Impact of the use of phytochemicals on rumen microbial function and enteric methane amelioration

Yutaka UYENO

Shinshu University, Nagano, Japan, ytkuyeno@shinshu-u.ac.jp

Introduction

The complexity of the rumen microbial ecosystem supports the efficient conversion of various carbohydrates to volatile fatty acids for fulfilling host energy requirement via stepwise disposal of H through the reduction of CO_2 to methane. The archaeal component of the ecosystem, which is thought to be represented exclusively by methanogens, is responsible for the removal of hydrogen, which places the rumen archaea into essentially one functional group in this organ. Methane production in ruminants has attracted a great deal of attention in relation to its contribution to greenhouse gas effect and global warming. Various strategies have been therefore considered for its mitigation. Promising means have been suggested for reducing methane emissions by decreasing the number or activity of rumen methanogens. However, these interventions can be achieved but sometimes can result in unintended consequences. Thus, trials have indicated the need for further consideration of the composition, function, and microbial interactions within the ecosystem. To establish a more efficient way for the mitigation of methane emission, systematic intervention in the rumen microbial populations by a combination of biological and chemical means may also be feasible. Here provides background regarding the diversity and plasticity of functions of the rumen bacterial and methanogenic communities, as well as some of the methane abatement options that aim to manipulate the rumen community, including the use of phytochemicals and other concepts.

Rumen bacterial and archaeal community

Ruminant animals harbor a complex microbial community consisting of a diverse array of bacteria, archaea, protozoa, and fungi in the rumen. These different microbes interact with one another and play important role in digestion of fibrous plant material and anaerobically fermenting them into end products, which are in turn used as energy source by the host. This nutritional change is a unique step in the energy metabolism of ruminants. Among ruminal microbes, bacteria decompose the feed into short-chain (C1 – C5) fatty acids, amino acids, hydrogen, carbohydrates, etc. Ruminal bacteria and archaea are obligate anaerobes. For many years, descriptions of bacterial diversity in the rumen were based mainly on the use of anaerobic culture techniques. More than 200 bacterial species have been isolated from the rumen, and many of these have been characterized both phylogenetically and physiologically (Dehority, 2003). Although culture dependent studies have improved our understanding of rumen microbiology, there are still methodological obstacles to the precise and rapid monitoring of the entire ruminal microbial community, as many of the rumen microbes have yet to be cultured. There is renewed interest in this question due to the development of various molecular techniques, especially those based on the 16S rRNA genes. The extreme bacterial molecular diversity was uncovered in such investigations, which reflect the complex metabolic network in which rumen bacteria are involved. We succeeded in monitoring the community members predominating in ruminal ecosystems using a probe set coupled with an RNA-based quantitative detection method (Uyeno *et al.,* 2007). The results were generally consistent with previous observations of the quantitative detection of the target groups, the genera *Bacteroides/Prevotella*, *Ruminococcus flavefaciens* and *Ruminococcus albus*, and the genus *Fibrobacter.* Also, archaeal methanogens accounted for greater proportions of the microbiota in our study. The coverage with the probe set was 71–78% of the total bacterial 16S rRNA. Kong *et al.* (2010) also investigated the composition and distribution of bacterial populations associated with liquid and solid rumen contents from ruminal cannulated Holstein dairy cows fed grass hay or barley silage diets with or without flaxseed using fluorescence in situ hybridization (FISH). *Bacteroidetes* and *Firmicutes* were abundant in the rumen fractions in these studies. In their study, the classes *Deltaproteobacteria* and *Gammaproteobacteria* were also major constituents of the rumen microbial community. At the same time, both analyses showed a certain unknown proportion that none of the probes could determine. This implies difficulty in producing a complete description of the whole community by these methods, mainly due to the genetically diverse structure of the bacteria in rumen ecosystems.

The archaeal component of the ecosystem, which is thought to be represented exclusively by methanogens, is implicated in the removal of hydrogen. This makes the rumen archaea an indispensable part of this organ. The population of these methanogens has been estimated by various molecular studies, and was within the range of 0.3% to 3.3%. Archaea have been shown to already occupy an ecological niche in the rumen of young ruminants no more than eight weeks of age (unpublished data). Cultivation-based analyses have identified *Methanobrevibacter*, *Methanomicrobium*, and *Methanobacterium* as predominant methanogenic genera in the rumen. Due to the fastidious growth requirements of rumen methanogens, the information has been too limited in both quantity and quality to determine the true activity and potential of these microbes compared to other rumen bacteria. Tajima *et al.* (2001) succeeded in recovering a wide range of the rumen archaeal molecular diversity as well as several 16S rRNA sequences that did not cluster with known methanogens using two different sets of archaeal primers, which have not been used previously in culture-based microbiological studies. Studies of rumen archaea yielded 5 species, *Methanobacterium formicicum*, *Methanobacterium ruminantium*, *Methanosarcina barkeri*, *Methanosarcina mazei*, and *Methanosarcina mobile*, all of them methanogens. Rumen protozoa are also involved in methanogenesis because of their ecto/endosymbiotic association with methanogenic archaea that utilize hydrogen produced during fermentation. The protozoa-associated population consists mainly of the family *Methanobacteriaceae* and accounts for about 90% of all rumen methanogens, while the remainder consists of free-living organisms represented by the *Methanomicrobiales*.

The ruminal microbial community is viewed as an ecosystem that can be perturbed, for example, by altering diet. The effects of diet on changes in the diversity and number of a wide range of bacterial species in the rumen are known. Limited genera and/or species comprising the methanogenic ecology may play important role in contributing to the difference in methane gas emission between animals with different feed efficiencies. An application of more high-resolution detection technique is warranted to learn more about the linkage between the microbial ecology of methanogens and feed efficiency in cattle especially the energy-harvesting mechanism.

Enteric methane emission and mitigation

For several decades, the importance of methanogen archaea in rumen function has been studied intensively with regard to methane emission and its suppression. More than 95% of the methane emitted by ruminants is produced in the rumen, and associated with 2–15% of gross energy loss from the ruminants, which contributes to 13–19% of global greenhouse gas. This shows the role of these organisms in global warming contributed from domesticated livestock. Methane generation from the rumen is inevitable due to the normal function of the rumen, but efforts to mitigate the generation of methane have been made to increase energy deposition in the hosts' body and to lower greenhouse gas emission from ruminants. Cattle with higher feed efficiencies are reported to produce 20% to 30% less methane, clearly indicating that large amounts of ingested energy are usually discarded as methane in ruminant production. The level of feed intake, forage processing, type of carbohydrate, and addition of lipids and ionophores are few important factors associated with methane production and lead a change in rumen fermentation pattern. More the carbohydrate digestion in rumen, more disposal of reducing power in the form of methane is required. Given their critical role in interspecies hydrogen transfer, a more intricate issue is how to counteract hydrogen in the rumen when methanogens are inhibited. A principal concept of the manipulation of methane production is a means of diverting hydrogen away from methane formation. For example, propionate production from succinate is an alternative metabolic pathway to dispose of reducing power. An inverse relationship has been described long back between methane and propionate production in rumen, which reflects competitiveness of both processes for the net consumption of reducing power.

Various strategies aim at inhibition of enteric methane generation either directly or indirectly. Compounds that inhibit the activity of methanogens are likely to reduce or eliminate methane production. Mitigating rumen methane excretion by applying functional microbes ("probiotics") may represent an interesting ecological tool. For example, hydrogen utilization and acetate production by a ruminal acetogenic bacterial isolate have been shown to be improved *in vitro* by the addition of a yeast strain, even in the presence of methanogens. Redirection of hydrogen for acetate production instead of methane production would also be favorable for energy generation for the host, as acetate is an important energy source. Researchers have mainly focused on manipulating the numbers and/or activities of rumen methanogens. Approaches are promising but the diversity and plasticity of functions of the rumen bacterial and methanogenic communities may be limiting factors for their successful application. Substantial decreases in methane reduction by these approaches would be difficult to achieve without compromising production. However, accompanying benefits (for example, decrease in rumen protein degradability and increase in post-rumen protein availability) are expected. A number of abatement options can fit animal production systems in the immediate or near future, many of which are likely to be cost effective.

Tannins in methane abatement

Direct ruminal interventions aim to manipulate any of the steps, where possibility appears for the mitigation of methane emission in ruminants. Several efforts have focused on decreasing enteric methane emission through the addition of lipids, plant compounds, monensin, and other organic compounds, or by otherwise controlling diet composition. Recently, there is increasing interest in the use of plants and plant extracts to mitigate enteric ruminal methane emissions (Hook *et al.,* 2010). The use of plant secondary metabolites (PSM) in many parts of the tropics to reduce livestock methane emission and thereby improving animal performance is increasing. The PSM are well recognized as antimicrobial agents, which act against bacteria, protozoa, and fungi, (i.e. do not necessarily target archaea) as a substitute for chemical feed additives. Tannins and saponins constitute the major classes of PSM that are currently under investigation in a number of laboratories (e.g., Guo *et al.,* 2008). They are widely distributed in nutritionally important forage trees, shrubs, legumes, cereals, and grains. The antimicrobial actions and effects on rumen fermentation of these compounds depend on their nature, activity, and concentration in a plant or plant product. Beneficial effects of tannins (HT and CT) and saponins (Malik and Singhal, 2008a) have been observed in *in vitro* and *in vivo* studies.

Tannins are polyphenol substances of diverse molecular weights and variable complexity, which can form complexes mainly with proteins due to the presence of a large number of phenolic hydroxyl groups. Based on their structure and chemical properties, tannins are classified into two groups. Hydrolyzable tannins (HT), which consist a central sugar to which a number of phenolic carboxylic acids are bound by esters of gallic acid (gallotannin) or ellagic acid (ellagitannins), another group of tannins is condensed tannins (CT) or proanthocyanidins, represents the large group of natural polyphenols widely distributed in the plant kingdom. HT are widely distributed in oak, and acacia species, especially in the browse, with a level up to 200 g/kg on DM basis. They are large molecular weight compounds (500 – 20,000 Da), which are composed of chains of flavan-3-ols unit. They can also be toxic, especially when large quantities are given to ruminants with insufficient time for microbial adaptation. However, once animals are adapted to HT containing diets, acceptable levels of production can be achieved with appropriate feed management. CT are complex polymers derived by condensation of flavan-3-ol (catechin) or flavan-3,4-diol (epigallocatechins or delpinidins) subunits linked through interflavan bonds. Differences in degree of polymerization produce a large variety of chemical structures which contributes to variation in their biological properties of CT (Patra and Saxena, 2011). Chestnut tannins are the most common HT extracted from temperate plants; and quebracho and mimosa are CT extracted from tropical plants, which sometimes have levels of 170 g CT/kg DM (Puchala *et al.*, 2005).

When ruminants are fed tannin-rich forage, methane production from ruminal fermentation decreases up to 50% (Patra and Saxena, 2011). The antimethanogenic activities of HT or CT have been extensively demonstrated in several *in vitro* and *in vivo* studies (e.g., Hess *et al.*, 2004; Malik and Singhal, 2008b). However, it has not been determined that which type of tannins are more effective in suppression of methane production. Different types of tannins show differences in affinity for bacterial and plant proteins. It should be noted that not all types of tannins produce beneficial nutritional and environmental responses. CT from various plant species shows different magnitudes of effect on rumen fermentation, which could be related to their different chemical structures and molecular weights. Generally, tannins with low molecular weight have greater inhibitory effects on rumen microbes, because of their higher protein-precipitating capacities than high molecular weight polymeric tannins while it has been reported that CT fractions with highest MW had greatest inhibitory effect on methane production. Although a direct antimethanogenic activity of CT has been suggested, mechanisms for inhibition of methanogenesis by CT are largely hypothetical.

Feeding with tannin sometimes results in a decrease in ammonia concentration, exhibiting the efficient use of volatile fatty acid (VFA) for microbial protein synthesis. Multiple phenolic hydroxyl groups of tannins can react with proteins, forming tannin–protein complexes and, thus preventing the degradation by proteases and binding proteins at ruminal pH. Thereafter, they can allow protein release at the abomasum level. The effects of CT on ruminal N metabolism are well documented (e.g., Waghorn, 2008). Tannins commonly make a shift in N excretion from urine to feces during nutrition conversion in the host. Sometimes, methane production is reduced with CT supplementation, and there are no negative impacts of feeding CT on VFA concentration or NDF digestibility. Therefore, CT contents of diet from low to moderate (20–40 g/kg) level may have beneficial effects on protein metabolism and further improvement in animal performance, such as body weight, milk yield, and reproduction because of increased small intestinal absorption of amino acids. Accordingly, the effects of tannins on ruminant productivity depend on the quality (e.g. degradability and composition of essential amino acids) and quantity of dietary protein, requirements of amino acids, and status of other nutrients. Consequently, some tannins are known to produce excessive escape of CP from rumen.

Impact of PSMs on rumen microbial ecology and host nutrition

Tannins are also regarded as antimicrobial compounds, exert inhibitory effects on bacteria through complex formation. Inhibition of archaeal growth is mainly due to the bacteriostatic and bactericidal effects of CT. The formation of complexes with bacterial cell wall membrane components causes morphological changes and secretion of extracellular enzymes. Either interaction is likely to inhibit the transport of nutrients into the cell and/or retard the growth of the organism. Tannin induced membrane disruption, direct action on microbial metabolism, deprivation of substrates for microbial growth, and chelation of cations by tannins; all reduces its availability to microbes. Tannins lower methane production probably by directly inhibiting the activities of methanogenic archaea and/or reducing fiber digestion in the rumen.

The effects of tannins on ruminal bacteria were reported to be dependent upon the species of microorganism and type or source of tannin. Tannins could reduce fiber digestion by complexing with lignocellulose and preventing microbial digestion and/or by directly inhibiting cellulolytic microorganisms due to such antinutritional effects. Within PSMs, mimosa tannins showed a marked inhibitory effect on microbial fiber degradation in the rumen. Inhibition of methane emission by CT may primarily be attributed to the reduction in cellulolytic and/or total bacteria numbers that take part in nutrient digestion, and as a result decreased rumen hydrogen production. Thus, on feeding of tannins from quebracho or mimosa, sumach, and chestnut, ruminal acetate concentration decreased. Synchronized increase in molar proportion of propionate with less hydrogen production from enteric fermentation also affects the methane production negatively. Rumen digestion of readily fermentable carbohydrate and hemicellulose is also reduced albeit compensated by increased postruminal digestion.

The effects of PSM on ruminal fermentation are desirable if, they lead an increase or do not alter the VFA concentration, and decrease both ammonical N and methane production. Many studies have shown that forages containing CT reduces methane emissions in ruminants (Hess *et al.,* 2004; Animut *et al.,* 2008), but in most cases the reduction in methane production was accompanied by negative effects on digestibility. Presumably, tannins at a level of higher than 5–6% of DM are generally regarded as antinutritional factor for livestock, showing reduced voluntary feed intake, crude protein and fiber digestibility, and growth of ruminants. Voluntary feed intake and performance of animals may depend on the type of CT present in forage. High dietary CT concentrations also depress voluntary feed intake probably because of an interaction between diets and chemical characteristics of CT (and other secondary metabolites), with a subsequent reduction of palatability of diets. The concentration range of tannins that show *in vivo* antimethanogenic effects without decreasing organic matter digestibility in animals has yet to be determined.

CT can decrease methane through reduction of fiber digestion (indirect effect), while HT appear to act through inhibition of the growth and/or activity of methanogens and/or hydrogen-producing microbes (direct effect). However, there have been very few studies for investigating the effect of tannins on rumen archaea. Bhatta *et al.* (2009) evaluated the effects of 6 commercially available natural sources of tannins (3 sources of HT and 3 sources of CT in different combinations) on total archaea using mixed cultures. They found that CT reduced methane production by 5.5% and suppressed the population of methanogenic archaea by 12.0%. The total archaeal population was lower with the combination of HT and CT than with HT alone. The different modes of action of two kinds of tannins may explain why the effects of HT+CT on total gas and methane production were greater than those of HT alone. Mohammed *et al.* (2011) evaluated the effects of dry corn distillers grain with solubles (DDG) and CT from *Acacia mearnsii* added to the diet of beef cattle on rumen methanogens using PCR denaturing gradient gel electrophoresis and quantitative real time (qRT)-PCR. Their findings indicated that inclusion of DDG or a mixture of DDG and CT altered methanogenic diversity without altering total copy numbers of methanogenic 16S rRNA gene, and they suggested that the total methanogen population in the ruminal digesta was similar among diets. Since the symbiosis of protozoa with methanogenic bacteria in the rumen is well established, a reduction in methanogens would probably affect the protozoal population. The number of protozoan cells generally decreases when CT is added. Reduction in rumen protozoal counts may decrease archaeal counts as well, which may indirectly impact on methane emission.

Future prospects

It has been demonstrated that rumen microbial composition can be altered by various factors, including diet, age, and stress. Any changes in the microbiota composition have the potential to influence energy expenditure, satiety, and food intake of the host. Host health and productivity may therefore be determined as the ability to maintain a balance within the rumen ecosystem. Functional interactions between microbes and relations between microbes and host cells are warranted as a fundamental aspect of future research. For example, beneficial effects of nutritional supplementation have been observed by *in vitro* studies, and *in vivo* studies with animal hosts are required to determine the feasibility of these materials as feed additives or ingredients in ruminant diets to mitigate methane production without detrimental effects on the animal. Although some of the methane abatement strategies have shown efficacy *in vivo*, comprehensive studies with animal hosts are required to confirm the potential and feasibility as feed additives or ingredients in ruminant diets. Moreover, further efforts and combined physiological and ecological approaches in addition to nutritional approaches are required for setting up suitable conditions for domestic ruminants for their best performance.

Conclusion

For many years, researchers have tried to manipulate the numbers and/or activities of rumen methanogens to improve the efficiency of ruminant production. Ruminal interventions sometimes are achieve with unintended consequences. For instance, complete blockage of eructation could result in an unhealthy build-up of hydrogen in the animals. Mitigation approaches have been shown to favorably modulate rumen fermentation, but consistent beneficial effects of rumen modulation and animal performance have not been observed. This indicates that the essential features have yet been determined at present. Abatement strategies are often limited by the diet fed, the management conditions, physiological state and use of the animals, as well as government regulations, resulting in difficulties applying an optimum approach to the problem of enteric methane mitigation. All of the present strategies appear to be promising, either singly or in combination, but more research is needed to validate these approaches.

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