

# Chapter 11

## Saponins: Effects on Rumen Microbial Ecosystem and Metabolism in the Rumen

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**Abstract** Saponins are triterpene and steroidal glycoside compounds in which aglycone (sapogenins) are attached to one or more sugar moieties. They are polar compounds and have great diversity in their chemical structures. Different aqueous or polar solvents have been used to extract saponins. Saponins exhibit several physical and biological properties such as the formation of a stable foam, haemolysis, antimicrobial activity and defaunation of the rumen; however, there is little correlation among these properties. This paper describes the methods of saponin extraction, their chemical diversity and the effects on rumen microbial ecosystem along with metabolism of saponins in the rumen. Recent studies on methane emissions by ruminants provide evidence that saponins may have potential to be used as an antimethanogenic agent; however, the inclusion level of saponin from each source should be tested to get the optimum result. The practical utility of saponins or saponin containing plants as feed additives in sustainable and environmental friendly ruminant production warrants further investigation.

**Keywords** Saponins • Rumen • Microbial ecosystem • Metabolism of saponins • Extraction • Microbial diversity

### Abbreviations

NMR    nuclear magnetic resonance  
VFA    volatile fatty acid  
DGGE    denaturing gradient gel electrophoresis

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PCR	polymerase chain reaction
CLA	conjugated linoleic acid
VA	vaccenic acid
RNA	ribonucleic acid
MPS	microbial protein synthesis

## 11.1 Introduction

Saponins are a structurally diverse family of plant secondary metabolites. In general, saponins consist of an aglycone attached to one or more sugar moieties. They are classified as triterpene saponins and steroid saponins based on their aglycones which can be in the form of triterpene (30 C-atoms) or steroid (27 C-atoms), respectively. Recently, Vincken et al. (2007) proposed a new classification of saponins based on the biosynthesis of carbon skeletons of the aglycone. An enormous diversity found in saponin structures was the reason for this new classification. According to this classification, 11 main classes of saponins were distinguished, i.e. dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes, and steroids. Out of these structures, oleanane is the most common aglycone occurring in plants with glucose, arabinose, rhamnose, xylose and glucuronic acid as the common sugars attached to it. Most saponins are defined as monodesmosides if they contain one saccharide chain attached to C3 atom of their aglycones or bidesmosides, if an additional saccharide chain is attached to C26 (furanstanol saponins) or C28 (triterpene saponins) (Güçlü-Üstündağ and Mazza 2007). Saponins can also be classified as neutral if the sugar attached to sapogenin is a common monosaccharide (glucose, xylose, arabinose etc.) or acidic if the sugar moiety contains uronic acid or one or more carboxylic groups (Lasztity et al. 1998). The aglycone component of the saponin is hydrophobic whereas the conjugated sugars are hydrophilic. Their unique structures allow saponins to give stable foams in water or aqueous solutions.

There are several recent reviews on the effect of secondary compounds including saponin or saponin containing plants on rumen function and animal production (Hart et al. 2008; Patra and Saxena 2009a). This review describes in more details saponin extraction methods, the structural diversity of saponins and their effect on rumen microbes and rumen fermentation. The information presented here could provide wider opportunities for the utilization of saponins and saponin-containing plants as feed additives in sustainable and environmental friendly ruminant production.

## 11.2 Biological Properties of Saponin

The most important property of saponins is their ability to form very stable foam as a consequence of their surfactant ability. The presence of polar groups in the sugar moiety together with the non polar character of the aglycone moiety enables saponins

to lower surface tension in aqueous systems. Traditionally, saponin-containing plants have been used as natural washing agents.

Other biological properties of saponins include their ability to haemolyse the red blood cells, to depress protozoal populations in the rumen, and to inhibit the growth of microbes, especially fungi. The same saponin extract can exhibit several of these biological properties.

The methanol extract of pericarp of *Sapindus rarak* fruit could make a stable foam, haemolyse the red cells (Wina, unpublished) and also depress the protozoa population both *in vitro* and *in vivo* (Wina et al. 2005a, 2006a). Saponins extracted from guar meal in 100% methanol had the ability to haemolyse the red cells and also showed antibacterial activities against the pathogenic bacteria, *Staphylococcus aureus*, *Salmonella typhimurium* and *Escherichia coli* (Hassan et al. 2010a, b). Gestetner et al. (2006) found that the lucerne saponin exhibited haemolytic property and also antifungal activity. Owing to these various phenomena, several authors have tried to link the biological activities of saponins as antifungal, antiprotozoal, antimicrobial or cytotoxic activity to haemolytic activity or foaming ability (Chwalek et al. 2006; Wang et al. 2007). The foaming test and haemolysis test for saponin are simple tests. Therefore, it would be faster and easier to screen many different saponins by either foaming test or haemolysis test and to relate the result to other biological activities of saponins, then, to choose the potential saponins for further use as feed additives or medicinal application.

The result of our study with 23 saponin-containing plants, however, did not find any correlation between height of foam or haemolytic activity to the antiprotozoal activity (Wina, unpublished). Wang et al. (2007) have disclosed from their study with 63 steroidal saponins that there was no correlation between haemolytic activity and cytotoxicity.

Lin and Wang (2010) studied the haemolytic mechanism of saponins using molecular dynamic simulation technique. Saponins first penetrate easily into the lipid membrane, and then accumulate in the lipid raft micro-domain. Saponins bind cholesterol in the lipid membrane and prevent the cholesterol from the interaction with sphingomyelin. Saponin-cholesterol micelles destabilize the structure of the lipid raft micro-domain and cause disruption of the lipid bilayer, which eventually lead to the haemolysis of red cells (Lin and Wang 2010; Baumann et al. 2000). The mechanism of protozoal lysis by saponins is similar to the mechanism of haemolysis of red blood cells by saponins. However, some aglycones still showed their ability to haemolyse the red cells but lost their activity toward protozoa. The presence of intact sugar moiety is very important for its activity against protozoa. Muetzel et al. (2005) reported that the aglycone of *Sapindus rarak* saponins (hederagenin) did not show its ability to depress protozoa population.

From several studies, it can be concluded that antiprotozoal, antimicrobial or cytotoxicity activity of saponins cannot be predicted from the height of foam or its haemolytic activity. Direct screening of the saponin extracts or saponin containing plants based on its specific biological property is recommended.

### 11.3 Biodiversity of Saponins in Plant Materials

Saponins are a complex group of compounds and different species or parts of plant synthesize different types of saponins (Lasztity et al. 1998; Mahato and Garai 1998). Diversity can also occur in one location of the plants. Several saponins in one plant usually have the same aglycone but different sugar moieties. Several species of *Sapindus* trees are found in different parts of the world such as *Sapindus rarak*, *Sapindus emarginatus*, *Sapindus mukorossi* and *Sapindus saponaria*. Their fruits contain foaming substances: it was reported that 20 different monodesmosidic saponins have been isolated and structural elucidated in *Sapindus rarak* fruit's pericarp (Hamburger et al. 1992; Asao et al. 2009).

Hederagenin is the aglycone found in these saponins. When arabinose is directly attached to hederagenin and rhamnose and arabinose attached to arabinose, this saponin is called sapindoside (Fig. 11.1). When arabinose attached to hederagenin and acetyl group attached to arabinose or rhamnose, this saponin is called rarasaponin. Asao et al. (2009) have isolated and identified six different structures of rarasaponin (I to VI) from *Sapindus rarak* fruit's pericarp. Other *Sapindus*, *Sapindus emarginatus* (Kanchanapoom et al. 2001), *Sapindus mukorossi* (Huang et al. 2008) and *Sapindus saponaria* (Tsuzuki et al. 2007) fruit's pericarp contained similar saponins to *Sapindus rarak* with hederagenin as the aglycone. Huang et al. (2008) named the isolated saponin as sapinmusaponin, which was almost identical with rarasaponin in *Sapindus rarak*. They also found damarane type of saponin in the gall of *Sapindus emarginatus*. Kanchanapoom et al. (2001) isolated saponins with acetyl hederagenin and oleanolic acid as the aglycone in *Sapindus emarginatus*. Lemos et al. (1992, 1994) found other saponins in *Sapindus saponaria* with glucose directly attached to hederagenin and rhamnose and arabinose linked to the glucose. The complexity of these saponins has not yet received any attention in relation to their individual activity on rumen microbes.

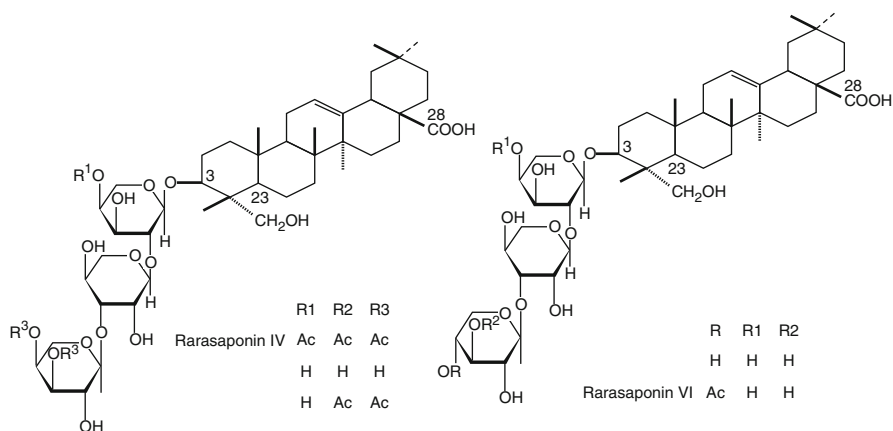


Fig. 11.1 Some saponin structures in the fruit's pericarp of *Sapindus rarak* (Asao et al. 2009)

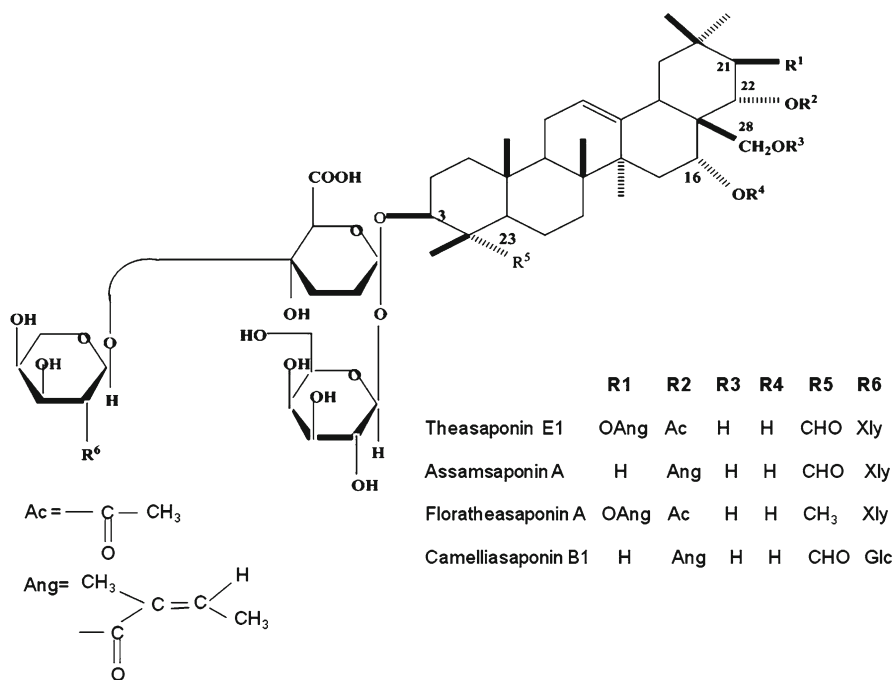


Fig. 11.2 Some saponin structures in tea (*Camellia sinensis*) seed (Yoshikawa et al. 2005)

Several structures of saponin have been identified in tea (*Camellia sinensis*) seeds, leaves and flowers. Saponin extract from tea seed is commercially available and used for killing small fish in shrimp ponds. Recently, it has been tested for a feed additive for ruminants and as a medicinal use. Yoshikawa et al. (2005) reported that 16 different structures of saponins have been identified in tea seeds. Further, they elucidated 12 new saponins in tea seeds (Yoshikawa et al. 2007). Therefore, in tea seeds, 28 different saponins have been isolated and identified as theasaponins, camelliasaponins, floratheasaponins, etc. (Fig. 11.2). Yoshikawa et al. (2005, 2007) showed that the position of acyl group that attached to the aglycone (theasapogenol) and different groups in the sugar moieties are important from a pharmacological point of view since different positions or groups attached to the aglycone or sugar moiety cause different activities.

*Yucca schidigera* stem contained as much as 10% of steroidal saponins which consisted of 28 different structures of spirostanol and furostanol glycosides. *Yucca* saponins have several aglycones, i.e. sarsapogenin, markogenin, smilagenin, samogenin, gitogenin and neogitogenin. They can be monodesmosides with one sugar chain attached at 3-O (Fig. 11.3, 1–4) and bidesmosides with two sugar chains attached at 3-O and 26-O positions (Fig. 11.3, 5–7). The predominant saponins in *yucca* are spirostanol saponins which primarily are glycosides of sarsapogenin (66%). Thus, the major property of *Yucca* saponins as described by Cheeke et al. (2006) is determined by spirostanol saponins (Oleszek et al. 2001).

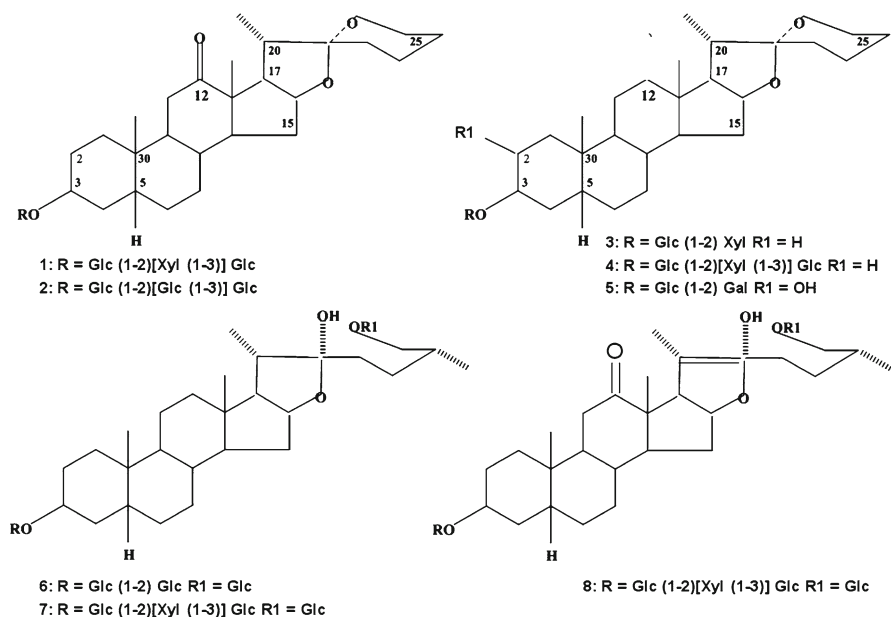


Fig. 11.3 Some saponins of *Yucca schidigera* bark (Cheeke et al. 2006; Oleszek et al. 2001)

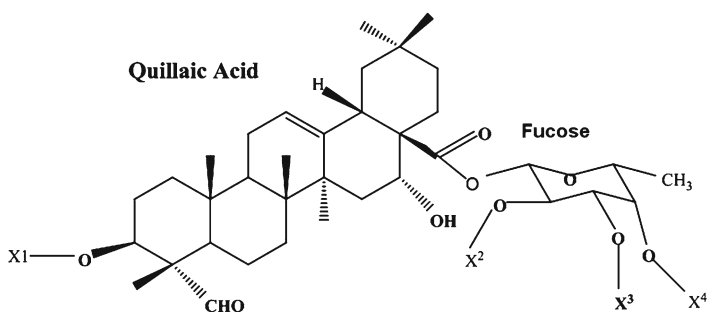


Fig. 11.4 The common basic saponin structure of *Quillaja saponaria* (Bankefors 2006)

*Quillja saponaria* Molina extract contains 10% total saponins, and more than 20 different structures of triterpenoid saponin have been structurally elucidated by NMR studies (Guo et al. 2000) Further, Bankefors (2006) did structural classification of 47 *Quillaja* saponins including minor compounds by electrospray ionisation ion trap multiple-stage mass spectrometry in combination with multivariate analysis from the chromatographic fractions. All studied structures are bidesmosides and consist of quillaic acid (as the aglycone) which is substituted with di or tri-saccharides at C-3 and a branched oligosaccharide at C-28. Bankefors (2006) found that saponin profiles in the young plants were different compared to older specimen; hence the biological and chemical activity differed between batches from different specimen (Fig. 11.4).

## 11.4 Extraction of Saponins

Saponins from plant materials can be extracted using different techniques and solvents. The conventional techniques for saponin extraction used soxhlet, liquid-liquid or solid-liquid extraction (Berhow et al. 2002; Hassan et al. 2010a, b). These methods consume a lot of solvent, time and may lead to potentially deleterious degradation of labile compounds (Kerem et al. 2005). Therefore, in recent years, new extraction techniques include accelerated solvent extraction, supercritical fluid extraction, solid-phase microextraction, sonication, extraction with supercritical or subcritical water, and microwave-assisted extraction have been developed and are considered to be more efficient than the conventional methods (Wu et al. 2001; Kerem et al. 2005; Ligor et al. 2005; Güçlü-Üstündağ and Mazza 2007). Ultrasonication-assisted extraction of ginseng saponins was about three times faster than the liquid-liquid extraction and can be carried out at lower temperature (Wu et al. 2001). Kerem et al. (2005) reported that methanol- microwave assisted method to extract saponin of chickpea proved to be faster and more efficient than soxhlet extraction.

Since saponins are polar compounds, many saponin extraction methods used water, aqueous methanol or ethanol, absolute methanol, ethanol or n-butanol (Kaur and Arora 2009; Xu et al. 2010; Tsuzuki et al. 2007; Zhang et al. 2005). The type of aglycone, type of sugar moiety and functional group attached to aglycone or sugar moiety, the concentration of saponin influence the saponin's ability to dissolve in different solvents, therefore different extracts exerted different activities.

Our study with *Sapindus rarak* fruit's pericarps showed that different extracts had different activity on suppressing protozoa population in the *in vitro* rumen (Wina, unpublished). The 50% methanol extract caused the highest suppression on protozoa population in the *in vitro* rumen fermentation, followed by 70% methanol and ethanol extract. While Kamra et al. (2006) and Agarwal et al. (2006) showed that the methanol extract of *S. mukorossi* was highly detrimental to protozoa in the *in vitro* rumen followed by ethanol and water extracts. Goel et al. (2008a) reported that either 50% methanol or 95% methanol extract of Fenugreek or *Sesbania sesban* gave similar effect in depressing protozoa, while water extract of *Sesbania sesban* showed lower activity to depress protozoa compared to 50% or 95% methanol extract. Patra et al. (2006) found that the ethanol extract of five plants (*Acacia concinna* pod, *Terminalia chebula*, *Terminalia belerica*, *Embllica officinalis* seed pulp and *Azadirachta indica* seed kernel) was more active than methanol or water extract in decreasing protozoa population and *in vitro* methane production. Sirohi et al. (2009) reported that the acetone extracts of several plants have more effective antimicrobial property than methanol or aqueous extracts, whereas aqueous extract of *Sapindus mukorossi* was the best inhibitor of methane production among other extracts. Hassan et al. (2010b) showed that saponins of guar meal that dissolved in 100% methanol had antibacterial activities against *Staphylococcus aureus*, *Salmonella typhimurium* and *Escherichia coli*, while only those dissolved in 20% and 60% methanol stimulated *Lactobacillus* spp. growth. These above reports

showed that different extracts of a specific plant had different activities against microorganism growth or population.

Unless it is purified, the saponins present in an extract may be in conjunction with other compounds. These compounds may or may not have an effect on the rumen fermentation. Soluble carbohydrates (simple sugars) are usually present in the methanol extract and these compounds may contribute to the increase of total production of short chain fatty acid during rumen fermentation (Wina et al. 2005b). Studies with a subfraction of methanol extract of *Sapindus rarak* showed that the ethyl acetate subfraction decreased protozoa numbers in the *in vitro* rumen fermentation much less than the aqueous subfraction (16% vs. 62%, respectively), and reduced total gas production 10% while no effect of aqueous subfraction was observed (Wina, unpublished). Beside saponins, a sesquiterpene glycoside, mukurozioside IIb has been identified in the fruit pericarp of related plant, *Sapindus mukorossi* and *S. emarginatus* (Kanchanapoom et al. 2001). This compound might dissolve in the ethyl acetate subfraction and not in the aqueous subfraction of *S. rarak* methanol extract

Different techniques or solvents used for saponin extraction may affect the purity of the extract. This was shown by different contents of sarsaponin in four commercial products of *Yucca schidigera* extract (Singer et al. 2008). *Y. schidigera* powder contained not only saponins but also phenolics (resveratrol, yuccaol) (Piacente et al. 2005), glycoprotein and stilbenes. These other compounds were present in non-butanol extractable fraction and exert several properties such as antiinflammatory, antioxidant (Cheeke et al. 2006), reducing air ammonia concentration and fecal odour (Piacente et al. 2005). Therefore, different results on the use of *Yucca* extract in the ruminant feed can be influenced by the presence of other compounds in the extract.

The use of pure saponin would allow the study of the effect of saponin on the rumen without any confounding effects from other impurities. The purification of the crude saponin extract usually requires a sequential approach from extraction, precipitation, adsorption, ultrafiltration and chromatography (Güçlü-Üstündağ and Mazza 2007)

## 11.5 Saponin Structure Activity Relationship

Studies on structure activity relationship mainly have been conducted to identify compounds for medical treatment or health. These findings would be useful in understanding the various results found in many animal experiments using saponins since no structure activity relationship of saponins to their activity toward ruminal microbes has been elucidated.

Saponin from *Sapindus mukorossi* and its monodesmosides, resulting from the partial degradation of saponin have exhibited an activity in reducing fungal growth (Saha et al. 2010). Further removal of the sugar moiety yielded a complete loss in activity. Esterification of the hydroxyl group has been found to influence the antifungal activity (Saha et al. 2010). Another study with steroidal saponins found



that antifungal activity against *Candida neoformans* was influenced by the aglycone moiety, number and structure of sugar moieties. The sugar moiety of four or five monosaccharide units displayed remarkable antifungal activity and when the sugar moiety contained less than 4 monosaccharides units, the activity was lost (Yang et al. 2006). The spirostanol type of steroidal saponin has more antifungal activity compared to the furanstanol type (Zhang et al. 2005). Conversely, Barile et al. (2007) found that furastanol saponin has higher activity than spirostanol.

Tomatidine, a steroidal alkaloid has inhibitory activity against *Saccharomyces cerevisiae* (Simons et al. 2006). The removal of a single sugar from the tetrasaccharide chain of  $\alpha$ -tomatine (the aglycone) resulted in a substantial reduction in antimicrobial activity. But, the complete loss of sugars thus forming tomatine (the aglycone) led to enhanced antifungal activity. In contrast, the triterpene aglycones i.e. oleanic acid,  $\beta$ -amyrin, and hederagenin, did not exhibit any inhibitory activity (Simons et al. 2006).

Saponins from several plant sources have nematicidal activity. The partial hydrolysis of saponins showed more nematicidal activity than the related saponins at the same dose. The aglycones exerted nematicidal activity with hederagenin being the most active followed by medicagenin and bayogenin (Argentieri et al. 2008).

The haemolytic activity of saponins is dependent on the nature of aglycone and the glycoside chain (sugar moiety) including the configuration of the interglycosidic linkages, the substitution pattern and the type of sugar units involved (Chwalek et al. 2006).

The different plant source and structure of saponins, structure of aglycone, length and position of sugar moiety may contribute various activities toward bacteria, fungi, protozoa and further studies are warranted.

## 11.6 Effect of Saponins on Ruminal Microorganisms

Wina et al. (2005a) compiled the data published from 1987 to 2004 on the saponin effect on rumen fermentation both *in vitro* and *in vivo* while Hart et al. (2008) modified the same data by adding the data of total production of volatile fatty acid and methane production. Recently, Patra and Saxena (2009a) presented the *in vivo* data published from 1987 to 2009 on saponin effects on rumen fermentation and the performance of animals. Tables 11.1 and 11.2 present the data published from 2005 to 2010 on the effect of saponin on rumen fermentation *in vitro* and *in vivo*, respectively.

### 11.6.1 Protozoa

Saponins depress the protozoa population and activity in the rumen (Wallace et al. 2002). The mechanism proposed is that saponin reduces the protozoa due to the interaction of saponins with cholesterol in the outer membrane of the protozoa

**Table 11.1** Responses in the *in vitro* system due to the addition of different saponin extracts or saponin containing plants

No	References	Saponin	Concentration	<i>in vitro</i> system	Feed	Response
1	Wina et al. (2005a)	Methanol extract of <i>S. rarak</i> pericarps	0.25, 0.5, 1.0, 2.0–4.0 mg/ml	Gas test – holstein steers rumen liquor	Elephant grass: wheat bran (65:35 w/w)	Decreased: Protozoal numbers (11–49%); Ammonia production (8–16%) Increased: Gas production (2–7%), Total VFA production (2–8%), Molar proportion of propionate (1–45%), Microbial protein synthesis (4–42%)
2	Hu et al. (2005)	Tea ( <i>Camellia sinensis</i> ) saponin extract	0.2–0.4 mg/ml of buffered RL	Gas test- sheep rumen liquor	50% corn meal and 50% grass meal	Decreased: Gas production (4–10%), Protozoal numbers (13–16%); Ammonia production (19%); Methane production (12.7–14%) No effect on total VFA production Interactions between tea saponin and protozoa were mostly weak
3	Busquet et al. (2005)	<i>Trigonella foenum graecum</i> extract, 180 g/kg sarsaponin	2.2 mg/l of buffered RL = 7.1 mg/day	Continuous culture-dairy cow rumen liquor	Alfalfa hay 594, barley grain 201, corn grain 124, SBM 69 g/kg	No effect on total VFA, ammonia, propionate
4	Cardozo et al. (2004)	<i>Yucca schidigera</i> extract contained 80 g/kg saponins	0, 0.3, 3, 30, 300 mg/l buffered RL	Fermentor tubes-heifers fed forage: concentrate (1:9)	Forage: concentrate (1:9)	At pH 7: no effect acetate, propionate, butyrate Decreased ammonia (14–43%), Increased branched VFA (29–100%) At pH 5: decreased acetate (21.7–27.3%), Increased butyrate (12.7%)

5	Kamra et al. (2006)	Ethanol extract of <i>Sapindus mukorossi</i>	0.5 ml/30 ml buffered RL	Buffalo	Wheat flour: wheat straw (80:20)	Decreased: Protozoal numbers (76%); Methane production (96%), Ammonia production (2.7%), pH (1.7%), acetate (18.7%) Gas production (11%) Increased molar proportion of propionate (37%)
6	Pen et al. (2006)	<i>Yucca schidigera</i> extract contained 80–100 g/kg saponins	2, 4, and 6 ml/l buffered RL	Continuous-dairy cow rumen liquor	Oat and alfalfa hay (1:1,w/w)	Reduced: Protozoal numbers (29–56%), Ammonia production (26–48%), Methane production (17–42%) No effect on total VFA Increased: Molar proportion of propionate (16–54%) CO <sub>2</sub> production (0–28%)
7	Pen et al. (2006)	<i>Quillaja saponaria</i> extract contained 50–70 g/kg saponins	0, 2, 4, and 6 ml/l buffered RL	Continuous-dairy cow rumen liquor	Oat and alfalfa hay (1:1,w/w)	Decreased: Protozoal numbers (34–40%), Ammonia production (21–20%), Increased: Molar proportion of propionate (15–19%) CO <sub>2</sub> production (1.5–28%) No effect on total VFA, methane production

(continued)

Table 11.1 (continued)

No	References	Saponin	Concentration	<i>in vitro</i> system	Feed	Response
8	Patra et al. (2006)	Water, methanol and ethanol extracts of <i>Sapindus mukorossi</i>	0.5 ml/30 ml buffered RL	Buffalo	Wheat flour: wheat straw (80:20)	All extracts decreased protozoal count and activity, ethanol extract gave the highest inhibition to: Methane production (22.7%) Total gas production (11.5%) and 0%, Acetate and butyrate reduced, DMD decreased Increased propionate by 26%, 57.6% and 37% with water, methanol and ethanol extracts, respectively
9	Goel et al. (2008a)	<i>Trigonella foenum-graecum</i> seed	0, 66 mg/40 ml buffered RL	Gas test -hay (concentrate) fed Holstein cattle	Hay (concentrate)	Depressed: Protozoal numbers (50%-hay, 56%-conc.) Methane production (5%-hay, 10%-conc.)
10	Goel et al. (2008a)	<i>Sesbania sesban</i> leaves	0, 66 mg/40 ml buffered RL	Gas test -hay (concentrate) fed Holstein cattle	Hay (concentrate)	Decreased: Protozoal numbers (48%-hay, 50%-conc.) Methane production (11%-hay, 12%-conc.)

11	Goel et al. (2008b)	80% methanol extract of <i>Sesbania sesban</i> leaves	0, 10.9 and 21.2 mg/40 ml buffered RL	Gas test- conc: hay fed holstein cattle	Concentrate: hay (1:1)	Decreased: Protozoal numbers (14–36%) Methanogens (78%), fungi (38%) Methane production (4.69–6.14%) Increased: Fibrobacter (45%) Ruminococcus (26%) No effect on total VFA, propionate Depressed Protozoal numbers (15–39%) Methanogens (22%), Fungi (65%) Methane production (1.82–1.97%) Increased: Fibrobacter (42%) Ruminococcus (40%) No effect on total VFA, propionate Reduced protozoal numbers (14–25%), methane production (5.50–6.43%) Increased: Fibrobacter (24%), ruminococcus (18%) No effect on total VFA, propionate. Methanogens (21%), fungi (30%)
12	Goel et al. (2008b)	80% methanol extract of <i>Trigonella foenum-graecum</i> seed	0, 5.62 and 11.54 mg/40 ml buffered RL	Gas test-conc: hay fed holstein cattle	Concentrate: hay (1:1)	
13	Goel et al. (2008b)	80% methanol extract of <i>Knautia arvensis</i> leaves	0, 3.88 and 7.76 mg/40 ml buffered RL	Gas test -conc: hay fed holstein cattle	Concentrate: hay (1:1)	

(continued)

Table 11.1 (continued)

No	References	Saponin	Concentration	<i>in vitro</i> system	Feed	Response
14	Soliva et al. (2008)	<i>Sesbania sesban</i> leaves 10865, <i>Sesbania sesban</i> leaves 15019	300 mg/syringe	Gas test -conc (0.5 kg), hay ad lib. fed brown Swiss cow	No other substrate	<i>S. sesban</i> 10865: Methane 0.75 ml, methane/total gas 0.086 v/v; Protozoal count $3.01 \times 10^7$ /ml, Bacteria $1.21 \times 10^9$ /ml, ammonia 10.28 mmol/l, Total VFA 47.6; propionate 19.6 mmol/l; <i>S. sesban</i> 15019: Methane 6.56 ml; methane/total gas 0.144 v/v; Protozoal count $3.77 \times 10^4$ /ml, Bacteria $1.29 \times 10^9$ /ml; ammonia 16.13 mmol/l; Total VFA 78.0 mmol/l; propionate 20.1 mmol/l
15	Guo et al. (2008)	Tea saponin	0.4 mg/ml buffered rumen liquor	Bottle-gas test- rumen sheep fed alfalfa hay : concentrate (60:40)	Corn meal and grass meal (750 mg; 50:50, w/w)	Depressed: Protozoal relative abundance (51 %), Fungal relative abundance (79%), Methane production (8%), Expression of <i>mcrA</i> gene (76%) Increased: Molar proportion of propionate (11%) <i>F. succinogenes</i> (41%) No effect on total VFA production, methanogens, <i>R. flavefaciens</i>

16	Soliva et al. (2008)	<i>Sapindus saponaria</i> fruit, <i>Enterolobium cyclocarpum</i> fruit	300 mg/syringe	Gas test -conc (0.5 kg), hay <i>ad lib.</i> fed brown Swiss cow	No other substrate	<i>S. saponaria</i> : Methane 5.14 ml, methane/total gas 0.12 v/v; Protozoal count $1.86 \times 10^9$ /ml, Bacteria $1.03 \times 10^9$ /ml, ammonia 18.17 mmol/l, Total VFA 92.5 mmol/l; propionate 21.7 mmol/l; <i>E. cyclocarpum</i> : Methane 12.71 ml; Protozoal count $0.175 \times 10^9$ /ml, Bacteria $1.41 \times 10^9$ /ml; ammonia 9.57 mmol/l; Total VFA 102.7 mmol/l; propionate 26.5 mmol/l
17	Holtshausen et al. (2009)	<i>Yucca schidigera</i> whole plant powder (6% saponin)	15, 30, 45 mg/g substrate	Batch culture -holstein cow rumen liquor	51:49 forage (barley silage):concentrate	Depressed: Total gas (2–4%), ammonia (81–100%) and Methane production (8–26%) Increased molar proportion of propionate (10–36%) No effect on total VFA production

**Table 11.2** Responses in the *in vivo* system due to the addition of different saponin extracts or saponin containing plants

No	References	Saponin	Concentration	Animal	Feed	Response
1	Lila et al. (2005)	Sarsaponin ( <i>Yucca schidigera</i> )	5–10 g/kg DM = 11.2–22.4 g/day	Holstein steers	Sudangrass hay: concentrate mixture (1.5:1)	Depressed: Protozoal numbers (67–71%), Methane production (8–12%), Total VFA production (2–2%), Acetate (2.5–3.8%)  Increased: Propionate (4–7%), butyrate (7–10%), Ammonia production (5.7–10%)
2	Wina et al. (2006a)	Methanol extract <i>S. rarak</i> fruits pericarps	0.24, 0.48, 0.72 g/kg BM – daily for 100 days = 4, 8, 12 g/day	Thin tail sheep	Elephant grass: wheat bran (65:35 w/w)	Reduced: Protozoal numbers (34–80%), Ammonia production (12–18%) Increased propionate (14–19%) No effect on total VFA production, fungi, Methanogens, MCP
3	Wina et al. (2006b)	Methanol extract <i>S. rarak</i> pericarps	0.48–0.72 g/kg BM – daily feeding for 27 days	Thin tail sheep	Elephant grass: wheat bran (65:35 w/w)	Decreased: Protozoal numbers (76–78%), Ammonia production (21–29%) Increased propionate (13–14%) No effect on total VFA production, MCP
4	Wina et al. (2006b)	Methanol extract <i>S. rarak</i> pericarps	0.48–0.72 g/kg BM-interval feeding 3 days for 27 days	Thin tail sheep	Elephant grass: wheat bran (65:35 w/w)	Depressed: Protozoal numbers (69–64%), Ammonia production (7–21%) Increased propionate (6–7%) No effect on total VFA production, MCP



5	Lovett et al. (2006)	<i>Yucca schidigera</i>	0, 25, 50 g/head/day	Steer	Maize silage: grass silage: concentrate (61:23:15 DM)	Depressed: Protozoal numbers (19–34%), Propionate (23–19%), Total VFA (10–6%) Reduced total VFA (18%) Increased digestible energy (4%), ammonia (20%) No effect on methane emission, CO <sub>2</sub> , MCP, Total energy loss Reduced: Total VFA (12%), Ammonia (11%) Increased digestible energy (6%) No effect on protozoal numbers, methane emission, CO <sub>2</sub> , MCP, digestible energy
6	Pen et al. (2007)	<i>Yucca schidigera</i> extract (directly fed to rumen)	1.31–1.64 g saponin/day	Cheviot weather	Concentrate: Italian ryegrass hay (2:3 w/w)	Reduced: Total VFA (12%), Ammonia (11%) Increased digestible energy (6%) No effect on protozoal numbers, methane emission, CO <sub>2</sub> , MCP, digestible energy
7	Pen et al. (2007)	<i>Quillaja saponaria</i> extract (directly fed to rumen)	0.8–1.3 g saponin/day	Cheviot weather	Concentrate: Italian ryegrass hay (2:3 w/w)	Reduced: Total VFA (12%), Ammonia (11%) Increased digestible energy (6%) No effect on protozoal numbers, methane emission, CO <sub>2</sub> , MCP, digestible energy
8	Santoso et al. (2007)	<i>Biophytum petersianum</i> Klotch aqueous extract	0, 13, 19.5 and 26 mg saponin/kg BW	Kacang goat	Elephant grass silage: grain-based concentrate (70:30 DM)	Reduced: Protozoal numbers (35–41%), Total VFA (9–20%), Ammonia (26–32%) Acetate, butyrate, iso acids reduced Increased propionate (16–33%) and MCP (2–38%)
9	Baah et al. (2007)	<i>Quillaja saponaria</i> extract (100 g saponins/kg)	8 g/kg diet DM	Jersey heifer	Rolled barley grain (885), barley silage (92), canola meal (11)	Reduced protozoal numbers (34%) No effect on ammonia production, total VFA production, Propionate, enzyme activities

(continued)

Table 11.2. (continued)

No	References	Saponin	Concentration	Animal	Feed	Response
10	Koenig et al. (2007)	<i>Enterolobium cyclocarpum</i> leaves (saponin 0.8 mg/g leaves)	16 g/100 g diet	Canadian arcott weathers	Barley silage (600 g/kg) and a barley grain and soyabean meal pelleted concentrate (400 g/kg)	Reduced: protozoal numbers (25%), Ammonia production (42%), OM digested in the rumen (7%) Higher pH, total VFA (28%) and isoacids Lower propionate (9%) No effect on acetate, butyric No effect on protozoal numbers, ammonia production, Total VFA, propionate
11	Benchaar et al. (2008)	<i>Yucca schidigera</i> extract (saponin 100 mg/g)	60 g/day	Holstein lactating cow	Grass silage 39.6, Corn, 15.9, Beet pulp 12.0, CGM 9.9, Wheat bran 9.8, SBM 48% CP 8.8, NaHCO <sub>3</sub> 1.5, Limestone 1.1	Increased molar proportion of propionate (P=0.08) A tendency decreased ammonia No effect on protozoal numbers and pH
12	Singer et al. (2008)	<i>Yucca schidigera</i> extract (sarsaponin 95.4 mg/g)	0, 50, 100, 150 g/cow/day	Holstein cow	Alfalfa hay 361, barley grain 111, corn grain 166, DDGS 34, SBM 20.7, cottonseed 95.6, beet pulp 55, almond hull 120, fat 8 g/kg	No effect on protozoal numbers, ammonia, methane production, total VFA, propionate
13	Holtshausen et al. (2009)	<i>Yucca schidigera</i> whole plant powder (6% saponin)	10 g/kg DM	Holstein cow	51:49 forage (barley silage):concentrate	No effect on protozoal numbers, ammonia, methane production, total VFA, propionate
14	Holtshausen et al. (2009)	<i>Quillaja saponaria</i> whole plant powder (3% saponin)	10 g/kg DM	Holstein cow	51:49 forage (barley silage):concentrate	No effect on protozoal numbers, ammonia, methane production, total VFA, propionate

15	Poungchompu et al. (2009)	<i>Sapindus saponaria</i> fruit (15.7% saponin) + Mangosteen ( <i>Garcinia mangostana</i> ) peels (12.7% tannin):4:51	40 g/kg feed	Holstein friesian heifers	Rice straw and concentrate (cassava chip 66%, rice bran 5%, brewer grains 9%, cottonseed meal 13%, urea 3%)	Decreased protozoal numbers and fungal numbers, acetate Increased VFA, propionate and methanogens No effect on ammonia, butyrate and total bacteria
16	Suharti (2010)	Methanol extract <i>S. rarak</i> whole fruit	100, 200 mg/kg BW	Crosbred ongole cattle	Native grasses: concentrate (70:30 DM)	Decreased: Protozoal numbers (not significant) MCP(not significant), Ammonia (43% at high level <i>S. rarak</i> ), Ratio acetate:propionate (13–14%) Increased: Total VFA (46%, 31%) Molar: proportion of propionate (11–13%) No effect on digestibility, acetate or butyrate
17	Mao et al. (2010)	Tea ( <i>Camellia sinensis</i> ) saponin extract	3 g/day	Huzhou lamb	Concentrate: Chinese rye (40:60)	Reduced: Protozoal numbers (41%), Ammonia production (15%), Methane production (28%), <i>R. flavefaciens</i> (51%) Enhanced: Total VFA (13%), MCP (22%) No effect on propionate, fungi, <i>F. succinogenes</i> , Methanogens

(continued)

Table 11.2 (continued)

No	References	Saponin	Concentration	Animal	Feed	Response
18	Zhou et al. (2010)	Tea ( <i>Camellia sinensis</i> ) saponin extract	3 g/day	Hu lamb	Concentrate: Chinese rye (40:60). Concentrate : a mixture of ground corn 626, wheat bran 114, soybean meal 104, and rape seed meal 156	<p><i>Refaunated</i>: Decreased: Protozoa (43%), <i>F. succinogenes</i> (79%) Ammonia production (13%), Methane production (11%),</p> <p>Increased: Propionate (11%), MCP (16%) No effect on total VFA, methanogen, fungi, Ruminococcus</p> <p><i>Defaunated vs. control (refaunated)</i>: Decreased: Protozoa (100%), <i>F. succinogenes</i> (100%) Ammonia (34%), Methane (18%)</p> <p>Increased: Propionate (34%), MCP (36%) No effect on total VFA, methanogens, fungi, Ruminococcus</p>

making a pore and, hence, lysing the protozoa membrane (Wallace et al. 2002). Another mechanism proposed suggests that saponins with its ability to reduce surface tension has a similar effect to detergent causing toxicity to protozoa (Cheeke 2000; Francis et al. 2002). The sugar moiety has an important role in depressing protozoa; once, the saponin is completely hydrolysed, the inhibitory effect on protozoa was reduced or completely disappeared (Teferedegne et al. 1999; Wang et al. 2000; Muetzel et al. 2005). Partial acid hydrolysis of lucerne saponins decreased protozoa numbers in sheep rumen (Lu and Jorgensen 1987) suggesting that partial acid hydrolysed saponins might have some sugars attached to the aglycone, thus still retained its activity to inhibit protozoa.

Most of *in vitro* experiments in either batch or continuous systems showed that the reduction in protozoa population caused by saponins occurred very fast and was not specific. Koenig et al. (2007) showed that the protozoal numbers in sheep rumen markedly reduced 2 h after feeding *Enterolobium cyclocarpum*. *Entodinium* protozoa were present in the rumen as the dominant protozoal species, *Diplodinium*, *Isotricha* or *Dasytricha* were also present in the *in vitro* and *in vivo* experiments. In these experiments, addition of saponins did not influence the proportion of protozoa species (Baah et al. 2007; Koenig et al. 2007; Suharti et al. unpublished). However, when the level of saponin increased, all protozoa species decreased (Ivan et al. 2004; Wina et al. 2005a; Suharti et al. unpublished). Saponins reduced not only the numbers but also the diversity of protozoa. This was shown by a denaturing gradient gel electrophoresis (DGGE) study which revealed a lower diversity of protozoa after 21 days feeding of 3 g of tea saponin to sheep (Wang et al. 2010). *Eremoplastron dilobum* band on DGGE gel disappeared on the tea saponin treatment while *Entodinium furca monolobum* which was indicated as a faint band on the control treatment, appeared as a strong band in the saponin treatment (Wang et al. 2010; Zhou et al. 2010). It could be concluded that saponins may depress only specific protozoa species. Predation among protozoa may also explain the higher growth of one species of protozoa than the others (Dehority 1998; Ohene-Adjei et al. 2007). More studies should be conducted to confirm whether saponins affect specific protozoa species or if there is any predation among the protozoa or both. Table 11.1 shows that the reduction of protozoa numbers by saponin in most of *in vitro* experiments varied from 11% to 80% while in the *in vivo* experiments, the reduction varied from 0% (no reduction) to 71%. The variation among the experiments could be due to the different diet, saponin source, type of saponin, level of saponin and animal species used in the different studies.

Several studies showed that the presence of saponins in the rumen caused only partially defaunation. In the *in vitro* fermentation, *Enterolobium cyclocarpum* and *Sesbania sesban* which both contain saponins showed inhibiting effect on protozoa population (Leng et al. 1992; Teferedegne et al. 1999). When supplementing *Enterolobium cyclocarpum* to sheep, however, the antiprotozoal effect of *E. cyclocarpum* was only transient as the protozoa numbers started to increase after 12 days (Ivan et al. 2004; Koenig et al. 2007). When sheep received *Sesbania sesban* supplementation, protozoa numbers also increased after several days of supplementation (Newbold et al. 1997). Protozoa numbers in sheep rumen remained lower

compared to that in control sheep when fed *S. rarak* pericarp's extract at the level of 0.48–0.72 g/kg body mass for 100 days (Wina et al. 2006a). However, protozoal numbers were not significantly reduced when saponin extract of *S. rarak* whole fruit was fed to local cattle at the level 200 mg/kg BW/day for 90 days (Suharti 2010). The *S. rarak* saponins do not always depress protozoa numbers in the rumen perhaps due to the difference in composition of diet, the ratio of forage to concentrate, the animal breed, the level or type of saponin extract. The inconsistent defaunation effect of saponin between *in vitro* and *in vivo* experiments occurred when using other saponin sources such as in *Yucca* and *Quillaja* saponins (Pen et al. 2006, 2007; Lovett et al. 2006; Baah et al. 2007). Probably adaptation of the ruminal microbes, the nutrient flow or dilution effect might be responsible for the different results observed in the *in vitro* and *in vivo* studies (Benchaar et al. 2008).

### 11.6.2 Methanogens

Recently, methanogens and their diversity have been the subject of increasing interests due to the methane production by ruminants (Firkins et al. 2008). Much research is still in progress to study methanogens in order to mitigate methane emission from ruminants.

Beside living freely in the liquid mat or living associated with particles in the rumen, some methanogens remain associated with ruminal protozoa. It was estimated that 10–20% of total methanogens exist in close association with the protozoa either on the surface (ectosymbiosis) or inside the protozoal cell (endosymbiosis). Oligotrichs have endosymbiotic methanogens and entodiniomorph protozoa have ectosymbiotic methanogens (Ohene-Adjei et al. 2007). Using fluorescent-microscopic technique, Vogels et al. (1980) showed that nine genera of protozoa from the family of *Ophryoscolecidae* (order *Entodiniomorpha*) associated with methanogens. Physical structure (pellicle, surface structure and interior structure of cell cortex) of protozoa may affect this association. Study using molecular technique revealed that about 99% of protozoa-associated methanogens belong to the family of *Methanobacteriaceae* (Karnati et al. 2009a), and 20 novel sequences which differed from sequences previously known for protozoa-associated methanogens were obtained from rumen samples of goat, sheep and cow (Regensbogenova et al. 2004; Morgavi et al. 2006). DGGE profile also showed that inoculation of different species of protozoa to defaunated sheep resulted in different association of methanogens (Ohene-Adjei et al. 2007). The above findings indicate that various cultured and uncultured methanogens and their association with protozoa require further study.

Protozoa hydrolysed carbohydrate to produce hydrogen which then be used directly by associated methanogens to produce methane. Ushida et al. (1997) showed the occurrence of interspecies hydrogen transfer between the rumen ciliate *Polyplastron multivesiculatum* and the methanogenic archebacterium, *Methanosarcina barkeri*. Therefore, reducing protozoa will also reduce ruminal methanogens, thus reducing methane production. Tables 11.1 and 11.2 show that the studies mostly reported the

effects of saponins on ciliate protozoa, nitrogen metabolism, and methane production but only few studies reported the effect of saponins on methanogens. It was shown that saponins in the *S. rarak* extract at the level of 2 mg/ml in the *in vitro* fermentation did not affect methanogens RNA concentration (Wina et al. 2005a). The same result was observed with the feeding of *S. rarak* at a dose of 0.48 g/kg body to sheep (Wina et al. 2006a). In contrast, Goel et al. (2008a) found that the addition of saponin extracts from sesbania, fenugreek or knautia decreased methanogens by 78%, 22% and 21%, respectively in the *in vitro* rumen fermentation.

Tea saponins did not inhibit *M. ruminantium* as pure culture (Guo et al. 2008), but at the level of 0.4 mg/ml in the *in vitro* rumen fermentation, it inhibited significantly the activity of the methyl coenzyme-M reductase (*mcrA*) gene of *Methanobrevibacter ruminantium* by 76% (Guo et al. 2008). The *mcrA* is crucial to the final step of methanogenesis where it is involved in the reduction of the methyl group bound to coenzyme-M. Tea saponin has been reported to decrease methanogens diversity (Zhou et al. 2010), without having any effect on the relative abundance of methanogens in sheep (Mao et al. 2010). The study on the use of different saponin containing plants related to the expression or activity of *mcrA* gene in the rumen is very limited. Further studies are warranted to unravel the mechanism by which saponins reduce the ruminal methane production.

### 11.6.3 Bacteria

Beside the antiprotozoal activity, many published reports showed that saponins are antimicrobial or antifungal agents. These properties were observed when saponins were tested on pure cultures. Wang et al. (2000) showed that saponins isolated from *Yucca schidigera* reduced the growth of *Streptococcus bovis* and *Ruminobacter amylophilus*. Wallace et al. (1994) previously reported that yucca extract reduced the growth of *S. bovis* and *Butyrivibrio fibrosolvens*. Yucca saponins also reduced filter paper and endoglucanase activities of *Ruminococcus albus* and *R. flavefaciens* but not those of *Fibrobacter succinogenes* (Wang et al. 2000). *Selenomonas ruminantium* but not *Prevotella bryantii* growth, was enhanced by yucca saponins (Wang et al. 2000) while the reverse result was reported by Wallace et al. (1994). The inconsistency of these results may be due to the isolation procedure and type of saponin.

The response of the mixed rumen microbes to saponins may be due to a direct effect of saponin on bacteria and an indirect effect of saponin reducing protozoal numbers. As the protozoal population decreased due to the use of saponins, the total bacteria numbers increased (Wina et al. 2005a; Pen et al. 2006; Goel et al. 2008a). However, the bacteria increase depended on the level of saponins added to the substrate. Wina et al. (2005a) found that total ruminal bacteria numbers increased significantly when *S. rarak* extract was included at the level of 1 mg/ml in the *in vitro* fermentation system, but the bacterial numbers decreased when the inclusion of saponin was increased to twofold to fourfold. It was further reported that saponins in the *in vitro* fermentation system caused a shift in bacterial population which was

shown by different intensity of bands on DGGE gel (Suharti et al. unpublished). Further analysis of three intensified bands show that the sequences of these bands have high similarity with sequences from *Prevotella ruminicola* (98–100%) *Pseudobutyrvibrio ruminis* or *Butyrvibrio fibrosolvans* (99%) and *Coprococcus eutactus* (99%) (Suharti et al. unpublished). In dual-flow continuous culture fermenters, removing protozoa increased population of *Prevotella*, *Eubacterium* spp., *Ruminococcus* spp., *Butyrvibrio fibrisolvans* which were shown as increased intensity bands on DGGE gel (Karnati et al. 2009b).

On pure culture, the growth of *Prevotella ruminicola* was stimulated by the addition of yucca extract (Wallace et al. 1994). In the *in vitro* fermentation using cattle rumen, a significant increase of *Prevotella ruminicola* due to the addition of *S. rarak* saponin extract was also reported (Suharti et al. 2010) and this result confirmed a previous qualitative analysis by DGGE (Suharti 2010).

A preference of protozoal predation toward *Butyrvibrio fibrisolvans* (Williams and Coleman 1992) explains the increased intensity of band matched with *B. fibrisolvans* during partial defaunation. Saponins also affected three major fibrolytic bacteria, *Ruminococcus albus*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* in the rumen. *S. rarak* saponins reduced RNA concentrations of *Ruminococcus albus* and *R. flavefaciens* both in the *in vitro* fermentation and *in vivo* during short term feeding (6 days) (Wina et al. 2005a, 2006a), but this effect did not occur during a long term feeding trial on sheep (100 days). RNA concentration of *Fibrobacter succinogenes* was unaffected in sheep rumen during short and long term feeding of saponin (Wina et al. 2006a). Quantitative analysis by real time PCR showed that *S. rarak* extract did not inhibit *R. flavefaciens* and *F. succinogenes* population in the *in vitro* fermentation (Suharti et al. 2010). However, sesbania saponin in the *in vitro* fermentation increased *F. succinogenes* (21–45%) and *Ruminococcus flavefaciens* (23–40%) populations measured by real time PCR (Goel et al. 2008a). Tea saponin did not affect the number of *R. flavefaciens* but increased that of *F. succinogenes* in the *in vitro* system (Guo et al. 2008). The effect of tea saponin was different in the *in vivo* trial using Hu lambs. Mao et al. (2010) found no effect of tea saponin on relative abundance of *R. flavefaciens* and *F. succinogenes* while Zhou et al. (2010) reported a decrease of *F. succinogenes* but no effect on *R. flavefaciens*, *R. albus*, *Butyrvibrio fibrisolvans*. These inconsistent results still can not be explained and warrant more studies to be conducted.

#### 11.6.4 Fungi

Studies on the effects of saponins on fungi were mostly performed on fungi which caused disease problems in humans or plants (Zhang et al. 2005; Barile et al. 2007; Coleman et al. 2010). Steroidal saponins from *Tribulus terrestris*, saponins from *Allium minutiflorum*, plant saponins have shown potent activity against fluconazole-resistant fungi (Zhang et al. 2005), soil-borne and air-borne pathogenic fungi



(Barile et al. 2007) and *Candida albicans* (Coleman et al. 2010). Similar to what observed on protozoa, the mechanism of saponins inhibiting fungal growth could be the binding between saponins and sterol present in outer membrane of fungi causing its disruption (Armah et al. 1999). However, no correlation was found between antifungal activity and antiprotozoal or haemolytic activity of saponins as mentioned above. Studies on the effect of saponins on individual rumen fungi are of scarcely available. Pure culture study showed that saponins from *Yucca schidigera* completely inhibited the growth of ruminal fungi, *Neocallimastix frontalis* and *Piromyces rhizinflata* (Wang et al. 2000). *S. rarak* saponins reduced RNA concentration of ruminal fungi both in the *in vitro* fermentation (at the level of 2–4 mg/ml) and *in vivo* during short term feeding but this effect did not occur in a 100 days of feeding trial (Wina et al. 2005a, 2006a). Tea saponin showed activity to depress fungal growth in the *in vitro* system (Guo et al. 2008) and no such activity in the *in vivo* system (Mao et al. 2010; Zhou et al. 2010). Goel et al. (2008a) reported that knautia, sesbania and fenugreek extracts reduced the population of ruminal fungi in the *in vitro* fermentation by 30%, 38%, 65%, respectively. This fungal inhibition may or may not disappear when the extracts are given *in vivo* and thus further investigation is required

## 11.7 Adaptation and Resistance of Ruminal Microbes to Saponins

The transient effect of saponins on ruminal protozoa has been reported by several authors (Newbold et al. 1997; Ivan et al. 2004; Koenig et al. 2007). Beside transient effects, the varying effects of the same saponin on protozoa occurred. Feeding *Sapindus saponaria* fruits to sheep suppressed protozoal numbers as demonstrated by Diaz et al. (1993) and Navas-Camacho et al. (1993), but increased protozoal numbers in the study of Abreu et al. (2004). This variability of the anti protozoal effect exerted by saponins may be caused by an adaptation of mixed microbes to saponins although breed and exposure of the animals to different environment or diets may also contribute to the variability (Teferedegne 2000; Abreu et al. 2004). Adaptation mechanism of rumen microbes to saponins may occur. *F. succinogenes* was resistant to saponins. *F. succinogenes* is a Gram negative bacterium, with two membranes, and a thin surface carbohydrate coat of radiating fibers. The outer membrane contains of two polysaccharides, i.e. lower-molecular-mass fraction designated glycolipid and a high-molecular-mass capsular polysaccharide fraction. The presence of 2-aminoethylphosphonic acid on the surface of both polysaccharides which covalently linked to the membrane polymers, enhances membrane stability of *F. succinogenes* (Vinogradov et al. 2001). So, not only *F. succinogenes* but most of Gram negative bacteria may also exert resistance to saponins. Wang et al. (2000) reported that thickening the cell wall membrane as found in *Prevotella bryantii* is another adaptation mechanism of microbe to saponins.

## 11.8 Effect of Saponins on Rumen Fermentation

### 11.8.1 Methane Production

Recent reviews on the use of secondary metabolites including saponins to reduce methanogenesis (methane production) are available (Rochfort et al. 2008; Patra and Saxena 2009b).

The use of *Sapindus rarak* fruit's pericarp powder, the crude extract of the fruit's pericarp or that of the whole fruit suppressed *in vitro* methane production by 21%, 31% (Thalib 2004) and 10%, respectively (Suharti 2010); however there are no reports yet on the effect of *S. rarak* saponin on *in vivo* methane production. Similar effect of suppression was obtained by adding aqueous extract of *Sapindus mukorossi* to the *in vitro* fermentation (Kamra et al. 2008; Sirohi et al. 2009). Goel et al. (2008b) found reduced methane production in the *in vitro* fermentation with the addition of sesbania, fenugreek or knautia leaves but not with their extracts. Saponins from *Yucca schidigera* or *Quillaja saponaria* reduced methane production in the *in vitro* fermentations (Pen et al. 2006; Holtshausen et al. 2009) but not in the *in vivo* experiments (Pen et al. 2007; Holtshausen et al. 2009). Depression on methane production occurred both in the *in vitro* and *in vivo* experiments was reported when using sarsaponin (Lila et al. 2003, 2005), and tea saponins (Hu et al. 2005; Mao et al. 2010; Zhou et al. 2010). But the depression was significant only in faunated rumen fluid, suggesting that this effect was mediated through associated effects on protozoa. In the partial defaunation by saponins, the population of methanogens that associated with protozoa would decline, hence the methane production reduced. However, saponin rich extracts of *S. sesban* leaves, *Knautia arvensis* leaves and fenugreek seeds did not decrease methane production *in vitro* although these extracts reduced protozoa number and methanogen population (Jayanegara et al. 2010) The lack of inhibition on methane production with decreased methanogens could be caused by changes in composition of the methanogen community and their increased efficiency of methane production (Jayanegara et al. 2010).

The formation of methane in the rumen requires the involvement of several microbes and enzymes. Reducing the activity of the methyl coenzyme-M reductase (*mcrA*) gene of *Methanobrevibacter ruminantium* by saponin (Guo et al. 2008), indicated that tea saponin inhibited the rate of methanogenesis and not the numbers of methanogens. It is likely that H<sub>2</sub> availability rather than the number of methanogens controlled the methanogenesis. High proportion of H<sub>2</sub> in the rumen is produced by protozoa when digesting starch, but when protozoa are inhibited, H<sub>2</sub> production in the rumen is limited and thus methane production is reduced. Saponin may suppress the H<sub>2</sub>-producing bacteria (Wang et al. 2000) such as cellulolytic bacteria and bacteria that use pyruvate-ferredoxin oxidoreductase, hence reduce H<sub>2</sub> availability for methanogens.

Increasing H<sub>2</sub> utilization by organisms other than methanogens also reduce methane production. This process requires addition of an appropriate electron acceptor and an efficient type of rumen bacteria that can perfectly utilize such an acceptor

in the production of acetate or propionate, hence, reduced methane production (Abdl-Rahman et al. 2010).

Thalib and Widiawati (2008) suggested a combination of feeding *S. rarak* saponin extract with *Acetoanaerobium noterae*, an acetogenic bacteria that reduced *in vitro* methane production by 20%. Further, Thalib et al. (2010) showed that this combination also reduced methane production in sheep by 24%.

Caution should be taken in interpreting methane production data since methane production is expressed in different units. In the *in vitro* system, the units of methane production could be expressed as the total production per day or concentration relative to the total gas. Hess et al. (2004) showed that methane release could be expressed relative to metabolic weight or organic matter digested or energy intake or body nitrogen retained. Different units of methane release give various values which may lead to different conclusion. From several results, it can be suggested that saponins have potential to be used as a methane reducing agent, however, the inclusion level of saponin from each source should be tested to get the optimum result.

### 11.8.2 Biohydrogenation of Fatty Acid

Recently, there has been a renewed interest by several researchers in the study of biohydrogenation of fatty acid in the rumen after a growing demand for producing healthy animal products for human consumption. Conjugated linoleic acid (CLA) and vaccenic acid (VA) are fatty acid intermediates produced in the rumen. CLA is known as potentially health-promoting agent since many studies showed that it contributed to cancer prevention, decreased atherosclerosis and improved immune response (Pariza 2004; Palmquist et al. 2005). The most abundant *trans*-18:1 isomer, vaccenic acid (18:1 *trans*-11) was 6.6% of total fatty acids in protozoa and 2.0% of total fatty acids in bacteria. The *cis*-9, *trans*-11 CLA was 8.6-times greater in the protozoal fraction (1.32% of total fatty acids) than in the bacterial fraction (0.15%) (Or-Rashid et al. 2007). Proportion of CLA and VA in the rumen fluid of faunated 1.6–2.5 times higher than those of defaunated cattle (Sultana et al. 2011) The supply of CLA for post rumen absorption depend on the flow of protozoa from the rumen (Yanez-Ruiz et al. 2006). Only limited data of the effects of saponins on CLA and VA concentration in the rumen is available. Addition of quillaja saponins in the dual-flow continuous culture at the level of 500–1,000 mg/L did not affect the total or individual volatile fatty acid nor change the extent of biohydrogenation (Lourenço et al. 2008). No effect of saponin from *Yucca schidigera* on the rate of  $\alpha$ -linolenic acid biohydrogenation was reported when added at the level of 1.12% of dry matter feed in a Rumen simulation technique system (Khiaosa-Ard et al. 2009). Defaunation by using a certain size filter only slightly reduced biohydrogenation in the rumen (Karnati et al. 2009a) and, hence, the presence of protozoa was not necessary for biohydrogenation to occur. More studies need to be done on the effect of different saponins on biohydrogenation of fatty acid in the rumen and the production of *cis*-9, *trans*-11-CLA and VA (18:1 *trans*-11).

Only bacteria in the same group as the *B. fibrisolvens* group formed cis-9, trans-11-CLA or trans-11-18:1 as intermediates in the process of biohydrogenation of linoleic acid (Jenkins et al. 2008). The DGGE result to study rumen bacterial diversity showed that the addition of *S. rarak* saponin extract caused *B. fibrisolvens* to appear as a more intense band (Suharti et al. unpublished). Defaunation by other compounds also increased some *Butyrivibrio* (Karnati et al. 2009a). Further study to quantify *B. fibrisolvens* following saponin addition may explain about its role in the CLA production in the rumen. The effects of various phytochemicals on microbial biohydrogenation in the rumen have been discussed in details in Chap. 9.

### 11.8.3 Nitrogen Metabolism

Protein consumed by ruminants is partly degraded in the rumen to peptides, amino acids and finally to ammonia. Therefore, there is a relationship between ammonia concentration in the rumen and percentage of total protein in the diet (Eugene et al. 2004). Another source of ammonia in the rumen comes from the proteolysis of bacterial protein when protozoa engulf ruminal bacteria as their principal source of protein amino acids. Reduced ammonia concentrations in the rumen typically occurred when total defaunation was applied (Eugene et al. 2004). The reduction of protozoal numbers resulted in less predation of bacterial and, hence, less lysis of bacterial protein to ammonia. Therefore, the observed ammonia level in the rumen is affected by the rate of degradation of feed protein to ammonia, the rate of bacterial lysis by protozoa, and the uptake of ammonia for microbial protein synthesis. Fewer protozoa also could influence the ammonia concentration since protozoa contributed to 10–40% of the total rumen nitrogen. The excess of ammonia is diffused from the rumen, converted to urea and excreted from the animal.

Saponins which partially reduced protozoal numbers, also reduced ammonia concentration in the rumen both in the *in vitro* (Table 11.1) and *in vivo* experiments (Table 11.2). Experiments using tea saponin or *S. rarak* extract to partially defaunate the rumen either *in vitro* or *in vivo* showed lower ammonia production. Tables 11.1 and 11.2 showed inconsistency in the effect of saponin on ruminal ammonia production between the *in vitro* and *in vivo* experiments using the same source of saponins. Saponin extract of *Yucca schidigera* (Lila et al. 2005; Cardozo et al. 2004; Pen et al. 2006, 2007; Singer et al. 2008; Holtshausen et al. 2009) or *Quillaja saponaria* (Cheeke 2000; Pen et al. 2006, 2007; Holtshausen et al. 2009) caused different effect on ammonia production. Comparison of all the results is rather complicated because of the different source of saponin, different level of saponin, process of extraction, dietary ingredient of forage and concentrate, breed and physiology of the animal.

There is limited information on the effect of saponins on peptide and amino acids metabolism in the rumen. Bacteria are responsible for most peptide degradation within the rumen and among them, Gram-negative bacteria *Prevotella ruminicola* appears to be the most important peptidolytic bacteria (Broderick et al. 1991).

Suharti et al. (2010) found that addition of *S. rarak* whole fruit extract to *in vitro* fermentation increased the relative abundance of *Prevotella ruminicola*. Further investigation is required to prove whether saponins increase the production of peptides in the rumen. However, the inhibition of rumen proteolytic activity by yucca saponins has been observed by Wallace et al. (1994). The reduction of ruminal N digestion was also observed when sheep was fed *E. cyclocarpum* leaves (Koenig et al. 2007). However, Muetzel et al. (2005) did not find any effect of *S. rarak* saponin on protein degradation *in vitro*. There is no effect of quillaja or yucca saponin on deaminative enzymes (Hristov et al. 1999; Baah et al. 2007).

Fenugreek extract in the continuous culture system showed no effect on large peptides, small peptides and amino acids productions in the rumen (Busquet et al. 2005) while Yucca extract increased the average peptide N concentration by 26.2% but no effect on amino acid concentration and ammonia was found (Cardozo et al. 2004).

Accumulation of peptides in the rumen fluid would be beneficial to ruminants since it would increase microbial protein synthesis and the flow of dietary amino acids to the lower gut (Griswold et al. 1996). Microbial protein synthesis (MPS) was enhanced by quillaja saponin in the *in vitro* fermentation (Makkar et al. 1998), *S. saponaria* (Abreu et al. 2004) and tea saponin in the *in vivo* trials (Mao et al. 2010; Zhou et al. 2010). However, MPS *in vivo* was not affected by applying *S. rarak* extract (Wina et al. 2006a, b), Quillaja or Yucca saponin (Pen et al. 2007). The application of yucca saponin at low level (15 µg/ml) increased microbial protein synthesis, in contrast, MPS reduced at high level of saponins (75 µg/ml) (Wang et al. 2000). Efficiency of MPS improved by 13% in sheep fed a diet supplemented with *E. cyclocarpum* (Koenig et al. 2007) and by 51% when supplemented with *Biophytum petersianum* Klotzsch (Santoso et al. 2007).

From the rumen, the bacteria and protozoa flow to duodenum could be observed by measuring the markers, <sup>15</sup>Nitrogen and phosphatidylcholine (PC) in the rumen and duodenal digesta (Ivan et al. 2006). Not only the microbial protein synthesis, but also the flow of microbial protein to duodenum was enhanced by *Sapindus saponaria* (Abreu et al. 2004) and tea saponin (Mao et al. 2010; Zhou et al. 2010). These positive effects on the microbial protein synthesis, efficiency microbial protein synthesis, the microbial N flow and Non Ammonia Nitrogen (NAN) may help to improve dietary N utilization by ruminants (Busquet et al. 2005).

#### 11.8.4 Carbohydrate Metabolism

Interaction of several diverse species of bacteria, fungi and protozoa facilitates the breakdown of cellulose and other carbohydrate fractions in plant materials in the rumen. With the decrease of protozoal numbers, this interaction may be disturbed as some carbohydrate degrading enzymes have less activity. Wina et al. (2005b, 2006a) showed that the xylanase activity in the rumen decreased when *S. rarak* saponin extract was administered to *in vitro* fermentation or directly to sheep. The CMCase activity was not affected in the *in vitro*, however, it was depressed by

*S. rarak* extract entered the rumen of sheep. Protozoa also produced carbohydrate degrading enzyme including xylanase and CMCase (Williams and Withers 1991; Devillard et al. 1999) and a positive correlation was observed between protozoal numbers and both xylanase and CMCase activities in the sheep rumen (Wina et al. 2006a). Amylase activity in the *in vitro* rumen fermentation increased in the presence of saponin extract of *S. rarak* whole fruit (Suharti 2010). However, CMCase, xylanase, glucanase and amylase activities in heifer's rumen were not affected by Quillaja saponin extract (Baah et al. 2007). Eventhough the fibrolytic enzyme activity might be reduced in the rumen and, hence, reducing the fibre digestibility in the rumen, the total tract digestibility was not affected by saponin addition (Wina 2005).

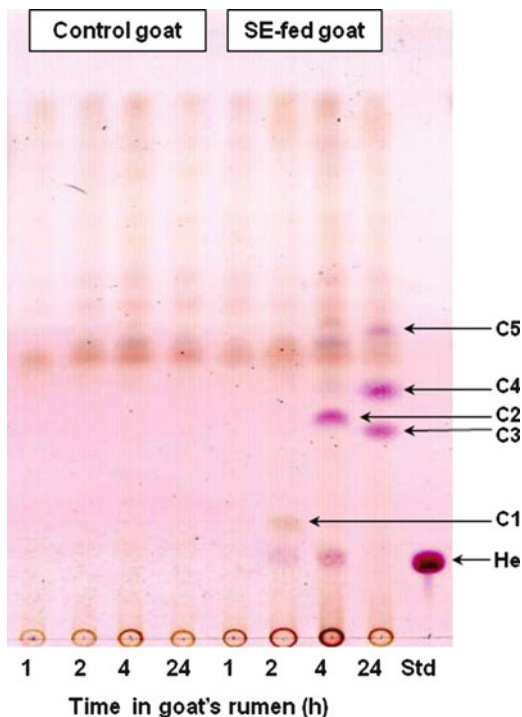
The products of carbohydrate degradation in the rumen would be volatile fatty acids. The higher total VFA production was observed by *S. rarak* extract addition in the *in vitro* (Wina et al. 2005b; Suharti et al. unpublished) and in cattle rumen (Suharti 2010) indicating that saponin improved the fermentation while many experiments showed no effect or depressed effect of saponin on total VFA production. Consistent results were seen in the *in vitro* system and both sheep and cattle feeding trials in that the molar proportion of propionate increased in the presence of *S. rarak* saponin extract (Tables 11.1 and 11.2). *Y. schidigera* and *Q. saponaria* extracts increased propionate production *in vitro* (Pen et al. 2006) but not in sheep rumen (Pen et al. 2007). There are other reports in agreement and showing an increase in the molar proportion of propionate in the presence of saponin although some did not agree with this result (see Tables 11.1 and 11.2). The higher molar proportion of propionate could be due to higher activity or numbers of *Prevotella ruminicola* to form propionate. *Prevotella ruminicola* in pure culture increased in the presence of *Y. schidigera* saponin fraction (Wallace et al. 1994) and its relative abundance as a percentage to the total bacteria increased significantly in the presence of *S. rarak* extract in the *in vitro* fermentation (Suharti et al. 2010). Lower acetate and butyrate production which were the major fermentation end products in isolated protozoa caused a shift in molar proportion of individual VFAs to a higher proportion of propionate and a reduced ratio of acetate to propionate. A higher molar proportion of propionate means a higher glucogenesis and would be beneficial for animals fed high roughage diets that are commonly found in tropical countries where available sources for feed are mainly roughages of low quality. The potential usage of saponin extract as a natural substance to improve propionate production requires more study.

## 11.9 Metabolism of Saponins in the Rumen

Rumen microbes produce a variety of intracellular or extracellular enzymes. These enzymes hydrolyse all substances at different rates. Saponins are glycosylated compounds and are highly soluble in water or aqueous medium. Saponins can dissolve easily in the rumen and therefore be readily degraded by the various glycosidases



**Fig. 11.5** Sapogenin fraction extracted from rumen of control and SE (saponin extract) fed goat taken at 1, 2, 4 and 24 h after feeding and separated on thin layer of Silica gel plate. C1–C5 represented the metabolites of saponins (in the form of sapogenin), *He* hederagenin, the main aglycone of *Sapindus rarak* saponins



produced by the different microbes. In the *in vitro* study, Makkar and Becker (1997) reported that 81% of quillaja saponins still undegraded in buffered rumen liquor up to 9 h of incubation and were degraded rapidly after this time. Wina (2005) studied the degradation of *S. rarak* saponin in the *in vitro* rumen and showed that after 6 h of incubation only one saponin compound could be detected by thin layer chromatography. With further incubation for 12 h, this compound was detected with lower intensity. Using Rusitec system, Wang et al. (1998) found that after 2 h of incubation, most of yucca saponins (93.5%) remained intact in the rumen but with longer incubation, the degradation process occurred quite rapid and more than 50% of saponins disappeared at 8 h of incubation (Wang et al. 2000). Different rates of saponin degradation are shown in the *in vitro* systems (Makkar and Becker 1997; Wang et al. 1998, 2000; Wina 2005) and the degradation rate seems faster in the *in vivo* system especially if the saponin was directly introduced to the rumen. Saponins from an extract of *Costus speciosus* rhizomes was rapidly hydrolysed at 1 h after dosing to the rumen (Meagher et al. 2001). However, Wina (2005) did not find any degradation product of *S. rarak* saponins 1 h after feeding. The degradation products (aglycone structures) appeared after 2 h of feeding (Fig. 11.5). Hederagenin (*He*) as the aglycone of *Sapindus rarak* saponins was detected up to 4 h after feeding and then, it disappeared and perhaps structurally changed to several other degradation products after 24 h of the saponin extract feeding.

Flåøyen et al. (2002) reported that the saponin from *Yucca schidigera* was hydrolysed in the rumen to its main algycone, sarsapogenin, but then they assumed that this sarsapogenin was oxidized and reduced at C-3 position to become the corresponding epi-sarsapogenins. This result was in agreement with their previous study with saponins from *Nartheicum ossifragum* which caused photosensitization to sheep (Flåøyen et al. 2001). Several free aglycones are formed in the rumen and the ring structure was not being degraded further in the rumen (Flåøyen et al. 2001, 2002). *Fibrobacter succinogenes* was reported to deglycosylate yucca saponin (Wang et al. 2000). There might be many other rumen microbes that have glycosidases activity and can hydrolyse saponin but have never been reported yet.

### 11.10 Summary of the Effects of Saponin on Rumen Fermentation

A scheme of effects of saponins on rumen microorganisms, activities and its products is presented in Fig. 11.6. Evidence obtained from numerous experiments suggests that the effects of saponins or saponin containing plants initially affected the microorganism in the rumen. Effect of saponin or its degradative products on microbial intestinal or caecum of ruminants, however, are limited known.

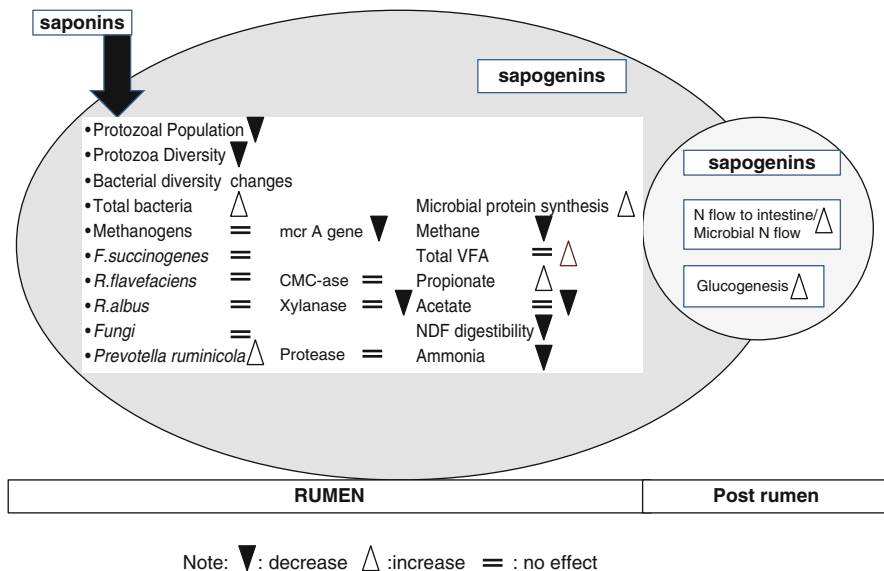


Fig. 11.6 Schematic diagram of possible effects of saponins or saponin containing plants on rumen microbes, activity and products



When saponins enter the rumen, the first effect is to reduce protozoal population (Fig. 11.6) Total bacterial numbers increase as less predation of bacteria by protozoa after saponins reduce protozoal population. The increase in total bacteria numbers means that the microbial protein synthesis enhances, hence, increases the microbial nitrogen flow to post rumen or intestine. Individual bacteria responses differently to saponins. Several studies show that the activity of methanogens but not methanogens population is reduced by saponins, resulting in a decrease in the methane produced in the rumen. Three major fibrolytic bacteria (*F. succinogenes*, *R. flavefaciens* and *R. albus*) are unaffected by saponin in the *in vivo* system Similar to fibrolytic bacteria, ruminal fungi are not affected by saponin. Protozoa and fungi possess carbohydrases (CMCase, xylanase), therefore partial defaunation by saponin causes a reduction in carbohydrate degrading enzymes. The reduced activity of these enzymes by saponin causes NDF digestibility in buffered rumen to decrease (Wina et al. 2005a). Saponin does not inhibit the activities of carbohydrate degrading enzymes in sheep or cattle rumen and, hence, does not impair total tract digestibility. Saponin increases VFA and propionate production in the rumen, therefore, it is expected that glucogenesis in post rumen is enhanced.

With reduced protozoal population, increase of total bacteria population, propionate production, microbial biomass production, microbial nitrogen flow and glucogenesis and without any effect of saponin on total tract digestibility, it is expected that the performance of ruminant is improved by saponin addition.

## 11.11 Conclusion

Various methods of extracting saponins from plant materials and the diversity of saponins influenced the activity of rumen microorganisms. Saponins could manipulate the rumen fermentation by depressing the protozoal population in the rumen. The consequences of reduced protozoal population leads to some changes in the rumen microbial composition and population, a shift in individual volatile fatty acids toward higher propionate and increased microbial nitrogen flow to duodenum. Saponins could also decrease methane production with or without decreasing the numbers of methanogenic archaea. Evaluation on the dose of specific saponins to be added into the diet and the interaction of saponins with different composition of diets require further studies so that saponins could be potentially used as a defaunating agent, a propionate enhancer and an inhibitor for methane production in the rumen.

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