Suppressing *In Vitro* Rumen Methanogenesis with *Alcaligenes faecalis* and a Low Concentration of Nitrate

M. O. Brien[1], M. Shoda[2], T. Nishida[1] and J. Takahashi[1]
[1]Department of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan, [2]Tokyo Institute of Technology, Yokohama, 226-8503, Japan

The objective of this study was to determine the effect of *Alcaligenes faecalis* (AF)and a low concentration of nitrate (NO_3; 2mM) on *in vitro* rumen fermentation and methane (CH_4) output. Methane is a harmful greenhouse gas and enteric fermentation is one of the principal sources. The use of NO_3 has been demonstrated *in vivo* to suppress enteric CH_4 production. However, the practical use of NO_3 is precluded by the toxicity associated with its reduced intermediate, nitrite (NO_2). To successfully reduce CH_4 emissions in livestock using lower concentrations of NO_3 would be a novel innovation. The *in vitro* gas quantification system was used to monitor CH_4 output during a 24 h incubation period. Ten grams of dried hay+concentrate (50:50) were weighted into individual 1 L fermentation vessels (3 or 4 vessels per experiment) together with 800 ml of a 4:1 volumetric mixture of buffer solution and rumen fluid inoculum, and 40 ml of each treatment. Rumen fluid was collected from two non-lactating fistulated cows and pooled. In experiment 1 (Exp. 1), to determine the effect of AF and NO_3 on *in vitro* rumen fermentation and CH_4 output, the four treatments were: (1) sterile water (SW; control), (2) AF culture, (3) SW + NO_3 and (4) AF culture + NO_3. In a second experiment (Exp. 2) to determine if an ingredient in the AFculture medium or a bacteriocin-like compound was responsible for suppressing CH_4 output, the three treatments were: (1) AF culture + NO_3, (2) AF cells + NO_3 and (3) cell-free supernatant + NO_3. For replication, Exp. 1 and 2 were repeated four and three times, respectively. In Exp. 1, CH_4 output (ml CH_4/24 h) was affected by treatment ($p<0.001$), with CH_4 output for NO_3 alone (37.8 ml) being lower ($p<0.05$) than either the control (51.0 ml) or AF culture (51.7 ml) treatments. AF culture + NO_3 treatment had a lower ($p<0.05$) CH_4 output (11.5 ml) than all other treatments. Total volatile fatty acids (VFA), individual VFA proportions (acetic (A), propionic (P) and butyric) and the A: P ratio was not affected by treatment ($p<0.05$). In Exp. 2, CH_4 output from AF culture + NO_3 (8.7 ml) and AF cells + NO_3 (7.8 ml) treatments was lower ($p<0.05$) than that from the cell-free supernatant + NO_3 (35.4 ml) treatment. In conclusion, a combination of AFand a low concentration of NO_3 (*i.e.* 2mM) can reduce methane output to a greater extent than NO_3 alone, without an adverse affect on fermentation. With lower concentrations of NO_3 required when combined with AF, the risk of NO_2 accumulation in the rumen would be greatly reduced. The reduction in CH_4 output with AF + NO_3 appears to have been due to the activity of the bacteria and NO_3, and not to either an ingredient of the culture medium or a bacteriocin-like compound excreted into the culture medium by AF. Further studies are required to elucidate the synergistic mechanism in which AF + NO_3 can reduce *in vitro* methanogenesis.

INTRODUCTION

Methane is a harmful greenhouse gas and enteric fermentation is one of the principal sources. Methane (CH_4) production during rumen fermentation is the result of the reduction of carbon dioxide (CO_2) with hydrogen (H_2) by methanogenic *Archaea*. Nitrate (NO_3) reduction is energetically more favourable than CO_2 reduction, and the presence of NO_3 in the rumen redirects H_2 from methanogenesis to NO_3 reduction, thereby decreasing CH_4 production. However, the practical use of NO_3 is precluded by the toxicity associated with its reduced intermediate, nitrite (NO_2). Nitrite accumulates in the rumen due to the more rapid reduction of NO_3 to NO_2 than the reduction of NO_2 to ammonia (NH_3) (Iwamoto et al., 1999). It would be desirable to be able to enhance the rate of nitrite reduction or alternatively, reduce the amount of nitrate added. There is some evidence to suggest AF No. 4 is capable of anaerobic denitrification (M. Shoda, pers. comm.), and thus may reduce NO_2 accumulation if introduced into the rumen. When this bacterium was initially introduced into the artificial rumen system with NO_3, there was no difference in the rate of NO_2 reduction compared to the control treatment. However, it became evident that a synergistic mechanism to reduceCH_4output was occurring with this bacterium and low concentrations of NO_3. Therefore, the objectives of this study were to demonstrate the synergistic effect of AF and $NO_3$to reduce *in vitro*CH_4 output (Experiment1) and to determine if an inhibitory substance from the AFculture medium or a bacteriocin-like compound excreted into the AF suspension during storage at 4ºC was responsible for suppressing CH_4 output(Experiment2).

MATERIALS AND METHODS

Preparation of *Alcaligenes faecalis* cells

The synthetic medium used was prepared by dissolving the following in 1 litre of distilled water: 14 g K_2HPO_4, 6 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 15 g trisodium citrate dehydrate, 0.2 g $MgSO_4.7H_2O$ and 2 ml of trace mineral solution. The trace mineral solution contained (per liter): 57.1g EDTA.2Na, 3.9 g $ZnSO_4.7H_2O$, 7 g $CaCl_2.2H_2O$, 5.1 g $MnCl_2.4H_2O$, 5 g $FeSO_4.7H_2O$, 1.1 g $(NH_4)_6Mo_7O_{24}.4H_2O$, 1.6 g $CuSO_4.5H_2O$ and 1.6 g $CoCl_2.6H_2O$. The preculture was prepared by inoculating 100 ml of medium with 1 ml of the stock culture in a shaking flask and incubating at 30ºC at 100 strokes per minute for 48 h. The preculture was then used to inoculate 5 litre of medium in a 10 litre fermentation vessel and incubated at 30ºC for 30 h. After incubation, the cells were concentrated by continuous centrifugation at 12,000 rpm for 1 h and the pellet solubilized in 100 ml of sterile distilled water (= AF suspension). *A. faecalis* suspensions were freshly prepared for each *in vitro* incubation period and stored at 4ºC until required.

In vitro fermentation

Rumen liquid.

The rumen liquid for this study was collected from two non-lactating fistulated Holstein cows, maintained on a daily diet of 10 kg of orchardgrass hay, with free access to clean drinking

water and a mineral block. The rumen liquid was collected from the cows just before morning feeding and strained immediately through a woven nylon cloth into a thermos flask and used as the source of inoculum.

Treatments.

To demonstrate the effect of AF suspension and NO_3 on *in vitro* methane output and other rumen fermentation variables, the following four treatments were used: (1)control, (2) AF suspension, (3) $NO_3$and (4) AFsuspension + NO_3 (Experiment 1). For Treatments 1 and 3, 40 ml of sterile distilled water was added and for Treatments 2 and 4, 40 ml of *A. faecalis*cells suspended in sterile distilled water was used. To attempt to identify the source of the inhibitory effect of AF (Experiment 2) the three treatments (40 ml) were: (1) AF suspension + NO_3, (2) AF cells + NO_3 and (3) cell-free suspension + NO_3.The cell-free suspension of AF was obtained by the centrifugation (8000 g for 15 min) of the AF suspension and then passing through a filter (0.45 µm pore size) into a sterile recipient. The pellet (AF cells) was washed three times in sterile phosphate buffer and re-suspended in sterile distilled water. Nitrate ($NaNO_3$) was added to the appropriate vessels to achieve a final concentration of 2 mM.

In vitro fermentation assay.

The *in vitro* gas quantification system (Sar et al., 2005) was used according to the protocol of Pen et al. (2006). A mixture of dried milled hay and concentrate (1:1, w/w) was used as the substrate. The substrate (10 g dry matter) was weighted into four individual 1 litre fermentation vessels together with 760 ml of a 4:1 volumetric mixture of sterile artificial saliva (McDougall, 1948) and rumen fluid inoculum from two cows (1:1, v/v) and into each fermentation vessel a treatment (40 ml) was added. To start each experiment, the vessels were sealed to ensure anaerobic conditions and fermentation was allowed to continue at 39 °C for 24 h. Methane and CO_2 output were continuously measured at 1-min intervals by the auto infrared CH_4 (EXA IR, Yokogawa Electric Corporation, Tokyo) and CO_2 (Model RI-555, Riken Keiki Co. Ltd, Tokyo) analyzers installed in the *in vitro* gas quantification system (Takasugi Seisakusho Co. Ltd, Tokyo). The pH and oxidation-reduction potential (ORP) were also continuously monitored in each fermentation vessel at 1-min intervals. All data were pooled into a computer through an interface with the analyzers. *In vitro* incubations were carried out on four and three separate days for Experiment 1 and 2, respectively. Therefore, the source of replication (n=4 or 3) in the experimental model was provided by rumen fluid inocula collected on the separate occasions for each experiment. Treatments were randomly assigned to incubation jars for each incubation period. At the end of each 24 h incubation period, all incubations were stopped, the contents were discharged, and the fermentation vessels were thoroughly washed and autoclaved prior to the next incubation. The fermentation vessels were then re-charged with fresh artificial saliva and inoculum(and *A. faecalis* cells) to begin the next 24 h incubation period.

Sample collection and analyses.
An aliquot from the fermentation medium at24 h were analysed for volatile fatty acids (VFA) concentrations. For individual VFA determination, samples were prepared as per Mwenya et al. (2005) and analysed by gas chromatographyas described by Pen et al. (2008).

Statistical analysis
Statistical analysis was carried out using the Statistical Package for the Social Sciences (SPSS) Version 16 for MS-Windows (SPSS Inc., Chicago, USA). The effects included in the General Linear Model for each variable were replication (day) and treatment effect. Differences among means were identified using Tukey`s multiple comparisons with $P<0.05$ being considered significant.

RESULTSAND DISCUSSION

For Experiment 1,the *in vitro*CH_4output and other fermentation variables are presented in Table 1. Cumulative CH_4 output (ml CH_4/24 h) for NO_3 alonewas lower ($p<0.05$) than either the control or AF suspensiononly treatments. *A. faecalis* suspension + NO_3 treatment had a lower ($p<0.05$) CH_4 output than all other treatments, which clearly demonstrates the occurrence of a synergistic effect to reduce CH_4 output. Propionic (P) acid concentration was higher in the AF suspension + NO_3 treatment compared to the control treatment, but there was no difference ($p>0.05$) between NO_3 alone and the control or *A. faecalis* suspension + NO_3 treatments. The higher propionic acid concentration in theAF suspension + NO_3 treatment does not agree with the literature because NO_3 is known to successfully compete with methanogenesis and propionogenesis for reducing equivalents, because of its higher affinity for H_2, resulting in a reduction in both CH_4 and propionate (Ungerfeld and Kohn, 2006). Cumulative CO_2 output, acetic (A) acid concentration, the A:P ratioand pH were not affected by treatment ($p>0.05$) which would indicate that the AF suspension + NO_3 treatment mainly affects methanogenesis. In Experiment 2, there was little evidence to suggest that CH_4 output was being suppressed due to an inhibitory substance carried over from the AF culture medium to the AF suspension or by the production of a bacteriocin-like compound excreted into the AF suspension during storage at 4ºC. Both of those possible modes of actions for AF to reduce CH_4 output could be eliminated because AF cells + NO_3 treatment reduced CH_4 output (8.7 ml) to the same extent as AF suspension + NO_3 treatment (7.8 ml), and CH_4 output from both treatments were lower ($p<0.05$) than that from the cell-free suspension + NO_3 treatment (35.4 ml).

CONCLUSION

a combination of AFand a low concentration of NO_3 can reduce methane output to a greater extent than NO_3 alone, without having an adverse affect on fermentation. With lower concentrations of NO_3 required when combined with AF, the risk of NO_2 accumulation in the rumen would be greatly reduced. The reduction in CH_4 output with AF suspension + NO_3

appears to be due to the activity of the bacterium, which is interacting with NO_3 to suppress methanogenesis. Further studies are required to elucidate the synergistic mechanism in which AF + NO_3 reduces*in vitro* methanogenesis.

Table 1 Effect of *Alcaligenus faecalis* and/or low nitrate concentration on *in vitro* rumen methane (CH_4) and other fermentation variables after 24 h incubation (Experiment 1)

	Treatments					
	Control	*A. faecalis* Suspension	Nitrate(2 mM)	*A. faecalis* Suspension + Nitrate(2 mM)	SEM	p
Cumulative CH_4 (ml)[1]	51[a]	51.7[a]	37.8[b]	11.5[c]	1.17	<0.001
Cumulative CO_2 (ml)[1]	774	856	750	718	43.7	NS[2]
VFA[3](mM)[1]						
Acetic acid (A)	21.4	24.8	24.8	24.0	1.14	NS
Propionic acid (P)	10.5[a]	12.5[ab]	11.4[ab]	13.0[b]	0.56	0.046
Butyric acid	3.1[ab]	3.6[a]	3.0[b]	3.2[ab]	0.14	0.051
Valeric acid	0.4	0.4	0.4	0.3	0.05	NS
Total VFA	35.4[a]	41.3[b]	39.6[ab]	40.5[b]	1.16	0.024
A:P ratio[1]	2.03	1.99	2.18	1.87	0.141	NS
pH[4]	6.99	6.92	6.93	6.89	0.064	NS
ORP (mV)[4]	-324.1[a]	-362.1[b]	-341.7[ab]	-351.4[ab]	8.34	0.054

[1]Mean values at 24 h (n=4)

[2]NS, not significant (p>0.05)

[3]Volatile fatty acids

[4]Mean values of seven sampling times (0, 2, 4, 6, 8, 12 and 24 h) (n=4).

[a–c]Means within a row with common superscripts do not differ (p<0.05).

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