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Plant components with specific activities against rumen methanogens

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A wide range of plant bioactive components (phytochemicals) have been identified as having potential to modulate the processes of fermentation in the rumen. The use of plants or plant extracts as natural feed additives has become a subject of interest not only among nutritionists but also other scientists. Although a large number of phytochemicals (e.g. saponins, tannins and essential oils) have recently been investigated for their methane reduction potential, there have not yet been major breakthroughs that could be applied in practice. A key tenet of this paper is the need for studies on the influence of plant components on methane production to be performed with standardized samples. Where there are consistent effects, the literature suggests that saponins mitigate methanogenesis mainly by reducing the number of protozoa, condensed tannins both by reducing the number of protozoa and by a direct toxic effect on methanogens, whereas essential oils act mostly by a direct toxic effect on methanogens. However, because the rumen is a complex ecosystem, analysis of the influence of plant components on the populations of methanogens should take into account not only the total population of methanogens but also individual orders or species. Although a number of plants and plant extracts have shown potential in studies in vitro*, these effects must be confirmed* in vivo*.*

Keywords: ruminants, methane, tannins, saponins, essential oils

Implications

This review demonstrates that plant phytochemicals can have important effects on rumen methanogens, either by affecting methanogens directly and/or indirectly by affecting rumen protozoa. Modulation of the rumen microbiota, including methanogens and protozoa, with plant extracts such as saponins, tannins and essential oils has implications in improvement of animal nutrition through decreased dietary energy loss, and in limitation of the negative impact on the environment through mitigation of methane production.

Characteristics of the rumen ecosystem in relation to methanogenesis

The rumen ecosystem is one of the richest microbial environments, inhabited by numerous microorganism species including roughly 10^{11} bacterial cells/ml of rumen fluid, roughly 10^6 protozoal cells/ml of rumen fluid, roughly 10^3 fungal cells/ml of rumen fluid and roughly 10^9 methanogen cells/ml of rumen fluid. Only ~10% of the microbial population of this ecosystem has been identified and described (Pers-Kamczyc *et al.*, 2011), mainly due to the difficulty with maintaining them in *in vitro* culture. According to the literature data, the population of methanogens is very important to rumen functioning. Together with bacteria and fungi, methanogens are the earliest colonizers of the rumen. *Archaea* can be found in the lamb rumen as soon as 30 h after birth (Morvan *et al.*, 1994). The population of these microorganisms in lambs in the 1st week of life was 10^4 cells/g of the rumen contents, whereas in the 3rd week of life it was 10^8 to 10^9 cells/g (Skillman *et al.*, 2004). It is estimated that the methanogen population accounts for ~3% to 5% of rumen microbial biomass. So far 113 species of methanogen have been described, and although there are many more in other ecosystems, only a few have been described in the rumen (Janssen and Kirs, 2008).

Methanogens appearing first in the rumen are of the genus *Methanobrevibacter* (Skillman *et al.*, 2004). In the case of *Methanobacterium*, representatives of the family colonize the rumen environment rapidly, but, in contrast to *Methanobrevibacter*, they disappear rapidly, that is, on the 12 to 19th day after birth (Zhu *et al.*, 2007). According to some literature data, predominant methanogens in the rumen ecosystem of farm animals include microorganisms of the genus *Methanobrevibacter* and *Methanosaricina*, and especially the following species: *Methanobacterium formicicum*, *Methanobrevibacter ruminantium*, *Methanosaricina barkeri*, *Methanosaricina mazei*

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and *Methanomicrobium mobile* (Stewart *et al.*, 1997; St-Pierre and Wright, 2012). Other data demonstrated predominance of only *M. ruminantium* (Leahy *et al.*, 2010), whereas other data indicated the order *Methanobacteriales* as predominant in the rumen (Jarvis *et al.*, 2000). However, the literature is still expanding and newer methanogens are being identified (Wright *et al.*, 2008; Zhou *et al.*, 2009; King *et al.*, 2011; Lee *et al.*, 2013).

There are also differences in the methanogen populations associated with rumen solid- and liquid phases. *M. mobile*, *Methanobacterium aarhusense* and *Methanosphaera stadtmanii* are species occurring only as free-living forms in rumen fluid, which account for a small percentage of the general *Archaea* population (Zhu *et al.*, 2007). *Archaea* associated with solid phases may be a much larger part of the total population of rumen methanogens (Tajima *et al.*, 2001). Zhu *et al.* (2007) named *Methanobrevibacter* spp., *Methanosphaera* spp. and unidentified methanogens as species characteristic for the solid fraction. However, Shin *et al.* (2004) showed that the most numerous group of *Archaea* inhabiting solid particles are the families *Methanomicrobiaceae* and *Methanobacteriaceae*. In the group of microorganisms related to the rumen wall, predominant methanogens include *Methanobrevibacter* spp. and *Methanosphaera* spp. (Zhu *et al.*, 2007). A considerable part of the population of methanogens in the rumen are of unknown taxonomy and properties.

Previous research demonstrated that quantitative as well as qualitative variability of microorganisms (including methanogens) in the rumen depends on many factors, such as: animal species, geographical location, feed and also on the use of feed additives that affect rumen fermentation (Szumacher-Strabel *et al.*, 2009; Hook *et al.*, 2010; Huang *et al.*, 2012; Popova *et al.*, 2012; Kumar *et al.*, 2013).

Analysis of the process of methanogenesis in the rumen requires understanding of interactions between hydrogen producers (bacteria, protozoa, fungi) and consumers (methanogens) in the rumen ecosystem in terms of the possibility of reducing the negative effects (both environmental and economic) of methane production in the rumen. Therefore, growth and development of *Achaea* directly depends on the population of microorganisms, for example, those utilizing cellulose, which leads to an increase in the concentration of hydrogen (substrate for the process of methanogenesis), a by-product of crude fiber hydrolysis (Morvan *et al.*, 1996; Wang *et al.*, 2011). Syntropic interaction was demonstrated between rumen bacteria and methanogens by Wolin *et al.* (1997). Bacteria provide a substrate for methanogenesis in the form of hydrogen and formic acid, whereas methanogens decrease the concentration of bacterial metabolism products (i.e. in the process of horizontal hydrogen transfer). High amounts of bacterial metabolism products may be harmful to other microorganisms inhabiting the rumen environment. Maintaining low (10^{-6} to 10^{-7} mole/dm^3) concentration of hydrogen favorably influences the development of particular groups of microorganisms that are important for a range of rumen processes (Wolin and Miller, 1988; Ushida and Jouany, 1996). Additionally, the hydrogen concentration in the rumen affects effectiveness of the process of methanogenesis. Acidification to pH 5.5 causes loss of the ability to bind H^+ by *Archaea*, leading to further acidification of the rumen and a reduction in the methanogen population (Russell *et al.*, 1988; Van Kessel and Russell, 1996).

Rumen archaea use large amounts of hydrogen for methane production (Hungate, 1967). The simplest reaction eliminating hydrogen from the rumen of ruminants is a combination of H_2 and CO_2 according to the following equation: $CO_2 + 4H_2 = CH_4 + 2H_2O$ (Whitmann *et al.*, 1992; Morgavi *et al.*, 2010). The theory of hydrogen utilization was confirmed in the study of Demeyer and De Graeve (1991), where hydrogen addition to rumen fluid caused an increase in methane production by 94% with little change in the production of volatile fatty acids (VFA). Methane may also come from other rumen reactions, including the reduction of formic acid, methanol, methylamine, diethylamine, triethylamine (Hungate *et al.*, 1970). It is likely that the scale of methane production in the above processes is modest (Wolin *et al.*, 1997).

The process of methanogenesis, and thereby the number of methanogens in the rumen has been directly correlated with the protozoal population (Newbold *et al.*, 1995). Some *in vitro and in vivo* studies demonstrated that the lack of the protozoal population in the rumen ecosystem has a significant effect on both the population of methanogens and the level of methane production (Cieslak *et al.*, 2009a; Morgavi *et al.*, 2012). The research also showed that sheep maintained without protozoa for more than 2 years have reduced methanogenesis in comparison with sheep kept without protozoa for only 2 months (Morgavi *et al.*, 2012). Similarly, earlier studies also demonstrated that short-term (up to 3 months) defaunation of the sheep rumen causes reduction of methane emission in comparison with the rumen of sheep inhabited by all groups of microorganisms and after long-term defaunation for ca. 1 year (Ranilla *et al.*, 2004). The results from other study emphasize the need to consider the different methanogen communities (free-living *v.* protozoa-associated methanogen) when developing strategies for mitigating methane emissions in ruminants (Tymensen *et al.*, 2012). Further, it is important to recognize that different archaeal phylotypes are associated with specific groups of protozoa (Ohene-Adjei *et al.*, 2007). A number of experiments have shown that the methanogens living in close symbiosis with rumen protozoa include: *M. formicicum*, *M. ruminantium*, *M. barkeri*, *M. mazei* and *M. mobile* (Stewart *et al.*, 1997). Regensbogenova *et al.* (2004) demonstrated that *M. mobile* occurs mainly in single-species cultures of *Metadinium medium*, *Entodinium furca monolobum* and *Diplodinium dentatum*, while Kamra (2005) named four species of rumen ciliates of the group *Entodiniomorpha*, which are in close symbiosis with the methanogens: *Entodinium elonginucleatum*, *Entodinium bursa*, *Eudiplodinium maggii* and *Eremoplastron bovis*. Other researchers (Finlay *et al.*, 1994; Kisidayova *et al.*, 2000; Cieslak *et al.*, 2006) added to the list *Dasytricha ruminantium*, *Entodinium caudatum*, *E. f. monolobum*,

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D. dentatum, *Eremoplastron dilobum*, *Epidinium ecaudatum* and *Ophryoscolex caudatus*. It is presumed that the species preferences of the methanogens result from differences in the protozoal cell surface (Vogels *et al.*, 1980). However, literature data are ambiguous mainly because of the difficulty of culturing methanogens *in vitro*.

This review focuses on the effects of plant components on rumen methanogens and an important first step in understanding responses, and variation in responses, is to consider the structure, biochemistry and chemical analysis of these compounds.

Standardization of plant material and extract for biological activity studies

Saponin sources

One of the most commonly researched plant materials for anti-methanogenic activity is *Yucca schidigera*. It was approved by Food and Drug Administration as a food and feed additive with the 'GRAS' label (Generally Recognized as Safe). Two different product of yucca are available on market, that is, yucca powder (YP) and yucca extract (YE). *Y. schidigera* contains as much as 10% of steroidal saponins on a dry matter (DM) basis, which makes this plant one of the richest commercial source of saponins. YP, which is just powdered yucca log also contains 10% saponins. YE is prepared by squeezing the juice present in yucca logs, followed by the condensation to produce a syrup, and may contain 15% to 19% of steroidal saponins. Acid-hydrolyzed fractions of these saponins contain both furostanol and spirostanol aglycones. These include sarsapogenin, markogenin, smilagenin, samogenin, gitogenin and neogitogenin (Kaneda *et al.*, 1987). In the plant, these can be found in a multi-component mixture of glycosides (Tanaka *et al.*, 2000; Oleszek *et al.*, 2001, Kowalczyk *et al.*, 2011). They can be found both as monodesmosides with one sugar chain attached at the 3-O- and bidesmosides with two sugar chains at the 3-O- and 26-O- positions. Tanaka *et al.* (2000) identified as many as 13 structurally different monodesmosidic saponins, giving them trivial names from YS-I to YS-XIII.

In the work of Oleszek *et al.* (2001) eight individual saponins were isolated and identified out of which five were known spirostanol and three new furostanol structures. However, monodesmosides made up about 93% of the total saponins present. Recent LC-MS analysis showed that there are substantial qualitative and quantitative differences in the concentration of monodesmoside and bidesmoside saponin components between YP and YE; YE contains predominantly monodesmosides, whereas YP has higher amount of bidesmosidic forms. The bidesmosides in YP result from the presence of yucca bark (Kowalczyk *et al.*, 2011).

The effects of *Y. schidigera* have been historically attributed to its saponin content. However, experiments performed with rats showed effects of both saponin-containing and non-saponin-containing fractions (Duffy *et al.*, 2001). Several mechanisms have been proposed for the mode of action of *Y. schidigera* extract, but none has been conclusively proven.

The above discussion shows the difficulty of standardizing batches of yucca product, and also the potential for large variation between batches. Typical quality control procedures for the yucca products involve acid hydrolysis of the butanol phase followed by GC-FID analysis of resulting sapogenins. No information is given on glycosidic composition. In fact, glycosidic composition rather than aglycone content determines the biological activity of these compounds.

Additional difficulties in interpretation of experimental data when YE is used relate to the composition of the rest of the product. Even if concentration of steroidal saponins is 20% of the sample, the other 80% still remains unknown. A high proportion of YE is made up of polysaccharides of unknown composition and their effects on extract activity is not known.

Similar problems arise with other saponin sources, for example, quillaya saponins, the second largest commercial source of triterpene saponins. The term quillaya saponins covers the multicomponent mixture of individual glycosides of high polarity and complex structural diversity. Most of the published work on this plant refers to one of the best known component (QS-21) of acylated bidesmosidic triterpene saponin. No analytical protocols are available to standardize these saponins and not much is known about their stability under different extraction/purification conditions. Usually acylation is very sensitive to temperature, pH or other extraction/purification treatments. Thus, the development of analytical protocols for standardization of quillaya saponins is essential.

Tannins and proanthocyanidins

Tannins are a complex mixtures of individual compounds having molecular weights ranging from 500 to over 3000 (gallic acid esters) and up to 20 000 (proanthocyanidins). Their standardization before *in vitro* or *in vivo* trials represents a unique analytical problem. They are usually standardized using a spectrophotometric technique with Folin–Ciocalteu reagent, with results expressed as gallic acid equivalents. In fact this method is not specific, measuring total reducing capacity. There is a need for more specific analytical approaches, such as HPLC for procyanidins and GCMS for proanthocyanidins.

Essential oils

Widely occurring in plants and animals, the essential oils may consist of volatile constituents of terpenoid or non-terpenoid origin. Under this group, hundreds of large or small molecules can be present, consisting of hydrocarbons and their oxygenated derivatives. Some of these compounds may additionally contain nitrogen or sulfur. They may exist in the form of alcohols, acids, esters, epoxides, aldehydes, ketones, amines and sulfides but in some cases halogenated compounds are also found. The composition of essential oils is usually characteristic for the particular plant species and responsible for its fragrance. Each of the compounds may have different effects on organisms causing damage to the plant tissue (e.g. herbivorous insects or microorganisms).

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As with other groups of secondary metabolites, the level and composition of essential oils change depending on the variety, environmental factors, time of harvest, etc. A further source of variation in commercially available essential oils is differences in the technology used for their isolation, as well as storage. The most effective analytical method for essential oils remains GS-MS. More advanced techniques, such as GC/FT-IR and NMR, are required for identification of new components of essential oils.

Effect of plant components on rumen methanogens

Plant components (e.g. saponins, tannins, essentials oils) may affect methanogenesis by inhibiting growth, development and activity of the population of methanogens both indirectly (by reducing the number of protozoa associated with methanogens) and directly, by affecting methanogens. Moreover, plant components may also case a shift toward propionate production, which affects methanogenesis through reduced competition for hydrogen.

Saponins

Although saponins have reduced the amount of enteric methane production by up to 50% in some studies (Szumacher-Strabel and Cieslak, 2010; Patra and Saxena, 2010; Bodas *et al*., 2012), these effects need to be confirmed in more *in vivo* studies. There is some ambiguity in the literature concerning the mechanism of saponins action to reduce methanogens and methanogenesis. According to Guo *et al.* (2008), mitigation of methanogenesis using tea saponins results from decreased activity of the *mcrA* gene (an indicator of the methanogenic activity of the methanogen population), without changing the total methanogen numbers. This effect was noted when using a mixed rumen culture for *in vitro* studies, but not when pure cultures of *M. ruminantium* were tested. Other researchers used 3 g/day of tea saponins in sheep diets and concluded that there was no effect on the populations of methanogens (Mao *et al*., 2010; Zhou *et al*., 2011). Earlier *in vitro* research had suggested mitigation of the process of methanogenesis without reduction in the number of methanogens with the use of saponins from *Sapindus saponaria* or tea saponins (Hess *et al*., 2003; Hu *et al*., 2005). It seems likely that the reduction in methanogenesis was related to a reduction in *Archaea* associated with protozoa as a result of partial defaunation. A reduction in the protozoal population decreases the amount of hydrogen available in the rumen for the process of methanogenesis (Szumacher-Strabel and Cieslak, 2010).

Saponins, due to their structure (hydrophobic sapogenin, i.e. aglycone, and a hydrophilic sugar part – glycone, which may comprise glucose, arabinose, xylose, galactose) can interact with cholesterol present in eukaryotic cell membranes and thereby cause destruction of this cell type (Cheeke, 1996; Wina *et al*., 2005). This may explain also a lack of direct saponin effect on methanogen cells. Wina *et al.* (2005) found that lower doses (1 mg/ml) of methanol extract of *Sapindus rarak* containing saponins, used in *in vitro* research, did not reduce the concentration of methanogen RNA. The reduction was observed only for higher concentrations (4 mg/ml). Similar conclusions were drawn by other authors (Staerfl *et al*., 2010; Wang *et al*., 2011; Zmora *et al*., 2012b and 2012c). Moreover, Bodas *et al.* (2012) demonstrated that low saponin concentrations indirectly influence methane production in the rumen by reducing the number of protozoa, whereas higher saponin concentrations have direct negative effect on methanogens.

As the rumen is a highly dynamic ecosystem, examination of the influence of phytofactors on microorganisms involved in the process of methanogenesis should take into account factors that can deactivate biological properties of saponins, including degradation, hydrolysis, deglycosylation, detoxication of saponins (Miles *et al*., 1992; Makkar and Becker 1997; Odenyo *et al*., 1997; Wang *et al*., 1998; Teferedegne *et al*., 1999). According to other authors, the duration of saponin administration and the ratio of forage to concentrate may have a significant influence on their effect (Newbold *et al*., 1997, Teferedegne *et al*., 1999; Goel *et al*., 2008).

Tannins

Research on the effects of tannins on the populations of methanogens has been carried out both under *in vitro* and *in vivo* conditions, however, results were not always unequivocal (Tavendale *et al*., 2005; Bhatta *et al*., 2009; Szumacher-Strabel *et al*., 2011; Cieslak *et al*., 2012). The range of mitigation of methane production by using tannins is quite broad, from 2% to 58% in comparison with analyzed control groups (Patra and Saxena, 2010a; Bodas *et al*., 2012), and factors responsible for the mitigation are various, for example, tannin type, plant source. Tavendale *et al.* (2005) suggested that inhibition of methanogen growth is due to the bacteriostatic and bactericidal effects of condensed tannins (CT). The study demonstrated deactivation of methanogens (*M. ruminantium*), linked to reduction of produced methane (Tavendale *et al*., 2005). Similar conclusions were drawn by Pellikaan *et al.* (2011).

Other researchers analyzed six commercial sources of tannins containing hydrolysable tannins (HT) or HT and CT, and showed greater potential for mitigation of methanogenesis after using a mixture of HT and CT (Bhatta *et al*., 2009). The mixture of HT and CT suppresses methanogenesis by reducing the methanogenic populations in the rumen either directly or by reducing the protozoal population, thereby reducing methanogens symbiotically associated with the protozoal population (Bhatta *et al*., 2009). However, Goel and Makkar (2012) showed that HT led to a larger reduction in the population of methanogens or microorganisms providing them with H_2 than CT. Other authors noted not only a direct tannin effect on methanogens but also a direct influence on protozoa associated with them (Patra and Saxena, 2009). Research with dairy cattle showed that addition of CTs from *Vaccinium vitis idaea* at 2 g/kg dietary DM caused mitigation of methanogenesis mainly resulting from a reduction in protozoal numbers without a negative effect on the digestibility of organic matter and VFA production (Cieslak *et al*., 2012).

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CT extracted from *Leucaena leucocephala* caused a linear reduction in total methanogens (up to 99%) and total protozoa (up to 83%) with increasing levels (from 20 to 60 mg/g DM of substrate) of CT in an *in vitro* study (Tan *et al.*, 2011). However, the reduction in protozoal population was not always proportionally related to the decrease in methanogen population. Another study also confirmed that tannin effects on the protozoal population are varied, probably because some tannins have a direct effect on methanogens, which are not associated with protozoa (Bhatta *et al.*, 2012). Other researchers have demonstrated a decline in the methanogen population associated with protozoa, for example, species belonging to *Methanobacteriaceae*, and simultaneous increase in the number of free-living *Methanobacteriales*, after inhibition of protozoa (Goel and Makkar, 2012). A reduction in the number of protozoa is not always accompanied by a reduction in the number of methanogens. Limitation of the population of one methanogen may cause an increase in the populations of the others. As phytofactors may increase populations of some microorganisms by decreasing others, the analysis of rumen microorganisms should include also the quantitative and qualitative analysis of other organisms in the rumen (Zmora *et al.*, 2012a).

Interpretation of results with extracts containing one bioactive phytofactor, even in a predominant concentration, is difficult because of the complexity of structures, as well as the possibility of interactions between individual bioactive extract components or bioactive extract components and feed components. Soltan *et al.* (2012) also suggested that the potential methanogenic properties of feed containing tannins may be related not only to the tannin content, but also to other factors. Another cause of lack of the effect of feed additives containing tannins on the number of methanogens in rumen fluid under *in vitro* conditions may be the use of too low concentrations of tannins in the supplement (Szumacher-Strabel *et al.*, 2011). Jayanegara *et al.* (2012) stressed that methane declined when dietary tannins increased, however, when the amount of tannin (in the batch culture system) is too high (more than 100 g tannin/kg DM) the accuracy of estimates of the impact of tannins on methanogenesis decreased.

Evaluation of the effects of tannins on rumen methanogenesis should also include other responses whose modulation may indirectly cause mitigation. For example, the use of extracts or feed containing considerable amount of tannins may limit feed intake or reduces digestibility of organic matter, and therefore decreases the amount of methane produced (e.g. Carulla *et al.*, 2005).

There is scarce information about the direct effect of tannins on methanogens. Smith *et al.* (2005) demonstrated a number of mechanisms improving tolerance of bacteria to the unfavorable environment produced by tannins. These include modification of the cell membrane, secretion of a protective exo-polysaccharide layer around the cells, and degradation/modification of tannins.

Essential oils

Essential oils are mixtures of terpenoids, low-molecular-weight aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters, and/or lactones (Dorman and Deans, 2000), and are usually extracted from plant material by water or aqueous alcohol steam distillation. Antimicrobial effects of essential oils are manifested in their high affinity for microbial cell membranes (Jouany and Morgavi, 2007). However, the effect of individual essential oils depends on their structure, which results from chemical composition and type of functional group, for example, terpenoids or phenols. They cause disturbances of ion transport (electrons) through the cell membrane, mitigate protein translocation, phosphorylation and enzyme-dependent reactions taking place in the membrane (Jouany and Morgavi, 2007). Essential oils affect individual groups of microorganisms differently because of differences in the structure of the cell membrane, for example, between bacteria and methanogens.

In some studies, essential oils stimulated some protozoa species, for example, *Isotricha* spp. or *Dasytricha* spp. and this led to an increase in associated *Methanobrevibacter smithii*. However, in many studies authors showed decreased rumen methane production in response to essential oil supplements, without alterating protozoa population (Evans and Martin, 2000; Busquet *et al.*, 2005). The direct influence of essential oils on methanogen cells may be related to the structure and properties of the oil used or secondary plant metabolites contained therein. In an experiment carried out by Busquet *et al.* (2005), garlic oil (*Allium sativum*) was used as the supplement. In contrast to the essential oils that are active only against Gram-positive bacteria, garlic oil is active against Gram-negative and Gram-positive bacteria, fungi, viruses and parasites, and the main mechanism of action is related to the ability to react with –SH groups (O'Ghara *et al.*, 2000). Similar results of a decrease in total methanogens were obtained by Kongmun *et al.* (2011) after using the addition of 7% coconut oil with 100 g/day of garlic powder in rumen fistulated swamp buffalo bulls. In their study, there was no relationship between the protozoal population and the methanogen population, probably because methanogens are found in a wide range of environments in the rumen: (i) free in the rumen fluid; (ii) attached to particulate material and rumen protozoa and (iii) attached to the rumen epithelium (Janssen and Kirs, 2008). According to Goel *et al.* (2009), decreases in methanogen populations can be correlated with decreased bacteria populations, for example *Ruminococci* that produce significant amounts of hydrogen. However, these effects may depend on the concentration of the specific treatment factor used and, according to McIntosh *et al.* (2003), ruminal methanogens seem to be affected only at high concentrations of essential oils. In their experiment, EO blend (containing thymol, eugenol, vanillin and limonene) of the concentration up to 160 ppm did not alter the *M. smithii* population, which was inhibited only at a concentration of 1000 ppm.

Essential oils can modulate either the rumen methanogen population or methanogen activity and they do not always influence the total number of methanogens inhabiting the rumen, instead affecting the distribution among methanogen species. This thesis is confirmed by the results of Ohene-Adjei *et al.* (2008), where an increase in the diversity

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Table 1 *Effects of saponin sources on methane production, methanogen and protozoa populations in the rumen*

Reference	Saponin sources	Diet/substrate	Test system/dosage	Methane	Methanogen	Protozoa
Narvaez *et al.* (2013)	*Yucca schidigera* extract (153 mg smilagenin equivalent per g DM of steroidal sapogenins)	Total mixed ration (forage/concentrate total mixed ration barley silage–barley grain) 65 : 35	Serum bottle/650 μg per ml	−15%	−52%	NA
Li and Powers (2012)	Yucca saponin (8.5% saponin) (YS)	Total mixed ration (C) (concentrate/forage) 54 : 46	Holstein steers/		NA	NA
	Quillaja saponin (3.6% saponin) (QS)		Exp1/			
			C + 0.64% YS	−15%		
	Tea saponin (21.6% saponin) (TS)		C + 1.5% QS	−9%		
			Exp2/			
			C + 0.25 TS	NE		
			Exp3/			
			C + 1.5 QS	+5%		
			C + 0.5 TS	−18%		
			C + 1.5% YS	NE		
Zhou *et al.* (2011)	Tea saponin (60% triterpenoid saponins)	Chinese wild rye/concentrate 60 : 40	Refaunated or defaunated sheep/ 3 g per day	−11% refaunated, −18% defaunated	NE refaunated, NE defaunated	−43% refaunated, −100% defaunated
Wang *et al.* (2011)	Gynosaponins powder (98% gynosaponins)	Rice straw 100	Batch culture/100 and 200 mg/l	−73%, −89%	−42%, −73%	NA
Mao *et al.* (2010)	Tea saponins (60% saponins)	Wild rye/concentrate 60 : 40	Growing lambs/3 g per day	−27%	NE	−41%
Holtshausen *et al.* (2009)	*Yucca schidigera* (6% saponin)	Barley silage/concentrate 51 : 49	Batch culture/15, 30 and 45 mg/g substrate	−8%, −15%, −26%	NA	NA
	Quillaja saponaria (6% saponin)	Barley silage/concentrate 51 : 49	Dairy cows/10 g per kg of DM	NE, −11%, −12%		
	Yucca schidigera (6% saponin)			NE		
	Quillaja saponaria (6% saponin)			NE		
Wang *et al.* (2009)	*Yucca schidigera* extract (Desert King International Product, United States of America)	Hay/concentrate 75 : 35	Sheep/170 mg per day	−15%	NA	NA
Guo *et al.* (2008)	Tea saponin (60% triterpenoid saponin)	Grass meal/corn meal 50 : 50	Serum bottle/0.4 mg per ml	−8%	NE	−50%
Pen *et al.* (2008)	*Quillaja saponaria* extract (QSE) (5% to 7% saponins)	Oat hay/concentrate 50 : 50	Continuous culture fermentation vessels/ 2 and 4 ml(QSE)/l	NE	NA	−62%, −75%
	Yucca schidigera extract (YSE) (8% to 10% saponins)		Continuous culture fermentation vessels/ QSE : YSE, 2 : 0 ml/l, 2 : 2 ml/l, and 2 : 4 ml/l	−14%, −16%, NE		−50%, −75%, −75%
Goel *et al.* (2008)	*Sesbania sesban* leaves extract (63.5% saponins)	Hay/concentrate 50 : 50	HGT/10.9 and 21.2 mg	NE	−78%	−14%, −36%
	Knautia arvensis leaves extract (82.4% saponins)		HGT/3.88 and 7.76 mg		−21%	−14%, −25%
	Trigonella foenum-graecum seeds extract (34.5% saponins)		HGT/5.62 and 11.54 mg		−22%	−15%, −39%
Pen *et al.* (2007)	*Yucca schidigera* extract	Italian ryegrass hay/concentrate 60 : 40	Sheep/1.31 to 1.64 g saponin/day	NE	NA	NE
	Quillaja saponaria		Sheep/0.8 to 1.3 g saponin/day			
Pen *et al.* (2006)	*Yucca schidigera* Extract (80–100 g/kg saponins)	Oat hay/concentrate 50 : 50	Serum bottle/2, 4 and 6 ml/l	−17%, −29%, −42%	NA	−29%, −55%, −56%
	Quillaja saponaria (50–70 g/kg saponins)			NE		−34%, −41%, −40%
Hu *et al.* (2005)	Tea saponins (60% saponins)	Grass meal/corn meal 50 : 50	HGT/0.2 and 0.4 mg/ml	−13%, −16%	NA	−13%, −16%

DM = dry matter; HGT = hohenheim gas test system; NE = no effect; NA = not analyzed; –decrease; +increase.

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Table 2 *Effects of tannin sources on methane production, methanogen and protozoa populations in the rumen*

Reference	Tannin sources	Diet/substrate	Test system/dosage	Methane	Methanogen	Protozoa
Hassanat and Benchaar (2013)	*Acacia mearnsii* extract (82% CT)	Total mixed ration (forage/concentrate) 65 : 35	Serum bottle/4, 10, 20, 30 and 40 mg	NE, −12%, −21%, −32%, −38%	NA	NA
	Schinopsis balansae extract (90.4% CT)			NE, NE, −23%, −34%, −40%		
	Castanea sativa extract (5.7% CT and 75.5% HT)			NE, −13%, −23%, −31%, −40%		
	Quercus aegilops extract 8.0% CT and 71.2% HT)			NE, −11%, −19%, −26%, −36%		
Soltan *et al.* (2012)	*Acasia saligna* leaves (6.3% CT)	*Acasia saligna* 100	Serum bottle/500 mg	−38%	NA	−9%
	Laucaena leucocephala leaves (4.6% CT)	Laucaena leucocephala 100		−36%		−23%
	Prosopis juliflora leaves (0.04% CT)	Prosopis juliflora 100		NE		34%
	Atriplex halimus leaves (0.02% CT)	*Atriplex halimus* 100		NE		10%
Niderkorn *et al.* (2011)	*Onobrychis viciifolia* sainfoin (1.52% CT)	*Onobrychis viciifolia*/600 mg	Serum bottle/600 mg/3.5 h	NE	NA	NA
	Cocksfoot/sainfoin 50 : 50	Cocksfoot plus sainfoin/600 mg		NE		
	Ryegrass/sainfoin 50 : 50	Ryegrass plus sainfoin/600 mg		NE		
	Onobrychis viciifolia sainfoin (1.52% CT)	*Onobrychis viciifolia*/600 mg	Serum bottle/600 mg/24 h	NE		
	Cocksfoot/sainfoin 50 : 50	Cocksfoot plus sainfoin/600 mg		+8%		
	Ryegrass/sainfoin 50 : 50	Ryegrass plus sainfoin/600 mg		NE		
Tan *et al.* (2011)	*Leucaena leucocephala* extracts (100% CT)	Guinea grass 100	HGT/10, 15, 20, 25 and 30 mg	−33%, −47%, −57%, −59%, −63%	−25%, NE, −99%, −94%, −95%	−86%, −83%, −62%, −55%, −55%
Hariadi and Santoso (2010)	*Acacia mangium* (5.4% tannins)	Elephant grass/ *Accacia mangium* 80:20	Glass syringes/60 mg	−29%	NA	NE
	Biophytum petersianum (4.3% tannins)	Elephant grass/*Biophytum petersianum* 80 : 20		−25%		NE
	Psidium guajava (3.5% tannins)	Elephant grass/*Psidium guajava* 80 : 20		−18%		NE
	Phaleria papuana (3.1% tannins)	Elephant grass/*Phaleria papuana* 80:20		NE		−31%
	Persea americana (2.4% tannins)	Elephant grass/*Persea americana* 80 : 20		NE		NE
	Sesbania grandiflora (1.9% tannins)	Elephant grass/*Sesbania grandiflora* 80 : 20		NE		−55%
Grainger *et al.* (2009)	*Acacia mearnsii* extracts (603 g CT/kg DM)	Grazing ryegrass/cracked triticale grain 89 : 11	Dairy cows/0.9 or 1.8% DMI	−14%, −29%	NA	NA
Bhatta *et al.* (2009)	Mimosa tannins (7.78% HT plus 1.5% CT)	Timothy hay/concentrate 65 : 35	HGT/5, 10, 15, 20 and 25% tannin-containing samples of the basal diet DMI	−11%, −14%, −27%, −32%, −41%	NE	NE, −27%, +26%, −15%, −9%
	Quebracho tannin (7.62% HT plus 3.67% CT)			NE, −31%, −37%, −45%, −45%	−20%, −27%, −27%, −27%, −35%	−4%, −16%, −32%, −28%, −55%
	Quebracho tannin (3.94% HT plus 1.33% CT)			−13%, −23%, −26%, −31%, −38%	NE, −34%, −26%, −22%, −30%	−8%, −20%, −34%, −33%, −34%
Ramirez-Restrepo *et al.* (2010)	*Salix* spp. (34% CT)	Ryegrass/white clover	Sheep (5th or 11th weeks)/grazed willow (*Salix* spp.) fodder blocks 12 g CT/kg DMI	−19% (5th week) NE (11th week)	NA	NA
Animut *et al.* (2008a)	*Lespedeza striata* forage (151 g CT/kg DM)	*Lespedeza striata*/ *Sorghum bicolor* (33 : 67, 67 : 33, 100)	Goats/200, 447 and 613 g/day	−33%, −47%, −58%	NA	−42%, −56%, −69%
Animut *et al.* (2008b)	*Lespedeza striata* forage (140 g CT/kg DM)	*Lespedeza striata*/*Lespedeza cuneata* 100 : 0, 50 : 50, 0 : 100	Goats/720, 719 and 745 g/day	−49%, −54%, −51%	NA	−34%, −22%, −51%
	Lespedeza cuneata forage (151 g CT/kg DM)					
Beauchemin *et al.* (2007)	Quebracho tannins (91% CT)	Barley silage/concentrate 70 : 30	Beef cattle/1 or 2% of DMI	NE	NA	NA
Zeleke *et al.* (2006)	*Acacia angustissima* 459	*Brachiaria humidicola* grass/*Acacia angustissima 20 : 80*	Rusitec/2.8 g DM/day	−12%	NA	NA
	Sesbania sesban 10865	Brachiaria humidicola grass/*Sesbania sesban* 20 : 80		−37%		

CT = condensed tannins; HT = hydrolysable tannins; DM = dry matter; DMI = dry matter intake; HGT = hohenheim gas test system; NE = no effect; NA = not analyzed; –decrease; +increase.

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Table 3 *Effects of essential oil sources on methane production, methanogen and protozoa populations in the rumen*

Reference	Essential oil source	Diet/substrate	Test system/dosage	Methane	Methanogens	Protozoa
Lin *et al.* (2012a)	Combination of essential oil (809 g/kg eugenol in thyme oil; 837 g/kg carvacrol in oregano oil; 855 g/kg cinnamaldehyde in cinnamon oil; 801 g/kg limonene in lemon oil) plus monosodium fumarate	Ground maize/ground *Leymus chinensis* hay 50 : 50	Serum bottles/500 mg/l of essential oil plus 0, 5, 10 and 15 mM monosodium fumarate	−51%, −63%, −80%, −56%	−38%, −48%, −41%, −48%	−95%, −95%, −94%, −94%
Lin *et al.* (2012b)	Combination of essential oil (eugenol, carvacrol, citral, cinnamaldehyde; purity >99%) plus monosodium fumarate	Ground corn kernels/ ground *Leymus chinensis* hay 50 : 50	IVGPS (24 h)/200 mg/l essential oil plus 0, 5, 10 and 15 mM of monosodium fumarate	−31%, −76%, −84%, −65%	−23%, −16%, −34%, −16%	−88%, −85%, −88%, −82%
Manh *et al.* (2012)	Eucalyptus leaf meal powder	Concentrate 0.5% of BW/rice straw ad libitum	Dairy cows/100 and 200 g/day)	−16%, −26%	NA	NE, −22%
Chaves *et al.* (2012)	Cinnamon leaf (eugenol; 0.76 v/v)	Barley silage	Serum vials (6, 12 and 24 h)/37.5, 75 and 120 mg/kg silage DM	6 h: −47%, −29%, NE; 12 h: NE; 24 h: NE	NA	NA
	Oregano (carvacrol, thymol; >0.6 v/v)			6 h: NE, −24%, −24%; 12 h: NE24 h: NE		
	Sweet orange (limonene; >0.95 v/v)			6 h: NE; 12 h: NE; 24 h: NE		
Patra and Yu (2012)	Clove oil	Ground alfalfa hay/concentrate 50 : 50	Serum bottles/0.25, 0.50 and 1.0 g/l Fermentation medium	−11%, −17%, −34%	−6%, −3%, −12%	+2%, −6%, −27%
	Eucalyptus oil			−26%, −8%, −17%	−1%, −0.4%, −6%	+1%, −2%, −7%
	Garlic oil			−22%, −28%, −42%	−8%, −14%, −16%	−0.2%, +3%, −7%
	Origanum oil			−12%, −38%, −86%	−15%, −20%, −38%	−11%, −32%, −35%
	Peppermint oil			−8%, −20%, −25%	−15%, −20%, −20%	−1%, −11%, −33%
Sallam *et al.* (2011)	*Achillea santolina* (16-dimethyl 15-cyclooactdaiene; 60.5%)	Concentrate/forage 50 : 50	IVGPS (24h)/25, 50 and 75 μl/75 ml rumen fluid	+37%, +56%, −30%	NA	−9%, −5%, −45%
	Artemisia judaica (piperitone and camphor; 49.1% and 34.5%)			+46%, +43%, −4%		NE
	Mentha microphylla (piperitone oxide and *cis*-piperitone oxide; 46.7% and 28%)			−92%, −100%, −100%		−12%, −21%, −49%
	Schinus terebinthifolius (γ-muurolene and α-thujene; 45.3% and 16.0%)			NE		NE, −21%, −41%
Araujo *et al.* (2011)	Carvacrol (2-methyl-5-isopropyl-1-phenol) Eugenol (2-methoxy-4-(2-propenyl)-phenol) 1,8-cineol (1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane)	Concentrate/forage 80 : 20	Serum bottles/50 mg/l	−95%, −67%, −13%	NA	NA
Patra *et al.* (2010)	*Foeniculum vulgare* seed extracts (ethanol and methanol)	Wheat straw/concentrate 50 : 50	HGT (24 h)/ethanol and methanol extracts of 0.5 ml/30 ml	−39%, −71%	NA	−41%, −24%
	Syzygium aromaticum flower bud extracts (ethanol and methanol)			−47%, −86%		−43%, −48%
Sallam and Abdelgaleil (2010)	Citrus essential oil (DL-limonene and γ-terpinene; 83.9% and 10.75%)	Roughage/concentrate 50 : 50	SAGPT (24 h)/25, 50 and 75 μl/75 ml rumen fluid	NE, −16%, −33%	NA	NE, −35%, −35%
	Limonene (100%)			+14%, −20%, −10%	NA	−47%, −48%, −40%
Wang *et al.* (2009)	Oregano extract *Ropadiar*	Hay/concentrate 75 : 25	Sheep/250 mg per day	−12%	NA	NA
Sallam *et al.* (2009)	*Eucalyptus oil* eucalyptol (1,8-cineole)	Roughage/concentrate 50 : 50	IVGPS (24 h)/25, 50, 100 and 150 μl/75 ml	−26.5%, −47%, −77%, −85%	NA	−23%, −36%, −59%, −64%
Agarwal *et al.* (2009)	Peppermint oils–*Mentha piperita*	Wheat straw/concentrate mixture 50 : 50	HGT (24 h)/0.33, 1.0 and 2.0 μl/ml	NA	+105%, −82%, −80%	−30%, −78%, −88%
Soliva *et al.* (2008)	Pine oil–*Pinus mugos* (monoterpene hydrocarbons α-pinen, β-pinen, limonellen, ω3-caren, β-phellandren with proportions of 22%, 13%, 12%, 25% and 14%)	Mixed forage/concentrate 50 : 50	Rusitec(10 days)/0.008 g DM/day	NE	NA	−8%

Table 3 *Continued*

Reference	Essential oil source	Diet/substrate	Test system/dosage	Methane	Methanogens	Protozoa
Macheboeuf *et al.* (2008)	Carvacrol (2-methyl-5-isopropyl-1-phenol; >980 g/kg)	Corn/soybean/hay 45 : 30 : 25	Batch (16 h)/1.5, 2, 3 and 5 mM	−13%, −32%, −85%, −98%	NA	NA
	Cinnamaldehyde ((*E*)-3-phenyl-2-propenal; >980 g/kg)		Batch (16 h)/1, 2, 3 and 5 mM	NE, −13%, −19%, −94%		
	Thymol (5- methyl-2-isopropyll-phenol; >980 g/kg)		Batch (16 h)/1, 2, 3 and 6 mM	NE, −32%, −84%, −99%		
	Anethum graveolens (400 g/kg carvone, 320 g/kg limonene)		Batch (16 h)/2.5, 5, 10 and 25 mM	NE, NE, −47%, −76%		
	Cinnamomum verum (790 g/kg cinnamaldehyde)		Batch (16 h)/1, 3, 5 and 10 mM	NE, −26%, −98%, −98%		
	Thymus vulgaris (470 g/kg thymol, 200 g/kg terpinene, 200 g/kg p-cymene)		Batch (16 h)/0.5, 1, 2 and 3 mM	NE, NE, −62%, −94%		
	Origanum vulgare (890 g/kg carvacrol, 50 g/kg thymol)		Batch (16 h)/1, 2, 3 and 5 mM	NE, NE, −63%, −97%		
	Origanum vulgare thymol chemotype (210 g/kg carvacrol, 350 g/kg thymol)		Batch (16 h)/0.5, 1, 2 and 3 mM	NE, NE, −60%, −95%		

HGT = hohenheim gas test, IVGPS = *in vitro* gas production system; DM = dry matter; SAGPT = semi-automatic gas production technique; NE = no effect; NA = not analyzed; − decrease; + increase.

of methanogenic *Archaea* (*Methanosphaera stadtmanae*, *M. smithii* and some uncultured groups) was observed in response to treatment with cinnamaldehyde, garlic and juniper berry oil, without alteration of the total rumen methanogenic capacity.

Therefore, inhibition of the process of methane production in the rumen is not always accompanied by changes in the diversity of methanogens or a decrease in their population. Twofold increase in the number of rumen methanogens resulting from using the addition of 0.33 μl peppermint oil per ml of incubation medium *in vitro* was followed by 20% decrease in methane production, while using higher concentrations of peppermint oil (1 or 2 μl/ml) decreased methanogen populations (on average by 82%) and methanogenesis by 61% (Agarwal *et al.*, 2009). A similar effect was observed in an *in vitro* experiment where limonene, the main component of fir oil (*Abies alba*), at 40 or 400 mg/l (Cieslak *et al.*, 2009b) was used. The addition of limonene in greater amounts caused reduction of the population of methanogens (on average by 25%) with simultaneous mitigation of the process of methanogenesis (on average by 28%; Cieslak *et al.*, 2009b). The lower level had no effect on methanogen numbers or methane production. This confirmed earlier observations that medium and high concentrations of essential oils can affect the number of methanogens (Cieslak *et al.*, 2009b). The authors also do not exclude that the addition of limonene, indirectly reducing the number of microorganisms providing the substrate for methanogens in the form of H_2 and CO_2, can reduce the amount of methane produced in the rumen.

The possible mechanism of action of essential oils on methane production in the rumen may be the result of direct inhibition of methanogens (Calsamiglia *et al.*, 2007). Essential oils may affect the unique methanogen cell membrane structure (isoprenoid unit) that leads to cell destruction. Studies with essential oils have not considered the possibility that methanogen species may change in response to treatments as an adaptive response. This would complicate targeted efforts to minimize the methane production (Ohene-Adjei *et al.*, 2008). In addition to studies of effects of essential oils on methanogens, a number of other studies have made indirect observations based on measurements of VFA production and VFA proportions (Szumacher-Strabel and Cieslak, 2010; Vasta and Bessa, 2012).

Several studies have investigated effects of saponins, tannins and essential oils on rumen fermentation, including methane production, but only a few have specifically determined their effects on methanogen population. The most recent results are presented in Table 1 for saponins, Table 2 for tannins and Table 3 for essential oils.

Conclusions

Studies of the influence of plant bioactive components (phytochemicals) on methane control in ruminants should be performed using as far as possible standardized samples. Natural products like saponins, tannins or essential oils occur

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in the plant material as multicomponent mixtures. The composition of the mixture and quantitative relations of individual compounds may change under different circumstances. The plant variety, time of harvest, climate, water availability or even sample treatment after plant harvest may influence qualitative/quantitative relations of the sample.

We can generally conclude that the saponins mitigate methanogenesis mainly by reducing the number of protozoa; CTs act both by reducing the number of protozoa and by a direct toxic effect on methanogens, whereas essential oils act mostly by a direct toxic effect on methanogens. However, because the rumen is the complex ecosystem, analysis of influence of plant components on the populations of methanogens should take into account not only the total population of methanogens but also individual orders or species. Most of tested plant and plant components have presented their antimicrobial activity in *in vitro* research when tested at high doses. The limited scientific information available from long-term *in vivo* trials suggested that benefits associated with bioactive components *in vitro* are not always obtained *in vivo* or are diminished over time due, for example, microbial adaptation. Hence, there is an urgent need to establish a clear definition of the optimal active dose of plant components that can be used as additives for ruminants.

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