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Manipulation of rumen methanogenesis by the combination of nitrate with β1-4 galacto-oligosaccharides or nisin in sheep

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Abstract

Effects of β1-4 galacto-oligosaccharides (GOS) or nisin in combination with nitrate were assessed on rumen methanogenesis, intoxication by nitrate, and metabolic rate in nitrate-treated sheep. Four rumen-fistulated wethers were allocated in a 4 × 4 Latin square design. Nitrate (1.3 g $NaNO_3$/$kg^{0.75}$ of body weight (BW)), with and without β1-4 galacto-oligosaccharides or nisin was administered into the rumen through a fistula 30 min after the morning meal as a single dose, whereas, β1-4 galacto-oligosaccharides (20 g per day) or nisin (3 mg/$kg^{0.75}$ of BW per day) was supplemented by sprinkling it onto the feed. Physiological saline was used as control treatment. When compared to saline, nitrate alone markedly lowered rumen methane production and caused a marked accumulation of rumen and plasma nitrite, and formation of blood methemoglobin consequently reduced oxygen consumption, carbon dioxide production, and metabolic rate. As compared to nitrate alone, simultaneous administration of nitrate with β1-4 galacto-oligosaccharides or nisin lowered the concentration of rumen and plasma nitrite and methemoglobin, while keeping rumen methanogenesis at a low level. A relatively higher rate of oxygen consumption, carbon dioxide production, and metabolic rate of sheep given nitrate plus nisin was observed versus that in sheep given nitrate alone. Therefore, an appropriate combination of nitrate with β1-4 galacto-oligosaccharides or nisin might be effective manipulators to abate rumen methanogenesis without nitrate intoxication in ruminants.

Keywords: Rumen methanogenesis; Nitrate; β1-4 galacto-oligosaccharide; Nisin; Sheep

Abbreviations: BW, body weight; DM, dry matter; Gal, galactose; Glc, glucose; GOS, β1-4 galacto-oligosaccharides; NH_3–N, ammonia nitrogen; ORP, redox potential; S.E.M., standard error of the mean; VFA, volatile fatty acids

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1. Introduction

Methane produced during ruminal fermentation represents a loss of 2–12% of the gross energy consumed by ruminants, and it is also a greenhouse gas that has been implicated as a contributor to the global warming (Johnson and Johnson, 1995). Thus, much research has been carried out on manipulation of the rumen fermentation to inhibit rumen methanogenesis with a view to increase energetic efficiency (Moss, 1993). Alternative hydrogen sinks in the rumen such as nitrate markedly suppress methanogenesis (Takahashi et al., 1997). When ruminants consume excess dietary nitrate, an acute and subclinical toxicity of nitrate occurs due to ruminal nitrite accumulation causing methemoglobin formation in blood (Takahashi et al., 1998). However, β1-4 galacto-oligosaccharides (GOS) were reported to decrease rumen methanogenesis and blood methemoglobinemia in nitrate-treated sheep (Sar et al., 2002). To date, nisin produced by certain strain of *Lactococcus lactis* subsp. *lactis* has received a Generally Recognized as Safe (GRAS) status for use as an antimicrobial preservative in food (FDA, 1988). Besides its antibacterial activity mainly against lactic acid bacteria and other Gram-positive bacteria, nisin at high concentrations inhibit *Escherichia coli* (Gram-negative bacteria) growth (Carneiro De Melo et al., 1996). In addition, as reported by Callway et al. (1997), nisin causes a decrease in the in vitro rumen methanogenesis and the acetate to propionate ratio.

In an attempt to manipulate rumen methanogenesis, the present experiment was conducted to determine the effects of combinations of nitrate with β1-4 galacto-oligosaccharides or nisin on rumen methanogenesis, intoxication by nitrate, and the metabolic rate in sheep.

2. Materials and methods

2.1. Experimental design

2.1.1. General

Four rumen-fistulated wethers with an initial live weight of 50.8±8.5 kg were individually housed in metabolic crates equipped with a ventilated respiratory collection hood and fed on the basal diet of chopped timothy hay, alfalfa hay cube, and concentrate (40:40:20 as dry matter (DM) basis) at a maintenance energy level. The experimental design was a 4×4 Latin square with 1 week allowed between treatments to assure no carry-over effects of the previous treatment. Each period consisted of 8 days with 7 days for acclimatization to feeds and 1 day for measurement of respiratory gas exchange and metabolic rate as well as simultaneous collection of rumen fluid and blood. The four treatments consisted of saline, nitrate, nitrate plus GOS, and nitrate plus nisin. Physiological saline was administered as the control treatment.

To test the suppressing effects of GOS or nisin on the nitrate-induced poisoning, respiratory gaseous exchange, metabolic rate, and characteristics of rumen fermentation were determined. Nitrate with and without GOS or nisin was administered into the rumen through the rumen fistula 30 min after the morning feeding as a single dose, whereas, GOS and nisin were administered by sprinkling onto the feed. The sheep were weighed weekly prior to the beginning of each period to determine daily allowance of feed and dosages of nitrate and

Table 1
The structure and composition of GOS (Oligomate 55)

Components	Oligosaccharide structures	Unit (g/100 g)
Monosaccharide	Glc, Gal	18.3
Disaccharides	Galβ1-4 Glc	22.2
	Galβ1-3 Glc	9.2
	Galβ1-2 Glc	3.8
	Galβ1-6 Glc	1.6
	Others	2.0
Trisaccharides	Galβ1-4 Galβ1-4 Glc	12.2
	Galβ1-4 Galβ1-3 Glc	4.3
	Others	6.6
Tetrasaccharides	Galβ1-4 Galβ1-4 Galβ1-4 Glc	9.2
	Galβ1-4 Galβ1-4 Galβ1-3 Glc	2.2
	Others	4.3
Penta- and hexasaccharides		4.51
Water		3.7

Glc, glucose; Gal, galactose (*Source:* Yakult Central Institute for Microbiological Research, Tokyo, Japan).

nisin. Respiratory gases were monitored from 1 h before to 9 h after feeding the morning meal. Rumen fluid and blood were collected either through the rumen fistula every hour or via a jugular catheter after 1, 3, 5, 7, and 9 h of administration of the chemicals. The experimental protocol was approved by Obihiro University of Agriculture and Veterinary Medicine Committee for Animal Use and Care.

2.1.2. Feeding and supplements

Animals were fed twice daily (08:00 and 17:00 h) with the basal diet with chopped timothy hay, alfalfa hay cubes, and concentrate (40:40:20 as DM basis) at a maintenance energy level (55 g DM/kg of body weight (BW) per day). Each sheep had free access to water and a block of NaCl throughout the experiment.

A 30% (w/v) aqueous solution of 1.3 g $NaNO_3$/$kg^{0.75}$ of BW considered to be a subclinical nitrate toxicity (Takahashi and Young, 1991) was administered via rumen fistula 30 min after the morning feeding on the day of sample collection. GOS (Oligomate 55) provided by the Yakult Central Institute for Microbiological Research (Tokyo, Japan) was administered to sheep by sprinkling it onto the feed twice a day at half amount of the daily allowance (20 g per day) for 7 days during each treatment. The structure and composition of GOS are in Table 1. Nisin was purchased from Sigma (St. Louis, MO, USA). Nisin (3 mg/$kg^{0.75}$ of BW per day) was determined and administered to sheep twice daily for 7 days during each treatment.

2.2. Experimental measurement

2.2.1. Respiratory gas exchange and metabolic rate

Oxygen consumption as well as carbon dioxide and methane production 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). Rate of methanogenesis in the rumen was estimated from respiratory methane. Carbon dioxide, oxygen, and methane concentration was measured as reported by Takahashi et al. (1998). Data were collected and entered to a computer from the analyzers through an interface at 1 min intervals, and then automatically standardized at 0 °C, 1013 hPa and zero water vapor pressure.

2.2.2. Rumen fluid

The value of pH and redox potential (ORP) in rumen fluid were measured using a pH and ORP meter (HM-21P, TOA Electronics Ltd., Tokyo, Japan), and then each sample was frozen for later determination of rumen nitrate, nitrite, ammonia N, and volatile fatty acids (VFA). Nitrite in rumen was determined colorimetrically by the diazo-coupling method of Horwitz (1975) after deproteinisation and dilution of sample fluid by using three volumes of lead acetate (50 g/l) and one volume of a saturated $Na_3PO_4{\cdot}12H_2O$ solution (Prins et al., 1980). Nitrate was similarly determined in the manner described for nitrite except that a pinch of powdered zinc was added after the addition of mercuric chloride. Ammonia N concentration was estimated by 1 ml sample using the modified micro-diffusion method of Conway and O'Mally (1942). The concentrations of VFA were analyzed by gas–liquid chromatography (Shimadzu GC-14A, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column (ULBON HR-52, 0.53 mm i.d. × 30 m 3.0 μm) 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). Analysis of degradability rate of GOS in rumen fluid was conducted by HPLC using a Shodex RI SE-61 detector set at a Shodex KS802 column (8.0 mm × 300 mm) operated at 80 °C and at a flow rate of 0.5 ml/min. The mobile phase was H_2O.

2.2.3. Blood

Venous blood collected from jugular catheter was determined for hemoglobin (Nescauto Hemokit-N, Azwell Inc., Osaka, Japan) and methemoglobin (Evelyn and Malloy, 1938). Plasma nitrate and nitrite were determined by the diazo-coupling method, as for rumen fluid.

2.3. Statistical analysis

According to the general linear models procedures of the Statistical Analysis Systems Institute (SAS, 1994), data were analyzed by repeated measure analysis of variance for a Latin square arrangement of treatments. Treatment means were compared using Duncan's multiple-range test. Statistical significance of differences was accepted at $P < 0.05$.

3. Results

3.1. Ruminal methane emission in nitrate-treated sheep with and without GOS or nisin

One hour after administration of nitrate, the rate of ruminal methane production in nitrate-treated sheep was lower ($P < 0.01$) than that in saline sheep (Fig. 1). Compared to

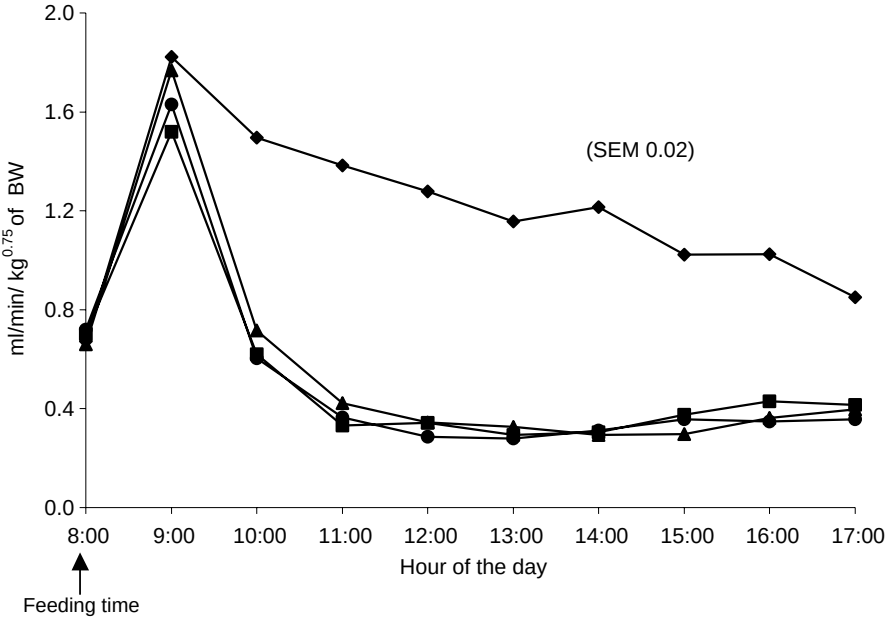

Fig. 1. Diurnal changes in methane emission from sheep administered with saline (◆), nitrate (■), nitrate plus GOS (▲), and nitrate plus nisin (●).

nitrate-treated sheep, the rate of ruminal methane production in sheep administered with nitrate and GOS or nisin was not affected.

3.2. Ruminal nitrate disappearance, ruminal nitrite accumulation, and plasma nitrite formation in nitrate-treated sheep with and without GOS or nisin

Simultaneous administration of nitrate with GOS gave ($P > 0.05$) lower levels of ruminal nitrate concentration and an increase in the rate of disappearance of ruminal nitrate when compared with that in the administration of nitrate alone (Fig. 2(a)). A peak ruminal accumulation of nitrite was observed 4 h after administration of nitrate (Fig. 2(b)). Simultaneous administration of nitrate with GOS or nisin tended to decrease ($P > 0.05$) peak and mean values of ruminal nitrite concentration when compared to those in the administration of nitrate alone. Plasma nitrite concentration peaked 5 h after the administration of nitrate (Fig. 2(c)). Compared to the administration of nitrate alone, a ($P < 0.05$) decrease in the mean plasma nitrite concentration was observed in the simultaneous administration of nitrate plus GOS or nisin.

3.3. Blood methemoglobin, oxygen consumption, carbon dioxide production, and metabolic rate in nitrate-treated sheep with and without GOS or nisin

There was no detectable concentration of methemoglobin in the blood of the saline sheep (Fig. 3(a)). The peak methemoglobin level of total hemoglobin occurred 7 h after

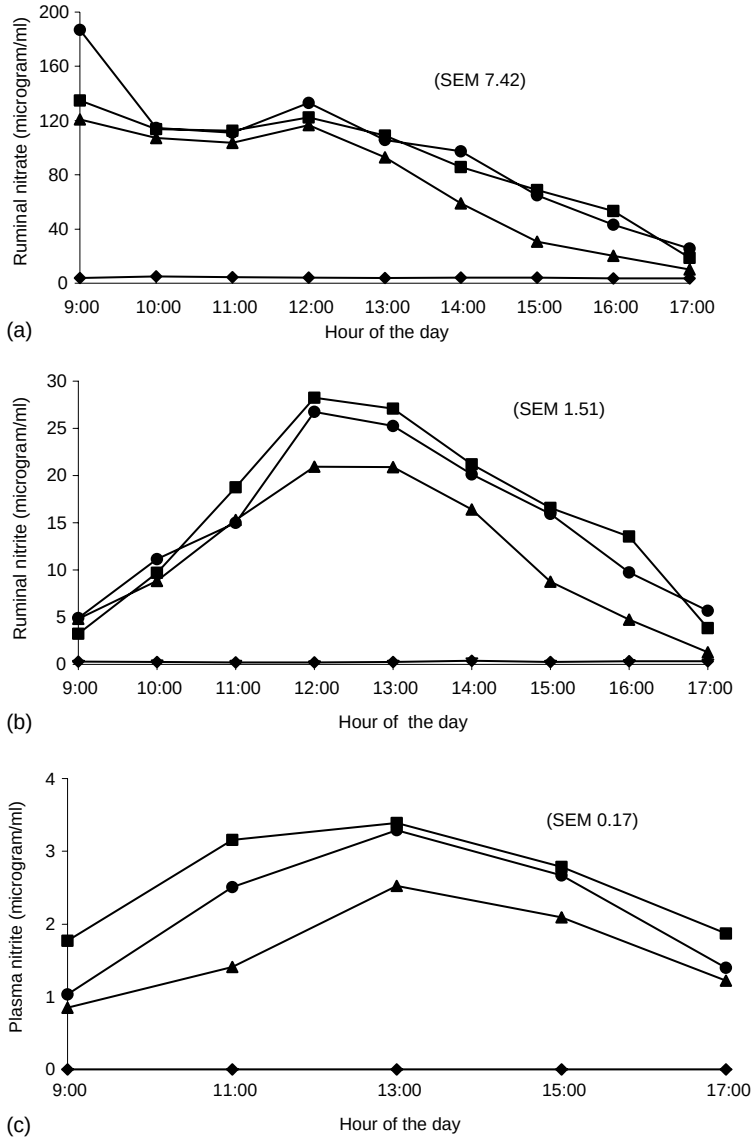

Fig. 2. Ruminal nitrate disappearance (a); ruminal nitrite accumulation (b); and plasma nitrite accumulation (c) in sheep administered with saline (◆), nitrate (■), nitrate plus GOS (▲), and nitrate plus nisin (●).

administration in sheep treated with nitrate. Peak values of blood methemoglobin for sheep given nitrate with GOS or nisin tended to decrease ($P > 0.05$) when compared to nitrate-treated sheep. A lesser ($P > 0.05$) difference in oxygen consumption was observed between saline (control) sheep and nitrate-treated sheep after 4–6 h of administration of nitrate. A decrease ($P > 0.05$) in carbon dioxide production in nitrate-treated sheep was

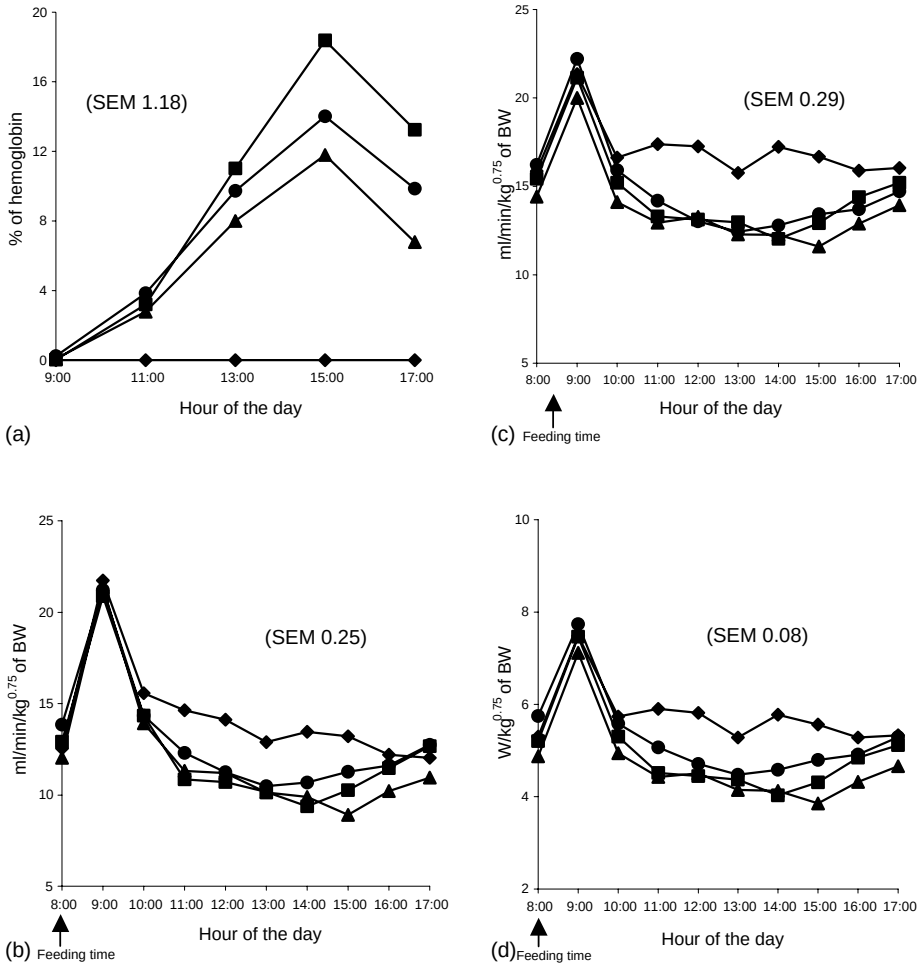

Fig. 3. Formation of blood methemoglobin (a); diurnal changes in oxygen consumption (b); carbon dioxide production (c); and metabolic rate (d) in sheep administered with saline (◆), nitrate (■), nitrate plus GOS (▲), and nitrate plus nisin (●).

observed after 4–5 and 7–8 h of administration of nitrate. The metabolic rate in nitrate-treated sheep decreased ($P > 0.05$) after 4–8 h of the administration of nitrate.

3.4. Rumen fermentation characteristics in nitrate-treated sheep with and without GOS or nisin

Mean ruminal pH in nitrate-treated sheep was lower ($P > 0.05$) than that in saline administered sheep. The mean values of ORP in sheep given nitrate alone and nitrate with GOS or nisin was higher ($P > 0.05$) than that in saline sheep. Mean ruminal ammonia

nitrogen (NH_3–N) was increased ($P > 0.05$) after administration of nitrate. Simultaneous administration of nitrate with GOS or nisin tended to increase ($P > 0.05$) mean ruminal NH_3–N when compared to the administration of nitrate alone. Increased ($P > 0.05$) mean total VFA in the administration of nitrate occurred when compared to saline administration. Compared to the saline administration, administration of nitrate alone increased ($P > 0.05$) molar proportion of acetate, and ($P > 0.05$) decreased molar proportion of propionate and butyrate.

4. Discussion

Following administration of nitrate, in vitro and in vivo methanogenesis by rumen microbes have been reported to be markedly decreased (Jones, 1972; Allison et al., 1981; Takahashi and Young, 1991). Results obtained from the present experiment are in good agreement with these studies. Allison and Reddy (1990) suggested that this decrease was due to nitrate reduction in the rumen acting as the electron acceptors, and so, effectively competing with rumen methanogenesis as sinks for electrons generated during fermentation. When GOS or nisin was added with nitrate, rumen methanogenesis seemed not to be suppressed further compared to the administration of nitrate alone. These may be due to nitrate having a more potent effect than GOS or nisin on rumen methanogenesis.

It is established that when nitrate was applied in excess to suppress methane emission from ruminants, its intoxication is apparently observed in the host because of the toxic accumulation of nitrite in the rumen inducing production of blood methemoglobin (Takahashi and Young, 1991, 1992). Results in Fig. 2(b) and (c) show that the toxic accumulation of nitrite in rumen and plasma is at peak 4 and 5 h after sheep were given nitrate, respectively, and these agree with previous findings (Takahashi and Young, 1991). However, when GOS was added to nitrate, a tendency of decrease in ruminal nitrate concentration, and an increase in the rate of disappearance of ruminal nitrate as well as a drop in the peak values of ruminal and plasma nitrite concentration was observed in comparison to that in the sheep administered with nitrate alone. GOS is produced from lactose by the action of β-galactosidases having transgalactosylation activity (Yanahira et al., 1995), and is a mixture of two galactose (Gal) units and one glucose (Glc) unit (Matsumoto et al., 1990). Glucose had been reported to accelerate in vitro reduction of ruminal nitrate and nitrite (Sapiro et al., 1949). Thus, results suggest that glucose derived from the degradation of GOS (Fig. 4) stimulates a series of reductions of nitrate and nitrite in the rumen, and consequently the toxic accumulation of nitrite in rumen and plasma are abated. It has been known that nisin has displayed antimicrobial activity against a wide range of Gram-positive bacteria (Hurst, 1981), contain unsaturated amino acids (dehydroalanine and β-methyldehydroalanine) and thioether amino acids (lanthionine and β-methyllanthionine) (Ingram, 1970), and that nisin is rapidly degraded by mixed rumen bacteria (Lee et al., 2002). When nisin was administered with nitrate, both peak and mean nitrite concentration in rumen and plasma tended to be lower when compared to those in the administration of nitrate alone. These may be due to nisin exerting an effect on some of the ruminal nitrate-reducing bacteria such as *Sarcina ventriculi*, *Eubacterium ruminantium*, *Propionibaterium acnes* (Ogimoto and Sochi, 1981), *Staphylococcus xylosus*, *Staphylococcus saprophyticus*, and *Staphylococcus gallinarum* (Laukoá

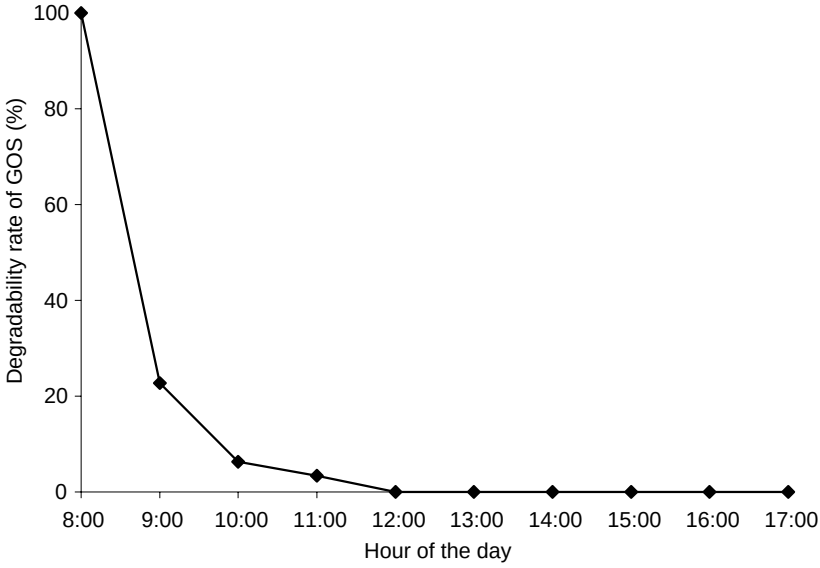

Fig. 4. Degradability rate of GOS in the rumen in the presence of nitrate.

and Marounek, 1992), all being Gram-positive. It was found that nisin bound to Lipid II, a membrane-anchored precursor in peptidoglycan synthesis, in the pore formation process to kill Gram-positive bacterial cells (Breukink and Kruijff, 1999). Another possible explanation is that the sulfur group of L-cysteine, derived from lanthionine and β-methyllanthionine in nisin, inhibited nitrate reduction by rumen microbes and so resulted in decrease of ruminal nitrite accumulation through binding of sulfide-S generated from L-cysteine with molybdenum (Takahashi et al., 1989). This binding is known to interfere with nitrate reduction in the rumen (Takahashi and Young, 1991).

A nitrate-induced methemoglobin content of about 20% of total hemoglobin is considered subclinically toxic to sheep (Bodansky, 1951). A methemoglobin level of 18.4% of total hemoglobin was produced by the administration of nitrate (Table 2) which is comparable with Bodansky (1951). A marked decrease in mean values of oxygen consumption, carbon dioxide production, and metabolic rate is due to development of nitrate-induced blood methemoglobin, and these results are consistent with those reported by Takahashi et al. (1998) who also reported that every 10% of blood methemoglobin formation instead of oxyhemoglobin reduced oxygen consumption by 10.3%. When GOS was administered with nitrate, both mean and peak blood methemoglobin levels tended to decrease compared to that in the administration of nitrate alone, confirming the previous reports (Sar et al., 2002), possibly due to the abatement of ruminal and plasma nitrite accumulation by GOS. An increase in the metabolic rate due to simultaneous administration of nitrate with nisin, compared to administration of nitrate alone was attributed to a tendency of decrease in nitrate-induced blood methemoglobin level by nisin.

Takahashi (1989) reported that an increase in ruminal pH was observed by the administration of nitrate in an in vitro study using orchardgrass forage as the substrate. In contrast

Table 2
Ruminal nitrite, plasma nitrite, blood methemoglobin, oxygen consumption, carbon dioxide production, methane production, and metabolic rate in sheep given nitrate with GOS or nisin

Parameters	Treatment				S.E.M.[a]	*P*-value
	Saline	Nitrate	Nitrate plus GOS	Nitrate plus nisin		
Ruminal nitrite concentration (μg/ml)						
Mean of all values	0.28[b] a	15.79 b	11.33 b	14.95 b	1.51	<0.001
Mean of maximums	0.22 a	28.25 b	20.94 b	26.76 b	3.15	0.002
Plasma nitrite concentration (μg/ml)						
Mean of all values	0.00 a	2.67 b	1.63 c	2.18 d	0.16	<0.001
Mean of maximums	0.00 a	3.39 b	2.52 b	3.29 b	0.33	0.001
Methemoglobin concentration (% of Hb)						
Mean of all values	0.00 a	9.23 b	5.89 b	7.53 b	1.18	<0.001
Mean of maximums	0.00 a	18.37 b	11.79 b	14.01 b	2.96	0.020
Oxygen consumption[c] (ml/(min $kg^{0.75}$ of BW))	14.23 a	12.59 b,c	11.99 b	12.97 c	0.25	<0.001
Carbon dioxide production (ml/(min $kg^{0.75}$ of BW))	17.01 a	14.57 b,c	13.77 b	14.86 c	0.29	<0.001
Methane production (ml/(min $kg^{0.75}$ of BW))	1.19 a	0.59 b	0.56 b	0.52 b	0.02	<0.001
Metabolic rate ($W/kg^{0.75}$ of BW)	5.74 a	4.96 b	4.70 c	5.29 d	0.08	<0.001

[a] Standard error of the mean.
[b] Means within rows with different letters (a–d) differ significantly ($P < 0.05$). Each value indicates mean of four animals.
[c] All values are mean of 9 h observation.

to our results, mean ruminal pH was relatively lower (6.34 versus 6.61) with the nitrate treatment alone versus saline. This may be due to differences in the type of feed with the previous finding and this study. When GOS was added with nitrate, mean ruminal pH tended to be higher compared to the administration of nitrate alone. The apparent effect of GOS on ruminal pH is clear which is in agreement with Santoso et al. (2003). As reported in the in vitro experiment of Jalč and Lauková (2002), ruminal pH was not affected by nisin. Results in this study showed that nisin tended to increase mean ruminal pH because when nisin was added to nitrate, the mean ruminal pH seemed to be higher compared to that obtained by nitrate administration alone. No reason for this difference between this study and previous reports is apparent. A relative increase in mean ruminal NH_3–N by administration of nitrate in our study could confirm the findings of others (Lewis, 1951a; Farra and Satter, 1971), and this is as a result of reduction of nitrate and nitrite in the rumen (Lewis, 1951a). When GOS was added to nitrate, a mean ruminal NH_3–N seemed to increase further and this could be due to ruminal nitrate and nitrite reduction by microbes stimulated by GOS (shown in Fig. 2(a) and (b)). A slightly increased mean ruminal NH_3–N with simultaneous administration of nitrate with nisin compared to the administration of nitrate alone is contrary to our assumption that when nisin is added, it inhibits ruminal nitrate reduction resulting in decrease in ruminal nitrite concentration, and consequently ruminal NH_3–N should be low.

The increase in molar proportion of acetate and decrease in molar proportion of propionate and butyrate with the administration of nitrate (Table 3) could reflect the electron sink of nitrate in rumen fermentation (Farra and Satter, 1971). Molar ratios of VFA, altered by nitrate, tended to improve by the addition of GOS. These are comparable with the fact that GOS increased short chain fatty acids in cecal contents (Kikuchi-Hayakawa et al., 1997).

Table 3
Ruminal pH, redox potential (ORP), the concentration of ruminal ammonia nitrogen (NH_3–N), total VFA, and the molar proportions of VFA in sheep given nitrate with GOS or nisin

Parameters	Treatment				S.E.M.[a]	*P*-value
	Saline	Nitrate	Nitrate plus GOS	Nitrate plus nisin		
Ruminal pH[b]	6.61[c] a	6.34 b	6.42 b	6.38 b	0.03	<0.001
Ruminal ORP (mV)	−307 a	−230 b	−243 b	−235 b	8.13	<0.001
Total VFA (mM)	55.49 a	77.24 b	76.75 b	76.21 b	1.92	<0.001
Molar % of VFA						
Acetate (A)	77.21 a	79.62 b	78.65 b	78.62 b	0.47	0.006
Propionate (P)	14.36 a	13.52 b	13.86 a,b	14.20 b	0.22	0.040
Butyrate	6.99 a	5.16 b	5.56 b	5.22 b	0.23	<0.001
Others[d]	1.44 a	1.68 a,b	1.70 a,b	1.94 b	0.10	0.010
A:P ratio	5.42 a	6.07 b	5.74 a,b	5.69 a,b	0.12	0.007
Ruminal NH_3–N (mg/l)	187 a	330 b	339 b	332 b	8.19	<0.001

[a] Standard error of the mean.
[b] All values are means of nine sampling times.
[c] Means within rows with different letters (a and b) differ significantly ($P < 0.05$). Each value indicates means of four animals.
[d] Including valeric, *iso*-valeric and *iso*-butyric fatty acid.

With simultaneous administration of nitrate with nisin, a tendency of increase in the molar proportion of propionate and butyrate in compensation for decrease in that of acetate was observed compared to the administration of nitrate alone. This indicates that molar ratios of VFA are affected by nisin, confirming results of Jalč and Lauková (2002). In comparative approaches to the utilization efficiency of VFA as the sole source of energy for maintenance of ruminants, acetate has been reported to be used less efficiently than propionate and butyrate (Armstrong et al., 1957). With the administration of nitrate, an increase in the acetate to propionate ratio was observed, although, rumen methanogenesis was markedly decreased. However, Moss and Givens (2002) reported that rumen stoichiometry could not explain the change in methane production. When GOS or nisin is added with nitrate, the acetate to propionate ratio tend to decrease compared to the administration of nitrate alone. This is consistent with the ability of GOS or nisin to decrease the methane production (Fig. 1) and probably divert reducing equivalents to other disposal mechanisms (e.g., propionate). Thus, GOS or nisin tends to improve some of the rumen fermentation parameters which are adversely affected by nitrate administration.

5. Conclusions

Addition of β1-4 galacto-oligosaccharides or nisin to nitrate abates rumen methanogenesis with a decrease in nitrate/nitrite toxicity and tend to improve some rumen fermentation parameters. Thus, simultaneous administration of nitrate with β1-4 galacto-oligosaccharides or nisin might be effective manipulators on rumen methanogenesis with abatement of nitrate/nitrite toxicity in ruminants. However, dosage of nitrate (1.3 g $NaNO_3$/kg$^{0.75}$ of BW) was chosen in this study so as to induce sufficient blood methemoglobinemia to cause subclinical toxicity (Takahashi and Young, 1991). In order to avoid nitrate/nitrite toxicity to ruminant when nitrate is potentially applied as an inhibiting manipulator of rumen methanogenesis, future studies should evaluate minimizing of nitrate amount as well as the importance of the optimal combination between the minimum level of nitrate with β1-4 galacto-oligosaccharides or nisin.

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