

## REVIEW

### Prophylactic Modulation of Methane and Nitrous Oxide Emitted from Ruminants Livestock for Sustainable Animal Agriculture

J. Takahashi

Graduate School of Animal Science, Obihiro University of Agriculture and Veterinary Medicine  
Obihiro 080-8555, Japan,  
College of Environmental Science and Engineering, Tongji University  
Shanghai 200092, China

Department of Nutrition and Feed Technology, Faculty of Animal Science, Bogor Agricultural University  
Kampus IPB Darmaga, Bogor 16680, Indonesia

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## ABSTRACT

Major greenhouse gases (GHG) attributed to animal agriculture sector are methane ( $\text{CH}_4$ ) and nitrous oxide ( $\text{N}_2\text{O}$ ), either generated from enteric fermentation or manure. The abatement mechanism of rumen  $\text{CH}_4$  emission may be divided to direct and indirect suppression to methanogens in the rumen. The most significant strategy to mitigate ruminal  $\text{CH}_4$  emission in indirect manner is to promote alternative metabolic pathway to dispose of the reducing power, competing with methanogenesis for  $\text{H}_2$  uptake. This includes prebiotics and probiotics (mostly propionate enhancers) which consume metabolic hydrogen ( $\text{H}_2$ ) compete with methanogens and abate rumen methanogenesis in indirect manner. With regard to mitigate GHG emissions from manure, such waste has been proposed as a renewable energy and nitrogen sources through biogas plant. Furthermore, in advanced new biogas system, the ammonia stripping from digested slurry of livestock manure in biogas plant has been examined to apply to nitrogen recycling-options mitigating  $\text{N}_2\text{O}$  emission. These options are: (1) ammonolysis on fiber-rich feedstuffs, (2) saccharification of the  $\text{NH}_3$  treated cellulose biomass to produce bio-ethanol, and (3) reformed hydrogen into  $\text{NH}_3$  fuel cell to generate electricity with proton exchange membrane fuel cell (PEM).

**Key words:** rumen methane, nitrous oxide, probiotics, prebiotics, ammonia stripping

## ABSTRAK

Gas rumah kaca (GRK) yang terkait dengan sektor peternakan adalah metana ( $\text{CH}_4$ ) dan di-nitrogen oksida ( $\text{N}_2\text{O}$ ), baik yang dihasilkan melalui proses fermentasi enterik maupun manur. Mekanisme mitigasi emisi metana dapat dibagi menjadi penghambatan secara langsung dan tidak langsung terhadap populasi metanogen di rumen. Strategi utama menurunkan emisi metana dari rumen secara tidak langsung adalah dengan cara menyediakan jalur alternatif penggunaan  $\text{H}_2$  selain dari metanogenesis. Strategi ini diantaranya adalah melalui pemanfaatan prebiotik dan probiotik (umumnya peningkat propionat) yang juga mengkonsumsi hidrogen metabolismik sehingga berkompetisi dengan metanogen. Adapun upaya mengurangi GRK dari manur adalah melalui instalasi biogas. Dalam sistem biogas yang canggih, emisi  $\text{N}_2\text{O}$  dari manur didaur ulang menjadi amonia ( $\text{NH}_3$ ). Amonia yang dihasilkan kemudian digunakan untuk: (1) ammoniasi pakan berserat tinggi, (2) sakarifikasi dari biomassa tinggi selulosa yang telah diamoniasi untuk menghasilkan bioetanol, dan (3) reformasi hidrogen menjadi sel bahan bakar berbasis  $\text{NH}_3$  untuk menghasilkan energi listrik.

**Kata kunci:** metana, dinitrogen oksida, probiotik, prebiotik, amonia

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\*Corresponding author:  
E-mail: junichi@obihiro.ac.jp

## INTRODUCTION

The mitigation of anthropogenic four GHG, carbon dioxide ( $\text{CO}_2$ ), methane ( $\text{CH}_4$ ), nitrous oxide ( $\text{N}_2\text{O}$ ) and sulphur hexafluoride ( $\text{SF}_6$ ) and two groups of GHG, hydrofluorocarbons (HFCs) and perfluorocarbons (PFCs) have been established as legally binding commitments in The Kyoto Protocol (IPCC, 1996). Important GHG attributed to animal agriculture are  $\text{CH}_4$  and  $\text{N}_2\text{O}$ . Rumen fermentation of ruminant livestock and anaerobic fermentation of agricultural organic waste including animal manures are major contributors of  $\text{CH}_4$  emission as anthropogenic sources (Moss, 1993).

To abate the GHG, the development of mitigation methods of rumen  $\text{CH}_4$  is the most significant issue in the world ruminant livestock production (Van Nevel & Demeyer, 1996).  $\text{CH}_4$  emitted from ruminants is generated in the rumen by hydrogenotrophic methanogens that utilize hydrogen to reduce  $\text{CO}_2$  and is a significant electron sink in the rumen ecosystem (Klieve & Hegarty, 1999).  $\text{CH}_4$  contains 892.6 kJ combustible energy per molecule at 25 °C and 1013hPa, while not contributing to the total supply of metabolic energy to ruminants (Takahashi *et al.*, 1997). As reported by Leng (1991),  $\text{CH}_4$  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 (Chalupa, 1984; Hopgood & Walker, 1967). However, these ionophores may depress fiber digestion and protozoal growths (Chen & Wolin, 1979). In addition, some resistant bacteria will appear in the rumen from the results of long term use of the ionophores. Thus, 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  $\text{H}_2$ , the major substrates for  $\text{CH}_4$  formation or an increase in the utilization of  $\text{H}_2$  and formate by organisms other than methanogens (Figure 1). However, direct inhibition of  $\text{H}_2$ -forming

reactions may depress fermentation in microorganisms that produce  $\text{H}_2$ , including main cellulolytic bacteria such as *Ruminococcus albus* and *Ruminococcus flavefaciens* (Belaich *et al.*, 1990; Wolin, 1975). Therefore, a reduction in  $\text{H}_2$  production by the enhancement of reactions that accept electrons is desirable (Stewart & Bryant, 1988).

The prompt increase of atmospheric  $\text{N}_2\text{O}$  since last century is closely related to abrupt expansion of human and animal population after an innovation of Haber-Bosch process. Severe environmental pollutions were caused at the same time though the reactive nitrogen withdrawn from atmosphere as stable paired nitrogen brought about prosperous food production. To secure food production preventing environmental catalyses by global warming sustainable development of animal agriculture should be sought in not only developed but also developing countries as an alternative way. Inventories of emitters and their abatements are accurately assessed in both GHG. The key element of these recycling must be low-input for sustainable animal agriculture in developing countries. Carbon and nitrogen recycling in the agricultural biomass as renewable energy and nitrogen resources might contribute mitigation of  $\text{CH}_4$  and  $\text{N}_2\text{O}$  (Takahashi *et al.*, 2003; 2004; Takahashi, 2007; Takahashi & Uemura, 2009).

The present paper deals with perspective on GHG control and possible uses of biomass towards sustainable animal agriculture.

## MITIGATION OF RUMEN METHANE EMISSION WITH PROBIOTICS AND PREBIOTICS

In the rumen, metabolic  $\text{H}_2$  is produced during the anaerobic fermentation of glucose. This  $\text{H}_2$  can be used during the synthesis of volatile fatty acids and microbial organic matter. The excess  $\text{H}_2$  from NADH is eliminated primarily by the formation of  $\text{CH}_4$  by methanogens, which are microorganisms from the *Archea* group that are normally found in the rumen ecosystem (Baker, 1999). The stoichiometric balance of VFA,  $\text{CO}_2$  and  $\text{CH}_4$  indicates that acetate and butyrate promote  $\text{CH}_4$  production whereas propionate formation conserves  $\text{H}_2$ , thereby reducing  $\text{CH}_4$  production (Wolin & Miller, 1988). Therefore, a strategy to mitigate ruminal  $\text{CH}_4$  emission is to promote alternative metabolic pathway to dispose of the reducing power, competing with methanogenesis for  $\text{H}_2$  uptake. As assimilate nitrate reduction in the rumen

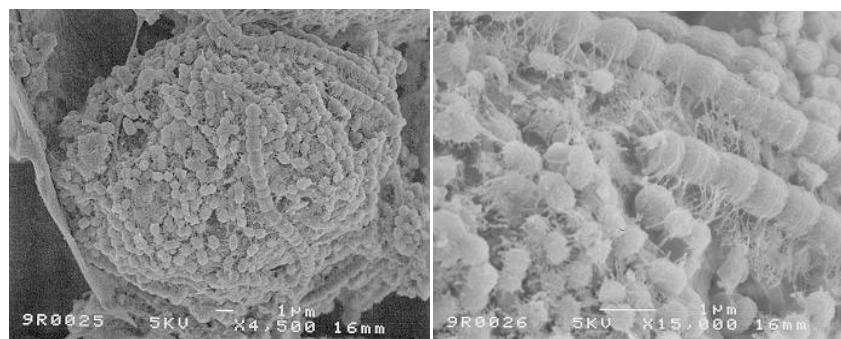


Figure 1. Electric scanning microscopy of symbioses of methanogens on the surface of ciliate protozoa

which shows strong redox potential is relatively higher affinity to H<sub>2</sub> than hydrogenotrophic methanogenesis, the administration of nitrate remarkably suppressed ruminal methanogenesis (Takahashi & Young, 1991; 1992).

Table 1 shows that the formation of toxic nitrite reduced from nitrate is successfully prevented by L-cysteine (Takahashi & Young, 1991; 1992; Takahashi *et al.*, 1989; 1998; 2000; 2002), the effective mitigation of ruminal CH<sub>4</sub> emission is safely achieved by simultaneous administration of nitrate and L-cysteine without nitrate intoxication (Takahashi, 2001). 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 2).

Figure 2 shows mitigating effect of Wild-type *E. coli* W3110 and the construction of *E. coli* nir-Ptac on rumen CH<sub>4</sub> emission in sheep. CH<sub>4</sub> emission was abated by the inoculation of wild-type *E. coli* W3110 or *E. coli* nir-Ptac, although the underlying mechanism has not been elucidated (Figure 3).

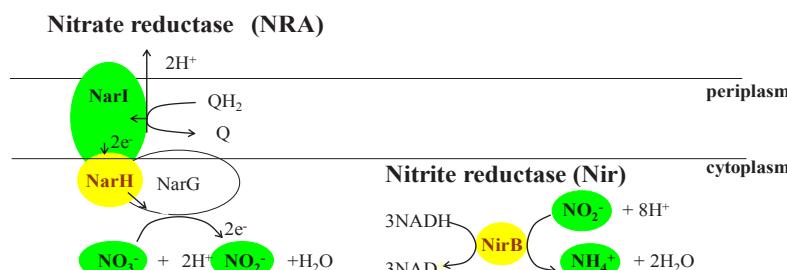
Rumen manipulation with ionophores such as monensin has been reported to abate rumen methanogenesis (Mwenya *et al.*, 2005). However, there is an increasing interest in exploiting prebiotics and probiotics as natural feed additives to solve problems in animal

Table 1. The suppressing effect of L-cysteine (0.21 g S kg<sup>-0.75</sup> body weight) on nitrate-induced production of methaemoglobin (expressed as a percentage of total haemoglobin) and rumen methane production (ml min<sup>-1</sup> kg<sup>-0.75</sup> body weight) in sheep 4h after intra-ruminal administration of nitrate) 1.3 g NaNO<sub>3</sub> kg<sup>-0.75</sup> body weight.

	Treatment			
	Control	NO <sub>3</sub>	NO <sub>3</sub> +CYS	CYS
Methaemoglobin (%)	0.5±0.4 <sup>a</sup>	16.1±3.0 <sup>b</sup>	3.9±2.4 <sup>a</sup>	0.5±0.4 <sup>a</sup>
CH <sub>4</sub> production (mL min <sup>-1</sup> kg <sup>-0.75</sup> BW)	2.1±0.2 <sup>a</sup>	0.3±0.0 <sup>b</sup>	0.7±0.2 <sup>c</sup>	1.2±0.2 <sup>ab</sup>

Note: Means in the same row with different superscripts differ significantly (P<0.05).

### 1. Wild-type *E. coli* W3110



### 2. Construction of *E. coli* nir-Ptac by replacement of self-promoter (*nir*) in *E. coli* W3110 by *tac* promoter (Ptac) (Ajinomoto Co. Inc., Kawasaki, Japan)

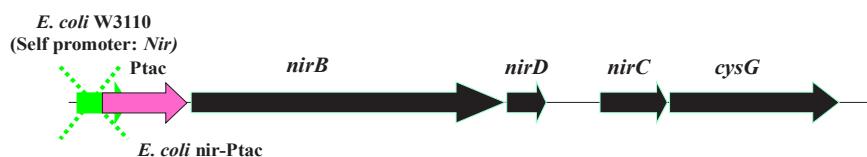


Figure 2. Wild-type *E. coli* W3110 and the construction of *E. coli* nir-Ptac

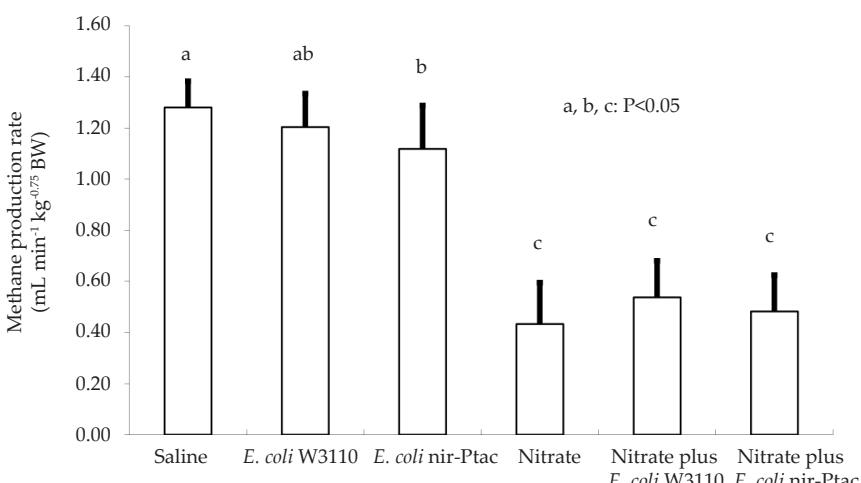


Figure 3. Effect of wild-type *E. coli* W3110 and the construction of *E. coli* nir-Ptac on rumen CH<sub>4</sub> emission in sheep

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* saponins have a steroid nucleus, whereas *Q. saponaria* saponins are triterpenoid in structure. 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; Wang *et al.*, 2000; Takahashi *et al.*, 2000; Santoso *et al.*, 2004a; Mwenya *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.

$\beta$ 1-4 Galacto-oligosaccharides (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 *Bifidobacteria* (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; Mwenya *et al.*, 2004b; 2004c; 2004d: 2005; Santoso, 2004a; Sar *et al.*, 2002; 2004b; 2004c; Takahashi *et al.*, 2002; 2003). However, the efficacy of GOS with the probiotics on different diets and animal species remains to be elucidated.

#### 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. The saponins contained in *Y. schidigera* have a steroid nucleus, whereas *Q. saponaria* saponins are triterpenoid in structure.

There has been a growing interest in utilizing antimicrobial substances to selectively target rumen methanogens to reduce CH<sub>4</sub> emissions (Nollet *et al.*, 1998; Less *et al.*, 2002; Asa *et al.*, 2010). Figure 5 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<sub>4</sub> and CO<sub>2</sub> 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 6 shows DGGE band patterns of archaea and eubacteria. All fluorescence brightness of methanogens

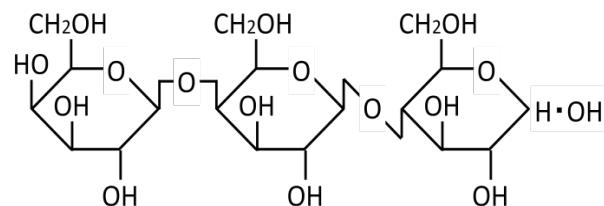


Figure 4. Chemical structure of  $\beta$ 1-4 galacto-oligosaccharides

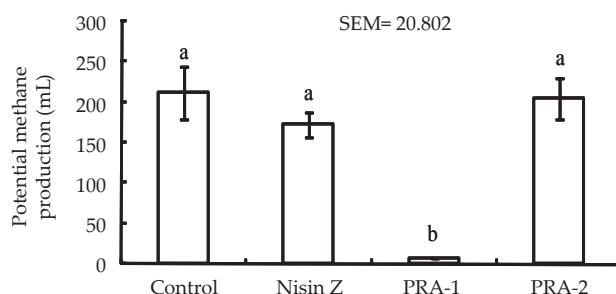


Figure 5. Effect of PRA on potential methane production. Control: *Lactococcus lactis* ATCC19435 (non-antibacterial substances), Nisin-A: *Lactococcus lactis* NCIMB702054, PRA-1: *Lactobacillus plantarum* TUA1490L, and PRA-2: *Leuconostoc citreum* JCM9698. Vertical bars represent standard deviation (n=4). 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 archaea 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*, *Pseudobutyryvibrio* sp., *Prevotella* sp., *Succinivibrio dextrinosolvens* and *Schwarzia succinivorans* in Gram negative bacteria were decreased by adding PRA-1. The supernatant of *Lactobacillus plantarum* TUA1490L (LP) reduced in vitro CH<sub>4</sub> production, but the non-proteinaceous anti-microbial substance (PRA-1) was not identified, but has subsequently been shown to be hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (J. Takahashi, pers. Comm.).

The anti-microbial effect of H<sub>2</sub>O<sub>2</sub> has been attributed to its strong oxidizing effect on bacterial cells and to the destruction of the molecular structure of cell proteins (Ito *et al.*, 2003; Zalán *et al.*, 2005). However, there is no information available on the effect of H<sub>2</sub>O<sub>2</sub> on rumen fermentation. Thus, it was necessary to assess if methanogenesis was more sensitive to H<sub>2</sub>O<sub>2</sub> in the LP supernatant than primary and secondary rumen fermentation processes that produce volatile fatty acids (VFA). The effect of supernatant of *Lactobacillus plantarum* TUA1490L on in vitro rumen CH<sub>4</sub> output was investigated minutely with

in vitro gas quantification system (Takasugi Seisakusho, Tokyo, Japan, Takahashi & Kawabe, 2011) installed auto infrared CH<sub>4</sub> (EXA IR, Yokogawa, Tokyo, Japan) and CO<sub>2</sub> (Model RI-555, Riken Keiki, Tokyo, Japan) analyzers (O'Brien, 2013). In consequence, H<sub>2</sub>O<sub>2</sub> was detected in the supernatant and CH<sub>4</sub> output was reduced by 72%. However, the supernatant has an adverse effect on total VFA concentration. Follow-on studies will examine if lower additions of chemically-synthesized H<sub>2</sub>O<sub>2</sub> can reduce CH<sub>4</sub> output without significantly reducing total VFA production.

#### CREATION OF RENEWABLE ENERGY (BIOGAS) FROM ANAEROBIC FERMENTATION (BIOGAS PLANT) OF ANIMAL MANURES AND THE INNOVATIVE REUSE OF THE DIGESTED SLURRY TO MITIGATE N<sub>2</sub>O

The increased emissions of CH<sub>4</sub> and N<sub>2</sub>O from decomposing unmanaged and bio-based industrial wastes along with the expansion of human activities contribute climate change as GHG. The conventional biogas system based on anaerobic fermentation of the organic wastes, however, is not a nitrogen recycling but carbon recycling one. Therefore, isometric fertilization of the digested slurry after anaerobic fermentation may not be a solution of current issue on excess nitrogen abatement, although nitrous oxide emission is almost completely suppressed during anaerobic fermentation (Figure 7).

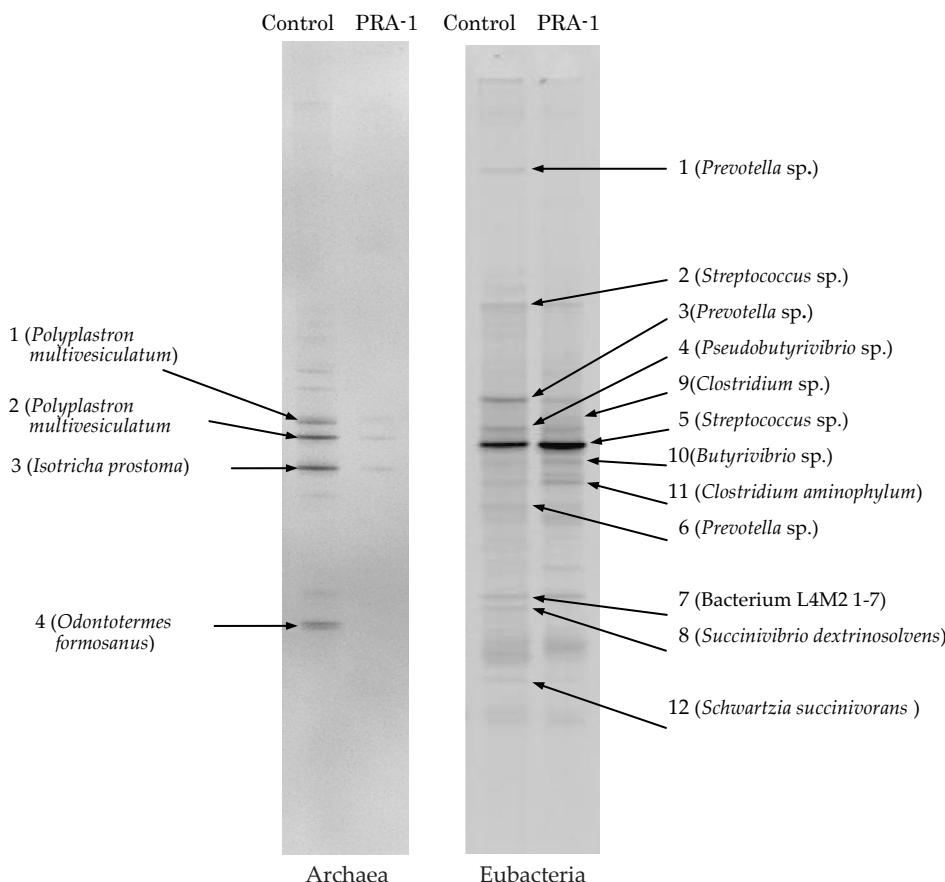


Figure 6. DGGE band patterns of rumen archaea and eubacteria

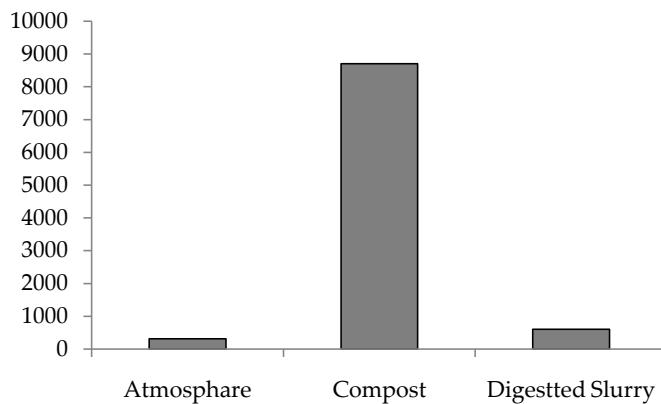
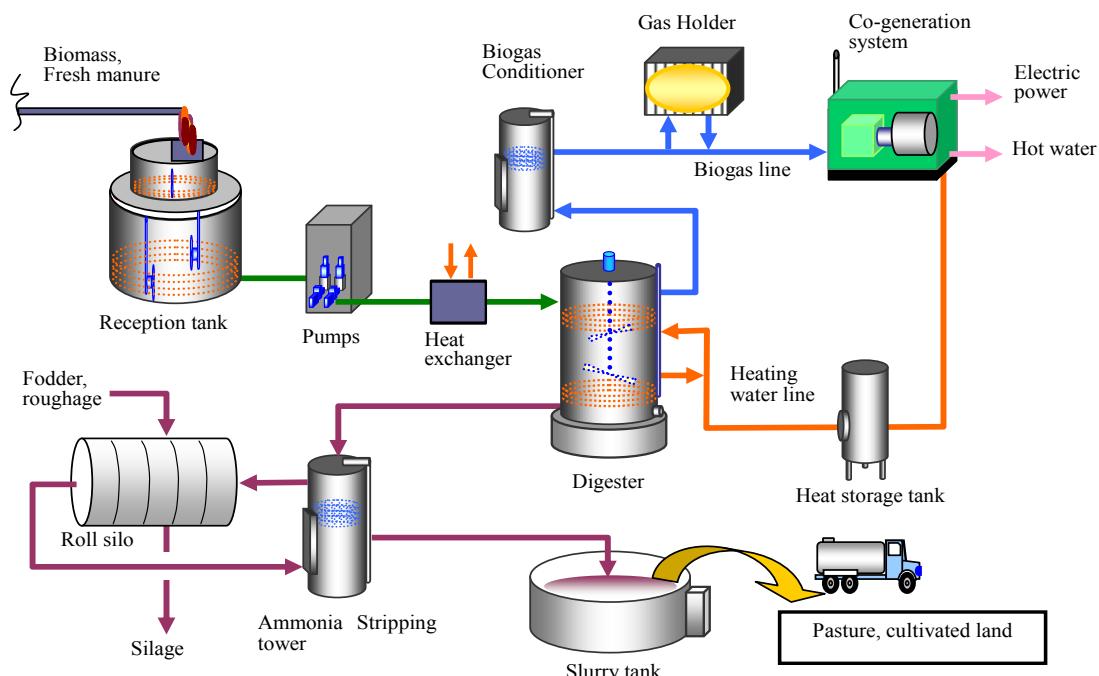
Figure 7. N<sub>2</sub>O emission from different manure treatments

Figure 8. Advanced biogas system-high efficient wastesrecyclingandco-generation

It causes not only methane emission, but also nitrate leaching and N<sub>2</sub>O emission from soil (Takahashi, 2006). The introduction of ammonia stripping from digested slurry of thermophilic biogas plant might be a solution to reduce total nitrogen content of the slurry as a liquid fertilizer containing suitable nitrogen and eventually can contribute the mitigation of N<sub>2</sub>O emission as a new concept of biogas system (Figure 8). Furthermore, the stripped ammonia can be put to practical use as a low-input and renewable nitrogen resource without energy supply from outside, because abundant amount of organic wastes exist in developing countries and the energy required for ammonia stripping can be supplied from biogas plant attached to the ammonia stripping apparatus. The following three options have been examined for future nitrogen recycling. Production of high quality feed from cellulose biomass in agricultural waste with ammonia stripping process from digested slurry of biogas plant (Takahashi, 2006; 2007). Saccharification of

soft cellulose biomass to create bio-ethanol and hydrogen using ammonolysis by stripped ammonia from effluent and hydrolysis of rumen bacteria (unpublished). Ammonia fuel cell with ammonia stripping from digested slurry (Takahashi & Uemura, 2009; FOCUS, April 14, 2009).

## CONCLUSION

The amplifying competition of metabolic H<sub>2</sub> with probiotics may be a key factor in the regulation of rumen methanogenesis. However, direct mitigating 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. Thus, the mechanism for international accreditation of manipulators must be established to mitigate global CH<sub>4</sub> emission.

By contrast, the biogas plant produce biogas including combustible CH<sub>4</sub> as renewable energy using unused resources like animal manures, can provide fuel, heat and electricity, and minimize the impact on the environment thus reducing the amount of pollutants discharged. Furthermore, the new advanced biogas system equipped with ammonia stripping apparatus will contribute to mitigate N<sub>2</sub>O recycling excess amount of ammonia from the digested slurry.

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