

## Effects of Combination of Nitrate with $\beta$ 1-4 Galacto-oligosaccharides and Yeast (*Candida kefyr*) on Methane Emission from Sheep

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**ABSTRACT :** The objective of the present study was to determine whether  $\beta$ 1-4 galacto-oligosaccharides (GOS) and *Candida kefyr* combined with nitrate as manipulators could suppress rumen methanogenesis without nitrate poisoning in sheep. Four rumen fistulated wethers were allocated to a 4x4 Latin square design. Nitrate (1.3 g NaNO<sub>3</sub> kg<sup>0.75</sup> body weight) with and without GOS and *Candida kefyr* were administered into the rumen through fistula as a single dose 30 min after the morning meal. GOS and *Candida kefyr* were supplemented by sprinkling onto the feed and through rumen fistula, respectively. The four treatments consisted of saline, nitrate, nitrate plus GOS and nitrate plus GOS plus *Candida kefyr*. Physiological saline was used as the control treatment. Compared to saline treatment, the administration of nitrate alone resulted in a very marked decrease in rumen methanogenesis and an increase in rumen and plasma nitrite production and blood methaemoglobin formation consequently causing a decline in oxygen consumption, carbon dioxide production and metabolic rate. When compared to nitrate alone, the simultaneous administration of nitrate with GOS decreased nitrite accumulation in rumen and plasma and nitrate-induced methaemoglobin, while retaining low methane production. However, GOS could not fully restore metabolic parameters reduced by nitrate. When compared to the simultaneous administration of nitrate with GOS, the simultaneous administration of nitrate with GOS plus *Candida kefyr* lowered rumen methanogenesis to a negligible level, but did not decrease rumen and plasma nitrite accumulation as well as blood methaemoglobin formation. Thus, these results suggest that combination of nitrate with GOS may be a potent manipulator to suppress rumen methanogenesis with abating the hazards of nitrate-nitrite toxicity in ruminants. (*Asian-Aust. J. Anim. Sci.* 2004, Vol 17, No. 1 : 73-79)

**Key Words :** Rumen Methanogenesis, Nitrate,  $\beta$ 1-4 Galacto-oligosaccharide, *Candida kefyr*, Methaemoglobin

### INTRODUCTION

Methane is an important greenhouse gas second only to carbon dioxide in its contribution to global warming due to its high absorption of infrared in the radiation from the sun (IPCC, 1994). The world population of ruminants is an important source of methane, contributing approximately 15% of the total atmospheric methane flux (Sahoo et al., 2000). Methane is generated by methanogenic archaea that utilise hydrogen to reduce carbon dioxide, and is a significant electron sink in the rumen ecosystem (Klieve and Hegarty, 1999). The control of rumen methanogenesis can be mostly achieved by developing alternative hydrogen sinks in order to divert hydrogen away from methanogens (Joblin, 1999). Alternative hydrogen sinks in rumen, such as nitrate, markedly suppress rumen methanogenesis (Takahashi, 2001). However, consumption by ruminants of forages or water containing high levels of nitrate results in an acute toxicity syndrome due to toxic rumen nitrite accumulation inducing a relatively severe methaemoglobinemia (Jones, 1972), and these are attributed to a relatively higher reduction rate of nitrate than that of

nitrite in the rumen (Takahashi and Young, 1991, 1998). Thus, the acceleration of nitrite reduction is an effective way to depress the nitrite accumulated. To reduce any adverse contamination, the combination of nitrate with safe natural compounds and probiotics must be sought as manipulators to regulate rumen methanogenesis.

$\beta$ 1-4 galacto-oligosaccharide is a mixture of two galactose units and one glucose unit (Tanaka et al., 1983), and readily utilized by many enteric *Bifidobacterium* strains (Matsumoto et al., 1990).  $\beta$ 1-4 galacto-oligosaccharides are known to stimulate short-chain fatty acids (SCFAs) in cecum contents in rats (Kikuchi-Hayakawa et al., 1997).  $\beta$ 1-4 galacto-oligosaccharides tended to increase rumen total volatile fatty acid (VFA) and decreased rumen methane production in *in vitro* experiments using orchardgrass silage as substrate (Gamo et al., 2002). However, no effect of the supplementation of  $\beta$ 1-4 galacto-oligosaccharides on total VFA (Santoso et al., 2003b) and rumen methanogenesis (Santoso et al., 2003a) in cows fed orchardgrass silage alone or mixed with alfalfa silage were observed. On the other hand, Takahashi et al. (2002) reported that  $\beta$ 1-4 galacto-oligosaccharides tended to decrease *in vitro* rumen nitrite accumulation, although there were no effects on rumen nitrate reduction rate.

Recently, it has been reported that yeast culture supplements can have a significant impact on the performance of ruminants (Dawson et al., 1990).

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Received May 21, 2003; Accepted October 11, 2003

**Table 1.** Composition of  $\beta$ 1-4 galacto-oligosaccharides

Sample grade: 80% grade (4-GOS-80)	
Galactooligosaccharides purity >80% (Analysis by HPLC)	
Sugar composition:	
GOS	
Penta- and hexasaccharides	14%
Tetrasaccharides	24%
Trisaccharides	40%
Disaccharides	10%
Others	
Lactose	11%
Glucose	1%

Sourced from Yakult Central Institute for Microbiological Research (Tokyo, Japan).

Preliminary studies by Gerald et al. (1984) showed that the yeast culture did not have an effect on nitrate toxicity in sheep or cattle. However, *in vitro* experiments using cultured strains of yeast, *Trichosporon sericeum* and *Candida kefyr* in an attempt to abate nitrate toxicity suggested that *Candida kefyr* culture tended to lower rumen nitrite formation (Takahashi et al., 2002). Hence, the ability of *Candida kefyr* to decrease rumen nitrite accumulation should be clarified.

The objective of the present study, therefore, was to determine whether  $\beta$ 1-4 galacto-oligosaccharides and *Candida kefyr* combined with nitrate as manipulators could suppress rumen methanogenesis without nitrate poisoning in sheep.

## MATERIALS AND METHODS

### Experimental design, animals and diets

Four rumen-fistulated wethers with initial weight of 43.8-59.3 kg were allocated to four dietary treatments in a 4×4 Latin square design. Animals were fed twice a day (08:00 and 16:00 h) on the basal diet with chopped timothy hay and alfalfa hay cube (50:50) at maintenance level (55 g DM kg<sup>-0.75</sup> body weight day<sup>-1</sup>), and had free access to water and a block of NaCl throughout the experiment. All animals were individually maintained in metabolism cages each equipped with a ventilated respiratory head cage. Each test period consisted of 8 days with 7 days for adaptation to the feed and 1 day for gaseous measurement and rumen and blood collection. An interval of a week was allowed between each of the four trials to make sure of no carry-over (lag) effects of previous treatment. To test the effects of dietary treatment on gaseous exchanges, metabolic rate and nitrate-induced poisoning, nitrate with and without  $\beta$ 1-4 galacto-oligosaccharides (GOS) and *Candida kefyr* was directly administered into the rumen via fistula as a single dose 30 min after the morning feeding. GOS and *Candida kefyr* were supplemented by sprinkling onto the feed and through rumen fistula, respectively. The four treatments consisted of saline, nitrate, nitrate plus GOS and nitrate plus

GOS plus *Candida kefyr*. Physiological saline (0.9% NaCl) was given as the control treatment. All animals were weighed weekly before the onset of each period to calculate daily allowance of feed and dosages of nitrate.

### Treatments and sample collection

Dosage of a subclinical level of nitrate. 1.3 g NaNO<sub>3</sub> kg<sup>-0.75</sup> body weight as a 30% (W/V) aqueous solution was administered to animals via fistula after the morning feeding on the day of sample collection (Takahashi et al., 1991). GOS provided by Yakult Central Institute for Microbiological Research (Tokyo, Japan) was offered to animals twice a day at half amounts of daily allowance (10 g day<sup>-1</sup>) for 7 days during each treatment. The composition of GOS was shown in Table 1. *Candida kefyr* strain, used as a yeast culture supplement in this trial, was extracted from naturally-fermented milk "Laban" produced from sheep milk in Yemen. *Candida kefyr* was cultured in liquid autoclaved (15 min, 121°C) YM medium containing 5 g of peptone from casein, 3 g of yeast extract, 3 g of malt extract, and 3 g of D (+) Glucose per litre. 100 ml of this culture supplement (1×10<sup>7</sup> cfu/ml) was given to animals through rumen fistula.

Respiratory gaseous exchanges were monitored from 1 hour before to 8 h after treatments. Venous blood samples were collected via a jugular catheter 1, 2, 3, 4, 5 and 6 h and rumen fluid was withdrawn via rumen fistula 1, 2, 3, 4, 5, 6 and 7 h, to check the development of nitrate-nitrite poisoning physiologically.

### Measurements

*Respiratory gas exchange and metabolic rate* : Oxygen consumption, carbon dioxide and methane production were monitored by the fully automated open-circuit respiratory system using a hood over the animal's head (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 were measured as reported by Takahashi et al. (1998). Those data were taken and pooled into the computer from the analyzers through an interface at 1 min intervals, and then automatically standardized at 0°C, 101 kPa and zero water vapour pressure.

*Rumen fluid* : Nitrite in rumen was determined colorimetrically by the diazo-coupling method (Horwitz, 1975) after deproteinisation and dilution of sample fluid by using three volumes of lead acetate (50 g l<sup>-1</sup>) and one volume of a saturated Na<sub>3</sub>PO<sub>4</sub>·12H<sub>2</sub>O solution (Prins et al., 1980). Nitrate was similarly determined after reducing to nitrite by zinc powder. Analysis of degradability rate of GOS in rumen fluid was conducted by HPLC using Shodex RI SE-61 detector set at an Shodex KS802 column (8.0×300

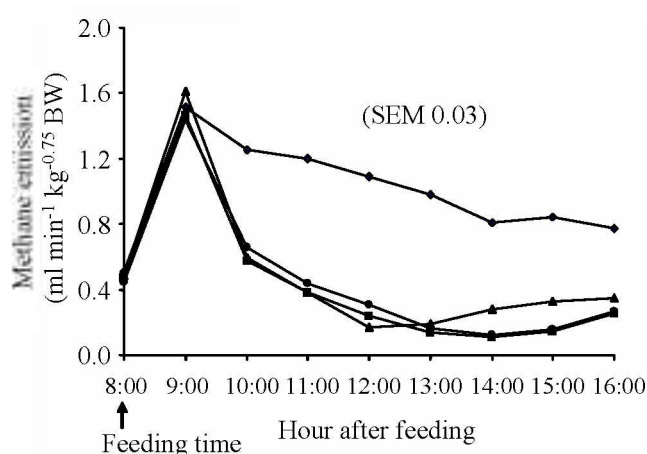
**Table 2.** Methane emission, oxygen consumption, carbon dioxide production and metabolic rate in sheep given nitrate with and without  $\beta$ 1-4 galacto-oligosaccharides (GOS) and *Candida kefyri*

Parameters	Treatment				SEM <sup>3</sup>	P
	Saline	Nitrate	Nitrate plus GOS	Nitrate plus GOS plus <i>Candida kefyri</i>		
Methane emission <sup>1</sup> (ml min <sup>-1</sup> kg <sup>-0.75</sup> BW)	0.99 <sup>a</sup>	0.42 <sup>b</sup>	0.49 <sup>b</sup>	0.44 <sup>b</sup>	0.03	0.0001
Oxygen consumption (ml min <sup>-1</sup> kg <sup>-0.75</sup> BW)	13.67 <sup>a</sup>	11.20 <sup>b</sup>	10.83 <sup>b</sup>	11.34 <sup>b</sup>	0.21	0.0001
Carbon dioxide production (ml min <sup>-1</sup> kg <sup>-0.75</sup> BW)	12.74 <sup>a</sup>	10.60 <sup>b</sup>	10.75 <sup>b</sup>	11.40 <sup>c</sup>	0.22	0.0001
Metabolic rate (W kg <sup>-0.75</sup> BW)	4.65 <sup>a</sup>	3.84 <sup>b</sup>	3.95 <sup>b,c</sup>	4.06 <sup>c</sup>	0.07	0.0001

<sup>1</sup>All values are mean of 8 h observation.

<sup>2</sup>Means within rows with different superscripts (a, b, c) differ significantly ( $p < 0.05$ ). Each value indicates means of four animals.

<sup>3</sup>Standard error of the mean.



**Figure 1.** Diurnal changes in methane emission from sheep given saline (♦), nitrate (■), nitrate plus  $\beta$ 1-4 galacto-oligosaccharides (GOS) (▲) and nitrate plus GOS plus *Candida kefyri* (●).

mm) operated at 80°C and at a flow rate of 0.5 ml/min. The mobile phase was H<sub>2</sub>O.

**Blood:** Venous blood collected from a jugular vein catheter was measured for haemoglobin (Nescauto Hemokit-N, Azwell Inc.) and methaemoglobin (Evelyn and Malloy, 1938). Plasma nitrate and nitrite were determined by the diazo-coupling method as for rumen fluid.

### Statistical analysis

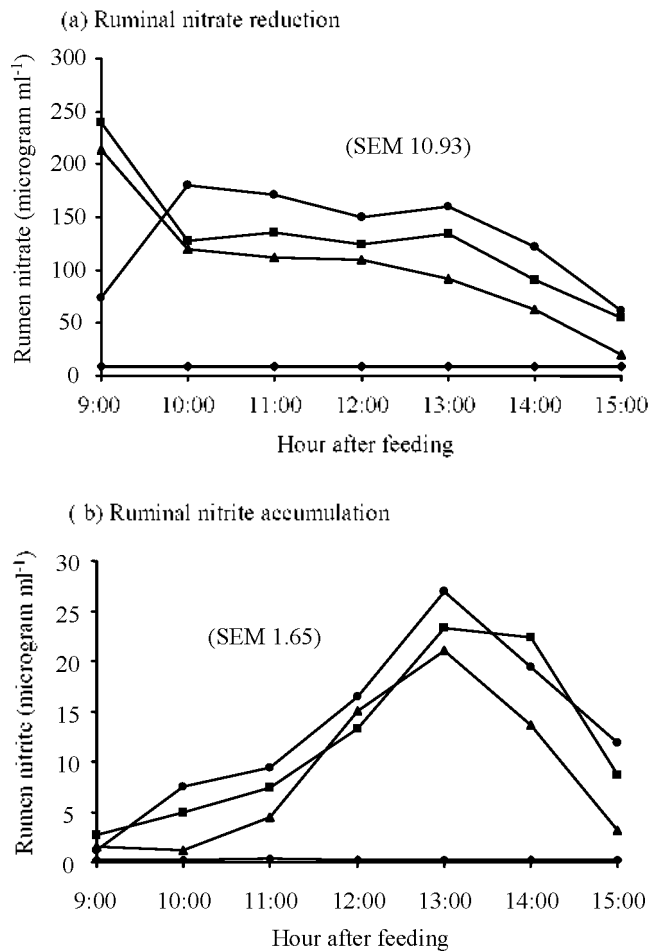
Data were analysed by repeated measures analysis of variance according to the General Linear Models Procedures of the Statistical Analysis Systems Institute (Cary, NC) for a Latin square arrangement of treatments. Treatment means were compared using Duncan's multiple range tests. Statistical significance of differences was accepted at  $p < 0.05$ .

## RESULTS AND DISCUSSION

### Rumen methane emission

A sharp decline in rumen methane emission in sheep was observed after nitrate administration (Figure 1). Total methane production in sheep given nitrate was significantly ( $p < 0.01$ ) decreased, compared to those given saline (Table

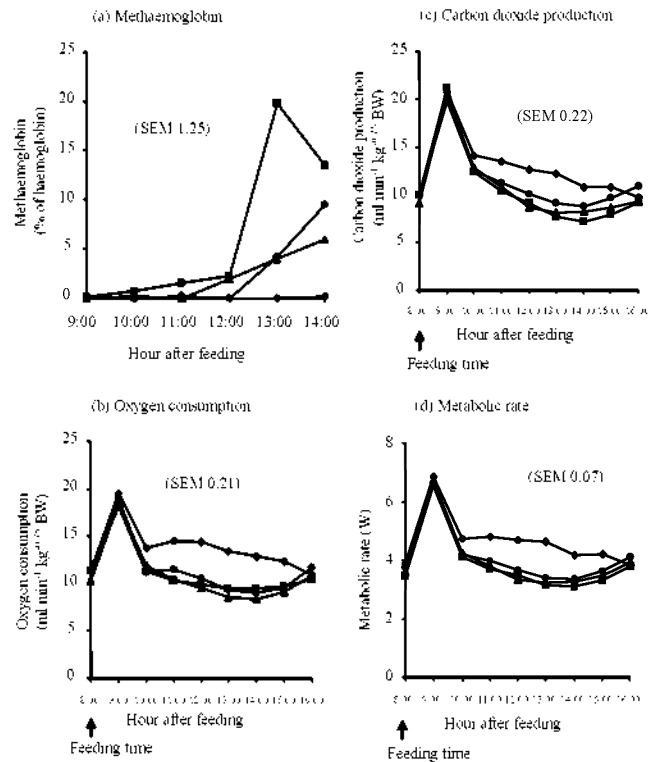
2). This is in agreement with the established fact that nitrate reduction acts as a highly competitive hydrogen sink against rumen methanogenesis (Allison and Reddy, 1990).  $\beta$ 1-4 galacto-oligosaccharides (GOS) have been reported to cause an increase in lactate, acetate, propionate and butyrate in cecal contents of rats (Kikuchi-Hayakawa et al., 1997). Some hindgut bacteria species can utilize lactate by the succinate pathway, producing propionate and acetate (Gottshalk, 1986). The accumulation of succinate and lactate, which are electron-sink products, was observed in the inhibition of methanogenic and sulfate-reducing bacteria (Gibson et al., 1993). Additionally, the *in vitro* study using orchardgrass silage as substrate showed that rumen methanogenesis was decreased by GOS supplementation (Gamo et al., 2001). On the contrary, GOS had no an inhibiting effect on rumen methane production in cows fed orchardgrass silage alone or mixed with alfalfa silage (Santoso et al., 2003a). Results in Table 2 indicate that rumen methanogenesis in sheep given nitrate with GOS is decreased to a similar level as those in sheep given nitrate alone (averaging 0.49 vs. 0.42 ml min<sup>-1</sup> kg<sup>-0.75</sup> BW). This may be due to nitrate having a more potent effect than GOS on rumen methanogenesis, and consequently rumen methanogenesis is not further decreased by the addition of GOS to nitrate. Another explanation is that the amounts of GOS supplemented were not enough to stimulate growth of *Bifidobacterium* species to produce lactate in the rumen. In the rumen, lactate is the main intermediate during conversion of starch to propionate, and the cellulolytic bacteria *Fibrobacter succinogenes* is the major propionate producer through the succinate pathway on roughage-based diet (Moss et al., 2000). As far as the role of *Bifidobacteria* in the rumen is concerned, Trovatelli and Matteuzzi (1976) indicated that the *Bifidobacteria* proliferate in the rumen when animals are fed concentrate diets rather than roughage diets. Also, Table 2 indicates that the tendency of decrease in rumen methanogenesis in sheep given nitrate with GOS plus *Candida kefyri* is observed when compared to those in sheep given nitrate with GOS (averaging 0.44 vs. 0.49 ml min<sup>-1</sup> kg<sup>-0.75</sup> BW). *Candida kefyri* per se has an influence on rumen methanogenesis, which is consistent with the *in vitro* previous finding (Gamo et al., 2001).



**Figure 2.** Ruminal nitrate reduction (a) and nitrite accumulation (b) in sheep given saline (♦), nitrate (■), nitrate plus β1-4 galacto-oligosaccharides (GOS) (▲) and nitrate plus GOS plus *Candida kefir* (●).

#### Nitrate reduction and nitrite accumulation in rumen and nitrite formation in plasma

When excess nitrate was used to suppress methane emission from ruminants, its toxicity was observed in the host because of the accumulation of nitrite in the rumen inducing the production of blood methaemoglobin (Takahashi and Young, 1991). Ruminal nitrate reduction and nitrite accumulation are shown in Figure 2. There was no nitrate concentration detected in rumen of saline treated sheep. The ruminal accumulation of nitrite reached a peak at 5 h after administration of nitrate alone (Figure 2 (b)) and this is fairly consistent with the previous report (Takahashi et al. 1998). GOS are produced from lactose by the action of β-galactosidases which have transgalactosylation activity (Yanahira et al., 1995), and is a mixture of two galactose units and one glucose unit (Tanaka et al., 1983). Transgalactosylated oligosaccharide caused a significant decrease in the activity of cecum nitrate reductase in rats (Rowland and Tanaka, 1993). However, nitrate-nitrite



**Figure 3.** Blood methaemoglobin (a) and diurnal changes in oxygen consumption (b), carbon dioxide production (c) and metabolic rate (d) in sheep given saline (♦), nitrate (■), nitrate plus β1-4 galacto-oligosaccharides (GOS) (▲) and nitrate plus GOS plus *Candida kefir* (●).

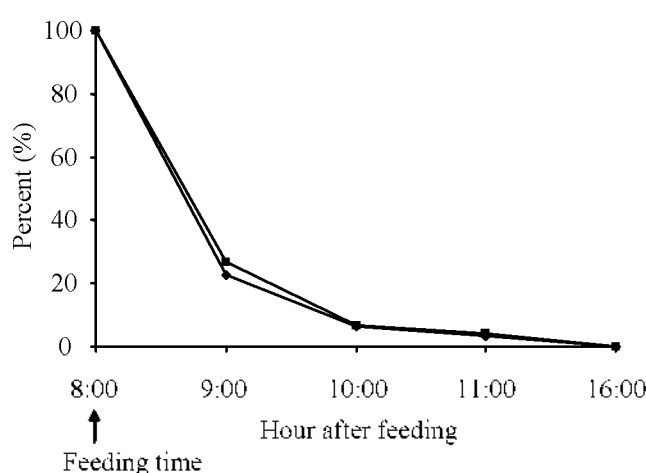
toxicity may be prevented by an adequate supply of lactate when ruminants ingest diets containing high levels of nitrate (Asanuma and Hino, 2002). Additionally, glucose has been reported to stimulate the *in vitro* reduction of nitrate and nitrite (Sapiro, 1949; John et al., 1957). Figure 2 also shows that the simultaneous administration of nitrate with GOS stimulates ruminal nitrate reduction and decreases ruminal nitrite accumulation at 5 h in the comparison with the administration of nitrate alone. Also, a decline in mean nitrite concentration in rumen ( $p > 0.05$ ) and plasma ( $p < 0.05$ ) are observed (Table 3). Thus, these results suggest that GOS accelerates a series of reductions of nitrate and nitrite in the rumen due to lactate specifically serving as an effective electron donor for nitrite reduction (Iwamoto et al., 2001) and glucose from GOS degradation (Figure 4). Table 3 also shows that when *Candida kefir* is added to mixed administration of nitrate with GOS, mean concentration of nitrite and maximum concentration of nitrite in rumen tend to be higher when compared to the simultaneous administration of nitrate with GOS. Also, a marked increase in mean plasma nitrite concentration and maximum plasma nitrite concentration are observed. These may be due to nitrate-reducing bacteria such as *Selenomonas ruminantium*, *Veillonella parvula*

**Table 3.** Concentration of nitrite in rumen and plasma and methaemoglobin (% of total blood haemoglobin) in sheep given nitrate with and without  $\beta$ 1-4 galacto-oligosaccharides (GOS) and *Candida kefyr*

Parameters	Treatment				SEM <sup>2</sup>	P
	Saline	Nitrate	Nitrate plus GOS	Nitrate plus GOS plus <i>Candida kefyr</i>		
<b>Rumen nitrite</b>						
Mean of all values, $\mu\text{g ml}^{-1}$	0.27 <sup>1, a</sup>	11.82 <sup>b</sup>	8.58 <sup>b</sup>	13.26 <sup>b</sup>	1.65	0.0001
Mean of maximums, $\mu\text{g ml}^{-1}$	0.28	23.28	21.03	27.00	31.89	NS
<b>Plasma nitrite</b>						
Mean of all values, $\mu\text{g ml}^{-1}$	0.00 <sup>a</sup>	1.98 <sup>b</sup>	1.04 <sup>c</sup>	1.70 <sup>b</sup>	0.20	0.0001
Mean of maximums, $\mu\text{g ml}^{-1}$	0.00 <sup>a</sup>	3.46 <sup>b</sup>	0.93 <sup>a</sup>	2.33 <sup>c</sup>	0.30	0.0009
<b>Methaemoglobin</b>						
Mean of all values, % of Hb	0.04 <sup>a</sup>	5.98 <sup>b</sup>	2.37 <sup>a, b</sup>	2.68 <sup>a, b</sup>	1.25	0.002
Mean of maximums, % of Hb	0.00	19.75	4.00	4.25	6.14	NS

<sup>1</sup>Means within rows with different superscripts (a, b, c) differ significantly ( $p < 0.05$ ). Each value indicates means of four animals.

<sup>2</sup>Standard error of the mean. NS: not significant.

**Figure 4.** Degradability rate of  $\beta$ 1-4 galacto-oligosaccharides (GOS) in the rumen in the presence of nitrate ( $\blacklozenge$ ) and nitrate with *Candida kefyr* ( $\blacksquare$ ).

(*alcalescens*) and *Wollinella succinogenes* (Stewart and Bryant, 1988) probably enhanced by *Candida kefyr*.

#### Blood methaemoglobin, oxygen consumption, carbon dioxide production and metabolic rate

Blood methaemoglobin production and diurnal changes in oxygen consumption, carbon dioxide production and metabolic rate in sheep are shown in Figure 3. Figure 3 (a) shows no detectable concentration of methaemoglobin in blood of the saline sheep and the maximum methaemoglobin level reached 5 h after nitrate administration in sheep. A methaemoglobin content of about 20% of total haemoglobin is considered subclinical toxicity (Bodansky, 1951). Table 3 shows that both maximum and mean methaemoglobin in the blood tend to be not significantly higher in sheep given nitrate alone than in sheep given nitrate with GOS. These are attributable to the inhibition of rumen and plasma nitrite accumulation by GOS. Also, Table 3 shows no additional effect of *Candida*

*kefyr* to the combination of nitrate and GOS on the suppression of methaemoglobin formation. This result could agree with previous preliminary studies of Gerald et al. (1984) that yeast culture did not decrease nitrate-induced methaemoglobin formation in the sheep and cattle in which only methaemoglobin was used as the assessment parameter of nitrate toxicity exposure.

Table 2 demonstrates that when compared to saline sheep, oxygen consumption ( $p < 0.05$ ), carbon dioxide production ( $p < 0.05$ ) and metabolic rate ( $p < 0.05$ ) in sheep given nitrate alone declined. These may be due to the progressive formation of nitrate-induced blood methaemoglobin (Takahashi and Young, 1991; 1992). Moreover, Takahashi et al. (1998) reported that every 10% of methaemoglobin formation instead of oxyhaemoglobin reduced oxygen consumption by 10.3% in sheep. Table 2 shows that oxygen consumption, metabolic rate and carbon dioxide production ( $p < 0.05$ ) in sheep given nitrate with GOS plus *Candida kefyr* tended to be higher although blood methaemoglobin formation did not decrease, when compared to the simultaneous administration of nitrate with GOS. However, these treatments may not fully restore nitrate toxicity-relating metabolic parameters to baseline levels of saline treatment.

#### CONCLUSION

This study suggests that the addition of  $\beta$ 1-4 galacto-oligosaccharides to nitrate may be a potential manipulator of rumen methanogenesis by abating the hazards of nitrate-nitrite toxicity in ruminants. Clearly, 1.3 g  $\text{NaNO}_3 \text{ kg}^{-0.75}$  body weight nitrate induced subclinical toxicity in sheep although rumen methanogenesis was remarkably suppressed at this dose of nitrate. Thus, minimizing nitrate amount to manipulate rumen methanogenesis should be evaluated to avoid the nitrate-nitrite toxicity in ruminants. Takahashi et al. (1998) reported that a reduced dose of nitrate (0.75 g  $\text{NaNO}_3 \text{ kg}^{-0.75}$  body weight) still caused a

slight risk in sheep due to 7% of blood methaemoglobin formed, while suppressing rumen methanogenesis. The optimal combination of the minimum level of nitrate with  $\beta$ 1-4 galacto-oligosaccharides should be assessed to suppress rumen methane emission while avoiding nitrate-nitrite toxicity.

### ACKNOWLEDGEMENTS

The authors are grateful to X. Zhou, B. Mwenya, A. Senda, M. Mii and A. Koyama for their assistance during the experiment.

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