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Lactic Acid Bacteria and Mitigation of GHG Emission from Ruminant Livestock

Junichi Takahashi

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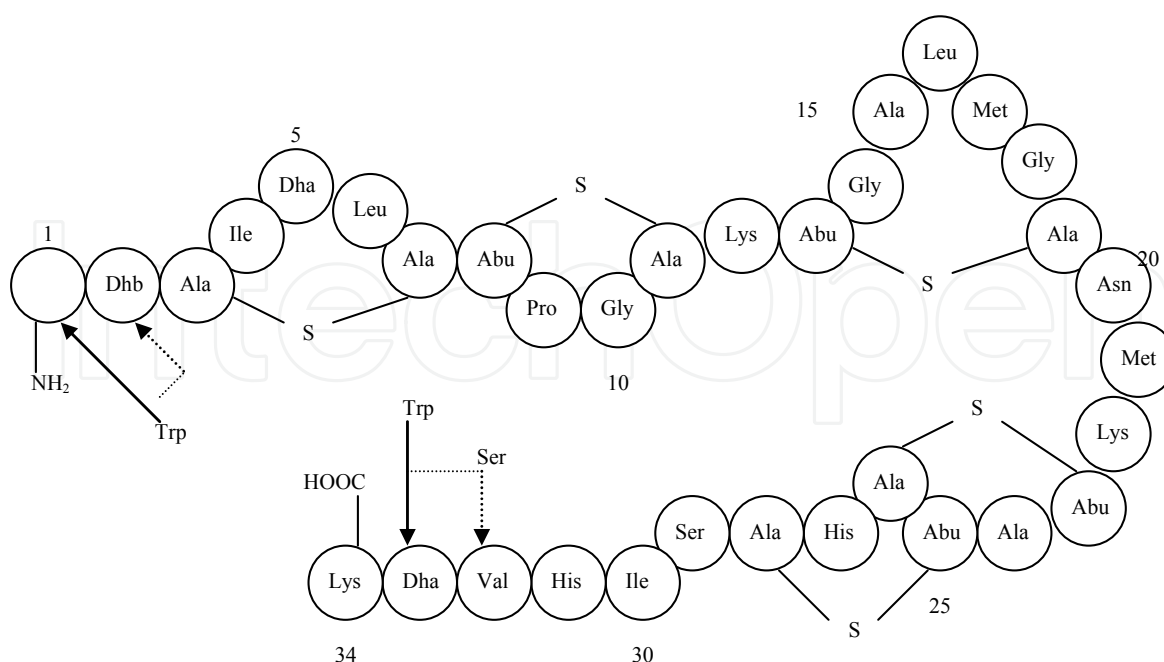
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1. Introduction

The gases which bring greenhouse effect are water vapor and trace gases in atmosphere, carbon dioxide (CO₂), methane (CH₄), nitrous oxide (N₂), sulfur hexafluoride (SF₆), hydrofluorocarbons (HFCs), and perfluorocarbons (PFCs). Global warming due to increases in the atmospheric concentration of greenhouse gases (GHG) is an important issue. The worldwide trends of carbon dioxide have shown an increase in the greenhouse effect on global warming (Houghton, 1994). However, CH₄ is an important greenhouse gas second only to CO₂ 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 15-18% of the total atmospheric CH₄ flux. The control of CH₄ emission is a logical option since atmospheric CH₄ concentration is increasing at a faster rate than carbon dioxide (Moss, 1993). CH₄ emitted from ruminants is mainly 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), although acetotrophic methanogens may play a limited role for rumen methanogenesis (McAllister, 1996). Methane 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), 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 (Chalupa, 1984; Hopgood and Walker, 1967). 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* (Belaich *et al.*, 1990; Wolin, 1975). 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). By contrast, reductive methanogenesis might contribute to mitigate methane (Immig *et al.*, 1996). Therefore, a strategy to mitigate ruminal CH_4 emission is to promote alternative metabolic pathway to dispose the reducing power, competing with methanogenesis for H_2 uptake. 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. β 1-4 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 *et al.*, 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 mechanism for accreditation of manipulators must be established to mitigate global CH_4 emission.



Dha = dehydroalanine, Dhb = dehydrobutyryne, Ala-S-Ala = lanthionine, Abu-S-Ala = β -methyllanthionine. (adapted from Breukink *et al.*, 1998).

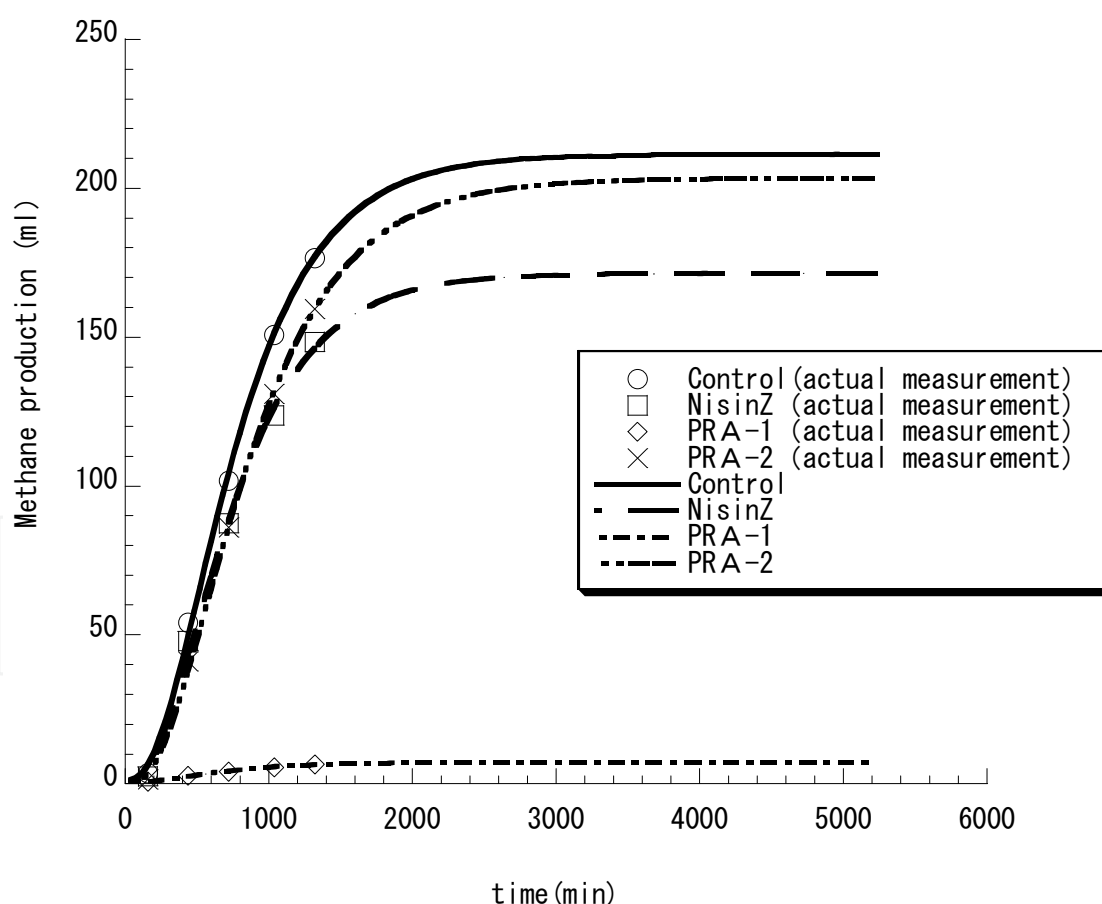
Figure 1. Primary structure of nisin.

2. Possible control of indirect action of lactic acid bacteria as probiotics on rumen methanogenesis

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). Particular interest concerning bacteriocins which produced by lactic acid bacteria has increased recently.

Bacteriocins, antimicrobial proteinaceous polymeric material substances, are ubiquitous in nature being produced by a variety of Gram-negative and Gram-positive bacteria, and typically narrow spectrum antibacterial substances under the control of plasmid. Nisin is produced by *Lactococcus lactis* ssp. *lactis* which is an amphiphilic peptide composed by 34 amino acids with two structural domains that are connected by a flexible hinge (Breukink *et al.*, 1998; Montville and Chen, 1998), and is classified into the group of lantibiotics. Nisin has a mode of action similar to ionophores, which show antimicrobial activity against a broad spectrum of Gram-positive bacteria and is widely used in the food industry as a safe and natural preservative (Delves-Broughton *et al.*, 1996). It 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. Recent works have indicated that *Lactococcus lactis* subsp. *lactis* produce nisin Z, which has been identified from Korean traditional fermented food “Kimchi” besides nisin A (Park, 2003). They have similar antibacterial ability to mitigate methane emission (Mwenya *et al.*, 2004; Santoso *et al.*, 2004; Sar *et al.*, 2006), to inhibit growth both of *Clostridium amoniphilum*, which is obligate amino-acid fermenting bacteria (Callaway *et al.*, 1997) and lactic acid-producing ruminal Staphylococci and Enterococci (Lauková, 1995). *Leuconostoc mesenteroides* ssp. *mesenteroides*, *Leuconostoc lactis* and *Lactococcus lactis* ssp. *lactis* were isolated from “Laban” which was a traditional fermented milk product in Yemen and determined the mitigating effect on in vitro rumen methane production. These strains isolated from Laban enhanced propionate production and decreased acetate/propionate ratio. In consequence, they reduced methane production remarkably (Gamo *et al.*, 2002). For *Leuconostoc mesenteroides* ssp. *mesenteroides*, in particular, the mitigating effect was amplified with GOS, which was degradable about 80% within 1 hour incubation in the artificial rumen fluid due to the stimulation of reduction reactions consuming metabolic hydrogen. However, direct involvement of bacteriocin or lower molecular substances produced by the strain on rumen methanogenesis remains to be elucidated.



Where, $y(\text{ml})$ = gas produced at time t (min), a = first gas production, b = second gas production and c = fractional rate gas production, using Kaleida Graph (Version 3.6, Synergy Software, Reading, PA, USA).

Figure 2. Effect of PRA on the cumulative methane production extrapolated by nonlinear regression analysis; $y = a + b(1 - e^{-ct})^3$.

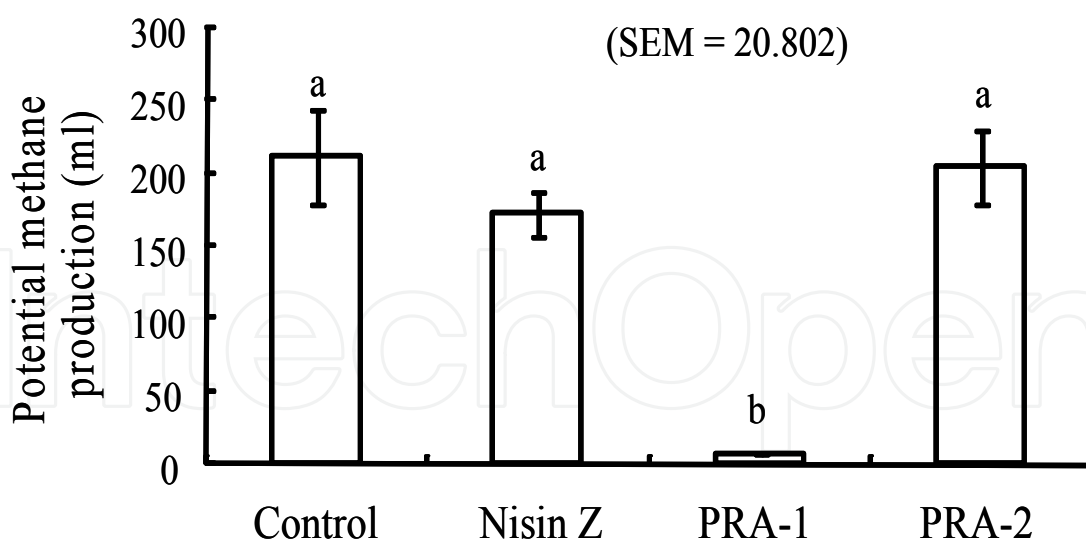


Figure 3. Effect of PRA on potential methane production. Control: *Lactococcus lactis* ATCC19435 (non-antibacterial substances), Nisin-A: *Lactococcus lactis* NCIMB702054, PRA-1: *Lactobacillus plantarum* TUA1490L, and PRA-2: *Leuconostoc citreum* JCM9698. Vertical bars represent standard deviation (n = 4). Means with different letters differ significantly (p<0.01).

3. Abatement of rumen methanogenesis by direct action of lactic acid bacteria as probiotics producer

For low molecular compounds, small amounts of volatile fatty acids (acetic acid, formic acid), hydrogen peroxide, β -hydroxy-propionaldehyde (reuterin) are produced by lactic acid bacteria as antibacterial substances in addition to lactic acid. Because lactic acid bacteria themselves don't have a group of catalase, considerable amount of hydrogen peroxide accumulates in the bacterial cells. Many strains of the genus *Lactobacillus* are commonly referred to as having high ability to produce hydrogen peroxide (Jaroni and Brashears, 2000; Aroucheva *et al.*, 2001; Gardiner *et al.*, 2002).

Its antimicrobial activity is effective against numerous Gram-positive bacteria. Although it has been reported that nisin suppress rumen methanogenesis, the suppressing efficacy of nisin on rumen methanogenesis may not be sustained, because proteinaceous nisin is degradable in the rumen due to bacterial protease (Sang *et al.*, 2002). Several strains of lactic acid bacteria produce different types of protease resistant antimicrobial substance (PRA). In our research, the strain of lactic acid bacteria that produce PRA were screened on MRS agar plates containing Umamizyme G (protease mixture from *Aspergillus oryzae*, amino Enzyme Inc, Nagoya, Japan) as follows: 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 plates were then overlaid with Bacto Lactobacilli agar AOAC (Becton, Dickinson and Company, NJ. USA) containing an indicator strain, *Lactobacilli 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 and

Leuconostoc citreum JCM9698 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. GYEKP medium to prepare inoculants for PRA-1, PRA-2, nisin Z and control were used for the culture of lactic acid bacteria. Each strain of lactic acid bacteria was inoculated into a shaking flask containing GYEKP, and was cultivated for 20 h at 30°C using SILIKOSEN (Shin-Etsu polymer, Tokyo), which was culture plug for aeration cultivation after confirmation of the stationary phase. The cells were removed by centrifugation at $8,000 \times g$ at 4°C and filtration with 0.45 µm membrane filter. The supernatants were used as PRA inoculants in the in vitro gaseous quantification trials. Methane mitigating effects of PRA-1 from *Lactobacillus plantarum* TUA1490L and PRA-2 from *Leuconostoc citreum* JCM9698 isolated from foods were determined in comparison with *Lactococcus lactis* ATCC19435 which did not produce any antibacterial substances as a negative control and *Lactococcus lactis* NCIMB702954 which produced nisin-Z as a positive control using in vitro continuous incubation system equipped with automated infra-red quantification apparatus (Takahashi *et al.*, 2005). Fig.2 shows effects of PRA-1 and PRA-2 produced by *Lactobacillus plantarum* and *Leuconostoc citreum* on cumulative methanogenesis extrapolated by nonlinear regression analyses. PRA-1 remarkably decreased cumulative methane production. For PRA-2, there were no effects on CH₄ and CO₂ production and fermentation characteristics in mixed rumen cultures. Fig. 3 shows the effect of PRA on potential methane production which estimated from non-linear regression analysis of cumulated methane production. It has been suggested that PRA-1 significantly decreases potential methane production by rumen methanogens (Asa *et al.*, 2010). 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. Fig. 4 shows DGGE band patterns of archaea and eubacteria. All fluorescence brightness of methanogens 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 or parasitic methanogens sticking on protozoan surface (Fig.5). 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*, *Pseudobutyrvibrio* sp, *Prevotella* sp, *Succinivibrio dextrinosolvens* and *Schwartzia succinivorans* in Gram negative bacteria were decreased by adding PRA-1.

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.*, 1994), olives (Jimenez-Diaz *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, methane suppressing activity of PRA-1 was not inactivated by treatments Umamizyme G and protease K. Moreover, aeration cultivation is an essential procedure for activation of PRA-1 to abate methanogenesis. Therefore, possible mechanism of PRA-1 produced by *Lactobacillus plantarum* TUA1490L on rumen methane production might be assumed as resulting from the direct involvement of low molecule substance such as hydrogen peroxide due to the requirement of aeration for the preparation.

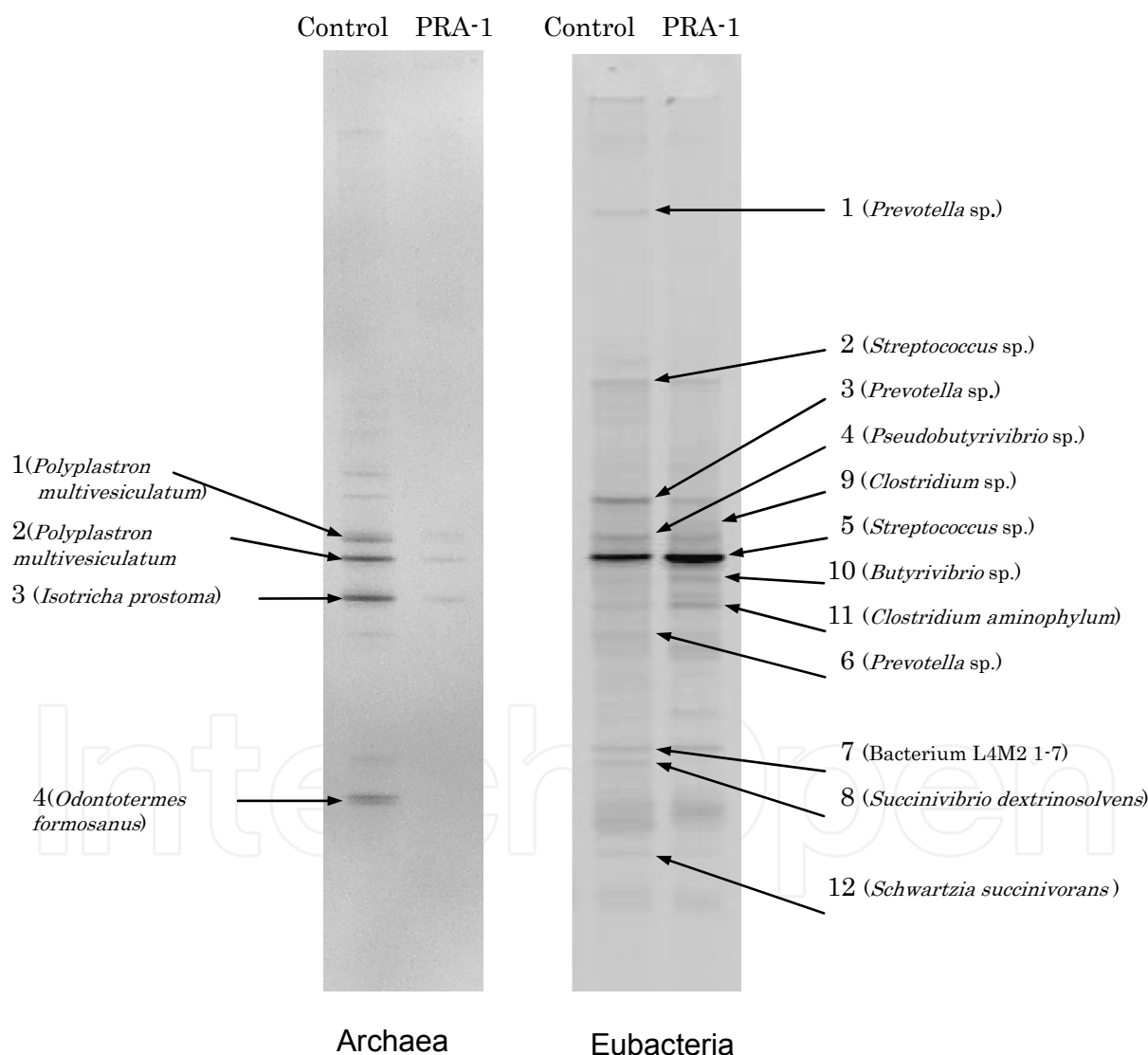


Figure 4. DGGE band patterns

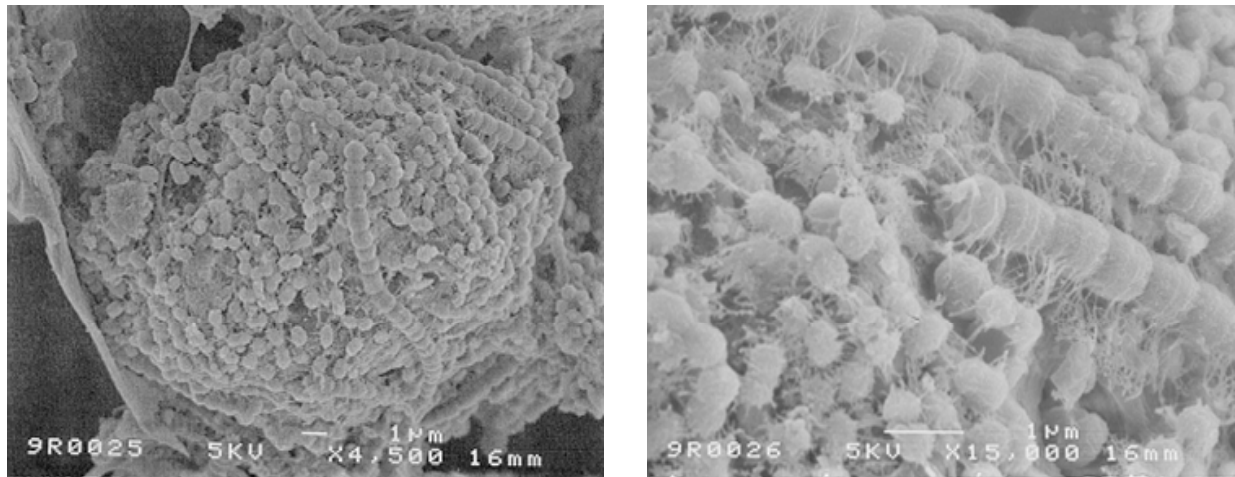


Figure 5. Electric scanning microscopy of symbioses of methanogens on the surface of Ciliate Protozoa.

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