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Effect of ruminal administration of *Escherichia coli* wild type or a genetically modified strain with enhanced high nitrite reductase activity on methane emission and nitrate toxicity in nitrate-infused sheep

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The effects of two kinds of *Escherichia coli* (*E. coli*) strain, wild-type *E. coli* W3110 and *E. coli* nir-Ptac, which has enhanced NO_2 reduction activity, on oral CH_4 emission and NO_3 toxicity in NO_3-treated sheep were assessed in a respiratory hood system in a 4 × 6 Youden square design. NO_3 (1·3 g $NaNO_3$/kg$^{0.75}$ body weight) and/or *E. coli* strains were delivered into the rumen through a fistula as a single dose 30 min after the morning meal. *Escherichia coli* cells were inoculated for sheep to provide an initial *E. coli* cell density of optical density at 660 nm of 2, which corresponded to 2×10^{10} cells/ml. The six treatments consisted of saline, *E. coli* W3110, *E. coli* nir-Ptac, NO_3, NO_3 plus *E. coli* W3110, and NO_3 plus *E. coli* nir-Ptac. CH_4 emission from sheep was reduced by the inoculation of *E. coli* W3110 or *E. coli* nir-Ptac by 6 % and 12 %, respectively. NO_3 markedly inhibited CH_4 emission from sheep. Compared with sheep given NO_3 alone, the inoculation of *E. coli* W3110 to NO_3-infused sheep lessened ruminal and plasma toxic NO_2 accumulation and blood methaemoglobin production, while keeping ruminal methanogenesis low. Ruminal and plasma toxic NO_2 accumulation and blood methaemoglobin production in sheep were unaffected by the inoculation of *E. coli* nir-Ptac. These results suggest that ruminal methanogenesis may be reduced by the inoculation of *E. coli* W3110 or *E. coli* nir-Ptac. The inoculation of *E. coli* W3110 may abate NO_3 toxicity when NO_3 is used to inhibit CH_4 emission from ruminants.

***Escherichia coli* W3110: *Escherichia coli* nir-Ptac: Methane emission: Nitrate**

The interaction between ruminant animals and ruminal micro-organisms is clearly symbiotic (Van Kessel & Rusell, 1996). The animal provides the micro-organism with a habitat for growth, and the micro-organisms, in turn, provide the animal with fermentation acids and microbial protein (Hungate, 1966). CH_4 is also a major end product of ruminal fermentation and represents the loss of 2–12 % of the gross energy consumed by ruminants (Johnson & Johnson, 1995). Additionally, CH_4 is a greenhouse gas implicated as a contributor to global warming (Moss, 1993). An inhibition of CH_4 production by ruminants would therefore have significant economical and environmental benefits (Van Nevel & Demeyer, 1996).

Attempts to identify specific chemical inhibitors of CH_4 production have largely been unsuccessful (Van Nevel & Demeyer, 1996). An alternative strategy to reduce ruminal methanogenesis is to promote alternative metabolic pathways to dispose of the reducing power, competing with methanogenesis for H uptake (López *et al.* 1999). The administration of NO_3 remarkably inhibited ruminal methanogenesis *in vitro* (Jones, 1972; Sar *et al.* 2005*a*) and *in vivo* (Takahashi & Young, 1991, 1992). However, elevated levels of NO_3 in feeds and water could pose a serious threat to animals owing to its conversion to toxic NO_2 in the rumen, subsequently causing methaemoglobin formation in the blood (Takahashi & Young, 1991, 1998; Sar *et al.* 2004*a*). The accumulation of ruminal toxic NO_2 is often the result of a usually faster reduction of NO_3 to NO_2 than of NO_2 to NH_3 (Takahashi *et al.* 1998). Stimulating ruminal NO_2 reduction to NH_3 is therefore an effective way of preventing NO_2 toxicity.

Wild-type *Escherichia coli (E. coli)* W3110 is known to have a certain NO_2 reductase activity in which NO_2 reductase has two subunits encoded by the *nirBD* operon. This enzyme, involved in NO_3 respiration, is induced in O_2-limited conditions (Gennis & Stewart, 1996). *Escherichia coli* nir-Ptac, constructed by replacing the promoter of the *nirBD* in *E. coli* W3110 by the *tac* promoter (Ajinomoto Co. Inc., Tokyo, Japan), showed twice as high an NO_2 reductase activity as was seen in *E. coli* W3110. Later, Sar *et al.* (2005*b*) indicated that *E. coli* W3110 and *E. coli* nir-Ptac inhibited toxic NO_2 accumulation and decreased CH_4 production in mixed ruminal cultures supplemented with NO_2, but did not inhibit it in mixed ruminal cultures supplemented with NO_3, although a decrease in CH_4 production was observed.

The objective of this study was therefore to evaluate the effects of two kinds of *E. coli* strain, wild-type *E. coli* W3110 and *E. coli* nir-Ptac (which has enhanced NO_2 reduction activity), on ruminal fermentation characteristics, CH_4 emission and NO_3 toxicity in NO_3-infused sheep.

Abbreviations: OD660, optical density at 660 nm; ORP, redox potential; VFA, volatile fatty acid.

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Materials and methods

Experimental design, animal feeding and additives

Four ruminally fistulated Cheviot wethers (53·25 (SD 4·01) kg) were individually kept in metabolic crates equipped with a ventilated respiratory collection hood and allocated in a 4×6 Youden square design. The animals were fed with twice daily (08.00 and 17.00 h) with a maintenance level of energy (55 g DM/$kg^{0·75}$ body weight per d) with a basal diet comprising Italian ryegrass, alfalfa hay cubes and a concentrate (in g/kg: dry matter (DM) 881·6; organic matter (OM) 940·3; dietary crude protein (CP) 192·6; acid detergent fibre (ADF) 75·0; neutral detergent fibre (NDF) 224·0; acid detergent lignin (ADL) 22·7; gross energy (GE) 19·00 MJ/kg DM) in the ratio 40:40:20 on a DM basis. Each sheep had free access to water and a block of trace mineralised salt (Fe 1232, Cu 150, Co 25, Zn 500, I 50, Se 15, Na 382 mg/kg). Each period lasted 8 d, comprising 7 d for acclimatisation to feeds and 1 d for the measurement of respiratory gas exchange and metabolic rate, as well as the simultaneous collection of ruminal fluid and blood. One week was allowed between treatments to assure that there were no carry-over effects of previous treatment.

The six treatments consisted of saline, inoculated wild-type *E. coli* W3110, inoculated *E. coli* nir-Ptac, NO_3, NO_3 plus *E. coli* W3110 and NO_3 plus *E. coli* nir-Ptac. Physiological saline (0·9 % NaCl; same volume as that of infused NO_3) administered as the control treatment was infused into the rumen through the ruminal fistula 30 min after the morning feeding. A 300 g/l aqueous solution of 1·3 g $NaNO_3$/$kg^{0·75}$ body weight, considered to be able to induce a subclinical NO_3 toxicity (Takahashi & Young, 1991), was administered via the ruminal fistula 30 min after the morning feed. To test the suppressant effects of wild-type *E. coli* W3110 and *E. coli* nir-Ptac on the NO_3-induced poisoning, respiratory gaseous exchange, metabolic rate and characteristics of ruminal fermentation, both *E. coli* strains (wild-type *E. coli* and *E. coli* nir-Ptac) and/or NO_3 were administered into the rumen through the ruminal fistula as a single dose 30 min after the morning feeding once on the sampling day. The cells of *E. coli* W3110 or *E. coli* nir-Ptac were inoculated for sheep to provide an initial *E. coli* cell density of an optical density at 660 nm (OD660) of 2 (Sar *et al.* 2005*a*), which corresponded to approximately 2×10^{10} cells/ml.

The sheep were weighed weekly prior to the beginning of each period to determine the daily allowance of feed and dosages of NO_3. The oral exchange of respiratory gases was monitored from 1 h before to 9 h after feeding the morning meal. Ruminal fluid was collected at 1, 2, 3, 4, 5, 6, 7, 8 and 9 h through a rumen fistula equipped with a valve to avoid any losses of gases during sampling using a hand syringe. Blood was collected via a jugular catheter 1, 3, 5, 7 and 9 h after an administration of the chemicals and inoculation of *E. coli* strains. The experimental protocol was approved by Obihiro University of Agriculture and Veterinary Medicine Committee for Animal Use and Care.

Genetic modification

Escherichia coli nir-Ptac was constructed by replacing the promoter region upstream of the chromosomal *nirBD* genes of wild-type *E. coli* W3110 by the *tac* promoter. First, a 3 kbp DNA fragment of the *nirBD* gene was amplified by PCR using *E. coli* W3110 chromosomal DNA as the template and oligonucleotides 5′-AAA AGA ATTCGAGGCAAA AATGAGCAA AGT-3′ and 5′-CCCCAA GCT TCATGCAAA AAGGGGAGGCAT-3′ as the primers, and was cloned into the *Eco*RI-*Hind*III site of an *E.coli* expression vector pKK223-3 (Amersham Pharmacia Biotech, New York, USA), containing the *tac* promoter so that the *nirBD* gene was expressed under the regulation of the *tac* promoter. Then, using this plasmid as the template, PCR was performed using oligonucleotides 5′-CGGGGTACCTTC TGGCGTCAGGCAGCCAT-3′ and 5′-ACATGCATGCCGTCTACGCCCAGCAGTTTC-3′ as the primers, which gave a 2 kbp DNA fragment with 200 bp of the pKK223-3 vector-derived sequence containing the *tac* promoter, followed by a 1·8 kbp sequence of the *nirBD*. The primers were designed so that the amplified DNA fragment had a *Kpn*I site at its 5′ end (at the end of the vector-derived region) and a *Sph*I site at its 3′ end (at the end of the *nirBD* gene-derived region). The amplified DNA fragment was digested with *Kpn*I and *Sph*I and was designated fragment 1.

Another PCR was performed using *E. coli* W3110 chromosome DNA as the template, and oligonucleotides 5′-CGGAATTCGTATGAAGGGCGTCAGCGCG-3′ and 5′-CGGGGTACCTTCTTA AGTCACGGA ATTGT-3′ as the primers, which gave a 1 kbp DNA fragment with its 3′ end 121 bp upstream of the start codon of the *nirB* gene. The primers were designed so that the amplified DNA fragment had an *Eco*RI site at its 5′ end (at the end furthest from the *nirB* gene) and a *Kpn*I site at its 3′ end (the end closer to the *nirB* gene). The amplified DNA fragment was digested with *Eco*RI and *Kpn*I, and was designated fragment 2.

Fragments 1 and 2 were ligated into the *Eco*RI and *Sph*I site of *E. coli* vector plasmid pHSG299 in the order of *Eco*RI–fragment 2–fragment 1–*Sph*I. The constructed plasmid was designated pHSG–*nir*-P*tac*. From pHSG-*nir*-P*tac*, the inserted DNA fragment was cut out with *Hind*III and ligated into *Hind*III sites of temperature-sensitive vector plasmid, pMAN997, which is a derivative of vector plasmid pMAN031 (Matsuyama & Mizushima, 1985). The resulting plasmid was designated pMAN-*nir*-P*tac*. Wild-type *E. coli* W3110 was transformed with pMAN–*nir*-P*tac* and cultured at a non-permissive temperature, and the clones with the promoter upstream of *nirBD* replaced by *tac* promoter were selected. The clone was designated nir-Ptac.

To measure the NO_2 reductase activity of the nir-Ptac, the *E. coli* nir-Ptac cells were cultured in a 500 ml flask containing 20 ml media (40 g glucose/l, 1 g $MgSO_4·7H_2O$/l, 24 g $(NH_4)_2SO_4$/l, 1 g KH_2PO_4/l, 10 mg $MnSO_4·7H_2O$/l, 10 mg $FeSO_4·7H_2O$/l, 2 g yeast extract/l, 30 g $CaCO_3$/l, 10 mM-KNO_2; pH 7·0) with constant shaking at 37°C for 11 h. The OD660 and NO_2 concentration in the culture media were measured every 1–2 h, and the NO_2 reductase activity (reduced NO_2, mM/h per g dry cells) was calculated by dividing the volumetric rates of NO_2 reduction by the respective values for cell mass. The dry cell weight was calculated from the OD660 by an experimentally obtained formula:

$$\text{Dry cell weight} = \text{OD660} \times 0{\cdot}67 + 0{\cdot}002$$

Escherichia coli W3110 or *E. coli* nir-Ptac cells were anaerobically grown on the Luria-Bertani broth agar (Sanko Junyaku Co. Ltd. Tokyo, Japan) at 37°C for 10 h and inoculated into each 5000 ml flask containing 1000 ml Luria-Bertani broth (10 g tryptone, 5 g yeast extract, 10 g NaCl/l) and cultured for 16 h at 37°C with constant shaking (120 rpm). Stationary-phase cells were harvested by centrifugation (15 000 ***g***, 8 min, 4°C), washed in sterile buffer solution (pH 6·8; McDougall, 1948) and resuspended in the sterile buffer solution.

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Experimental measurements

CO_2 and CH_4 production and V_{O_2} were monitored by a fully automated open-circuit respiratory system using a hood over the sheep's head, as reported by Takahashi *et al.* (1998). Metabolic rate (W) was calculated using the equation of Brouwer (1960). The rate of methanogenesis in the rumen was estimated from respiratory CH_4. CO_2, O_2 and CH_4 concentrations were measured as reported by Takahashi *et al.* (1998). Data were collected and entered into a computer from the analysers through an interface at 1 min intervals and then automatically standardised at 0°C, 1013 hPa and zero water vapour pressure.

The values of pH and redox potential (ORP) in ruminal fluid were measured using a pH and ORP meter (HM-21P; TOA Electronics Ltd. Tokyo, Japan), and then each sample was not acidified and was frozen at −20°C for later determination of ruminal NO_3, NO_2, NH_3-N, and volatile fatty acids (VFA). NO_3 and NO_2 concentration in the rumen were measured using the NO_3/NO_2 Assay Kit-C (Colorimetric; Dojindo, Kumamoto, Japan). The absorption coefficient was measured using a microplate reader (ELSIA Reader; Otsuka Electronics Co. Ltd, Osaka, Japan). NH_3-N concentration in the rumen was estimated as previously described (Sar *et al.* 2004*b*). The concentrations of VFA in the rumen were analysed by GLC (Shimadzu GC-14A; Shimadzu, Kyoto, Japan) equipped with a flame-ionisation detector and a capillary column (ULBON HR-52, 0·53 mm inner diameter × 30 m; Shinwa, Kyoto, Japan) by using 2-ethyl-*n*-butyric acid as the internal standard. Values were calculated automatically using a Chromatopac data processing system (C-R 4A; Shimadzu).

Ruminal juice for ciliate protozoal enumeration was collected 5 h after feeding and supplementation. Ruminal fluid (1 ml) was diluted with 9 ml methylgreen–formaline–saline solution, and ciliate protozoa were enumerated using a Fuchs-Rosenthal counting chamber (Hausser Scientific Partnership, Horsham, PA, USA) as previously described (Ogimoto & Imai, 1981).

Venous blood collected from a jugular catheter was used to determine concentrations of Hb (Nescauto Hemokit-N; Azwell Inc., Osaka, Japan) and methaemoglobin (Evelyn and Malloy, 1938). Plasma NO_2 concentration was measured as described for ruminal fluid.

Statistical analysis

Statistical analysis for Youden square experiments using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA) examined the effects of sheep, period and supplementary treatment in the model. Excluding data on protozoa, all data were analysed using the MIXED SAS procedure for repeated measures (Littell *et al.* 1998). Data on protozoa were analysed by ANOVA using the MIXED SAS procedure. Differences between treatments were determined using the least squares means procedure (PDIFF option) of SAS. Statistical significance of differences was taken as $P<0·05$, and trends were considered when $0·05 < P<0·10$ unless otherwise indicated.

Results

Methane emission

Table 1 shows that mean CH_4 production in sheep was decreased ($P<0·001$) by the addition of NO_3 compared with saline-infused sheep. Compared with saline-infused sheep, the mean CH_4 production in sheep was decreased by the inoculation of *E. coli* W3110 ($P=1·109$) or *E. coli* nir-Ptac ($P=0·004$). The inoculation of *E. coli* W3110 or *E. coli* nir-Ptac did not change the effect of NO_3 on mean CH_4 production in sheep. Fig. 1 shows that the rate of CH_4 production in sheep given NO_3 declined rapidly ($P<0·001$) compared with that in saline-infused sheep 1 h after the administration of NO_3. Although significant differences were not observed, the rate of CH_4 production in sheep inoculated with *E. coli* W3110 compared with saline-infused sheep decreased numerically ($P>0·05$) after 1 h, 3–5 h and 7 h after inoculation of *E. coli* W3110. Compared with saline-infused sheep, the rate of CH_4 production in *E. coli* nir-Ptac inoculated sheep decreased after 1–2 h ($P>0·05$) and 4–7 h ($P=0·10$) of the inoculation of *E. coli* nir-Ptac. When *E. coli* W3110 or *E. coli* nir-Ptac was inoculated into NO_3-infused sheep, the rate of CH_4 production was unaffected ($P>0·05$) compared with sheep infused with NO_3 alone.

Nitrate disappearance and nitrite accumulation in the rumen, plasma nitrite formation and blood methaemoglobin

Fig. 2 (A) shows the disappearance of NO_3 in the rumen. Compared with sheep given NO_3 alone, the NO_3 concentration was decreased ($P=0·095$) by the inoculation of *E. coli* nir-Ptac to NO_3-infused sheep, a further decrease ($P=0·008$) being observed with the inoculation of *E. coli* W3110 to NO_3-infused sheep. Fig. 2 (B) shows the accumulation of NO_2 in the rumen.

Table 1. Blood methaemoglobin, O_2 consumption, CO_2 and CH_4 production, and metabolic rate in sheep given NO_3 and/or *Escherichia coli* W3110 or *E. coli* nir-Ptac

	Treatment							
Parameters	Saline	*E. coli* W3110	*E. coli* nir-Ptac	NO_3	NO_3 plus *E. coli* W3110	NO_3 plus *E. coli* nir-Ptac	SEM	Statistical significance of effect: *P*
Methaemoglobin (% Hb)								
Mean of all values*	$0·00^{a}$	$0·00^{a}$	$0·00^{a}$	$12·67^{b}$	$7·89^{c}$	$13·66^{b}$	1·234	0·0001
Mean of maximum	$0·00^{a}$	$0·00^{a}$	$0·00^{a}$	$27·55^{b}$	$18·91^{c}$	$33·21^{b}$	2·590	0·0001
O_2 consumption† (ml/min per $kg^{0·75}$ BW)	$13·96^{a}$	$14·25^{a}$	$13·97^{a}$	$13·49^{a}$	$15·07^{b,c}$	$14·58^{a,b}$	0·463	0·0714
CO_2 production† (ml/min per $kg^{0·75}$ BW)	$14·40^{a}$	$13·07^{b,c}$	$14·46^{a}$	$11·69^{b}$	$13·34^{b,c}$	$13·38^{c}$	0·485	0·0004
CH_4 production† (ml/min per $kg^{0·75}$ BW)	$1·28^{a}$	$1·20^{a,b}$	$1·12^{b}$	$0·43^{c}$	$0·54^{c}$	$0·48^{c}$	0·042	0·0001
Metabolic rate† (Watt/$kg^{0·75}$ BW)	4·92	4·89	4·84	4·60	5·16	5·03	0·159	0·2256

BW, body weight.
* Value is mean of five sampling times.
[abc] Mean values within a row with unlike superscript letters were significantly different ($P < 0·05$) each value indicates mean of four animals.
† Value is the mean of 9 h observation.

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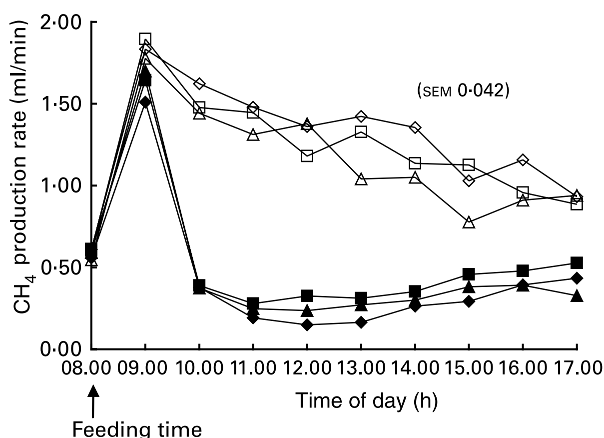

Fig. 1. Diurnal changes in CH_4 emission from sheep given saline (◇), *E. coli* W3110 (□), *E. coli* nir-Ptac (△), NO_3 (◆), NO_3 plus *E. coli* W3110 (■) and NO_3 plus *E. coli* nir-Ptac (▲).

The accumulation of NO_2 peaked 4 h after NO_3 had been administered to the sheep. When *E. coli* W3110 was inoculated into NO_3-infused sheep, both peak ($P=0·049$) and mean ($P<0·001$) NO_2 accumulation decreased compared with the values in sheep given NO_3 alone. Compared with sheep given NO_3 alone, NO_2 accumulation in sheep given NO_3 plus *E. coli* nir-Ptac decreased initially and then declined rapidly after the 3 h peak had been obtained, and the mean NO_2 accumulation also decreased ($P=0·075$).

Figure 2 (C) shows NO_2 formation in the plasma. Plasma NO_2 concentration peaked 5 h after NO_3 had been administered to the sheep. Compared with sheep given NO_3 alone, a decrease in both peak ($P<0·001$) and mean ($P=0·014$) plasma NO_2 concentration was observed in sheep given NO_3 and *E. coli* W3110. When *E. coli* nir-Ptac was inoculated to NO_3-infused sheep, both peak and mean plasma NO_2 concentrations were unaffected compared with the sheep given NO_3 alone.

Figure 2 (D) shows the formation of blood methaemoglobin in sheep. Blood methaemoglobin concentration in any of the saline-infused groups of sheep was below the detection level. The methaemoglobin level of total blood Hb peaked 7 h after the sheep had been infused with NO_3. When *E. coli* W3110 was inoculated into the NO_3-infused sheep, peak values of blood methaemoglobin decreased ($P<0·001$) compared with sheep given NO_3 alone. The inoculation of *E. coli* nir-Ptac to the NO_3-infused sheep did not affect the peak values of blood methaemoglobin compared with sheep given NO_3 alone.

Oxygen consumption, carbon dioxide production and metabolic rate

Table 1 shows that compared with saline-infused sheep, the addition of NO_3 to sheep caused a decline ($P<0·001$) in CO_2 production and a numerical decrease ($P>0·05$) in O_2 consumption and metabolic rate. When *E. coli* W3110 was inoculated to NO_3-infused sheep, an increase ($P=0·019$) in O_2 consumption and a numerical increase ($P>0·05$) in CO_2 production and metabolic rate were observed compared with sheep infused with NO_3 alone.

Ruminal fermentation characteristics

Ruminal fermentation characteristics are shown in Table 2. Ruminal pH did not decrease in sheep given NO_3 alone compared

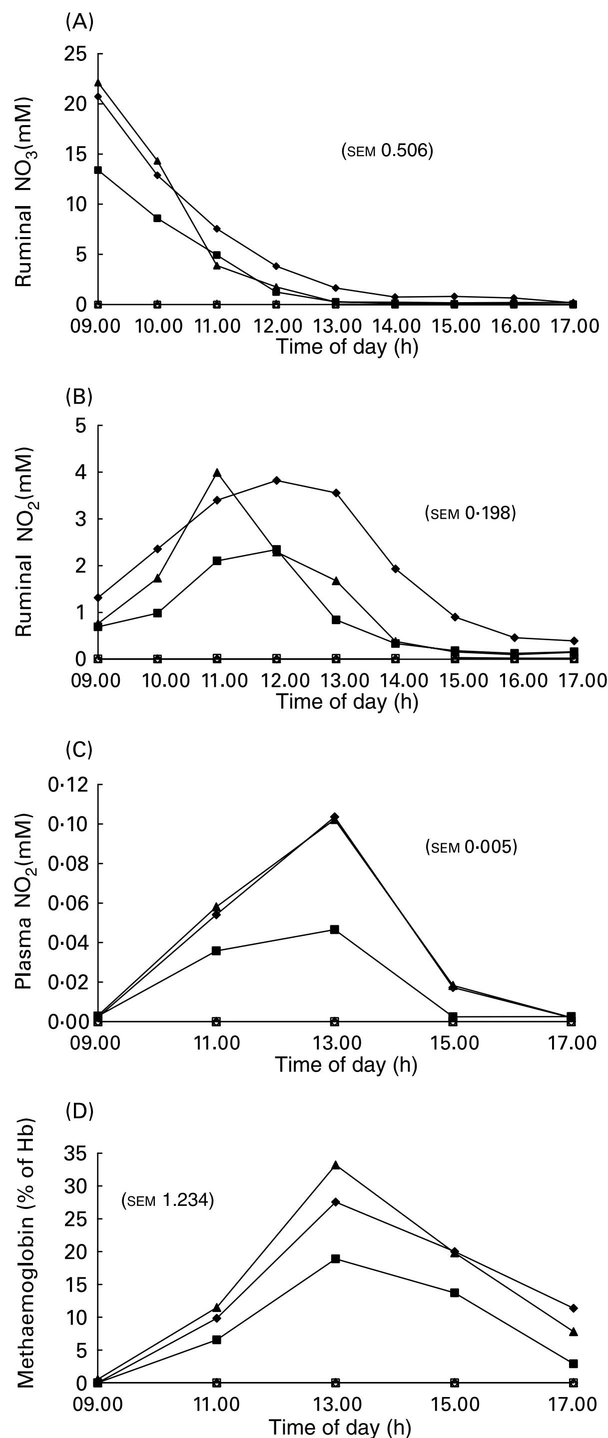

Fig. 2. Ruminal NO_3 disappearance (A) and NO_2 accumulation (B), plasma NO_2 formation (C), and formation of blood methaemoglobin (D) in sheep given NO_3 (◆), NO_3 plus *E. coli* W3110 (■) and NO_3 plus *E. coli* nir-Ptac (▲).

with saline-infused sheep. Compared with saline-infused sheep, ruminal pH decreased ($P=0·044$) in sheep inoculated with *E. coli* W3110 but increased ($P<0·001$) in sheep inoculated with *E. coli* nir-Ptac. The ORP in sheep given NO_3 increased ($P=0·0048$) compared with saline-infused sheep. When NO_3 was added to sheep, ruminal NH_3-N concentration increased ($P<0·001$) compared with saline-infused sheep. Compared with

sheep given NO_3 alone, ruminal NH_3-N concentration was unaffected by the inoculation of *E. coli* W3110 to NO_3-infused sheep. *Escherichia coli* nir-Ptac caused an increase in NH_3-N concentration in NO_3-infused sheep. Compared with saline-infused sheep, an increase in total VFA concentration was observed in sheep given NO_3 alone ($P=0{\cdot}016$) and sheep inoculated with *E. coli* W3110 ($P=0{\cdot}007$) or *E. coli* nir-Ptac ($P=0{\cdot}008$). The administration of NO_3 to sheep caused an increase ($P<0{\cdot}001$) in the molar proportion of acetate and a decrease ($P<0{\cdot}001$) in the molar proportions of propionate and butyrate. The inoculation of *E. coli* W3110 or *E. coli* nir-Ptac to NO_3-infused sheep decreased ($P<0{\cdot}001$) the molar proportion of acetate and increased the molar proportions of propionate ($P<0{\cdot}001$) and butyrate ($P>0{\cdot}05$). Compared with saline-infused sheep, a numerical decrease ($P>0{\cdot}05$) in ciliate protozoa was observed in sheep given NO_3 and in sheep inoculated with *E. coli* W3110 or *E. coli* nir-Ptac. Compared with sheep given NO_3 alone, the inoculation of *E. coli* W3110 to sheep given NO_3 numerically increased ($P>0{\cdot}05$) the ciliate protozoa.

Discussion

Several reports have shown that CH_4 production in the rumen was efficiently reduced by the administration of NO_3 (Jones, 1972; Takahashi & Young, 1991; Anderson & Rasmussen, 1998; Sar *et al.* 2004*a*). Our results showed that CH_4 emission was remarkably inhibited when the sheep were administered with NO_3, which is in agreement with these reports. This inhibition has been known to be due to NO_3 reduction in the rumen effectively competing with ruminal CH_4 production for electrons generated during fermentation (Allison & Reddy, 1990). Additionally, Allison & Reddy (1990) also reported that as the free-energy change ($\Delta G\acute{o}$) in the reactions concerned is as follows:

$$NO_3^- + 2H^+ + 4H_2 \rightarrow NH_4^+ + 3H_2O; \Delta G\acute{o} = -598\,\text{kJ/reaction}$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O; \quad \Delta G\acute{o} = -131\,\text{kJ/reaction}$$

then NO_3 reduction acts as a highly competitive H_2 sink against ruminal methanogenesis. The ORP of microbial cultures is a measure of the degree of anaerobiosis in the growth environment (Marais *et al.* 1988). For normal growth, the predominant ruminal micro-organisms require a low ORP value in the ambient medium, and ORP values of −300 mV or less have been reported for the ruminal contents of cattle (Smith & Hungate, 1958) and for the growth of methanogens (Stewart & Bryant, 1988). This study showed that when NO_3 was administered to sheep, the value of ORP increased to −290 mV compared with saline-infused sheep. Another, albeit less likely, reason for the inhibition of CH_4 emission from sheep by NO_3 may be, therefore, that the very low ORP required for the metabolism of ruminal methanogens is not sustained.

When ruminants consume forage or water containing high levels of NO_3 NO_3 intoxication was observed in the host because of the toxic accumulation of NO_2 in the rumen and plasma, inducing the production of blood methaemoglobin (Takahashi & Young, 1991, 1992; Sar *et al.* 2004*a*). This study showed that the toxic accumulation of NO_2 in the rumen and plasma respectively was at its peak 4 h and 5 h after the sheep were given NO_3 (Fig. 2 (B) and (C)), which is consistent with previous findings (Takahashi & Young, 1991; Sar *et al.* 2004*b*). An increase in rate of ruminal NO_3 disappearance, as well as a marked decrease in both peak and mean toxic NO_2 accumulation in the rumen and plasma, were observed in sheep given NO_3 plus *E. coli* W3110 compared with sheep given NO_3 alone. This result is contrary to *in vitro* findings (Sar *et al.* 2005*a*) that *E. coli* W3110 did not decrease toxic NO_2 accumulation in mixed ruminal cultures supplemented with NO_3 using the basal diet of orchard grass hay (in g/kg; DM 873·3 OM 989·8 %, CP 14 hrs %, ADF 388·4 , NDF 732·6 , ADL 41·0 %, GE: 4·45 Mcal, as DM basis), but supports our expectation that concentrate containing in diet supplies electrons for *E. coli* W3110 to accelerate NO_3/ NO_2 reduction in the rumen.

However, the inoculation of *E. coli* W3110 to the NO_3-infused sheep did not change the effect of NO_3 on CH_4 production. This may be due to NO_3 having a more potent effect than *E. coli* W3110 on ruminal methanogenesis. When *E. coli* nir-Ptac was inoculated to NO_3-infused sheep, NO_2 accumulation seemed to be unaffected, although it was initially decreased and then rapidly declined after the peak values at 3 h obtained compared with sheep given NO_3 alone and sheep given NO_3 plus *E. coli* W3110. These results do not support the hypothesis that *E. coli*

Table 2. Ruminal fermentation characteristics in sheep given NO_3 and/or *Escherichia coli* W3110 or *E. coli* nir-Ptac*

	Treatment							
Parameters	Saline	*E. coli* W3110	*E. coli* nir-Ptac	NO_3	NO_3 plus *E. coli* W3110	NO_3 plus *E. coli* nir-Ptac	SEM	Statistical significance of effect: *P*
Ruminal pH	6·47[a]	6·37[b]	6·63[c]	6·48[a]	6·43[a]	6·43[a]	0·038	0·0001
Ruminal redox potential (mV)	−319[a]	−316[a]	−319[a]	−294[c]	−308[a, b]	−290[b, c]	8·008	0·0004
Total VFA (mM)	84·64[a]	95·48[b]	94·76[b]	87·81[b]	86·81[a, b]	103·89[c]	3·004	0·0001
Molar % of VFA								
Acetate	75·72[a]	76·87[a]	74·81[b]	79·15[c]	76·56[d]	77·14[a]	0·343	0·0001
Propionate	12·42[a]	12·91[c]	12·76[c]	11·24[b]	14·16[c]	14·06[a, c]	0·258	0·0001
Butyrate	10·05[a]	9·05[b]	10·37[a]	6·77[c]	7·45[c]	7·00[c]	0·188	0·0001
Other VFA†	1·81[a, e]	1·16[a, d]	2·07[b]	1·17[c, d]	1·83[a, e]	1·79[e]	0·157	0·0001
Acetate:propionate ratio	6·29[a]	6·23[b]	6·03[a, b]	8·09[c]	5·50[a, b]	5·57[a, b]	0·243	0·0001
NH_3-N (mg/l)	181[a]	179[a]	190[a]	289[b]	287[b]	316[c]	7·817	0·0001
Ciliate protozoa ($\times 10^5$/ml)‡	12·80	9·95	9·50	5·44	9·81	8·33	2·278	0·2334

VFA, volatile fatty acids.

* All values are mean of nine sampling times unless otherwise indicated.

[abcde] Mean values within a row with unlike superscript letters were significantly, different ($P<0{\cdot}05$). Each value indicates means of four animals.

† Including valeric, isovaleric and isobutyric fatty acids.

‡ Value indicates the mean of sampling times at 5 h.

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nir-Ptac, via a potential of *tac* promoter, has a greater ability than wild-type *E. coli* W3110 to inhibit toxic NO_2 accumulation. This may be due to *E. coli* nir-Ptac not accelerating the activity of NO_3 reductase compared with *E. coli* W3110. Another reason is that as NO_3 reduction was accelerated by the inoculation of *E. coli* nir-Ptac (Fig. 2 (A)), NO_2 reduction in *E. coli* nir-Ptac may be limited by an insufficient electron supply in the present experiment. It has been reported that NO_2 reduction in *E. coli* strains was promoted by electron donors such as formate (Abou-Jaoudé *et al.* 1977) and especially pyruvate when strains of *E. coli* were deficient in NADH-NO_2 reductase activity and unable to produce formate from pyruvate owing to a lack of pyruvate formate-lyase activity (acetyl-CoA: formate *C*-acetyltransferase; EC 2.3.1.54; Pascal *et al.* 1981).

The administration of NO_3 to sheep in the present study produced a methaemoglobin level of 27·5 % of total Hb, which is comparable with the previous finding (Bodansky, 1951) reporting that an NO_3-induced methaemoglobin content of about 20 % of total Hb is considered subclinically toxic to ruminants. O_2 consumption, CO_2 production and metabolic rate in sheep given NO_3 alone were numerically decreased compared with saline-infused sheep, which is consistent with the findings of others (Takahashi & Young, 1991, 1992). These may result from the progressive production of NO_3-induced blood methaemoglobin. It has been reported that every 10 % replacement of oxyhaemoglobin with methaemoglobin reduced O_2 consumption by 10·3 % in sheep (Takahashi *et al.* 1998). The inoculation of *E. coli* W3110 into NO_3-infused sheep decreased both peak and mean blood methaemoglobin production, which may be explained by the fact that *E. coli* W3110 accelerated NO_3/NO_2 reduction in the rumen; consequently, toxic NO_2 accumulation in the rumen as well as in the plasma was decreased, as described earlier. Numerical increases in O_2 consumption, CO_2 production and metabolic rate in sheep given NO_3 plus *E. coli* W3110 compared with sheep given NO_3 alone were due to the decrease in blood methaemoglobin production resulting from the ability of *E. coli* W3110 to reduce NO_2 to NH_3. When *E. coli* nir-Ptac was inoculated into NO_3-infused sheep, blood methaemoglobin production was unaffected compared with sheep given NO_3 alone; this explains the lack of decline in ruminal and plasma NO_2 accumulation observed in sheep given NO_3 plus *E. coli* nir-Ptac.

An increased ruminal NH_3-N concentration associated with NO_3 supplementation has previously been reported (Lewis, 1951), confirmed by the result in the present study. This is the result of NO_3 reduction to NH_3. The inoculation of *E. coli* W3110 to NO_3-infused sheep did not affect ruminal NH_3-N concentration compared with sheep given NO_3 alone, and this does not support the hypothesis that when *E. coli* W3110 was inoculated to NO_3-infused sheep, it accelerated ruminal NO_3 and NO_2 reduction, causing the fall in ruminal toxic NO_2 accumulation; consequently, ruminal NH_3-N concentration should be high.

It was reported that the reduction in the number of protozoa could explain the higher total VFA concentration (Nollet *et al.* 1998) although a considerable number of reports showing the negative effects of defaunation on VFA production have been published (Williams & Coleman, 1995). The stimulation of total VFA concentration by *E. coli* W3110 or *E. coli* nir-Ptac in this study may account for the decrease in the number of ciliate protozoa. The inoculation of *E. coli* W3110 or *E. coli* nir-Ptac decreased CH_4 emission from sheep by 6 % and 12 %, respectively (Table 1), which is consistent with our *in vitro* results (Sar *et al.* 2005*b*). This decrease may account for a decrease in number and/or activity of ciliate protozoa by the inoculation of *E. coli* W3110 or *E. coli* nir-Ptac. It has been reported that ruminal methanogens are known to be associated with ciliate protozoa (Stumm *et al.* 1982; Finlay *et al.* 1994), and a disruption of this cohabitation may decrease CH_4 production (Nollet *et al.* 1998).

The increase in the molar proportion of acetate, and the decrease caused by NO_3 supplementation in the molar proportions of propionate and butyrate, in the present study confirmed previous reports (Farra & Satter, 1971; Nakamura *et al.* 1981; Takahashi *et al.* 1989; Sar *et al.* 2004*b*), and these could reflect the electron sink of NO_3 in rumen fermentation (Farra & Satter, 1971). Although the addition of *E. coli* W3110 did not change the effect of NO_3 on ruminal CH_4 production in sheep, a decrease in acetate:propionate ratio was observed. Moss and Givens (2002) reported that rumen stoichiometry could not explain the change in CH_4 production.

Conclusion

The present study has shown that NO_3 inhibited CH_4 emission from sheep via its reduction directing reductant away from CH_4. CH_4 emission from sheep was abated by the inoculation of wild-type *E. coli* W3110 or *E. coli* nir-Ptac, although the underlying mechanism has not been elucidated. Compared with sheep given NO_3 alone, the inoculation of *E. coli* W3110 to NO_3-infused sheep abated ruminal and plasma toxic NO_2 accumulation and blood methaemoglobin production, while keeping methanogenesis low. Ruminal and plasma toxic NO_2 accumulation and blood methaemoglobin production were unaffected by the inoculation of *E. coli* nir-Ptac. It is suggested that CH_4 emission from sheep may be reduced by the inoculation of wild-type *E. coli* W3110 or *E. coli* nir-Ptac. The inoculation of wild-type *E. coli* W3110 may abate NO_3/NO_2 toxicity when NO_3 is potentially applied to inhibit CH_4 mission from ruminants. Further research is required, however, to verify the long-term efficacy of wild-type *E. coli* W3110 as well as *E. coli* nir-Ptac on NO_3/NO_2 reduction *in vitro* and *in vivo*.

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