Chapter 12
Effect of Plant Secondary Metabolites on Rumen Methanogens and Methane Emissions by Ruminants

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Abstract Methanogenesis occurs in the rumen to take care of reducing power generated during fermentation of feed and accounts for a significant loss of energy offered to the ruminants as feed. Once carbon dioxide is reduced to methane, it cannot be oxidized to release energy under the anaerobic conditions prevailing in the rumen. To save this energy loss, several chemicals have been tested and some of them are very effective in selectively inhibiting methanogenesis, but these chemicals cannot be used in practical feeding of livestock due to their adverse effects on other rumen microbes, health of the animals and the quality of livestock products. Therefore, plants containing secondary metabolites might be superior feed additives to control methanogenesis without affecting other microbes of the rumen. *In vitro* screening experiments conducted in many laboratories have indicated that methanogenesis can be inhibited by inclusion of plants/plant extracts in the substrate. Some of the plants which showed *in vitro* methane inhibition are : *Allium sativum, Azadirachta indica, Emblica officinalis, Eugenia jambolana, Ficus benghalensis, Foeniculum vulgare, Lotus pedunculatus, Mangifera indica, Ocimum sanctum, Populus deltoides, Psidium guajava, Quercus incana, Sapindus mukorossi, Sapindus rarak, Sesbania sesban, Syzygium aromaticum, Trachyspermum ammi, Terminalia chebula* and *Yucca schidigera,* but some of them do have adverse effects on rumen fermentation and feed digestibility. Several of the above plants have been tested *in vivo* as feed additives in different ruminants either alone or in a combination and have shown significant decrease in *in vivo* methane emission and no adverse effect on feed utilization when used at the rate of 1–2% of dry matter intake. There is a need to screen larger number of plants containing secondary metabolites and to study the effect of feeding these compounds on the feed utilization and the quality of livestock products.

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A.K. Patra (ed.), *Dietary Phytochemicals and Microbes*,
DOI 10.1007/978-94-007-3926-0_12,

Keywords Plant secondary metabolites • Essential oils • Saponins • Tannins • Ruminal microbiota

Abbreviations

ADF	Acid detergent fibre
ADG	Average daily gain
CH_4	Methane
CO_2	Carbon dioxide
CT	Condensed tannins
D	Dalton
DDM	Digested dry matter
DGGE	Denaturing gradient gel electrophoresis
DM	Dry matter
DMI	Dry matter intake
EMP	Embden-Meyerhof pathway
EO	Essential oils
EOm	Essential oil mixture
HAP	Hyper ammonia producing
MW	Molecular weight
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NDF	Neutral detergent fibre
NH_3-N	Ammonia nitrogen
NO_3	Nitrate
OM	Organic matter
PCR	Polymerase chain reaction
PSM	Plant secondary metabolites
rDNA	Recombinant deoxyribonucleic acid
SO_4	Sulphate
TS	Tea saponins
VFA	Volatile fatty acids

12.1 Introduction

Ruminants have a unique capability of converting a non utilizable energy source of feed (lignocellulosic agro-industrial by-products) to an utilizable form of energy i.e. volatile fatty acids, which the ruminants can use as a source of energy. The other animals like monogastrics or non ruminant herbivores either are not able to digest lignocellulose or they do it only partially and inefficiently. The process is completed in three steps: cellulose and hemicellulose are released from the lignocellulosic

feeds by the activity of esterases; cellulose and hemicellulose are depolymersized to monomers (hexoses and pentoses) by the activity of glucanases and finally these monomers are partially oxidized to volatile fatty acids with a release of carbon dioxide and hydrogen. The capability of any animal to digest fibrous lignocellulosic feed depends upon the volume of fermentation sac (rumen, caecum or colon) where these fibre degrading microbes harbour and help in anaerobic oxidation of feed. In ruminants (cattle, buffalo, sheep, goat, deer etc.), such fermentation sac, the rumen is the first and the largest compartment of complex stomach, making up about 80% of total stomach volume and around 15–20% of the body weight of animals. Therefore, the ruminants are ideal animals to convert agricultural wastes like straws, stovers, oil cakes, brans and green fodders into edible meat and milk products and do not face any competition with the human beings for their feed.

When hexoses and pentoses are fermented anaerobically as in the rumen, reduced cofactors like NADH and NADPH are produced through EMP pathway. For re-use in the anaerobic ecosystem, these reduced cofactors have to be oxidized to NAD^+ and $NADP^+$ by electron transfer to acceptors other than oxygen like CO_2, SO_4, NO_3 etc., as a result these electron acceptors are reduced to methane, hydrogen sulphide and ammonia, respectively. If this reducing power concentrated in the reduced cofactors is not utilized for reduction of an oxidized compound, further fermentation of carbohydrates will be stopped and no release of energy from feed will be possible. Therefore, to continue release of energy for the animals, methane (by reduction of carbon dioxide by the reduced cofactors) has to be generated in the rumen. But in this process about 5–15% of gross energy intake by the animals is wasted in the form of methane (Johnson and Johnson 1995). Therefore, research efforts are needed to suppress methane emission for eco-friendly and economic livestock production.

There are many possibilities of mitigation of methane emission by the livestock e.g. use of chemicals, selective removal of ciliate protozoa, elimination of high methane producing or unproductive animals, microbiological and biotechnological interventions to inhibit growth of methanogens in the rumen, but each one of the methods listed above have one or the other limitation or have toxic effects on fermentation of feed in the rumen. Therefore, their practical application in the field is not visible in the near future. During the last one decade, the researchers have shown interest in using plants containing secondary metabolites (PSM) as feed additives to control methanogenesis. In this chapter efforts have been made to compile information on these PSM to assess the possibility of using them as feed additives to inhibit methanogenesis and their effect on productivity of the livestock.

12.2 Plant Secondary Metabolites

The plant secondary metabolites (PSM) are a vast variety of chemical compounds synthesized in plants that are not involved in the primary biochemical processes of growth and reproduction. These bioactive compounds, which have anti-microbial

activities, are meant for protection of the host plant against invasion by the foreign particles including pathogenic microbes. Therefore, these compounds have been used as medicine in traditional system of medicare in India, China, Srilanka, Japan and other Asian and African countries, for preservation of foods and as spices in kitchen in many parts of the world since time immemorial. More than 200,000 defined structures of plant secondary compounds have been identified. These PSM can generally be classified into three major groups: saponins, tannins and essential oils (EO).

Plant secondary metabolites might inhibit methane emission by the following modes:

- They might directly inhibit methanogens as these compounds have anti-microbial activities against different microbial groups.
- The plant secondary metabolites might have anti-protozoal activity, which might indirectly result in reduced number of methanogens. As the ciliate protozoa and methanogens have an ecto-symbiotic relationship, the latter might lose their symbiotic partners and hydrogen supply due to killing of ciliates by the plant secondary metabolites and therefore, might result in reduced production of methane.
- As the plant secondary metabolites have anti-microbial activity, which might reduce the numbers of bacteria and fungi and thus result into lower digestibility of feed and consequently cause a decrease in methanogenesis as the feed degradation and methane production are directly related to each other.

12.2.1 Tannins

The word "Tannin" was originally coined by Seguin to describe substances present in vegetable extracts, which are responsible for converting animal skin into leather. In plant extracts, these substances exist as polyphenols of varying molecular sizes and complexities. One of the most appropriate definitions of tannins was given by Horvath (1981), "Any phenolic compound of sufficiently high molecular weight containing sufficient hydroxyl and other suitable groups like carboxyl to form effective strong complexes with protein and other macromolecules under the particular environmental conditions being studied".

Bate–Smith (1972) defined tannins as water soluble phenolic compounds having a molecular weight between 500 and 3,000 D. These polyphenols contain a large number of hydroxyl or other functional groups (1–2 per 100 D) and therefore are capable of forming cross–linkages with protein and other macromolecules. Tannins are usually subdivided into two major groups: hydrolysable and condensed tannins and are considered to have both adverse and beneficial effects depending upon their concentration and nature besides other factors such as animal species, physiological state of animal and composition of diet (Makkar 2003).

Hydrolysable tannins split into sugars and phenolic carboxylic acids both in acid and alkaline conditions (White 1957). These are further classified according to the

products of hydrolysis, into gallo-tannins (gallic acid and glucose) and ellagi–tannins (ellagic acid and glucose) (McLeod 1974). Condensed tannins are often referred to as proanthocyanidins because they produce red anthocyanidins when heated in acid (Haslam 1982). Proanthocyanidins are phenylpropanoid polyphenols and are categorized by the type of monomers they contain–flavan-3-ols or flavan-3, 4-diols–into catechins or leucanthocyanidins (Horvath 1981). Besides hydrolysable tannins and proanthocyanidins, a group called beta–tannins also exists (Swain 1979; Horvath 1981), which are protein precipitating compounds and are insoluble in water. They form very stable bonds with protein and have lower molecular weight than other tannins.

The molecular weight (MW) and chemical structures of CT play a key role in their biological activity. The CT fractions with the highest MW had the highest inhibition (62% lower than the control) (Huang et al. 2011). Inclusion of 15 mg of CT/500 mg DM reduce CH_4 production by 47%, total methanogens and total protozoa number decreased with increasing levels of CT (Tan et al. 2011). However, higher CT inclusions, with further reduction in CH_4 emissions, have substantive negative effects on DM digestibility.

Methane production was completely inhibited in pure cultures of methanogens incubated with big trefoil (*Lotus pedunculatus*) compared with alfalfa, suggesting that these phenolics directly inhibit methanogen metabolism (Tavendale et al. 2005). In contrast, Beauchemin et al. (2007) reported that the diet of growing beef cattle supplemented with 18 g CT /kg DM from quebracho Colorado trees had no effect on enteric CH_4 emissions or DM digestibility. The effects of forage legumes containing condensed tannins on methane and ammonia production in continuous cultures of mixed ruminal microorganisms did not influence apparent digestibility of dry matter or neutral detergent fiber (Williams et al. 2011).

Terminalia belerica (bahera, 23.6–37.4% tannins) and *Terminalia chebula* (harad, 30–32% tannins in dry fruit pulp) contain tannins as secondary metabolites and the methanol extracts of both the plants showed antimethanogenic activity but harad (almost complete methane inhibition) was more effective as compared to bahera. The methane inhibition in this case was also associated with reduction in protozoa population and feed degradability (Patra et al. 2006b). The results indicated that tannins inhibited rumen methanogenesis through interaction with the rumen microbes. Adverse effect of tannins on feed digestion, microbial population and enzyme activity has been demonstrated in many studies (McSweeney et al. 2001; Hristov et al. 2003). As a mixture of both types of tannins (in extracts or ground samples of plant parts) has been used in most of the studies, it is difficult to ascertain which individual part is more effective against methanogenesis in the rumen. The condensed tannin rich leguminous fodders have anti-methanogenic activity, hence, it might be possible that condensed tannins are more detrimental for methanogens (Puchala et al. 2005). As the tannins have direct effect on methanogens, there might be some adverse effects on fiber degradation and lower hydrogen production.

Different sources of tannins have different effects on gas production and methane production. In an *in vitro* study with three condensed tannins (quebracho, grape seed, and green tea tannins) and four hydrolysable tannins (tara, valonea, myrabolan, and

chestnut tannins), Quebracho, valonea, myrabolan and grape seed decreased gas production and the maximum rate of CH_4 production, whereas addition of chestnut, green tea and tara tannins neither affected total gas nor CH_4 production (Pellikaan et al. 2011).

Findings from PCR-DGGE and RT-PCR analysis suggest that inclusion of CT altered the diversity of rumen methanogens without affecting total methanogen number (Mohammed et al. 2011). They also reported that *Methanobrevibacter smithii* B181, *Methanosphaera stadtmanae*, Methanogenic archaeon LGM-AFM09 and *Methanobrevibacter smithii* strain ALI-A increased while *Methanobrevibacter* sp. WBY1 and *Methanobrevibacter millerae* strain ZA-10 decreased with an increase in the proportion of CT in the diet.

12.2.2 Saponins

As the name indicates saponins are soapy in nature and are glycosides consisting of a steroid (C27) or a triterpenoid (C30) sapogenin nucleus with one or more carbohydrate branches. The degradability of saponins in the rumen depends upon many factors including its structure. Makkar and Becker (1997) reported that quillaja saponins are degraded by mixed microbial population of the rumen of cows and that of alfalfa (lucerne) were rapidly released into the rumen fluid and extensively degraded in the digestive tract of sheep, but the final products of degradation have not been identified (Mathison et al. 1999).

Methanogen population was decreased in the presence of *Sesbania sesban* saponins by 78%, Knautia saponins by 21% and fenugreek saponins by 22% in *in vitro* fermentation from cattle rumen liquor (Goel et al. 2008). The addition of 0.14 and 0.29 g/l of *Trigonella foenum-graecum* seed extract containing 34.5% saponins did not reduce methanogen numbers in an *in vitro* gas production test (Goel et al. 2008). It has been suggested that saponins may decrease methanogen populations through a reduction in the numbers of protozoa. Methanogens associated with protozoa may account for decreased methane production by 9–25% (Newbold et al. 1995) and as much as 37% (Finlay et al. 1994).

Saponins have anti-protozoal activity and affect the rumen fermentation significantly. Thalib et al. (1996) reported that methanol extract of *Sapindus rarak* fruit (0.07% of body weight, every 3 days) resulted in 57% reduced protozoal population, 69% increased bacterial numbers, significantly reduced ammonia nitrogen, greater daily body weight gain and improved feed conversion efficiency. It has been observed that ethanol extract of soapnut was superior to methanol and water extracts as far as the inhibition of protozoa and methane are concerned (Agarwal et al. 2006).

The saponins affect different bacteria of the rumen differently as evidenced by Wang et al. (2000) and confirmed by Hess et al. (2003) who reported that total bacteria and methanogens were not affected adversely by *Sapindus saponaria*, but rumen protozoa were significantly lowered. It was interesting to note that methanogenesis was reduced by 20% without affecting the degradation of fiber.

Y*ucca schidigera* and *Quillaja saponaria* containing 4.4% and 10% saponins (Wang et al. 1998) decreased polysaccharide degrading enzymes (carboxymethylcellulase and xylanase) considerably and inhibited protozoa to the extent of 42% and 54% respectively (Hristov et al. 1999, 2003). Wang et al. (1998) studied the effect of yucca extract (0.5 mg/ml buffer) on rumen fermentation in RUSITEC and reported no effect on dry matter digestibility, gas production and volatile fatty acid production, but the protozoa numbers were significantly reduced, while the number of bacteria was not affected.

Lila et al. (2003) studied the effects of different concentrations of sarsaponins of *Yucca schidigera* on ruminal microbial methane production using different substrates. Ammonia nitrogen and the numbers of protozoa were decreased with increasing dose of saponins. Total volatile fatty acids and gas production were increased. Molar proportion of acetate was decreased and propionate was increased with a corresponding decrease in acetate: propionate ratio. There was a decrease in methane production from 20% to 60% on different substrates.

The modified or partially degraded saponins, the sapogenins are degraded more slowly, but are not toxic for the ciliate protozoa. Interestingly in the experiment of Newbold et al. (1997) too the protozoa numbers reached the initial levels within 9 days. Thus the anti-protozoa effect is not consistent. This is not attributed to the fact that protozoa become resistant, but some other microbial group becomes active in degrading the anti-protozoal component of the extract. *Equisatum arvense* and *Salvia officinalis* inhibited methanogenesis and methane reduction was not associated with numbers of protozoa in the rumen liquor (Broudiscou et al. 2000).

Tea saponins (TS) and soybean oil had an inhibitory effect on methane production in growing lambs when they were added to the diets, but they showed different action against the protozoa, methanogens and other rumen microbes involved in feed degradation. Lambs fed diets with TS showed decreased daily methane production by 27.7%. The concentrations of total volatile fatty acids and microbial protein were increased with addition of TS with little effect on fungal population but protozoa populations relative to total bacterial 16S rDNA were decreased. Addition of TS with Soybean Oil had an inhibitory effect on the population of methanogens, fibrolytic microbes including *R. flavefaciens* and *F. succinogenes* (Mao et al. 2011). Tea saponins had similar effect on methane reduction as that of defaunation (2.1 L/day) with TS to (2.5 L/day) with defaunation (Zhou et al. 2011). A detailed discussions on thee effects of saponins on rumen microbial populations and fermentation characteristics have also been presented in Chap. 11.

12.2.3 Essential Oils

Essential oils (EOs) are steam-volatile or organic-solvent extracts of plants and are present in different parts of plants such as flowers, leaves, stems, bark, fruit pulps, roots and seeds. The concentrations of EOs might vary with the type of plant, stage of growth, plant health and environmental factors such as light, temperature, moisture and stress (Hart et al. 2008). Like other plant secondary metabolites, the

EOs too protect the plants against bacterial, fungal or insect invasion. Their use as food preservative and in traditional medicine is also very well known only because of their antimicrobial effects. In addition, they also possess biological activities as antioxidants, as hypocholesterolemics, as stimulant of digestive systems and as liver function enhancer (Craig 1999; Ramakrishna et al. 2003; Hernandez et al. 2004).

The most commonly occurring EOs are classified in two groups depending upon their chemical structure: terpenoids (monoterpenoids and sesquiterpenoids) and phenylpropanoids, which are synthesized through the mevalonate and shikimic acid metabolic pathways, respectively (Gershenzon and Croteau 1991; Calsamiglia et al. 2007). The terpenoids and phenylpropanoids act against bacteria through interaction with the cell membrane (Dorman and Deans 2000), which causes conformational changes in the membrane structure, resulting in its fluidification and expansion. The loss of membrane stability results in the leakage of ions across the cell membrane and causes a decrease in the trans-membrane ionic gradient. In most cases, bacteria can counter balance these effects by using ionic pumps and cell death does not occur, but large amount of energy is diverted to this function and bacterial growth is slowed down (Griffin et al. 1999; Ultee et al. 1999; Cox et al. 2001).

Essential oils have been examined for antimicrobial properties against rumen bacteria and many of them have been found to have strong activities. Therefore, research to exploit EOs as feed additive in animal nutrition has been accelerated in search for an acceptable feed additive, which can be used as a rumen modifier. McIntosh et al. (2003) reported that a mixture of EOs inhibited the growth of some hyper-ammonia producing (HAP) bacteria (*Clostridium sticklandii* and *Peptostreptococcus anaerobiosus*), but other HAP bacteria (*Clostridium aminophilus*) were less sensitive. This inhibition of HAP bacteria is diet dependent as reported in a study by Wallace (2004), where the number of HAP bacteria reduced by 77% in sheep receiving a low protein diet supplemented with EOs at 100 mg day^{-1}, but that EOs had no effect on HAP bacteria when sheep were fed a high-protein diet. At low doses, EOs could selectively inhibit the HAP bacteria, but all micro-organisms are adversely affected at higher concentrations (Wallace 2004). Similarly, Evans and Martin (2000) also reported that thymol selectively inhibited the growth of *Selenomonas ruminantium* at 90 mg L^{-1}, but not *S. bovis,* while at 400 mg L^{-1} all rumen microbes were inhibited. Individual EOs had different effects on mixed ruminal bacteria. Monoterpene hydrocarbons were less toxic and sometimes stimulatory to microbial activity compared with the corresponding oxygenated compounds, the monoterpene alcohols and aldehydes (Oh et al. 1967, 1968, Table 12.1).

There are several reports which indicate that essential oils do not have any adverse effect on ciliate protozoa in the rumen. McIntosh et al. (2003) observed that the bacteriolytic activity of rumen ciliate protozoa was unaffected in dairy cows supplemented with 1 g day^{-1} of mixed EOs and Newbold et al. (2004) and Benchaar et al. (2007a) reported that ruminal protozoa counts were not affected when sheep and dairy cows were fed 110 and 750 mg day^{-1} of a mixture of EOs, respectively. The extract of fennel (containing essential oils) had no effect on protozoa as reported by Patra et al. (2010).

Mentha piperita and *Eucalyptus globulus* oils, however, adversely affected ciliate protozoa *in vitro,* where the adverse effect increased with increasing concentration

Table 12.1 Essential oils, their active principles and effect on rumen fermentation and the animal

Plant part	Active principle	Effects	Reference
Trachyspermum copticum (ajwain) oil	Thymol [5-methyl-2-(1-methylethyl) phenol; $C_{10}H_{14}O$]	Active against a wide range of Gram-positive and negative bacteria, reduced methane production	Evans and Martin (2000), Pawar (2011)
Cinnamomum zeylanicum (cinnamon) oil	Cinnamaldehyde (3-fenil-2-propenal phenol; C_9H_8O)	Inhibits peptidolysis and methanogenesis, reduces acetate to propionate ratio	Cardozo et al. (2004), Busquet et al. (2005a), Pawar (2011)
Eugenia (Syzygium) aromaticum (clove) oil	Eugenol (4-allyl-2-methoxyphenol; $C_{10}H_{12}O_2$)	Inhibits methanogenesis, ammonia and VFA production	Davidson and Naidu (2000), Patra et al. (2009)
Allium sativum (garlic) oil	Allicin ($C_6H_{10}OS_2$), diallyl sulfide, diallyl disulfide and allyl mercaptan	Antiparasitic, insecticidal, anticancer, antioxidant, immunomodulatory, anti-inflammatory, hypoglycemic, anti-methanogenic	Reuter et al. (1996), Busquet et al. (2005b), Patra et al. (2009)
Eucalyptus globulus (eucalyptus) oil	Cineole, pinene and other terpenes, phellandrene	Effective against bacteria, methane inhibition	Kumar et al. (2009)
Mentha piperita (peppermint) oil	Menthol ($C_{10}H_{12}O$)	Methane inhibition, adverse effect on VFA production	Craig (1999), Agarwal et al. (2009)
Origanum vulgare (oregano) oil	Carvacrol	Increased butyrate, reduced propionate	Busquet et al. (2006), Evans and Martin (2000)
Pimpinella anisum (anise) oil	Anethol	Decreased VFA, acetate and propionate	Davidson and Naidu (2000), Busquet et al. (2006)

of essential oils in the medium (Agarwal et al. 2008; Kumar et al. 2009). It has also been observed that clove extract containing EOs decreased total numbers of protozoa, small entodiniomorphs and holotrichs, but did not affect large entodiniomorphs (Patra et al. 2010). In addition to *in vitro* studies, Ando et al. (2003) also reported *in vivo* that feeding 200 mg day^{-1} of peppermint oil (*Mentha piperita* L.) to Holstein steers decreased total number of protozoa and that of *Entodinium, Isotricha* and *Diplodinium.* However, Cardozo et al. (2006) observed that addition of a mixture of cinnamaldehyde (180 mg day^{-1}) and eugenol (90 mg day^{-1}) to the diets of beef heifers increased number of holotrichs and had no effect on entodiniomorphs, but there was no effect on numbers of these protozoa species when the mixture contained higher concentrations of cinnamaldehyde (600 mg day^{-1}) and eugenol (300 mg day^{-1}). Recently, Yang et al. (2010b) also observed that cinnamaldehyde supplemented at the rate of 0.4–1.6 g day^{-1} in steers did not affect total protozoa as well as *Isotricha, Dasytricha* and *Entodinium* sp.

The rate of fermentation of feed in the rumen is affected by several factors. Any prominent change in fermentation pattern is reflected first of all by variation in pH of the rumen liquor. Benchaar et al. (2006b) observed that ruminal pH was increased (6.50 *vs*. 6.39) by the addition of essential oils (Crina Ruminants; 2 g/day) and it tended to increase in lactating dairy cows fed essential oils. Beauchemin and McGinn (2006) observed that feeding essential oils (Crina Ruminants; 1 g/day) in cattle had no effect on ruminal pH.

The VFA concentration was not affected in lactating cows fed on alfalfa silage based diet, but were decreased when fed on the corn-silage based diet with the addition of 0.75 g day^{-1} of an EO mixture (Benchaar et al. 2007a). The acetate to propionate ratios were increased (Benchaar et al. 2007b; Macheboeuf et al. 2008; Agarwal et al. 2009) or some times were not affected (Wang et al. 2009; Kumar et al. 2009). Similarly, Yang et al. (2007) reported that the pH, concentration of ammonia nitrogen and total VFA were not affected by dietary supplementation of garlic oil (5 g/cow/day). In contrast, some studies showed an increase in concentrations of total VFA in the rumen liquor due to supplementation of cinnamaldehyde at the rate of 0.2 g kg^{-1} DM intake (Chaves et al. 2008a, b) and EO extract from oregano at 0.25 g kg^{-1} DM intake (Wang et al. 2009).

Thymol (0.4 g L^{-1}), the main component of EOs derived from Thymus and Origanum plants, a strong inhibitor of *in vitro* methane production (Evans and Martin 2000), caused a reduction in methane to the extent of 99% at 6 mM concentration (Macheboeuf et al. 2008). Anethole at 20 mg L^{-1} of medium caused an inhibition of methane *in vitro* (Chaves et al. 2008c). Other EOs like, Juniper berry EOs and cinnamon oil (Chaves et al. 2008c) and peppermint oil (Tatsouka et al. 2008; Agarwal et al. 2009) have been shown to have a strong inhibitory effect on methanogenesis. The active component of cinnamon oil i.e. cinnamaldehyde caused a depression in methane production to the extent of 94% at 5 mM (Macheboeuf et al. 2008). Methanol and ethanol extracts of fennel seeds and clove buds inhibited *in vitro* methane production (Patra et al. 2010). Eucalyptus oil inhibited methane production up to 58% at 1.66 mL L^{-1} (Kumar et al. 2009), 90.3% at 2 mL L^{-1} (Sallam et al. 2009) and 70% at a dose of 0.33 g of α-cyclodextrin-eucalyptus oil complex

(Tatsouka et al. 2008). The component of eucalyptus oil, p-cymene decreased methane by 29% at a concentration of 20 mg L^{-1} (Chaves et al. 2008c), however, α-cyclodextrin cineole did not influence methane up to a concentration of 0.33 g L^{-1} (Tatsouka et al. 2008).

Sallam et al. (2011) used four different EO *in vitro* isolated from *Achillea santolina, Artemisia judaica, Schinus terebinthifolius* and *Mentha microphylla*. The main components of the EO were piperitone (49.1%) and camphor (34.5%) in *A. judaica,* 16-dimethyl 15-cyclooactdaiene (60.5%) in *A. santolina,* piperitone oxide (46.7%) and cis-piperitone oxide (28%) in *M. microphylla*, and g-muurolene (45.3%) and α-thujene (16.0%) in *S. terebinthifolius*. The EO from *A. santolina* and *A. judaica* at all levels increased the gas production significantly, but *S. terebinthifolius*, *A. santolina* at different levels and all levels of *M. microphylla* decreased gas production significantly in comparison to that in control. The highest levels of *A. santolina, A. judaica* and *M. microphylla* inhibited the methane production along with a significant reduction in true degradation of dry matter, organic matter, protozoa count and NH_3-N concentration. It can be suggested that the EO has a promising methane mitigation effect. Whereas in another study with eucalyptus oil (*Eucalyptus citriodora*), methane emission (mL/g DM) was reduced by 53% and 57% with eucalyptus (Sallam et al. 2010).

The *in vivo* studies of Wang et al. (2009) showed that inclusion of 0.25 g day^{-1} of EO mixture from oregano plants in the diet of sheep for 15 days lowered methane emission. However, *in vivo* studies of Beauchemin and McGinn (2006) with EO mixture fed to beef cattle (1 g day^{-1}) for 21 days did not reveal any effect on methanogenesis. Many a times it has been observed that some essential oils have an inhibitory effect on methane production *in vitro*, but when tested *in vivo*, a similar effect might or might not be observed. There might be many reasons for that as *in vitro* tests usually have many limitations (Flachowsky 2009).

No effect on dry matter intake (DMI) was observed when lactating dairy cows were fed a mixture of essential oils (750 mg/day; Crina Ruminants) or on supplementation of a mixture of essential oils (1 g/head/day) (Beauchemin and McGinn 2006). Similarly, Yang et al. (2007) also reported no change in intakes of dry matter (DM), organic matter (OM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) by dietary supplementation of garlic oil or juniper berry oil (2 g/cow/day) in cows. However, Kung et al. (2008) observed that dry matter intake was greater in cows fed a blend of essential oils (1.2 g/cow/day) compared with cows fed control diet (28.3 *vs.* 26.4 kg/day). Benchaar et al. (2006a) reported that feeding higher amounts (2 and 4 g/head/day) of a blend of essential oil compounds (Vertan; IDENA, Sautron, France) increased DMI of growing beef cattle fed silage based diets. But Cardozo et al. (2006) reported that supplementation of a mixture of cinnamaldehyde (0.6 g/day) and eugenol (0.3 g/day) oils decreased DMI, whereas, feeding capsicum oil (1 g/day of capsicum extract containing 15% capsaicin) increased DMI in Holstein heifers.

The retention of nitrogen was not affected in lactating dairy cows or in beef cattle (Benchaar et al. 2006a) fed different doses of a mixture of essential oil compounds. In contrast, Yang et al. (2007) observed that ruminal digestibility of nitrogen increased with the dietary supplementation of garlic oil (5 g/cow/day). There is not

much information available on effects of EOs or their compounds on performances of ruminants. Bampidis et al. (2005) observed no change in average daily gain (ADG) and feed conversion efficiency when growing lambs were fed diets supplemented with oregano leaves (*Origanum vulgare* L.) providing 144 or 288 mg of oregano oil (850 mg g^{-1} of carvacrol) per kilogram of dietary DM. Similarly, Beauchemin and McGinn (2006) observed no change in ADG of cattle supplemented with a mixture of essential oils (Crina Ruminants; 1 g/day). Benchaar et al. (2006b) reported no change in ADG of beef cattle fed a silage-based diet supplemented with 2 or 4 g day^{-1} of a mixture of EOs consisting of thymol, eugenol, vanillin and limonene. However, the EO mixture had a quadratic effect on feed conversion with a dose of 2 g day^{-1} improving feed conversion efficiency as compared to the dose of 4 g day^{-1}. Also, Yang et al. (2010) observed that dietary supplementation of cinnamaldehyde at 400, 800, or 1,600 mg/steer/day improved growth performance in steers. However, higher ADG (250 or 254 *vs.* 217 g day^{-1}) was observed when cinnamaldehyde or juniper berry EO was added to a barley-based diet at a similar concentration (0.2 g kg^{-1} of dietary DM). Soltan (2009) assigned 100 Holstein male calves to investigate the effect of essential oil mixture (eucalyptus oil, menthol crystal, mint oil; EOm) supplementation in milk replacer (0, 94, 187 and 281 mg/calf/day) during 8 weeks (pre-weaning period) and in drinking water (0, 15.6, 31.2 and 46.8 mg/l) for the next 16 weeks (post-weaning period). The results showed EOm supplementation at different levels in milk replacer had no effect on body weight gain when compared with the control which was attributed to lower concentrate intake by the treated calves during the whole period of experiment. However, during post-weaning period, calves administrated 15.6 mg of EOm/l of drinking water improved daily body weight gain, reduced feed intake and improved FCR as compared to control. Thus, it appears that the influence of EO on growth performance is diet dependant.

12.3 Selection of Plants Containing Secondary Metabolites

In the last one decade a large number of laboratories, throughout the globe have screened a lot of plants containing secondary metabolites with different objectives to:

- inhibit methanogenesis and ciliate protozoa,
- stimulate the activity of fiber degrading anaerobic fungi or bacteria to extract more energy from lignocellulosic feeds,
- inhibit ammonia production in the rumen for economic utilization of nitrogen by the animal,
- inhibit protein degradation in the rumen so that intact protein is available in the lower part of the gastro-intestinal tract or
- detoxify anti-nutritional factors if these are present in the feed offered to the animals.

The degree of success achieved in each of the experiments is variable. A large number of plant extracts have been screened for their potential to inhibit

methanogenesis and ciliate protozoa growth in the rumen of buffalo (Kamra et al. 2006, 2008, 2009). In addition to these two important parameters, fibre degrading enzyme profile, *in vitro* feed digestibility, ammonia production, volatile fatty acid production and sometimes microbial profile were also studied to get as much information as possible on each of the plant products so that possibility of their use as a rumen modifier could be explored. Out of 93 plant extracts tested, 11 inhibited *in vitro* methanogenesis to the extent of 25–50% and nine plant extracts inhibited more than 50%. Among these 20 extracts exhibiting antimethanogenic activity, nine were ethanol extracts, ten were methanol extracts and only one was water extract. Some of these plant extracts inhibited ciliate protozoa as tested by microscopic examination and ^{14}C-labelled radio-isotopic technique, but the protozoa inhibition was not correlated with methane inhibition, indicating that the methanogens sensitive to plant secondary metabolites might or might not be having any symbiotic relationship with ciliate protozoa. Methane inhibition was accompanied with a drastic fall in the number of methanogens as determined by real time PCR. Plants that appeared to have some potential as feed additives to control methanogenesis by the ruminants are: (i) seed pulp of *Sapindus mukorossi* (rich in saponins) and *Terminalia chebula* (rich in tannins), (ii) leaves of *Populus deltoides, Mangifera indica,* and *Psidium guajava* (rich in tannins and essential oils) and (iii) flower buds of *Syzygium aromaticum* and bulb of *Allium sativum* (rich in essential oils). The anti-methanogenic activity of most of these plants, especially the spices has been reported for the first time (Patra et al. 2006a, b). Some of these results have been summarized in Table 12.2. Most of this work was conducted in a multi-locational project funded by International Atomic Energy Agency, Vienna, Austria.

Similarly another large experiment on screening of plants containing secondary metabolites has been completed in the "RUMEN-UP" project in Europe. Primarily the foliage plants (450 in number) were screened *in vitro* for their potential to inhibit methanogenesis by the rumen microbes. The selection of plants was restricted to those which were growing or could be grown in European countries, therefore excluding most of the plants growing in the tropical regions of the world. Out of 450 plants examined in this project, 35 plants inhibited methane by more than 15% and only six plants (*Carduus pycnocephalus* L., *Populus tremula* L., *Prunus avium* L., *Quercus robur* L., *Rheum nobile* Hook. F. and Thoms., and *Salix caprea* L.) inhibited methane by more than 25% (Bodas et al. 2008). These plants did not have any adverse effect on any of the fermentation parameters tested, indicating that the secondary metabolites present in these plants were selective inhibitors of methanogenic archaea and did not affect any other rumen microbe at the level used in this experiment. These plants are reported to contain essential oils like hexadecanoic acid in *Carduus* sp. (Esmaeili et al. 2005), flavenoids in *Pycnocephalus* sp. (El Lakany et al. 1997), flavenoids and anthranoid derivatives in *Rheum nobile* (Iwashina 2003), some non-protein amino acids and cyanogenic glycosides in *Prunus avium* and phenolics and isoprenoids in *Populus* sp. and *Salix* sp. (Ikonen et al. 2002). These secondary metabolites have been proposed to be the major principles responsible for anti-methanogenic activities of these plants.

Table 12.2 Effect of seed pulp, tree leaves, spices and their extracts (in water, WE; methanol, ME and ethanol, EE) on inhibition of methane, *in vitro* true digestibility and ciliate protozoa in buffalo rumen liquor

Botanical name	Common name and part of the plant	CH_4 inhibition (%)[a]	Protozoa inhibition (%)	IVTD Reduction (%)	Reference
Cannabis indica	Bhang, leaves	34.42 (EE)	28.05	0.90	Kamra et al. (2008)
Eugenia jambolana	Jamun, leaves	24.27 (ME)	50.21	19.78	Kamra et al. (2008)
Ficus benghalensis	Banyan, leaves	20.17 (P)	7.14	8.24	Zadbuke (2009)
Mangifera indica	Mango, leaves	35.67 (ME)	55.74	−2.71	Kamra et al. (2008)
Ocimum sanctum	Tulsi, leaves	24.81 (ME)	11.26	4.66	Patra et al. (2008)
Populus deltoides	Poplar, leaves	85.86 (ME)	17.91	−2.63	
Psidium guajava	Guava, leaves	81.79 (EE)	52.76	5.30	Kamra et al. (2008)
Quercus incana	Oak banjh, leaves	37.12 (P)	5.81	8.12	Zadbuke (2009)
Quercus semicarpipholia	Oak khurson, leaves	30.00 (P)	5.48	3.17	
Allium sativum	Garlic, bulb	69.73 (ME)	−17.42	5.31	Kamra et al. (2008)
Curcuma longa	Turmeric, root	21.89 (ME)	0.90	5.04	Zadbuke (2009)
Foeniculum vulgare	Fennel, seeds	70.72 (ME)	23.86	9.77	Patra et al. (2006b)
Syzygium aromaticum	Clove, flower bud	85.61 (ME)	52.42	27.39	Patra et al. (2006b)
Trachyspermum ammi	Ajwain, seeds	42.28 (EE)	29.95	0.28	Pawar (2011)
Azadirachta indica	Neem, seeds	34.59 (EE)	14.56	7.71	Patra et al. (2006a)
Emblica officinalis	Amla, seed pulp	27.68 (ME)	−16.50	9.21	
Sapindus mukorossi	Soapnut, seed pulp	95.80 (EE)	52.29	47.64	Agarwal et al. (2006)
Terminalia belerica	Bahera, seed pulp	28.11 (ME)	24.27	3.00	Patra et al. (2006a)
Terminalia chebula	Harad, seed pulp	99.79 (ME)	37.86	14.13	

P powdered, *WE* water extract, *ME* methanol extract, *EE* ethanol extract

[a]Only those plants have been included which showed methane inhibition more than 20% in comparison to their respective controls. Methane inhibition has been calculated in terms of ml of methane produced/g DM

12.4 *In vivo* Feeding Trials

Most of the above studies have been conducted in *in vitro* conditions. There are only a few experiments conducted to report methane inhibition *in vivo*. The results indicate that there are many plants which contain secondary metabolites and are active against rumen methanogenesis. Many times methane inhibition by secondary metabolites in *in vitro* conditions might not be translated into similar effects in *in vivo* conditions. This might happen due to improper selection of the dose of these metabolites in the ration of animals.

In an *in vivo* experiment in sheep with tea saponins it has been reported that saponins inhibit protozoa, methane emission and improved rumen fermentation, where the reduction of methane emission was mediated through inhibitory effect on protozoa (Zhou et al. 2010).

Some experiments indicate positive results of including plants/plant extracts on inhibition of methanogenesis. In one experiment *Terminalia chebula, Allium sativum* and the mixture of two were fed to sheep at the rate of 1% of DMI, resulted in a decreased ($p = 0.09$) methane production by 24%, 11% and 23.5% in *T. chebula, A. sativum* and the mixture of the two, respectively, when expressed as L/kg digestible DM intake (Patra et al. 2010). *T. chebula* is a rich source of tannin (4.89% of DM), whereas, *A. satium* is rich in essential oils. The data indicated that *T. chebula* was more effective as compared to garlic. The reason for low *A. sativum* activity might be due to the instability of allicin, the main secondary metabolite responsible for antimicrobial activity of *A. sativum*.

Murrah buffaloes fed on a diet of wheat straw and concentrate mixture (50:50) and supplemented with a feed additive (a mixture of *Allium sativum,* 1% and *Mentha piperita* oil, 0.1% of DMI) (Mix 1) on every alternate day resulted in 7% reduction in methane emission (l/kg DMI), but this reduction in methane emission was attributed to reduction in dry matter intake (Verma et al. 2009). There was no adverse effect on rumen fermentation pattern, enzyme and microbial profiles.

In another experiment, a mixture of three plants (Mix 3) fed to buffalo calves at the rate of 1%, 2% and 3% of DMI, resulted in a dose dependent inhibition in methane emission (l/kg DDM) since per cent inhibition increased with an increase in dose of the feed additive (Chaudhary et al. 2009) without affecting dry matter digestibility at any of the levels of feed additives tested. The VFA and fibre degrading enzyme activities were not affected, whereas, there were a few changes in the rumen microbial profile as estimated by real time-PCR, but these were not responsible for any significant change in rumen fermentation.

As discussed above there are many plants which have a potential to inhibit methanogenesis in the rumen, but that is not the end. Therefore, screening of plants should be a continuous process to search for more useful ones, which can be used for rumen manipulation. In the secondary screening process, only selected plants should be tested in *in vivo* experiments to examine their potential for practical application.

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