c}	0.259^{c}	0^{c}	0.018^{c}	0^{b}
N + S + Mo +	8.33^{c}	0.299^{c}	0^{c}	0.024^{c}	0^{b}

* Within columns, means with a common suffix do not differ significantly

Table 4. Incubation medium pH, ttotal protozoa numbers in incubation medium of different treatments

Treatment	Incubation medium pH	Total protozoa count (× 10^4/mL)	Nitrite – N (µg/mL)	Conversion rate of Nitrate-N** to Nitrite - N (%)
N – S – Mo –	6.54^c	2.67^a	0^c	0^c
N – S + Mo +	6.58^b	4.17^a	0^c	0^c
N – S + Mo –	6.57^b	3.92^a	0^c	0^c
N – S – Mo +	6.61^b	4.33^a	0^c	0^c
N + S – Mo +	6.63^a	1.67^b	423^b	65.3^b
N + S + Mo –	6.64^a	1^b	485^a	74.9^a
N + S – Mo –	6.67^a	1.92^b	519^a	80.1^a
N + S + Mo +	6.63^a	1.67^b	447^b	68.9^b

* Within columns, means with a common suffix do not differ significantly

** Calculated Nitrate N concentration in incubation medium is 648 ug/mL

While not quantitative, a qualitative assessment of bacterial biomass was made based on visual assessment while counting protozoa and all of the nitrate supplemented incubations were noted as having a large bacterial biomass, while none of the nitrate free incubations had this characteristic. This warrants further investigation.

Reference

Getachew G, Blummel M, Makkar HPS, Becker K (1998) In vitro gas measuring techniques for assessment of nutritional quality of feeds: a review. *Animal Feed Science and Technology* **72**, 261–281.

Tilley JMA and Terry RA (1963) A two stage technique for the in vitro digestion of forage crops. *Journal of the British Grassland Society* **18**, 104.

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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.

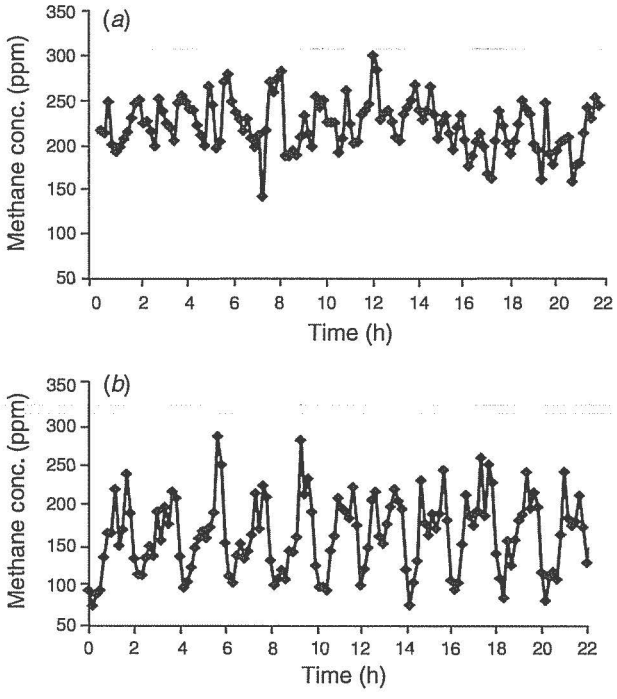

Fig. 1. Time-course of methane concentration in respiration chambers associated with 2-hourly feeding of iso-nitrogenous diets; (*a*) control diet with urea but no added KNO_3 and (*b*) diet with 4% added KNO_3.

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.

Acknowledgements

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References

Akunna J, Bizeau C, Moletta R, Bernet N, Heduit A (1994) Combined organic carbon and complete nitrogen removal using anaerobic and aerobic upflow filters. *Water Science and Technology* **30**, 297–306.

Allison MJ, Reddy CA (1984) Adaptations of gastrointestinal bacteria in response to changes in dietary oxalate and nitrate. In 'Current perspectives in microbial ecology: Proceedings of the 3rd international symposium of microbial ecology'. (Eds MJ Klug, CA Reddy) pp. 248–256. (American Society for Microbiology: Washington, DC)

Allison MJ, Reddy CA, Cook HM (1981) The effects of nitrate and nitrite on VFA and CH_4 production by rumen microbes. *Journal of Animal Science* **53**, 391–399.

Bird SH, Hegarty RS, Woodgate R (2008) Persistence of defaunation effects on digestion and methane production in ewes. *Australian Journal of Experimental Agriculture* **48**, 152–155. doi:10.1071/EA07298

Carver LA, Pfander WH (1974) Some metabolic aspects of urea and/or potassium nitrate utilization by sheep. *Journal of Animal Science* **38**, 410–416.

Davidson B, Doughty JL, Boulton JL (1941) Nitrate poisoning of livestock. *Canadian Journal of Comparative Medicine* **5**, 303–313.

Downes AM, McDonald IW (1964) The chromium-51 complex of ethylenediamine tetraacetic acid as a soluble rumen marker. *The British Journal of Nutrition* **18**, 153–162. doi:10.1079/BJN19640015

Faichney GJ (1975) The effect of formaldehyde treatment of a concentrate diet on the passage of solute and particle markers through the gastrointestinal tract of sheep. *Australian Journal of Agricultural Research* **26**, 319–327. doi:10.1071/AR9750319

FAO/IAEA (2003) 'The technique for the estimation of microbial N supply in ruminants from purine derivatives in urine. Training package.' (Joint Division FAO/IAEA: Vienna)

Farra PA, Satter LD (1971) Manipulation of the ruminal fermentation. III. Effect of nitrate on ruminal volatile fatty acid production and milk composition. *Journal of Dairy Science* **54**, 1018–1024. doi:10.3168/jds.S0022-0302(71)85965-9

Guo WS, Schaefer DM, Guo XX, Ren LP, Meng QX (2009) Use of nitrate-nitrogen as a sole dietary nitrogen source to inhibit ruminal methanogenesis and to improve microbial nitrogen synthesis *in vitro*. *Asian-Australasian Journal of Animal Sciences* **22**, 542–549.

Hegarty RS (2004) Genotype differences and their impact on digestive tract function of ruminants: a review. *Australian Journal of Experimental Agriculture* **44**, 459–467. doi:10.1071/EA02148

Hegesh E, Gruener N, Cohen S, Bochkovsky R, Shuval HI (1970) A sensitive micromethod for the determination of methemoglobin in blood. *Clinica Chimica Acta* **30**, 679–682. doi:10.1016/0009-8981(70)90260-3

Hungate RE (1966) 'The rumen and its microbes.' (Academic Press: New York)

Leng RA (1970) Formation and production of volatile fatty acids in the rumen. In 'Physiology of digestion and metabolism in the ruminant'. (Ed. AT Phillipson) pp. 406–421. (Oriel Press: Newcastle upon Tyne, UK)

Leng RA (2008) The potential of feeding nitrate to reduce enteric methane production in ruminants. Report to Department of Climate Change, Commonwealth Government, Canberra. Available at http://www.penambulbooks.com/Downloads/Leng-Final%20Modified%20%2017-9-2008.pdf [Verified 17 July 2010]

López S, Hovell FD, MacLeod NA (1994) Osmotic pressure, water kinetics and volatile fatty acid absorption in the rumen of sheep sustained by intragastric infusions. *The British Journal of Nutrition* **71**, 153–168. doi:10.1079/BJN19940123

McDonald I (1981) A revised model for the estimation of protein degradability in the rumen. *Journal of Agricultural Science, Cambridge* **96**, 251–252. doi:10.1017/S0021859600032081

NGGI (2009) 'National greenhouse gas inventory.' (Australian Government, Department of Climate Change: Canberra)

Ørskov ER, McDonald I (1979) The estimation of protein degradability in the rumen from incubation measurements weighted according to the rate of passage. *Journal of Agricultural Science, Cambridge* **92**, 499–503. doi:10.1017/S0021859600063048

Stefanovski D, Moate PJ, Boston RC (2003) WinSAAM: a windows-based compartmental modelling system. *Metabolism: Clinical and Experimental* **52**, 1153–1166. doi:10.1016/S0026-0495(03)00144-6

Sutherland TM (1977) The control and manipulation of rumen fermentation. *Recent Advances in Animal Nutrition in Australia* **1**, 110–129.

Trinh Phuc Hao, Ho Quang Do, Preston TR, Leng RA (2009) Nitrate as a fermentable nitrogen supplement for goats fed forage-based diets low in true protein. *Livestock Research for Rural Development* **21**, article 10. Available at http://www.lrrd.org/lrrd21/1/trin21010.htm [Verified 10 August 2010]

Ungerfeld EM, Kohn RA (2006) The role of thermodynamics in the control of rumen fermentation. In 'Ruminant physiology: digestion, metabolism and impact of nutrition on gene expression, immunology and stress'. (Eds K Sejrsen, T Hvelplund, MO Nielsen) pp. 55–85. (Wageningen Academic Publishers: Wageningen, The Netherlands)

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A safe short-term approach to establishing the extent and time course of enteric methane suppression by dietary nitrate

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Introduction

Methane production in the rumen is a major sink for hydrogen and its associated electrons produced during anaerobic fermentation, and this hydrogen removal is essential to maintain efficient rumen fermentation. One approach to reducing methane emission is to supplement the animal's diet with alternative electron sinks such as nitrate. The administration of nitrate inhibits methane production by microbes in vitro (Sar et al., 2005) and in vivo (Tillman et al. 1965, Farra and Satter, 1971; Nolan et al. 2010; Van Zijderveld et al. 2010).

The purpose of this trial was to evaluate a new technique for studying the extent of the suppression in CH_4 production and the time course of CH_4 inhibition in sheep and to determine the reduction in methane output in sheep given nitrate containing diets.

Materials and Methods

Animals and feeding

The experimental protocol was approved by the UNE Animal Ethics Committee of the University of New England.

Four merino wethers (59.3 kg ± SD 4.2 kg) were held in individual metabolism cages for 10 days while they were acclimated to a basal diet of chopped lucerne hay (*Medicago sativa*) with 0.44 % added NO_3. They were subsequently moved into respiration chambers for 4 d to enable their CH_4 production to be monitored. The quantity of feed offered (800 g/d) was calculated to meet their maintenance requirements (55g DM/kg$W^{0.75}$.day). The chemical composition of the lucerne chaff used in the diet is given in Table 1.

Table 1: Chemical composition of the lucerne hay

Component	%
Dry matter (%)	91.8
Crude protein (%)	21.6
Neutral detergent fiber (%)	40
Acid detergent fiber (%)	21
Metabolisable energy (MJ/kg DM)	9.3

Each day's ration (800 g/d as fed; 734 g DM/d) was offered to the sheep in equal 100 g portions every 3 h by means of an overhead belt feeder during the acclimation period. The sheep had continuous access to fresh drinking water throughout the experiment. There were 4 dietary treatments, *viz.* the basal diet of lucerne containing 0.44 % NO_3, and three NO_3-enriched diets containing 1.33, 2.21 and 3.1% NO_3. The NO_3 supplemented feed was prepared by dissolving Calcinit ($Ca(NO_3)_2 4H_2O$), which contains 62% NO_3, in a minimal volume of water and sprinkling the solution onto the lucerne hay which was then well mixed and dried before being used.

Methane emissions from individual sheep were estimated in the final 4 days of the 14-day experimental period in 4 open-circuit methane chambers. One of the 4 diets was allocated to one of the 4 chambers for the whole 4-day period and sheep were moved to a new chamber each day. A dietary treatment consisted of 7 meals offered at 3 h intervals over a 21-h period. Each meal was either the 0.22% NO_3 diet followed by the same diet, or one of the three NO_3 enriched diets in an alternating sequence (see Table 2). This feeding schedule minimised any risk of NO_3-NO_2 toxicity, the average daily NO_3 concentration in the diet being less than 1.1% NO_3. All sheep were moved into a chamber each day before 08:00 h when they were immediately offered a meal of the 0.44% NO_3 diet. They were removed from the chamber the next day between 09:00h and 10:00h when the chambers were cleaned and the floor and tray were washed.

Table 2. Example, for one sheep, of how the lucerne diet containing 0.44 %,d with was alternated with lucerne diets containing 1.33, 2.21 and 3.1% NO_3 across 8 meals per day over the 4-day measurement period. (The 08:00h meal was given each day, but sheep were removed from the chambers soon after 09:00h on the following day, except on Day 4 when they were removed after 11:00h).

Days	Chamber	09:00h	11:00h	13:00h	17:00h	20:00h	23:00h	02:00h	05:00h	08:00h
1	1	0.44	1.3	0.44	1.3	0.44	1.3	0.44	1.3	0.44
2	2	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44
3	3	0.44	2.2	0.44	2.2	0.44	2.2	0.44	2.2	0.44
4	4	0.44	3.1	0.44	3.1	0.44	3.1	0.44	3.1	0.44

At the time each animal was removed from the chamber, a rumen fluid sample (15 ml) was taken by oesophageal intubation to be used for protozoal identification and counting, and analysis for VFA concentration.

Feed analysis

Feed composition was determined by Near Infrared Spectrometry using calibration equations developed by (NSW Agriculture, Wagga Wagga)

Methane measurements

The four open-circuit respiration chambers used were made of polycarbonate (1.21 ± 0.13 m^3 in volume) with ~120 L/min of air, as measured by a dry gas meter (AL800; American Meter Co., PA, USA), drawn from outside the animal house through the chamber by a side channel blower (Uni-jet 40CE; ESAM, Parma, Italy). Air within the chamber was continuously circulated by a 30 cm diameter fan inside the chamber and above the sheep. Water was easily accessible from a long

trough fitted along one side within the respiration chamber that was filled with water at the start of a measurement day. A feed bin was fitted inside the front end of the metabolism crate. Outside the chamber, above the internal feed bin, there was a computer-controlled automatic meal dispenser which dropped pre-weighed meals into the feed bin every hour via a sealed tube. Faeces and urine passed through a grated floor into a tray in each chamber.

Sub-samples of the gas in the outlet from each chamber were analysed to determine CH_4 concentration at 14 min intervals throughout the period when sheep were housed in the chamber, by passing it through an Innova 1312 photo-acoustic multi-gas analyser (Air Tech Instruments, Denmark). The resulting data were logged to a computer. In addition, gas from within each chamber was continuously pumped into a gas collection (Tedlar) bag for the 21 h period while sheep were in the chamber and the CH_4 concentration of this gas was determined using the same gas analyser. Air from the inlet to the chambers was also pumped into a Tedlar bag over the 21 h period and similarly analysed. At the start of 21-h measurement, the analyser was calibrated using analytical standard gases (200 ppm CH_4 and 5000 ppm CO_2).This determines the absolute range (0–200 ppm for CH_4). A CH_4 recovery test was made by injecting a dose of pure CH_4 into each chamber. Recovery in the chambers was 98-104% suggesting that reliable estimates of emissions from animals were obtained during this experiment.

Methane production was calculated as gas flow out of the chamber multiplied by the average CH_4 concentration in the gas outflow corrected for background CH_4 concentration in the gas drawn into the chamber, determined both from the 14 min samples and from the 22-h bag sample. Methane volume was adjusted to standard temperature and pressure (pressure, temperature and humidity were measured every 2 min with analogue sensors whose responses were processed by a datalogger (EASY sense) communicating with a computer).

These give 6 x 3h periods over the 4 days on each basal diet for each sheep and CH_4 production response to a given level of NO_3 in the previous meal.

Statistical analyses

Methane production in each chamber (L/day) was monitored each day at 14 min intervals over a period of 21 h while the sheep were in the chambers. During each 21 h period, the 4 sheep on were given 3 hourly meals of one of the 4 higher NO_3 diets alternated with meals of the 0.44% NO_3 containing diet. Over the 4 days of CH_4 measurements, each sheep had 1 d in each chamber on each diet.

The methane measurements were analysed using a generalised linear model (GLM). The model used was:

CH_4 production = μ + Diet + Firstday + Firstday(2).Diet + Sheep + Day

---

Fixed model Random model

where:

μ = the underlying constant for these data
Diet = 0.44, 1.3, 2.2, 3.1% NO_3 (for each sheep, these diets were tested in the same order (i.e. 0.44, 3.1, 2.2, 1.3).
Firstday = a dummy factor (Y/N) created to identify those diets delivered on the first day of the trial (i.e. no preceding diets) from the diets that followed different diets (with possible carryover effects).
Firstday(2) = the remaining diets that had preceding diets

Sheep = the four sheep used for this study.
Through the fixed model, the analysis first examined the effect of Diet on CH_4 production (i.e. Diet) was examined; then it was determined if sheep receiving one of those diets on the first day in the chambers produced different results to those on days 2, 3 and 4 (i.e. Firstday). Finally, whether there was any carryover effect from diets received on previous days, for those diets not given on day 1 (i.e. Firstday(2).Diet) was determined. Placing Sheep and Day in the random model included the variation between sheep and between days. For the pairwise comparisons, lsd ($p = 0.05$).

To determine the trend in CH_4 produced over the 22 h in each one day period, a linear mixed model was fitted with 4 levels of diet, time of day and their interactions as fixed effects. To estimate the differences in CH_4 production between meals of 0.44% lucerne and higher NO_3 diets (4 meals/d), a 2-level factor (test diet v. 0.44% diet) and its interaction with diet was included. Random terms were a spline term to model the overall smooth non-linear trends in the data, and the interaction (spline term x diet) to model smooth non-linear trends for each of the diets. Other random terms accounted for variation due to the effects of sheep. Random terms for 'chamber' were not included, 'chamber' being equivalent to diet, as each test diet was only fed in one chamber.

To determine the trends in CH_4 produced over the 21 h in each 1-d period, a linear mixed model was fitted with diet (0.44%, and 1.3%, 2.2% and 3.1%), time of day and their interactions as fixed effects. To estimate change in CH_4 production between the higher NO_3 test diets and the adjacent 0.44% NO_3 diet, a 2-level factor was included, *viz.* on/off diet and its interaction with diet. Random terms were a spline term to model the overall non-linear trends in the data and the interaction of spline term x diet, to model smooth non-linear trends for each diet. Other random terms accounted for variation between sheep. Random terms for 'chamber' were not included, 'chamber' being equivalent to 'diet'.

The average CH_4 production in the 3-hour periods after sheep were offered meals containing the 4 levels of NO_3 were also modelled as follows:

CH4day = μ. diet*time !r spl(time) diet.spl(time) sheep, sheep.time sheep.spl(time)

where 'time' is h from start of each 3-h period (i.e., 0, 1, 2 or 3).

Data were analysed in ASREML 2.0. VSN International Ltd., Hemel Hempstead, UK (Gilmore et al. 2006).

Results

The reductions in CH_4 production from sheep in the chambers in periods after ingesting diets with 1.3%, 2.2% or 3.1% NO_3 after meals of 0.44% NO_3 are given in Table 1.

Table 1. Mean reductions in CH_4 outputs from sheep over the 3 h after they ingested meals of NO_3 enriched lucerne (0.44, 1.3%, 2.2%, 3.1% NO_3) compared with CH_4 outputs after their previous meals of lucerne chaff (0.44% NO3-N)

Diet (%NO3)	Reduction in CH_4 output (L/d)	SED[≠]
0.44%	1.65	0.372
2.2%	3.22	0.380
3.1%	4.73	0.372

[≠]SED: Overall Standard Error of Difference = 0.375.

Methane productions of sheep in the chambers were fitted to show the average rates of production over the 3-hour periods after meals containing one of the 4 levels of NO_3 were offered, and the results are given in Fig. 2.

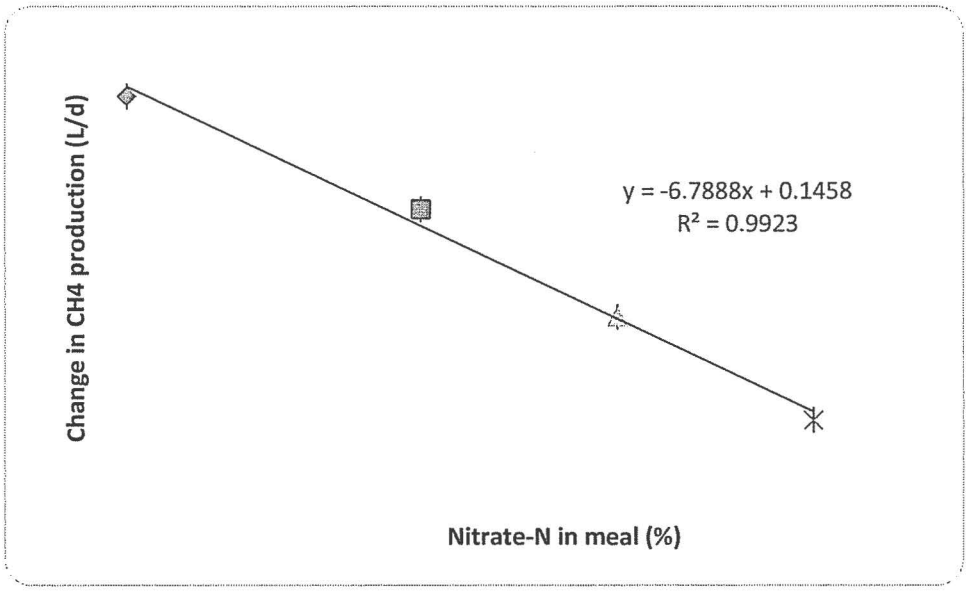

Fig. 1. Reduction in CH_4 emissions from sheep when ingesting meals with increasing NO_3 concentrations (means ± SE).

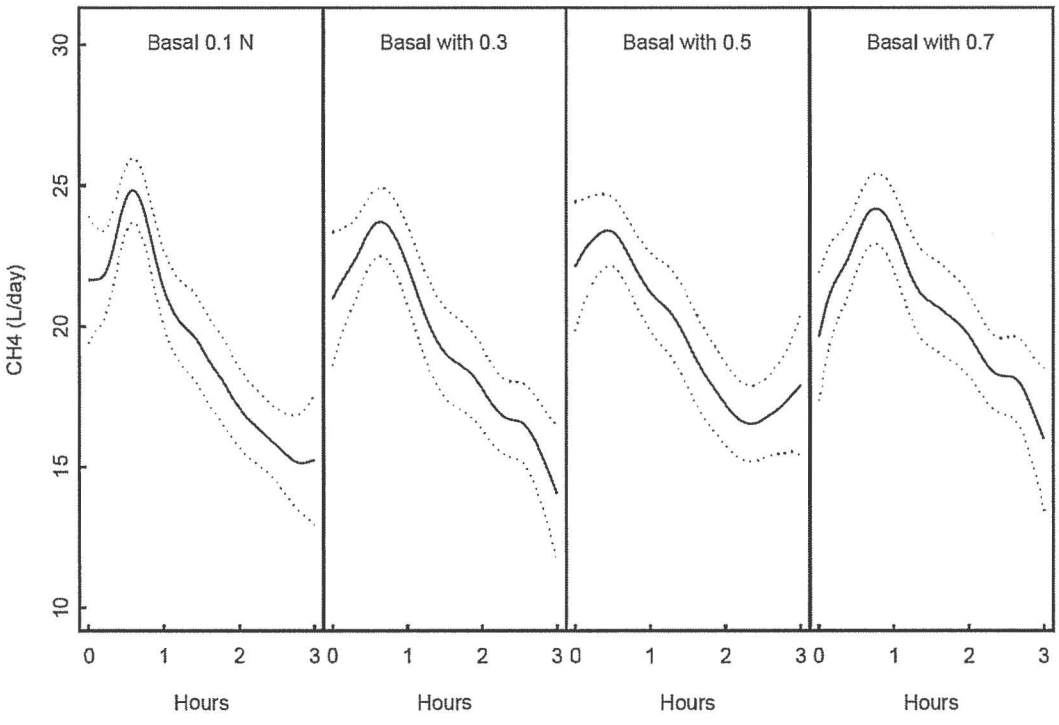

Fig. 2. Patterns of CH_4 production between meals of 0.44%NO_3 diet followed by meals containing higher concentrations of NO_3.

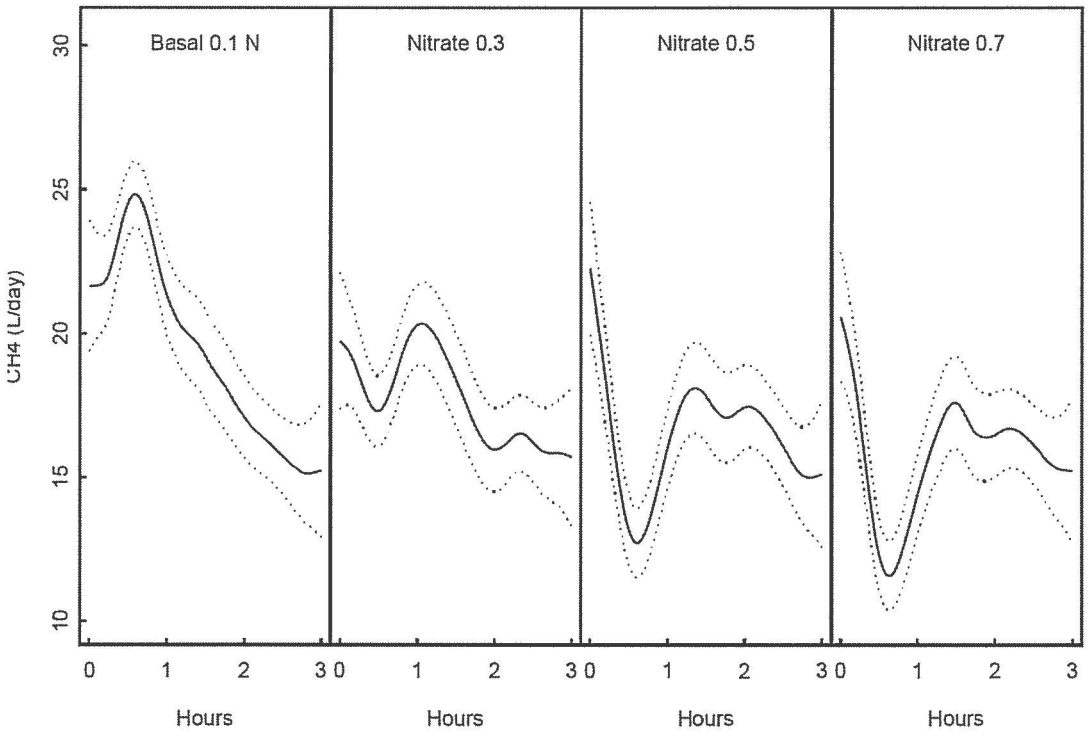

Fig. 2. Patterns of CH_4 production between meals containing increasing levels of added NO3-N following after a meal containing the the 0.44%NO_3 diet.

After ingesting a meal containing 0.44% NO_3, CH_4 output increased after feeding for about 40 min, then declined until the next meal. With the higher levels of NO_3, CH_4 production showed a more pronounced decline during the 1-2 h after the meal was delivered, but then increased before again decreasing and all diets tended to be similar for the last hour before the next meal.

Discussion

This study has demonstrated that inclusion of additional NO_3 in the diet of sheep acclimated to a diet with a low concentration of NO_3, effectively suppresses CH_4 production by sheep. The suppression effect of nitrate has confirmed in vivo earlier studies (Farra and Satter 1971; Sar et al. 2004; Nolan et al. 2010; Trinh Phuc Hao et al 2009). The period of methane suppression in the present study in which NO_2 was provided in small meals every 3 h was relatively short; CH_4 production was greatest in the 2 h following ingestion of NO_3 containing meals, and appeared to cease during the third hour when NO_3 was probably no longer present. It appears that fermentation rate and CH_4 production increased almost immediately after each meal of lucerne, but when NO_3 was present in the meal, the increase due to fermentation was counteracted until there was no longer any oxidisable NO_3 or NO_2 to remove electrons produced during the fermentation and until electrons again became available to reduce CO_2 to CH_4.

The short-term effect in this study differs from earlier findings Sar et al. (2004) in sheep feed twice daily where the NO_3 was given as a single intraruminal dose (1.3 g NaNO3/kg$W^{0.75}$). cf. Daily dosing (Van Zijderveld et al. 2010).

Stoichiometry shows that reduction of 1 mol NO_3 to NH_3 uses the same amount of H_2 (and high potential electrons) as does the reduction of 1 mol CO_2 to CH_4, i.e 1 mol NO_3 should theoretically reduce CH4 output by 1 mol/d (22.4 L/d). Intakes of NO_3 on the 4 diets were 0.06, 0.17, 0.29 and 0.4 and the calculated stoichiometric reductions in CH_4 production were 1.34, 3.84, 6.39 and 8.96 L/d, respectively (Fig 3). The slope of the line indicates the reduction in CH_4 production was ??% of

the theoretical stoichiometric reduction. This compares with 78% in Nolan et al. (2010). Reasons could be absorption of NO_2 and incomplete conversion of NO_2 to NH_3. The less-than-predicted reduction may have been a consequence of a greater ruminal fermentation rate, which increased H_2 availability in NO_3-supplemented sheep, as evidenced by a tendency for a higher total VFA concentration and a higher acetate percentage in the VFA.

References

Farra PA, Satter LD (1971) Manipulation of the ruminal fermentation. III. Effect of nitrate on ruminal volatile fatty acid production and milk composition. *Journal of Dairy Science* **54**, 1018–1024. doi:10.3168/jds.S0022-0302(71)85965-9

Guo WS, Schaefer DM, Guo XX, Ren LP, Meng QX (2009) Use of nitrate nitrogen as a sole dietary nitrogen source to inhibit ruminal methanogenesis and to improve microbial nitrogen synthesis *in vitro*. *Asian-Australasian Journal of Animal Sciences* **22**, 542–549.

Gilmour AR, Cullis BR, Harding SA Thompson R (2006). ASReml Update. What's new in Release 2.00. VSN International Ltd. Accessed 19/12/2011. http://www.animalgenome.org/bioinfo/resources/manuals/ASReml/UpdateR2.pdf

Leng RA (2008) The potential of feeding nitrate to reduce enteric methane production in ruminants. Report to Department of Climate Change, Commonwealth Government, Canberra. Available at http://www. penambulbooks.com/Downloads/Leng-Final%20Modified%20%2017-9-2008.pdf [Verified 17 July 2010].

Nolan JV, Hegarty RS, Hegarty J, Godwin I and Woodgate R (2010) Effects of dietary nitrate on rumen fermentation, methane production and water kinetics in sheep. *Animal Production Science* **50**, 801-806.

Sar C, Santoso B, Gamo Y, Kobayashi T, Shiozaki S, Kimura1 K, Mizukoshi H, Arai I, Takahashi J (2004) Effects of Combination of Nitrate with β1-4 Galacto-oligosaccharides and Yeast (*Candida kefyr*) on Methane Emission from Sheep. *Asian-Australasian Journal of Animal Science* **17**, 73-79)

Trinh Phuc Hao, Ho Quang Do, Preston TR, Leng RA (2009) Nitrate as a fermentable nitrogen supplement for goats fed forage-based diets low in true protein. *Livestock Research for Rural Development* **21**, article 10. Available at http://www.lrrd.org/lrrd21/1/trin21010.htm [Verified 10 August 2010]

Van Zijderveld S.M, Gerrits W.J J, Apajalahti J A, Newbold J R, Dijkstra J, Leng R A and Perdok H B 2010a Nitrate and sulfate: effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. Journal of Dairy Science. Volume 93, 5856-5866 http://download.journals.elsevierhealth.com/pdfs/journals/0022-0302/PIIS0022030210006387.pdf

An initial investigation on rumen fermentation pattern and methane emission of sheep offered diets containing urea or nitrate as the nitrogen source

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Abstract. The effects of dietary nitrate and of urea on rumen fermentation pattern and enteric methane production were investigated using 4-month-old ewe lambs. Ten lambs were allocated into two groups (n = 5) and each group was offered isonitrogenous and isoenergetic diet containing either 1.5% urea (T1) or 3% calcium nitrate (T2). Methane production was estimated using open-circuit respiration chambers after 6 weeks of feeding. No difference in Nitrogen (N) balance, apparent digestibility of N or microbial N outflow existed between treatments (P >0.05). The difference in ME intake estimates between treatments was non-significant (P >0.05). Animals offered T2 diet lost less energy through methane than did those fed T1 diet (P=0.04). Total VFA concentration, molar proportion of propionate, and molar ratio of acetate to propionate in rumen fluid were not affected by dietary N source. Compared to urea inclusion, nitrate inclusion caused a significantly higher acetate and lower butyrate percentage in rumen VFA. Nitrate supplementation tended to lower methane production by about 7.7 L/d relative to urea supplementation (P=0.06). Methane yield (L/kg DM intake) was reduced (P=0.02) by 35.4% when 1.5% urea was replaced by 3% calcium nitrate in the diet. Emission intensity (L methane/kg liveweight gain) was approximately 17.8% lower in the nitrate-supplemented sheep when compared with urea fed sheep, however, the reductions were not statistically significant (P=0.48). This study confirms that the presence of nitrate in the diet inhibits enteric methane production. As no clinical symptoms of nitrite toxicity were observed and sheep receiving nitrate-supplemented diet had similar growth performance compared with those consuming urea-supplemented diet, it is concluded that 3% calcium nitrate can replace 1.5% urea as a means of meeting ruminal N requirements and of reducing enteric methane emissions from sheep, provided animals are adjusted to nitrate gradually.

Running title: Methane emission in sheep offered urea and nitrate supplementation
Additional keywords: feed intake, liveweight gain, rumen VFAs concentration, methaemoglobin

Introduction

The methane resulting from enteric methanogenesis leads to a loss of 2 to15% digested energy from the animal (Blaxter and Clapperton 1965; Johnson and Ward 1996; McCrabb and Hunter 1999) and contributes 11% of Australia's total greenhouse gas emissions (NGGI 2009). Therefore, practical feeding strategies to reduce methane emissions from sheep and cattle are required. Previous *in-vitro* and *in-vivo* studies have shown that there is a potential for nitrate to be used as a nitrogen (N) source by the ruminal biota and this is associated with inhibition of methanogenesis (Guo *et al.* 2009; Nguyen *et al.* 2010; Nolan *et al.* 2010; van

Zijderveld *et al.* 2010). However, nitrate supplementation is not well accepted as a N supplement due to the risk of toxicity from nitrite it generates in the rumen, and there is a need to investigate the effects of long-term feeding of a nitrate source on animal growth performance and health. In contrast, urea supplementation is accepted as a common practice in the extensive/grazing industries (Entwistle and Knights 1974;Farrell and Knights 2001). A comprehensive review conducted by Leng (2010) implied that nitrate could replace urea as a source of fermentable N in the rumen given the N source is mixed in the feed with the expectations of reduced enteric methane production. There is a need to assess whether nitrate can replace urea as a N supplement to achieve moderate to high levels of animal production over a relatively long feeding period.

This study investigates methane production, rumen fermentation pattern and growth performance of sheep offered isonitrogenous & isoenergetic diets containing either 1.5% urea or 3% calcium nitrate. Our hypothesis was that changing the N source from urea to calcium nitrate salt would reduce methane emission from enteric fermentation without jeopardising animal health or affecting growth performance. This study is a further step in the evaluation of nitrate as potential nitrogen supplement for the Australian sheep industry to complement or replace urea.

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 10/090).

Ten weaned ewe lambs (Dorset sire × Dohne ewe, LW = 33.5 ± 0.66 kg from 'Nordale', Delungra, NSW) were selected from a commercial farm at approximately 4 months of age. Lambs were allocated to two dietary treatment groups by stratified randomisation within weight ranges and individually penned. The two dietary treatments were inclusion of 1.5% urea (T1) or 3% calcium nitrate (T2, 1.9% nitrate), with the N source included in the dietary pellet. Both diets were formulated to be iso-nitrogenous and iso-energetic (on ME basis) but subsequent analysis showed slight differences in measured N content and ME content (Table 1). During the whole trial period, representative samples of the feed offered were taken and combined for later analysis.

Table 1. Analysed nutrient composition of experimental diets fed to sheep

	T1- 1.5% urea	T2-3% Calcium nitrate
Dry matter (%)	88.02	88.43
CP % as fed	12.12	11.11
ME (MJ/kg) as fed	9.77	9.90

The experiment lasted for 54 days and consisted of 2 periods, being an initial 35-day *ad libitum* feeding period and a 19-day period in which lambs were restricted fed at 1.5 × $ME_{maintenance}$. The maintenance requirement (M, MJ ME/day) of each sheep was calculated according to the equation used in SheepExplorer (2003). The average liveweight of sheep in each group obtained at Day 35 was used to calculate average ME requirement for all sheep in that group for the restricted feeding period.

The feeding regime and experimental events for the 35-day *ad libitum* feeding period were as follows: T1 diet required no adaptation; therefore, lambs were on full diet from Day 1. Lambs offered T2 were gradually adapted to diet over a 7-day period, progressively changing the ration from 100% T1 on Day 1 to 100% T2 on Day 7. Once sheep from both groups were on diet, growth performance was monitored (Day 8 – Day 35). During the 35-day *ad libitum* feeding period feed was offered twice daily in two equal portions

at 10:00 and 16:30 h. Daily feed intake for each lamb was recorded and all animals had access to fresh water at all times.

After the growth study was completed (Day 8 – Day 35), animals were moved to metabolism cages and offered limited feed (1.5M) in preparation for the methane and nutrient balance measurement. The purpose of the restricted feed availability was to allow comparison of the effects of dietary treatments on methane production and nutrient balance, without the potential confounding effect of variation in daily feed intake. The fixed feeding level of 1.5M was approximately 60% of the average feed intake of sheep before restriction. Methane production was measured for 22h on days 42-45 of the trial in open circuit respiration chambers then sheep returned to their metabolism cages. Total collection of faeces urine and refusals were made over days 49-54 to determine N and DM digestibility.

Methaemoglobin (MetHb) concentration in blood

Blood was sampled 3 h after morning feeding on days 0, 14 and 28. Blood samples (~ 8 mL) were taken from the jugular vein, using lithium heparinised vacutainers (BD Frankin Lakes NJ, USA). MetHb concentration in blood was determined within 30 min of blood collection as described by Hegesh *et al.* (1970).

Rumen fluid pH, volatile fatty acid (VFA) concentrations and protozoo enumeration

A 15 mL rumen fluid sample was taken (using an oesophageal tube) from each animal 1 h before feeding on days 15 and 31. Rumen pH was measured immediately using a portable pH meter (Orion 230 Aplus, Thermo Scientific, Beverly, MA, USA). A 10 mL subsample was placed in wide-neck McCartney bottle acidified with 0.3 mL 18M sulphuric acid and then stored at – 20°C for subsequent VFA analysis. All VFA analyses and calculations were conducted by the Department of Agriculture Western Australia, Perth, Australia (Method Reference: VFA - GC Separation of C2-C5. Supelco Bulletin No.749D). Another 4 mL subsample of rumen fluid was mixed with 16 mL of 4% formalised saline and stored in wide-neck McCartney bottle at room temperature for later protozoa counts. Total protozoa numbers were counted using a Hawksley Cristalite B.S. 748 counting chamber (Sussex, UK).

Methane production

Total methane production (L/d at STP) was measured over a 2 × 22-h period for each lamb as described by Bird *et al.* (2008), with the daily ration (1.5M) being offered at 11:00 and 16:30 h in the chamber. Chambers were approximately 1200 L in volume, the external air was drawn through the chamber at ~100 L/min, and so the mean retention time of air in the chambers was approximately 12 min. Methane concentration was measured by an Innova 1312 Photoacoustic multi-gas analyser with inbuilt moisture correction (Innova Airtech Instruments, Ballerup, Denmark). Recovery of a dose of pure methane introduced to each chamber was determined and the measured emission data adjusted for 100% recovery. Methane production was calculated as air flow (L/day) × methane concentration in air immediately after leaving the chamber (ppmv) and adjusted for methane concentration in the incoming air and the temperature and atmospheric pressure in the chamber.

DM digestibility, nitrogen and energy utilisation

Faeces and urine outputs from animals were collected daily over a 6-day period. During this period, feeds were again offered twice daily in equal amounts. The total daily faecal output was well mixed, sub-sampled (10%, w/w), and subsamples pooled over 6 d and stored at – 20°C. Urine was collected into buckets containing approximately 500 mL of 1.8M 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. A daily

representative sample (3%, w/w) of diluted urine was taken, pooled over 6 d, and stored at – 20°C. Feed offered and any feed refused were sub-sampled daily (25%, w/w), pooled and stored at – 20°C until analysis.

DM concentration of experimental diets, feed refusals and faeces were determined by drying a subsample at 80°C in a forced draught oven to a constant weight. Total N in the feed, faecal and urine samples was determined using an automated Organic Nitrogen Determinator (FP-2000, Leco Corporation, USA). The mean yield of microbial N from the rumen was estimated from mean daily allantoin excretion in the urine collected over a 6-day period. The concentration of allantoin in the urine was determined using the colorimetric 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). Gross energy content of feed, faeces and urine were determined using a bomb calorimeter (Calorimeter C7000 with Cooling System C7002, IKA Werke, Germany). The energy loss through methane was calculated assuming an energy content of methane of 40.5 J/L methane after Brouwer (1965). Metabolisable energy was determined from the gross energy consumed less the measured energy losses through faeces, urine and methane.

Statistical analysis

Results for feed intake, liveweight gain, DM digestibility, N utilisation and methane production were assessed by one-way ANOVA. The effects of treatment and measurement day on rumen fermentation parameters and protozoa numbers and their interactions were assessed by the general analysis of variance procedure using Genstat 12.1 (VSN International Ltd). If a difference between means existed, a Duncan's multi-range test was used to compare means from within a fixed factor. Unless stated otherwise, the results were expressed as mean ± standard error of difference of mean (s.e.d.). The significance level was set at $P < 0.05$, and trends were noted if $0.05 \leq P < 0.10$.

Results

Feed intake (FI) and live weight gain (LWG) during the 4-week ad libitum feeding period

The average FI, LWG and feed conversion ratio (FCR) results are summarised in Table 2. During the 4-week *ad libitum* feeding period, no difference in FI (mean 1661 g/d, as fed) was observed between the treatments. No difference in LWG (mean 277 g/d) or feed conversation ratio (mean 6.1 g FI/g LWG) occurred between treatments.

Table 2. Average daily feed intake (*as fed*), daily liveweight gain (LWG) and FCR (g feed intake/g LWG) during 4-week *ad libitum* feeding of a urea or nitrate-supplemented pelleted diet

	Treatment			
	T1 - 1.5% urea	T2 - 3% calcium nitrate	s.e.d.	*p* value
	n = 5	n = 5		
FI (g/d)	1776	1546	127	0.108
DM intake (g/d)	1563	1367	112	0.119
LWG (g/d)	306	247	35.6	0.136
FCR (g FI/ g LWG)	5.8	6.4	0.43	0.198

Whole tract dry matter (DM) digestibility, nitrogen (N) and energy utilisation

While formulated to be isonitrogenous and isoenergetic, the mean N and ME content of T1 and T2 feeds were not identical (Table 1). Estimates of DM digestibility, N and energy utilisation are given in Table 3. Throughout the period of the total collection, whole tract DM digestibility was not affected by diet ($P > 0.05$). Sheep offered T1 diet consumed significantly more ($P < 0.001$) N than did those fed T2 diet; this

could have been due to the discrepancy between the calculated and actual N content in the experimental diet (Table 1). No difference in N balance, apparent digestibility of N or microbial N outflow existed between treatments ($P > 0.05$). The difference in ME intake estimates between treatments was non-significant ($P > 0.05$). Animals offered the T2 diet lost less ($P = 0.044$) energy through methane than did those fed the T1 diet.

Table 3. Measures (means ± s.e.d.) of DM digestibility, N and energy utilisation of sheep fed two pelleted diets – T1 supplemented with 1.5% urea or T2 supplemented with 3% calcium nitrate. The measurements were made during a 6-d period of collection of urine and faeces and animals were offered 1.5 × ME $_{maintenance}$ ration over this period.

	T1- 1.5% urea	T2-3% Calcium nitrate		
	n = 4	n = 4	s.e.d.	*p* value
DM intake (g/d)	915.0	861.0	22.80	0.056
DM digestibility in the whole GI tract (%)	70.0	68.1	2.750	0.511
N intake (g/d)	20.2	17.3	0.459	<.001
Faecal N (g/d)	4.48	3.95	0.362	0.196
Urinary N (g/d)	6.84	8.09	1.715	0.494
N retention (g/d)	8.86	5.27	1.884	0.106
Apparent N digestibility (%)	77.8	77.1	1.988	0.742
Microbial N supply (g/d)	6.52	8.01	1.632	0.4
Gross energy intake (MJ/d)	16.1	15.1	0.401	0.057
Energy loss in faeces (MJ/d)	4.82	4.67	0.0616	0.677
Energy loss in urine (MJ/d)	0.194	0.271	0.0616	0.259
Energy loss in methane (MJ/d)	0.903	0.531	0.1462	0.044
Energy loss in methane/Gross energy intake (%)	5.62	3.54	0.9659	0.075
Metbolisable energy (MJ/d)	10.2	9.7	0.652	0.479
ME (MJ/kg DM)	11.1	11.2	0.523	0.863

Rumen pH, ruminal VFA concentration and total protozoa count

During the 35-day *ad libitum* feeding, a significant decline in rumen pH was observed in both treatments (Fig.1).

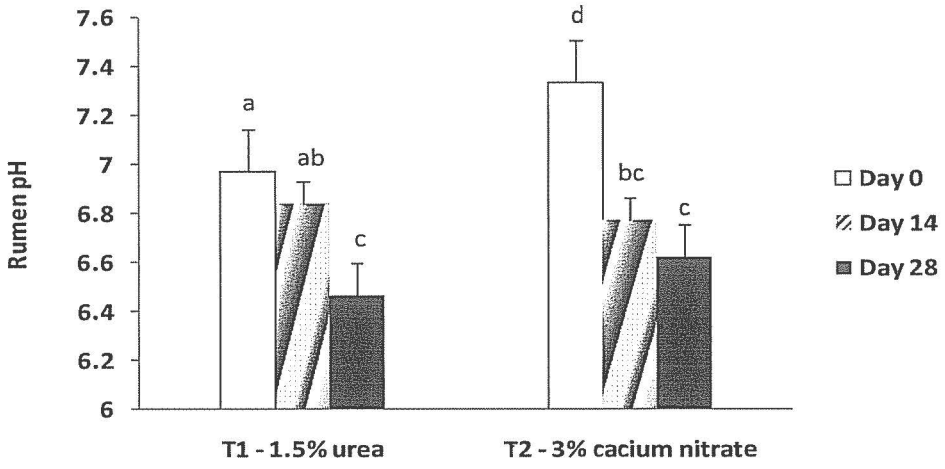

Fig. 1. Rumen pH in *ad libitum* fed sheep receiving the urea-supplemented (T1) or nitrate containing diet (T2) over a period of 35 days. Error bars indicate s.e.d. and a common suffix above error bars indicates non-significant difference.

Concentrations of VFA, molar proportions of acetate and propionate, and molar ratio of acetate to propionate in ruminal fluid of sheep on both treatments were not affected by feeding urea or nitrate (Table 4). Nitrate supplemented sheep tended to have a lower molar percentage of butyrate than urea supplemented sheep ($P = 0.051$). The total protozoa population was not affected by inclusion of urea or nitrate in the feed. The population consisted of small entodiniomorphs (90%) and large entodiniomorphs (4%) and holotrich (5.6%) protozoa.

Table 4. Total VFA concentrations, VFA molar proportions, total protozoa numbers in rumen contents of growing ewe lambs fed *ad libitum* 1.5% urea or 3% calcium nitrate-supplemented diets. Measures were made on day 15 (D15) or day 31 (D31) of feeding.

	T1 - 1.5% urea		T2 - 3% Ca nitrate					
Variable	n = 5		n = 5			*P* value		
	D15	D31	D15	D31	s.e.d.	Treatment Effect	Day Effect	Treatment × Day
Total volatile fatty acids (mM)	90.5 [a]	135.8 [b]	96.0 [a]	109.9 [a]	15.96	0.379	0.019	0.184
Acetate (mol %)	58.9 [a]	54.8 [a]	56.9 [a]	61.2 [b]	2.71	0.271	0.975	0.044
Propionate (mol %)	25.9 [a]	34.4 [b]	30.5 [b]	31.6 [b]	3.06	0.679	0.040	0.108
Butyrate (mol %)	12.7 [a]	9.4 [ab]	10.3 [a]	5.7 [b]	2.06	0.051	0.017	0.666
Acetate : propionate ratio	2.4 [a]	1.6 [b]	1.9 [b]	2.0 [b]	0.37	0.759	0.193	0.075
Total protozoa (× 10^5/ml)	31.9 [a]	7.3 [b]	28.6 [a]	16.6 [a]	12.79	0.745	0.060	0.495

Within rows, means with a common suffix do not differ significantly.

Enteric methane production

The restricted ration (1.5M) that was offered to sheep while they were in the respiration chambers resulted in similar DM intake in all treatments (Table 5). Nitrate supplementation tended (P = 0.06) to lower methane production by 7.7 L/d relative to the urea supplementation. Methane yield (L/kg DM intake) was reduced (P = 0.02) by 35.4% when 1.5% urea was replaced by 3% calcium nitrate in the diet. When this methane yield was multiplied by the feed intake of lambs and LWG over days 1-42, emission intensity (L methane/kg LWG) was calculated to have been reduced by approximately 17.8% in the nitrate-supplemented sheep (P = 0.52) when compared with urea fed sheep, however, this was not statistically different.

Table 5. Dry matter intake, methane yield of sheep offered 1.5M ration of pelleted diet supplemented with 1.5% urea or 3% calcium nitrated over a 6-day methane measurement period

Variable	T1 - 1.5% urea	T2 - 3% Ca nitrate		
	n = 5	n = 5	s.e.d.	*P* value
DMI (g/d)*	862	882	62.5	0.757
Methane production (L/d)	22.4	14.7	3.54	0.061
Methane yield (L/kg DMI)	25.7	16.6	3.17	0.021
DLWG (kg/d)**	0.31	0.25	0.036	0.136
Methane intensity (L/kg DLWG)	76.2	63.0	19.43	0.515

*DMI – dry matter intake was the average value of methane measurement day's intake and the previous day's intake.
**LWG – daily liveweight gain was calculated as (LW at Day 35 – LW at Day 7)/28 days, when sheep were *ad libitum* fed but adjusted to diet.

Methaemoglobin (MetHb) concentration in blood

Blood MetHb concentrations in nitrate-supplemented animals were significantly higher than those of urea-supplemented sheep on Day 14 and 28 (0.71 *vs.* 0.36%, $P < 0.001$). There was a significant increase ($P < 0.001$) in blood MetHb concentrations in nitrate-fed sheep (0.36% on Day 14 *vs.* 0.76% on Day 28). None of the sheep had blood MetHb concentrations higher than 1.2% during the 4-wk feeding of the nitrate containing diet.

Discussion

Effect of nitrate feeding on Methaemoglobin (MetHb) concentration in blood

A major constraint to inclusion of nitrate as a N source in ruminant feeds has been the risk of nitrite toxicity. In ruminants, methaemoglobinaemia could occur if MetHb concentration reaches 20% (Cockrum *et al.* 2008), but methaemoglobinaemia can be managed and avoided by slow adaptation to a dietary source of nitrate. A series of early experiments demonstrated that animals can be gradually adapted to 3-4% KNO_3 with little or no harmful effects in the rumen (Sinclair and Jones 1964;Clark *et al.* 1970;Nolan *et al.* 2010). The adaptation is probably associated with the rumen microorganisms, the liver and the kidney (Alexander

et al. 1972). For instance, the work of Cheng *et al.* (1985) showed that a large number of rumen microorganisms are capable of reducing nitrate or nitrite to ammonia and their population densities increase if nitrate is present in the diet. Based on our findings that the sheep showed no signs of ill health, it was evident that the rumen of sheep gradually adapted to the pelleted diet containing 21 g NO_3/kg DM without ill effect within 4 weeks.

Effects of feeding calcium nitrate on feed intake (FI) and live weight gain (LWG)

One of the major limitations to progress in using nitrate instead of urea as alternative N source has been the lack of information on the effects of long-term feeding a nitrate source on production parameters, bodyweight gain or milk production in ruminants.

As hypothesized, there was no difference in LWG between lambs fed the two iso-nitrogenous treatments, which is consistent with recent research findings of Huyen *et al.* (2010) and van Zijderveld *et al.* (2010). Nguyen *et al.* (2010) fed growing goats (10 kg initial weight) a sugar cane-based diet supplemented with either 5.0% calcium nitrate or 2.6% urea for about 12 weeks. They observed that calcium nitrate supported the same growth rate as urea when used as the principle source of N. (van Zijderveld *et al.* 2010) found that feeding 26 g nitrate/kg DM to crossbred Texel lambs (43 kg initial weight) for 4 weeks had no adverse effects on feed intake and liveweight gain. Additionally, Bruning-Fann and Kaneene (1993) suggested that only when sheep received more than 30 g/kg nitrate in their diet and beef cattle more than 10 g/kg in the diet did they exhibit reduced feed intake and other possible nitrate toxilogical symptoms. While the current study also showed no significant effect of nitrate (21 g nitrate/kg DM) on feed intake relative to urea, there was a 19% decline in LWG explained by a corresponding 13% (though non-significant) difference in intake. Further study of nitrate impacts in the rumen that may affect feed intake is warranted.

Effect of nitrate feeding on whole tract dry matter (DM) digestibility, nitrogen (N) and energy utilisation

Results obtained from the current study showed that nitrate supplementation did not affect DM or N digestibility, relative to urea supplemented sheep. Therefore, it is reasonable to conclude that nitrate was efficiently used as a N source for microbial growth in the rumen.

The fact nitrate supplemented sheep had 13% less feed intake yet produced 23% greater microbial protein outflow to the intestine suggests there may be a greater efficiency of N capture in the nitrate supplemented than the urea supplemented rumen. Nolan *et al.* (2010) also reported that as percent of nitrate increased from 0 to 4%, ruminal microbial N outflow numerically increased from 9.3 to 11.8 g N/d. The apparent increase in microbial N production, though not statistically different, should not be ignored. As mentioned above, sheep consuming the nitrate-supplemented diet ingested more N than did those on urea-supplement diet, however, N retention results showed no diet effect on apparent N digestibility.

In considering energetic efficiency, the energy loss through methane as percent of gross energy intake ($EL_{methane}/GE_{intake}$) when sheep were fed 1.5M urea-supplemented diet was 5.6%, which falls into the range of 5.5–6.5% of gross energy intake lost via methane for the world's cattle, sheep and goats (Johnson and Ward 1996). We observed that energy loss in methane was reduced to 3.5% when sheep consumed the nitrate-supplemented diet. Despite nitrate-supplemented diet having lower energy loss through methane as a percentage of gross energy, ME intake did not significantly differ between the treatments. The difference in ME intake estimates between treatments was non-significant. van Zijderveld *et al.* (2010) found that sheep fed nitrate had lower heat production (sheep on all treatment consumed the same amount of gross energy during the heat production measurement) and they argued that the reduced heat production resulted from a reduced ME; however, ME intake was not measured in the study of van Zijderveld *et al.* (2010); therefore, it is difficult to reconcile with our results.

Effect of nitrate feeding on rumen pH, Ruminal VFA concentration and total protozoa count

The significant decline in rumen pH observed (Fig. 1) indicate the rumen had not reached a stable ecological and chemical status by day 14 or 15. Warner (1962) reported that at least 10 days were required

for the microbial populations to stabilise after changing a single sheep from roughage to a concentrate diet. Stable rumen pH observed after 4-week-adaptation indicates that urea or nitrate-supplemented diet did not cause dramatic changes in rumen conditions, which suggests that the most important alternations and modifications in the rumen ecology occurred during the adaptation period. The above claim is further supported by the measurements of ruminal VFA concentrations and protozoa counts (Table 4). The total protozoa population was not affected by inclusion of urea or nitrate in the feed. This supports the findings of Nolan *et al.* (2010) and van Zijderveld *et al.* (2010).

It appeared that nitrate was rapidly reduced, causing a diversion of electron from butyrate formation to nitrate reduction. Acetate followed the expected pattern, with acetate increasing following feeding nitrate (Nolan *et al.* 2010); however, feeding nitrate did not affect molar proportion of propionate, which is consistent with previous observation (van Zijderveld *et al.* 2010).

Effect of nitrate feeding on enteric methane production

Leng (2010) reviewed the literature at length and concluded that the reduction in methane production varied from 16 to 50%, depending on inclusion rate of nitrate and type of diet used in the previous studies. Stochiometrically, 1 mol nitrate fed per sheep per day should reduce methane production by 1 mol or 22.4 L per day in nitrate-supplemented sheep. In our study, sheep on the nitrate treatment consumed approximately 0.354 mol nitrate per day, which theoretically should reduce methane production by 7.9 L per day. The actual observed reduction in methane production was 7.7 L per day, which is 97.5% of the expected stoichiometric potential.

Conclusion

Feeding strategies, such as calcium nitrate supplementation, reduce enteric methane emissions, hence, minimise the energy loss via enteric methane production. In terms of liveweight gain and feed conversion ratio, animals receiving 3% calcium nitrate had similar performance to those fed 1.5% urea as a source of fermentable N. From the point view of reducing greenhouse gas emissions from sheep, nitrate could replace urea as a source of fermentable N supplements for the sheep industry, provided nitrate was fed at appropriate levels and animals were adjusted to nitrate gradually.

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References

Alexander M, Army TJ, de Serres FJ, Frink CR (1972) Accumulation of Nitrate. In 'Hazards of nitrate, nitrite and nitrosamines to man and livestock.' (Eds A Board, DoBa Agriculture and NR Council) pp. 1-95. (National Academy of Sciences: Washington D. C.)

Bird SH, Hegarty RS, Woodgate R (2008) Persistence of defaunation effects on digestion and methane production in ewes. *Australian Journal of Experimental Agriculture* **48**, 152-155.

Blaxter KL, Clapperton JL (1965) Prediction of the amount of methane produced by ruminants. *British Journal of Nutrition* **19**, 511-522.

Brouwer E Report of sub-committee on constants and factors In 'Third Symposium Energy Metabolism. European Association of Animal Production', 1965, London, UK. (Ed. KL Blaxterd), pp. 441-443

Bruning-Fann CS, Kaneene JB (1993) The effects of nitrate, nitrite, and N-nitroso compounds on animal health. *Veterinary and Human Toxicology* **35**(3), 237-253.

Chen XB, Chen YK, Franklin MF, Ørskov ER, Shand WJ (1992) The effect of feed intake and body weight on purine derivative excretion and microbial protein supply in sheep. *Journal of Animal Science* **70**, 1534-1542.

Cheng KL, Phillippe RC, Kozub GC, Majak W, Costerton JW (1985) Induction of nitrate and nitrite metabolism in bovine rumen fluid and the transfer of this capacity to untreated animals. *Canadian Journal of Animal Science* **65**, 647-652.

Clark JL, Pfander WH, Bloomfield RA, Krause GF, Thompson GB (1970) Nitrate containing rations for cattle supplemented with either urea or soybean meal. *Journal of Animal Science* **31**(5), 961-966.

Cockrum R, Austin K, Ludden P, Cammack K (2008) Effects of elevated dietary nitrate on production and reproduction parameters in Suffolk ewes. *American Society of Animal Science* **59**.

Entwistle KW, Knights G (1974) The use of urea-molasses supplements for sheep grazing semi-arid tropical pastures. *Australian Journal of Experimental Agriculture and Animal Husbandry* **14**, 17-22.

Farrell R, Knights G (2001) 'Livestock nutrition - Supplementary feeding using the Toorak urea block.' In (Primary Industries and Fisheries, Queensland Government) Available at http://www2.dpi.qld.gov.au/sheep/8073.html

Guo WS, Schaefer DM, Guo XX, Ren LP, Meng QX (2009) Use of nitrate-nitrogen as a sole dietary nitrogen source to inhibit ruminal methanogenesis and to improve microbial nitrogen synthesis in vitro. *Asian-Australian journal of animal science.* **22**(4), 542-549.

Hegesh E, Gruener N, Cohen S, Bochkovskf R, Shuval HI (1970) A sensitive micromethod for the determination of metheemoglobin in blood. *Clinica Chimica Acta* **30**, 679-682.

Huyen LTN, Do HQ, Preston TR, Leng RA (2010) 'Nitrate as fermentable nitrogen supplement to reduce rumen methane production.' In Livestock Research for Rural Development (Fundación CIPAV: Cali,Colombia) Available at http://www.lrrd.org/lrrd22/8/huye22146.htm [Verified 02/03/2011]

IAEA (1997) 'Estimation of rumen microbial protein production from purine derivatives in urine.' In (Vienna, Austria) Available at http://www.iaea.org/programmes/nafa/d3/public/tecdoc-945.pdf [Verified January 2010]

Johnson DE, Ward GM (1996) Estimates of animal methane emissions. *Environmental Monitoring and Assessment* **42**(1-2), 133-141.

Leng RA (2010) Further considerations of the potential of nitrate as a high affinity electron acceptor to lower enteric methane production in ruminants. In 'International Conference on Livestock, Climate Change and Resource Depletion. ' (Eds R Preston and B Ogle) pp. 9-11: Champasack University, Pakse, LAO PDR)

McCrabb GJ, Hunter RA (1999) Prediction of methane emissions from beef cattle in tropical production systems. *Australian Journal of Agriculture Research* **50**, 1335-1339.

NGGI (2009) National greenhouse gas inventory. In. ' (Australian Government, Department of Climate Change: Canberra)

Nguyen NA, T.H. K, Duong NK, Preston TR (2010) Effect of calcium nitrate as NPN source on growth performance and methane emissions of goats fed sugar cane supplemented with cassava foliage. In

'Mekarn Conference on Livestock Production, Climate Change and Resource depletion. ' (Eds TR Preston and B Ogle): Pakes, Laos)

Nolan JV, Hegarty RS, Hegarty J, Godwin IR, Woodgate R (2010) Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. *Animal Production Science* **50**, 801-806.

SheepExplorer (2003) 'The excel spreadsheet SheepExplorer of GRAZPLAN.' In Available at http://www.pi.csiro.au/grazplan/ [Verified Accessed April 2005]

Sinclair KB, Jones DIH (1964) Nitrate toxicity in sheep. *Journal of the Science of Food and Agriculture* **15**(10).

van Zijderveld SM, Gerrits WJJ, Apajalahti JA, Newbold JR, Dijkstra J, Leng RA, Perdok HB (2010) Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *Journal of Dairy Science* **93**, 5856-5866.

Warner ACI (1962) Some factors influencing the rumen microbial population. *Journal of General Microbiology* **28**, 129-146.

Delivery of dietary nitrate through nitrate containing lick-blocks

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Objectives:

1. Quantify the effect of calcium nitrate inclusion on voluntary feed intake of lick-blocks by sheep.
2. Quantify the methane mitigation arising from voluntary consumption of nitrate enriched lick-blocks
3. Determine the voluntary intake of nitrate enriched lick-blocks in sheep grazing pasture.

Methods and Materials:

A three-part study was undertaken to assess the intake of dietary nitrate enriched lick-blocks (NEBs) and the impact of this consumption on enteric methane production.

In an initial study, mature fine-wool Merino sheep (n = 8) were individually housed and adapted to a diet of blended (1:1 w:w) oaten/lucerne chaff. (9.3 MJ ME/kg DM; 11.9%CP), fed at 1.4x Maintenance/d based on liveweight with intake adjusted for LW weekly. Sheep adapted to this diet were then offered free access a lick-block (17 kg) which was placed in a secondary feed bin in the pen with each sheep. The experiment was composed of 4 x 8d feeding periods. In each feeding period, all sheep were offered iso-nitrogenous blocks, being either urea-containing blocks (n=4) or nitrate containing blocks (n=4). The block was provided for 8d, with blood samples being taken on day 2,5 and 8 of block access and a sample of rumen fluid collected on day 8 by esophageal intubation. Blocks were weighed daily to determine consumption. In period 1, blocks containing 0.78%N as either urea or nitrate were offered, while in periods 2, 3 and 4, blocks offered contained 1.41%, 2.82%, and 4.23%N respectively were offered. All blocks were manufactured by Olsson's stock feeds (Brisbane, Queensland).

In study 2, the methane production of sheep offered the same basal feed as study one and the average voluntary intake of lick-block providing either 2.82% nitrate-N or 2.82% urea-N from study 1 was determined. For 3d, all sheep were again offered chaff at 1.4x maintenance energy intake and were supplemented with 55g of either powdered urea or powdered NEB containing 2.82%N. In order to ensure the powdered block was fully consumed, the powdered blocks were dissolved in aqueous slurries (kept refrigerated) and aliquots taken daily and sprinkled on the basal feed then mixed in thoroughly by hand. No refusals occurred in the feeding prior to methane measurement. After 3d acclimation (sheep had been consuming 4.23%N blocks for the previous 8 days), sheep were placed in individual open circuit chambers for 21h and offered their feed (mixed with slurried block) on chamber entry. On day 3, two urea supplemented sheep and two nitrate supplemented sheep were placed in respiration chambers and on day 4 the two remaining sheep on each treatment were placed in respiration chambers. There were some refusals from nitrate-fed sheep while in the respiration chambers, so these sheep were placed in respiration chambers for a further 21h commencing day 5

Study 3 consisted of a field assessment of the voluntary intake of urea containing or iso-nitrogenous nitrate containing lick-blocks by grazing sheep. Fifty merino sheep were drawn from a larger flock to provide a set of animals of uniform starting liveweights. The animals were

allocated into two groups by stratified randomization on liveweight and then randomly allocated to paddock. A 5ha paddock of predominantly *Danthonia* with annual ryegrass invasion was divided into two equal portions using electric fencing. One lick-block (either 2.82%N as urea or 2.82%N as nitrate) was placed under a roof (to shield from rain) in each paddock. In order to overcome possible differences in pasture availability or quality affecting block intake, the blocks, together with the sheep consuming them, were swapped between paddocks after two weeks and allowed a further 2 weeks to graze after relocation. Blood samples were taken from all sheep throughout this study.

Results

Experiment 1.

A fixed allocation of basal ration was offered so there was no effect of block type or N inclusion on intake of the basal diet, with all animals eating >98%of feed offered (P>0.05).

Block intake showed a N-source x N level interaction (P=0.018), such that while intake of urea containing blocks increased as N content increased (25g to 76g/d), intake of nitrate containing blocks decreased (81g to 21g/d) as N content rose (Figure 1).

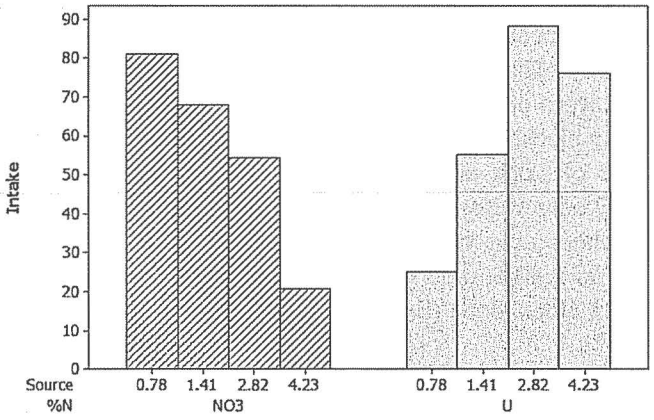

Figure 1. Voluntary daily intake of lick block containing either nitrate (at one of 4 levels) or urea (at one of 4 levels). Each value is the mean of four sheep.

Blood methaemoglobin (MHB) concentration was significantly higher in nitrate supplemetned sheep (1.37%) than in nitrate supplemented sheep 0.55% MHB. The levels of MHB in blood of nitrate supplemented sheep, however, were very low and posed no theat to animal health.

Experiment 2.

Exact feed intakes for the respiration chamber studies are unavaialble at present (student absconded with data), however calculations assuming no refiusals are provided below.

When sheep were fed their average voluntary daily intake of the nitrate blocks containing 2.82%N as supplement (55g/d), methane yield (g CH_4/kg DM) was significantly reduced realtive to emission from sheep fed an equal quanitty fo supplementary nitrogen as urea (24.2 g/kg DMl v 31.23 g/kgDM).

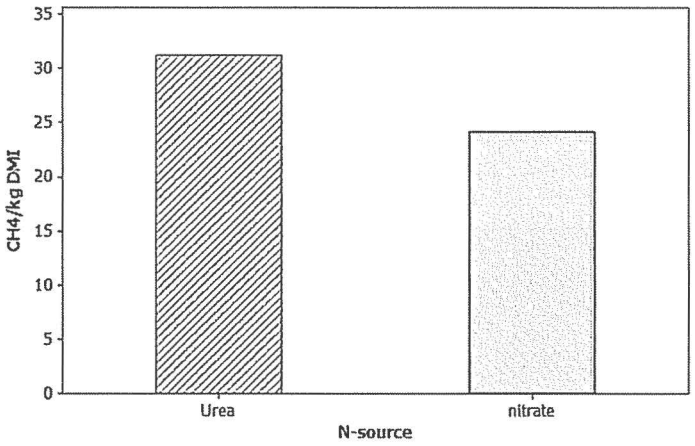

Figure 2. Methane yield of sheep fed either 55g of block containing 2.82% nitrate-N. Or an equal amount of nitrogen provided by a block containing urea.

Experiment 3.

This 'split paddock' trial was characterised by shep growing faster in one padock than another. In the first 2 weeks, urea supplemented sheep grew faster than nitrate fed sheep, but after the sheep were swapped between paddocks, the reverse was true ($P<0.05$), indicating that one paddock used provided superior nutrition than the other.

Average daily intake of lick block by sheep was significanlty less for nitrate containing blocks (mean 19.7 g/d) than for isonitrogenous urea containing blocks (34 g/d; $P<0.05$; Figure 2).

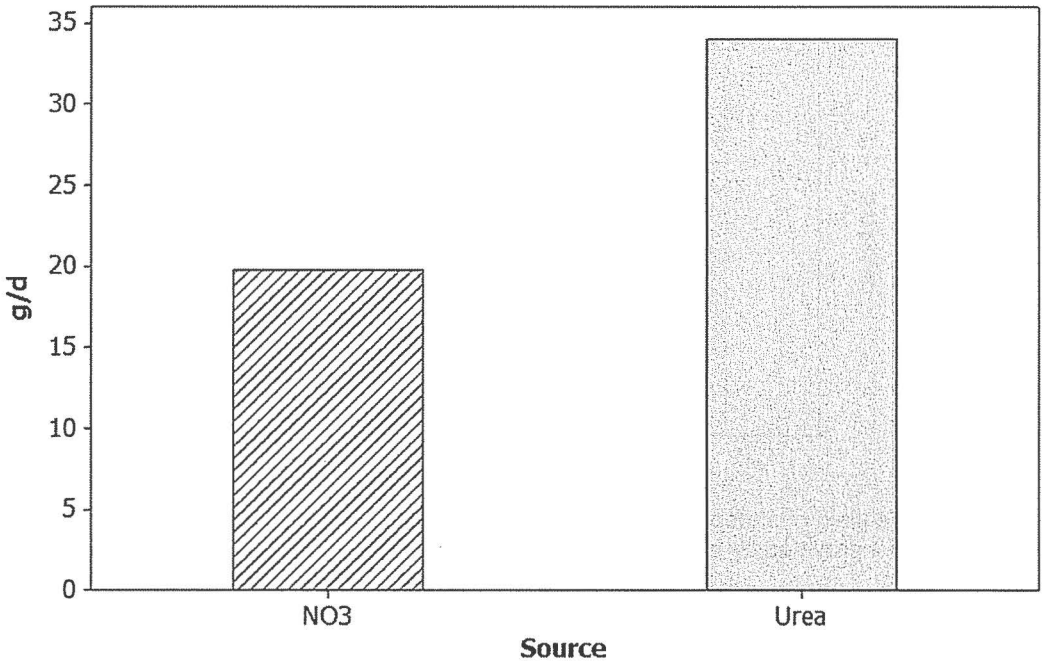

Figure 3. Average daily intake of lick block per sheep within flocks of 25 grazing sheep provided with either 1 nitrate block or 1 urea block containing 2.82%N as the additive

As in experiment 1, nitrate supplemented sheep tended to exhibit a higher blood MHB concetration than did urea supplemented sheep ($P<0.10$) but there was significant sample-day variaion in MHB concentration as well. Also in keeping with previous observations, nitrate supplementation stimulated wool growth (9.5 mm v 8.8mm per month $P<0.05$) relative to that on the urea supplement.

Conclusions:

This experiment demonstrates a number of important facts around delivery of nitrate in the field when delviered through lick-blocks.

- Firstly, at no stage did blood MHB concentration exceed 10% or approach levels which could be anticipated to affect animal health.
- Block intake declined with increasing calcium nitrate inclusion. This is likely to be a palatability issue since increasing inclusion of urea (which is also metabolised to rumen nitrate) increased intake of those blocks.
- The average intake of nitrate occuring with *ad-libitium* intake of blocks containing 2.82% Nitrate-N was suificient to cause a significant (22%) decrease in daily methane production of sheep.
- The average *ad-libitum* intake of the same (nitrate) containing blocks in grazing sheep over 4 weeks was less than half of that in pen studies (20 g'd v 54 g/d) and would be expected to deliver an 8% reduction in daily methane emissions across the flock.

- The intake of block was not affected by the quality of pasture available, resulting in no period effects on block intake in this study. This is indicative that lick-block intake may be robust over time and so its mitigation effect can also be anticipated to be stable over season.

Acknowledgements

The provision of nitrate and urea containing blocks by Olsson's is appreciated, as is the leadership of Prof. Ron Leng.

Elimination of Rumen Protozoa as a Mitigation and Production-Enhancing Technology

Anaerobic ciliate protozoa are natural inhabitants of the gastrointestinal tract of most animals and all grazing ruminants. They are however not necessary for rumen function or animal survival, and ruminants free of enteric ciliate protozoa have been produced through caesarian section and isolation of sheep (Ivan 1988), through rumen emptying and treatment of digesta (Morgavi et al., 2008), and through chemical treatment of the rumen (Bird and Leng 1978). Ciliate protozoa are a major cause of bacterial lysis in the rumen and their absence increase the quantity of feed- and microbial-protein available to the host animal for absorption. This gives productivity advantages to animals in which production constrained by protein availability (eg. Wool production and weaner growth). A range of endo- and ecto-symbiotic microorganisms including methanogens live in or on rumen ciliates, and literature has indicated elimination of protozoa reduces methane production (eg. Kreuzer 1986; Hegarty 1998). However, more recent studies in sheep with long term stable protozoa-free rumens have not shown a reduction in methane production or a change in VFA balance.

The purposes of this component of BCCH1010 were to quantify the productivity and methane emissions of cattle rendered protozoa free by chemical treatment as summarized below:

By 1 December 2011 identify and evaluate effective anti-protozoal chemicals on methane reduction and productivity in cattle for commercial development, specifically:

. The impact of defaunation on methane production and productivity of cattle determined

. A suite of chemical families with high efficacy against rumen protozoa evaluated

These objectives were achieved by:

(1) Establishing an in-vitro screening procedure to identify compounds with high protozoal activity but causing an otherwise minimal disturbance to rumen fermentation.
(2) Managing and measuring emissiosn from cattle known to be protozoa-free.

The details of these procedures and investigations are described in the following sections:

In-vitro Investigations of Compounds for Activity against Rumen Protozoa

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Abstract

Eighteen compounds, representing 11 different modes of predicted antiprotozoal action, were tested at concentrations varying from 300 to 5 ppm.

In general, 5-nitroimidazoles produced the greatest decrease in protozoa count and in methane production but with a concomitant reduction in overall gas production. For example, ronidazole at 100 ppm concentration resulted in a protozoa count of 7% relative to that of the negative control and gas production of 40% relative to control.

When the major protozoan species present was *Entodinium exiguum*, 5-nitroimidazoles were ineffective.

Miltefosine at 50 ppm was found to cause a reduction in protozoa number to about 60% of control with little reduction in total gas production and was also found to have activity in the experiment in which 5-NIs produced no reduction in protozoa count.

Combination treatments with ronidazole (the most effective 5-nitroimidazole against protozoa) and miltefosine may prove to have optimum effectiveness and should be investigated.

The 5-nitroimidazole, C17, produced the largest reduction in the volume of methane of all compounds tested and the lowest reduction in total gas production of the 5-NIs and should be investigated as a potential treatment for reducing methane emissions. Other 2-styryl-5-nitroimidazoles should be synthesised and investigated with the aim of further increasing selectivity for toxicity to methanogens.

Introduction

The role of rumen protozoa

Due to their relatively large size, ciliate protozoa may constitute almost half of the microbial mass in the rumen, despite their population being less than one millionth of that of rumen bacteria. Protozoa play roles in the fragmentation of food particles, digestion of cellulose, maintenance of rumen pH and removal of oxygen from the rumen. They are also a source of hydrogen used by methanogens in the formation of methane. Removal of protozoa (defaunation) has been shown to result in an increase in number and a change in the species profile of rumen bacteria and a reduction in methane production and rumen ammonia content.

There have been mixed results concerning the effect of defaunation on the growth of the host animal but there is evidence that defaunation improves the growth of animals fed a poor quality diet.

Thus, an efficient method of defaunation could prove valuable both for reduction of methane emissions by livestock and for increased animal productivity. This project investigated the use of a number of chemical classes for their activity against rumen protozoa *in vitro*.

Classes of compounds tested and their proposed modes of action

The aim of the research was to find compounds capable of reducing or eliminating rumen protozoa while causing minimal disruption to other rumen organisms and having no ill effects on the mammalian hosts. To this end biosynthetic pathways, either unique to protozoa or implemented differently in protozoa, were targeted. Examples included the inability of protozoa to synthesis choline, making them reliant on dietary choline and thus susceptible to choline transport inhibitors and their lack of substrate specificity in nucleotide phosphorylation allowing purine analogues to be incorporated into and interrupt nucleotide interconversion. Test compounds were selected based on:

- reported activity against rumen protozoa;
- previous or proposed use as medical antiprotozoal agents, particularly against anaerobic protozoa such as *Plasmodium, Trichomonas* and *Leishmania*;
- reported activity as choline transport inhibitors;
- chemical structure.

A list of compounds tested and their expected modes of action is given in Table 1.

Table 1. Compounds tested in this work

Chemical class	Mechanism	Compounds tested
5-nitroimidazoles	formation of toxic radicals under reducing conditions	metronidazole, ornidazole, ronidazole, C17
purine analogues	interfere with purine nucleotide interconversion and purine salvage	allopurinol, toyocamycin, 6-methyladenine
quaternary ammonium compounds	prevent choline uptake	hemicholinium-3, hexadecyltrimethyl ammonium bromide (HDTAB), dimethylaminoethanol (DMAE)
phospholipid analogues	inhibits phosphatidylcholine synthesis	miltefosine
triazines	probably dihydrofolate reductase inhibitors	anilazine
polyphenols	histone acetyltransferase inhibitor, promotes of reactive oxygen formation	curcumin
acridine derivative	unproven but possibly targets protozoal cell membrane	quinacrine
benzimidazoles	inhibit □-tubulin synthesis	fenbendazole
detergent	disrupt cell membrane	alkanate (30% sodium lauryl diethoxy sulphate (SLDS))
polypeptide antibiotics	blocks dephosphorylation of C_{55}-isoprenyl pyrophosphate	bacitracin
nitrofurans	DNA crosslinkers	furazolidone

Method

A number of trial experiments were run to optimise experimental conditions, including incubation vessel, incubation medium, nutrients and methods of assessing protozoal viability (see previous milestone report for details).

The final method for *in-vitro* testing of antiprotozoal activity was as follows:

Incubations of rumen fluid were conducted in 60 ml plastic syringes (Terumo: Plate 1).

Each syringe was fitted with a three-way luer-lock tap (BD-Connecta) and ground lucerne chaff (500□m sieve size; 200 ± 10 mg chaff/syringe) was weighed directly into the syringe. Syringes were pre-warmed in a 39°C water bath until inoculation.

Rumen fluid was collected from sheep by rumen cannula approximately 30 minutes prior to the commencement of inoculations and maintained at 39°C. A vessel, fitted with one tube to allow withdrawal of incubation medium and another tube to allow constant bubbling of CO_2 through the incubation medium, was placed in a 39°C water bath and charged with a modified Soliva-Hess buffer (220 ml) while flushing with CO_2 (BOC anaerobic grade). Approximately 15 minutes prior to commencement of incubations, rumen fluid (110 ml) was added via syringe to the vessel to provide a buffered rumen fluid inoculum for dispensing into incubation syringes.

Test compounds (10 mg) were dissolved in DMSO (Fluka) (2.0 ml) to give a stock solution of 5,000 ppm concentration. Miltefosine was found not to be readily soluble in DMSO and was used as an aqueous solution instead. Stock solutions were stored at 4° when not in use.

Immediately prior to the commencement of incubations, the appropriate volume of stock solution (measured by Epindorf Multipette) was added to the syringe and a corresponding volume of DMSO, to give a consistent total volume of 500□L DMSO/syringe, was added. The buffered incubation medium (10 ml) was then drawn up into the syringe, the syringe shaken well, the initial volume of gas read

from the scale on the syringe and the syringe returned to the shaking water bath for incubation. The duration of incubations was measured from the time of drawing up incubation solution. Incubations continued at 39°C for 18 h, with incubations commenced (and completed) in a staggered program using three minute intervals.

The position of each syringe in the water bath (and hence order of commencement of incubation) was assigned on a randomised basis. In general, treatments were duplicated and four negative controls (DMSO only) were used in each run. All runs also incorporated a positive control (50 or 100 ppm metronidazole). Concentrations of test compounds ranged from 5 to 300 ppm.

After 18 h. the syringe was removed from the shaking water bath and the final gas volume read. The tap was then removed from the syringe and the liquid expelled into a 25 ml plastic vial and a clean tap fitted to the syringe. The syringe was set aside for later measurement of the gas composition.

The pH of the liquid was measured (Eutech EcoScan 5/6) and then two aliquots (each 0.67 mL) were removed, using a Gilson pipettor fitted with a wide orifice tip, for VFA analysis and protozoal count.

Analysis of Gas Composition.

Gas samples were pumped directly from the incubation syringe, through two 17 mm syringe filters (Chromacol, PTFE, 0.45□m), into a Varian CP4900 Gas Chromatograph, fitted with thermal conductivity detectors. Three channels consisting of a Molsieve 5Å (MS5A) column, with argon as carrier, a Molsieve 5Å column with helium as carrier and a CP-PoraPLOT U (PPU) column with helium as carrier were used to determine hydrogen and methane, methane and carbon dioxide respectively. In general duplicate measurements were conducted on each gas sample, with the exception of Run 4 in which only syringes 1-5 and 24 had duplicate injections.

Volatile Fatty Acid Analysis

At the completion of each incubation period, an aliquot of the incubation medium (0.5 ml) was pipetted into a 1.5 ml Eppindorf microcentrifuge tube containing an isocaproic acid internal standard solution (1.0 ml). The samples were centrifuged (13000g x 10 minutes) and the supernatant transferred to autosampler vials for analysis.

The samples were chromatographed on a Varian CP-3800 Gas Chromatograph, using Flame Ionisation detection. The instrument has duplicate injectors, columns and detectors allowing near concurrent injections of samples. Duplicate injections of each sample were made into each column with a 1.5 min delay between injection into the front and middle columns. Injector temperature was 200° C and detector temperature was 220° C. The carrier gas was nitrogen with a flow rate of 38 ml/min. The column oven used the following temperature program*:

temp(°C)	Rate(°C/min)	Hold(min)	Total (min)
115	0	1.5	1.5
120	5	3	5.5
140	10	3.5	11
150	10	2.5	14.5

* A temperature program was required due to the presence of DMSO in the samples. DMSO undergoes some thermal decomposition under the acidic conditions used, resulting in the formation of products which eluted at the beginning of the run, overlapping the acetate and propionate peaks. The use of temperature programming reduced this overlap.

(Note this is the temperature program experience by samples injected into the front injector. Due to the 1.5 minute delay before injection into the middle injector, those samples would experience a slightly different temperature program.

Under the chromatographic conditions used DMSO was found to co-elute with isocaproic acid, preventing normalisation between injections by means of the internal standard. Repeated runs indicated that reproducibility was acceptable without the use for normalisation. The concentrations of each VFA determined by reference to a commercial standard VFA mixture (Supelco).

Protozoal Counts

At the completion of a run, an aliquot of the incubation medium (0.5 ml) was pipetted into a 5 ml plastic vial containing a formaldehyde/sodium chloride solution (0.25 ml). One drop of Brilliant Green stain was added and after standing for 1-2 hours, the solution was diluted with 30% glycerol (1.5 ml) and protozoal numbers determined on a counting slide by visual enumeration.

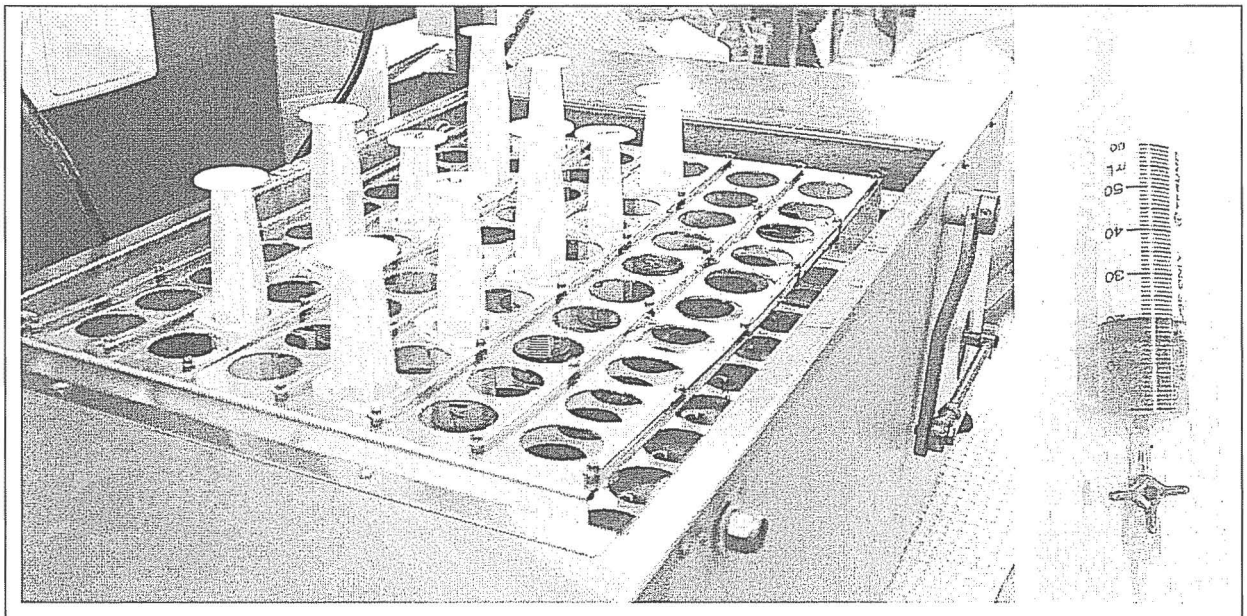
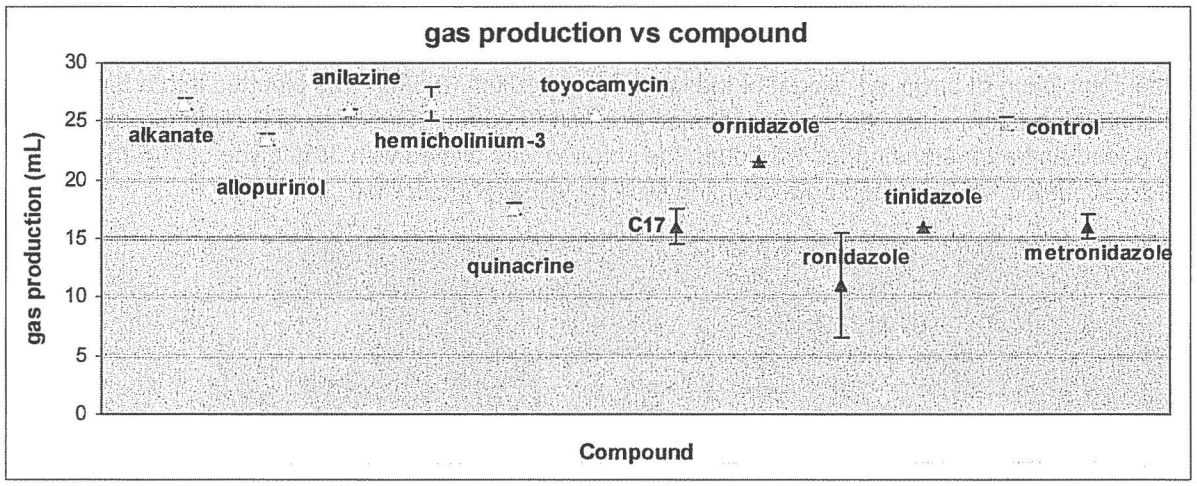


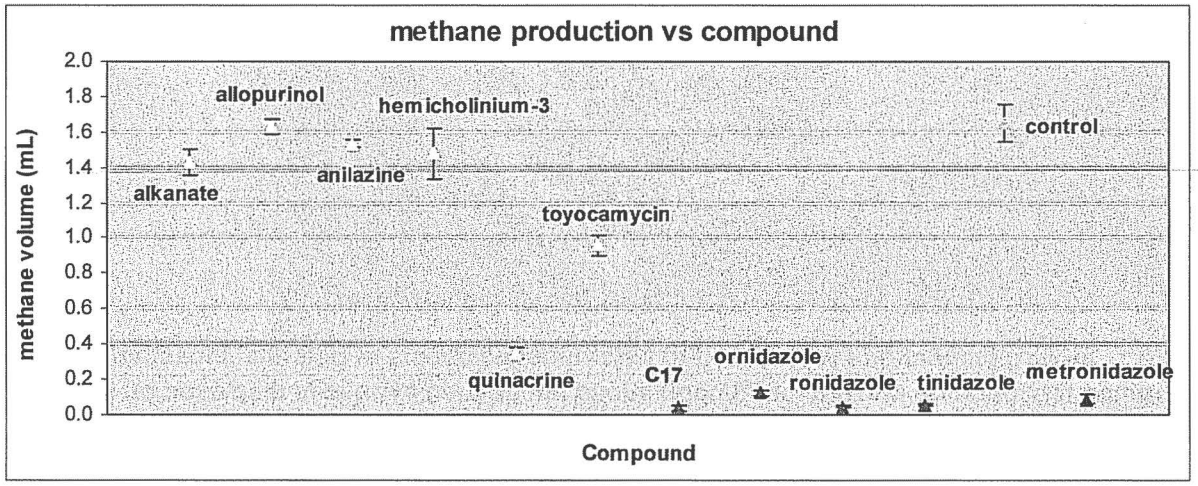
Plate 1. In-vitro incubation system used to determine the effects of potential bioactive agents on rumen fermentation and microbial populations. Incubation syringe is displayed on right.

Results and Discussion

Preliminary Observations

In all 19 experiments were run. Of these 6 were trial experiments to establish optimum experimental conditions and 13 experiments were actual bioassays, each usually measuring the effects of 10 compounds, in duplicate, as well as four controls and two incubations with metronidazole as a positive control. Figure 1 shows the results of a typical experiment (Experiment 14).





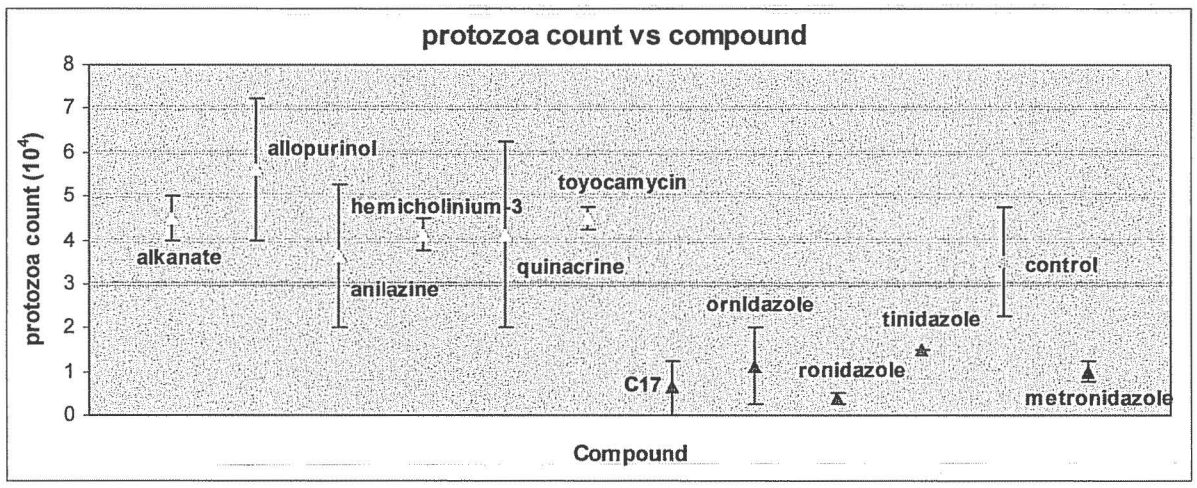


Figure 1. Results from a typical bioassay showing total gas production (top), methane production (middle) and protozoa number (bottom). 5-nitroimidazoles are denoted by red triangles. Error bars indicate the range of two measurements (four for negative controls). The concentration of all test compounds was 100 ppm.

Initially compounds were tested at 100 ppm concentration. Compounds showing good activity at this concentration were subsequently tested at lower concentrations (down to 5 ppm, while compounds inactive at 100 ppm were further tested at higher concentrations (up to 300 ppm).

In general, the 5-nitroimidazoles had the greatest effect on total gas production, methane production and on protozoa numbers. As a result the 5NI, metronidazole, was used as a positive control for all later runs.

There was a large variation from experiment to experiment in the measurements for the negative (DMSO) and positive (metronidazole) controls, particularly in the protozoal count (see Figure 2 for variation in the 8 most recent runs).

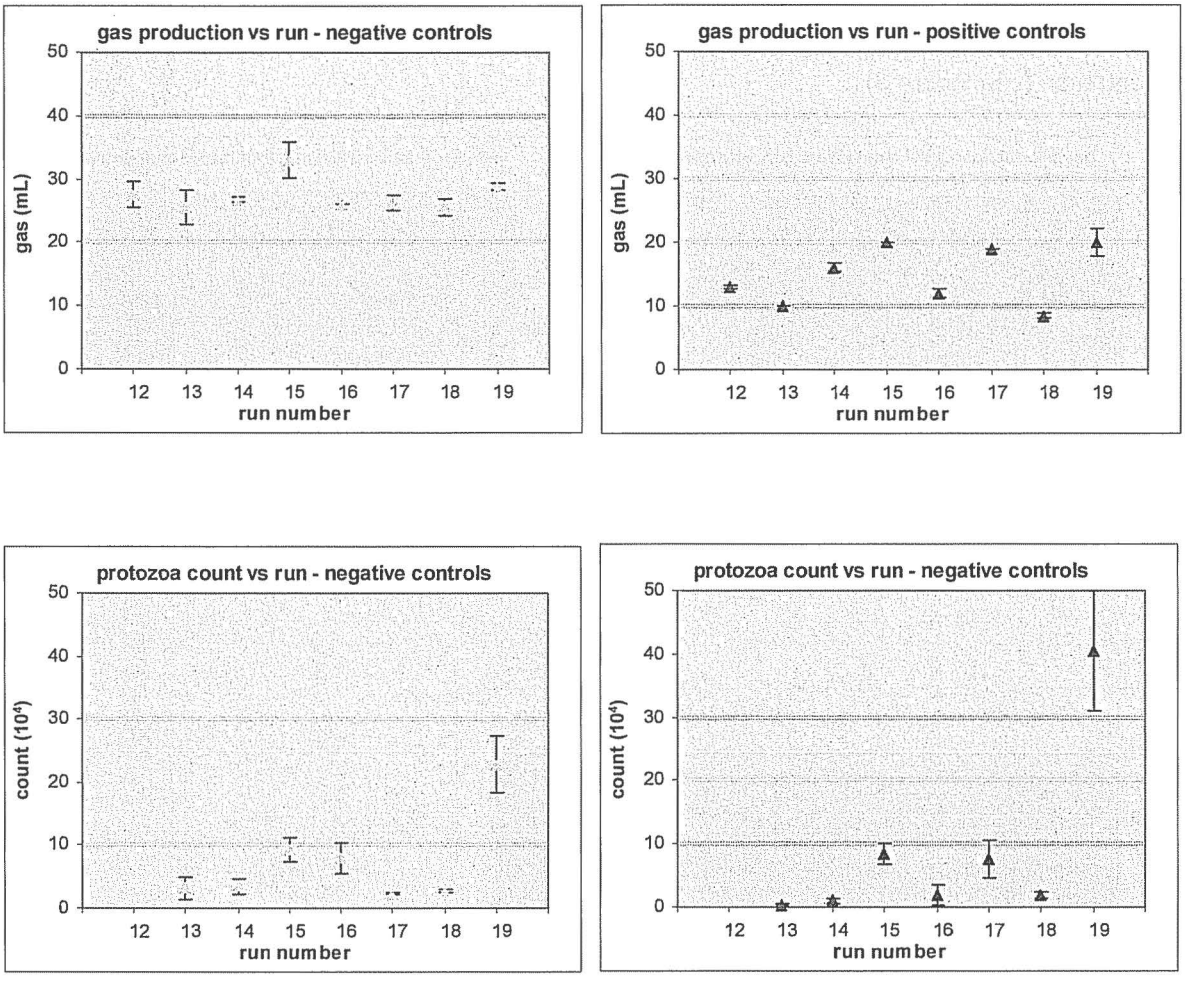


Figure 2. Run-to- run variation in total gas production (top) and protozoa number (bottom) for negative (left) and positive (right) controls. Error bars indicate the range of two measurements for positive controls (50ppm metronidazole) and +/- one standard deviation of four measurements in the case of negative controls (DMSO). There are no protozoa counts for run 12.

It can also be seen that in two runs (17 and 19) the positive control has a higher protozoa count than the negative control, while run 15 shows little difference between positive and negative controls. In the case of run 19, observation of the rumen fluid under the microscope prior to its use in the

experiment revealed that the only protozoa present were very small entodiniomorphs, probably *E. exiguum*, whereas more typically the rumen fluid used contained a mixed population, including *E. caudatum* and other similar sized entodiniomorphs, larger entodiniomorphs, *Dasytricha* species and *Isotricha* species. For the other two runs no identification of the protozoa was attempted prior to the experiment but the counting statistics indicated only small entodiniomorphs were present. In the counting procedure, no distinction is currently made between *E. exiguum* and the other slightly larger species, which are still categorised as small entodiniomorphs (two categories of entodiniomorph (large and small) and two of isotrich are differentiated during counting). It seems possible that 5-nitroimidazoles (5-NIs) have little or no effect on *E. exiguum* and, in populations consisting largely of this species, these compounds perform no better as antiprotozoal agents than the negative control. The elevated count for metronidazole relative to the control in these cases may be due to the reduction in bacteria numbers in the incubation (this is consistent with the typical reduction in gas production seen in these anomalous runs). It seems possible that *E. exiguum* may compete with bacteria, possibly for nutrients, and the reduction of bacterial numbers by the 5-NIs allows a higher population of protozoa. A similar effect may account for the dose response characteristics observed for metronidazole (see figure 3) in one experiment (Exp. 9), where the protozoa population at the lower concentrations tested are higher than those of the negative control, whereas the gas production is reduced relative to control at all concentrations and is directly proportional to the logarithm of the concentration (see Figure 3).

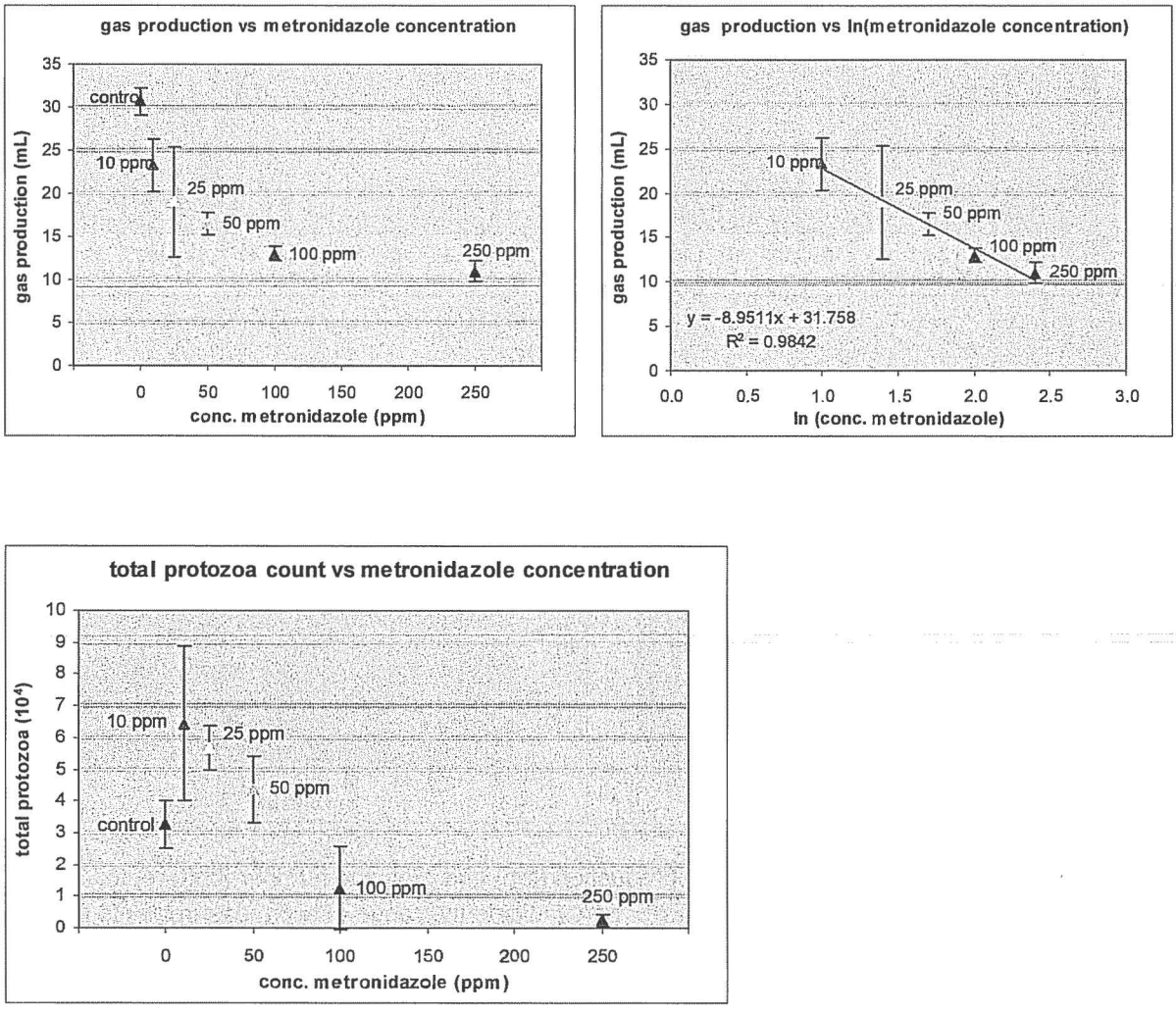


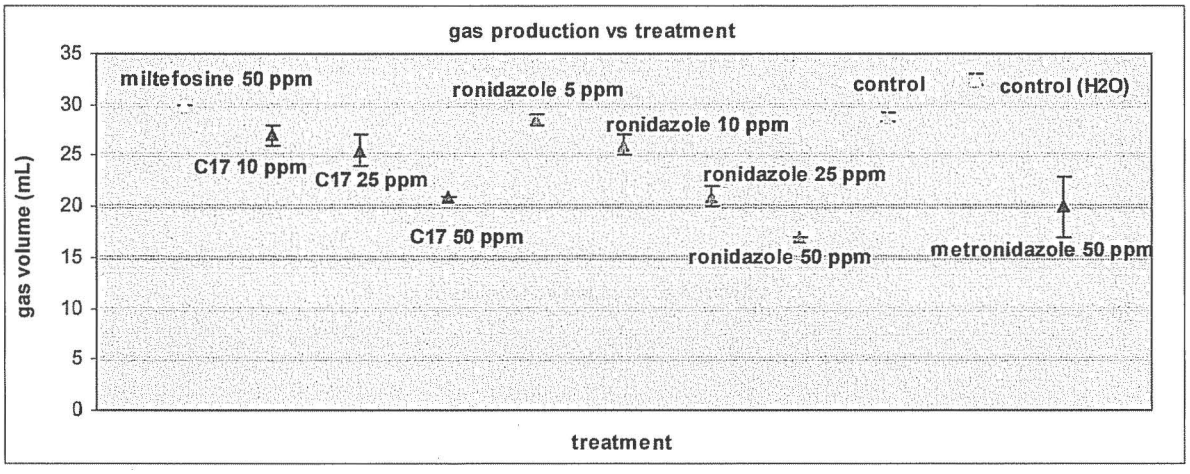
Figure 3. Experiment 9 - Dose response study for metronidazole
Top left: gas production vs concentration, top right: gas production vs ln(concentration)

Bottom: protozoa count vs concentration. Points are the average of 4 measurements for metronidazole and 6 measurements for the negative control.
Error bars are +/- one standard deviation.

A similar effect is observed for all 5-nitroimidazoles in Run 19. While they exhibit roughly linear dose-response relationships for gas production and methane production (see Figure 4, in the case of protozoa count both C17 and ronidazole result in higher counts than the control at all concentrations, with the dose-response curves having maximum counts at 25 ppm. This too supports the proposition that the elevated protozoa count is due to reduction of bacterial numbers. At concentrations below 25 ppm the antibacterial effect is reduced until the count approaches that of the control.

Total VFA production in experiment 19 also showed little variation with treatment, whereas in Experiment 18, 5-nitroimdazoles caused a substantial decrease in total VFA production (See Figure 5). 5-nitroimidazoles also caused a large reduction in protozoa number in Experiment 18 (see Figure 5, bottom graph), suggesting that VFA production in these *in vitro* experiments is strongly related to protozoa numbers. This is supported by a comparison of the total VFA concentrations for the negative controls in Experiments 18 and 19, where the VFA concentration in the latter experiment is double that in the former and of the protozoa numbers, where there is an eight-fold difference. A graph of VFA concentration against protozoa numbers for the combined data from Experiments 18 and 19 revealed an approximately logarithmic relationship between the two quantities (see Figure 6). On the other hand total gas production is very little influenced by protozoa numbers with a variation of only about 10% between the two runs.

A similar effect is observed for the VFA composition where in Experiment 18 (and other experiments where 5-NIs exhibit antiprotozoal activity) the acetate percentage is considerably increased by the action of 5-NIs whereas in Experiment 19 there is little variation due to treatment (see Figure 7). The increase in acetate observed in Experiment 18 and other experiments is contrary to the usual observation that the ratio of acetate to propionate decreases upon defaunation and may reflect the effect of antibacterial activity in addition to reduction in protozoa numbers.



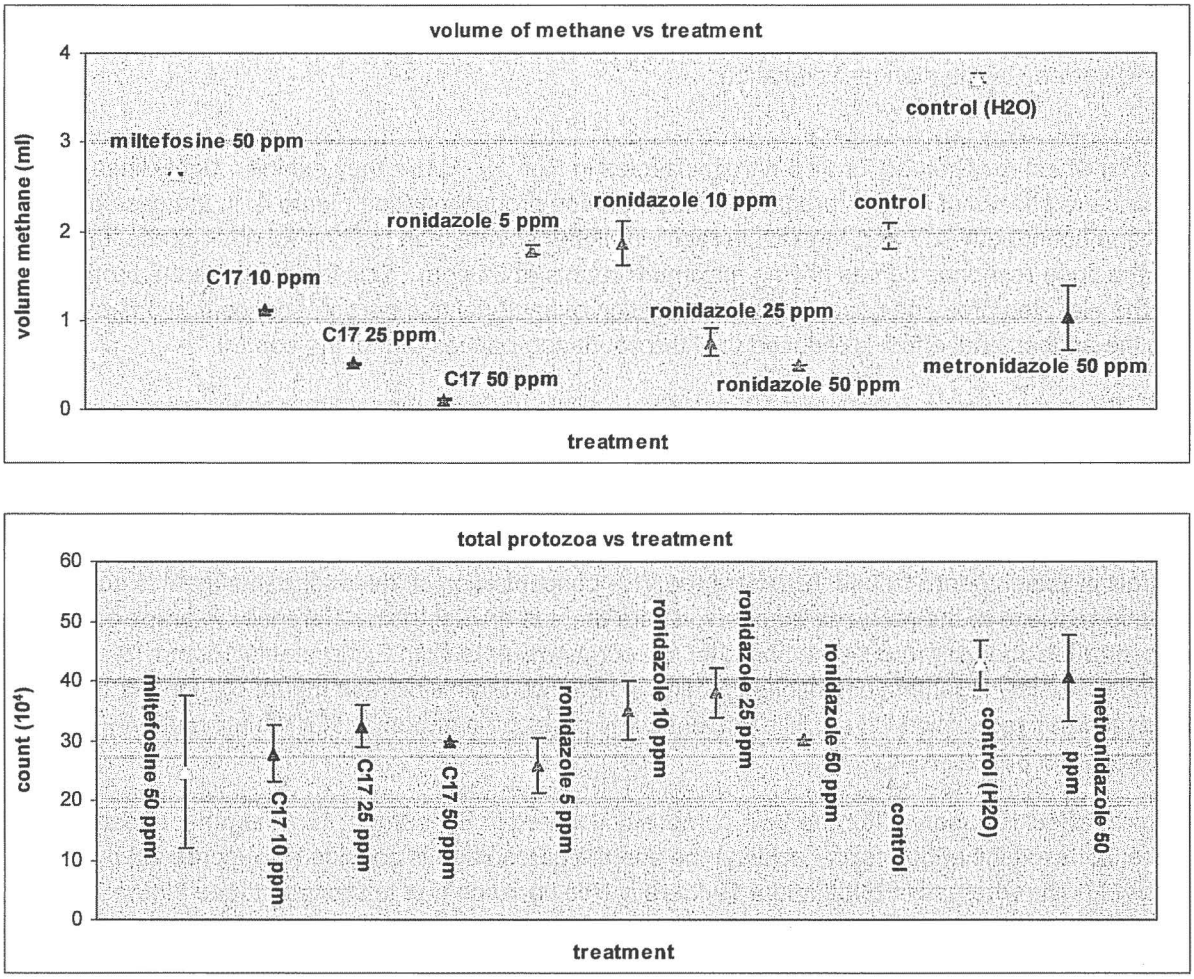
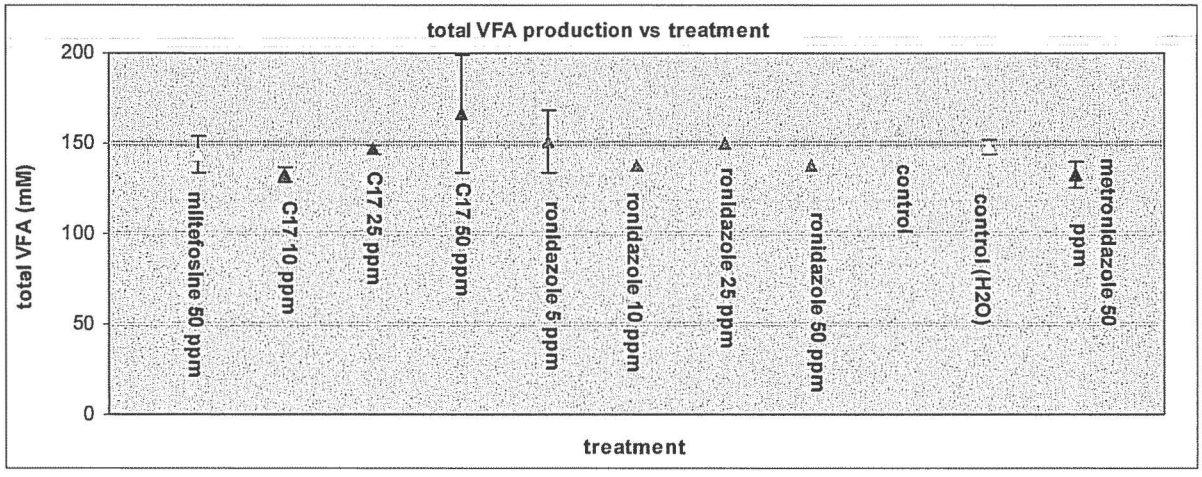


Figure 4. Results from bioassay number 19 showing total gas production (top), methane production (middle) and protozoa number (bottom). 5-nitroimidazoles are denoted by red or orange triangles. Error bars indicate the range of two measurements (+/- one standard deviation of four measurements for negative controls).



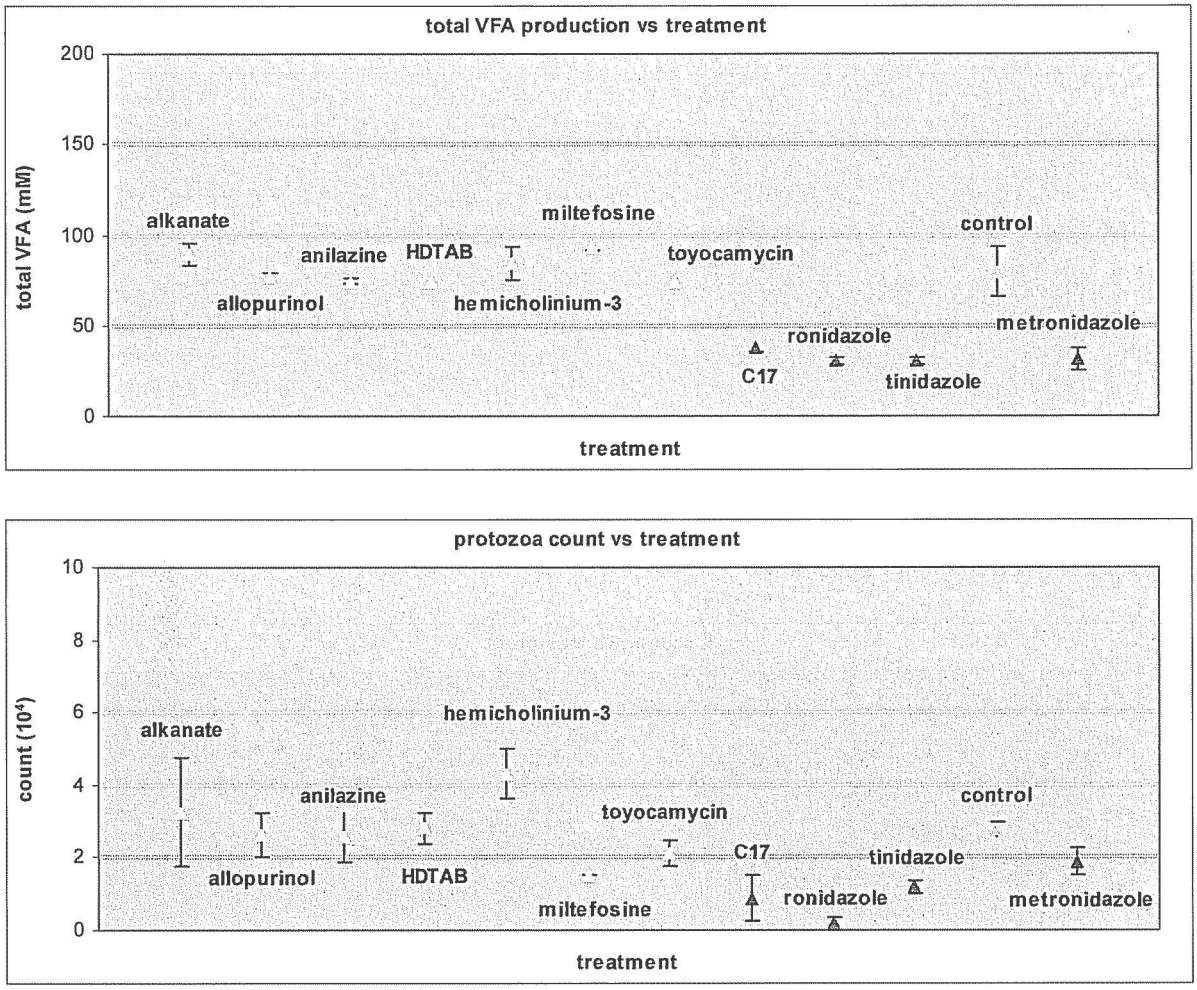


Figure 5. VFA production vs treatment for bioassay number 19 (top) and 18 (middle) and protozoa count for experiment 18 (bottom). 5-nitroimidazoles are denoted by red or orange triangles.

Error bars indicate +/- one standard deviation of two measurements (four measurements for negative controls).

Concentrations used in experiment 19 are:

alkanate: 300 ppm allopurinol: 300 ppm anilazine: 300 ppm HDTAB: 50 ppm hemicholinium-3: 300 ppm miltefosine: 100 ppm toyocamycin: 300 ppm C17: 50 ppm ronidazole: 50 ppm tinidazole: 100 ppm metronidazole: 50 ppm

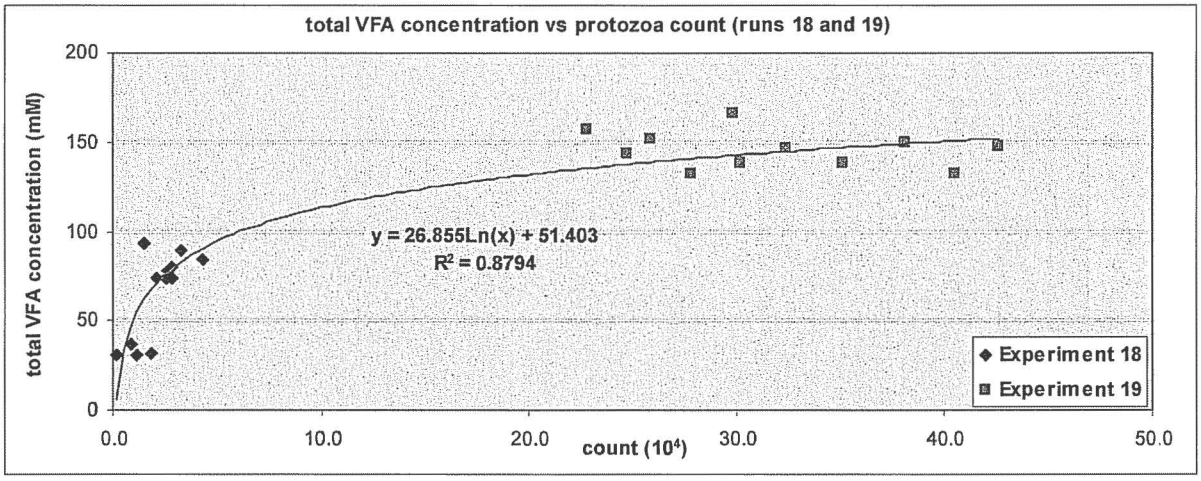


Figure 6. VFA production vs protozoa count for bioassay number 19 and 18 showing an approximately logarithmic relationship.

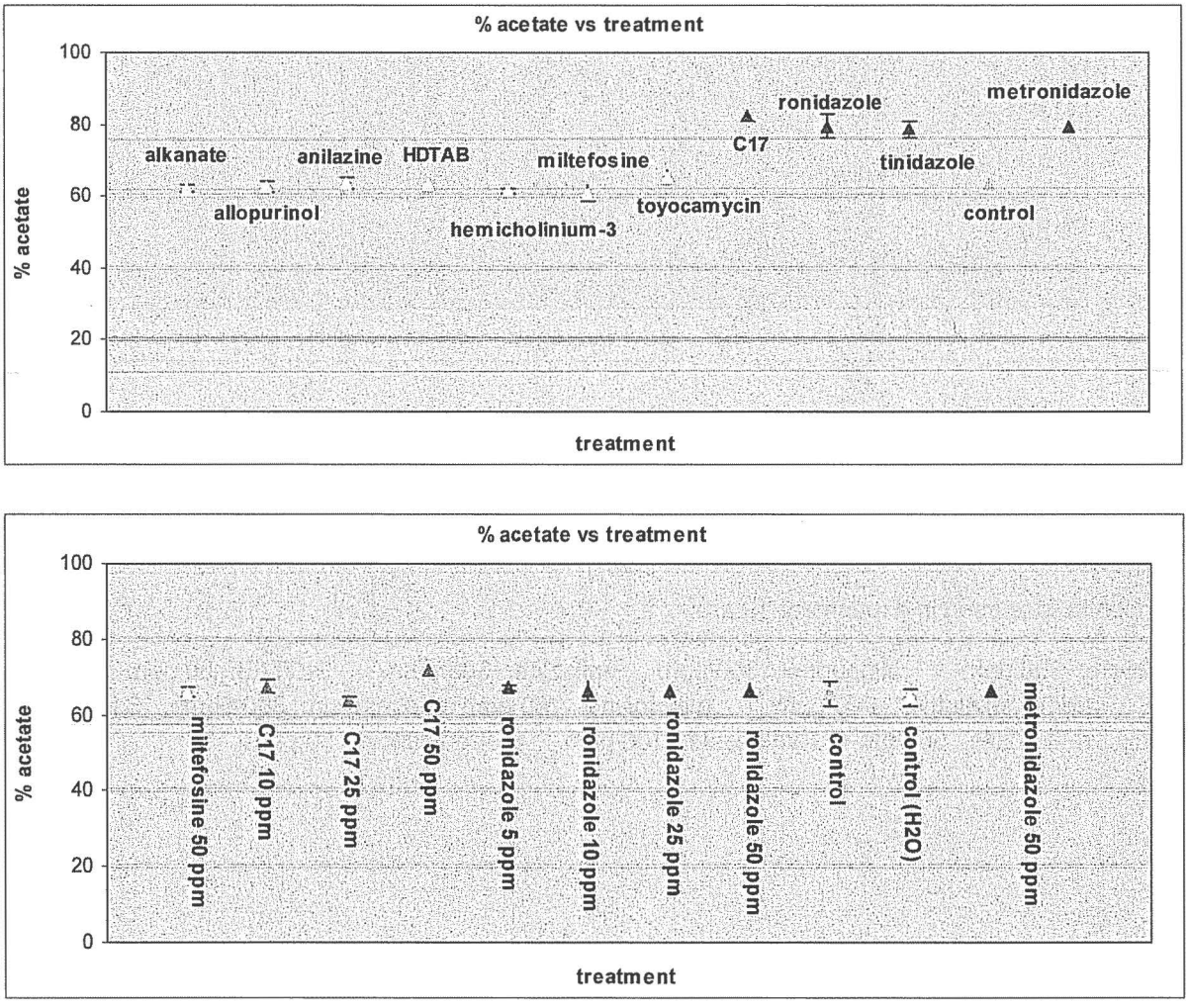


Figure 7. % acetate vs treatment for bioassay number 18 (top) and 19 (bottom). 5-nitroimidazoles are denoted by red or orange triangles. Error bars indicate +/- one standard deviation of two measurements (four measurements for negative controls). Concentrations used in experiment 19 are: alkanate: 300 ppm; allopurinol: 300 ppm anilazine: 300 ppm;HDTAB: 50 ppm; hemicholinium-3: 300 ppm miltefosine: 100 ppm toyocamycin: 300 ppm; C17: 50 ppm; ronidazole: 50 ppm tinidazole: 100 ppm metronidazole: 50 ppm

Activities of compounds tested at 100 ppm concentration

A summary of the results for all classes of compounds are given in table 2. The data are given for compounds measured at 100 ppm concentration. For antiprotozoal activity, the results are averages from Experiments 13-18, excluding Experiment 16 (and 19), the experiments where the positive controls gave higher counts than the negative controls. For total gas production and methane production the data is averaged from Experiments 12-19. In all cases the data was converted to a percentage of the control for that run before being averaged across runs.

As total gas production was considered to be an indicator of fermentation efficiency (and presumably translates to an indicator of rumen function in an *in vivo* situation), treatments were sought which produced good reduction in protozoa count or methane production with minimum reduction in overall gas production.

5-nitroimidazoles, in particular ronidazole, typically gave the lowest protozoa counts and methane production but also the lowest overall gas production. C17 (a 2-dibromostyryl 5-nitroimidazole), while having less antiprotozoal activity than ronidazole, showed a large reduction in methane production with a relatively lower loss of total gas production. As can be seen by comparing the results for ronidazole and C17, there is considerable variation in activity depending on the chemical structure and the investigation of further synthetic variations of the C17 structure appear warranted.

Miltefosine showed reasonable antiprotozoal activity (about 50% reduction in count) but no methane reducing activity relative to the DMSO control. However as described in the next section, when compared to a control consisting of water only, miltefosine also resulted in some reduction of methane production. Results for miltefosine from earlier runs were disregarded as they showed anomalously low activity when compared to later runs. In those runs DMSO was used as a solvent and it seems likely that little of the compound had dissolved for transfer to the syringes used as the bioassay vessel.

Hexadecyltrimethyl ammonium bromide (HDTAB) showed good activity, comparable to that of 5-NIs, when tested at 100 ppm but when tested at 50 ppm showed little effect (see Figure 5 for antiprotozoal results). Further testing is required to ascertain whether this is a true dose response effect or due to run-to-run variation.

Quinacrine showed a reduction in gas production and methane production comparable to that of 5-nitroimidazoles but little antiprotozoal activity, suggesting that its effect is purely antibacterial.

Toyocamycin shows little effect on total gas production or on protozoa numbers but produces a substantial reduction (to about 70% of control on average) in methane production. This suggests that toyocamycin may be showing selective activity against methanogens and warrants further investigation.

Other compounds tested had poor activity including anilazine, which had previously been shown to be active against rumen protozoa at 250 ppm.

Investigations at other concentrations

Due to their considerable activity at 100 ppm concentration, the 5-nitroimidazoles were tested at greater dilution, resulting in considerable reductions in methane production and total gas production at concentrations down to 5 ppm (see Figure 4). Interestingly, methane production in the 50 ppm C17 treatment was less than in the 50 ppm ronidazole treatment and considerably less than in the 50 ppm metronidazole positive control. The 10 ppm C17 treatment resulted in similar methane production to the positive control. On the other hand, total gas production for C17 was roughly the same or better than the other two 50 ppm 5-nitroimidazole treatments. As there was no antiprotozoal activity shown

in this run, this suggests that 5-NIs can reduce methane production irrespective of their effect on protozoa, i.e. they possess activity against methanogens directly. Furthermore, as the different 5-NI structures have different relative activities in the three tests, it seems likely that the antiprotozoal, anti-methanogen and antibacterial activity may operate though somewhat different mechanisms. This lends further credence to the possibility of being able to further manipulate chemical structure to maximise the desired activity while reducing the effect on overall fermentation.

Figure 4 also shows that miltefosine at 50 ppm exhibits little difference compared to the negative control (DMSO) with respect to total gas production or protozoa count and a higher rate of methane production. However miltefosine is insoluble in DMSO and so an aqueous solution of miltefosine was used in the last two bioassays rather than a DMSO solution, as was the case for all other compounds tested. When compared to a control consisting of deionised water only (in experiment 19 only), miltefosine showed considerable effect in reducing methane production and protozoa numbers. The reduction in protozoa count with 50 ppm miltefosine relative to the aqueous control was similar to that of the 100 ppm solution relative to a DMSO control, suggesting that the true value for 100 ppm miltefosine should be considerably lower and is probably comparable to that of 5-NIs.

The effectiveness of miltefosine in Experiment 19 suggests this compound may have use in killing protozoa that are not affected by 5-nitroimidazoles (assumed to be *E. exiguum*) and a combined treatment of miltefosine and a 5-nitroimidazole may be a more effective antiprotozoal agent.

The difference between the aqueous and DMSO controls suggests that DMSO itself has some antiprotozoal and methane reducing properties, having implications for the many bioassays that use this solvent.

As they had failed to show the anticipated activity at 100 ppm, anilizine, allopurinol, hemicholinium-3 and alkanate were tested at a 300 ppm concentration. Anilizine, allopurinol and toyocamycin displayed a slight increase in antiprotozoal activity when tested at 300 ppm (relative to the results at 100 ppm) but the counts were still only 80-90% of that of the negative control. Hemicholinium-3 and alkanate still showed no effect at this concentration. The increase in concentration had no effect on the methane production for toyocamycin, still being similar to that for the 100 ppm solution and casting doubts on the suggested potential (previous section) that this compound may have potential against methanogens. Methane production was not reduced relative to control for any of the other compounds when tested at 300 ppm.

Table 2. Summary of results for treatments at 100 ppm

Chemical class	Compound	Protozoa Count*	Methane Production*	Total Gas Production*	n**
5-nitroimidazole	metronidazole	26	8	51	2,4,4
	ornidazole	32	15	68	1,3,3
	ronidazole	7	6	41	2,4,4
	C17	18	4	69	1,3,3
purine analogue	allopurinol	123	119	102	2,4,4
	toyocamycin	129	70	102	1,2,2
	6-methyladenine	107	76	91	1,1,1
quaternary ammonium compound	hemicholinium-3	118	93	103	1,3,3
	hexadecyltrimethyl ammonium bromide (HDTAB)	13	23	59	2,2,2
	dimethylaminoethanol (DMAE)	91	100	103	2,2,2
phospholipid analogue	miltefosine***	54	135	105	1,3,3
triazine	anilazine	92	82	98	2,4,4
polyphenol	curcumin	94	103	105	2,3,3
acridine derivative	quinacrine	118	32	76	1,3,3
benzimidazole	fenbendazole	no visible effect – preliminary experiments only			
detergent	alkanate (30% sodium lauryl diethoxy sulphate (SLDS))	112	94	98	2,4,4
polypeptide antibiotic	bacitracin	large effect on gas production, no antiprotozoal activity – preliminary experiments only			
nitrofuran	furazolidone	141	100	103	1,1,1

*percentage of negative control (DMSO)

**number of runs averaged (for protozoa count, for methane production, for total gas production) – treatment duplicated in each run

*** values for miltefosine were 58%, 71%, 92% when compared to a control containing water only

Conclusion

Large variations from run to run in the results for both the positive and negative controls, particularly for protozoa numbers, made interpretation of the results difficult.

A major cause for the variation appeared to be differences in the species makeup of the rumen protozoa population. A number of variables, including difference between diet (pasture or grain), differences between individual sheep, time spent in the animal house prior to collection of rumen fluid and seasonal differences, may have contributed to this variation. Ideally the sheep used as donors for rumen fluid would have been housed and fed under identical conditions (or a single sheep used as the source of rumen fluid) but the long time frame of the research (about 20 months) effectively precluded this.

Despite these limitations, the research revealed a number of important findings:

- 5-nitroimidazoles (5-NIs), which had previously been used for treatment of human diseases caused by protozoa, also have a profound effect on rumen protozoa with ronidazole at a 100 ppm concentration reducing the protozoa population to 7% of that of the negative control. These compounds also cause a substantial reduction in methane production but at the same time result in a large decrease in overall gas production (considered to be a guide to the likely effect on rumen function if the compounds were to be used *in vivo*).

- Different 5-NIs performed with differing relative effectiveness in each of the tests, suggesting that antibacterial and antiprotozoal activity may not be caused by exactly the same mechanism and hence that greater selectivity for the desired outcomes may be achieved by further modification of the 5-nitroimidazole structure. 5-NIs are believed to act via reduction of the nitro group of the compound by the pyruvate:ferredoxin oxidoreductase complex contained in the organism, with formation of toxic radicals. The reduction potential of 5-NIs has been shown to vary with chemical structure and the ease of reduction has been found to be of significance for mammalian cell toxicity. However, the variation in relative toxicity to particular organisms, viz. protozoa, methanogens or bacteria, suggested by these results indicate that factors other than reduction potential, perhaps differing susceptibility to structurally different radical anions, must also be important.

- Miltefosine showed good antiprotozoal activity, although 2-6 times less active than the 5-NIs, with very little reduction in total gas produced. Importantly, miltefosine was active against the protozoa species that appeared to be unaffected by 5-NIs, suggesting a combined treatment of miltefosine and ronidazole may prove to be a very effective antiprotozoal agent.

- The potential of toyocamycin for methane reduction and hexadecyltrimethylammonium bromide for antiprotozoal activity was unclear because of discrepancies between runs but these compounds warrant further investigation.

- The suggested antiprotozoal and methane reducing activity exhibited by the control, DMSO, may have masked weak activity in those compounds that appeared to be inactive, as well as leading to a small underestimate of the potency of those compounds that did show activity.

The results from this project suggest a number of topics which should be further investigated:

- the likelihood that some protozoa species have different susceptibilities to antiprotozoal agents, which can be researched by growing axenic cultures of a number of species and bioassaying compounds against single species;
- the possibility that miltefosine and ronidazole may form a more potent treatment used in combination;
- modification of the C17 chemical structure to further optimise the selectivity for toxicity towards methanogens, compared to fermentative rumen bacteria, that has already been observed.

Effects of ronidazole on animal performance, rumen fermentation and microbial growth in sheep

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Executive Summary

- A randomized block design experiment was conducted using twenty 5-month-old lambs (LW 32.5 ± 0.95 kg). Lambs (n=4) were allocated to either the control treatment (T1) or the treatments receiving compounds (T2 – Rumensin and T3 – Ronidazole). Rumen parameters were measured before, during and after a 15d medication period.
- Rumensin showed greater anti-protozoal action and methane reduction than Ronidazole
- Ronidazole showed minimal impact on the sheep or on rumen protozoa, volatile fatty acid concentrations and proportions, or methane production and yield.
- It is concluded that Ronidazole was not the most promising agent to reduce protozoa or mitigate methane of two compounds tested. No further study on this compound will be pursued.

Materials and methods

Animals and diets

All protocols for the care of the animals used in this trial were approved by the University of New England Animal Ethics Committee (AEC 11/088).
Twelve wether lambs (1st Coolalee, LW = 32.5 ± 0.95 kg) from 'Nordale', Delungra, NSW were selected at 5 months of age. On arrival, all sheep were treated with Q-drench (1 mL/5 kg LW; Jurox, Australia) and housed in group pen for three days before being allocated to the individual pens.

Lambs were allocated by stratified randomisation within weight ranges, to three treatment groups: the negative control group (T1), the positive control group (T2 – Rumensin @ 1 mg/kg LW) and the treatment group receiving Ronidazole at 2 mg/kg LW (T3). The sheep were allowed 12 days for acclimation to the feeding regime (morning pellets and afternoon chaff) and environment, followed by a 15-day medication period and finished with a 6-day non-medication period. A diagram describing this program of work is shown below (Figure 1).

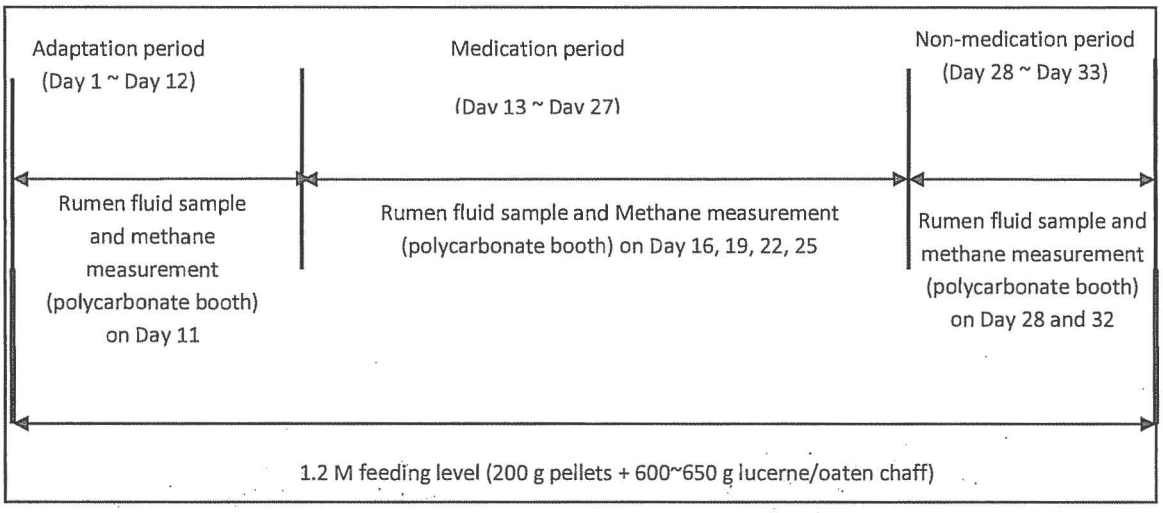

Figure 1: Experimental schedule showing the diet adaptation, medication and post-medication periods.

During the whole trial period, the pellet diet (Table 1) with or without the additive was fed at the rate of 200 g/sheep daily. Chopped mixed oaten/lucerne chaff (Table 2) at the rate of 600 ~ 650 g /sheep was supplied 5 h later. The amount of chaff fed to each sheep was restricted to meet the1.25 × $ME_{maintenance}$ requirement of each sheep. The maintenance requirement (M, MJ ME/day) of each sheep was calculated according to the equation used in SheepExplorer (2003). The average liveweight of sheep in each group obtained at two-week interval was used to calculate average $ME_{maintenance}$ requirement for all sheep in that group for that period.

Table 1. The ingredients and chemical composition of the experimental pellet diet

Introductory sheep pellet	
Ingredients	% as fed
Barley	50
Lucerne chaff	30
Oaten chaff	10
Cotton seed meal	10
plus Lime	1.27
plus Salt	0.5
plus mineral supplement	0.125
Total	100
Chemical composition	
Dry matter (%)	91.7
Neutral detergent fibre (%)	30
Acid detergent fibre (%)	16
Crude protein (%)	17
Dry matter digestibility (%)	76
Digestible organic matter in the dry matter (%)	75
Inorganic ash (%)	7
Organic matter (%)	93
Metabolisable energy (MJ/kg DM)	11.7
Crude fat (%)	2.0

Table 2. The ingredients and chemical composition of the experimental chaff diet

Oaten/lucerne chaff	
Ingredients	% as fed
Lucerne chaff	50
Oaten chaff	50
Total	100
Chemical composition	
Dry matter (%)	89.8
Neutral detergent fibre (%)	51
Acid detergent fibre (%)	32
Crude protein (%)	12.2
Dry matter digestibility (%)	64
Digestible organic matter in the dry matter (%)	61
Inorganic ash (%)	8
Organic matter (%)	92
Metabolisable energy (MJ/kg DM)	9.3

The purpose of the restricting feed intake was to allow comparison of the effects of dietary additives on methane production and rumen fermentation pattern, without the potential confounding effect of variation in daily additive intake. During the whole trial period, representative samples of the feed offered were taken and pooled together for later analysis. Daily feed intake for each lamb was recorded and all animals had access to fresh water at all times. The animals were weighed on Day 1, 8, 12, 22, 30 and 32 before morning feeding. Animals were checked twice daily for signs of abnormal feed behaviour, alertness and diarrhoea occurrence.

Administration of compounds

The amount of each compound added to the 200 g pellet was calculated on the basis of the recommended dosage and the average liveweight of sheep in each group.

The test compound was precisely weighed into Bijou bottle and then dissolved with 85% ethanol (5 mg compound/mL 85% ethanol). The dissolved compound was then sprayed onto the pellets using Hill 500 mL trigger sprayer (Figure 2). After draining the content in the spray bottle, small amount of 85% ethanol was used to rinse off the possible residue in the bottle and sprayed onto the feed. Treated feed was left in the fume cupboard for 5 h to let ethanol evaporate completely before the treated feed was weighed accurately into the individual bags for feeding the next day.

Figure 2. Treated pellets were left in the fume cupboard for 5 h before weighing into individual bags.

Methane production

Methane production (L/h) was measured on days 11, 16, 19, 22, 25, 28 and 32 using confinement chambers over a 1-h period for each lamb as described by Goopy *et al.* (2011). Initial testing for gas leakage was done by injecting 1 L of 99% CH_4 (BOC, Sydney, Australia) into each chamber and the CH_4 concentration was monitored for the next 2 hrs at an hour interval. The estimated recovery was determined, which was typically 98 ~ 100% of injected CH_4.

One hour after consuming the pellets, the sheep was confined inside the chamber for one hour. All gases exhaled and eructed during the 1 h collection period were trapped. After sheep were confined for 1 h, methane concentration (ppm) was measured by a MicroFID flame ionization detector (FID, ENVCO, Wellington, New Zealand) fitted with a 20 cm flexible silicon sampling tube, which was introduced to each chamber through a 3 mm diameter sampling port located on the top panel. One minute after the methane measurement, carbon dioxide concentration (%) was also monitored to check if the chamber had any gas leakage. The typical CO_2 concentration was approximately 2%.

The net gas volume inside the chamber was estimated by the following equation: Net gas volume (L) = Volume of chamber (L) – Volume occupied by the sheep (1 L/kg LW)*

* Volume of sheep was estimated by assuming that the volume occupied by the sheep was equal to 1 L/kg LW

Methane production during the 1-h confinement was calculated as:

CH_4 (L/h) = (CH_4 concentration – CH_4 concentration background) × Net gas volume

The volume of CH_4 produced was adjusted to standard temperature and pressure (STP).

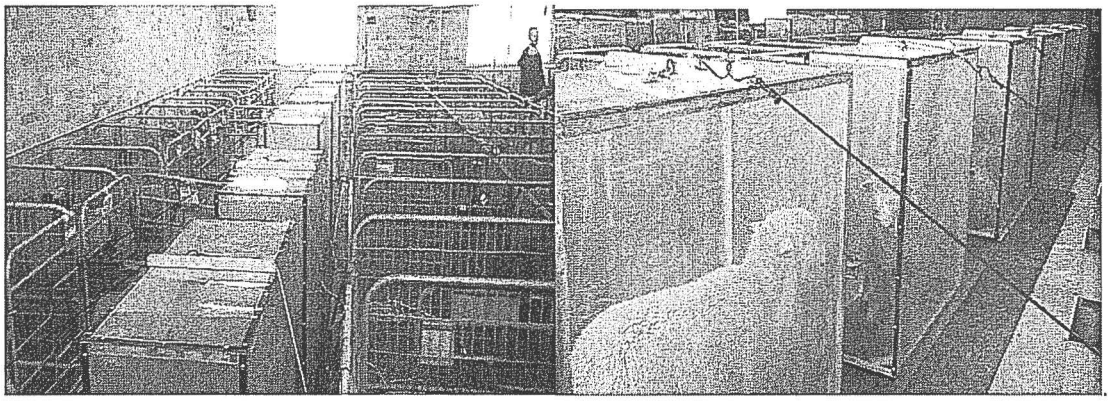

Figure 3. Short-term confinement chambers set-up used to measure methane production over 1h. These was used every 3d to monitor the impact of the medication on enteric methane emissions throughout the experiment.

Rumen fluid pH, volatile fatty acid (VFA) concentrations and protozoa enumeration

A 20 mL rumen fluid sample was taken (using an oesophageal tube) from each animal immediately after sheep was removed from confinement chamber on days 11, 16, 19, 22, 25, 28 and 32. Rumen pH was measured immediately using a portable pH meter (Orion 230 Aplus, Thermo Scientific, Beverly, MA, USA). A subsample (10 ml) was placed in wide-neck McCartney bottle acidified with 5 drops 18M sulphuric acid and then stored at – 20 °C for later VFA analysis. The samples were chromatographed on a Varian CP-3800 Gas Chromatograph. Peak areas were normalised with respect to that of the internal standard and the concentrations of each VFA determined by reference to a commercial standard VFA mixture (LB68687, Supelco). Another 4 mL subsample of rumen fluid was mixed with 16 mL of 4% formalised saline and stored in wide-neck McCartney bottle at room temperature for later protozoa counts. Total protozoa numbers were counted using a Hawksley Cristalite B.S. 748 counting chamber (Sussex, UK).

Statistical analysis

The effects of compound on feed intake, liveweight gain, rumen fermentation parameters, protozoa numbers and methane production were assessed by one-way ANOVA using Genstat 12.1 (VSN International Ltd). Unless stated otherwise, the results were expressed as mean ± least significant difference of means (l.s.d.). The significance level was set at $P < 0.05$, and trends were noted if $0.05 \leq P < 0.10$.

Results

Total feed intake and live weight gain (LWG) during the 33-day restricted feeding period

Results are summarised in Table 3. T2 had highest final LW and total FI of any treatment ($P < 0.05$). No difference in LWG (average 1.34 kg) or feed conversation ratio (average 14.4 kg FI/kg LWG) was observed among any of the treatments.

Table 3. Measures (means ± l.s.d.) of total feed intake (FI), LWG and feed conversion ratio (FCR, kg feed intake/kg LWG) of sheep fed 1.25 × ME $_{maintenance}$ ration throughout the entire 33-d trial period.

	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW	l.s.d.	p value
Initial LW (kg) *	32.6	33.8	32.5	1.40	NS
Final LW (kg) **	34 a	35.12 b	33.75 a	0.91	0.017
LW gain (kg)	1.38	1.38	1.25	1.265	NS
Total FI (kg)	19.26 a	19.38 b	19.19 a	0.099	0.007
Total DM intake (kg)	17.38 a	17.49 b	17.33 a	0.089	0.007
FCR (kg FI/kg LWG)	13.8	17.8	11.5	14.30	NS

* Initial LW was measured on Day 0; ** Final LW was measured on Day 33

Rumen pH

Data are summarised in Table 4. Rumen pH in all treatments was within the normal range for the rumen 2 h after feeding (6.55 ~ 6.87). For treatment T1 and T2, medication did not affect rumen pH. A significant decrease in rumen pH was observed, on the 1st and 5th day off the medication, in those sheep receiving T3 treated pellets ($P < 0.05$), and these sheep The sheep from T3 group sustained low rumen pH to the same level, even after they were off the medication for 5 days.

Table 4. Rumen pH measurements in sheep receiving 1.25 × ME $_{maintenance}$ ration treated with T1, T2 and T3 throughout a 15-day medication period followed by a 6-day non-medication period. Within columns, means with a common suffix do not differ significantly ($P > 0.05$).

Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	6.60	6.83	6.8 c
4th day on medication	6.75	6.80	6.81 c
7th day on medication	6.55	6.84	6.71 abc
10th day on medication	6.57	6.85	6.76 bc
13th day on medication	6.65	6.87	6.69 abc
1st day post medication	6.69	6.77	6.65 ab
5th day post medication	6.64	6.71	6.62 a
l.s.d.	0.232	0.194	0.129
p value	NS	NS	0.048

Ruminal VFA concentrations

Concentrations of VFA, molar proportions of acetate, propionate and butyrate, molar ratio of acetate to propionate and molar ratio of acetate plus butyrate to propionate in ruminal fluid of sheep on five treatments are summarised in Table 5.

The addition of T2 to the diet decreased molar proportion of acetate, but increased molar proportion of propionate, hence lowered acetate/propionate and acetate plus butyrate/propionate ratio on the 4^{th}, 7^{th} and 10^{th} medication day, respectively ($P < 0.001$).

The addition T3 to the diet did not affect total VFA concentration, molar proportion of acetate, propionate, butyrate or molar ration of acetate to propionate ($P > 0.05$).

Table 5. Total VFA concentrations and VFA molar proportions in rumen contents of growing lambs receiving 1.25 × ME $_{maintenance}$ ration treated with T1, T2 and T3 throughout a 15-day medication period followed by a 6-day non-medication period. Within columns, means with a common suffix do not differ significantly (*P > 0.05*).

Total VFA (mM)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	83.4	75.2	71.6
4th day on medication	87.2	67.4	75.2
7th day on medication	75	61.9	77.7
10th day on medication	77.4	72.9	78.1
13th day on medication	78.1	70.1	80.9
1st day off medication	76.5	73.1	74.4
5th day off medication	83.6	77.5	79.6
l.s.d.	16.61	12.9	17.45
p value	NS	NS	NS
Acetate (mol %)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	66.5	66.9 de	64.3
4th day on medication	67.2	59.1 a	65.4
7th day on medication	62.8	61.6 ab	66.3
10th day on medication	63.3	63.6 bc	66.6
13th day on medication	64.3	64.2 bcd	67.5
1st day off medication	64.1	65.3 cde	66.4
5th day off medication	66.4	67.5 e	67.0
l.s.d.	3.32	2.9	2.25
p value	0.064	<.001	NS
Propionate (mol %)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	22.4	19.8 ab	22.6
4th day on medication	20.7	31.1 d	21.6
7th day on medication	24.9	26.6 c	20.3
10th day on medication	23.4	24.6 c	20.2
13th day on medication	21.8	23.8 bc	19.8
1st day off medication	22.7	22.6 abc	20.7
5th day off medication	19.8	18.6 a	20.2
l.s.d.	4.14	4.49	2.40
p value	NS	<.001	NS

Butyrate (mol %)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	9.3	11.1	11.1
4th day on medication	10.3	8.5	11.0
7th day on medication	10.5	10.2	11.4
10th day on medication	11.3	10.1	11.2
13th day on medication	12.1	10.2	11.0
1st day off medication	11.6	10.5	11.1
5th day off medication	12	12.1	11.1
l.s.d.	2.78	2.84	2.333
p value	N5	N5	N5
Acetate/propionate ratio	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	3	3.4 cd	2.8
4th day on medication	3.3	1.9 a	3.1
7th day on medication	2.6	2.3 ab	3.3
10th day on medication	2.7	2.6 b	3.3
13th day on medication	3	2.7 b	3.4
1st day off medication	2.9	3.0 bc	3.2
5th day off medication	3.4	3.7 d	3.3
l.s.d.	0.58	0.65	0.44
p value	N5	<.001	N5
(Acetate + Butyrate) /Propionate ratio	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	3.4	4 cd	3.34
4th day on medication	3.76	2.2 a	3.58
7th day on medication	3.08	2.73 ab	3.84
10th day on medication	3.25	3.03 b	3.88
13th day on medication	3.54	3.16 b	4.00
1st day off medication	3.39	3.44 bc	3.76
5th day off medication	3.96	4.37 d	3.87
l.s.d.	0.739	0.816	0.561
p value	N5	<.001	N5

Protozoa enumeration

The effects of treatments on protozoa numbers during and post medication period are shown in Table 6.

T2 reduced total protozoal numbers on the 4th and 7th day of receiving medicated pellets, largely owing to a significant drop in the numbers of small Entodinium protozoa. On the 5th post-medication day, the numbers of total protozoa and small Entodinium protozoa in the rumen fluid of sheep receiving T2 were larger than that observed on Day 0.

T3 had practically no effect on the number of total protozoa or small Entodinium during the medication period.

Table 6. Protozoa numbers in rumen contents of growing lambs receiving 1.25 × ME $_{maintenance}$ ration treated with T1, T2 and T3 throughout a 15-day medication period followed by a 6-day non-medication period. Within columns, means with a common suffix do not differ significantly (*P > 0.05*).

Total protozoa (× 10^4/ml)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	73.75	44.79 bc	74.06
4th day on medication	101.04	14.27 a	85.42
7th day on medication	115.94	20.83 ab	123.75
10th day on medication	92.19	28.85 abc	78.12
13th day on medication	83.23	34.38 abc	65.31
1st day off medication	98.65	51.04 c	96.35
5th day off medication	97.81	77.92 d	94.9
l.s.d.	75.546	26.673	108.916
p value	NS	0.001	NS

Large Ento. (× 10^4/ml)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	0.94	1.46	0.83
4th day on medication	0.31	0.94	1.35
7th day on medication	0.83	0.10	1.25
10th day on medication	2.08	0.94	0.21
13th day on medication	2.50	1.56	0.73
1st day post medication	3.02	0.62	0.52
5th day post medication	1.46	1.35	1.04
l.s.d.	2.390	1.927	2.140
p value	NS	NS	NS

Small Ento. (× 10^4/ml)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	72.50	43.33 bc	70.52
4th day on medication	100.10	12.71 a	83.02
7th day on medication	115.10	20.42 ab	120.1
10th day on medication	89.79	27.08 abc	76.67
13th day on medication	80.21	32.08 abc	62.5
1st day post medication	95.52	47.92 c	92.5
5th day post medication	95.63	75.73 d	91.46
l.s.d.	76.205	25.661	112.897
p value	NS	0.001	NS

Large Iso. ($\times 10^4$/ml)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	0.31	0.00	0.1
4th day on medication	0.31	0.52	0.31
7th day on medication	0.00	0.10	0
10th day on medication	0.31	0.10	0
13th day on medication	0.52	0.10	0
1st day post medication	0.10	0.94	0
5th day post medication	0.73	0.10	0
l.s.d.	0.762	0.830	0.366
p value	NS	NS	NS

Small Iso. ($\times 10^4$/ml)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	0.00	0.00	2.60
4th day on medication	0.00	0.10	0.73
7th day on medication	0.00	0.21	2.40
10th day on medication	0.00	0.73	1.25
13th day on medication	0.00	0.63	2.08
1st day post medication	0.00	1.56	3.33
5th day post medication	0.00	0.73	2.40
l.s.d.	Not applicable	2.160	6.541
p value	NS	NS	NS

Enteric methane production

Data are summarised in Table 7. The restricted ration (1.25M) that was offered to sheep resulted in similar DM intake in all treatments (738 ± 11.8 g/d). Daily DM intake was deployed in the following equation to calculate methane yield (g/kg DMI).

Methane yield (g/kg DMI) = methane production (g/h) × 24 h / DMI (kg/d)

Addition of T2 to the diet significantly reduced methane production (g/h) and methane yield (g methane/kg DMI) throughout the medication period and even on the 1st post-medication day ($P < 0.001$). The methane production (g/h) was lowered in the T3 sheep on the 4th day of receiving medicated pellets ($P < 0.05$).

T3 did not affect methane production or methane yield during the medication or post-medication period ($P > 0.05$).

Table 7. Methane production (g/h), methane yield (g/kg DMI) of growing lambs receiving 1.25 × ME $_{maintenance}$ ration treated with T1, T2 and T3 throughout a 15-day medication period followed by a 6-day non-medication period. Within columns, means with a common suffix do not differ significantly (*P > 0.05*).

CH4 (g/h @ STP)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	0.67	0.78 b	0.76
4th day on medication	0.70	0.58 a	0.75
7th day on medication	0.65	0.60 a	0.78
10th day on medication	0.74	0.64 a	0.79
13th day on medication	0.75	0.64 a	0.75
1st day post medication	0.78	0.67 a	0.76
5th day post medication	0.80	0.82 b	0.83
l.s.d.	0.117	0.098	0.099
p value	NS	<.001	NS

CH4 (g/kg DMI)	Treatment No.		
Days medication and post-medication	T1 - Negative control	T2 – Positive control – Rumensin @ 1 mg/kg LW	T3 – Ronidazole @ 2 mg/kg LW
Day 0 (before medication)	22.2	25.8 b	25.2
4th day on medication	23.2	18.7 a	24.9
7th day on medication	21.7	19.2 a	25.8
10th day on medication	24.6	20.3 a	26.1
13th day on medication	25.0	20.5 a	24.8
1st day post medication	24.9	21.6 a	25.3
5th day post medication	25.7	26.1 b	26.4
l.s.d.	3.87	3.15	3.29
p value	NS	<.001	NS

* DMI (kg/d) – dry matter intake was the value of methane measurement day's intake

**Methane production (L/d) – daily methane production was calculated as Methane production (L/h) × 24 h

Discussion and Conclusion

Ronidazole had been found to be the most effective compound tested by *in-vitro* screening for reducing protozoal number and methane production in incubated rumen fluid (previous reports). However, when efficacy was determined *in-vivo* in this study using sheep, ronidazole showed no impact on rumen protozoal population, on methane production or methane yield. In contrast, monensin showed a sustained efficacy in reducing the population of small entodiniomorph protozoa and in reducing the production rate and yield (g/kg feed) of enteric methane over the two weeks of medication. This leads to a disquieting conclusion that ronidazole does not have a future in chemical defaunation or methane mitigation in ruminant livestock.

Table 8 Summary of effects of compounds on rumen pH, protozoa numbers, rumen volatile fatty acids and enteric methane production

Note: data for each parameter measured on Day 0 were shown as it is; for the rest of measuring time points, the data was present as the percent of reduction, which was calculated as: (Data $_{\text{measured during the medication or post-medication period}}$ – Data $_{\text{measured on Day 0}}$)/Data $_{\text{measured on Day 0}}$ × 100. If values for the percent of reduction highlighted in bold red are significant ($P < 0.05$).

Compound ID	Number of days on medication and post-medication	Rumen pH	Total protozoa ($\times 10^4$/mL)	Total VFA (mM)	Acetate/Propionate ratio	Methane production (g/hr)	Methane yield (g/kg DMI)
T1 - Negative control	Day 0 (before medication)	6.60	73.8	83.4	3	0.67	22.2
		% reduction relative to Day 0					
	4th day on medication	2%	37%	5%	10%	4%	5%
	7th day on medication	-1%	57%	-10%	-13%	-3%	-2%
	10th day on medication	0%	25%	-7%	-10%	10%	11%
	13th day on medication	1%	13%	-6%	0%	12%	13%
	1st day off medication	1%	34%	-8%	-3%	16%	12%
	5th day off medication	1%	33%	0%	13%	19%	16%
T2 – Positive control – Rumensin @ 1 mg/kg LW	Day 0 (before medication)	6.83	44.79	75.2	3.4	0.78	25.8
		% reduction relative to Day 0					
	4th day on medication	0%	-68%	-10%	-44%	-26%	-28%
	7th day on medication	0%	-53%	-18%	-32%	-23%	-26%
	10th day on medication	0%	-36%	-3%	-24%	-18%	-21%
	13th day on medication	1%	-23%	-7%	-21%	-18%	-21%
	1st day off medication	-1%	14%	-3%	-12%	-14%	-16%
	5th day off medication	-2%	74%	3%	9%	5%	1%
T3 – Ronidazole @ 2 mg/kg LW	Day 0 (before medication)	6.80	74.06	71.6	2.8	0.76	25.2
		% reduction relative to Day 0					
	4th day on medication	0%	15%	5%	11%	-1%	-1%
	7th day on medication	-1%	67%	9%	18%	3%	2%
	10th day on medication	-1%	5%	9%	18%	4%	4%
	13th day on medication	-2%	-12%	13%	21%	-1%	-2%
	1st day off medication	-2%	30%	4%	14%	0%	0%
	5th day off medication	-3%	28%	11%	18%	9%	5%

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References

Goopy JP, Woodgate R, Donaldson A, Robinson DL, Hegarty RS (2011) Validation of a short-term methane measurement using portable static chambers to estimate daily methane production in sheep. Animal Feed Science and Technology 166-167, 219-226.

SheepExplorer (2003) 'The excel spreadsheet SheepExplorer of GRAZPLAN.' In Available at http://www.pi.csiro.au/grazplan/ [Verified Accessed April 2005]

In-vivo methane mitigation and productivity of beef cattle

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These assessments were intended to follow on from a previous project that was scheduled to deliver a herd of faunated and defaunated cattle for (this) further research by June 2009. Unfortunately, this preceding project was not successful in establishing a protozoa-free herd by that scheduled date and so BCCH1010 was delayed access to investigate methane and productivity effects of defaunation. Cattle were only confirmed protozoa-free by Mid-2011 and so available for emissions evaluation late in 2011.

The preliminary work that led to these cattle being established as protozoa-free is summarised below:

Initial attempts to permanently remove protozoa from the rumen of heifers were only partially successful. Despite the creation of an extended period of harsh conditions (for protozoa and other microbes) in the rumen through a combination of feeding high levels of cottonseed oil followed by a 4 day period of starvation and 3 daily drenches of an anti-protozoal detergent (Teric) only three out of twenty animals treated remained free of protozoa 90 days after treatment. Although most of the heifers were not permanently defaunated with any of the treatments used in the first study most if not all protozoa in the rumen were killed by a combination of a dietary oil supplement and an oral drench of an anti-protozoal chemical. Therefore a second study was designed using a similar protocol but with the following changes with respect to the first study:

1. The grain-based ration was replaced with a low quality cereal straw. The straw diet could be expected to support much lower populations of protozoa in the rumen (Abe et al 1973) and hopefully significantly reduce the number of protozoa in the omasum as little or no soluble carbohydrate will leave the rumen of animals fed this ration.

2. The cottonseed oil was replaced with coconut oil which is reported to have superior anti-protozoal activity.

3. The oral drench of Teric was replaced with Alkanate 3SL3 detergent which is known to have superior anti-protozoal activity.

Establishment of protozoa-free herd

Twenty Brangus heifers (220 kg) were allocated on the basis of weight to one of two groups. Both groups of animals were initially held in group pens for a 7 day acclimatization period and fed lucerne chaff. The diet was then changed to cereal straw and the treatment group of animals were moved to individual pens in a cattle shed. A supplement of coconut oil was gradually introduced into the diet of the treatment group of heifers. After a week of feeding the straw and oil ration a rumen fluid sample was collected to check the protozoa status of the treated animals. Feed was then withheld from the treatment group for 3 days while the animals received a daily drench of Teric (40g/d). Following the drenching program the treated animals were fed lucerne chaff and coconut oil was again gradually introduced into the ration. This feeding regime was

continued for 14 days when another rumen fluid sample was collected to check the protozoa status of the treated animals. The results are shown in Table 1.

Table 1. The effect of coconut oil supplementation and oral drenching with Teric on the population of protozoa in heifers.

Trial 1	Sample collection time		
	Pre –experimental	Post oil feeding	Post Teric drenching
Protozoal population *	206	5	164

* Protozoa cell count measured in a set volume of rumen fluid.

The dietary supplement of coconut oil reduced the numbers of protozoa by more than 95% indicating the high anti-protozoal activity of this oil. Surprisingly the Teric drenching program that was expected to remove the remaining protozoa was totally ineffective with protozoal numbers rapidly increasing in all animals from the low post-oil feeding period to a level that was only 20% lower than the pre-experimental level (Table 1). With the return of the protozoa in the treated animals both groups of animals were returned to the paddock for a four week period before a second study was initiated. At the end of the 4 week period the treatment group of animals were returned to the cattle shed and fed cereal straw and coconut oil for a 14 day period (7 days longer than the initial trial). At the completion of this period protozoal numbers had been reduced to less than 1% of the original population (protozoa were not detected in 6 of the heifers). Feed was then withheld from the treatment group for 3 days while the animals received a daily drench of Alkanate 3SL3 (45g/d). Protozoa were detected in one animal 3 weeks after the completion of the defaunation treatment and this animal received a second defaunation treatment. All treated animals remained free of protozoa for a further 5 months after which time they were tested for methane production.

Table 2. The effect of coconut oil supplementation and oral drenching with Alkanate on the population of protozoa in heifers.

Trial 2	Sample collection time		
	Pre –experimental	Post oil feeding	Post Alkanate drenching
Protozoal population *	233	1	0

* Protozoa cell count measured in a set volume of rumen fluid.

Measurement of methane production

Methane emissions were determined for both faunated (control) and defaunated heifers over two periods separated by a week. The duration of each measurement period was 48 h. Animals were

fed a mixed ration of lucerne and cereal straw (1:1) during the test period and the level of feeding was restricted to 1.2 X Maintenance. There were no feed refusals during the test period. Methane production was not different between the two groups of animals indicating that the removal of protozoa had no effect on methane production (Table 3). This result is consistent with sheep studies that also demonstrated that defaunation had no effect on methane production.

Table 3 Methane yield (CH_4/kgDMI) from faunated and defaunated heifers. The difference was not significant (P>0.05)

DEFAUNATED		FAUNATED	
Heifer	CH_4/KgDMI	Heifer	CH_4/KgDMI
13	24.0	16	26.5
32	31.1	18	29.5
39	24.7	25	28.5
101	26.8	30	27.2
214	26.3	31	26.1
238	22.4	41	30.5
243	25.7	106	24.8
254	28.1	223	26.8
256	27.2	228	26.7
		252	27.2
Mean	**26.2**		**27.4**

Measurement of cattle productivity

An assessment of digestive function in the faunated and defaunated herds has been completed in the past 5 days. No analysis of this work has been completed as yet. The only available information is that since defaunation was completed in March 2011, the defaunated cattle have continuously grown faster than faunated cattle, and the mean liveweights of the faunated and defaunated cattle are 341 kg and 384/kg respectively in December 2011 (Figure 1); so an approximate 40kg LW difference is apparent in favour of defaunated cattle. While this may in part be confounded with compensatory response to treatment, the difference is large and sustained and indicative that significant productivity benefits can accrue from defaunation of cattle.

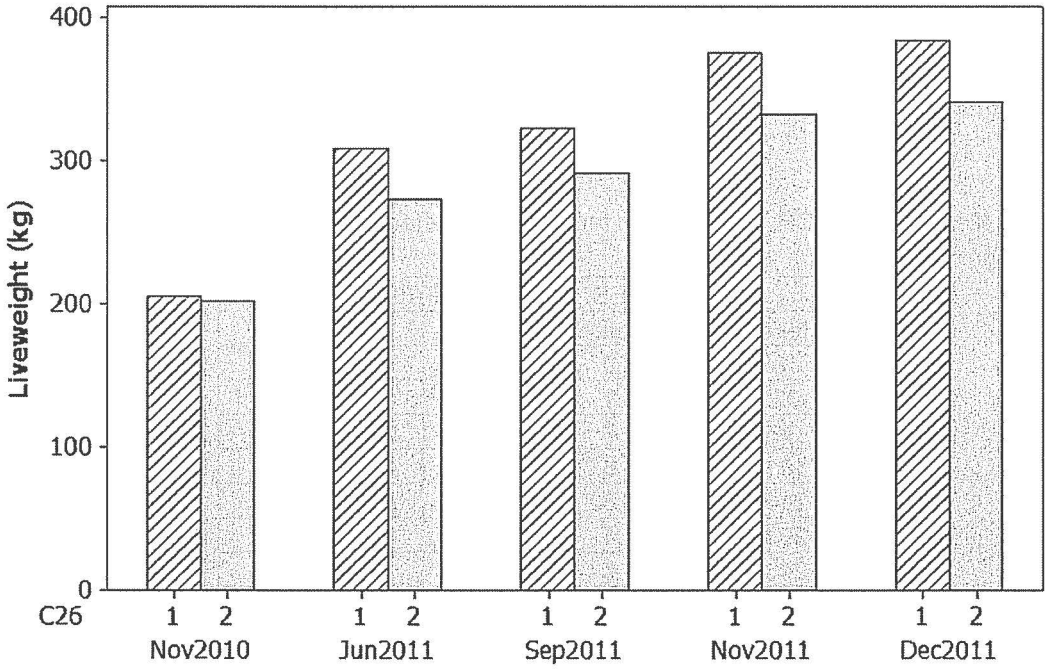

Figure 1. Liveweight of untreated (shaded) and defaunated heifers (hatched) from the time of defaunation (Nov 2010) until completion of research (Dec 2012)