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Comparisons of *In vitro* Nitrate Reduction, Methanogenesis, and Fermentation Acid Profile among Rumen Bacterial, Protozoal and Fungal Fractions*

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ABSTRACT : The objectives were to compare the ability of various rumen microbial fractions to reduce nitrate and to assess the effect of nitrate on *in vitro* fermentation characteristics. Physical and chemical methods were used to differentiate the rumen microbial population into the following fractions: whole rumen fluid (WRF), protozoa (Pr), bacteria (Ba), and fungi (Fu). The three nitrogen substrate treatments were as follows: no supplemental nitrogen source, nitrate or urea, with the latter two being isonitrogenous additions. The results showed that during 24 h incubation, WRF, Pr and Ba fractions had an ability to reduce nitrate, and the rate of nitrate disappearance for the Pr fraction was similar to the WRF fraction, while the Ba fraction needed an adaptation period of 12 h before rapid nitrate disappearance. The WRF fraction had the greatest methane (CH_4) production and the Pr fraction had the greatest prevailing H_2 concentration ($p<0.05$). Compared to the urea treatment, nitrate diminished net gas and CH_4 production during incubation ($p<0.05$), and ammonia-N (NH_3-N) concentration ($p<0.01$). Nitrate also increased acetate, decreased propionate and decreased butyrate molar proportions ($p<0.05$). The Pr fraction had the highest acetate to propionate ratio ($p<0.05$). The Pr fraction as well as the Ba fraction appears to have an important role in nitrate reduction. Nitrate did not consistently alter total VFA concentration, but it did shift the VFA profile to higher acetate, lower propionate and lower butyrate molar proportions, consistent with less CH_4 production by all microbial fractions. (**Key Words :** Nitrate, Rumen, Protozoa, Bacteria, Fungi, Methane)

INTRODUCTION

The main sources of nitrate intake by ruminants are feeds and water. Nitrate in forage has been reported to make up about 37% of total nitrogen (Miyazaki, 1977), especially in green forage. However, nitrate cannot be used in the rumen as a nitrogen source directly; it must be reduced by certain ruminal microbes to ammonia which is a preferred nitrogen source. Ruminant methane production is both a loss of 2-12% of the feed gross energy (Johnson and Johnson, 1995) and a source of greenhouse gas. Inhibition of methane production by ruminants would have significant economic and environmental benefits (Van Nevel and Demeyer, 1996). Reports from *in vitro* (Anderson and Rasmussen, 1998; Guo et al., 2009) and *in vivo* (Takahashi and Young, 1991) experiments have reported that addition of nitrate dramatically inhibited ruminal methanogenesis and increased microbial crude protein (MCP) production. Hydrogen is a principal substrate for ruminal methanogenesis, and also for nitrate reduction. Additionally, nitrate-reducing microbes compete with methanogens for H_2, and have a competitive advantage (Jones, 1972). *Selenomonas ruminantium*, *Veillonella parvula* and *Wollinella succinogenes* reduce nitrate and nitrite (Stewart and Bryant, 1988). Sar et al. (2005a, 2005b, 2006) reported that a mixed culture consisting of ruminal microbes, *Escherichia coli* W3110 and its derivative (*E. coli* nir-Ptac) was also effective in reducing nitrate and nitrite. Rumen protozoa have been reported to accelerate nitrate reduction when co-cultured with bacteria (Yoshida et al., 1982). However there is little research available concerning different microbial fractions responsible for nitrate reduction and methane production in the rumen. Therefore,

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the objective of this study was to compare the ability of ruminal bacteria, protozoa and fungi to reduce nitrate as well as to determine the effect of nitrate on *in vitro* methane production and VFA profile of each of these fractions of ruminal microbes.

MATERIALS AND METHODS

All procedures involving animals were conducted under approval of the China Agricultural University Institutional Animal Care and Use Committee.

In vitro substrates

Three substrate mixtures were formulated as described previously (Guo et al., 2009). Sodium nitrate (S5506, Sigma-Aldrich Chemical Co., St Louis, MO) or urea (U4128, Sigma-Aldrich) was the sole N source, while the control mixture had no N source added. Soluble starch (S4251, Sigma-Aldrich) and Avicel (GH-9471, Fluka, Chemie GmbH) were used to balance the dietary N content (12.5% CP) and sodium chloride (NaCl) was added to maintain equivalent Na concentrations (0.09%, W/V) as shown in Table 1.

Culture medium

Rumen fluid was withdrawn from three Limousin× Fuzhou crossbred steers (average body weight = 400 kg), each fitted with a permanent rumen cannula. The animals were fed two equal meals daily (08:00 and 16:00 h). The ration (12.6% CP) consisted of (DM basis) 60% corn silage and 40% mixed concentrate (DM basis, 62.5% corn grain, 36.0% soybean meal, 0.75% salt, 0.5% limestone, and 0.25% vitamin and trace mineral premix). Water was freely accessible to animals. Since there was no exogenous addition of nitrate to the diet or water consumed by these steers, their rumen microbial population was considered to be unadapted to nitrate reduction.

All rumen fluid inocula were obtained 5 h after the morning feeding. Ruminal contents were strained through four layers of cheesecloth and pooled together, then brought immediately to the laboratory. In order to compare the ability for nitrate reduction, four microbial fractions were prepared from the strained rumen fluid according to the method described by Lee et al. (2000) with a minor modification as follows:

- Whole rumen fluid (WRF): Rumen contents from three steers were obtained, strained through four layers of cheesecloth, and pooled together.
- Protozoa (Pr): To WRF were added antibacterial agents (streptomycin sulfate, penicillin G, potassium, and chloramphenicol (0.100 mg/ml each)), and antifungal agents (cychloheximide (0.05 mg/ml) and nystatin (200 U/ml)).
- Bacteria (Ba): WRF was centrifuged at 500×*g* for 5 min at 4°C to collect supernatant containing bacteria and fungi and then the antifungal agents were added.
- Fungi (Fu): WRF was centrifuged at 500×*g* for 5 min at 4°C to collect supernatant containing bacteria and fungi and then the antibacterial agents were added.

All antibiotics and chemicals were from Sigma (Sigma-Aldrich Chemical Co., St Louis, MO).

Table 1. Ingredients and chemical composition of the experimental treatments

Items	Treatment		
	Control[1]	UND[2]	NND[3]
Ingredients			
Soluble starch (% DM)	57.4	57.4	57.4
Avicel (% DM)	30.0	30.0	30.0
Urea (% DM)	-	4.5	-
Sodium nitrate (% DM)	-	-	12.6
Sodium chloride (% DM)	12.6	8.1	-
Chemical composition[4]			
Crude protein (% DM)	0.4	12.6	12.2

[1] Control = No N added. [2] UND = Urea-N diet.
[3] NND = Nitrate-N diet. [4] Actual determination.

Incubation and sampling methods

Incubations were conducted anaerobically for 24 h using the gas production method of Menke et al. (1979). Rumen fluid filtrates were pooled into an anaerobic buffer solution under a constant flow of O_2-free CO_2 (the ratio of rumen fluid: buffer = 1:2). Then, 30 ml or 50 ml of inoculated culture medium were pipetted with an automatic pump into replicate glass syringes (HFT000025, Häberle Maschinenfabrik GmbH, Germany) which were prewarmed to 39°C. The syringes were incubated in a shaking water bath at 39°C. Blank syringes which contained only inoculated culture medium with no substrate addition were simultaneously incubated. Each microbial fraction had its own three blank syringes. Aliquots of 30 ml of inoculated culture medium were incubated for gas composition measurement, and 50 ml aliquots were incubated for nitrate and nitrite determinations and for fermentation parameter measurement. Each microbial fraction at each sampling time had three syringes for determining gas composition; however, all collections for analyzing fermentation parameters were from the same syringe which had 50 ml of initial inoculated culture medium.

Sampling and analysis

Three milliliters were collected at 6, 12 and 24 h to immediately determine pH with a pH meter equipped with a glass electrode (Model PHS-3C, Shanghai Leici Scientific Instrument Co., Ltd., China). Then each sample was

centrifuged for 15 min at 3,000×g, 4°C. The supernatants were divided into three equal parts for later determination of nitrate (NO_3-N), nitrite (NO_2-N), ammonia-N (NH_3-N), and volatile fatty acid (VFA) concentrations. The first part of the supernatant was mixed with 25% phosphoric acid, and then frozen at -20°C overnight. After thawing, the acidified samples were centrifuged for 15 min at 6,000×g and 4°C, and the supernatant was analyzed for NO_3-N and NO_2-N concentrations using an ion chromatograph (Model Dionex-2500, Dionex Co., Ltd, USA) with an Ionpac AS11-HC 2-mm analytical column. The eluent system was multi-concentration eluent flow at 1.2 ml/min with 22.5 mmol/L NaOH for 6.7 min, 40 mmol/L NaOH between 6.7-12.0 min and 22.5 mmol/L NaOH from 12.1 min to 13.0 min. Standard solutions were obtained from National Information Infrastructure for Certified Reference Material (Beijing).

The second part of the supernatant was also mixed with 25% phosphoric acid which contained 2 g/L 2-ethyl-n-butyric acid as the internal standard, and was then frozen at -20°C overnight for VFA concentration measurement. After thawing, the centrifugation procedure was similar to that for nitrate and nitrite measurement. The detailed procedure for VFA determination was described by Guo et al. (2009). The third part of the supernatant was measured for NH_3-N concentration (Broderick and Kang, 1980). Net concentrations of VFA and NH_3-N are reported, following subtraction of concentrations found in corresponding control incubations.

Gas composition (hydrogen, nitrogen, methane and carbon dioxide) of the headspace gas of each microbial fraction was determined at 6, 12 and 24 h incubation. A gas sample was obtained from each syringe using a 1 ml glass syringe (Guo et al., 2009), and then analyzed for composition by gas chromatograph (TP-2060T, Beifen Ruili Analytical Equipment Co., Beijing, China) equipped with a TC detector (column: TDX-01, 1 m×3 mm×2 mm, column temperature: 120°C, detector temperature: 150°C, injector temperature: 150°C, carrier gas: argon; gas flow: 50 ml/min, injection volume: 1 ml). When nitrogen was detected, it was assumed to be an atmospheric contaminant of the headspace and not a product of the incubation. The contribution of nitrogen to total gas volume was removed by calculation. Gas volumes were converted to micromolar amounts using the ideal gas law. The formula for calculation of moles of gas was as follows:

Gas (μmol) = P×((total gas volume×individual percentage)-(blank gas volume×individual percentage))/(R×T), which is based on a temperature of 312°K and pressure of atmosphere and plunger weight.

Statistical analysis

All data were statistically analyzed as a completely randomized design using two-factor mixed procedures of SAS (SAS Institute, 2008). The model was $Y_{ijk} = \mu + X_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{11k} + \varepsilon_{12k} + \varepsilon_{21k} + \varepsilon_{22k}$, in which i is nitrogen source (nitrate vs. urea), j is ruminal microbial fraction (WRF, Pr, Ba and Fu), and k is tube. Means were analyzed using least squares means (LSMEANS) procedure.

RESULTS

NO_3-N and NO_2-N

Figure 1 shows nitrate disappearance and nitrite

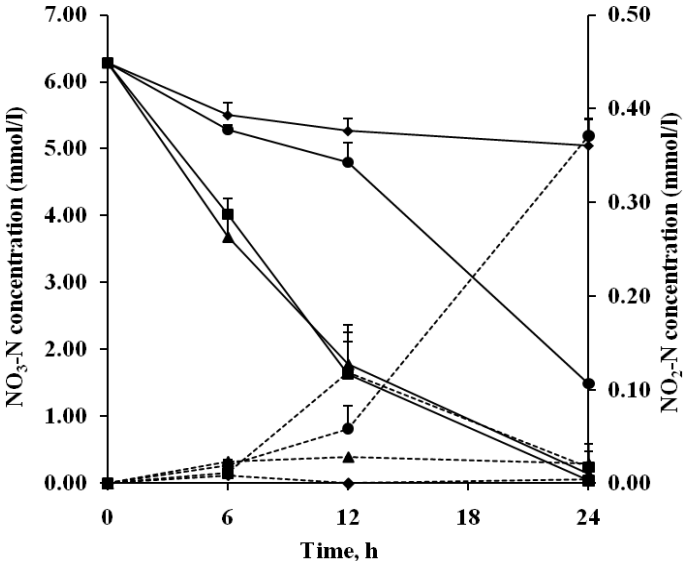

Figure 1. The concentration of nitrate and nitrite in ruminal microbe fractions at 6, 12 and 24 h *in vitro* incubation. Solid line represents nitrate concentration during incubation periods; concentration of nitrite is shown as a short dashed line on the right y-axis; WRF (■), whole ruminal fluid; Pr (▲), protozoa; Ba (●), bacteria; Fu (♦), fungi.

Table 2. Effect of nitrogen source (urea or nitrate) and rumen microbial fraction (whole rumen fluid (WRF), protozoa (Pr), bacteria (Ba), and fungi (Fu)) on gas production and composition at 24 h incubation *in vitro*[1]

Items	Urea				Nitrate				SEM		p-value		
	WRF	Pr	Ba	Fu	WRF	Pr	Ba	Fu	Nitrogen source (N)	Microbial fraction (MF)	N	MF	N*MF
Gas volume (ml/0.2 g DM)	57.4^{ACa}	29.4^{AEc}	47.3^{ADb}	7.4^{AFe}	41.8^{BCa}	16.2^{BEd}	22.8^{BDd}	3.6^{BFf}	1.56	2.99	<0.01	<0.01	<0.01
Gas proportion (%)													
H_2	0.020^{BEd}	0.377^{BDc}	0.012^{BEd}	0.959^{BCb}	0.082^{AEc}	0.335^{ADc}	0.005^{ADd}	1.625^{ACa}	0.04	0.08	<0.01	<0.01	<0.01
CH_4	15.0^{ACa}	15.5^{ACa}	8.5^{ADb}	7.5^{AEb}	8.7^{BCb}	8.6^{BCb}	3.9^{BDc}	1.9^{BEc}	0.26	0.40	<0.01	<0.01	0.02
CO_2	85.0^{BDc}	84.1^{BDc}	91.5^{BCb}	91.5^{BCb}	91.2^{ADb}	91.0^{ADb}	96.1^{ACa}	96.5^{ACa}	0.24	0.37	<0.01	<0.01	0.01
Net gas (μmol)													
H_2	0.636^{D}	8.619^{C}	0.319^{E}	2.063^{C}	2.382^{D}	6.153^{C}	0.064^{E}	3.832^{C}	0.74	1.65	0.83	<0.01	0.07
CH_4	383^{ACa}	205^{ADb}	176^{ADb}	16.3^{AEd}	81.9^{BCc}	-13.6^{BDd}	26.9^{BDc}	4.5^{BEd}	8.40	25.1	<0.01	<0.01	<0.01
CO_2	$2{,}058^{ACa}$	$1{,}038^{AEb}$	$1{,}851^{ADa}$	293^{AFd}	$1{,}700^{BCa}$	695^{BEc}	943^{BDc}	149^{BFe}	55.8	61.7	<0.01	<0.01	<0.01

[1] The first letter in each superscript series is associated with the largest mean value.

[A, B] Means with a different superscript denote the effect of nitrogen source ($p<0.05$).

[C, D, E, F] Means with a different superscript denote the effect of microbial fraction ($p<0.05$).

[a, b, c, d, e] Means with a different superscript denote the effect of nitrogen source×microbial fraction ($p<0.05$).

accumulation at 6, 12 and 24 h *in vitro* incubation by different fractions of ruminal microbes from cattle not adapted to exogenous dietary nitrate. The original concentration of sodium nitrate supplemented into the NND substrate mixture was 6.28 mmol/L. There was no difference ($p>0.91$) in nitrate disappearance between WRF and Pr fractions during the incubation. In the Ba fraction, NO_3-N disappeared slowly before 12 h incubation, but sharply thereafter, resulting in lower NO_3-N concentration than for the Fu fraction (6, 12 and 24 h incubation: 5.29, 4.80, 1.49 mmol/L for Ba fraction; 5.50, 5.27, 5.05 mmol/L for Fu fraction, $p<0.01$). Furthermore, the residual NO_3-N in WRF and Pr fraction (6, 12 and 24 h incubation (4.02, 1.63, 0.04 mmol/L in WRF fraction; 3.68, 1.78, 0.14 mmol/L in Pr fraction) was less than for Ba and Fu fractions ($p<0.01$). The decline in NO_3-N concentration was only 20.0% in the Fu fraction during 24 h of incubation.

Regarding nitrite accumulation by the microbial fractions, no difference was found at 6 ($p>0.47$) and 12 h ($p>0.13$) incubation among the four fractions. At 24 h incubation, the concentration of NO_2-N in the Ba fraction was greater ($p<0.01$) than for the other fractions. Overall, the concentration of NO_2-N was under 0.10 mmol/L among fractions during the incubation, except for the Ba fraction at 24 h which was 0.37 mmol/L.

Gas production

Net gas volume as well as the gas composition (hydrogen, methane and carbon dioxide) at 24 h of incubation are presented in Table 2. Since both gas volume and gas composition in various combinations over incubation are affected by nitrogen source and microbial fraction, emphasis will be placed on evaluation of treatment effects on quantity of total and individual gases.

Nitrogen source : When sodium nitrate was added, the net gas volume was less as was the quantity of CH_4 ($p<0.05$). Nitrate addition suppressed the accumulation of CO_2 after 24 h ($p<0.01$). Hydrogen accumulation was not affected ($p>0.10$) by nitrogen source.

Rumen microbial fraction : The greatest net gas production occurred in WRF, followed by Ba, Pr and then Fu ($p<0.01$). Hydrogen accumulation was greatest for the Pr fraction ($p<0.05$) and typically did not accumulate to greater than 4 μmol for WRF, Ba and F. The quantities of CH_4 and CO_2 were influenced by microbial fractions ($p<0.01$). WRF had the highest CH_4 and CO_2 accumulations. The CH_4 quantity in the Pr fraction was negative at 24 h because the result was calculated by difference using blank incubations.

Fermentation characteristics

The effects of nitrate on *in vitro* fermentation parameters for WRF and Pr, Ba and Fu fractions at 6, 12 and 24 h are presented in Table 3, 4 and 5.

Across all incubation durations, supernatant pH and total VFA concentration were inversely affected by microbial fraction. The WRF had the lowest pH and highest total VFA concentration while Fu was the inverse ($p<0.01$).

Concentrations of NH_3-N were higher for the urea than nitrate treatment ($p<0.01$) at all incubation times. The Pr fraction and WRF had similarly high NH_3-N concentrations at 6 h and 24 h, respectively. There was an interaction between nitrogen source and microbial fraction at 24 h incubation ($p = 0.04$). The lowest net NH_3-N concentration was recorded for the Fu fraction incubated with NND.

Nitrate affected VFA molar proportions by elevating acetate, and suppressing propionate and butyrate ($p<0.01$) at all incubation times. An interaction between nitrogen source and ruminal microbial fraction was observed for the molar proportions of acetate, propionate and butyrate at 12 and 24 h ($p<0.01$). Specifically, the most extreme effects of nitrate were observed for the Pr fraction.

Table 3. Effect of nitrogen source (urea or nitrate) and rumen microbial fraction (whole rumen fluid (WRF), protozoa (Pr), bacteria (Ba), and fungi (Fu)) on fermentation parameters at 6 h incubation *in vitro*[1]

Items	Urea				Nitrate				SEM		p-value		
	WRF	Pr	Ba	Fu	WRF	Pr	Ba	Fu	Nitrogen source (N)	Microbial fraction (MF)	N	MF	N*MF
pH	6.66^{E}	6.94^{D}	6.92^{DE}	7.39^{C}	6.90^{E}	7.02^{D}	6.89^{DE}	7.11^{C}	0.04	0.06	0.97	<0.01	0.06
NH_3-N (mmol/L)	9.4^{AD}	13.9^{AC}	8.2^{AD}	13.8^{AC}	3.8^{BD}	4.6^{BC}	3.6^{BD}	8.0^{BC}	0.67	0.95	<0.01	<0.01	0.15
Total VFA (mmol/L)	76.1^{C}	57.7^{D}	60.7^{D}	36.7^{E}	70.4^{C}	53.6^{D}	48.7^{D}	39.3^{E}	2.32	3.28	0.26	<0.01	0.65
VFA molar proportion (%)													
Acetate	60.7^{BCD}	61.0^{BC}	58.9^{BD}	61.7^{BCDb}	63.8^{ACD}	67.8^{AC}	60.8^{AD}	62.9^{ACD}	0.63	0.89	0.01	0.04	0.20
Propionate	21.0^{ADc}	19.1^{AEde}	27.3^{ACa}	17.4^{AFf}	20.3^{BDcd}	18.2^{BEef}	22.9^{BCb}	17.9^{BFeg}	0.21	0.29	<0.01	<0.01	<0.01
Butyrate	15.2^{ACDab}	16.2^{ADa}	11.7^{ADcd}	15.3^{ACab}	12.6^{BCDc}	10.4^{BDd}	13.4^{BDbc}	15.1^{BCab}	0.32	0.45	0.01	0.02	<0.01
Valerate	1.1	1.3	0.8	1.2	1.1	1.3	0.9	1.1	0.09	0.13	0.99	0.19	0.81
Isobutyrate	0.7	0.9	0.5	1.1	1.8	0.9	0.6	0.7	0.08	0.11	0.64	0.15	0.33
Isovalerate	1.3^{D}	1.5^{D}	0.9^{D}	3.2^{C}	1.4^{D}	1.5^{D}	1.4^{D}	2.4^{C}	0.25	0.36	0.89	0.04	0.65
A:P	2.9^{BDb}	3.2^{BCb}	2.2^{BEb}	3.6^{BCa}	3.2^{ADb}	3.7^{ACb}	2.7^{AEb}	3.5^{ACab}	0.04	0.06	<0.01	<0.01	0.02

[1] The first letter in each superscript series is associated with the largest mean value.
[A, B] Means with a different superscript denote the effect of nitrogen source ($p<0.05$).
[C, D, E, F] Means with a different superscript denote the effect of microbial fraction ($p<0.05$).
[a, b, c, d, e, f, g] Means with a different superscript denote the effect of nitrogen source×microbial fraction ($p<0.05$).

Table 4. Effect of nitrogen source (urea or nitrate) and rumen microbial fraction (whole rumen fluid (WRF), protozoa (Pr), bacteria (Ba), and fungi (Fu)) on fermentation parameters at 12 h incubation *in vitro*[1]

Items	Urea				Nitrate				SEM		p-value		
	WRF	Pr	Ba	Fu	WRF	Pr	Ba	Fu	Nitrogen source (N)	Microbial fraction (MF)	N	MF	N*MF
pH	6.63^{Ee}	6.76^{Dcd}	6.73^{DEde}	7.07^{Ca}	6.76^{Ecd}	6.89^{Dbc}	6.77^{DEcd}	6.91^{Cb}	0.02	0.03	0.26	<0.01	0.02
NH_3-N (mmol/L)	10.1^{A}	13.2^{A}	6.5^{A}	11.1^{A}	3.1^{B}	4.1^{B}	2.0^{B}	5.3^{B}	1.04	1.47	<0.01	0.06	0.50
Total VFA (mmol/L)	93.5^{ACa}	71.5^{ADbc}	77.8^{ADb}	48.4^{AEd}	96.1^{BCa}	69.1^{BDbc}	62.5^{BDc}	36.7^{BEe}	1.24	1.75	0.01	<0.01	0.06
VFA molar proportion (%)													
Acetate	60.2^{BDcd}	62.0^{BCcd}	55.9^{BFe}	62.9^{BEc}	66.8^{ADb}	72.3^{ACa}	60.7^{AFcd}	59.1^{AEd}	0.47	0.66	<0.01	<0.01	<0.01
Propionate	21.2^{ADc}	18.2^{AFe}	31.4^{AEa}	18.3^{ACe}	19.6^{BDd}	16.6^{BFf}	25.1^{BEb}	18.7^{BCde}	0.20	0.28	<0.01	<0.01	<0.01
Butyrate	15.5^{ADab}	15.9^{AEa}	11.0^{AEcd}	14.5^{ACb}	10.4^{BDd}	7.6^{BEe}	12.1^{BEc}	16.3^{BCa}	0.21	0.29	<0.01	<0.01	<0.01
Valerate	1.0^{BDcd}	1.4^{BCb}	0.5^{BEe}	1.1^{BCbc}	1.0^{ADcd}	1.3^{ACbc}	0.7^{AEde}	1.8^{ACa}	0.05	0.07	0.05	<0.01	0.01
Isobutyrate	0.7^{D}	0.9^{CD}	0.5^{D}	0.9^{C}	0.7^{D}	0.9^{CD}	0.6^{D}	1.4^{C}	0.09	0.13	0.24	0.06	0.47
Isovalerate	1.4^{D}	1.6^{D}	0.9^{C}	2.4^{E}	1.6^{D}	1.4^{D}	0.8^{C}	2.8^{E}	0.12	0.17	0.75	<0.01	0.63
A:P	2.8^{BDc}	3.4^{BCb}	1.8^{BEe}	3.5^{BDb}	3.4^{ADb}	4.4^{ACa}	2.4^{AEd}	3.2^{ADb}	0.04	0.06	<0.01	<0.01	0.01

[1] The first letter in each superscript series is associated with the largest mean value.
[A, B] Means with a different superscript denote the effect of nitrogen source ($p<0.05$).
[C, D, E, F] Means with a different superscript denote the effect of microbial fraction ($p<0.05$).
[a, b, c, d, e, f] Means with a different superscript denote the effect of nitrogen source×microbial fraction ($p<0.05$).

The molar proportion of valerate was increased by nitrate ($p = 0.05$) at 12 h and there were no other effects of nitrate ($p\geq0.13$) on molar proportions of isobutyrate and isovalerate.

Nitrate addition increased the acetate to propionate ratio ($p<0.01$). The Pr fraction had the greatest ratio, followed by Fu, WRF, and Ba ($p<0.01$). At 12 and 24 h, the Pr fraction in the nitrate treatment had the highest acetate to propionate ratio ($p\leq0.01$).

DISCUSSION

The disappearance of NO_3-N concentration was equally rapid for WRF and Pr fractions and the Pr fraction of this non-nitrate-adapted microbial population accounted for earlier onset of nitrate disappearance than the Ba fraction (Figure 1). There is very little information available for nitrate reduction by ruminal protozoa. Yoshida et al. (1982) reported that bacterial nitrate reduction in the rumen was accelerated by the existence of protozoa. Pfister (1988)

Table 5. Effect of nitrogen source (urea or nitrate) and rumen microbial fraction (whole rumen fluid (WRF), protozoa (Pr), bacteria (Ba), and fungi (Fu)) on fermentation parameters at 24 h incubation *in vitro*[1]

Items	Urea				Nitrate				SEM		p-value		
	WRF	Pr	Ba	Fu	WRF	Pr	Ba	Fu	Nitrogen source (N)	Microbial fraction (MF)	N	MF	N*MF
pH	6.67^{BE}	6.64^{BD}	6.68^{BCD}	6.71^{BC}	6.50^{AE}	6.65^{AD}	6.72^{ACD}	6.82^{AC}	0.01	0.02	<0.01	<0.01	0.30
NH_3-N (mmol/L)	11.8^{ACa}	9.6^{ACab}	6.9^{ADcd}	8.9^{ADbc}	7.8^{BCbc}	8.0^{BCbc}	5.4^{BDd}	2.8^{BDe}	0.50	0.71	<0.01	<0.01	0.04
Total VFA (mmol/L)	119^{C}	78.4^{D}	83.6^{D}	45.2^{E}	120^{C}	85.4^{D}	73.1^{D}	33.4^{E}	1.35	1.91	0.15	<0.01	0.05
VFA molar proportion (%)													
Acetate	59.4^{BDde}	64.3^{BCc}	54.2^{BFf}	62.4^{BEcd}	68.7^{ADb}	73.8^{ACa}	60.1^{AFde}	58.2^{AEe}	0.61	0.86	<0.01	<0.01	<0.01
Propionate	20.7^{ADc}	16.3^{AEef}	33.6^{ACa}	19.2^{ADcd}	18.3^{BDde}	15.0^{BEf}	25.8^{BCb}	19.2^{BDcd}	0.37	0.52	<0.01	<0.01	<0.01
Butyrate	16.1^{ADa}	15.0^{ADab}	10.4^{ADcd}	14.5^{ACab}	9.4^{BDcd}	7.4^{BDd}	12.0^{BDbc}	17.1^{BCa}	0.50	0.70	0.01	0.01	<0.01
Valerate	1.1^{CD}	1.7^{C}	0.6^{D}	1.0^{C}	1.1^{CD}	1.3^{C}	0.7^{D}	1.5^{C}	0.1	0.15	0.76	0.03	0.28
Isobutyrate	1.0^{Dab}	0.9^{Cb}	0.5^{Ed}	0.9^{Cb}	0.6^{Dc}	0.9^{Cab}	0.6^{Ec}	1.1^{Ca}	0.02	0.03	0.70	<0.01	<0.01
Isovalerate	1.8^{D}	1.9^{D}	0.7^{E}	2.0^{C}	2.0^{D}	1.6^{D}	0.8^{E}	3.1^{C}	0.11	0.15	0.13	<0.01	0.06
A:P	2.9^{BDc}	4.0^{BCb}	1.6^{BEe}	3.3^{BDc}	3.8^{ADb}	5.0^{ACa}	2.3^{AEd}	3.0^{ADc}	0.07	0.11	<0.01	<0.01	0.01

[1] The first letter in each superscript series is associated with the largest mean value.
[A, B] Means with a different superscript denote the effect of nitrogen source ($p<0.05$).
[C, D, E, F] Means with a different superscript denote the effect of microbial fraction ($p<0.05$).
[a, b, c, d, e, f] Means with a different superscript denote the effect of nitrogen source×microbial fraction ($p<0.05$).

cited the work of Marinho who was not able to detect a significant change in protozoal populations in the rumen fluid from sheep fed nitrates and suggested that protozoa are important for nitrite reduction in the rumen. It will be challenging to discern whether nitrate disappearance is due specifically to protozoal metabolism or to the symbiotic relationship between protozoa and associated bacteria. Nevertheless, the conclusion from our results is that nitrate disappearance by the WRF is due to the Pr fraction. Further research is warranted to understand nitrate metabolism by the Pr fraction. Microbial reduction of nitrate to nitrite, and nitrite to ammonia accelerates in response to nitrate exposure, but the microbial population requires 3 to 5 d to acclimatize (Pfister, 1988). The rapid decline in the nitrate concentration in the Ba fraction after 12 h incubation is consistent with a requirement by the ruminal bacterial population to adapt to the presence of nitrate. It appears that the Pr fraction does not have the same adaptation requirement. The Fu fraction accounted for little disappearance of nitrate, suggesting that it conducts little nitrate reduction in the unadapted state.

Nitrite did not accumulate during the incubations, except in the Ba incubations. The NO_2-N concentration of the Ba fraction at 24 h was greater than for the Pr fraction suggesting that the nitrite-reducing activity in the Pr fraction may be stronger in the unadapted state than for the Ba fraction. It is noteworthy for WRF that NO_2-N accumulated transiently at 12 h, presumably due to the onset of NO_3-N reduction by the Ba fraction, and then by 24 h the Pr fraction accounted for reduction of NO_2-N generated by the Ba fraction.

In this study, the pH values were above 6.5, which represent a normal fermentation. Geurink et al. (1979) and Johnson et al. (1983) have reported that the maximum nitrate reduction occurs when the rumen pH is 6.5 and nitrite reduction is maximal at pH 5.6. Thus, another reason for a lower nitrite concentration in WRF at 24 h may correspond to lower pH values.

These WRF, Pr and Ba *in vitro* incubations were rumen-like during the incubation in that total gas volume, CH_4, CO_2, and total VFA accumulated, incubation pH remained stable or declined slightly, and H_2 did not accumulate. The greatest net gas production was observed for WRF, followed by Ba, Pr and Fu fractions (Table 2). It is similar to previous research by Zhang et al. (2007) who demonstrated that gas production in WRF was the largest, followed by Ba. The Fu incubation was weak as indicated by a small accumulation of total gas, CO_2, and total VFA. Zhang et al. (2007) reported greater gas production by Fu than Pr, but they used substrates that included ground or milled corn stovers, which were more fibrous than the starch and Avicel used here. Bauchop (1979) showed that fungi were more prevalent in ruminants fed high fiber than in those fed less fibrous diets.

Nitrate suppressed total gas, CH_4, and CO_2 production at 24 h. The suppression of CH_4 production was not associated with an increase in H_2 accumulation. In addition, the pattern of VFA produced became enriched in acetate and diminished in propionate and butyrate. Pathways by which these two VFA are produced result in the net consumption of reducing equivalents (Russell and Wallace, 1988), e.g. H_2. Data reported here indicate that this effect of nitrate was noted for WRF and the Ba fraction, and especially the Pr fraction. Rumen methanogens have been identified as Achaea, in which the major genera are a hydrogenotrophic group. Finlay et al. (1994) used the characteristic

fluorescent coenzyme F 420 to find that rumen ciliate protozoa have much more numerous intracellular bacteria which produce methane than those attached to the external cell surface of ciliates. Thus, after adding antibacterial and antifungal agents, *in vitro* methanogenesis in the protozoa fraction was not attributed to protozoa itself, but parasitic methanogens in the cytosol of protozoa which escaped from killing by antibacterial agents. Coleman (1975) reported that intracellular bacteria are able to metabolise soluble compounds ingested or released by the protozoa. What species are engulfed and survive in protozoa has not been reported clearly. No previous reports were available which clearly indicated that protozoa itself or bacteria inside protozoa survive or that the interaction of these two microorganisms have ability to reduce nitrate or not, so we assumed that the protozoa fraction, which consisted of protozoa and the bacteria in the cytosol of protozoa, may have the ability to use nitrate as an electron acceptor and then decreased methane emission. A lower total VFA concentration was detected in the nitrate treatment, which is similar to Guo et al. (2009). By adding nitrate, the pattern of fermentation is very different from urea. The molar proportion of acetate increased while levels of butyrate in fractions of WRF, Pr and Ba decreased due to nitrate. This result is similar to previous research (Farra and Satter, 1971; Guo et al., 2009). These results are consistent with the hypothesis that nitrate acts as a preferential electron sink relative to electron consumption for acetate and butyrate formation.

When nitrate is added to ruminal and other anaerobic fermentations, it is an alternative to CO_2 as a terminal electron acceptor (Farra, 1969). In the rumen, CO_2 is reduced to CH_4 and this is the principal means for H_2 disposal from the ruminal fermentation. Nitrate is reduced to ammonia in the rumen according to the following stoichiometry:

$4H_2+HCO_3^-+H^+ \rightarrow CH_4+3H_2O$;
$\Delta G_o' = -175$ KJ/reaction (Conrad and Wetter, 1990)

$NO_3^-+2H^++4H_2 \rightarrow NH_4^++3H_2O$;
$\Delta G_o' = -598$ KJ/reaction (Allison and Reddy, 1984)

Nitrate reduction to ammonia was anticipated for the NND treatment, yet net NH_3-N accumulation was less for NND than for UND, even though the two nitrogen sources were added isonitrogenously. Ammonia is an intermediate in the rumen fermentation. Rapid hydrolysis of ammonia from urea apparently accounted for a greater NH_3-N concentration than that from nitrate, which must be reduced by microorganisms to ammonia. A greater level of NH_3-N was detected in the Pr fraction than in fractions of WRF and Ba. This is consistent with the report by Veira (1986).

When CO_2 is not used for CH_4 production, an increase in CO_2 production is expected. Here a diminished CO_2 production was noted especially for the Ba, Pr and Fu fractions in the nitrate treatment. Since these fractions were not previously adapted to nitrate supplementation, it is not possible to discern whether our results are reflective of the unadapted state or the long-term microbial metabolic response.

Hydrogen and carbon dioxide are end products of carbohydrate fermentation and the main substrates of methanogenesis in the rumen (Hungate, 1966). In connection with decreased CH_4 production, there was an increased percentage of H_2 in fractions of WRF and especially Pr. These results are consistent with the research reported previously (Sar et al., 2005b; Guo et al., 2009).

Methane represents both the loss of feed energy and a major greenhouse gas. It is 20-30 times more potent than carbon dioxide in its greenhouse effect (Rodhe, 1990). For this reason, reduction of CH_4 emission from ruminants is receiving significant attention. In this trial, nitrate supplementation resulted in a significant decline in CH_4 production and a greater acetate to propionate ratio. This effect was most acute for the Pr fraction. These results indicate that the Pr fraction is an important component of nitrate reducing activity by WRF. Furthermore, alternative electron acceptors such as nitrate hold promise as a strategy for mitigating ruminal methane emissions though there are ramifications in terms of the VFA profile.

IMPLICATIONS

Compared with urea as dietary N sources, sodium nitrate addition suppressed CH_4 production, increased proportion of acetate, decreased proportion of propionate and butyrate. The protozoa fraction had greater ability for nitrate and nitrite reduction than the bacteria fraction, and methane inhibition of nitrate was greatest in the Pr fraction. The Pr fraction has potential value for using nitrate as an alternative electron acceptor to mitigate ruminal methane emission, but further research on what role microorganisms play in nitrate reduction activity in the Pr fraction should be addressed.

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