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 NO2 reduction activity, on oral CH4 emission and NO3 toxicity in NO3-treated sheep were assessed in a respiratory hood system in a 4 × 6 Youden square design. NO3 (1.3 g NaNO3/kg 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 × 1015 cells/ml. The six treatments consisted of saline, *E. coli* W3110, *E. coli* nir-Ptac, NO3, NO2, NO3 plus *E. coli* W3110, and NO2 plus *E. coli* nir-Ptac. CH4 emission from sheep was reduced by the inoculation of *E. coli* W3110 or *E. coli* nir-Ptac by 6% and 12%, respectively. NO3 markedly inhibited CH4 emission from sheep. Compared with sheep given NO3 alone, the inoculation of *E. coli* W3110 to NO3-infused sheep lessened ruminal and plasma toxic NO2 accumulation and blood methaemoglobin production, while keeping ruminal methanogenesis low. Ruminal and plasma toxic NO2 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 NO3 toxicity when NO3 is used to inhibit CH4 emission from ruminants.

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

The interaction between ruminant animals and ruminal microorganisms is clearly symbiotic (Van Kessel & Russel, 1996). The animal provides the microorganism with a habitat for growth, and the microorganisms, in turn, provide the animal with fermentation acids and microbial protein (Hungate, 1966). CH4 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, CH4 is a greenhouse gas implicated as a contributor to global warming (Moss, 1993). An inhibition of CH4 production by ruminants would therefore have significant economical and environmental benefits (Van Nevel & Demeyer, 1996).

Attempts to identify specific chemical inhibitors of CH4 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 NO3 remarkably inhibited ruminal methanogenesis in vitro (Jones, 1972; Sar et al. 2005a) and in vivo (Takahashi & Young, 1991, 1992). However, elevated levels of NO3 in feeds and water could pose a serious threat to animals owing to its conversion to toxic NO2 in the rumen, subsequently causing methaemoglobin formation in the blood (Takahashi & Young, 1991, 1998; Sar et al. 2004a). The accumulation of ruminal toxic NO2 is often the result of a usually faster reduction of NO2 to NO than of NO2 to NH3 (Takahashi et al. 1998). Stimulating ruminal NO2 reduction to NH3 is therefore an effective way of preventing NO2 toxicity.

Wild-type *Escherichia coli* (*E. coli*) W3110 is known to have a certain NO2 reductase activity in which NO2 reductase has two subunits encoded by the nirBD operon. This enzyme, involved in NO3 respiration, is induced in O2-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 NO2 reductase activity as was seen in *E. coli* W3110. Later, Sar et al. (2005b) indicated that *E. coli* W3110 and *E. coli* nir-Ptac inhibited toxic NO2 accumulation and decreased CH4 production in mixed ruminal cultures supplemented with NO3, but did not inhibit it in mixed ruminal cultures supplemented with NO3, although a decrease in CH4 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 NO2 reduction activity), on ruminal fermentation characteristics, CH4 emission and NO3 toxicity in NO3-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 401) kg) were individually kept in metabolic crates equipped with a ventilated respiratory collection hood and allocated in a 4 x 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, NO3, NO3 plus E. coli W3110 and NO3 plus E. coli nir-Ptac. Physiological saline (0.9% NaCl; same volume as that of infused NO3) 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 NaNO3/g 0.75 body weight, considered to be able to induce a subclinical NO3 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 NO3-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 NO3 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 cell density at 660 nm (OD660) of 2 (Sar et al., 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 NO3-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 NO3 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 cell density at 660 nm (OD660) of 2 (Sar et al., 2005a), which corresponded to approximately 2 x 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 NO3. 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 Ohiohi 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 ATTCGAGGCAA AATGAGCAA AGT-3' and 5'-CCCCCA GCT TATGCAGAA AAGGGGAGGCAT-3' as the primers, and was cloned into the EcoRI-HindIII site of an E.coli expression vector pK2223-3 (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'-CGGGTACCTTC TGGC GTACGAGCCAT-3' and 5'-ACATGCTAGCGCTATGCCG CTACGAGTTTC-3' as the primers, which gave a 2 kbp DNA fragment with 200 bp of the pK2223-3 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 KpnI site at its 5' end (at the end of the vector-derived region) and a SphI site at its 3' end (at the end of the nirBD gene-derived region). The amplified DNA fragment was digested with KpnI and SphI and was designated fragment 1. Another PCR was performed using E. coli W3110 chromosome DNA as the template, and oligonucleotides 5'-CGGAATTCGATTGAA GGGGGCGTAGCGCGG-3' and 5'-CGGGTACCTTC TGGC GTACGAGCCAAG-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 EcoRI site at its 5' end (at the end furthest from the nirB gene) and a KpnI site at its 3' end (at the end closer to the nirB gene). The amplified DNA fragment was digested with EcoRI and KpnI, and was designated fragment 2.

Fragments 1 and 2 were ligated into the EcoRI and SphI site of E. coli vector plasmid pHSG299 in the order of EcoRI–fragment 2–fragment 1–SphI. The constructed plasmid was designated pHS–nir-Ptac. From pHSG-nir-Ptac, the inserted DNA fragment was cut out with HindIII and ligated into HindIII 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-Ptac. Wild-type E. coli W3110 was transformed with pMAN–nir-Ptac 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 NO3 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 MgSO4·7H2O/l, 24 g (NH4)2SO4/l, 1 g KH2PO4/l, 10 mg MnSO4·7H2O/l, 10 mg FeSO4·7H2O/l, 2 g yeast extract/l, 30 g CaCO3/l, 10 mM-KNO2· H2O/ l, pH 7.0) with constant shaking at 37°C for 11 h. The OD660 and NO3 concentration in the culture media were measured every 1-2 h, and the NO3 reductase activity (reduced NO3, µmol/h per g dry cells) was calculated by dividing the volumetric rates of NO3 reduction by the respective values for cell mass. The dry cell weight was calculated from the OD660 by an experimentally obtained formula:

\[ \text{Dry cell weight} = \text{OD660} \times 0.67 + 0.002 \]

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 (15000 g, 8 min, 4°C), washed in sterile buffer solution (pH 6.8; McDougall, 1948) and resuspended in the sterile buffer solution.
Experimental measurements

CO₂ and CH₄ production and VO₂ 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 CH₄, CO₂, O₂, and CH₄ 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 NO₃, NO₂, NH₃-N, and volatile fatty acids (VFA). NO₃ and NO₂ concentration in the rumen were measured using the NO₃/NO₂ Assay Kit-C (Colorimetric; Dojindo, Kumamoto, Japan). The absorption coefficient was determined using a microplate reader (ELSIA Reader; Otsuka Electronics Co. Ltd, Osaka, Japan). NH₃-N concentration in the rumen was estimated as previously described (Sar et al. 2004b). 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 x 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–formalin–saline solution, and ciliate protozoa were enumerated using a Fuchs-Rosenthal counting chamber (Hauser 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 NO₂ 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 CH₄ production in sheep was decreased (P<0.001) by the addition of NO₃ compared with saline-infused sheep. Compared with saline-infused sheep, the mean CH₄ 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 NO₃ on mean CH₄ production in sheep. Fig. 1 shows that the rate of CH₄ production in sheep given NO₃ declined rapidly (P<0.001) compared with that in saline-infused sheep 1 h after the administration of NO₃. Although significant differences were not observed, the rate of CH₄ 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 CH₄ 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 NO₃-infused sheep, the rate of CH₄ production was unaffected (P>0.05) compared with sheep infused with NO₃ alone.

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

Table 1. Blood methaemoglobin, O₂ consumption, CO₂ and CH₄ production, and metabolic rate in sheep given NO₃ and/or Escherichia coli W3110 or E. coli nir-Ptac

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Saline</th>
<th>E. coli W3110</th>
<th>E. coli nir-Ptac</th>
<th>NO₃</th>
<th>E. coli W3110</th>
<th>E. coli nir-Ptac</th>
<th>NO₂</th>
<th>E. coli W3110</th>
<th>E. coli nir-Ptac</th>
<th>NO₂ plus E. coli W3110</th>
<th>E. coli nir-Ptac</th>
<th>SEM</th>
<th>Statistical significance of effect: P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methaemoglobin (% Hb)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean of all values</td>
<td>0.00a</td>
<td>0.00b</td>
<td>0.00c</td>
<td>12.67</td>
<td>0.00d</td>
<td></td>
<td>7.89c</td>
<td>13.66d</td>
<td></td>
<td>1.234</td>
<td></td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Mean of maximum</td>
<td>0.00a</td>
<td>0.00b</td>
<td>0.00c</td>
<td>27.55</td>
<td>0.00d</td>
<td></td>
<td>18.91c</td>
<td>33.21b</td>
<td></td>
<td>2.590</td>
<td></td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>O₂ consumption (ml/min per kg⁻₀·⁷伍 BW)</td>
<td>13.96</td>
<td>14.25</td>
<td>13.97</td>
<td>13.49</td>
<td>15.07</td>
<td></td>
<td>14.58b</td>
<td>0.463</td>
<td></td>
<td>0.071</td>
<td></td>
<td>0.004</td>
<td></td>
</tr>
<tr>
<td>CO₂ production (ml/min per kg⁻₀·⁷伍 BW)</td>
<td>14.40</td>
<td>13.07</td>
<td>14.48</td>
<td>11.69</td>
<td>13.34</td>
<td></td>
<td>13.38c</td>
<td>0.485</td>
<td></td>
<td>0.0004</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH₄ production (ml/min per kg⁻₀·⁷伍 BW)</td>
<td>1.28</td>
<td>1.29</td>
<td>1.12</td>
<td>0.45</td>
<td>0.54</td>
<td></td>
<td>0.48</td>
<td>0.042</td>
<td></td>
<td>0.0001</td>
<td></td>
<td>0.2256</td>
<td></td>
</tr>
<tr>
<td>Metabolic rate (Watt/kg⁻₀·⁷伍 BW)</td>
<td>4.92</td>
<td>4.89</td>
<td>4.84</td>
<td>4.60</td>
<td>5.16</td>
<td></td>
<td>5.03</td>
<td>0.159</td>
<td></td>
<td>0.256</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BW, body weight.

a,b,c Mean values within a row with unlike superscript letters were significantly different (P<0.05) each value indicates mean of four animals.

† Value is mean of 9 h observation.

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The accumulation of NO2 peaked 4 h after NO3 had been administered to the sheep. When E. coli W3110 was inoculated into NO3-infused sheep, both peak ($P = 0.049$) and mean ($P = 0.001$) NO2 accumulation decreased compared with the values in sheep given NO3 alone. Compared with sheep given NO3 alone, NO2 accumulation in sheep given NO3 plus E. coli nir-Ptac decreased initially and then declined rapidly after the 3 h peak had been obtained, and the mean NO2 accumulation also decreased ($P = 0.075$).

Figure 2 (C) shows NO2 formation in the plasma. Plasma NO2 concentration peaked 5 h after NO3 had been administered to the sheep. Compared with sheep given NO3 alone, a decrease in both peak ($P < 0.001$) and mean ($P = 0.014$) plasma NO2 concentration was observed in sheep given NO3 and E. coli W3110. When E. coli nir-Ptac was inoculated to NO3-infused sheep, both peak and mean plasma NO2 concentrations were unaffected compared with the sheep given NO3 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 NO3. When E. coli W3110 was inoculated into the NO3-infused sheep, peak values of blood methaemoglobin decreased ($P < 0.001$) compared with sheep given NO3 alone. The inoculation of E. coli nir-Ptac to the NO3-infused sheep did not affect the peak values of blood methaemoglobin compared with sheep given NO3 alone.

**Oxygen consumption, carbon dioxide production and metabolic rate**

Table 1 shows that compared with saline-infused sheep, the addition of NO3 to sheep caused a decline ($P < 0.001$) in CO2 production and a numerical decrease ($P > 0.05$) in O2 consumption and metabolic rate. When E. coli W3110 was inoculated to NO3-infused sheep, an increase ($P = 0.019$) in O2 consumption and a numerical increase ($P > 0.05$) in CO2 production and metabolic rate were observed compared with sheep infused with NO3 alone.

**Ruminal fermentation characteristics**

Ruminal fermentation characteristics are shown in Table 2. Ruminal pH did not decrease in sheep given NO3 alone compared 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 NO3 increased ($P = 0.0048$) compared with saline-infused sheep. When NO3 was added to sheep, ruminal NH$_3$-N concentration increased ($P < 0.001$) compared with saline-infused sheep. Compared with
sheep given NO3 alone, ruminal NH3-N concentration was unaffected by the inoculation of E. coli W3110 to NO3-infused sheep. Escherichia coli nir-Ptac caused an increase in NH3-N concentration in NO3-infused sheep. Compared with saline-infused sheep, an increase in total VFA concentration was observed in sheep given NO3 alone (P=0.016) and sheep inoculated with E. coli W3110 (P=0.007) or E. coli nir-Ptac (P=0.008). The administration of NO3 to sheep caused an increase (P<0.001) in the molar proportion of acetate and a decrease (P<0.001) in the molar proportions of propionate and butyrate. The inoculation of E. coli W3110 or E. coli nir-Ptac to NO3-infused sheep decreased (P<0.001) the molar proportion of acetate and increased the molar proportions of propionate (P<0.001) and butyrate (P>0.05). Compared with saline-infused sheep, a numerical decrease (P>0.05) in ciliate protozoa was observed in sheep given NO3 and in sheep inoculated with E. coli W3110 or E. coli nir-Ptac. Compared with sheep given NO3 alone, the inoculation of E. coli W3110 to sheep given NO3 numerically increased (P>0.05) the ciliate protozoa.

Discussion

Several reports have shown that CH4 production in the rumen was efficiently reduced by the administration of NO3 (Jones, 1972; Takahashi & Young, 1991; Anderson & Rasmussen, 1998; Sar et al. 2004a). Our results showed that CH4 emission was remarkably inhibited when the sheep were administered with NO3, which is in agreement with these reports. This inhibition has been known to be due to NO3 reduction in the rumen effectively competing with ruminal CH4 production for electrons generated during fermentation (Allison & Reddy, 1990). Additionally, Allison & Reddy (1990) also reported that as the free-energy change (ΔG°) in the reactions concerned is as follows:

\[
\text{NO}_3^- + 2\text{H}^+ + 4\text{H}_2 \rightarrow \text{NH}_4^+ + 3\text{H}_2\text{O}; \Delta G^o = -598 \text{kJ/reaction}
\]

\[
4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}; \Delta G^o = -131 \text{kJ/reaction}
\]

then NO3 reduction acts as a highly competitive H2 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 NO3 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 CH4 emission from sheep by NO3 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 NO3 NO3 intoxication was observed in the host because of the toxic accumulation of NO2 in the rumen and plasma, inducing the production of blood methaemoglobin (Takahashi & Young, 1991, 1992; Sar et al. 2004a). This study showed that the toxic accumulation of NO2 in the rumen and plasma respectively was at its peak 4 h and 5 h after the sheep were given NO3 (Fig. 2 (B) and (C)), which is consistent with previous findings (Takahashi & Young, 1991; Sar et al. 2004b). An increase in rate of ruminal NO3 disappearance, as well as a marked decrease in both peak and mean toxic NO2 accumulation in the rumen and plasma, were observed in sheep given NO3 plus E. coli W3110 compared with sheep given NO3 alone. This result is contrary to in vitro findings (Sar et al. 2005a) that E. coli W3110 did not decrease toxic NO2 accumulation in mixed ruminal cultures supplemented with NO3 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 NO3/NO2 reduction in the rumen.

However, the inoculation of E. coli W3110 to the NO3-infused sheep did not change the effect of NO3 on CH4 production. This may be due to NO3 having a more potent effect than E. coli W3110 on ruminal methanogenesis. When E. coli nir-Ptac was inoculated to NO3-infused sheep, NO2 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 NO3 alone and sheep given NO3 plus E. coli W3110. These results do not support the hypothesis that E. coli...
The administration of NO3 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 NO3-induced methaemoglobin content of about 20% of total Hb is considered subclinically toxic to ruminants. O2 consumption, CO2 production and metabolic rate in sheep given NO3 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 NO3-induced blood methaemoglobin. It has been reported that every 10% replacement of oxyhaemoglobin with methaemoglobin reduced O2 consumption by 10.3% in sheep (Takahashi et al. 1998). The inoculation of E. coli W3110 into NO3-infused sheep decreased both peak and mean blood methaemoglobin production, which may be explained by the fact that E. coli W3110 accelerated NO3/NO2 reduction in the rumen; consequently, toxic NO2 accumulation in the rumen as well as in the plasma was decreased, as described earlier. Numerical increases in O2 consumption, CO2 production and metabolic rate in sheep given NO3 plus E. coli W3110 compared with sheep given NO3 alone were due to the decrease in blood methaemoglobin production resulting from the ability of E. coli W3110 to reduce NO2 to NH3. When E. coli nir-Ptac was inoculated into NO3-infused sheep, blood methaemoglobin production was unaffected compared with sheep given NO3 alone; this explains the lack of decline in ruminal and plasma NO2 accumulation observed in sheep given NO3 plus E. coli nir-Ptac.

An increased ruminal NH3-N concentration associated with NO3 supplementation has previously been reported (Lewis, 1951), confirmed by the result in the present study. This is the result of NO3 reduction to NH3. The inoculation of E. coli W3110 to NO3-infused sheep did not affect ruminal NH4-N concentration compared with sheep given NO3 alone, and this does not support the hypothesis that when E. coli W3110 was inoculated to NO3-infused sheep, it accelerated ruminal NO3 and NO2 reduction, causing the fall in ruminal toxic NO2 accumulation; consequently, ruminal NH3-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 deamination 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 CH4 emission from sheep by 6% and 12%, respectively (Table 1), which is consistent with our in vitro results (Sat et al. 2005b). 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 CH4 production (Nollet et al. 1998).

The increase in the molar proportion of acetate, and the decrease caused by NO3 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. 2004b), and these could reflect the electron sink of NO3 in rumen fermentation (Farra & Satter, 1971). Although the addition of E. coli W3110 did not change the effect of NO3 on ruminal CH4 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 CH4 production.

Conclusion

The present study has shown that NO3 inhibited CH4 emission from sheep via its reduction directing reductant away from CH4. CH4 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 NO3 alone, the inoculation of E. coli W3110 to NO3-infused sheep abated ruminal and plasma toxic NO2 accumulation and blood methaemoglobin production, while keeping methanogenesis low. Ruminal and plasma toxic NO2 accumulation and blood methaemoglobin production were unaffected by the inoculation of E. coli nir-Ptac. It is suggested that CH4 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 NO3/NO2 toxicity when NO3 is potentially applied to inhibit CH4 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 NO3/NO2 reduction in vitro and in vivo.

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References

Brouwer E (1960) On simple formulae for calculating the heat expenditure and quantities of carbohydrate and fat metabolized in ruminants from data on gaseous exchange and urine-N. In Energy Metabolism,


