Asian-Aust. J. Anim. Sci.
Vol. 24, No. 2 : 285 - 294
February 2011

www.ajas.info

Some Prophylactic Options to Mitigate Methane Emission from Animal Agriculture in Japan*

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ABSTRACT : The abatement of methane emission from ruminants is an important global issue due to its contribution to greenhouse gas with carbon dioxide. Methane is generated in the rumen by methanogens (archaea) that utilize metabolic hydrogen (H_2) to reduce carbon dioxide, and is a significant electron sink in the rumen ecosystem. Therefore, the competition for hydrogen used for methanogenesis with alternative reductions of rumen microbes should be an effective option to reduce rumen methanogenesis. Some methanogens parasitically survive on the surface of ciliate protozoa, so that defaunation or decrease in protozoa number might contribute to abate methanogenesis. The most important issue for mitigation of rumen methanogenesis with manipulators is to secure safety for animals and their products and the environment. In this respect, prophylactic effects of probiotics, prebiotics and miscellaneous compounds to mitigate rumen methanogenesis have been developed instead of antibiotics, ionophores such as monensin, and lasalocid in Japan. Nitrate suppresses rumen methanogenesis by its reducing reaction in the rumen. However, excess intake of nitrate causes intoxication due to nitrite accumulation, which induces methemoglobinemia. The nitrite accumulation is attributed to a relatively higher rate of nitrate reduction to nitrite than nitrite to ammonia via nitroxyl and hydroxylamine. The *in vitro* and *in vivo* trials have been conducted to clarify the prophylactic effects of L-cysteine, some strains of lactic acid bacteria and yeast and/or β1-4 galacto-oligosaccharide on nitrate-nitrite intoxication and methanogenesis. The administration of nitrate with β1-4 galacto-oligosaccharide, *Candida kefyr*, and *Lactococcus lactis subsp. lactis* were suggested to possibly control rumen methanogenesis and prevent nitrite formation in the rumen. For prebiotics, nisin which is a bacteriocin produced by *Lactococcus lactis subsp. lactis* has been demonstrated to abate rumen methanogenesis in the same manner as monensin. A protein resistant anti-microbe (PRA) has been isolated from *Lactobacillus plantarum* as a manipulator to mitigate rumen methanogenesis. Recently, hydrogen peroxide was identified as a part of the manipulating effect of PRA on rumen methanogenesis. The suppressing effects of secondary metabolites from plants such as saponin and tannin on rumen methanogenesis have been examined. Especially, yucca schidigera extract, sarsaponin (steroidal glycosides), can suppress rumen methanogenesis thereby improving protein utilization efficiency. The cashew nutshell liquid (CNSL), or cashew shell oil, which is a natural resin found in the honeycomb structure of the cashew nutshell has been found to mitigate rumen methanogenesis. In an attempt to seek manipulators in the section on methane belching from ruminants, the arrangement of an inventory of mitigation technologies available for the Clean Development Mechanism (CDM) and Joint Implementation (JI) in the Kyoto mechanism has been advancing to target ruminant livestock in Asian and Pacific regions. (**Key Words :** Methane, Nitrate, L-cysteine, Probiotics, Prebiotics, Sarsaponin)

INTRODUCTION

Global warming due to increases in the atmospheric concentration of greenhouse gases is an important issue. The worldwide trends of carbon dioxide have shown an increase in the greenhouse effect on global warming (Houghton, 1994). However, methane is an important greenhouse gas second only to carbon dioxide in its contribution to global warming due to its high absorption ability of infrared in the radiation from sun (IPCC, 1994). The world population of ruminants is important source of methane, contributing approximately 15% of the total atmospheric methane flux. The control of methane emission is a logical option since atmospheric methane concentration is increasing at a faster rate than carbon dioxide (Moss, 1993). Methane emitted from ruminants is generated in the rumen by hydrogenotrophic methanogens that utilize hydrogen to reduce carbon dioxide, and is a significant electron sink in the rumen ecosystem (Klieve and Hegarty, 1999). Methane contains 892.6 kJ combustible energy per molecule at 25°C and 1,013 hPa, while not contributing to

* This paper was presented at 2010 AAAP Animal Nutrition Forum of the 14th AAAP Animal Science Congress held in Pingtung, Taiwan during August 23-29, 2010.
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the total supply of metabolic energy to ruminants (Takahashi et al., 1997). As reported by Leng (1991), methane production from ruminants in the developing countries may be high since the diets are often deficient in critical nutrients for efficient microbial growth in the rumen. So far, a number of inhibitors of methanogenesis have been developed to improve feed conversion efficiency of ruminant feeds claimed to be effective in suppressing methanogens or overall bacterial activities (Chalupa, 1984). Attempts to reduce methanogenesis by the supplementation of chemicals such as ionophores (monensin and lasalocid), have long been made (Hopgood and Walker, 1967; Chalupa, 1984). However, these ionophores may depress fiber digestion and protozoal growths (Chen and Wolin, 1979). In addition, some resistant bacteria will appear in the rumen from the results of long term use of the ionophores. Therefore, development of manipulators to mitigate rumen methanogenesis must pay attention to secure safety for animals, their products and environment as alternatives of ionophores.

Theoretically, methanogenesis can be reduced by either a decrease in the production of H_2, the major substrates for methane formation or an increase in the utilization of H_2 and formate by organisms other than methanogens. However, direct inhibition of H_2-forming reactions may depress fermentation in microorganisms that produce H_2, including main cellulolytic bacteria such as *Ruminococcus albus* and *Ruminococcus flavefaciens* (Wolin, 1975; Belaich et al., 1990). Therefore, a reduction in H_2 production by the enhancement of reactions that accept electrons is desirable (Stewart and Bryant, 1988). In the rumen, metabolic H_2 is produced during the anaerobic fermentation of glucose. This H_2 can be used during the synthesis of volatile fatty acids and microbial organic matter. The excess H_2 from NADH is eliminated primarily by the formation of CH_4 by methanogens, which are microorganisms from the *Archea* group that are normally found in the rumen ecosystem (Baker, 1999). The stoichiometric balance of VFA, CO_2 and CH_4 indicates that acetate and butyrate promote CH_4 production whereas propionate formation conserves H_2, thereby reducing CH_4 production (Wolin and Miller, 1988). Therefore, a strategy to mitigate ruminal CH_4 emission is to promote alternative metabolic pathway to dispose of the reducing power, competing with methanogenesis for H_2 uptake. Allison et al. (1981) and Takahashi et al. (1983) showed that nitrate as a hydrogen (electron) acceptor competed with reducing steps in methane production and, consequently, markedly suppress methanogenesis by rumen microbes. However, elevated levels of nitrate in forages could pose a serious threat to animal due to its conversion to toxin nitrite. Ruminants are particularly vulnerable to nitrate intoxication as nitrate is primarily reduced to nitrite by NADPH-nitrate reductase (*EC* 1.6.6.3) of nitrate reducing bacteria in rumen, and then to ammonia via hydroxylamine. Subsequently, nitrite accumulated in the rumen is absorbed in the blood stream to produce methaemoglobin as a result of the way of oxidative properties of nitrite against ferrous forms of oxyhemoglobin (Allison and Reddy, 1984). With an advance of methaemoglobinemia, oxygen consumption of ruminants decreases because of disruption to oxygen transportation (Takahashi et al., 1983) and pulmonary of gaseous exchange and metabolic rate were altered, whereby indicating the extent of the physiological effects of nitrite on the animal as a whole (Takahashi and Young, 1991). Additionally, the reduction of nitrate in the rumen may alter the oxidation-reduction (redox) potential and the molecular proportion of volatile fatty acid (VFA) (Jamieson, 1958; Allison et al., 1981; Takahashi et al., 1983, 1989). An approach to the solution of this problem may be the depression of the nitrite formation rate by means of an inhibition of the enzymatic activity of nitrate reductase in the rumen microbes, requiring molybdenum (Metzler, 1977) to catalyze the primary step to nitrite in the assimilatory reduction of nitrate. To this end, in the studies using tungsten (W), it was clarified that the interfering incorporation of molybdenum into the bacterial nitrate reductase was the most efficient to inhibit nitrate reduction in the rumen (Prins et al., 1980; Korzeniowski et al., 1981; Marais et al., 1988; Takahashi et al., 1989). W may, however, have a slight application in animal feeding as a prophylactic against nitrate poisoning owing to its potential toxic properties (Takahashi and Young, 1991). Additionally, it has been reported in *in vitro* and *in vivo* experiments that the adverse effect of nitrate on ruminant physiology could be counteracted by L-cysteine. L-cysteine is degraded in rumen by a microbial cystathionine γ-lyase to generate sulphide, then, sulphur ion generated from sulphide can bind to W to form an insoluble inorganic compound. Consequently, the activity of nitrate reductase will be inhibited (Takahashi, 1989; Takahashi et al., 1989; Takahashi and Young, 1991; 1992). Also, as reported by Takahashi et al. (1997), methane emission in sheep was suppressed by L-cysteine. Thus, methanogenesis is possibly suppressed regulating assimilatory nitrate reduction by the combination between nitrate and inhibitors or enhancing nitrite reduction to prevent nitrite accumulation.

Oligosaccharides are naturally occurring carbohydrates with a low degree of polymerisation and consequently low molecular weight, being commonly found to perform in the various plant and animal sources. Galactooligosaccharides (GOS) are non-digestible carbohydrates, which are resistant to gastrointestinal digestive enzymes, but fermented by specific colonic bacteria. The products of fermentation of GOS in the colon, mainly short chain fatty acids, have a role in the improvement of the colonic environment, energy

supply to the colonic epithelium, and calcium and magnesium absorption (Sako et al., 1999). The indigestibility and stability of GOS to hydrolysis by α-amylase of human saliva, pig pancreas, rat small intestinal contents and human artificial gastric juice has been shown in several *in vitro* experiments (Ohtsuka et al., 1990; Watanuki, and Matsumoto, 1996). This is because GOS have β-configuration, whereas human gastrointestinal digestive enzymes are mostly specific for α-glycosidic bonds. From this point of view, expectedly, GOS will be readily degraded in the rumen as a result of the ruminal enzymes being specific for β-glycosidic bonds. Thus, lactic acid bacteria may consume GOS to promote propionate formation through acrylate pathway, and consequently the competition with methanogens for hydrogen will occur. Thus, the amplifying competition of metabolic H_2 with probiotics may be a key factor in the regulation of rumen methanogenesis. However, direct effects of prebiotics and secondary metabolites such as tannin, saponin and natural resin on methanogens and eubacteria in the rumen remain to be elucidated to secure the safety for animals, their products and environment.

The present paper deals with the possible accreditation of manipulators to mitigate CH_4 emission from ruminants as CDM.

MANIPULATORS TO MITIGATE RUMEN METHANOGENESIS AS ALTERNATIVE HYDROGEN SINKS - REGULATING ASSIMILATORY NITRATE REDUCTION OR ENHANCING NITRITE REDUCTION TO PREVENT NITRITE ACCUMULATION

In the rumen, metabolic H_2 is produced during the anaerobic fermentation of glucose. This H_2 can be used during the synthesis of volatile fatty acids and microbial organic matter. The excess H_2 from NADH is eliminated primarily by the formation of CH_4 by methanogens, which are microorganisms from the *Archea* group that are normally found in the rumen ecosystem (Baker, 1999). The stoichiometric balance of VFA, CO_2 and CH_4 indicates that acetate and butyrate promote CH_4 production whereas propionate formation conserves H_2, thereby reducing CH_4 production (Wolin and Miller, 1988). Therefore, a strategy to mitigate ruminal CH_4 emission is to promote alternative metabolic pathway to dispose of the reducing power, competing with methanogenesis for H_2 uptake. As assimilate nitrate reduction in the rumen which shows strong redox potential is relatively higher affinity to H_2 than hydrogenotrophic methanogenesis, the administration of nitrate remarkably suppressed ruminal methanogenesis (Takahashi and Young, 1991, 1992).

Figure 1 shows that the formation of toxic nitrite reduced from nitrate is successfully prevented by L-cysteine (Takahashi and Young, 1991, 1992; Takahashi et al., 1989, 1998, 2000, 2002), *i.e.* the effective mitigation of ruminal CH_4 emission is safely achieved by simultaneous administration of nitrate and L-cysteine without nitrate intoxication (Takahashi, 2001). Furthermore, to evaluate the prophylactic effect of GOS and two different types of probiotics (two strains of yeast and lactic acid bacteria) on rumen CH_4 production and nitrate reduction, $NaNO_3$ (NO_3, 10 mM) with or without GOS (200 mg/L), two types of cultured yeast.

Figure 2 shows cumulative methane production from 12 hours incubation was extrapolated by a non-linear regression model. Significantly ($p<0.05$) lower value of cumulative methane production was observed in all treatments compared to control incubation. The value in NO_3+GOS was the lowest among treatments. Moreover, the cumulative methane production was significantly ($p<0.05$) decreased when GOS and CYS were supplemented. Significantly ($p<0.05$) lower value of NO_2-N concentration in NO_3+CYS treatment was observed compared to NO_3

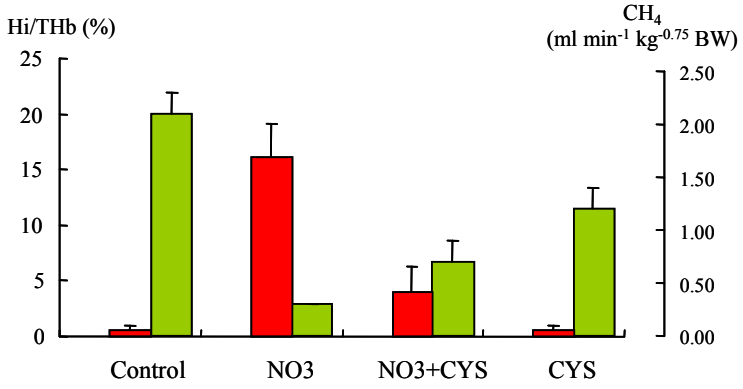


Figure 1. The suppressing effect of nitrate (1.3 g $NaNO_3$ $kg^{-0.75}$ body weight) on methane emission and prophylactic effect of L-cysteine (0.21 g S $kg^{-0.75}$ body weight) on nitrate-induced methemoglobinemia in sheep.

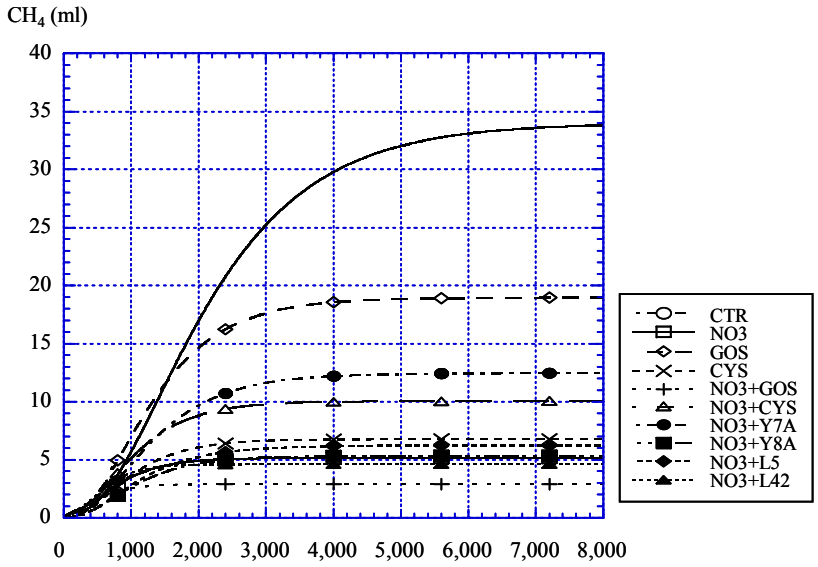


Figure 2. Effects of administration of nitrate, GOS, L-cysteine (CYS) and probiotics on a cumulative methane production CH_4 (ml) = $a+b(1-e^{-ct})^3$, t = min.

treatment whereas GOS, Y8A, and L42 tended to decrease nitrite formation ($p>0.05$). Although the possible mechanism was not determined, the administration of nitrate with β1-4 galactooligosaccharide (GOS), yeast (*Candida kefyr* strain), Lactic acid bacteria (*Lactococcus lactis subsp. lactis*) and L-cysteine were suggested to possibly control rumen methanogenesis and to prevent nitrite formation in rumen.

For *in vivo* trial, four rumen-fistulated wethers were allocated to four dietary treatments in a 4×4 Latin square design and fed on a basal diet of chopped alfalfa hay cube and timothy hay (50:50,w/w) at a maintenance level (55 g DM $kg^{-0.75}$ body weight). All animals were individually maintained in metabolic crate equipped with a ventilated hood system to capture respiratory methane. To examine the effect of GOS and yeast (*Candida kefyr* strain: Y8A) culture on the nitrate-induced poisoning, nitrate with or without GOS and Y8A was directly administered into the rumen via fistula as single dose 30 min after the morning feeding. GOS and Y8A were supplemented by sprinkling onto the feed and through rumen fistula, respectively. Physiological saline (0.9% NaCl) was given as the control treatment. Respiratory gaseous exchanges were monitored from 1 hour before to 7 hours after administration of the chemicals. Venous blood samples were collected via a jugular catheter at 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 after administration of nitrate to check the development of nitrate-nitrite poisoning physiologically. The maximum formation of methaemoglobin in haemoglobin was observed 5 h after administration of nitrate when the nitrate alone was given to animal. For GOS-treated animals, methaemoglobin were prevented though significant difference was not observed ($p>0.05$). Methaemoglobin concentration for nitrate with GOS+Y8A was also lower than that for nitrate alone ($p>0.05$).

Figure 3 shows the time course of change in methane production. Methane production decreased by 14% of the control value 5 h after administration of nitrate alone. Methane production for nitrate with GOS tended to be lower than that for nitrate alone 3-4 h and 5 h after administration. In addition, methane production for nitrate with GOS+Y8A treatment was lower than that for nitrates alone 2, 4.50 and 6.50 h after administration. However, for nitrate with GOS+Y8A treatment, methane production was not observed to lower than that for nitrate with GOS.

Figure 4 shows chemical structure of GOS. GOS are non-digestible carbohydrates in nonruminants and have a long history of research as a prebiotics food ingredient. GOS are resistant to gastrointestinal enzymes, but are selectively utilized *Bifidbacteria* (Sako et al., 1999). In the rumen, *Bifidobacteria* and *Lactobacillus* species utilize fructose, galactose, glucose and starch as substrates to produce lactate and acetate. Lactate is intermediate compound of a acrylate pathway during propionate production in the rumen.

Meanwhile, propionate production is indirect competition with methanogens for available hydrogen. As *Bifidobacteria* and *Lactobacillus* species in the rumen can utilize GOS and produce more lactate, ruminal methanogenesis have been suppressed by GOS with or without direct-fed microbe yeasts and lactic acid bacteria (Gamo, 2001; Sar et al., 2002; 2004b; 2004c; Takahashi et al., 2002; 2003; Mwenya et al., 2004b; 2004c; 2004d: 2005; Santoso, 2004a). However, the efficacy of GOS with the

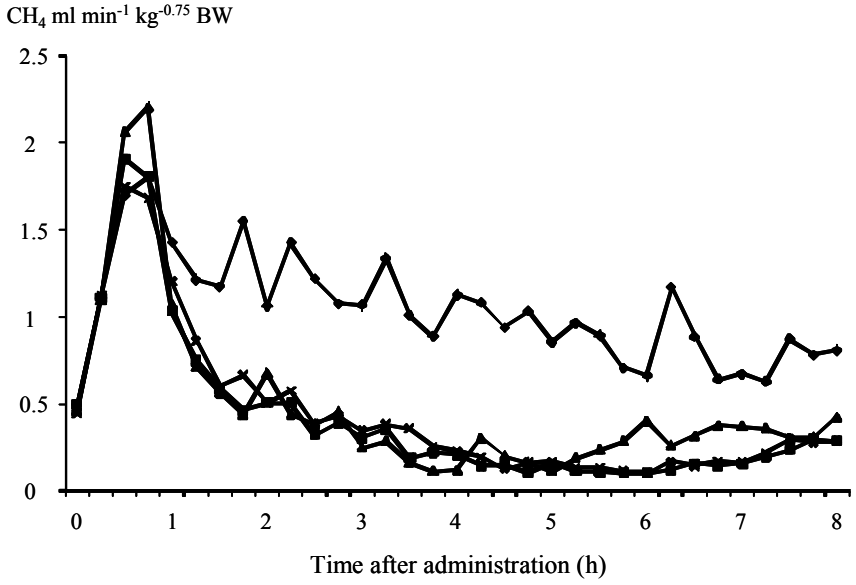


Figure 3. Time course of change of methane production after administration of saline control (♦), nitrate (■), nitrate+GOS (▲) and nitrate+GOS+Y8A (×). Each point indicates a mean of four animals.

probiotics on different diets and animal species remains to be elucidated. It has been suggested that for sheep treated with nitrate with GOS, GOS was readily degraded and consumed by lactic acid bacteria promoting propionate formation through acrylate pathway. Hence, methane production was inhibited as a result of propionate production being in direct competition with methanogenesis for available hydrogen. However, methane production for the administration of nitrate plus GOS was not observed to be lower than that for nitrate treatment alone because of nitrate possibly suppressed methanogenesis at the maximum level. Additionally, rates of nitrate reduction and nitrite accumulation in sheep given with nitrate plus GOS were decreased, possibly, as a result of GOS competed with nitrate reduction for hydrogen to produce propionate through acrylate pathway. In consequence, methanogenesis by rumen microbes and methaemoglobin formation will be suppressed. On the contrary, rates of nitrate reduction and nitrite accumulation with GOS were observed to be higher for sheep treated with nitrate plus GOS plus *Candida kefyr* than that without *Candida kefyr*, although methanogenesis was similarly low. Nitrate-reducing bacteria such as *Selenomonas ruminantium*, *Veillonella parvula* and *Wollinela succinogenes* are likely to be enhanced by

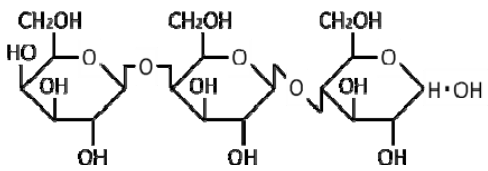


Figure 4. Chemical structure of GOS.

Candida kefyr. In conclusion, β1-4 galactooligosaccharide and *Candida kefyr* with nitrate may potentially suppress rumen methanogenesis and dysfunction attributed to nitrate as well as L-cysteine (Takahashi and Young, 1992; Takahashi et al., 1998).

Furthermore, *Escherichia coli* modified genetically was developed in an attempt to promote nitrite reduction abating ruminal methanogenesis (Sar et al., 2004a; 2005a; 2005b; 2005c) (Figure 5).

POSSIBLE CONTROL OF METHANOGENESIS BY HYDROGEN ACCEPTORS OR ALTERNATIVE MECHANISMS

Rumen manipulation with ionophores such as monensin has been reported to abate rumen methanogenesis (Mwenya, et al., 2005), However, there is an increasing interest in exploiting prebiotics and probiotics as natural feed additives to solve problems in animal nutrition and livestock production as alternatives of the antibiotics due to concerns about incidences of resistant bacteria and environmental pollution by the excreted active-antibacterial substances (Mwenya et al., 2006). Nisin and saponin-containing extracts of *Yucca schidigera* and *Quillaja saponaria* have been categorized as 'generally recognized as safe (GRAS)' for human consumption by US Food and Drug Administration. Nisin produced by *Lactococcus lactis* subsp. *lactis*, antimicrobial activity against spectrum of Gram-positive bacteria is characterized bacteriocin and performed mitigating effect on ruminal methane emission (Mwenya et al., 2004a; Santoso et al., 2004b; Sar et al., 2006). Saponins are natural detergents found in variety of plants. Yucca

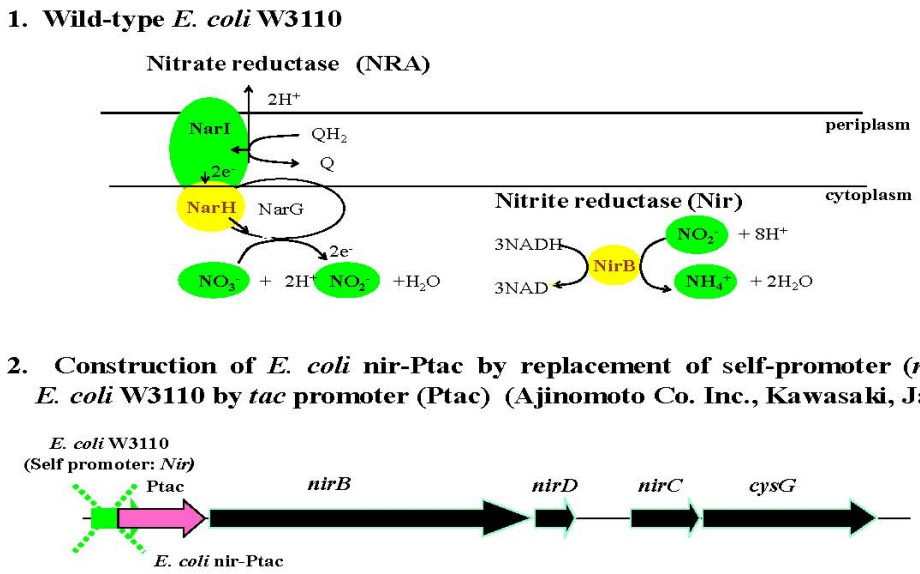


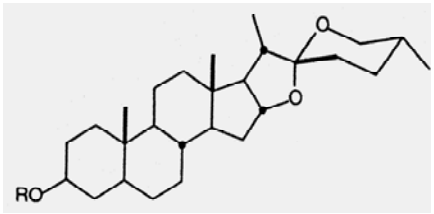
Figure 5. Wild-type *E. coli* W3110 and the construction of *E. coli* nir-Ptac.

saponins have a steroidal nucleus, whereas Quillaja saponins are triterpenoid in structure (Figure 6). Supplementation of saponin-rich plant extracts decreased ruminal protozoa counts and decreased methanogenesis accompanied by decrease in the ruminal acetate/propionate (A/P) ratio *in vitro* and *in vivo* (Wallace et al., 1994; Takahashi et al., 2000; Wang et al., 2000; Mwenya et al., 2004a; Santoso et al., 2004a; Pen, et al., 2006). However, Pen et al. (2007; 2008) showed in recent detailed examination that *Q. saponaria* had no effect on ruminal methanogenesis and A/P ratio, although it suppressed protozoa number.

Figure 7 shows that effects of protease-resistant antimicrobial substances (PRA) produced by *Lactobacillus plantarum* and *Leuconostoc citreum* on rumen methanogenesis were examined using the *in vitro* continuous methane quantification system (Asa, 2010). Four different strains of lactic acid bacteria, Control: *Lactococcus lactis* ATCC19435 (non-antibacterial substances), Nisin-Z: *Lactococcus lactis* NCIMB702054, PRA-1: *Lactobacillus plantarum* TUA1490L, and PRA-2: *Leuconostoc citreum* JCM9698 were individually cultured in GYEKP medium. An 80 ml aliquot of each supernatant was inoculated into phosphate-buffered rumen fluid. PRA-1 remarkably decreased cumulative methane production. For PRA-2, there were no effects on CH_4 and CO_2 production and fermentation characteristics in mixed rumen cultures. The results suggested that PRA-1 reduced the number of methanogens or inhibited utilization of hydrogen in rumen fermentation.

Figure 8 shows DGGE band patterns of archaea and eubacteria. All fluorescence brightness of methanogens

Yucca schidigera
(Steroidal saponins)



Quillaja saponaria
(triterpenoid saponins)

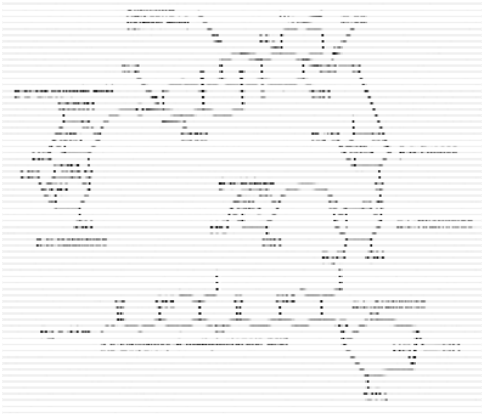


Figure 6. Chemical structure of *Yucca schidigera* and *Quillaja saponaria*.

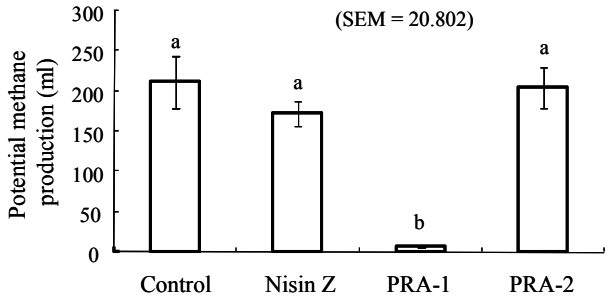


Figure 7. Effect of PRA on potential methane production. Control: *Lactococcus lactis* ATCC19435 (non-antibacterial substances), Nisin-A: *Lactococcus lactis* NCIMB702054, PRA-1: *Lactobacillus plantarum* TUA1490L, and PRA-2: *Leuconostoc citreum* JCM9698. Vertical bars represent standard deviation (n = 4). Means with different letters differ significantly ($p<0.01$).

bands of PRA-1 were remarkably light in color compared with control. Band No. 1 to No.3 in archaebacteria might be *Methanobrevibacter* sp. which is a Gram positive bacterium or parasitic methanogens sticking on protozoan surface. PRA-1 increased the fluorescence brightness of the band of the Gram positive bacteria and declined the fluorescence brightness of the band of the Gram negative bacteria. For Gram positive bacteria, *Streptococcus* sp., *Clostridium* sp., *Butyrivibrio* sp. and *Clostridium aminophilum* were increased, whereas *Prevotella* sp., *Prevotella ruminicola, Pseudobutyrivibrio* sp., *Prevotella* sp., *Succinivibrio dextrinosolvens and Schwartzia succinivorans* in Gram negative bacteria were decreased by adding PRA-1.

Figure 9 shows chemical structure of natural resin of cashew nut shell. The cashew nutshell liquid (CNSL) or cashew shell oil is a natural resin found in the honeycomb structure of the cashew nutshell. It consists of about 90% anacardic acids and 10% cardol. Both substances are dermatogenic,similarly to the oils of the poison ivy. It is a raw material of multiple uses in developing drugs, antioxidants, fungicides, etc. The anacardic acids have been used effectively *in vivo* against tooth abscesses due to their lethality to gram-positive bacteria. They are also active against a wide range of other gram-positive bacteria. Kobayashi and his colleagues have found CNSL is an effective suppressor to mitigate rumen methanogenesis (Unpublished).

The Kyoto protocol obligates Japan to mitigate 6% greenhouse gas emission in targeted year compared to 1990's emission in CO_2 equivalent. The benchmark 1990 emission levels in Kyoto Protocol is 1261.3 Tg in Japan

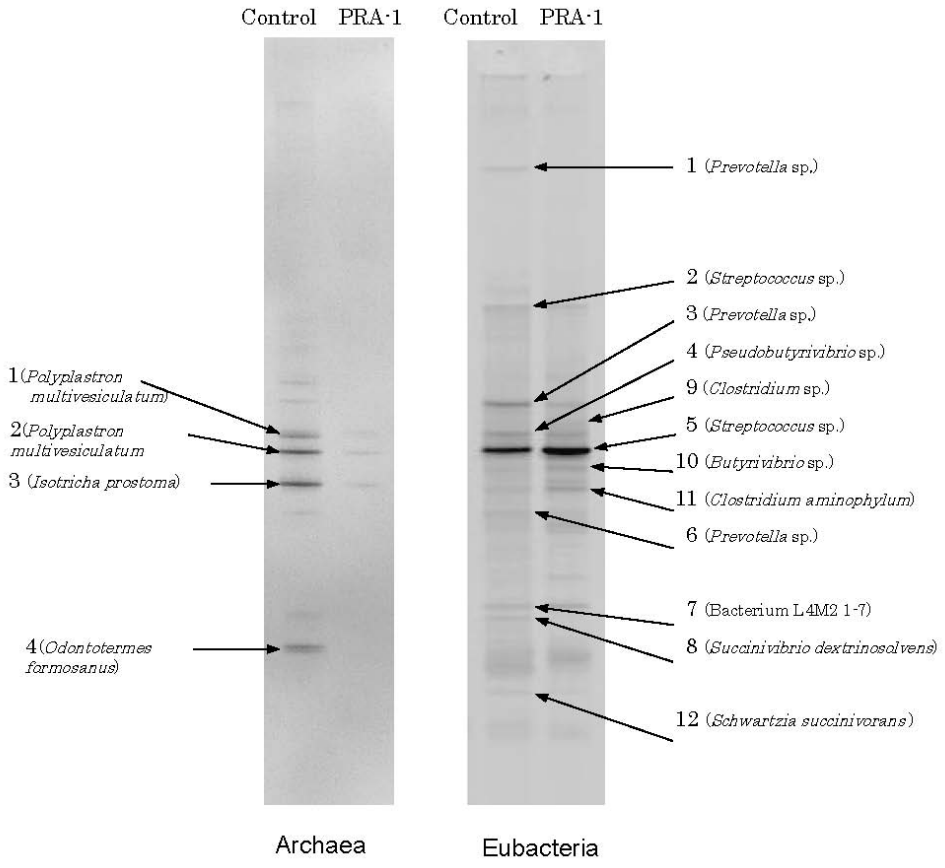


Figure 8. DGGE band patterns.

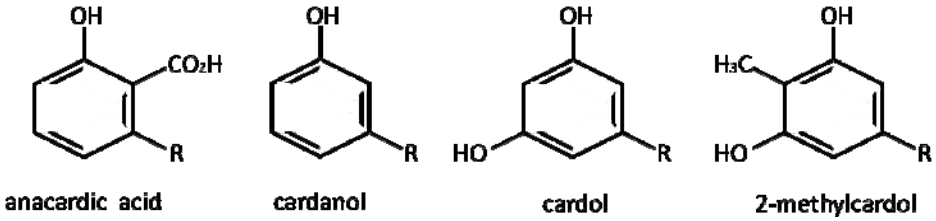


Figure 9. Chemical structure of natural resin of cashew nutshell.

(National Greenhouse Gas Inventory Report of Japan 2007). The 75.7 Tg is countable as the 6% reduction in Japanese obligation. In animal agriculture in Japan, however, total number of dairy and beef cattle account approximately 4.4 million (FAOSTST, http://faostat.fao.org/). Their total belching methane is counted 7.82 Tg/year in CO_2 equivalent, *i.e.* 10.3% of total load of GHG mitigation in Japan. Although the manipulators described in the present paper will not greatly contribute to mitigate GHG emission in Japan, the CDM and JI in Kyoto Mechanism might give highly economical and environmental incentives for the implementation in ruminant production of Asia and Pacific regions. Inventories of manipulators and their abatements are accurately assessed for the eligibility requirements as Kyoto Mechanism.

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