Asian-Aust. J. Anim. Sci.
Vol. 23, No. 6 : 700 - 707
June 2010

www.ajas.info

Effects of Protease-resistant Antimicrobial Substances Produced by Lactic Acid Bacteria on Rumen Methanogenesis

Reina Asa, Tanaka A., Uehara A.[1], Shinzato I.[1], Toride Y.[1], Usui N.[1], Hirakawa K.[1] and Takahashi J.*

Graduate School of Animal Science, Obihiro University of Agriculture and Veterinary Medicine
Obihiro, Japan 080-8555

ABSTRACT : 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. Four different strains of lactic acid bacteria, i) *Lactococcus lactis* ATCC19435 (Control, non-antibacterial substances), ii) *Lactococcus lactis* NCIMB702054 (Nisin-Z), iii) *Lactobacillus plantarum* TUA1490L (PRA-1), and iv) *Leuconostoc citreum* JCM9698 (PRA-2) 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, though propionate, butyrate and ammonia N decreased. 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. (**Key Words :** Methane Production, Lactic Acid Bacteria, *In vitro* Fermentation)

INTRODUCTION

Methane emitted from ruminants is one of the major greenhouse gases attributed to animal agriculture and represents an energy loss for the host animal of 2 to 12% of dietary energy (Johnson and Johnson, 1995). Therefore, abatement of enteric methane production is necessary not only from an economical viewpoint of livestock production but also from an environmental perspective. Although monensin is widely used as a feed additive to improve the feed efficiency of cattle, it has been reported to mitigate rumen methanogenesis (Sauer et al., 1998; Guan et al., 2006; Mwenya et al., 2006). There still remain public concerns on its residue in the environment, in livestock products or incidence of resistant organisms. Bacteriocins, bacterial proteinaceous products, are ubiquitous in nature being produced by a variety of Gram-negative and Gram-positive bacteria (Farkas-Himsley, 1980). Nisin is a polycyclic peptide antimicrobial bacteriocin produced by *Lactococcus lactis* which is generally recognized as safe (GRAS) and given international acceptance in 1969 by the joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (WHO, 1969). Its antimicrobial activity is effective against numerous Gram-positive bacteria (Delves-Broughton et al., 1996). It has been reported that nisin suppress rumen methanogenesis (Callaway et al., 1997; Santoso et al., 2004). However, the suppressing efficacy of nisin on rumen methanogenesis may not be sustained, because proteinacious nisin is degradable in the rumen due to bacterial protease (Sang et al., 2002). Several strains of lactic acid bacteria were isolated from fermented foods, which produce different types of protease resistant antimicrobial substance (PRA). Preliminary studies suggested that the PRA maintained their antimicrobial effects after incubation with proteases, while nisin lost its activity. Therefore, the PRA was hypothesized to be a more sustained agent than nisin for the mitigation of rumen methane emission.

The present study deals with mitigating effects of PRA produced by *Lactobacillus plantarum* and *Leuconostoc citreum* on rumen methanogenesis in comparison with nisin.

* Corresponding Author: J. Takahashi. Tel: +81-155-49-5421, Fax: +81-155-49-5421, E-mail: junichi@obihiro.ac.jp
[1] Fermentation & Biotechnology Laboratory, Amino Acids Company, Ajinomoto Co., Inc., Kawasaki, Japan 210-8681.
Received August 24, 2009; Accepted November 6, 2009

MATERIALS AND METHODS

Ruminal inocula

Two rumen cannulated non-lactating Holstein cows

were used as donors of rumen fluid. Cows were fed kleingrass (*Panicum coloratum* L.) (Table 1) at a maintenance level (55 g DM $kg^{-0.75}$) at 9:00 and 16:00, had free access to clean drinking water and a mineral block (1,232 mg Fe, 150 mg Cu, 25 mg Co, 500 mg Zn, 50 mg I, 15 mg Se and 382 mg Na in kg DM) and were cared for according to guidelines of the Obihiro University of Agriculture and Veterinary Medicine Committee for Animal Use and Care. The rumen fluid was collected through the rumen cannulae prior to the morning feeding and strained immediately through woven nylon cloth into a thermos flask with an O_2-free headspace. Two filtrates were combined on an equal volume basis and used to inoculate into the culture media.

Table 1. Chemical composition of concentrate mixture and kleingrass

	Concentrate[1]	Kleingrass
Dry matter (DM)	87.4	89.3
Organic matter (% DM)	93.2	90.5
Crude protein (% DM)	18.8	11.0
NDF (% DM)	16.5	68.6
ADF (% DM)	9.0	40.0
GE(Mcal/kg DM)	18.7	17.6

[1] Concentrate contained 540 g kg^{-1} of grains (heat treated corn, maize, heat treated oat and rye), 250 g kg^{-1} of oil seed meals (soybean meal and rapeseed meal), 130 g kg^{-1} of corn gluten feed, wheat bran and starch pulp, 80 g kg^{-1} others (alfalfa meal, molasses, calcium carbonate powder, salt, corn syrup, malt, yeast, lactic bacteria).

Preparation of PRA producing strains of lactic acid bacteria

The strains of lactic acid bacteria that produce PRA were screened on MRS agar plates (Becton, Dickinson and Company, NJ, USA) containing Umamizyme G (protease mixture from *Aspergillus oryaze*, Amano Enzyme Inc, Nagoya, Japan). Candidates were inoculated onto MRS agar with or without 1,000 IU ml^{-1} of Umamizyme G and incubated for 24 h at 30°C. The plate were then overlaid with Bacto Lactobacilli agar AOAC（Becton, Dickinson and Company, NJ, USA）containing an indicator strain, *Lactobacillus sakei* JCM1157T. The agar overlays were incubated for 24 h at 30°C and examined for zones of clearing. Protease degradable anti-microbial substances were decomposed by Umamizyme G, thus a clear zone did not form on the plate with Umamizyme G. Two strains of lactic acid bacteria, *Lactobacillus plantarum* TUA1490L (PRA-1) and *Leuconostoc citreum* JCM9698 (PRA-2) that produced almost the same size of clear zone on a Umamizyme G containing plate as that on a plate without Umamizyme G, were selected as PRA producers. *Lactobacillus plantarum* TUA1490L and *Leuconostoc citreum* JCM9698 were selected as PRA-1 and PRA-2 producers by courtesy of the culture collection of NODAI Culture Collection Centre, NRIC, Faculty of Applied Bioscience, Tokyo University of Agriculture.

***In vitro* gaseous quantification**

The *in vitro* continuous methane quantification system was adopted according to Sar et al. (2005). The system consisted of four 1,000-ml fermentation jars equipped with a stirring device, a thermister probe, a buffer input, a solid feed input, an inlet to draw sample, an input for nitrogen gas (N_2) infusion and an inlet for gas emission. Nitrogen gas was continuously infused into each jar at the rate of 20 ml min^{-1} to maintain anaerobic conditions in the jar and to transport gas generated from the jar to gas analysers.

Substrates for fermentation were added into each jar as follows: 640 ml of McDougall's artificial saliva (MacDougall, 1948), 160 ml of strained rumen fluid and 80 ml of each inoculum with 5 g of air-dried kleingrass (*Panicum coloratum* L.) and 5 g of concentrate (see Table 1). All incubations were conducted anaerobically for 24 hours at 39°C and at 38 rpm stirring speed. Four strains of lactic acid bacteria inocula were used according to a 4×4 Latin square arrangement as follows, i) *Lactococcus lactis* ATCC19435 which did not produce any anti-bacterial substances (control), ii) *Lactococcus lactis* NCIMB702054 (nisin-Z), iii) *Lactobacillus plantarum* TUA1490L (PRA-1), iv) *Leuconostoc citreum* JCM9698 (PRA-2). The CH_4 and CO_2 in the fermentation gas was continuously determined by automated infrared analyser (CH_4: EXA IR, YOKOGAWA, Tokyo; CO_2: MODEL RI-555, RIKRN KEIKI, Tokyo) installed in an *in vitro* continuous methane quantification system (Takasugi Seisakusho, Tokyo, Japan). Data were then pooled into the computer (Windows XP Professional 1-2 CPU, IBM Corporation, Tokyo, Japan) from the analyser through an interface at 1 min intervals.

The cumulative CH_4 and CO_2 production were extrapolated by nonlinear regression analysis as: $y = a+b\ (1-e^{-ct})$ [3], where y (ml) = gas produced at time *t* (min), a = first gas production, b = second gas production and c = fractional rate of gas production, using Kaleida Graph (Version 3.6, Synergy Software, Reading, PA, USA).

Analyses of fermentation characteristics

A 10 ml aliquot of culture fluid was anaerobically withdrawn from each fermentation jar (1 L vol.) and replaced with the same volume of new culture medium at 0, 4, 8, 12 and 24 h after incubation, and then stored at -30°C for analyses of volatile fatty acid (VFA) and ammonia N (NH_3-N) concentrations and protozoa number. The pH and oxidation-reduction potential (ORP) of the withdrawn fluid were determined immediately using a pH meter (HM-21P DKK, TOA Electronics, Tokyo, Japan) equipped with appropriate electrodes. The VFA concentrations were

analysed by gas-liquid chromatography (GC-14A, Shimadzu, Kyoto, Japan) using 2-ethyl n-butyric acid as an internal standard. The NH_3-N concentrations of incubation media were analysed according to a modified micro-diffusion method (Conway and O'Malley, 1942).

Preparation of nisin and PRA inoculants

GYEKP medium (glucose 10 g L^{-1}, yeast extract 20 g^{-1}, sodium citrate.2aq 2.0 g^{-1}, sodium acetate 5.0 g^{-1}, $MnSO_4$.5aq 0.05 g^{-1}, KH_2PO_4 12 g^{-1}, K_2HPO_4 11 g^{-1}) was used for the culture of lactic acid bacteria. Each strain of lactic acid bacteria was inoculated into a 500 ml shaking flask containing 100 ml GYEKP, and was cultivated for 20 hours at 30°C after confirmation of the stationary phase. The cells were removed by centrifugation at 8,000×g at 4°C and filtration with 0.45 μm membrane filter (DISMIC-25CS, ADVANTEC, Tokyo, Japan). The supernatants were used as PRA inoculants in the *in vitro* gaseous quantification trials.

Identification of eubacteria and archaebacteria in the culture fluids

Eubacteria and archaebacteria in the culture fluids were identified by DNA extraction, polymerase chain reaction (PCR) amplification, denaturing gradient gel electrophoresis (DGGE) analysis and sequencing. The samples for the microorganism analyses were obtained from culture fluid after 24 h of incubation of PRA-1 and the control. Total genomic DNA was extracted according to the method described by Yoshida et al. (2005). The primers for ruminal eubacteria and archaea in the rumen are listed in Table 2 (Muyzer, et al., 1993; Nakatsu et al., 2000; Green and Minz, 2005). Nested-PCR was carried out for analysis of the samples. 341F and 907R primers were used for the first-round PCR, and 341F-GC and 534R primers were used for the second-round of PCR in identification of eubacteria. 46F and 1100R primers were used for the first-round PCR, and 340FGC and 519R primers were used for the second-round of PCR in the identification of archaebacteria. First-round PCR conditions were as follows: 96°C for 5 min, 25 cycles at 96°C for 1 min, 55°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 5 min. Second-round PCR conditions were as follows: 96°C for 5 min, 25 cycles at 96°C for 1 min, 55°C for 30 s, and 72°C for 30s, and a final extension step at 72°C for 5 min. PCR amplification products were visualized on 1.5% (w/v) agarose gels prior to denaturing gradient gel electrophoresis (DGGE) analysis**.** DGGE was performed using the Bio-Rad DCode universal mutation-detection system, following the manufacturer's guidelines. PCR products (20 μl) were loaded onto 10% (w/v) polyacrylamide gels in 1× TAE (40 mM Tris base, 20 mM acetic acid and 1 mM EDTA) that contained a 30-70% denaturant gradient (100% denaturant, 7 M urea and 40% (v/v) deionised formamide). Electrophoresis was performed at a constant voltage (70 V) and temperature (60°C) for 16 h. Gels were then stained for 30 minutes with ethidium bromide.

After DGGE, the DNA of each band was extracted by TE buffer (pH8.0) from the gels.

Sequencing was performed with an automatic sequencer ABI Prism310 Genetic Analyser (Applied Biosystems, Foster City, USA) after sequencing reaction with a BigDye terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, USA). FASTA searches on each sequence were done against those in the DNA Data Bank of Japan (DDBJ) nucleotide sequence database.

Statistical analyses

In vitro continuous incubation of each treatment was carried out four replicates (n = 4). Means of results from treatments were analysed by one-way analysis of variance (ANOVA) using the General Linear Models Procedures of the Statistical Analysis Systems Institute (SAS, 1996). Means among treatments were compared with Tukey's Test.

RESULTS

Figure 1 to 4 show the effects of PRA on the cumulative and potential extent of production of CH_4 and CO_2 in the

Table 2. PCR primers

		Configuration
Archaea	46F	5'- YTAAGCCATGCRAGT -3'
	1100R	5'- YGGGTCTCGCTCGTTRCC -3'
	340FGC	5'- CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGG CACGGGGGGCCCTACGGGG(C/T)GCA(G/C)CAG -3'
	340F	5'- CCCTACGGGYGCASCAG -3'
Eubacteria	519R	5'- TTACCGCGGCKGCTG -3'
	341F	5'- CCTACGGGAGGCAGCAG -3'
	907R	5'- CCGTCAATTCCTTTRAGTTT -3'
	341FGC	5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC GGGGGGCCTACGGGAGGCAGCAG -3'
	534R	5'- ATTACCGCGGCTGCTGG -3'

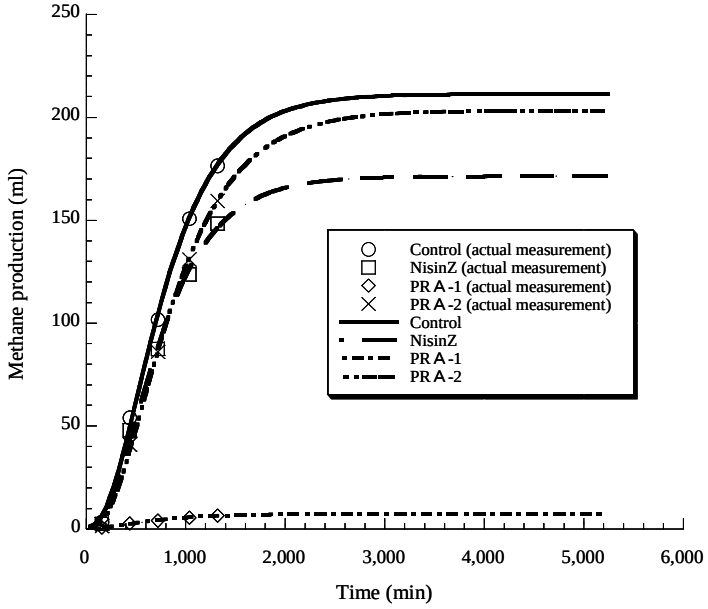

Figure 1. Effect of PRA on the cumulative methane production.

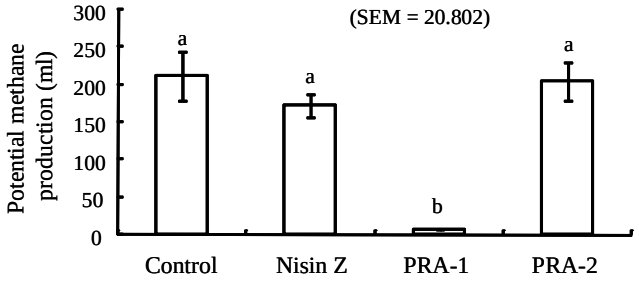

Figure 2. Effect of PRA on potential methane production. Vertical bars represent standard deviation (n = 4). Means with different letters differ significantly ($p<0.01$).

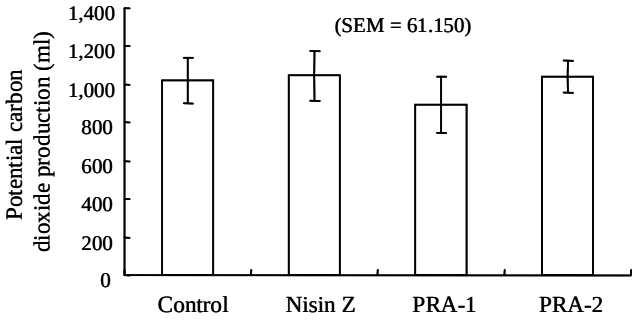

Figure 4. Effect of PRA on the potential extent of CO_2 production in cultures. Vertical bars represent standard deviation (n = 4).

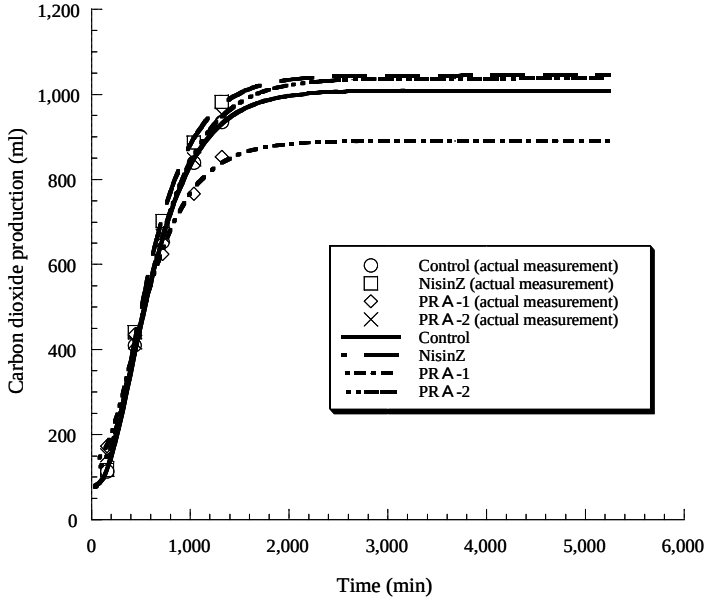

Figure 3. Effect of PRA on cumulative carbon dioxide production.

Table 3. Fermentation characteristics in continuous cultures of mixed rumen microbes

	Treatments				SEM	Significance level (P)		
	Control	Nisin Z	PRA-1	PRA-2		ctl×Nis	ctl×PRA1	ctl×PRA2
pH	6.9	6.9	7.1	6.9	0.03	0.9736	0.3189	0.9989
ORP[1] (mv)	-189.7	-222.3	-99.2	-211.6	16.07	0.7560	0.1054	0.9077
Total VFA[2] (mM)	37.6 [ab]	42.5 [a]	24.2 [b]	39.1[a]	2.28	0.7138	0.0819	0.9861
VFA proportion (mM)								
Acetate (A)	26.7	29.1	20.5	28.0	1.40	0.9016	0.4027	0.9808
Propionate (P)	8.5 [a]	11.6 [a]	2.7 [b]	9.8 [a]	0.97	0.2444	0.0200	0.8318
Butyrate	2.0 [a]	1.7 [b]	0.9 [c]	1.9 [ab]	0.11	0.0313	<0.0001	0.6321
A:P ratio	4.6 [ab]	3.8 [a]	6.4 [b]	5.0 [ab]	0.30	0.5359	0.071	0.9101
Ammonia N(μg/L)	50.8 [a]	40.6 [b]	21.4 [c]	45.1 [ab]	3.25	0.0118	<0.0001	0.1458

Values represent means (n = 4). Each value indicates a representative (mean) of 5 observations (0, 4, 8, 12, 24 h).
[1] ORP = Oxidation-reduction potential. [2] VFA = Volatile fatty acids.
[a, b, c] Mean values within a row with different superscript letters differ significantly with p-value in the same row.

culture fluids. PRA-1 significantly decreased cumulative methane production ($p<0.001$). However, PRA-2 and Nisin Z did not affect cumulative CH_4 and CO_2 production. Table 3 shows fermentation characteristics in the culture fluids. Propionate, butyrate and ammonia N in PRA-1 were significantly decreased ($p<0.05$, $p<0.0001$, $p<0.0001$). PRA-1 indicated a tendency to decrease total VFA concentration, and increase A/P ratio ($p<0.1$), though PRA-2 had no effect on the fermentation patterns. Nisin Z significantly decreased butyrate and ammonia N concentrations ($p<0.05$). Figure 5 shows DGGE band patterns of archaea and eubacteria. Identification of each band is shown in Table 4. Fluorescence brightness of all methanogen bands of PRA-1 was remarkably light in colour compared with the control. Band No. 1 to No.3 in archaebacteria might be *Methanobrevibacter sp.* which is a Gram positive bacterium or parasitic methanogen sticking on the surface of protozoa. PRA-1 increased the fluorescence brightness of the band of the Gram positive bacteria and decreased the fluorescence brightness of the

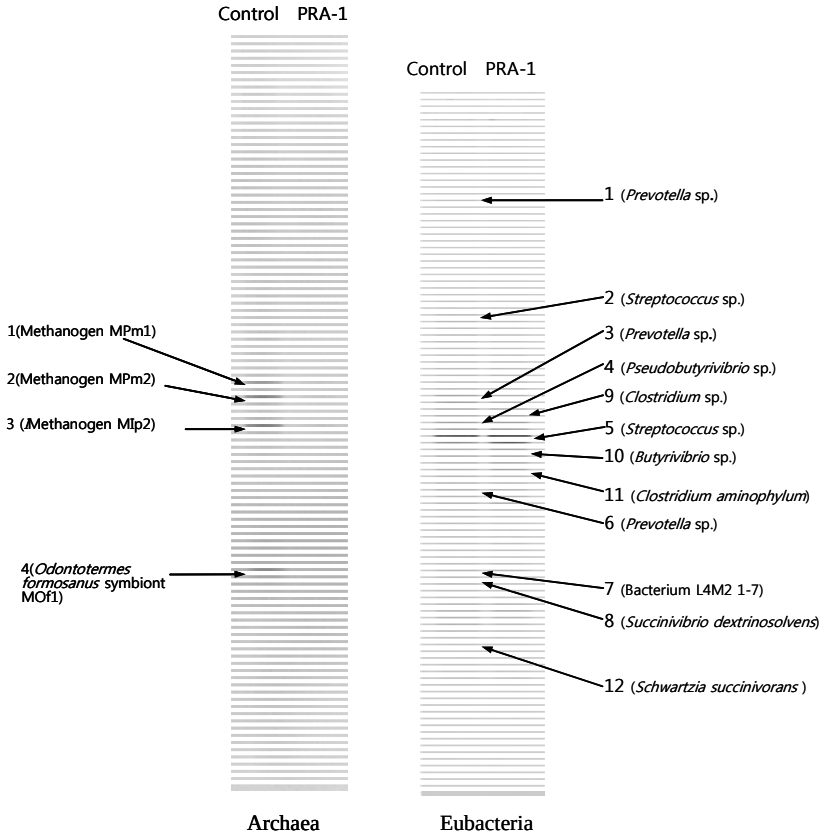

Figure 5. DGGE band patterns.

Table 4. Identification of archaea and eubacteria in Figure 5

	Band No.	Access No.	Strain	Identity (%)	Overlap (nt)
Archaea	1	AB026173	Methanogen MPm1 associated with *Polyplastron multivesiculatum* gene for 16S rRNA	100.0	93
	2	AB026174	Methanogen MPm2 associated with *Polyplastron multivesiculatum* 16S rRNA	100.0	93
	3	AB026169	Methanogen MIp2 associated with *Isotricha prostoma* gene for 16S rRNA	100.0	93
	4	AB009822	*Odontotermes formosanus* symbiont MOf1 gene for 16S rRNA	89.9	98
Eubacteria	1	AB003384	*Prevotella* sp. DNA for 16S rRNA	99.1	107
	2	M58835	*Streptococcus bovis* 16S ribosomal RNA	100.0	120
	3	AY699286	*Prevotella ruminicola* isolate L16 16S ribosomal RNA	100.0	109
	4	AF202262	*Pseudobutyrivibrio ruminis* strain pC-XS7 16S ribosomal RNA	100.0	102
	5	M58835	*Streptococcus bovis* 16S ribosomal RNA	100.0	127
	6	AF487886	*Prevotella* sp. Smarlab 121567 16S rRNA	96.2	130
	7	AY862595	Bacterium L4M2 1-7 16S ribosomal RNA	99.2	131
	8	Y17600	*Succinivibrio dextrinosolvens* 16S rRNA	100.0	107
	9	EF589958	*Clostridium perfringens* strain LNT6 16S ribosomal RNA gene	83.8	111
	10	AY699274	*Butyrivibrio fibrisolvens* isolate L8 16S ribosomal RNA	100.0	106
	11	DQ278862	*Clostridium aminophilum* isolate 152R-1b 16S ribosomal RNA	100.0	110
	12	Y09434	*Schwartzia succinivorans* 16S rRNA	99.2	125

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.

DISCUSSION

To mitigate rumen methanogenesis in safety, this study was conducted to determine the effects of protease-resistant antimicrobial substances produced by lactic acid bacteria on rumen methanogenesis *in vitro*. Some anti-microbial substances involved in rumen methane manipulation might exist in the inoculants of *Lactococcus lactis* used in the present study. The greenhouse effect attributed to ruminant eructation was suggested to be mitigated by 96% by PRA-1, whereas nisin-Z and PRA-2 from different sources of *Lactococcus lactis* had no significant effect on methane production. Ruminal fermentation of PRA-1 was clearly inhibited by antimicrobial substances produced by *Lactobacillus plantarum* TUA1490L. However, the effect of PRA-1 on methanogens and eubacteria of rumen microflora was analysed by PCR–DGGE method. The methanogens-specific PCR-DGGE bands were reduced by the addition of PRA-1 (Figure 5). This result suggests that PRA-1 has highly-specific antibacterial activity against methanogens, and suppressing effects against a few eubacteria such as *Prevotella sp.* and *Succinivibrio sp.* which are known as propionate producers. Thus, it is possible that the depression of propionate with PRA-1 was due to the disappearance of *Prevotella sp.* The present results suggested that PRA-1 reduced the number of methanogens or inhibited utilization of hydrogen in rumen fermentation.

Some bacteriocins produced by lactic acid bacteria have been identified as an alternative group of antimicrobes for manipulation of the rumen microbial ecosystem and characterized biochemically and genetically (Kalmokoff et al., 1996; Ennahar et al., 1999; Chen and Hoocver, 2003). Sang et al. (2002 b) reported that bovicin HC5, produced from *Streptococcus bovis* HC5, decreased the methanogenic population *in vitro*. Mantovani and Russell (2002) suggested that this bacteriocin inhibited a variety of Gram-positive bacteria and the spectrum of activity was similar to monensin. Nisin is widely utilized in the food industry for its highly effective and very strong antibacterial activity against many gram-positive bacteria (Yuan et al., 2004); it is nearly as potent a methane inhibitor as monensin and it was just as effective in decreasing the acetate to propionate ratio (Callaway, 1997).

Sar et al. (2006) reported that as the *in vitro* administration of nisin increased from 5 to 30 μmol L^{-1} methanogenesis was suppressed by 14-40%. Those nisin showed circulating nisin A. In this study, Nisin Z decreased methane production by 16%, and decreased the acetate to propionate ratio. Nisin is gene-encoded and ribosomally

synthesized and structural variants of nisin can be made by mutagenesis (McAuliffe et al., 2001), which could be used to provide information on its structure, property and function. Therefore, nisin Z and nisin A differed only in a single residue at position 27 without any changes in antimicrobial activity (Yuan et al., 2004). Thus, effects of nisin A on rumen fermentation may be similar to those of nisin Z.

Natural antimicrobial substances can be used alone or in combination with other novel preservation technologies to facilitate the replacement of traditional approaches (Brijesh, 2009). *Lactobacillus plantarum* produces bacteriocin from many foods including meat and meat products (Garriga et al., 1993; Enan et al., 1996; Aymerich et al., 2000), milk (Rekhif et al., 1995), Cheese (Gonzalez et al., 1994), fermented cucumber (Daeschel et al., 1990), olives (Jimenez-Diaz R et al., 1993; Leal et al., 1998), grapefruit juice (Kelly et al., 1996), Turkish fermented dairy products (Aslim et al., 2005), and sourdough (Todorov et al., 1999). PRA-1 was the antibacterial substance produced from a strain of *Lactobacillus plantarum* TUA1490L that was isolated from tomato in Japan. However, as the methane suppressing activity was not inactivated by treatments of Umamizyme G and proteinase K, identification of the substance and its protease resistant characteristics remain to be elucidated.

In conclusion, PRA-1 is able to effectively inhibit rumen methane production *in vitro*, but *in vivo* feeding trials remain to be elucidated to reconfirm safety in practical use of the antimicrobial substance as a ruminant feed additive, and specific RT-PCR should be adopted for more detailed microbial assessment on more sensitive target species.

ACKNOWLEDGMENT

We are deeply grateful to Dr. Sanae Okada, Tokyo University of Agriculture, for providing the TUA1490L strain.

REFERENCES

Aslim, B., Z. N. Yuksekdag, E. Sarikaya and Y. Beyatli. 2005. Determination of the bacteriocin-like substances produced by some lactic acid bacteria isolated from Turkish dairy products. LWT 38:691-694.

Aymerich, M. T., M. Garriga, J. M. Monfort, I. Nes and M. Hugas. 2000. Bacteriocin-producing lactobacilli in Spanish-style fermented sausages: characterization of bacteriocins. Food Microbiol. 17:33-45.

Brijesh, K. T., P. V. Vasilis, P. O. D. Colm, M. Kasiviswanathan, B. Paula and P. J. Cullen. 2009. Applivation of natural antimicrobials for food preservation. J. Agric. Food Chem. 57: 5987-6000.

Callaway, T. R., M. S. Alexandra, Carneiro De Melo and J. B. Russell. 1997. The effect of nisin and monensin on ruminal fermentations *in vitro*. Curr Microbiol. 35:90-96.

Chen, H. and D. G. Hoocver. 2003. Bacteriocins and their food applications. CRFSFS 12:82-99.

Conway, E. J. and E. O'Malley. 1942. Microdiffusion methods: ammonia and urea using buffered absorbents (revised methods for ranges greater than 10 μg N). Biochem. J. 36:655-661.

Daeschel, M. A., M. C. Mckenny and L. C. McDonald. 1990. Bacteriocidal activity of Lactobacillus plantarum C11. Food Microbiol. 7:91-99.

Delves-Broughton, J., P. Blackburn, R. Evans and J. hugenholtz. 1996. Applications of the bacteriocin, nisin. Antonie Van Leeuwenhoek 69:193-202.

Enan, G., A. A. El-Essawy, M. Uyttendaele and J. Debevere. 1996. Antibacterial activity of Lactobacillus plantarum UG1 isolated from dry sausage: characterization, production and bactericidal action of plantarcin UG1. Int. J. Food. Microbiol. 30:189-215.

Ennahar, S., K. Sonomoto and A. Ishizaki. 1999. Class IIa bacteriocins from lactic acid bacteria: Antibacterial activity and food preservation. J. Biosci. Bioeng. 87:705-716.

Farkas-Himsley, H. 1980. Bacteriocins-are they broad-spectrum antibiotics? J. Antimicrob. Chemother. 6:424-426.

Garriga, M., M. Hugas, T. Aymerich and J. M. monfort. 1993. Bacteriocinocinogenic activity of lactobacilli from fermented sausages. J. Appl. Bacteriol. 75:142-148.

González, B., P. Arca, B. Mayo and J. E. Suárez. 1994. Detection, purification and partial characterization of plantaricin C, a bacteriocin produced by a Lactobacillus plantarum strain of dairy origin. Appl. Environ. Microbiol. 6:2158-2163.

Green, S. J. and D. Minz. 2005. Suicide Polymerase Endonuclease Restriction, a Novel Technique for Enhancing PCR Amplification of Minor DNA Templates. Appl. Environ. Microbiol. 71:4721-4727.

Guan, H., K. M. Wittenberg, K. H. Ominski and D. O. Krause. 2006. Efficacy of ionophores in cattle diets for mitigation of enteric methane. J. Anim. Sci. 84:1896-1906.

Jiménez-Díaz, R., R. M. Rios-Sánchez, M. Desmazeaud, J. L. Ruiz-Barba and J. C. Piard. 1993. Plantaricin S and T, two new bacteriocins produced by Lactobacillus plantarum LPCO10 isolated from a green olive fermentation. Appl. Environ. Microbiol. 59:1416-1424.

Johnson, K. A. and D. E. Johnson. 1995. Methane emission from cattle. J. Anim. Sci. 73:2483-2492.

Kalmokoff, M. L., F. Bartlett and R. M. Teather. 1996. Are ruminal bacteria armed with bacteriocins? J. Dairy Sci. 79:2297-2306.

Kelly, W. J., R. V. Asmundson and C. M. Huang. 1996. Characterization of plantaricin KW30, a bacteriocin produced by Lactobacillus plantarum. J. Appl. Bacteriol. 81:657-662.

Leal, M. V., M. Baras, J. L. Ruiz-Barba, B. Floriano and R. Jimenez-Diaz. 1998. Bacteriocin production and competitiveness of Lactobacillus plantarum LPCO10 in olive juice broth, a culture medium obtained from olives. Int. J. Food. Microbiol. 43:129-134.

MacDougall, E. I. 1948. Studies on ruminal saliva 1. The composition and output of sheep's saliva. Biochem. J. 43:99-109.

Mantovani, H. C. and J. B. Russell. 2002. The ability of a bacteriocin of streptococcus bovis HC5 (bovicin HC5) to inhibit clostridium aminophilum, an obligate amino acid fermenting bacterium from the rumen. Anaerobe 8:247-252.

McAuliffe, O., R. P. Ross and C. Hill. 2001. Lantibiotics: structure, biosynthesis and mode of action. FEMS Microbiol. Rev. 25:285-308.

Muyzer, G., E. C. De waal and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59:695-700.

Mwenya, B., C. Sar, B. Pen, R. Morikawa, K. Takaura, S. Kogawa, K. Kimura, K. Umetsu and J. Takahashi. 2006. Effects of feed additives on ruminal methanogenesis and anaerobic-fermentation of manure in cows and steers. In: Greenhouse Gases and Animal Agriculture (Ed. C. R. Soliva, J. Takahashi and M. Kreuzer). Amsterdam. ELSEVIER B.V. pp. 209-212.

Nakatsu, C. H., V. Torsvik and L. Øvreas. 2000. Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. Soil Sci. Soc. Am. J. 64:1382-1388.

Ørskov, E. R. and I. McDonald. 1979. The estimation of protein degradability in the rumen from incubation measurements weighed according to rate of passage. J. Agric. Sci. Camb. 92: 499-503.

Rekhif, N., A. Atrih and G. Lefebvre. 1995. Activity of plantaricin SA6, a bacteriocin produced by Lactobacillus plantarum SA6 isolated from fermented sausage. J. Appl. Bacteriol. 78:349-358.

Sauer, F. D., V. Fellner, R. Kinsman, J. K. Kramer, H. A. Jackson, A. J. Lee and S. Chen. 1998. Methane output and lactation response in Holstein cattle with monensin or unsaturated fat added to the diet. J. Anim. Sci. 76:906-914.

Santoso, B., B. Mwenya, C. Sar, Y. Gamo, T. Kobayashi, R. Morikawa, K. Kimura, H. Mizukoshi and J. Takahashi. 2004. Effects of supplementing galacto- oligosaccharides, *Yucca schidigera* or nisin on rumen methanogenesis, nitrogen and energy metabolism in sheep. Livest. Prod. Sci. 91:209-217.

Sar, C., B. Mwenya, B. Santoso, K. Takaura, R. Morikawa, N. Isogai, Y. Asakura, Y. Toride and J. Takahasi. 2005a. Effect of *Escherichia coli* W3110 on ruminal methanogenesis and nitrate/nitrite reduction *in vitro*. Anim. Feed Sci. Technol. 118: 295-306.

Sar, C., B. Mwenya, B. Pen, R. Morikawa, K. Takaura, T. Kobayashi and J. Takahashi. 2005b. Effect of nisin on ruminal methane production and nitrate/nitrite reduction *in vitro*. Aust. J. Agric. Res. 56:803-810.

SAS. 1996. SAS/STAT® Software: Changes and Enhancements through release 6.11. SAS Institute Inc., Cary, NC, USA.

Sang, S. L., Jih-Tay Hsu, Hilario C. Mantovani and James B. Russell. 2002. The effect of bovicin HC5, A bacteriocin from Streptococcus bovis HC5, on ruminal methane production *in vitro*. FEMS Microbiol. Lett. 217:51-55.

Sang, S. L., Hilário C. Mantovani and James B. Russell. 2002. The binding and degradation of nisin by mixed ruminal bacteria. FEMS Microbiol. Ecol. 42:339-345.

Todorov, S., B. Onno, O. Sorokine, J. M. Chobert, I. Ivanova and X. Dousset. 1999. Detection and characterization of a novel antibacterial substance produced by *Lactobacillus plantarum* ST 31 isolated from sourdough. Int. J. Food Microbiol. 48:167-177.

WHO Expert Committee on Food Additives. 1969. Specifications for the identify and purity of food additives and their toxicological evaluation: some antibiotics. World Health Organ. Tech. Rep. Ser. No. 430.

Yoshida, N., N. Takahashi and A. Hiraishi. 2005. Phylogenetic characterization of a polychlorinated-dioxin-dechlorinating microbial community by use of microcosm studies. Appl. Environ. Microbiol. 71:4325-4334.

Yuan, J., Z.-Z. Zang, X.-Z. Chen and W. Yang. 2004. Site-directed mutagenesis of the hinge region of nisinZ and properties of nisinZ mutants. Appl. Microbiol. Biotechnol. 64:806-815.