STUDIES ON SAPINDUS RARAK DC AS A DEFAUNATING AGENT AND ITS EFFECTS ON RUMEN FERMENTATION

by

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ABSTRACT

The aim of the work described here was to examine the antiprotozoal activity of the pericarp from *Sapindus rarak* DC (Sapindaceae) for its potential to defaunate the rumen, either partially or completely, without having a detrimental effect on the bacterial population and on rumen fermentation, and hence enhancing rumen productivity.

An approach to improving microbial efficiency in the rumen is to eliminate protozoa (defaunation). Elimination of protozoa by chemical means is potentially the most convenient method. However, it is likely that some chemicals are not toxic specifically to the protozoa and probably kill other microorganisms and host cells in the rumen, therefore novel natural antiprotozoal agents are being sought.

A possible role for saponins has been of interest to many researchers as saponins induce marked reductions in rumen protozoa numbers, particularly when the animal is fed on high-concentrate diets. The susceptibility of rumen protozoa and lack of susceptibility of rumen bacteria to saponins is explained by the reaction of saponins with membrane sterols, which are present only in eukaryotic membranes and not in prokaryotic bacterial cells. Recent *in vitro* results suggest that *S. rarak* exhibited higher immobilising activity and produce higher activity to lyse the cells compared to other saponin-containing plants

A preliminary study on the antiprotozoal activity of the active agent present in the pericarp of fruits of *S. rarak*, well known for the high saponin content of its fruits, was carried out. An antiprotozoal assay was developed. The results were

consistent within experiments, but not between experiments, possibly due to the number of protozoa fluctuating widely with time. A study on the effect of *Sapindus rarak* on rumen bacteria, using a quantitative plating method, showed that there was no effect on rumen bacteria.

Raw material and extract of pericarp of *S. rarak* were screened for their effects on rumen protozoa and bacteria (with a view to predicting its safety as a feed supplement and for its potential to defaunate the rumen, either partially or completely). The pericarp of *S. rarak* was extracted using selective extraction of saponins with C_{18} support. The fractions were collected and monitored by Thin Layer Chromatography (TLC). The active compounds were tested *in vitro* for toxicity to rumen protozoa by visual assessment of protozoal viability (Nottingham studies) and by measuring the degradation of labelled bacterial protein by rumen protozoa (Aberdeen studies). The influence of methanol extract of *S. rarak* on growth of pure cultures of rumen bacteria was also examined (Aberdeen studies).

The results showed that the saponin fractions of *S. rarak* exhibit antiprotozoal activity as confirmed by visual assessment as well as by measurement of the breakdown of ^{14}C-leucine-labelled *Prevotella bryantii* in rumen fluid incubated *in vitro*. No evidence was found of protozoal resistance to *S. rarak*. Inclusion of methanol extract of *S. rarak* in the growth medium of pure cultures of rumen bacteria had no effect, except that cellulolytic bacteria showed susceptibility. Extracts appeared in this study to prolong the lag phase following inoculation of *Streptococcus bovis* or inhibit the growth of *Butyrifibrio fibrisolvens*, *Ruminococcos albus*, and *Ruminococcos flavefaciens*. It seems that antibacterial

properties were more pronounced against gram-positive bacteria, similar to the action of ionophores (McGuffey *et al.*, 2001).

A study on the effects of *S. rarak* on ruminal digestion, fermentation and ammonia concentration, using ruminally and duodenally cannulated dairy cows, showed that direct administration of *S. rarak* into the rumen did not decrease protozoal numbers *in vivo*. However, there were some indicators of selective activity against holotrichs. Changes in propionate concentration in the rumen and lack of change in microbial flow parameters suggest no adverse effects on other fermentation measures. Increased production of propionate is beneficial to the animal by affecting the capture of fermentation energy in the rumen. *S. rarak* caused a decrease in ammonia concentration. The observed ammonia levels represent a balance between the processes of degradation of feed protein and uptake of ammonia for synthesis of microbial protein. The lower ammonia levels could be due to higher incorporation of ammonia, peptide, or amino acids into microbial protein.

In conclusion, *S. rarak* tested in this study has a great potential for suppressing rumen ciliate populations, but exerted negligible general effects on ruminal fermentation. Therefore, controlling rumen ciliate protozoa would be expected to lessen the dependence on protein supplementation under high-production conditions and would also be beneficial under conditions where the quantity of protein absorbed from the post-ruminal gut limits animal productivity, which occurs frequently in animals receiving low-quality tropical forages.

CHAPTER 1

INTRODUCTION

Ruminant animals receive nutrients from a number of sources. A large proportion of the protein is supplied by rumen microbes; the rest is supplied through bypass protein, which is undegradable in the rumen and goes to the small intestine to be digested and absorbed along with microbial protein. Energy is produced mainly from microbial waste byproducts of the fermentation and digestion process called volatile fatty acids (VFAs). These acids are absorbed through the rumen wall.

The rumen is a vital organ in the nutrition of the ruminant where continuous feed fermentation takes place. As a complex environment that is composed of bacteria, protozoa and fungi, ruminal microbes work together to attack and digest the consumed feeds. Therefore, the microbial population in the rumen should be considered when feeding ruminant animals.

Lysis of bacteria in the rumen can severely reduce protein to energy ratio in the nutrients delivered to the animal for digestion and absorption (Leng and Nolan, 1984). Bacterial lysis may occur from death of bacteria when their substrate in the rumen is exhausted (Hespell, 1979). Degradation *in situ* of rumen bacteria also results through predation of bacteria by protozoa (Coleman, 1975). Although, microbial growth efficiency in the rumen can be high, the ratio of protein relative to energy that is available for digestion and absorption can be reduced markedly due to this lysis.

The rumen can be manipulated to improve the utilization of substrates and the efficiency of fermentation and to optimise the nutritional value of feeds. A potential approach to improving the production efficiency of ruminant animals is by maximizing microbial protein synthesis and flow to the duodenum by reducing the recycling of microbial N in the rumen (Koenig *et al.*, 2000). Rumen protozoa are actively involved in the degradation of dietary and microbial proteins in the rumen and may influence ruminal bacterial growth and intestinal flow of protein of both feed and bacterial origin (Jouany, 1996).

In vitro studies suggest that the engulfment and digestion of bacteria by protozoa is quantitatively the most important cause of bacterial turnover in the rumen. The rate of uptake of bacteria per protozoan was about 10^3/h based on the average rate of degradation of *Selenomonas ruminantium* protein of 2%/h per 10^5 protozoa per ml and assuming the total bacterial population was 5 x 10^9/ml (Wallace and McPherson, 1987). William and Coleman (1992) showed that all protozoa species engulf mixed rumen bacteria and they engulf those grown *in vitro* faster (1.5 to 17.6 times) that those grown *in vivo*. The variation in maximum uptake per unit protozoal volume for protozoa grown *in vitro* is from 1750 (with *Ostracodinium dilobium*) to 9235 (with *Entodinium longinucleatum*) and from 390 (with *Ophryoscolex caudatus*) to 3930 (with *Eremoplastron bovis*) for protozoa grown *in vivo*. Thus it is apparent that removing ciliate protozoa from the rumen (defaunating) avoids the recycling of nitrogen between bacteria and protozoa and thereby increases the efficiency of nitrogen metabolism in the rumen and stimulates the flow of microbial protein from the rumen (William and Coleman, 1992).

Most of the initial studies on rumen protozoa addressed two questions i.e., their specificity for this habitat, and whether the protozoa were essential for the host animal. Both questions were eventually answered in studies using defaunated or protozoa-free animals (Hespel *et al.*, 1997).

Leng *et al.* (1992) reported that the performance of fauna-free animals was better than that of faunated animals. Defaunation of the rumen is likely to be associated with a positive response in body weight gain when diet does not supply sufficient protein post-ruminally to meet animal requirements (Bird and Leng, 1983). Other positive production responses of defaunation include a highly significant increase in wool growth (Bird and Leng, 1984) and increased milk production from dairy cows (Moate, 1989).

The elimination of protozoa by chemical means is potentially the most convenient method to obtain fauna-free animals (Ushida *et al.*, 1991). However, since it is likely that chemicals are not specifically toxic to protozoa and probably kill other microorganisms and host cells and also because their antiprotozoal activity is inconsistent, new natural antiprotozoal agents have been studied. There is increasing interest in exploiting natural products to solve the problems in animal nutrition and livestock production following increasing awareness of hazards from consumers and health authorities on the use of chemical feed additives in the diets of farmed animals. Recently, some tropical plants were found to have the potential to be used to suppress or eliminate protozoa from the rumen (Diaz *et al.*, 1994; Navas-Camacho., 1993; Newbold *et al.*, 1997). These plants had the charateristic that they were rich in saponins.

A potential source of natural antiprotozoal agents is *Sapindus rarak* DC. *Sapindus rarak* DC (Sapindaceae) occurs naturally on the islands of Southeast Asia and produces fruits that are not used for food purposes. The genus *sapindus*, however, is well known for the high saponin content of its fruits. The pericarps of *Sapindus rarak* contain saponins, high-molecular-weight glycosides consisting of a sugar moiety linked to an aglycone (Hamburger *et al.*, 1992). Saponins have detergent or surfactant properties because they contain both water-soluble and fat-soluble components. Saponins, by virtue of their surfactant properties, have antiprotozoal activity by reacting with cholesterol present in protozoal cell membranes, causing cell lysis. The susceptibility of rumen protozoa, and lack of susceptibility by rumen bacteria, to saponins is probably explained by the presence of cholesterol in eukaryotic membranes but not in prokaryotic bacterial cells (Klita *et al.*, 1996).

Experimental Objectives

The aim of the work described here was to examine the antiprotozoal factor from the pericarp of *S. rarak* for its potential to defaunate the rumen, either partially or completely, without having a detrimental effect on the bacterial population and on rumen fermentation.

CHAPTER 2

REVIEW OF LITERATURE

2.1. Rumen Fermentation

Cattle and other ruminants are markedly different from monogastric animals in that they have four separate stomach compartments, known as the rumen, reticulum, omasum and abomasum, at the beginning of the gastrointestinal tract. The rumen allows ruminants to extract and absorb energy from structural components of plant material, which would not be available to mammalian enzymes. The rumen is essentially a fermentation chamber, where the resident microbial population digests any feed consumed by the animal. In sheep and cattle, the rumen represents 64-71% of the volume of the digestive tract and 9-13% of the total body volume (Van Soest, 1994). The products of microbial fermentation, mainly volatile fatty acids and microbial protein, will be available for absorption by the host animal. Volatile fatty acids can supply up to 80% of the animal's energy requirement, while microbial protein leaving the rumen can account for between 50 and 90% of the protein entering the small intestine (Beever and Siddon, 1986).

Maximising ruminant production involves meeting the requirements for both rumen microbial metabolism and mammalian metabolism in the tissues. In ruminants, fermentation activity appears to be a key component of the digestive process as nutrient inputs are subjected first to fermentative digestion by rumen micro-organisms in the rumen prior to gastric and intestinal digestion. The extent and type of transformation of feedstuffs determine the production performance of

the host animal. The interactions of the normal microbial flora with the host can be manipulated to improve the efficiency of nutrient utilization in ruminant animals (Mackie and White, 1990).

Two approaches are available to increase the efficiency of ruminants to use dietary energy. Firstly, increasing the efficiency to transform the energy of feed mainly fiber and starch into end-products of rumen fermentation, especially into volatile fatty acids (VFA); secondly, increasing the efficiency of utilising the end-products of fermentation for basal physiological processes and for the synthesis of animal products (Baran, 1997). Furthermore, manipulation of rumen digestion is dependent on factors such as the nature and level of feeding and the animal production system. High levels of animal production can be obtained by feeding high amounts of starch and protein with consequences that the rumen optimisation in this production system will be different from that of animals fed rations based on roughage and non-protein nitrogen. Rumen optimisation should be considered in terms of maximising reactions, such as the degradation of fibre to VFA, lactate fermentation, and conversion of non-protein nitrogen to microbial cell protein, or minimising reactions in the rumen, such as methane production, feed protein degradation, biohydrogenation of unsaturated fatty acids (Baran, 1997). Carbohydrates are hydrolysed by various routes to pyruvic acid, which is then fermented to acetic, propionic and butyric acids, The extent of breakdown and the proportions of these acids are determined by the nature of the feed. The volatile fatty acids are then absorbed through the rumen wall, while methane and carbon dioxide are the by-products of rumen fermentation. Dietary protein in the rumen are hydrolysed to peptides and amino acids which amino acids may be deaminated to yield ammonia. Microorganisms use these products,

and also non-protein sources of nitrogen to synthesise microbial protein. Microbial cells pass from the rumen to the abomasum and small intestine, where they are digested by the host animal's enzymes (Nagaraja *et al.*, 1997).

Methane produced during anaerobic fermentation in the rumen represents an energy loss to the host animals. If methane production is inhibited using methane inhibitors or by inhibition of methanogenesis and direct the hydrogen to a greater production of propionic acid, then fermentation efficiency could be increased (Ørskov, 1975). A reduction in any of the reactions involved in the conversion of protein to ammonia (proteolysis, peptide degradation, and amino acid deamination) will result in a higher supply of amino acids available for absorption which can increase animal productivity (Nagaraja et al., 1997). The reduction of hydrogenation can be approached either by reducing specific microbial activity with additives, or by protecting fats against microbial attack (Jouany et al., 2000).

Over the last five decades considerable efforts have been made to understand and ultimately manipulate the rumen microbial population to improve animal productivity (Nagaraja *et al.*, 1997). Modification of rumen microbial composition can be achieved by means of additives that selectively affect rumen symbiotes, or by genetically manipulating rumen microorganisms (Bello and Escobar, 1997).

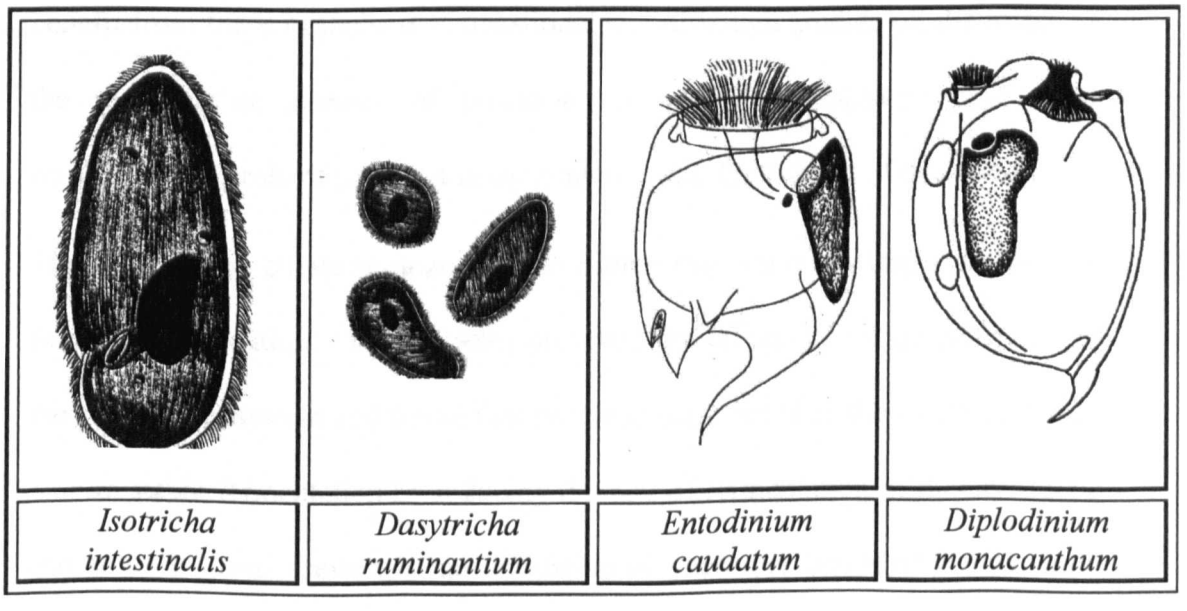
2.2. The Role of Protozoa in Ruminant Animals.

2.2.1. Protozoa in the rumen ecosystem

The rumen microbial ecosystem is characterised by an extreme variety and high density of microbial cells, which exist in a highly dynamic state. The total

population can change dramatically with factors such as feeding frequency and type of diet. Although bacteria are the most abundant micro-organisms (10^9 to 10^{10} per ml), protozoa (10^5 to10^6 per ml) may be equal in total microbial mass because protozoa are larger in size (Ogimoto and Imai, 1981). Protozoa can be divided into ciliates and flagellates, but most of those in the rumen are ciliate protozoa. Two groups of ciliates, entodiniomorphid (oligotrich) and the holotrich protozoa (Fig. 2.1), usually occur in the rumen.

Entodiniomorphids are characterized by the presence of a firm pellicle and by cilia situated only on the peristome and sometimes elsewhere. The holotrich protozoa have a more flexible pellicle, which is almost covered by cilia. The type and density of protozoa in the rumen are affected by factors, such as level of feeding and roughage-to-concentrate ratio, rumen pH, and physical form and particle size of the diet. Protozoa also transform an array of plant and bacterial constituents into cell components and metabolites that can be used by the host.



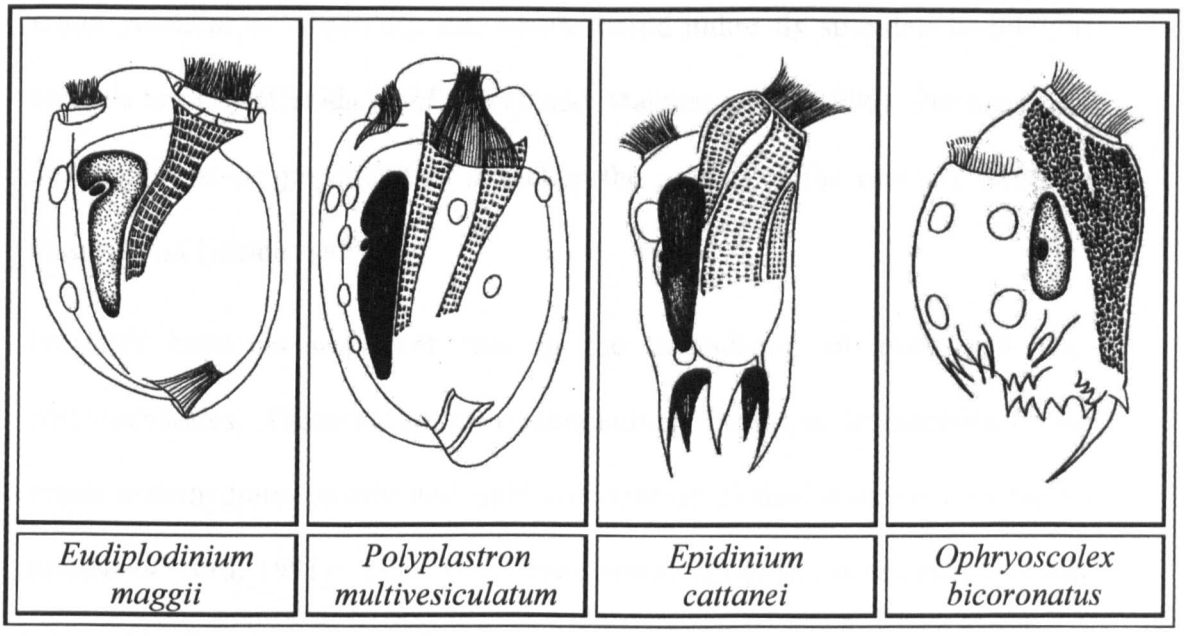


Figure 2.1. Some typical types of rumen protozoa (Dehority, 1993).

Although the rumen entodiniomorphid and holotrich ciliates both depend upon plant material, they do not compete directly for available nutrients as the holotrichs primarily use soluble carbohydrates, whereas the entodiniomorphid ciliates ingest and utilize particulate material (William and Coleman, 1988). They also ingest and digest bacteria in significant quantities (Rowe *et al.*, 1983). However, ruminants without protozoa are apparently normal and healthy, so the benefit from these organisms is questionable. Although studies on the effect of the presence or absence of protozoa on animal productivity have been undertaken, the role of protozoa in the rumen is still the subject of discussion.

The presence of ciliate protozoa in the rumen may serve an important role in ruminal fermentation. Veira (1986) observed the effects of ciliate protozoa on the ruminal ecosystem and found that protozoa contributed to the maintenance of a more stable fermentation by reducing the rate of fermentation of dietary starch and soluble sugar, preventing accumulation of excessive levels of lactate, thus giving an increase in acid tolerant, lactate-utilising bacteria and decreasing the

redox potential of rumen digesta, which should indirectly stimulate cellulolytic bacteria activity (Ushida *et al.*, 1991 and Mathieu *et al.*, 1996). Protozoa may also supply some peptides that stimulate the growth of the rumen microbiota (Jouany and Ushida, 1998).

Protozoa have an important role in the degradation of plant cell wall polysaccharides. Protozoa are also quantitatively important in digestion of the major carbohydrate, protein and lipid components of feed material ingested by ruminants (Bird, 1991). However, certain species of protozoa are preferentially retained in the rumen and only a small proportion of protozoal matter actually leaves the rumen (Leng *et al.*, 1980). Therefore, a reduction in net microbial synthesis and an increase in dietary protein degradation in the rumen are associated with an inefficiency of feed utilisation (Veira, 1986).

2.2.2. Ecological relationship between protozoa and other microorganisms

2.2.2.1. Interrelationships between protozoal species

The establishment of rumen protozoa is affected not only by the physiological status of the rumen caused by variations in the diet, but also by changes in the interrelationship between various ciliate species, either complementary or competitive. Ciliates are classified into two overall types. Type A includes *Polyplastron multivesiculatum*, *Diploplastron affine* and *Ophryoscolex tricoronatus*. Type B includes *Eudiplodinium maggii*, *Epidinium* sp., *Eremoplastron* sp., and *Ostracodinium* sp. Common species include *Entodinium* sp., *Isotricha* sp., and *Dasytricha ruminantium*. On various diets based either on forage or concentrate, or both, the ciliate population is made up of *Entodinium* sp. plus holotrichs, plus ciliates of type A, or *Entodinium* sp. plus holotrichs, plus

ciliates of type B. It is likely that the existence of the A and B type depends upon the predatory activity of *Polyplastron multivesiculatum* which engulfs and can completely eliminate *Eudiplodinium maggii*, *Epidinium* sp, *Eremoplastron* sp., and *Ostracodinium* sp. from a rumen (William and Coleman, 1992).

When the diet is made up of straw, there is complementarity between *Polypastron multivesiculatum* and *Entodinium* sp, but when it is rich in starch there is an incompatibility and *Entodinium* disappears (Bonhomme, 1990).

2.2.2.2. Protozoan-bacteria interactions

Qualitative and quantitative composition of the microfauna is affected by changes in the bacterial flora. Since protozoa are not able to make de novo amino acid synthesis from ammonia, bacteria are a vital source of protein. Protein or peptides from bacteria are used to synthesis their own (Jouany and Ushida, 1999). All rumen ciliates ingest bacteria and some digest them and release low molecular weight compounds, mainly amino acids, into the medium (Coleman, 1975). The level of ingestion of rumen bacteria examined *in vitro* varied from 20 to 58000 bacteria per protozoan per hour from a bacterial suspension of 10^9 ml^{-1} (Table 2.1), depending on the bacterial and protozoan species and on the numbers of ciliates and bacteria, with level of digestion varying from 0 to 3600 bacteria per protozoan per hour (Coleman and Sandford, 1979).

Entodinium sp selectively ingest bacteria of ruminal origin and rapidly digest two cellulolytic bacteria (*Butyrivibrio fibrisolvens* and *Ruminococcus flavefaciens*). *Butyrivibrio fibrisolvens* is ingested and digested more rapidly than other bacteria by ciliates (*Epidinium*, *Eremoplastron*, *Eudiplodinium*, *Ostracodinium* and *Entodinium*). Some ciliates, such as *Epidinium* sp., lyse the bacteria (*Bacillus*

megaterium, *Bacillus subtilis* and *Micrococcus lysodeikticus*) in the media before ingesting them (Coleman, 1980). *Isotricha prostoma* selects and ingests only certain rods, while *Dasytricha ruminantium* selects and ingests small cocci (Coleman, 1980).

Table 2.1. Uptake of bacterial species by *Entodiniomorphid* protozoa *in vitro* (Coleman and Sandford, 1979).

Bacteria	Bacteria engulfed per ciliate h^{-1} at 10^9 bacteria ml^{-1}
	Epidinium ecaudatum
Escherichia coli	700
Proteus mirabilis	6600
Butyrivibrio fibrisolvens	320
Streptococcus fragilis	1260
Mixed bacteria	3600
	Entodinium caudatum
Escherichia coli	1300
Proteus mirabilis	830
Butyrivibrio fibrisolvens	1400
Bacillus megaterium	370
Mixed bacteria	950
	Entodinium simplex
Escherichia coli	240
Proteus mirabilis	450
Butyrivibrio fibrisolvens	1100
Bacillus megaterium	20
Klebsiella aerogenes	710
Streptococcus bovis	190
	Polypastron multivesiculatum
Escherichia coli	9600
Proteus mirabilis	58000
Bacillus megaterium	1530
	Ophryoscolex sp.
Escherichia coli	21800
Proteus mirabilis	9200
Bacillus megaterium	4050

Numerous bacteria, predominantly *Streptococcus bovis* and *Ruminococcos albus*, are attached to the cell surface of protozoa (Imai and Ogimoto, 1978). Metabolic interactions between protozoan and bacteria are reflected in a specific somatic interaction between methanogenic bacteria and ciliates, especially *Entodinium*, *Polypastron*, *Diplodinium* and *Epidinium* (Stumm *et al.*, 1982). These bacteria are concentrated on the wall of the ciliates. Protozoa support the methanogenic activity of the attached methanogenic bacteria (Krumholz *et al.*, 1983). Shortly after feeding, the methanogens obtain hydrogen from outside the protozoa, whereas at low hydrogen pressures the protozoa cells are the predominant donors. Meanwhile, *holotrichs* are never associated with methanogens in spite of their hydrogen-producing metabolic pathways (Stumm and Zwart, 1986).

2.2.2.3. Protozoan-fungi relationships

Rumen protozoa obviously must interact with fungi in their ecosystem. According to Orpin (1984), protozoa and fungi may be complementary in the rumen. *Phycomycetes* may represent up to 8% of the microbial biomass (Orpin, 1981). They might be considered to have an ability to degrade the structural barriers in plants or to partially degrade and weaken the more resistant tissues, which relate to intake or passage of fibre through the intestinal tract. Fungal biomass in the rumen appears to be greater on high-fibre than on high-concentrate diets, which is contrary to findings in the ciliate protozoa. However, Bird and Leng (1985) observed a competitive interaction between these organisms with sheep fed straw-based diets. The concentration of viable zoospores in rumen fluid collected from defaunated sheep was 2-5 times higher than in rumen fluid from faunated sheep given high-roughage diets (Bird and Leng, 1984).

2.2.3. *Implications of protozoa for protein digestion*

Protozoa utilize bacterial and feed protein available in the rumen. Protozoa need to use proteins or peptides since they have no urease to use ammonia for synthesis of amino acids (Onodera *et al.*, 1977). Protozoa actively prey on bacteria. A single protozoan can take up $10^2 - 10^4$ bacteria h^{-1} (Coleman, 1975). Bacterial proteins are degraded within protozoa into small peptides and free amino acids, which are then incorporated into protozoal proteins. Protozoa excrete some peptides coming from bacterial proteins into rumen fluid as free amino acids. As a consequence, the total concentration of bacteria is lower and the concentration of amino acids and peptides is higher in the faunated than in the defaunated rumen (Hsu *et al.*, 1991).

The importance of protozoa on rumen protein degradation depends on the ratio between particulate proteins and soluble protein in the rumen. It is also related to the various protozoal genera and their concentration. Entodiniomorphid protozoa are particularly efficient in taking up insoluble proteins, while the soluble fraction is more degraded by holotrichs (Jouany and Ushida, 1999). Since protozoa depend on dietary and bacterial protein by degrading part of it to low molecular weight compounds, they reduce the amount of protein available to the host animal.

2.2.4. *Implications of protozoa for energy digestion*

Protozoa have an important role in the degradation of plant cell wall polysaccharides. Protozoa are also quantitatively important in digestion of the major carbohydrate, protein and lipid components of feed material ingested by the ruminants (Bird, 1991).

The impact of protozoa on rumen digestion depends on their concentration and the generic composition of their population (Jouany and Ushida, 1999). The number of protozoa is related to the energy content of the diet. The protozoa contribute to the nutrition of the host animal through their metabolic activities; the metabolites and cells formed represent a potential source of nutrients for the host.

The presence of protozoa in the rumen has been shown to influence the volume of the rumen and the retention time of digesta, the concentration and proportion of volatile fatty acids, the levels of other acidic metabolites and ammonia, rumen pH, and the numbers and type of rumen bacteria present (William and Coleman, 1992). The proportions of the main energy-yielding end products, the volatile fatty acids (VFA), are often changed by the presence or absence of protozoa (Jouany *et al.*, 1981).

2.3. Effects of Defaunation on the Rumen Ecosystem

Many studies have been carried out on the control of the rumen microbial ecosystem with the aim of improving its efficiency in terms of nutrient supply to ruminants. Some treatments were designed to alter the microbial ecosystem by eliminating protozoa (defaunation). Protozoa have positive and negative effects on digestion in the rumen. Protozoa may slow down the fermentation of dietary starch and soluble sugars and stabilize rumen pH when animals are fed diets rich in available starch. They may also supply some peptides that stimulate the growth of the rumen microbiota. However, protozoa also have several negative effects, such as the predation of bacteria, which increases protein turnover in the rumen, and their contribution to methane production (Jouany and Ushida, 1999). Therefore, removing ciliate protozoa from the rumen avoids a cycle of bacterial

protein breakdown and resynthesis in the rumen and increases the flow of protein to the animal (Lindsay and Hogan, 1972). Under conditions where the quantity of protein absorbed from the post-ruminal gut limits the productivity of the animal, which occurs frequently in animals receiving low-quality tropical forages, such a dietary manipulation would have enormous value.

2.3.1. Methods of defaunation

A number of methods have been employed to obtain fauna-free ruminants and these can be placed in four categories:

(1) **Isolation of newborn animals**. Since protozoa are not present in newborn animals, the removal of young animals from the dam and rearing in isolation will keep them free of protozoa. Jouany (1978) recommended the youngest age for separation is between 48 and 72 h after birth, which allows colostrum consumption for more disease-resistant young. During this short time, the young can get native bacterial populations (Fonty *et al.*, 1988) without ciliate contamination. The newborn animal acquires ciliates in the first weeks of life through maternal contact or by physical contact with others in the herd (Fonty *et al.*, 1988). Therefore, once defaunated, animals must be isolated from any contact with other ruminants. However, although this technique is reliable and perhaps the best method for producing ciliate-free animals, it is not flexible because the experimental animals need to be prepared well in advance. Also, Ushida *et al.* (1991) reported that defaunated animals prepared by this method did not develop a "normal" microbial population in the rumen, and this could introduce a bias into the results.

(2) **Chemical drenching**. Rumen protozoa are susceptible to various chemicals, especially to surface-active agents that can disrupt cell membranes.

Many chemicals have been used for this purpose, such as dioctyl sodium sulphosuccinate ("Manoxol OT") (Orpin and Letcher, 1984), nonyl phenol ethoxylate ("Terics") (Bird *et al.*, 1979) and sodium lauryl diethoxy sulphate ("Alkanate 3SL3") (Bird and Leng, 1984). Chemicals are introduced into the adult animal's rumen, either with an oesophageal probe or through a rumen fistula, in order to destroy protozoa. However, this method has some problems for experimental design. The removal of protozoa by this method is often followed by a loss of weight after the chemical drenching due to depressed feed intake (Bird, 1989). A problem may arise if experimental animals are treated, and then some are reinoculated with protozoa for use as the control animals. This method has been criticised because there is no guarantee that the re-inoculated animals are comparable to untreated animals. The chemicals used are not specifically toxic only to the protozoa but probably also to other micro organisms and the host cells in the rumen. Therefore, the development of a chemical defaunating agent that is specifically toxic to the protozoa and has no deleterious effects on the animal becomes very important.

(3) **Physical Treatment.** This consists of emptying the rumen and treating the contents before they are replaced. Eadie and Oxford (1957) used this procedure to eliminate holotrich protozoa by heating the rumen contents to 50^0 C for 15 min. Jouany and Senaud (1979) froze rumen contents, which completely kills all rumen ciliates. After the rumen is emptied, the animals are starved for about 40 h. The rumen mucosa is washed with water (39^0 C) and treated with a formaldehyde solution, which is then rapidly rinsed off. Although this method is reliable, up to 10% of treated animals required a second treatment for complete

defaunation. Incomplete washing of rumen mucosa after formaldehyde treatment may kill the host animals and the method is impractical at farm level.

(4) **Dietary manipulation**. Although defaunation by dietary manipulation is probably safer than chemical drenching, this method has not been widely used. Some plants produce secondary compounds as protective agents against insect, fungal, protozoal and microbial attack. Many secondary plant compounds, alkaloids, flavonoids, phenolic compounds, terpenoids and tannins are toxic to rumen microbes (Cowan, 1999; Reichling, 1999). Leng *et al.* (1992) surveyed tree forages with secondary plant compounds to study their potential to manipulate rumen protozoa and found that some of them, *Enterolobium cyclocarpum, Enterolobium timbouva, acacia sp, Desmanthus intortum, Indigofera schemperi and Lotus pedunculatus*, indicate a great potential as defaunating agents. Some tree legumes in sub-Saharan Africa have been investigated as potential supplements for ruminants because of their beneficial effect of increasing metabolisable energy intake, N intake and feed efficiency, and improving animal performance. Furthermore, the results also suggested that some plant materials might have a nutritional value beyond simply their nutrient content, i.e. as rumen-manipulating agents. The foliage of some tree legumes has been shown to be selectively toxic to rumen protozoa (Teferedegne, 2000).

2.3.2. Effects of defaunation on nutrient digestibility

Defaunation causes an increase in the total number of bacteria in the rumen (Eadie and Gill, 1971) because ciliates prey on bacteria as a source of nitrogen and nucleic acids. The number of amylolytic bacteria is also increased after defaunation as a consequence of elimination of nutrient competition between bacteria and protozoa for starch use, or by elimination of ciliates, which prey on

starch-adherent bacteria. Defaunation may yield improved N utilization by ruminants by increasing amino acid supply to the intestine and by reducing urinary N excretion. The turnover of microbial N may account for half of the microbial N formed in the rumen and results mainly from the predatory activity of protozoa toward bacteria and the retention of protozoa within the rumen (Demeyer and van Nevel, 1986).

Although protozoa are not able to produce methane, protozoa are active hydrogen producers, which are closely associated with methanogenic bacteria that transform hydrogen and carbon dioxide into methane. Therefore, defaunation could reduce methanogenesis (Ushida and Jouany, 1996).

However, protozoa must not be considered as a whole, some genera having specific effects in the rumen and their elimination can have detrimental effects on animals. For instance, some protozoa play an important role in the digestion of plant cell walls, Jouany and Ushida (1999) found that the presence of protozoa increased the digestion of plant cell wall by 15%. The positive effect of cellulolytic protozoa on cell wall digestion appears to be due to the ingestion and breakdown of plant material by the protozoa together with the removal and metabolism of starch and lactic acid which might have influence on the celullolytic activity in the rumen (Ushida *et al.*, 1989) or due to indirect effect of protozoa on rumen volume and particle retention (Demeyer, 1989), the size and nature of the bacterial population and the physico-chemical conditions of the ruminal environment (Williams and Withers, 1991). In most cases, elimination of these protozoa decrease the digestibility of plant cell wall (Demeyer, 1981;Ushida and Jouany, 1990:Chaudary *et al.*, 1995).

2.3.3. Effects of defaunation on animal performance

Although protozoa play a role in the production of energy utilizable by the host, the availability of metabolisable energy in defaunated animals was higher due to reduction of energy loss in methane production (Santra *et al.*, 1994).

Defaunation improved animal growth rate by 43% without an effect on feed intake (Bird and Leng, 1978). Eliminating protozoa from the rumen of lambs also resulted in an increase in wool growth by 37% (Bird and Leng, 1984). Moate (1989) examined the effects of defaunation on milk yield and milk composition in grazing dairy cows. It was found that the total number of protozoa was reduced by 95% in the rumen fluid of cows treated with the detergent Alkanate 3SL3 (sodium lauryl diethoxy sulphate). The yield of milk and milk protein content was increased from 20.0 l/cow/day in control cows to 22.7 l/cow/day in treated cows. Defaunation also increased the milk protein yield from 598 to 713 g/cow/day as well as increasing the milk protein : fat ratio from 0.69 to 0.78.

2.4. Studies on Antiprotozoal Agents

Many antiprotozoal agents have been studied experimentally, however, none has passed into routine use because of toxicity problems, either to the rest of the rumen microbial population or to the host animal (William and Coleman, 1997). It has been suggested that naturally occurring plant metabolites might be toxic to rumen protozoa, and some studies on the use of tropical plants as defaunating agents have appeared. A decrease in protozoal numbers was reported in the rumen of sheep infused with alfalfa saponin isolated by ethanol extraction and partial acid hydrolysis (Lu and Jorgensen, 1987) or fed on saponin-containing plants; *Enterolobium cyclocarpum* (Navas *et al.* 1993), *Sapindus saponaria* (Diaz *et al.*, 1994), *Sesbania sesban* (Newbold *et al.*, 1997; Odenyo *et al.*, 1997;

Teferedegne *et al.*, 1999), *Quillaja saponaria* and *Acacia auriculoformis* (Makkar *et al.*, 1998) and *Yucca schidigera* extract (Makkar *et al.*, 1998; Wang *et al.*, 1998). Inclusion of *E. cyclocarpum* increased the rate of body weight gain by 24 % (Leng *et al.* 1992) and 44 % (Navas *et al.* 1993), and wool growth by 27 % (Leng *et al.* 1992), which was attributed to a decrease in protozoal numbers. Foliage from *Sesbania sesban*, a multipurpose leguminous tree from sub-Saharan Africa, inhibited protozoal activity *in vitro* and transiently depressed the number of protozoa in the rumen of sheep (Newbold *et al.*, 1997; Teferedegne *et al.*, 1999). However, when *S. sesban* was placed directly into the rumen via a rumen cannula, protozoal numbers decreased significantly (Odenyo *et al.*1997).

2.4.1. Mechanism of selective action of antiprotozoal agents

Protozoa possess a more complex genome than the prokaryotes. As a result, they contain both structural and functional features that are not shared with the prokaryotes. These features could thus constitute specific target sites for antiprotozoal action. Antiprotozoal agents should have one or more of the following attributes:

1. Selective ability to penetrate protozoa, or to exert a toxic effect from the cell surface.
2. Activity limited to the protozoa.
3. Action directed against a unique protozoal target in the protozoa and active against all protozoal species and life cycle stages.
4. Where target sites are shared with other organisms (including perhaps the host animals) selectivity may result from accessibility of target sites in the

protozoa, which are more vulnerable than those in associated micro organisms (Van Demark and Batzing, 1987).

Generally, the mechanisms of antiprotozoal agents are related to:(1) Interference with energy metabolism; (2) Blocking cofactor synthesis; (3) Interference with protein synthesis; (4) Interference with nucleic acid metabolism and (5) Interference with membranes (Peter, 1985).

(1) Interference with energy metabolism

Some antiprotozoal compounds interfere with ATP production by disrupting the function of the glycerophosphate pathway which interferes with the ATP production. The specific example for this interference is shown by trypanocidal drug Suramin, the chemotherapeutic agent used against African sleeping sickness. This drug disrupts the function of the glycerophosphate pathway by interfering with the reoxidation of NADH and leading towards the production of glycerol and as a result in a reduction in ATP production (Peter, 1985).

(2) Blocking cofactor synthesis

The mechanism of action of some antiprotozoal compounds results from interference with uptake or incorporation of a number of different metabolites which reduce cofactor synthesis. For example, sulfonamides act as inhibitors of dihydropteroate synthetase (DHPS) by competing with para-aminobenzoic acid (PABA) in the biosynthesis of dihydrofolate. Dihydrofolate is required in the synthesis of thymidylate particularly but also purines, methionine, thiamine, pantothenate and formyl methionyl tRNA (Bryan, 1982).

(3) Interference with protein synthesis

A small group of antiprotozoal agents functions by interfering with protein synthesis. For example, chloramphenicol inhibits peptide bond formation on ribosomes by binding to the ribosome subunit (O'Leary, 1989).

(4) Interference with nucleic acid metabolism

A number of agents have a marked inhibitory action on: (1) nucleotide metabolism, such as azarine which inhibits purine biosynthesis (Bryan, 1982); (2) DNA and RNA synthesis, such as chloroquine (O'Leary, 1989).

(5) Interference with membranes

This kind of interference is most relevant to the present study. Some antiprotozoal compounds are known to act through interference with membrane structure. A number of studies have been conducted to remove protozoa from the rumen of sheep and cattle by using agents acting as ionophores, such as Alkanate 3SL3 (with lauryl ethyl sulphate as the active compound) and nonionic compounds, e.g. nonyl phenol ethoxylate (Teric GN9).

Cell membranes form an expandable barrier to control the entry and exit of solute molecules. These functions make the membrane vulnerable to a variety of agents (Hammond and Lambert, 1978). Essentially all membranes, whether from plants, animals, or micro organisms have a structural pattern based on a phospholipid bilayer (Fig.2.2). Phospholipids are amphiphilic with the hydrocarbon tail of the molecule being hydrophobic; its polar head hydrophilic.

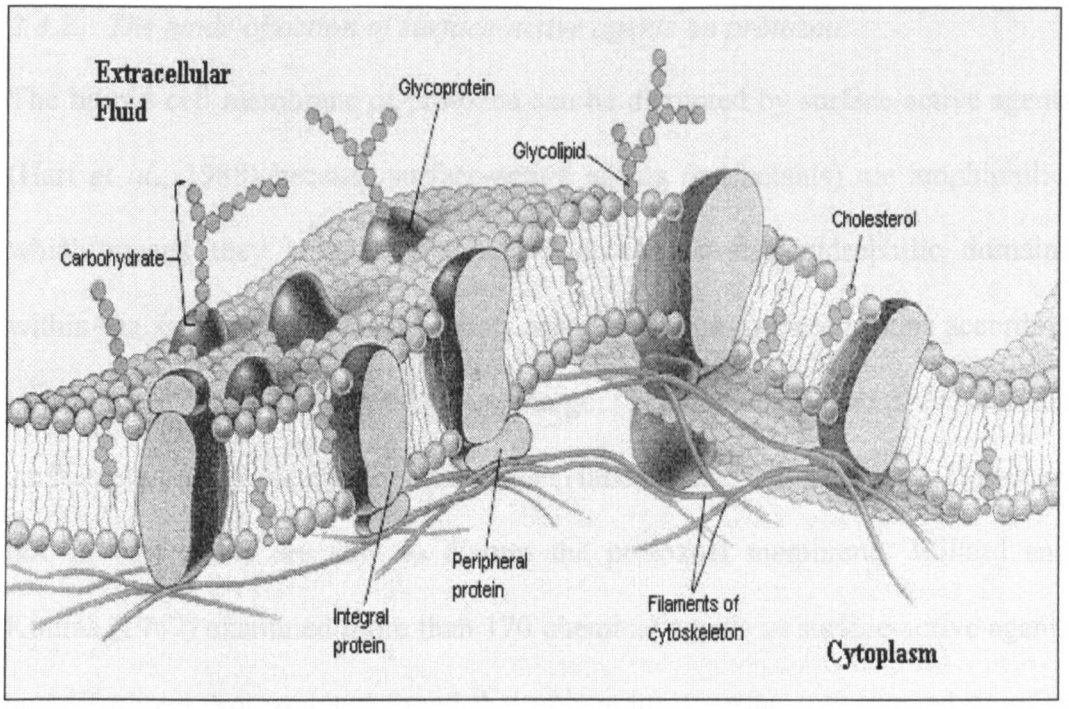


Figure 2.2. Structure of a typical cell membrane
(http://www.people.virginia.edu/~rjh9u/cellmemb.html)

Bacterial membranes differ from eukaryote membranes in several ways, notably in containing no cholesterol (Klita *et al.*, 1996). Cell membranes can be disrupted by surface-active compounds. These compounds bind to membrane sterols, such as cholesterol which they form insoluble complexes, and increase the permeability of the membrane to cations (Peter, 1985). This action initiates further deterioration (Pelczar *et al.*, 1986) causing breakdown of the membrane, cell lysis and death (Cheeke, 1999). Some of these surface-active compounds were tested for toxicity to rumen protozoa. Therefore, the susceptibility of rumen protozoa, and lack of susceptibility by rumen bacteria, to saponins is probably explained by the presence of cholesterol in eukaryotic membranes (including protozoa) but not in prokaryotic bacterial cells.

2.4.2. The mode of action of surface-active agents on protozoa.

The bilipid cell membrane of protozoa can be disrupted by surface-active agents (Hart *et al.*, 1988) because surface-active agents (surfactants) are amphiphilic, which means they contain separable hydrophobic and hydrophilic domains within the same molecule. Surfactants are classified into three groups according to the nature of the hydrophilic group charge: (1) anionic surfactants; (2) cationic surfactants and (3) nonionic surfactants (Hancock and Nicas, 1984). However, not all surfactants are able to disrupt the protozoal membrane. Willard and Kodras (1967) examined more than 170 chemical agents as surface-active agents against rumen protozoa and found that only anionic surfactants showed promise as antiprotozoal agents, while both cationic and nonionic surfactants were relatively ineffective.

Subsequent experiments conducted by Wright and Curtis (1976) found that the nonionic surfactants were also toxic to rumen protozoa, showing effects ranging from inhibition of motility to cell disintegration. "Teric GN9", nonyl phenol ethoxylate, and "Manoxol OT", dioctyl sodium sulphosuccinate, were found to have the most toxic effect on protozoa at concentrations as low as 0.01%.

Bird *et al.*, (1978) reported that Teric GN9, a nonionic surfactant given intraruminally, was an effective means of defaunating the rumen of lambs given low-protein-high-energy diets.

An *in vitro* study on the toxicity of some surfactants against rumen protozoa by Hart *et al.* (1988) showed that Teric GN9 killed protozoa at 250 ppm. Meanwhile, Alkanate 3SL3 an anionic surfactant, was less toxic than Teric GN9.

However, because of their toxicity to cells of the host animal, the commercial application of these surfactants is limited (Hart *et al.*, 1988)

2.5. Saponins as Antiprotozoal Agents

2.5.1. Background

Saponins are naturally occurring chemical compounds found in a wide variety of food, forage plants and a few marine animals. The definition of saponins is usually based on their surface activity; many saponins have detergent properties, give stable foams in water, show haemolytic activity, have a bitter taste and are toxic to cold blooded animals, especially fish and snails (Milgate and Roberts, 1995). The name saponins come from the Latin word 'sapo' (soap). Since some saponin-containing plants have been used as soaps, this fact is reflected in their common names: soapwort (*Saponaria officinalis*), soaproot (*Chlorogalum pomeridianum*), soapbark (*Quillaja saponaria*), soapberry (*Sapindus saponaria*) and soapnut (*Sapindus mukurossi*) (Hostettmann and Marston, 1995). Saponins have detergent or surfactant properties because they contain both water-soluble and fat-soluble components. Saponins, by virtue of their surfactant properties, have antiprotozoal activity by reacting with cholesterol present in protozoal cell membranes, causing cell lysis.

2.5.2. Saponins and ruminants

There are two reasons for investigating the effects of saponins on ruminants; to assess their potential as natural feed additives that alter rumen fermentation to improve animal productivity and to find natural antiprotozoal materials that might be used to suppress the growth of rumen ciliate protozoa. These two approaches have merged to some extent as the main effects of saponins on rumen fermentation are due to their toxicity to rumen ciliate protozoa. However, the

effects on rumen fermentation and their nutritional implications are not always consistent among different studies. Lu *et al.*, (1987) found that alfalfa saponins appeared to suppress fermentation in continuous culture. Subsequent *in vivo* studies confirmed a general decrease in fermentation activity, decreased VFA concentration and decreased cellulose digestion of the sheep fed alfalfa saponins (Lu and Jorgensen, 1987). In contrast, Van Nevel and Demeyer (1990) reported that the results of their *in vitro* study of sarsaponin, an extract from *Yucca schidigera*, showed no indication of toxic effects or effects on microbial growth or protein breakdown. Makkar *et al.* (1998) reported the beneficial effects of supplementing animal feed, particularly roughage-based diets, with extracts from *Yucca schidigera* and *Quillaja saponaria*, which led to higher microbial yields and lower emission of environment-polluting gases (CO_2 and CH_4).

2.5.3. Saponins and ruminal microorganisms

2.5.3.1.Rumen ciliate protozoa

El Hassan *et al.* (1995) screened seven multipurpose trees (MPT) for their antiprotozoal activity on rumen protozoa from sheep in Aberdeen, UK and from sheep in Debre Zeit, Ethiopia. The results showed that *Sesbania sesban* was highly toxic to rumen protozoa from sheep. Antiprotozoal activity was determined by measuring the degradation of labelled bacterial protein by protozoa based on the method of Wallace and McPherson (1987) to measure protozoal activity. It was found that tannins were not responsible for the observed antiprotozoal property of *S. sesban* after tannins were removed from solution by polyethylene glycol or polyvinyl pyrrolidine, while the precipitation of tannins had no effect on the antiprotozoal property of *S. sesban*. When an aqueous extract of *S. sesban* was extracted with n-butanol, the butanol extract

and the remaining aqueous phase were tested for their effects on the degradation of *S. ruminantium*; the antiprotozoal activity was moved to the butanol phase while the remaining aqueous extract had no effect. It was suggested that the component of *S. sesban* toxic to rumen protozoa was butanol-extractable and was likely to be part of the saponin fraction as butanol extraction causes the removal of saponins (Headon *et al.*, 1991). The observation that saponins are toxic to rumen protozoa is consistent with other studies. Navas-Camacho *et al.* (1993) showed that the antiprotozoal effects of plants, including *Sapindus saponaria*, were due to saponins. Eadie *et al.* (1956) showed that certain terpenes and other substances present in plant material had marked toxic properties on rumen protozoa, but it is unlikely that terpenes or alkaloids would be extracted into butonal phase by the method used. Akin (1982) observed that plant phenolic p-coumaric acid reduced the motility of entodiniomorphid protozoa, but it is unlikely that phenolic acids would be extracted from *S. sesban* into butanol. These results suggest that it is the saponin fraction of *S. sesban* that is toxic to rumen protozoa (El Hassan *et al.*, 1995).

2.5.3.2.Bacteria and fungi

Saponins have been shown to affect ruminal bacteria selectively. Newbold *et al.*, (1997) found that bacterial numbers increased when foliage from *Sesbania sesban* was introduced in to the diet.

Wallace *et al.* (1994) assessed an extract of the desert plant *Yucca shidigera* for its possible benefit in ruminal fermentation. Inclusion of *Y. shidigera* extract (1%, v/v) in the growth medium of the rumen bacterium *Streptococcus bovis* ES1 extended its lag phase, while growth of *Butyrivibrio fibrisolvens* (a fibre digester)

was arrested. The growth of *Prevotella* (Bacteroides) *ruminicola* B_14 was stimulated, and that of *Selenomonas ruminantium* Z108 was unaffected. The antimicrobial activities were unaffected by precipitating tannins with polyvinylpyrrolidone, but a butanol extract, containing the saponin fraction, retained its antibacterial and antiprotozoal effects. *Y. shidigera* extract, therefore, appears unlikely to influence ammonia concentration in the rumen directly, but its saponins have antiprotozoal properties, particularly in suppressing ciliate protozoa, which may prove beneficial to ruminal fermentation and may lead indirectly to lower ruminal ammonia concentrations by increasing microbial N from the rumen to the small intestine (Koenig *et al.*, 2000).

2.5.4. Chemical structure of saponins

Saponins are high-molecular-weight glycosides composed of carbohydrate and non-carbohydrate portions (Fig. 2.3). Specifically, saponins are made up of a hydrophobic aglycone (often referred to as genin or sapogenin) with a polymeric sugar side chain linked by a glycosidic bond to a hydroxyl group of the aglycone (Cheeke, 1983).

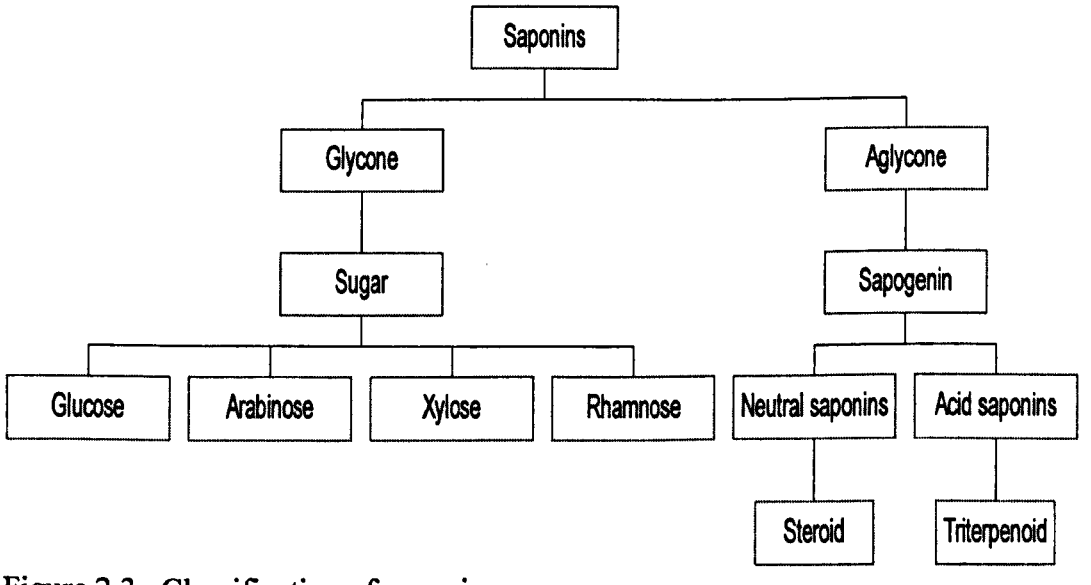


Figure 2.3. Classification of saponins

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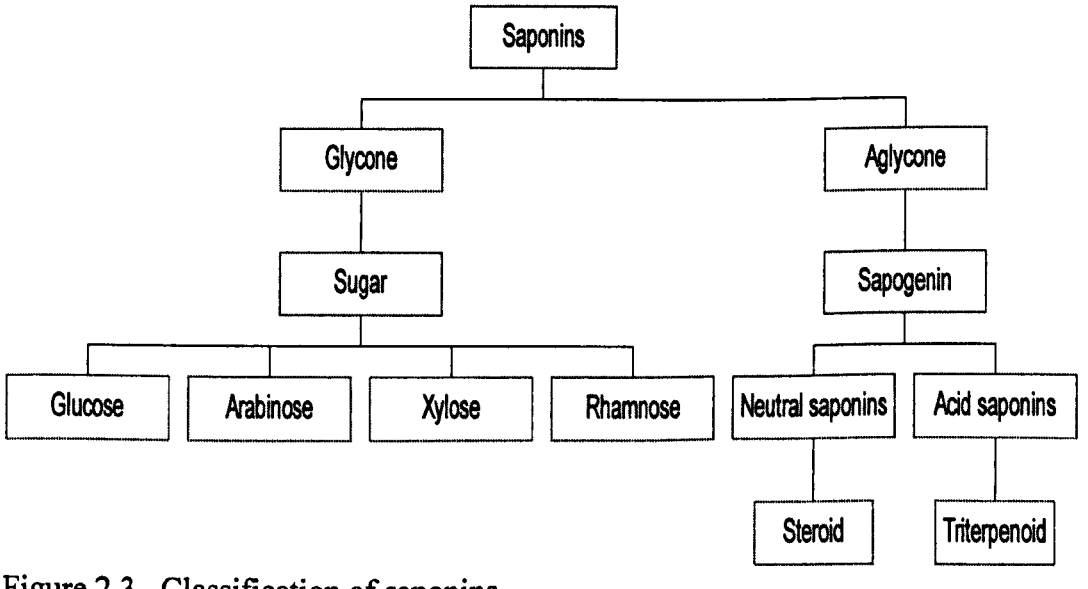


Figure 2.3. Classification of saponins

at C-3 and one attached through an ester linkage (acyl glycoside) at C-28 (Fig. 2.5). Bidesmosidic saponins are easily transformed into monodesmosidic saponins by, for example, hydrolysis of the esterified sugar at C-28 in triterpene saponins.

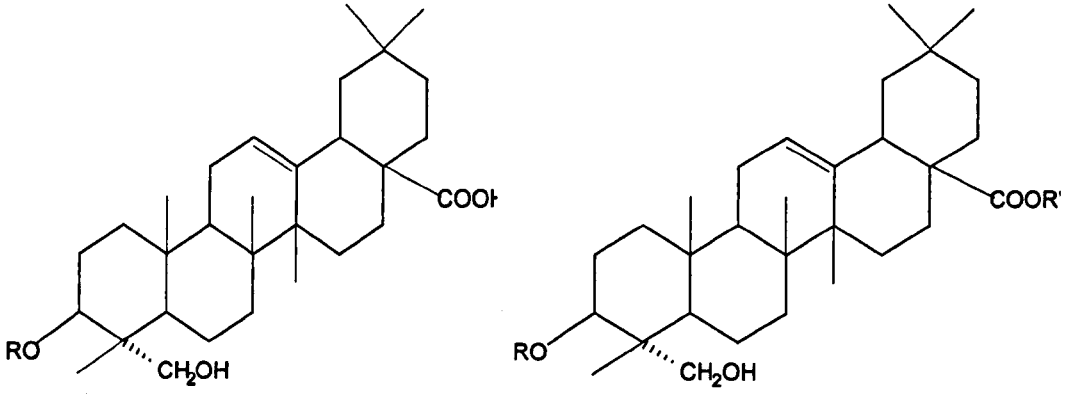


Figure 2.5. Monodesmosidic and bidesmosidic saponins (Hostettmann and Marston, 1995). R and R' represent branched glycosidic chains.

Monosaccharides that have been identified in the polymeric side chains are glucose, galactose, rhamnose, xylose, fucose and arabinose (Horber *et al.*, 1974; Cheeke, 1983). The saccharide moieties may be linear or branched and usually have relatively short sugar chains containing 2-5 monosaccharides residues. With the possibility of combination of numerous sapogenins with oligosaccharide side chains of various size and composition, an enormous range of saponins is possible and many variants of this general structure have been found to be present in plants (Oakenfull, 1981). It is well recognised that even one plant species may possess a number of individual saponins, e.g., alfalfa roots contain at least 25 medicagenic acid, hederagenin, zanhic acid, soyasapogenol and bayogenin glycosides with the attached number of sugars ranging from one to seven (Bialy *et al.*, 1999). These structure and their amounts may differ

depending on the plant part studied. This structural diversity and resulting wide range of polarities makes determination of individual saponins very difficult (Oleszek, 2002).

2.5.5. Isolation of saponins

Saponins are distributed in all parts of the plant: roots, stem, bark, leaves, seeds and fruits. Several tests have been reported for the identification and isolation of saponins, such as foaming capacity, toxicity to cold-blooded animals, bitter taste, complex formation with cholesterol and *in vitro* erythrocyte haemolytic activity (Birk and Peri, 1980; Cheeke, 1983; Agrawal *et al.*, 1985). Saponins may form a complex with protein that is insoluble in water; alternatively, the water-soluble saponins may be precipitated by complexing with cholesterol or other sterols (Cheeke, 1983). Since saponins have relatively large molecular weights and high polarity, their isolation is extremely difficult. Other problems involved in the isolation of pure saponins are the presence of complex mixtures of closely related compounds, either the nature of the aglycone or the sugar part (nature, number and position of attachment of the monosaccharides), and problems with labile substituents such as esters.

Extraction procedures have to be as mild as possible because certain saponins can undergo transformation, such as, enzymatic hydrolysis during water extraction (Domon and Hostettmann, 1984), esterification of acidic saponins during alcohol treatment or hydrolysis of labile ester groups. The most efficient extraction of dry plant material is achieved with methanol or aqueous methanol (Hostettmann and Marston, 1995).

2.5.6. Sapindus rarak *DC (Sapindaceae) as a potential source of antiprotozoal agents*

Sapindus rarak DC (Sapindaceae) (Fig. 2.6) occurs naturally on the islands of Southeast Asia and produces fruits that are not used for food purposes. The genus *sapindus*, however, is well known for the high saponin content of its fruits. The fruits are usually used for washing clothes in rural areas. Other species, such as *S. mukurossi* GAERTN. *and S. delavayi* RADLK, are well studied from a phytochemical viewpoint (Nakayama *et al.*, 1986a; Nakayama *et al.*, 1986b; Wong *et al.*, 1991; Diaz *et al.*, 1993). However, very limited investigations have been reported on *S. rarak* DC (Hamburger *et al.*, 1992).

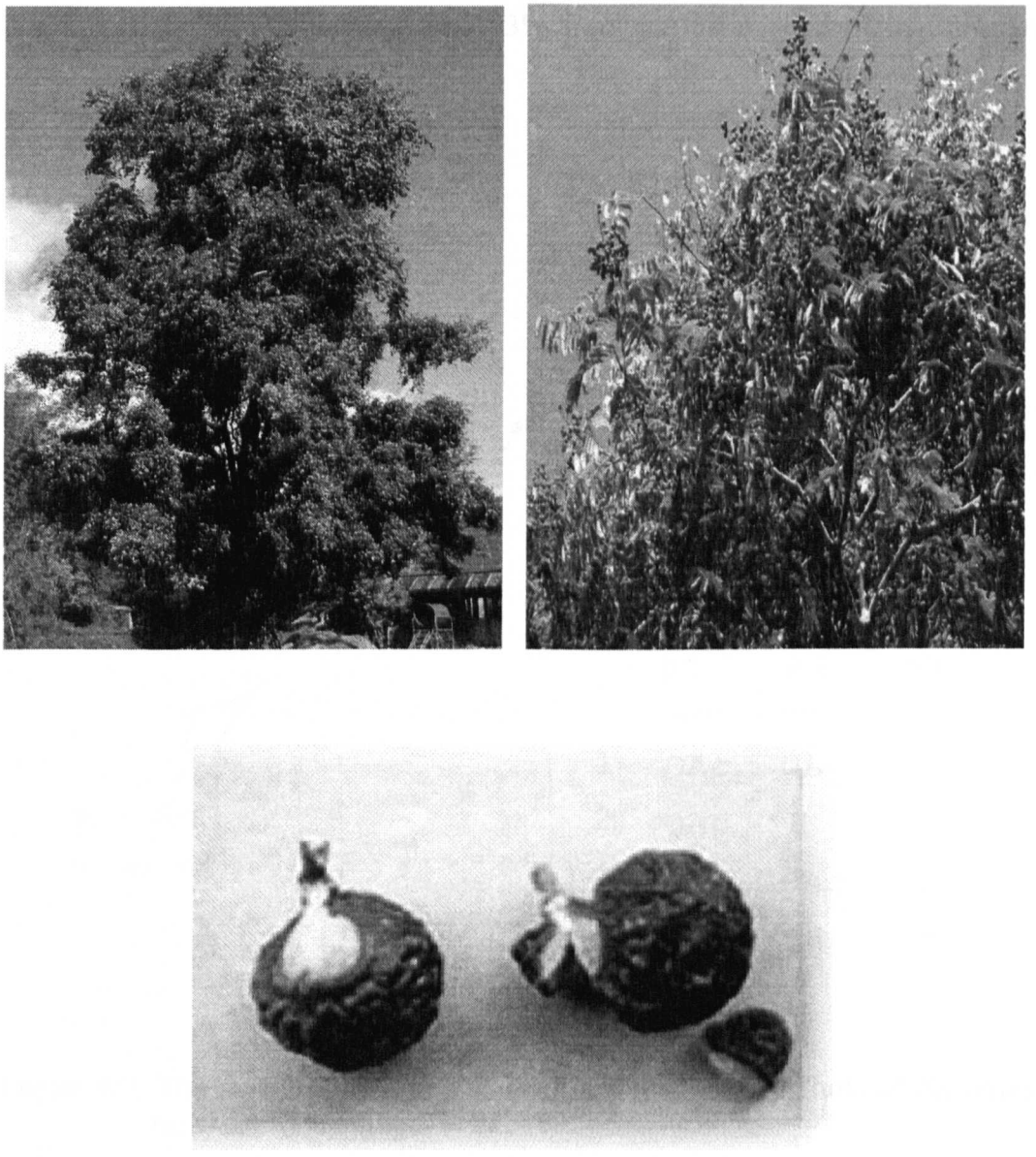
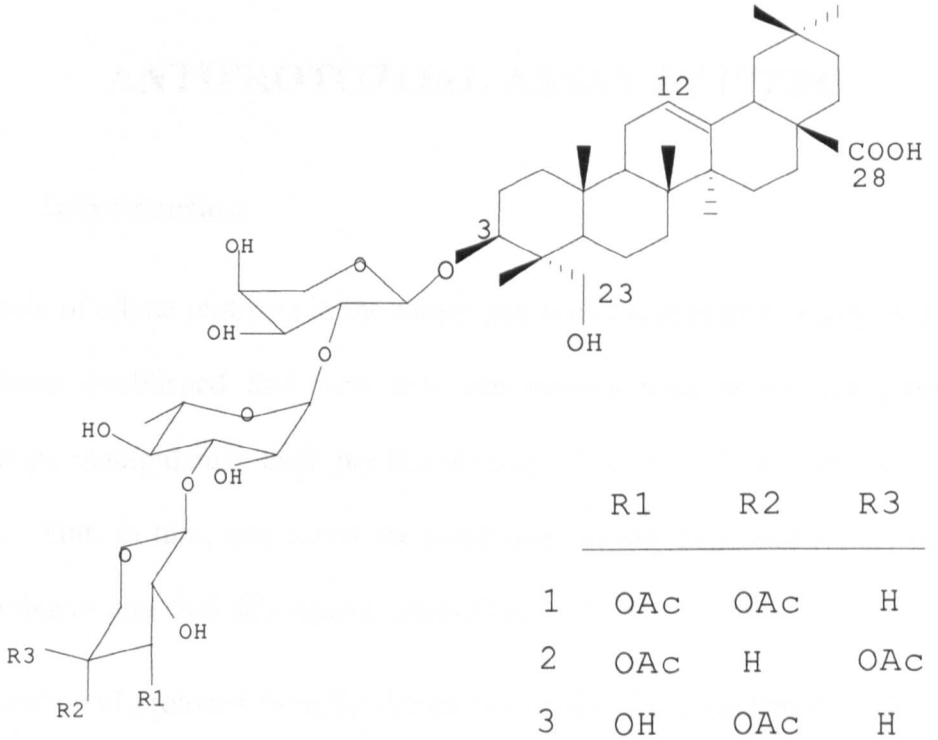


Figure 2.6. The tree and fruits of *Sapindus rarak* DC

Acid hydrolysis of a MeOH extract identified hederagenin as the triterpene moiety (Figure 2.7). Four monodesmosidic triglycosides, bearing up to two acetyl moieties on the terminal sugar, have been isolated. The position of the acetyl group and interglycosidic linkages have been established (Hamburger et al., 1992). Furthermore, Hamburger *et al.* (1992) reported that acetylated saponins from the pericarps of fruits of *S. rarak* exhibited molluscicidal activity at concentrations ranging from 6.25 to 12.5 ppm. The total saponins content

determined by HPLC analysis was 2.3% from the plant material investigated (Hamburger *et al.*, 1992).



	R1	R2	R3
1	OAc	OAc	H
2	OAc	H	OAc
3	OH	OAc	H
4	OH	OH	H

Figure 2.7. The structure of saponins from the pericarps of fruits of *Sapindus rarak* DC (Hamburger *et al.*, 1992).

S. rarak, like many other saponin-rich plants that has been found to improve growth, feed efficiency and reproduction in ruminants and has also been observed to kill protozoa, has provoked interest for exploitation as a natural antiprotozoal agent.

Therefore, the objectives of this work were to:

- Investigate the potential use of *S. rarak* as a safe and sustainable defaunating agent.
- Investigate further the effects of *S. rarak* on rumen fermentation, microbial protein synthesis and fibre digestion.

CHAPTER 3

DEVELOPING A STANDARD PROCEDURE FOR ANTIPROTOZOAL ASSAY *IN VITRO*

3.1. Introduction

The role of ciliate protozoa in the rumen has been the subject of many studies. It has been established that ruminants can survive with or without protozoa; however, manipulating their population may affect protein metabolism in the rumen. This, in turn, may affect the availability of microbial and dietary protein to ruminants and their subsequent production.

Elimination of protozoa from the rumen has been tested experimentally, but none of the elimination techniques is used routinely because of toxicity problems, either to the rest of the rumen microbial population or to the host animal (William and Coleman, 1992).

The identification of defaunating agents that are easily administered to ruminants would allow more extensive study of the contribution of rumen protozoa to productivity of ruminants.

When testing potential antiprotozoal agents, *in vitro* tests are valuable because they allow compounds to be tested quickly and require only small quantities.

The objectives of this study were, therefore, to construct and develop standard operational procedures for *in vitro* tests to study the effects of antiprotozoal compounds on rumen protozoa, based on visual assessment.

3.2. Materials and Methods

3.2.1. Experimental animal and basal diet

A rumen fistulated, non lactating Holstein cow was used as a source of rumen fluid. In the first experiment, the animal was fed twice daily at 08.30 and 16.00 with basal feeds consisting of 375 kg/tonne 1st Cut Grass Silage, 350 Kg/tonne Maize Silage and 275 kg/tonne Brewer Grains at the rate of 38.1 Kg for each cow. The animal was housed in a tether stall and had access to water *ad libitum*.

For the second experiment, the animal was fed with the same basal diet, plus a concentrate consisting of barley (40%), wheat (39%), soya (4 %), rape (4 %), urea (1 %), molassed meal (2.5%), megalac (2.5%), minerals/vitamins (2.5%), limestone (1.6%), C. Magnesite (0.6%) and fishmeal (2.%). The concentrates were fed at the rate of 7.5 kg for each cow/diet

3.2.2. Rumen fluid sampling

Rumen fluid (RF) was taken using a sampling probe. The sampling probe consisted of a filtering device made of a metal cage covered with 1mm nylon gauze and containing 4 mm plastic beads, aluminium pipe (i.d. 4 mm), a collection vessel (250 ml flask) controlled by a syringe acting as a manual suction pump (Figure 3.1). The filter was inserted into the ventral rumen via the rumen cannula. The air in the flask was withdrawn by suction with a syringe to create a vacuum. The reinforced tube was first introduced into the rumen and moved up and down a few times until a more fluid consistency was attained. Sampling of ruminal fluid was started by opening the valve, and because the flask was under vacuum, rumen fluid flowed automatically. The amount of sample collected was approximately 200 ml, after that the lid was put on to

prevent exposure to air. The fluid obtained was immediately placed in a container filled with warm water and kept in a water bath at 39^0C until used.

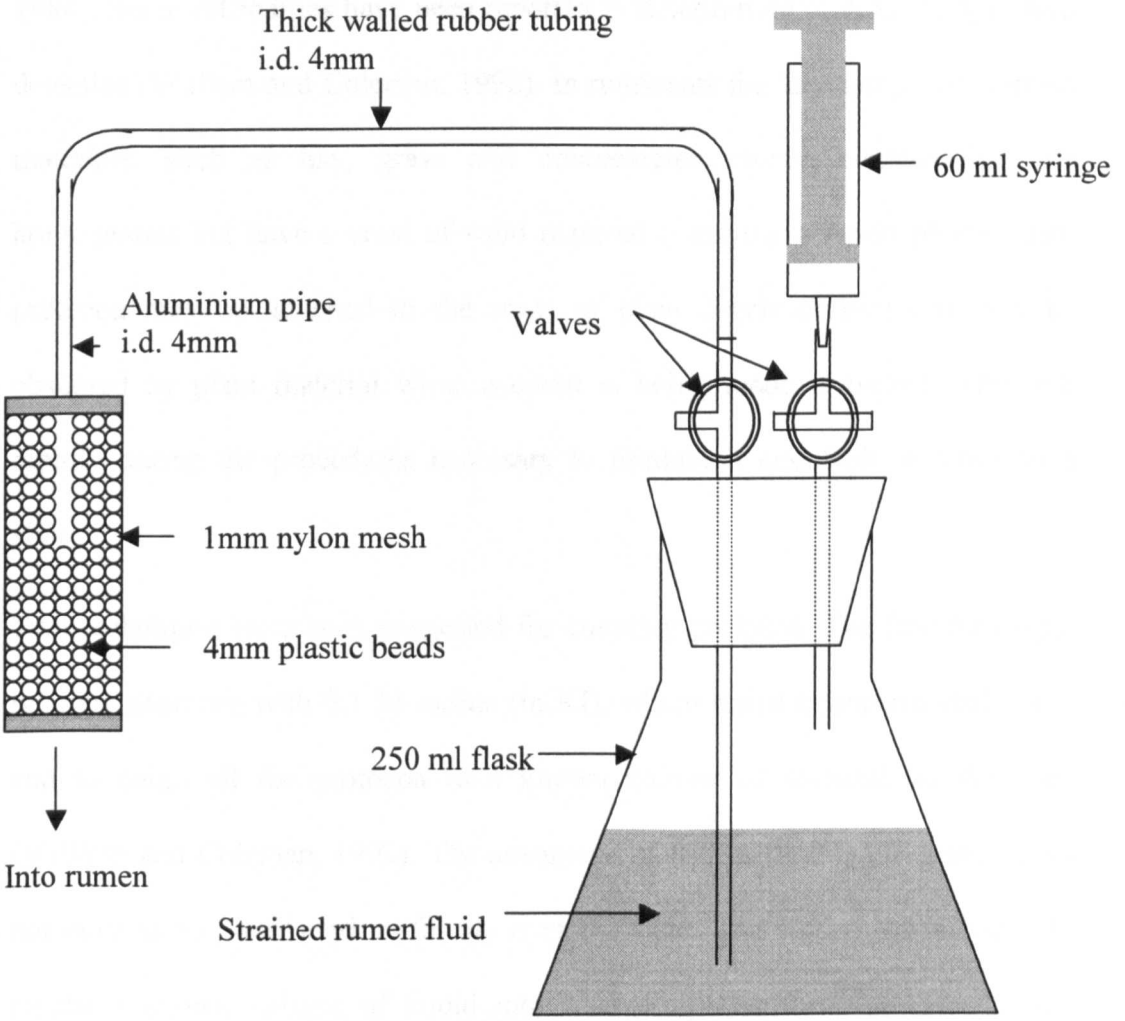


Figure 3.1. Rumen sampling device

3.2.3. Plant material

For this study, fruits of *Sapindus rarak* were obtained from West Sumatra, Indonesia. After oven drying at 60° C for 3 days, the pericarp of *S. rarak* fruits was ground and sieved through a 1 mm screen. The powder was then kept in a sealed plastic pot.

3.2.4. Observation of Protozoa

Numbers of protozoa can be significantly lower in rumen fluid than in the whole rumen contents depending on the time of sampling and the procedures (Dehority, 1984). Some difficulties have been reported in determining protozoal population densities (William and Coleman, 1992). In ruminants fed 'dry' temperate-climate materials, such as hay, grass and concentrates, rumen contents are not homogenous but have a crust of solid material overlying a liquid phase. Also, protozoa may be attached to the mass of plant debris or protozoa may be obscured by plant material when a count is being made. Protozoa settle out rapidly during the procedures necessary to produce a countable number on a slide.

Two techniques have been suggested for counting protozoa. The first technique is to fix protozoa with 0.1 M iodine (in KI), which stains organisms dark blue, and to count all the protozoa in a known volume of material on the slide (William and Coleman, 1992). The advantage of this method is the protozoa do not have to be distributed uniformly over the slide. The second technique is to pipette a known volume of liquid into a counting chamber and to count the number of protozoa in grids on the slide. Protozoa are preserved in formaldehyde and sometimes stained with brilliant green (Dehority, 1993) or methyl green (Ogimoto and Imai, 1981). The second technique was employed in this study as the advantage over the first that not all protozoa to be counted. A series of tests, such as sampling time and dilution time, was performed and developed as necessary prior to testing the effects of *S. rarak* on rumen protozoa.

Sampling Time. Rumen fluid was taken at two different times, i.e. before and after feeding. The aim was to determine the best sampling time to obtain a

representative sample because the validity of the results of all experiments depends upon the accuracy of the sampling method.

Dilution Time. The cell suspension was diluted with Hungate's salt to obtain a reading of 100-200 cells per counting chamber. The motility and number of rumen protozoa were observed and counted directly under a light microscope at 100 x power.

Observations of cell motility (Willard and Kodras, 1967) were made 1 h after the agent and strained ruminal contents were first mixed together (Fig. 3.2). Disintegrated cells were counted after adding methyl-green formal saline (MFS) to stain and fix the cells (Ogimoto and Imai, 1981).

Composition of methyl-green formal saline (MFS)

Formaldehyde solution 35%	100 ml
Distilled water	900 ml
Methyl green	0.6 g
Sodium chloride	8.0 g

The mixture was allowed to stand in the dark overnight to improve staining. Light exposure degrades methyl green into methyl violet resulting in poor staining. Only the nuclei of the ciliates are stained. Samples of rumen fluid were diluted with Hungate's salt.

Composition of Hungate's salt (Coleman, 1978)

NaCl	5.0 g/l
CH_3COONa	1.5 g/l
K_2HPO_4	1.0 g/l
KH_2PO_4	0.3 g/l

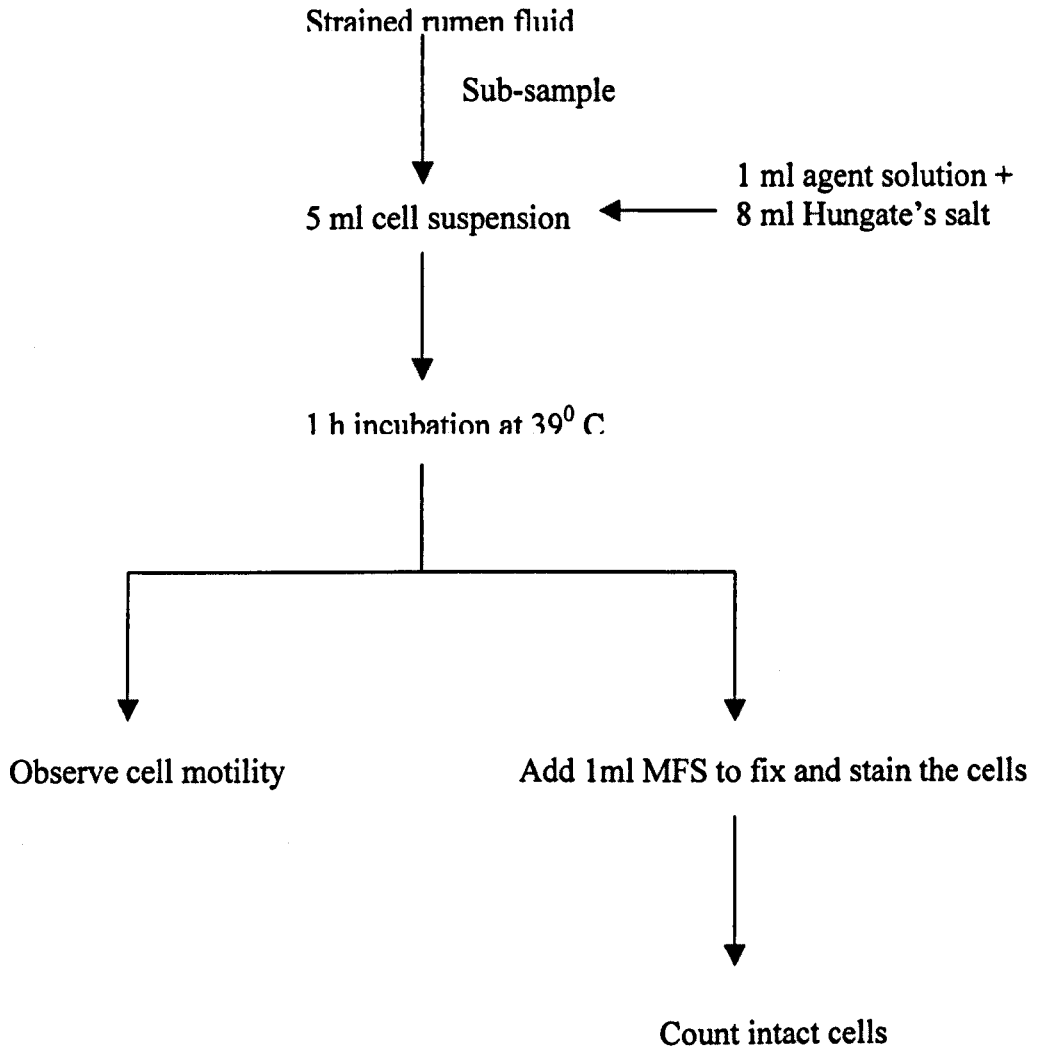


Figure 3.2. Flow diagram of antiprotozoal assays.

Treatments

A known amount of *S. rarak* solution prepared by diluting ground *S. rarak* with distilled water (concentration from 0 as control, 0.1, 0.3, 0.6, 0.9, 1.2, and 1.5 mg/ml of final concentration in 0.3 increments) was added to Hungate's salt to make a volume of 9 ml. Then 5 ml of strained rumen fluid was added to the mixture and incubated for 1h. Parameters

Antiprotozoal activity of *S. rarak* was assessed by two procedures: (1) measurement of decreased motility by subjective appraisal under the microscope and (2) measurement of the degree of disintegration of protozoa by microscopic examination. After thoroughly shaking the sample to mix the contents, a sub-sample was quickly withdrawn using a pasteur pipette and placed under a cover slip in the space between the grooves cut in the counting chamber.

The motility of protozoa was examined under a microscope at 100x power. The chamber was kept warm until used. Protozoa that exhibited no internal or external movement of cilia were considered dead (Willard and Kodras, 1967). Motility was estimated subjectively by comparing the motile cells in a test solution with the control.

The degree of disintegration of cells due to the activity of the agent was calculated after adding MFS to stain and fix the cells without any deterioration effects. Intact or disintegrating cells were distinguished under a microscope. The numbers of intact and disintegrating cells were counted in a Hawkesley Crystallite Counting Chamber (Fig. 3.3)

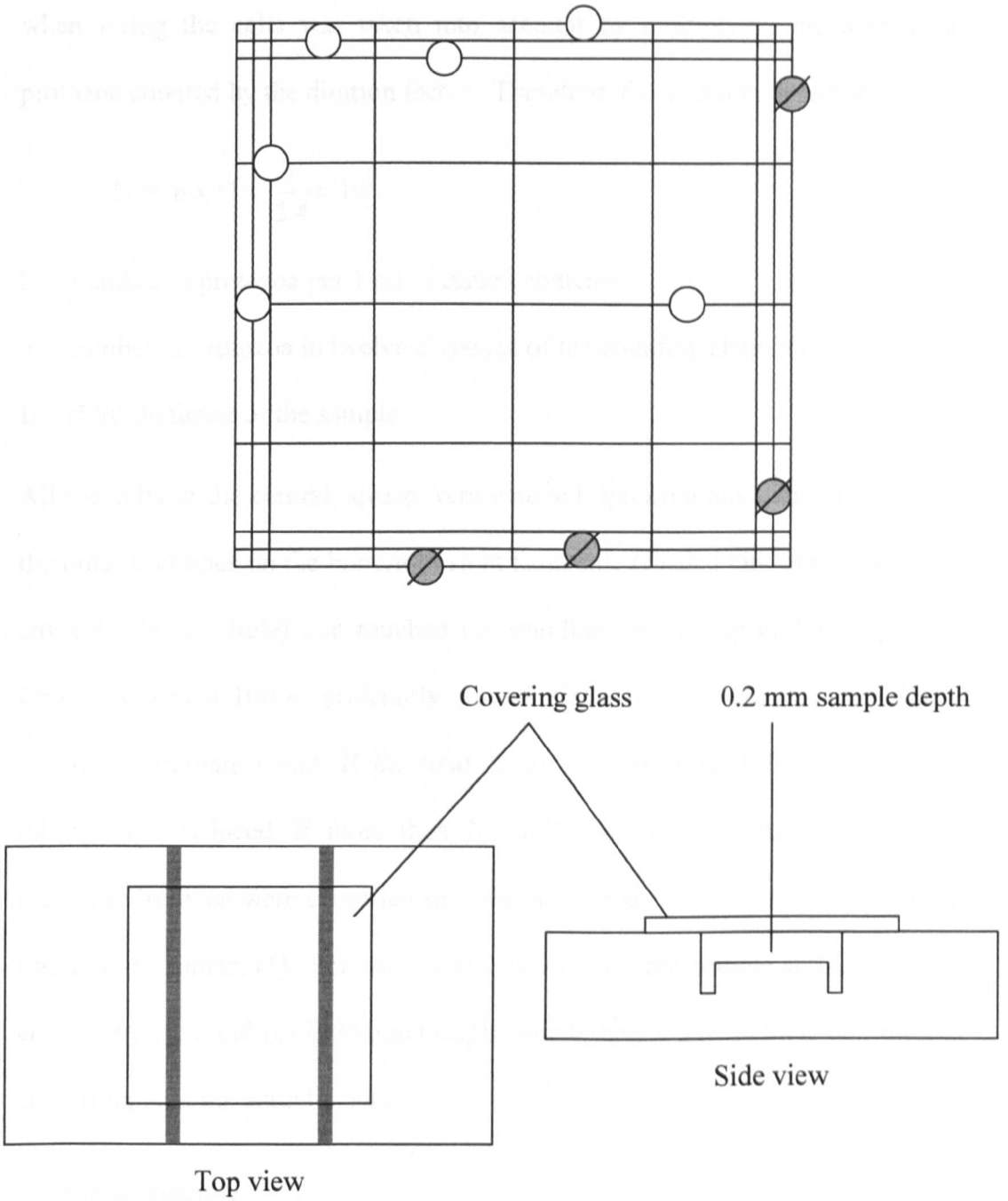


Figure 3.3. Hawkesley Crystallite Counting Chamber.

The counting chamber consisted of twelve divisions, 0.2 mm deep with the area of 12 mm^2 per division to give the total volume of 2.4 mm^3. One division consisted of 16 fields. Therefore, the number of protozoa per ml original solution could be calculated by counting the number of protozoa in twelve divisions multiplied by a factor of 10^3 and divided by 2.4. Dilution involved

when fixing the cells was taken into account by multiplying the number of protozoa counted by the dilution factor. Therefore, the equation used was:

$$N = n \times D \times \frac{1}{2.4} \times 10^3$$

N = number of protozoa per 1 ml of rumen contents

n = number of protozoa in twelve divisions of the counting chamber

D = dilution factor of the sample

All the cells in the central square were counted, ignoring any cells that touched the outer tramlines on the bottom or right hand side (shaded circle) but including any cells (clear circle) that touched the tramlines at the top or left (Fig. 3.3). Generally over a 100 or preferably around 200 cells were counted in order to ensure an accurate count. If the total count was less than 100 cells, then the dilution was reduced. If more than 200 cells per count, further dilution was required. Protozoa were classified into two basic body forms of ciliated protozoa found in the rumen, (1). Holotrich, which have a flexible pellicle and are covered entirely by cilia and (2) Entodiniomorph, which have a stiff pellicle and tufts of cilia, usually at the adoral position.

Statistical Analysis

A completely randomised design (CRD) was employed in this experiment with 7 different levels (0, 0.1, 0.3, 0.6, 0.9, 1.2, 1.5 mg/ml) of *Sapindus rarak* in 3 replicates. Data obtained were analysed using analysis of variance and regression to test the treatment effects.

3.3. Results and Discussion

Table 3.1. Sampling time and dilution rate.

Sampling time	Dilution	Total protozoa (10^5 ml^{-1})	SD
Before morning feeding	1:1	1.39	0.01
	1:2	1.37	0.006
	1:3	1.33	0.01
2 h after morning feeding	1:1	1.04	0.006
	1:2	1.09	0.008
	1:3	1.06	0.01

Note: Dilution is the ratio between rumen fluid and formal saline. Number of protozoa was the average of triplicate samples. SD is Standard Deviation.

Collecting rumen fluid 2 h after feeding in the morning provided clearer rumen fluid because less plant debris was present in the rumen. For counting protozoal cells microscopically, diluting rumen fluid with an equal volume of formal saline or twice as much as rumen fluid were equally acceptable. It was found essential to use a pipette with a wide mouth (in this case a cut-off pasteur pipette) to accommodate the bigger protozoa.

The number of protozoa before feeding was higher than 2 h after feeding. This is because, immediately after feeding, rumen fluid is diluted by water, saliva, etc., which causes the protozoal population density to fall.

Based on these preliminary observations, studies on the effects of *S. rarak* on rumen protozoa were carried out. Rumen fluid was collected 2 h after morning feeding from the same animals.

A known amount of *Sapindus rarak* solution (concentration from 0 up to 1.5 mg/ml of final concentration in 0.3 increments) was added to Hungate's salt to make a volume of 9 ml. Then 5 ml of rumen fluid was added to the mixture. After the effect on cell motility had been observed, 1 ml of MFS was added to each bottle to fix and stain the cells for enumeration. The number of cells and the ratio between holotrich and entodiniomorph were calculated (Table 3.2).

It was found that cell motility was slowed down in the presence of *S. rarak* at a concentration of 0.1 mg/ml, but inhibited totally at a concentration 0.3 mg/ml. In terms of protozoal numbers, there was a tendency for holotrichs to be more susceptible to *S. rarak* than entodiniomorphs. At a concentration of 0.3 mg/ml, all holotrichs were eliminated, whereas entodiniomorphs were not affected until a higher concentration (0.9 mg/ml). Lytic activity was measured by comparing the number in treated tubes with the control, assuming the control had 100% intact cells. In this experiment, 50% lysis was achieved at a concentration of approximately 0.9 mg/ml.

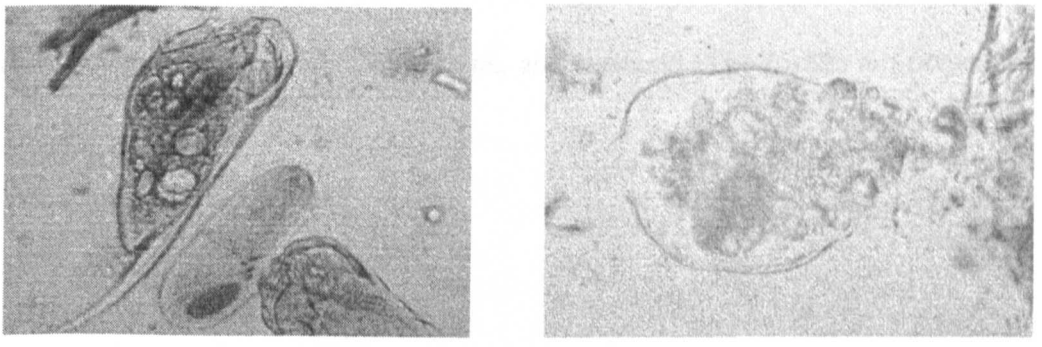


Figure 3.4. Microscopic observation of rumen ciliate protozoa showing intact and lysed protozoa.

Table 3.2. The effects of *S. rarak* on cell motility and degree of disintegration of rumen ciliate protozoa *in vitro*.

Concentration (mg ml^{-1})	Motility*	Entodiniomorph (10^5 ml^{-1})	Holotrich (10^5 ml^{-1})	Total (10^5 ml^{-1})	% Lysis
Control	0	0.8	0.5	1.3	0.0
0.1	x	0.8	0.4	1.2	9.1
0.3	x x x x	0.8	0	0.8	40.3
0.6	x x x x	0.8	0	0.8	40.3
0.9	x x x x	0.5	0	0.5	58.4
1.2	x x x x	0.7	0	0.7	45.5
1.5	x x x x	0.5	0	0.5	64.9

Note: Solution of *Sapindus rarak* (1 mg/ml) was prepared one day before. * In this assay, protozoal movement is reported relative to the control. The control appeared completely normal (100% of protozoa moving) and no disintegrating cells were observed. "0" indicates no visible effect on motility, the more "x" signs the greater the effect on motility.

A similar experiment was conducted with the same animal fed on the same basal diet, plus a concentrate consisting of barley, wheat, soya, rape, urea, molassed meal, megalac, minerals and vitamins, and fishmeal. The results are presented in Table 3.3.

Table 3.3. The effects of *S. rarak* on cell motility and degree of disintegration of rumen ciliate protozoa *in vitro*. Animal was fed with basal diet and concentrate.

Concentration (mg ml^{-1})	Motility*	Entodiniomorph (10^5 ml^{-1})	Holotrich (10^5 ml^{-1})	Total (10^5 ml^{-1})	% Lysis
Control	0	1.6	0.2	1.8	0.0
0.1	x	1.8	0.2	2.0	-10.0
0.3	x x	1.7	0.2	1.9	-5.0
0.6	x x	1.7	0.2	1.9	-5.0
0.9	x x x x	1.3	0.2	1.5	15.0
1.2	x x x x	1.4	0	1.4	20.0
1.5	x x x x	1.3	0	1.3	25.0

Note: * In this assay, protozoal movement reported is relative to the control. The control appeared completely normal (100% of protozoa moving) and no disintegrating cells were observed. "0" indicates no visible effect on motility, the more "x" signs the greater the effect on motility.

It was found that the effects of *S. rarak* on rumen protozoa were slightly different with this diet. The number of protozoa was 40% higher than in the previous experiment (Table 3.2). Cell motility was inhibited slightly at concentrations up to 0.6 mg/ml, but fully inhibited at a concentration of 0.9 mg/ml. At lower concentrations, the number of protozoa was not affected, but at concentrations of 0.9 mg/ml and higher, numbers were reduced markedly. However, even at the highest concentration tested (1.5 mg/ml), 50% lysis could not be achieved.

Results from these two experiments showed that *S. rarak* had antiprotozoal activity. The antiprotozoal factor in *S. rarak* might be associated with the

presence of saponins, high-molecular-weight glycosides. Numerous studies have demonstrated that saponins and saponin-containing plants have toxic effects on protozoa. Makkar *et al.* (1998) and Wang *et al.* (1998) have reported that *Yucca schidigera* extract has been shown to be toxic to rumen protozoa. Antiprotozoal effects of *Sapindus saponaria* were due to saponins (Navas-Camacho *et al.*, 1993). Saponins from *Quillaja saponaria* and *Acacia auriculoformis* (Makkar *et al.*, 1998) and foliage from *Sesbania sesban* (Newbold *et al.*, 1997) were also antiprotozoal *in vitro*. However, purified saponins from *Quillaja* bark and *Saponaria* sp. have been shown to be less toxic than the extract from *S. sesban* (Wallace *et al.*, 1994), suggesting that it is a specific type of saponin or saponin-like substance in *S. sesban* that is responsible for its antiprotozoal effect. The sensitivity of ciliate protozoa towards saponins may be explained by the presence of sterols in protozoal, but not in bacterial membranes (William and Coleman, 1992). The sterol binding capability of saponins most likely causes the destruction of protozoal cell membranes (Hostettmann and Marston, 1995).

The antiprotozoal activity was relatively higher in terms of inhibition of cell motility when the animal was fed only a basal diet compared with the later experiment when the cow was fed the basal diet and concentrate. This could be due to the changes in population of protozoa as the diet changed. William (1986) reported that the rumen ciliate population was determined by diet consumed besides generic composition, geographical location and protozoal interspecies antagonisms. The total number of protozoa in these experiments varied considerably from day to day, although rumen fluid was collected at the same time (2 hrs after feeding time) each day of collection. Precautions were taken when counting the number of protozoa under the microscope. An initial

observation was carried out to determine the appropriate dilution rates to reduce error. Hungate (1966) reported that there was a 10% error if the slides contain 5 protozoa per field. In this experiment the standard deviation of counting protozoa was maintained at less than 10% obtained from 3 replications for each counting.

3.4. Conclusion

In conclusion, a successful method for *in vitro* tests to study the effects of antiprotozoal compounds on rumen protozoa based on visual assessment was developed. The best conditions were animals fed the same ration throughout the experiment and rumen fluid were collected at the same time to avoid the high fluctuation in protozoa population. Fixation of cells with formaldehyde and staining with methyl green gave a satisfactory measure.

S. rarak exhibited antiprotozoal activity by affecting cell motility and cell integrity. In terms of kinds of protozoa affected by the tested compound, it was observed that holotrichs were more susceptible than entodiniomorphs. Although it is likely that it is the saponin fraction of *S. rarak* that is toxic to rumen protozoa, more work is required to identify the precise component.

CHAPTER 4

EFFECTS OF SAPONIN CONTAINING PLANTS ON RUMEN MICROBES

4.1. Introduction

It is apparent that protozoal predation of bacteria and their selective sequestration in the rumen accounts for a significant proportion of microbial protein turnover in the rumen and hence lower microbial growth efficiency and microbial cell flows to the intestine of the host (Demeyer and Van Nevel, 1986). Therefore, maximising microbial protein synthesis and flow to the duodenum by reducing the recycling of microbial N in the rumen will improve the production efficiency of ruminant livestock (Koenig *et al.*, 2000).

Removal of protozoa from the rumen has been shown to be associated with improved protein to energy ratio under certain dietary regimes and concomitantly beneficial effects on animal productivity (Ushida *et al.*, 1984). In turn, it is expected to lessen the dependence on protein supplementation under high-production conditions. It would also be beneficial under conditions where diets low in protein, but not limited in energy, are fed to animals with high protein requirements (Jouany and Ushida, 1999).

The elimination of protozoa by chemical means is potentially the most convenient method to obtain fauna-free animals (Ushida *et al.*, 1991). However, since it is likely that chemicals are not specifically toxic to protozoa and probably kill other microorganisms and host cells, and also because their antiprotozoal activity is inconsistent, it has been suggested to seek natural

antiprotozoal agents. Some preliminary studies on the use of tropical plants as defaunating agents have been reported (Leng *et al.*, 1992; Diaz *et al.*, 1993; Navas-Camacho *et al.*, 1993).

Plants produce a large number of chemicals, arbitrarily categorized as primary metabolites which perform essential roles in development and metabolism e.g., protein amino acids, cell wall sugars, hormones, and photosynthetic pigments, and secondary metabolites, which are essentially defence mechanisms for the plant against animal and insect predators and microbial infection. These include saponins, high-molecular-weight glycosides consisting of a sugar moiety linked to an aglycone, which have been reported to have antiprotozoal activity (Makkar *et al.*, 1998).

Saponins from *Yucca schidigera* have strong antiprotozoal activity and may serve as an effective defaunating agent for ruminants (Wallace *et al.*, 1994). Wang *et al.* (1998) also reported that the steroidal saponins in *Yucca schidigera* extract reduced protozoal number substantially ($P<0.01$). Triterpenoid saponin from *Quillaja saponaria* was reported to have antiprotozoal activity (Makkar and Becker, 1997).

The other potential source of natural antiprotozoal agents is *Sapindus rarak* as the pericarp of *Sapindus rarak* contains saponins (Hamburger *et al.*, 1992).

Some oat varieties also contain saponins with potential antiprotozoal effects. Although several oat-based products for human consumption have been developed for many years, most of the oats produced are used as animal feed. Oats have a high nutritional value and have become more popular in recent years as a result of their serum cholesterol lowering properties (Önning *et al.*, 1993).

These are mainly attributed to β-glucans and avenacosides A and B, a steroidal saponins (Price *et al.*, 1987). However, the main property behind these effects is probably the membranolytic activity of saponins.

The aims of the work described here were:

- to study diurnal variation in population density of protozoa in the rumen prior to investigating the antiprotozoal activity of some saponin-containing plants.
- to determine if saponins from *Sapindus rarak* are more potent to defaunate the rumen either partially or completely without having a detrimental effect on the bacterial population compared with other saponin-containing plants i.e. Oat, Naked Oat, Winter Naked Oat and *Quillaja saponaria.*
- to study the effect saponin-containing plants on the growth of mixed rumen bacteria.

4.2. Materials and Methods

4.2.1. Experimental animals

Four rumen fistulated, non lactating Holstein cows were used as sources of rumen fluid. The animals were fed twice daily with basal feeds and had access to water *ad libitum.*

4.2.2. Rumen Fluid Sampling

The apparatus used for obtaining rumen liquor consisted of a 0.5 ml diameter polythene tube with a metal tube and filter, a 250 ml flask with a two holed rubber stopper equipped with valves, and a 50 ml syringe (see Figure 3.1).

The amount of sample collected was approximately 200 ml. The fluid obtained was immediately placed in a water-bath at 39^0 C until used.

4.2.3. Experiments

Three experiments were performed in this study.

1. Observation of diurnal variation in density of protozoa in the rumen.

Rumen fluid (RF) was collected every 2 h starting from 2 h before feeding, 0, 2, 4, 6, and 8 hrs after feeding using a sampling probe (see Section 3.2.2). RF (200 ml) was transferred into a 250 ml erlenmeyer flask and kept in a shaker water bath set at 39°C until used.

2. Study on the effects of saponin-containing plants on rumen protozoa

Saponin-containing plants. *Sapindus rarak* DC, *Quillaja* sp, Oat, Naked Oat and Winter Naked Oat were observed for their effects on rumen protozoa with a view to predicting their potential antiprotozoal activity. Wheat and Barley were also included as controls. All plant materials had been oven dried, ground and sieved through a 1 mm screen.

Known amount of samples (0.5 mg) were first diluted in 25 ml Hungate's Salt to make a stock solution. After that, 5 ml of this stock solution was transferred into a universal bottle filled with 5 ml rumen strained fluid and 5 ml Hungate's Salt, mixed and incubated in a shaker water bath set at 39°C for 1 h. Observations of

cell motility were made 1 hr after the agent and cell solution were first mixed together and all samples were observed directly under a light microscope at 25, 100 and 400x power. Protozoal cells were counted after adding 5 ml methyl green formal saline (MFS) to the solution to stain and fix the cells without any deterioration effects and allowed to stand for at least four hours. Allowing to stand overnight generally resulted in better staining. After thoroughly shaking the sample to mix the contents, a sub-sample was quickly withdrawn using a cut-off pasteur pipette and placed under a cover slip in the space between the grooves cut in the counting chamber (Hawkesley Crystalite counting chamber).

Antiprotozoal activity of the saponin-containing plants was assessed by two steps: (1) measurement of decreased motility by subjective appraisal under the microscope and, (2) measurement of the degree of disintegration of protozoa by microscopic examination. The motility of protozoa was examined under a microscope at 100x power. The chamber was kept warm until used. Protozoa that exhibited no internal or external movement of the cilia were considered dead. Motility was estimated by comparing the motile cells in a test solution to the control. Intact or disintegrating cells were distinguished under a microscope. The number of intact cells was counted.

3. Study of the effects of Sapindus rarak on rumen bacteria *in vitro.*

The effects of *Sapindus rarak* on rumen bacteria were assessed by their influence *in vitro* on growth of mixed bacteria prepared from rumen contents. A known amount of sample (0.1 g) was incubated in 10 ml of strained rumen fluid. The solution was kept in a water bath for 1 h. After incubation, 1 ml of sample solution was diluted into 9 ml sterile Minimum Recovery Diluent (MRD) and

followed by a dilution series of up to 10^{-6}. The diluted sample was then plated over the surface of nutrient agar plate containing Brain Heart Infusion (BHI) and agar. The sample was spread on each plate using a sterile spreader. All samples (in duplicate) were incubated in an anaerobic jar in an inverted position at 39^0C for 24 hrs. After incubation, the colonies that developed were counted and expressed as colony-forming units (cfu).

Statistical Analysis

Results were analysed by a one-way ANOVA. Each animal was considered to be an experimental unit (n 4). A level of $P<0.05$ was chosen as the minimum for statistical significance.

4.3. Results and Discussion

Experiment 1. Observation of diurnal variation in density of protozoa in the rumen

Total protozoal numbers observed prefeed and postfeed in the rumen fluctuated. Diurnal variation in the population of holotrich and entodiniomorphid ciliates in the rumen differed (Figure 4.1). Entodiniomorphid protozoa are always dominant in the rumen, since they represent nearly 80% of the total ciliate number. Holotrichs develop in large numbers when soluble carbohydrates are readily available in the diet (Hungate, 1966). In this study, the number of holotrichs was less than 10^5 ml^{-1} of rumen contents, which represents as much as 20% of total ciliates.

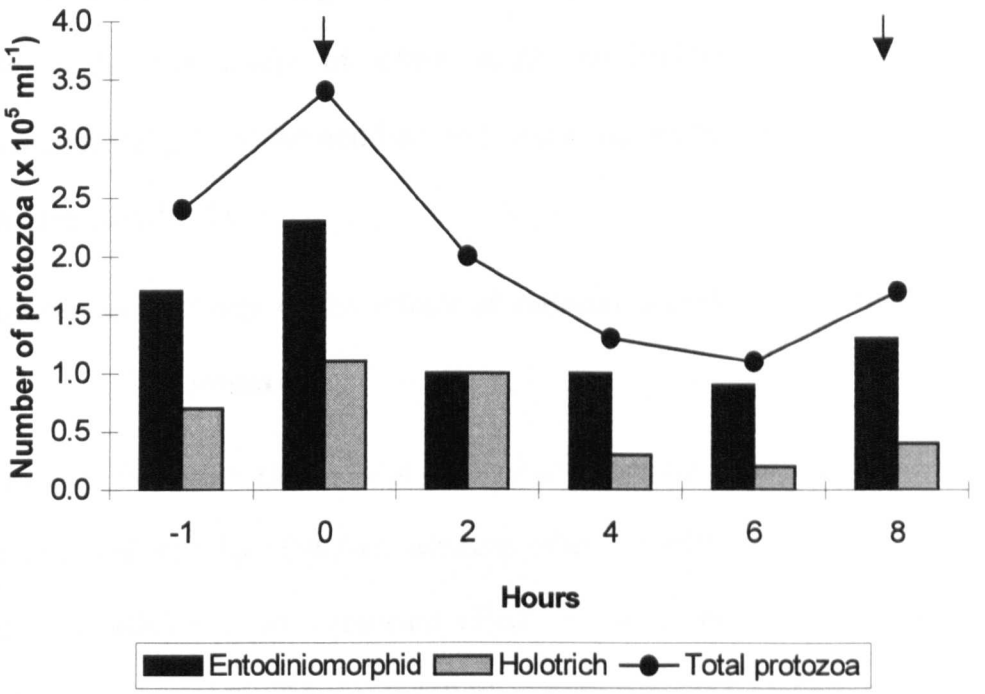


Figure 4.1. Diurnal variation in protozoal population density in the rumen fed twice a day (feeding times shown by arrows).

The number of protozoa was higher at feeding time in the morning than 1 h before feeding and subsequently decreased up to 6 h after feeding and then increased again when fed in the afternoon although the original prefeeding level was not achieved. Observation was carried out only up to 8 h. The marked decrease in holotrich and entodiniomorphid numbers after feeding probably related to an increase in rumen outflow rates. Immediately after feeding, rumen fluid is diluted by water and saliva, and this causes the protozoal population density to fall. The final increase in density just before the second feeding was probably due to division of protozoa, although it could be the result of a redistribution of protozoa within the rumen. Contraction of the reticulum could also cause a forced migration of some entodiniomorphid protozoa, as has been postulated to occur with the holotrich protozoa (William, 1986). The presence of protozoa in the rumen has been shown to influence the volume of the rumen, and

the retention time of the digesta, the concentration and proportion of the volatile fatty acids, the levels of other acidic metabolites and ammonia, the environmental pH and the numbers and type of rumen bacteria present (William and Coleman, 1992).

Experiment 2. Study of the effects of saponin-containing plants on rumen protozoa

In general, the results showed that *S. rarak* and *Quillaja saponin* exhibited strong antiprotozoal activity ($P<0.01$), whereas other saponin-containing plants (oats and beet pulp) had no significant effect on rumen protozoa compared with controls (Figure 4.2). Wheat and Barley were included as they are common feedstuffs but contain no saponins and, as expected, showed no antiprotozoal activity.

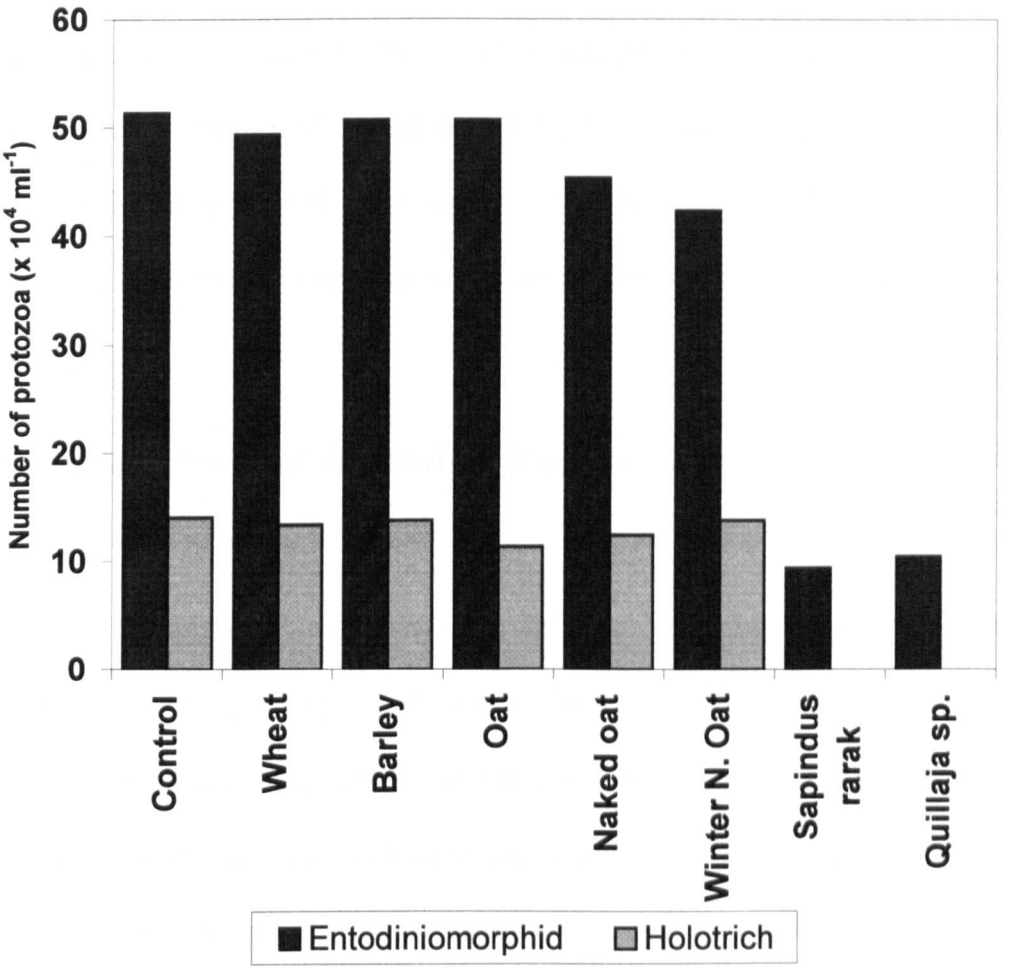


Figure 4.2. Effects of saponin-containing plants on rumen protozoa

Oat and Naked oat produced no antiprotozoal effect, but Winter naked oat reduced the number of entodiniomorphids. This may reflect the presence of different types or content of saponins among oat varieties. However, there was no attempt to identify the nature and quantity of saponins present. Two structures of oat saponins, avenacoside A and B, have been elucidated (Tschesche and Lauren, 1971). They have a steroid aglycone, nuatigenin, and two sugar chains containing glucose and rhamnose. Avenacoside B contains one more glucose residue than Avenacoside A.

Addition of *S. rarak* and *Quillaja saponaria* decreased the number of entodiniomorphids by 82% and 80% respectively, compared with control, and all

holotrichs were disrupted. Burgraaf and Leng (1980) reported that holotrichs were particularly sensitive to the effects of antiprotozoal surfactant detergents. Further characterisation of the antiprotozoal factor and evaluation of the ability of *S. rarak* to suppress ciliate protozoa *in vivo* for a longer period of time, their effects on other rumen microbes and overall effects on rumen fermentation is now required.

Experiment 3. Study of the effects of *Sapindus rarak* on rumen bacteria *in vitro*

Results of Experiment 3 are presented in Table 4.1. In terms of number of rumen bacteria (cfu), the pericarps of *S. rarak* did not exhibit antibacterial activity, whilst rumen protozoa were immobilised completely when the same concentration was applied. In addition, the number of bacteria tended to increase, although there was no significant difference ($P>0.05$) between treatment and control groups. These results suggest that saponins, the active agents present in the pericarp of *S. rarak*, could produce toxic effects on only rumen protozoa. This could be explained by their ability to complex with membrane sterols, which are present only in eukaryotic membranes and not in prokaryotic bacterial cells (Klita *et al.*, 1996; Osbourne *et al.*, 1996).

Table 4.1. Influence of *Sapindus rarak* on rumen bacteria incubated *in vitro*.

	Number of bacteria cfu ml^{-1} rumen fluid
S. rarak	2.26×10^7
Control	2.09×10^7
SED	0.49×10^7

4.4. Conclusion

In conclusion, *S. rarak* significantly reduced, though it did not eliminate, protozoal population but did not affect total bacterial numbers *in vitro*. Therefore, *S. rarak* may have value as a natural antiprotozoal agent for ruminant feeding. Furthermore, the possible use of natural products as a productivity enhancers provides potential benefit of the farmers and the environment and more consumer-acceptable alternatives to synthetic compounds.

Further observations will be required to examine the activity from the extract or more pure compounds from *S. rarak* to suppress ciliate protozoa, their effects on other rumen microbes and overall effects on rumen fermentation.

CHAPTER 5

SAPONIN FRACTIONS IN *SAPINDUS RARAK*: EFFECTS ON RUMEN MICROBES

5.1. Introduction

Microbial protein synthesised in the rumen is the major source of amino acids entering the small intestine and available for absorption in ruminants (Beever and Siddons, 1986). However, microbial protein turnover in the rumen may result in the net microbial protein outflow being less than half of the total amount synthesised (Nolan and Stachiw, 1979). Turnover could be partly due to autolysis of bacteria or other lytic factors. *In vitro* studies suggest that the engulfment and digestion of bacteria by protozoa is quantitatively the most important cause of bacterial turnover in the rumen (Wallace and McPherson, 1987). Thus it is apparent that removing ciliate protozoa from the rumen (defaunating) avoids recycling of nitrogen between bacteria and protozoa and thereby increases the efficiency of nitrogen metabolism in the rumen and stimulates the flow of microbial protein from the rumen (William and Coleman, 1992). Therefore, defaunation would be expected to lessen the dependence on protein supplementation under high-production conditions and would also be beneficial under conditions where the quantity of protein absorbed from the post-ruminal gut limits the productivity of the animal, which occurs frequently in animals receiving low-quality tropical forages.

There is increasing interest in the use of tropical plants that may have a nutritional value beyond simply their nutrient content, i.e. as rumen-manipulating agents as an alternative means to solve problems in animal nutrition and

livestock production. The non-nutrient bioactive principles in plants are essentially the secondary metabolites. It is generally accepted that secondary metabolites are an important means for plants to protect themselves from pathogens and herbivores. Saponin-containing plants and their extracts appear to useful as a possible means of suppressing the bacteriolytic activity of rumen ciliate protozoa and thereby enhancing total microbial protein flow from the rumen. Saponins are glycosides that are generally considered as ant-nutritional factors. The anti-nutritional effects differ depending on the digestive process of the ingesting animals. In ruminants, saponins are differentially toxic to rumen protozoa.

The aim of the work described here was to examine the antiprotozoal factor from the pericarp of *S. rarak* for its potential to defaunate the rumen, either partially or completely, without having a detrimental effect on the bacterial population.

5.2. Materials and Methods

Two series of studies were undertaken in Nottingham and Aberdeen. Series 1 (University of Nottingham) dealt with the extraction and fractionation of *S. rarak*, monitoring by TLC and screening for effects on rumen protozoa. Series 2 was under taken at the Rowett Research Institute, Aberdeen to investigate the influence of saponin extract of *S. rarak* on the activity of rumen protozoa by measuring the breakdown of ^{14}C-leucine-labelled *Prevotella (bacteroides) bryantii* B_14. The influence of saponin extract of *S. rarak* on the growth of individual species of rumen bacteria was also examined.

5.2.1. Nottingham Studies

Extraction and Fractionation

Saponins from the pericarp of *S. rarak* were extracted by selective extraction from a C_{18} solid-phase column with methanol-water solutions according to procedure of Oleszek (1988). The dried finely powdered pericarps of *S. rarak* were defatted with petroleum ether and extracted by refluxing for 1.5 h with 80% ethanol (Fisher, AR grade). Ethanol was removed in a rotary evaporator and the resultant brown aqueous solution was diluted with methanol to a final concentration of 35% (v/v). The solution was thoroughly mixed and filtered with a 0.2 μm PVDF filter. The residues contained no saponins (examined by TLC) and were discarded. The aqueous alcohol solution was applied to a C_{18} column (50 g, 3 x 10 cm) previously preconditioned with 35% MeOH. The column was washed first with 35% MeOH (250 ml) to remove sugars and phenolics and then with methanol (250 ml) to elute the saponins. Methanol was removed under N_2, due to a problem with the rotary evaporator, to yield a yellowish powder. The saponin powder was resuspended in distilled water and the suspension was loaded onto a C_{18} packed column (2.5 cm x 40 cm), previously equilibrated with water. The column was washed first with 100 ml of distilled water and then with 1 l of each of a series 0-100% methanol solutions with 20% increments. The saponin fractions were collected and monitored by TLC (Oleszek, 1988). TLC of saponins and reference substance (*quillaja* bark saponin) was performed on K6F Silica gel 60 A (20 x 20 cm, layer thickness 250 μm) developed with ethyl acetate/water/acetic acid (7:2:2 v/v). Plates were stained by dipping with Iodine

in chloroform (0.1%). Colour development (brown) in visible and UV light was observed. Fractions showing similar patterns were combined and evaporated to dryness.

Screening Antiprotozoal Activity

Saponin fractions were screened for antiprotozoal action based on immobilising activity and cell number following *in vitro* culture with rumen fluid. Activity was compared with that of a reference substance from *Quillaja* saponin.

5.2.2. Aberdeen Studies

The influence of saponin extract of *S. rarak* on activity of rumen protozoa.

Protozoal activity was measured by the breakdown of ^{14}C-leucine-labelled *Prevotella (bacteroides) bryantii* B_14 *in vitro* as described by Wallace and McPherson (1987).

Preparation of labelled bacteria

Prevotella bryantii B_14 was cultured on M8 media (modified Hobson's medium no 2, Table 5.1.). The medium contained 20% autoclaved and clarified rumen fluid from a cow at the Rowett Research Institute. It was labelled by growing overnight at 39^{0}C in Wallace and McPherson media containing [^{14}C]-Leucine as the sole N source (Table 5.1). The composition of Mineral I and II solution (McDougall, 1948) and vitamin solution N4a (Scott and Dehority, 1965) are presented in Table 5.2. Labelled bacteria were harvested by centrifugation (3000 g, 15 min) and washed once in 7 ml of fresh anaerobic Coleman's solution D containing 5 mmol unlabelled L-leucine to prevent re-incorporation of released

[^{14}C]-Leucine. Anaerobic conditions (100% carbon dioxide) were maintained throughout.

Bacteriolytic activity of protozoa

Rumen fluid was obtained approximately 2 h after feeding from three mature rumen-cannulated Dorset-cross sheep receiving 1 kg/d of a mixed diet of grass hay, barley, molasses, white fish meal and a mineral and vitamin mixture (500, 300, 100, 91 and 9.5 g/kg DM respectively) in two equal meals. Rumen fluid, withdrawn via the rumen cannula using hand pump, was strained through two layers of muslin and pre-incubated under CO_2 in a shaking water bath (Grant Instruments Ltd, Cambridge, 80 strokes/min) at 39^{0}C with 1, 5 and 10 g *S. rarak*/l for 1 h before adding 0.5 ml of [^{14}C]-Leucine labelled *P. bryantii* $B_1$4. Unlabelled L-leucine was included in all incubations at a final concentration of 5 mmol/l to prevent re-incorporation of released ^{14}C-leucine. Rate of bacterial protein breakdown was measured by removing 1 ml samples periodically at 0 h and at 1 h intervals up to 3 h into eppendorf tubes containing 0.25 ml trichloroacetic acid (25% TCA, w/v), and centrifuging the extract at 13,000 x g for 3 min. Samples of the supernatant fluid were counted by liquid-scintillation spectrometry (Packard 1900 CA, Berkshire, UK). The extent of degradation of [^{14}C]-Leucine labelled *P. bryantii* $B_1$4 at each incubation time was calculated from the radioactivity released into acid-soluble material and expressed as a percentage of the total DPM (disintegration per minute) present in labelled bacterial suspension. The rate of degradation per hour was calculated by linear regression of these values against incubation time.

Table 5.1. Composition of culture media for growth of rumen bacteria.

Ingredients	M8 medium	Wallace + McPherson medium
Bacto-casitone (g)	1	-
Yeast extract (g)	0.25	-
Glucose (g)	0.2	0.2
Maltose (g)	0.2	0.2
Cellubiose (g)	0.2	-
NaHCO3	0.4	0.4
Agar (g)	2	-
Rumen fluid (ml)	20	20
Mineral solution I (ml)	15	15
Mineral solution II (ml)	15	15
Resazurin (ml) (0.1% w/v)	0.1	0.1
Vitamins solution- N4a	-	10
Distilled water (ml)	49	40
Cysteine HCl (g)	0.1	0.1
[^{14}C]-leucine	-	1.26μCi/7ml

Notes: The ingredients were mixed and the solutions were boiled once and bubbled with O_2 free CO_2. Cysteine HCl was added last after boiling.

Table 5.2. Composition of mineral and vitamin solutions

Mineral Solution I		Vitamin solution $N4_a$	
KH_2PO_4	3 g	Pyridoxin HCl	0.2 g
Dist. H_2O	1 l	Riboflavine	0.2 g
Mineral Solution II		Thiamine HCl	0.2 g
KH_2PO_4	3 g	Nicotinamide	0.2 g
$(NH_4)SO_4$	6 g	Ca-D-pantothenate	0.2 g
NaCl	6 g	P-aminobenzoid acid	0.01 g
$MgSO_4$	0.6 g	Folic acid	0.005 g
$CaCl_2$	0.4 g	Biotin	0.005 g
Distilled H_2O	1 l	Vitamin B_{12}	0.0005g
		Distilled H_2O	100 ml

Two separate experiments were also carried to observe the effect of *S. rarak* on specific genera i.e. monoinoculated rumen (*Epidinium caudatum*) obtained from 2 isolated sheep, and on *holotrich* enriched rumen protozoa obtained by sedimentation and filtration of rumen fluid from mixed-fauna inoculated rumen (Fig. 5.1).

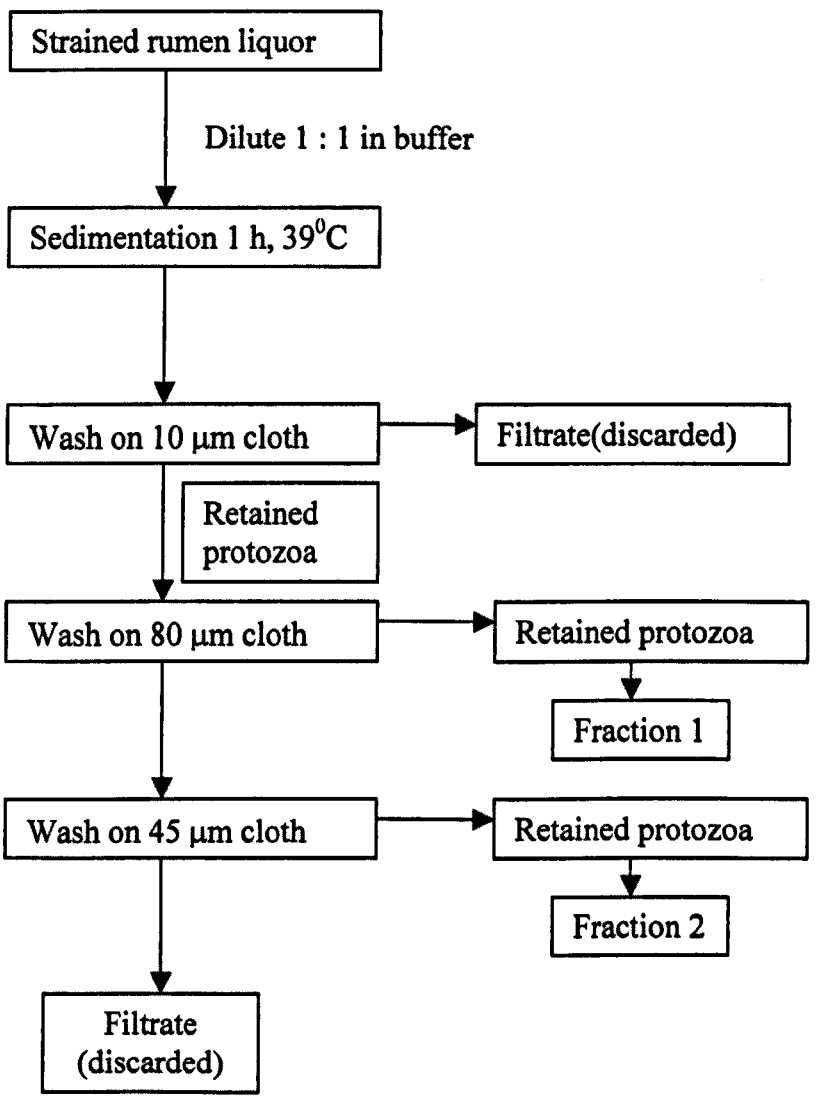


Figure 5.1. Outline of procedure used to isolate specific protozoal groups from a mixed protozoal population by differential filtration and sedimentation.

The influence of saponin extract of *S. rarak* on growth of pure cultures of ruminal bacteria

The effect of *S. rarak* on the growth of individual species of rumen bacteria (Ttable 5.3), isolated from sheep at the Rowett Research Institute, was examined by adding 0.5 ml methanolic extract of *S. rarak* to pure cultures of bacteria grown in Hungate tubes containing rumen fluid-containing medium 2 of Hobson (1969). The medium was dispensed under O_2-free CO_2 into Hungate tubes, sealed with butyl rubber stoppers. The medium was then autoclaved at 121^0C at 15 psi for 20 min. Individual tubes were then inoculated with 0.5 ml of pure cultures of rumen bacteria. The effect of saponin extract of *S. rarak* on bacterial growth was determined by monitoring optical density at 650 nm in a Nova spectrophotometer (Pharmacia Biotech) linked to a computer terminal using Hyper Terminal PE, version 5.0. The readings were recorded at 0 h, and every hour up to 10 h, 12, 24 and 36 h of incubation.

Table 5.3. Pure culture collection of rumen bacteria

No	Species	Code	Gram Reaction
1	*Megasphera elsdenii*	J1	-
2	*Veillonella parvula*	LS9	-
3	*Ruminobacter amylophilus*	WP109	-
4	*Selonomonas ruminantium*	HD4	-
5	*Prevotella bryantii*	$B_1$4	-
6	*Clostridium aminophilum*	23	+
7	*Lactobacillus casei*	L-caseii	+

8	*Streptococcus bovis*	ES1	+
9	*Streptococcus bovis*	C277	+
10	*Peptostreptococcus anaerobius*	27337	+
11	*Butyrifibrio fibrisolvens*	SH13	-
12	*Butyrifibrio fibrisolvens*	SH1	-
13	*Butyrifibrio fibrisolvens*	JW11	-
14	*Ruminococcos flavefaciens*	FD1	+

Statistical Analysis

Both antiprotozoal and antibacterial effects were conducted in duplicates. Results were analysed by a one-way ANOVA. Each sheep was considered to be an experimental unit. Following a significant *F* test ($P < 0.05$), significant differences between means were determined by Student's *t* test (Quinn and Keough, 2002).

5.3. Results and Discussion

5.3.1. Extraction and Fractionation of *S. rarak*

TLC of crude saponin extracts from the pericarp of *S. rarak* obtained from Java and Sumatra separated it into 5 to 6 spots. Fractions 1,2 and 3 were combined aqueous fractions which showed similar patterns from *S. rarak* from Sumatra, while fractions 4 and 5 were aqueous fractions from *S. rarak* from Java. Fractions 6 and 7 were combined methanolic fractions from *S. rarak* from Sumatra, and fractions 8 and 9 were combined methanolic fractions from *S. rarak* from Java. The TLC pattern of standard saponin indicated the presence of three main spots with retardation factor, R(f), 0.78, 0.82 and 0.85 (Fig.5.2).

Antiprotozoal activity was shown by aqueous (fraction 1-5)_and methanol fractions (fraction 6-9), indicating the presence of different types of saponins.

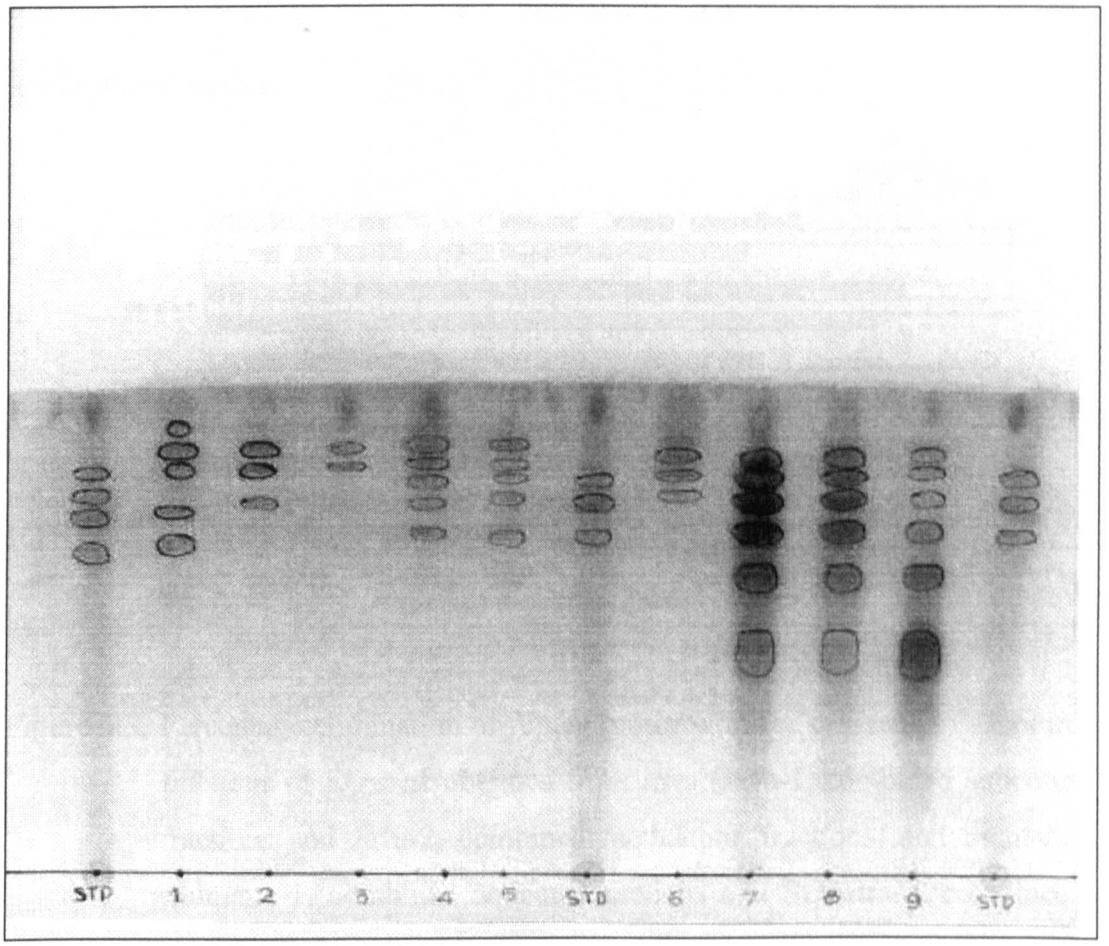


Figure 5.2. TLC of saponin fractions (1-9) and reference substance (*Quillaja* saponin). Plate was developed with ethyl acetate/water/acetic acid (7:2:2 v/v). Plates were stained by dipping with Iodine in chloroform (0.1%). Colour development (brown) in visible and UV light was observed.

When these fractions were tested on antiprotozoal assay, it appeared that all of these saponin fractions (aqueous and methanolic fractions) exhibited high immobilising activity and produced high activity to lyse the cells. *S. rarak* from Sumatra showed higher antiprotozoal activity compared to *S. rarak* from Java.

The number of Entodiniomorphid protozoa decreased by up to 83%, while all of the Holotrich protozoa were disrupted (Fig. 5.3). Burgraaf and Leng (1980) reported that the holotrichs were particularly sensitive to the effects of antiprotozoal agents.

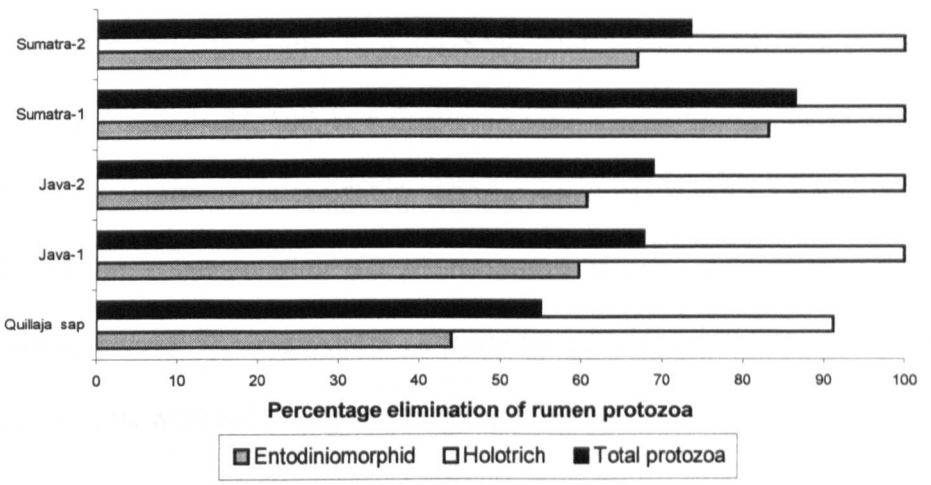


Figure 5.3. Percentage elimination of rumen protozoa in the presence of saponin fractions of *S. rarak* obtained from Java (Java-1, combined aqueous fractions and Java-2, combined methanolic fractions) and Sumatra (Sumatra-1, combined aqueous fractions and Sumatra-2, combined methanolic fractions).

5.3.2. The influence of saponin extract of *S. rarak* on activity of rumen protozoa.

The antiprotozoal effects of *S. rarak* (raw material and methanol extract) on different types of rumen protozoa (mixed rumen protozoa from 3 sheep, *E. caudatum* from 2 mono-inoculated sheep, and *holotrich* enriched rumen protozoa from 2 mixed faunated sheep) were measured by the breakdown of ^{14}C-leucine-labelled *Prevotella (bacteroides) bryantii* B_14 *in vitro* as described by Wallace and McPherson (1987). The screenings were carried out on different days with different

types of rumen protozoa, each with different protozoal population. To enable comparison of results, the data were presented as a percentage of the breakdown in the absence of *S. rarak* (Fig. 5.4 – 5.7).

The results showed that the raw material and methanol extracts of *S. rarak* caused a progressive decrease in the observed rate of release of ^{14}C-leucine from *P. bryantii* B_14, probably due to a physical interference with the predatory activity of the protozoa as well as a degree of toxicity. However, the results did not show a dose response relationship. The lowest concentration (1 g/l) for raw material, and the corresponding 0.5 g/l methanol extract, resulted in a rate of protein breakdown of the same order as those found with higher concentrations, up to 10 g/l of raw material or 1 g/l of methanol extract.

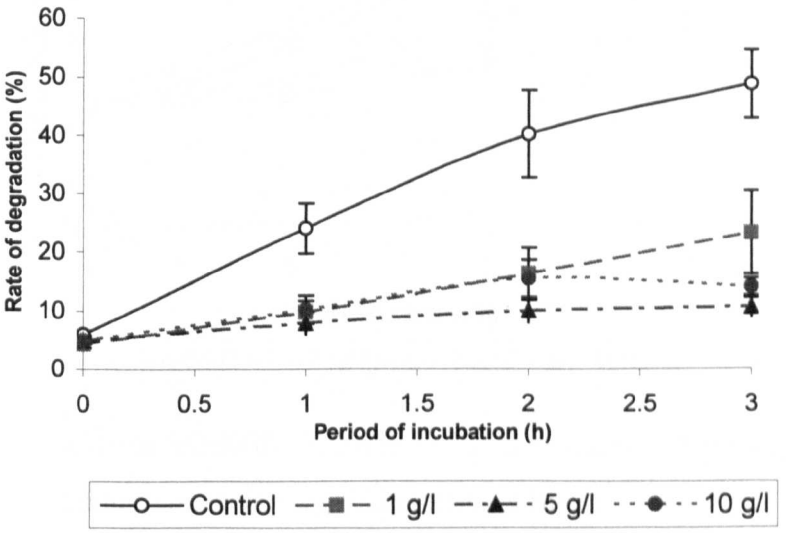


Figure 5.4. Influence of raw material of *S. rarak* on protozoal breakdown of ^{14}C-leucine-labelled *Prevotella (bacteroides) bryantii* B_14 in mixed population of protozoa *in vitro*.

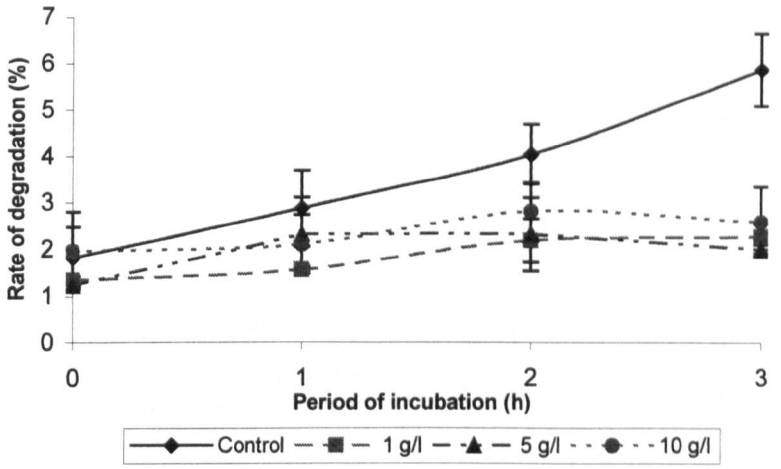


Figure 5.5. Influence of raw material of *S. rarak* on protozoal breakdown of ^{14}C-leucine-labelled *Prevotella (bacteroides) bryantii* B_14 in *E. caudatum*, mono-inoculated rumen fluid *in vitro*.

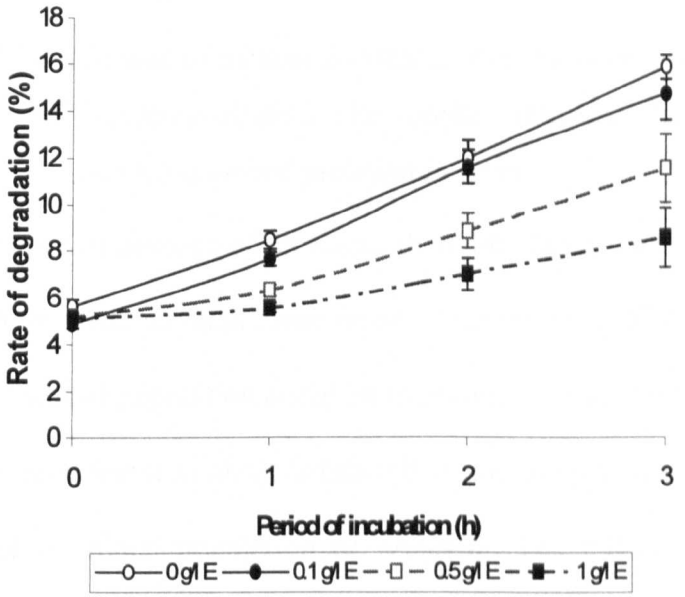


Figure 5.6. Influence of methanol extracts of *S. rarak* on protozoal breakdown of ^{14}C-leucine-labelled *Prevotella (bacteroides) bryantii* B_14 in mixed population of protozoa *in vitro*.

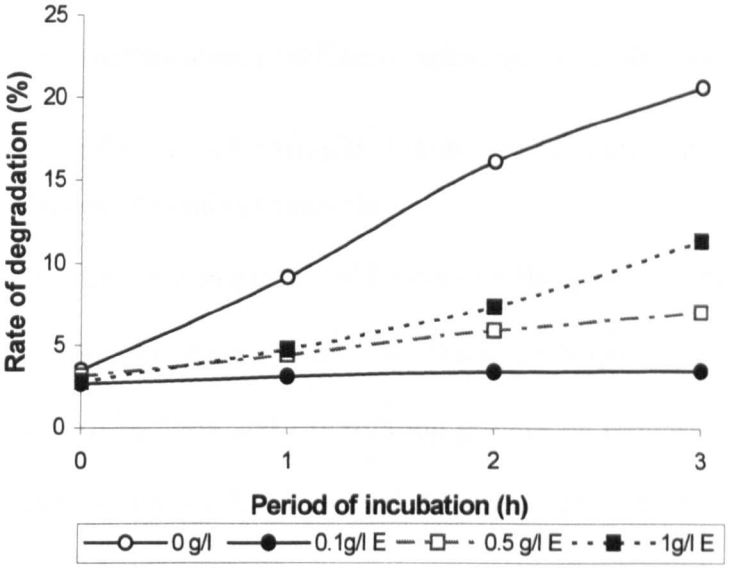


Figure 5.7. Influence of methanol extracts of *S. rarak* on protozoal breakdown of ^{14}C-leucine-labelled *Prevotella (bacteroides) bryantii* B_14 in *holotrich*-enriched protozoa *in vitro*.

The presence or absence of protozoa, therefore, has a major influence on the rate at which bacterial proteins break down in rumen fluid. The size and composition of the protozoal population could be expected to determine the magnitude of the effect. In an attempt to observe this effect, the samples used in the present study contained a unique population of protozoa. The ciliates were classified into mixed population, *E. caudatum* population from mono-inoculated rumen and holotrich-enriched population. Obviously these categories may contain different numbers of organisms, and hence widely different metabolic activities. There was no correlation between type of protozoa and the rate of bacterial protein degradation, although it was likely that holotrichs were more susceptible to the active compound. This result was in agreement with the results from microscopic

studies that the holotrich protozoa were burst and could not be traced under microscope, whereas some entodiniomorphid protozoa remained intact.

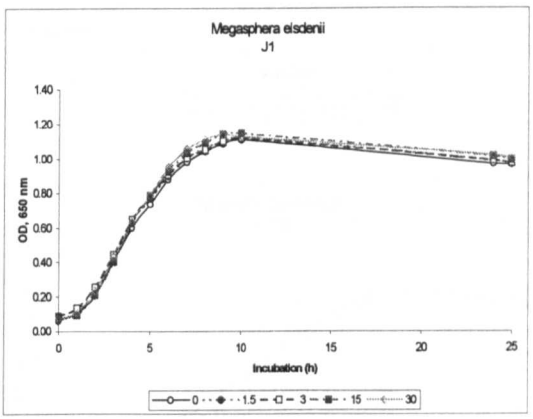
5.3.3. The influence of saponin extract of *S. rarak* on growth of pure cultures of ruminal bacteria

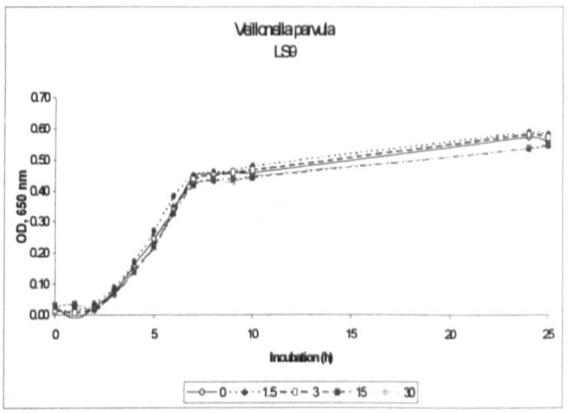
The influence of saponin extract of *S. rarak* on the growth of non-cellulolytic and cellulolytic bacteria was examined by adding methanolic extract of *S. rarak* to pure cultures of bacteria and determining growth by monitoring optical density. The saponin extract of *S. rarak* had no effect on growth of non-cellulolytic bacteria (Table 5.3). However, it appears in this study to have affected cellulolytic bacteria, by prolonging the lag phase following inoculation of *Streptococcus bovis* or abolishing the growth of *Butyrifibrio fibrisolvens*, *Ruminococcos albus*, and *Ruminococcos flavefaciens* (Fig. 5.8). Different susceptibilities to breakdown probably reflect only the intrinsic resistance of different species to physical breakdown. *B. fibrisolvens* would be expected to be most vulnerable due to its thin cell wall. The toxic effect of saponin extract of *S. rarak* against cellulolytic bacteria would be expected to have major consequences for fibre breakdown in ruminants. This result could explain why the cellulolytic bacterial population has been found to be lower in defaunated than in unaltered rumens (Jouany *et al.*, 1988).

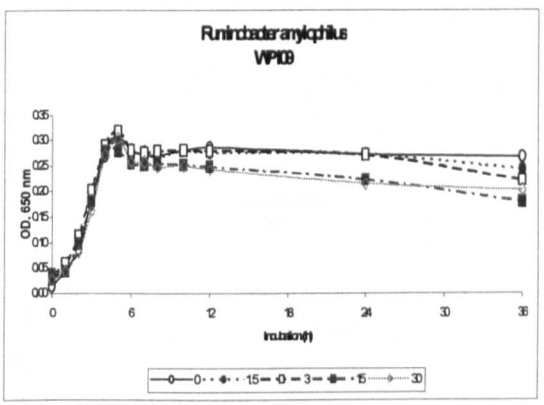
Table 5.3. Effects of *S.rarak* on bacterial growth rate

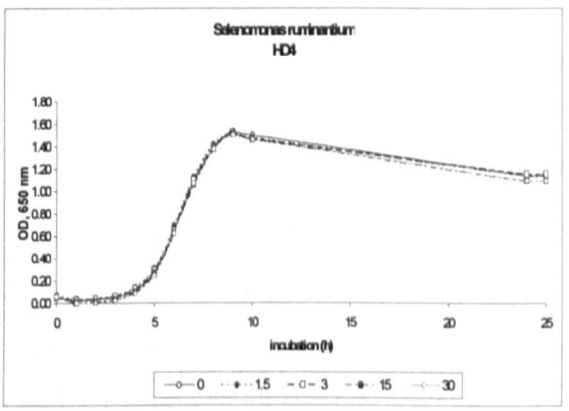
	Species	Rate of inclusion (mg/ml)					mean	SED	P
		0	1.5	3	15	30			
1	*Megasphera elsdenii* J1	0.301	0.311	0.342	0.336	0.359	0.330	0.017	P<0.05
2	*Veillonella parvula* LS9	0.380	0.446	0.366	0.346	0.390	0.386	0.055	ns
3	*Ruminobacter amylophilus* WP109	0.334	0.289	0.328	0.273	0.325	0.310	0.019	ns
4	*Selonomonas ruminantium* HD4	0.424	0.452	0.441	0.438	0.443	0.440	0.019	ns
5	*Prevotella bryantii* $B_1$4	0.404	0.446	0.440	0.441	0.487	0.443	0.022	P<0.05
6	*Clostridium aminophilum* 23	0.372	0.455	0.349	0.373	0.431	0.396	0.070	ns
7	*Lactobacillus caseii* L-caseii	0.409	0.431	0.435	0.314	0.285	0.375	0.043	P<0.05
8	*Streptococcus bovis* ES1	0.269	0.231	0.368	0.494	0.495	0.371	0.044	P<0.001
9	*Streptococcus bovis* C277	0.538	0.580	0.657	0.636	0.518	0.586	0.068	ns
10	*Peptostreptococcus anaerobius* 27337	0.248	0.274	0.263	0.238	0.160	0.237	0.026	P<0.01
11	*Butyrifibrio fibrisolvens* SH13	0.549	0.314	0.000	0.000	0.000	0.173	0.140	P<0.01
12	*Butyrifibrio fibrisolvens* SH1	0.597	0.383	0.390	0.000	0.000	0.274	0.055	P<0.001
13	*Butyrifibrio fibrisolvens* JW11	0.301	0.366	0.393	0.000	0.000	0.212	0.031	P<0.001
14	*Ruminococcos flavefaciens* 007	0.348	0.000	0.000	0.000	0.000	0.070	0.023	P<0.001

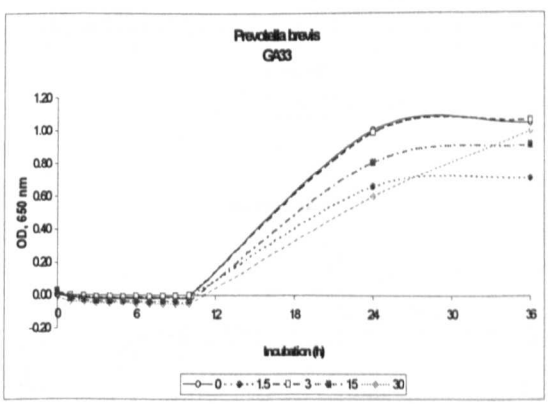
Figure 5.8. Influence of saponin extract of *S. rarak* on growth of individual species of rumen bacteria. Inclusion levels were 0, 1.5, 3, 15, 30 g of the saponins extract per litre.

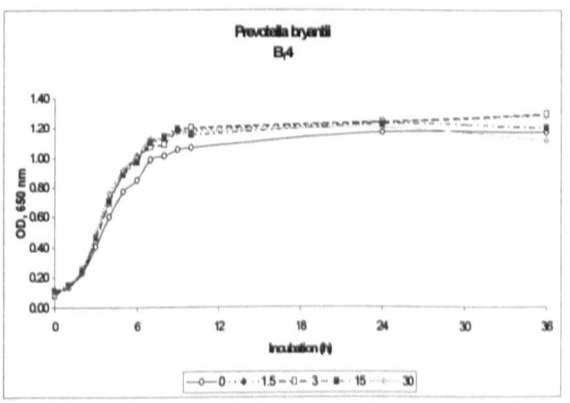


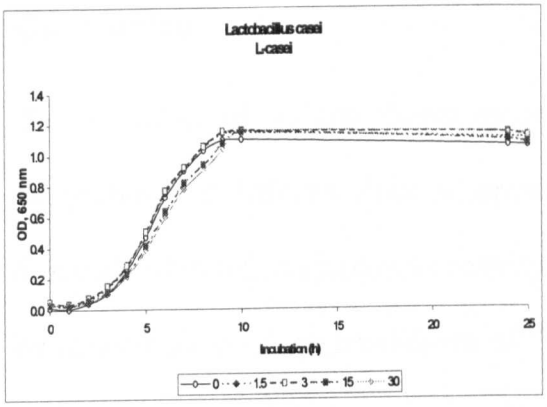


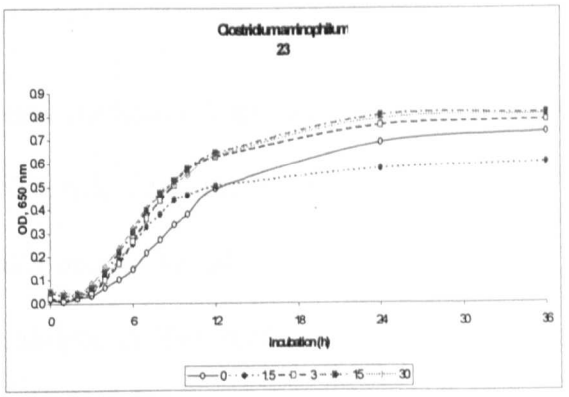


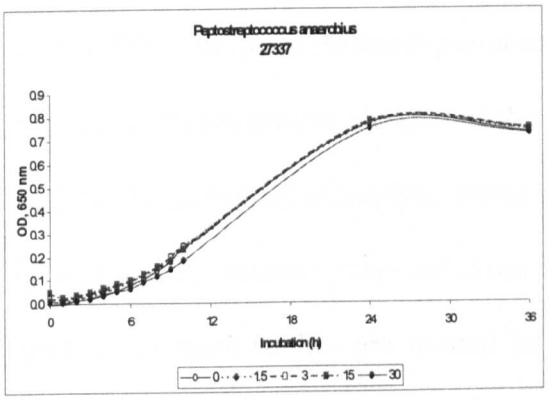


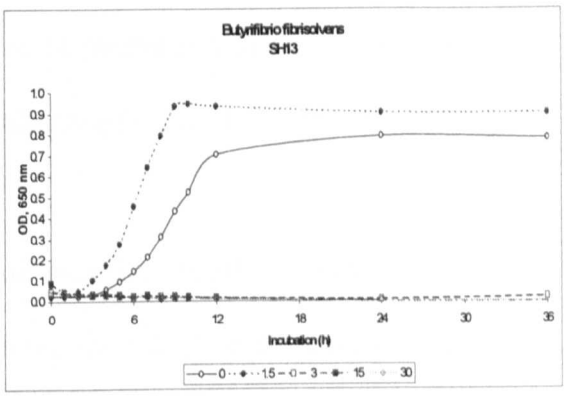


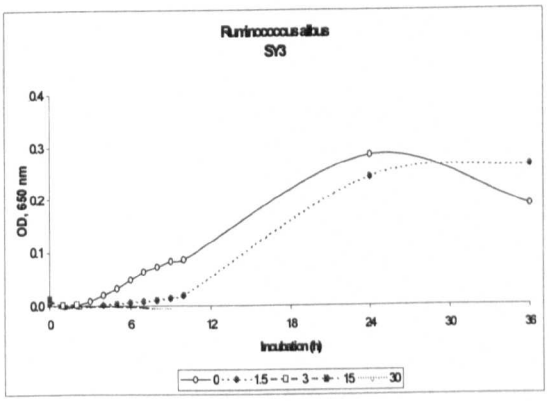


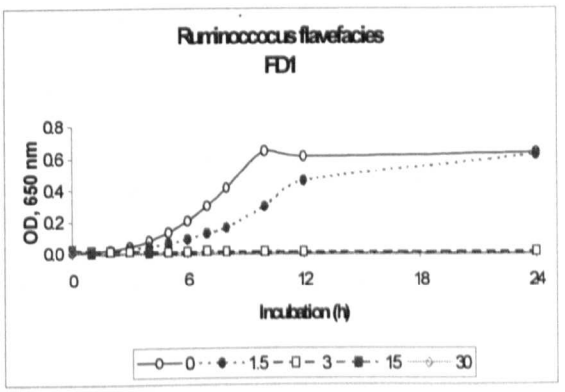

Lactobacillus casei
L-casei
OD, 650 nm
Incubation (h)
0 1.5 3 15 30

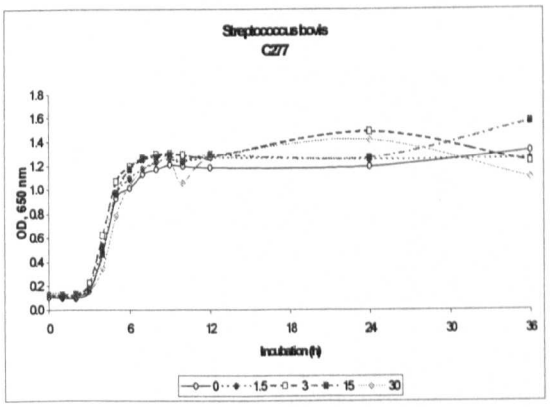

Clostridium aminophilum
Z3
OD, 650 nm
Incubation (h)
0 1.5 3 15 30


Peptostreptococcus anaerobius
27337
OD, 650 nm
Incubation (h)
0 1.5 3 15 30


Butyrifibrio fibrisolvens
SH13
OD, 650 nm
Incubation (h)
0 1.5 3 15 30


Ruminococcus albus
SY3
OD, 650 nm
Incubation (h)
0 1.5 3 15 30


Ruminococcus flavefacies
FD1
OD, 650 nm
Incubation (h)
0 1.5 3 15 30


Streptococcus bovis
C277
OD, 650 nm
Incubation (h)
0 1.5 3 15 30

Conclusion

The antiprotozoal activity shown by aqueous and methanol fractions indicated the presence of different types of saponins in *S. rarak*. The saponin fractions of *S. rarak* exhibited antiprotozoal activity, as confirmed by visual assessment and by measurement of the breakdown of ^{14}C-leucine-labeled *Prevotella bryantii* in rumen fluid incubated *in vitro*. No evidence was found of differential susceptibility between different protozoal genera. Inclusion of a methanol extract of *S. rarak* in the growth medium of the pure cultures of rumen bacteria inhibited slightly the growth of cellulolytic bacteria.

In conclusion, extracts prepared from *S. rarak* may be useful as defaunating agents, but more studies are needed to investigate the effect of the extracts on ruminal fibre digestion and other rumen fermentation characteristics.

CHAPTER 6

EFFECTS OF INTRARUMINAL INFUSION OF *SAPINDUS RARAK* ON RUMEN FERMENTATION, MICROBIAL PROTEIN SYNTHESIS AND FIBRE DEGRADATION

6.1. *Introduction*

Manipulation of ruminal fermentation could improve the utilisation of substrates and efficiency of fermentation to maximise the nutritional value of feeds. The aim should be to increase the rate of some beneficial processes, such as fibre degradation and conversion of non-protein nitrogen (NPN) to microbial cell protein, and to reduce the rate of detrimental process, such as methane production and protein degradation (Nagaraja *et al.*, 1997).

Approaches used to manipulate rumen fermentation have aimed to modify the composition and activities of the rumen microbial population with antibiotics, methane inhibitors or defaunating agents.

Reducing protozoal populations in rumen could improve nitrogen utilisation in the rumen and increase microbial protein flow to the intestine, thereby enhancing ruminant production. Rumen protozoa ingest and digest bacteria (William and Coleman, 1992). Consequently, protozoa reduce the efficiency of nitrogen utilization in the rumen by increasing protein turnover (Ushida *et al.*, 1991; William and Coleman, 1992).

Recently, there has been increased interest in plant components as a possible means of suppressing or eliminating protozoa in the rumen. It has been suggested that naturally occurring plant metabolites might be toxic to rumen protozoa. Plants produce a large number of chemicals, arbitrarily categorised as primary and secondary metabolites, which act as defence mechanisms for the plant against animal, insect predator and microbial infection (Haborne, 1989). Some studies on the use of tropical plants as defaunating agents have been reported (Navas-Camacho *et al.*, 1993; Thalib *et al.*, 1995; Newbold *et al.*, 1997; Odenyo *et al.*, 1997). Wallace *et al.* (1994) reported that saponins from *Yucca schidigera*, a desert plant, have strong antiprotozoal activity and may serve as an effective defaunating agent for ruminants.

Previous studies showed that the saponin fractions of *S. rarak* exhibit antiprotozoal activity, as confirmed by visual assessment as well as by measurement of the breakdown of ^{14}C-leucine-labelled *Prevotella bryantii* in rumen fluid incubated *in vitro*. Inclusion of a methanol extract of *S. rarak* in the growth medium of pure cultures of rumen bacteria had no effect, except that cellulolytic bacteria showed susceptibility. This may be because the bacteria had not previously been exposed to *S. rarak*, as results from other studies indicated that bacteria could develop resistance to the antibacterial agent.

Aim of Experiment

The aim of this experiment was to study the effects of *S. rarak* on ruminal digestion, fermentation and ammonia pattern using ruminally and duodenally cannulated dairy cows.

6.2. *Materials and Methods*

6.2.1. Experimental Animals

Four non-lactating dairy cows, weighing 600 kg and fitted with 100 mm rumen fistulae (Bar Diamond Inc, Idaho, USA), and a rigid polyethylene T-piece cannula (Ankom, Fairport, New York, USA) in the proximal duodenum, were used. They were individually housed in tethered stalls at the Dairy Metabolism Unit (DMU), University of Nottingham, with free access to water at all times and sawdust bedding; they were let out for exercise in a concrete yard.

6.2.2. Experimental Diets

The rations offered in this experiment were restricted so that all the animals consumed the same amount of food. The diets were formulated using the Ultramix-Plus Least Cost Feed Formulation and Modelling System (AGM Systems Ltd, Exeter, UK) using the ruminant database, which formulated to AFRC (1993) requirements. This calculated the metabolisable energy requirement as 72 MJ/day and 1003 g protein /day for a non-lactating/pregnant cow assuming that the cows weighed 600 kg.

Concentrates were formulated containing barley (825 g/kg), soya (Hi-Pro) (100 g/kg), molassed meal (50 g/kg) and minerals/vitamins (25 g/kg).

Cows were fed 8 kg hay/day (a common basal diet), supplemented with 2 kg concentrates offered twice daily at 08.00 and 15.30.

6.2.3. Experimental Design

Animals were allocated to treatment sequences in a 2 x 2 crossover design (Fig. 6.1) for each 21-d experimental period. In the first period, two cows were given *S. rarak* solution intraruminally and the two other cows acted as controls. After 14 d of adaptation to treatment, responses were measured over a seven-day sample collection period. A changeover period of seven days was allowed between treatments periods, so that any physiological effect of the previous treatment was eliminated. Treatments were reversed in the second period..

Cow ID	H	F	R	T
Period 1	T	T	C	C
Period 2	C	C	T	T

Figure 6.1. Allocation of cows to treatments in a 2 x 2 crossover design. T = Treatment; C = Control

6.2.4. Administration of *S. rarak*

After oven drying at 60^{0}C for 3 days, the pericarps of *S. rarak* were ground and sieved through a 1 mm screen. *S. rarak* suspension was prepared by adding 50 g of *S. rarak* powder into 1 l distilled water (50 g/l) then administered twice daily (1 l dose/day) into the rumen through the ruminal fistulae. *S. rarak* contains 23 g saponins/kg (Hamburger *et al.*, 1992). Administration of *S. rarak* was at the morning feeding (08:30) and at 16:00, which was approximately 0.5 h after cows commenced

the second feeding time to allow for synchronisation of *S. rarak* availability with increased ruminal NH_3.

6.3. Procedures and Analysis

6.3.1. Diet Sampling

The basal diet and concentrate were sampled weekly and combined for each period. Dry matter was determined by oven drying at 65^0C to constant weight. Samples were ground and sieved through a 1mm screen and analysed for NDF (Van Soest *et al.*, 1991) and crude protein.

6.3.2. Rumen Fluid Sampling

On days 1, 2, 3, and 4 of each sampling period, a 200 ml sample of strained rumen fluid was collected at 0, 2, 4, and 6 h after *S. rarak* dosing in the morning. Samples were taken using a filtering device placed in the rumen and a 60 ml syringe and a flask (Figure 3.1). After recording pH of the filtrate, sub-samples of filtrate were prepared for VFA and ammonia analyses. The filtrate was also preserved for enumeration of protozoa by combining 2.5 ml of filtrate with 5 ml of methylgreen:formalin:saline (MFS) solution (Ogimoto and Imai, 1981). Protozoa were counted using a Hawkesly counting chamber (see Section 3.2.4).

6.3.3. Faeces and Urine Collection

Urine was collected using a device that allowed complete separation of urine from faeces (Figure 6.2 and Figure 6.3). The apparatus consisted of a heat-sealed plastic bag designed to fit around the vulva and sit flush against the skin under the anus of

the cow. This allowed urine to pass from the bag down a plastic tube (12 mm diameter) into a collection vessel; faeces dropped past the bag onto the floor. The bag was attached using velcro tape (25 mm), the loop half of which was attached to the bag whilst the hook half was securely to the cow using Evo-Stik™ glue.

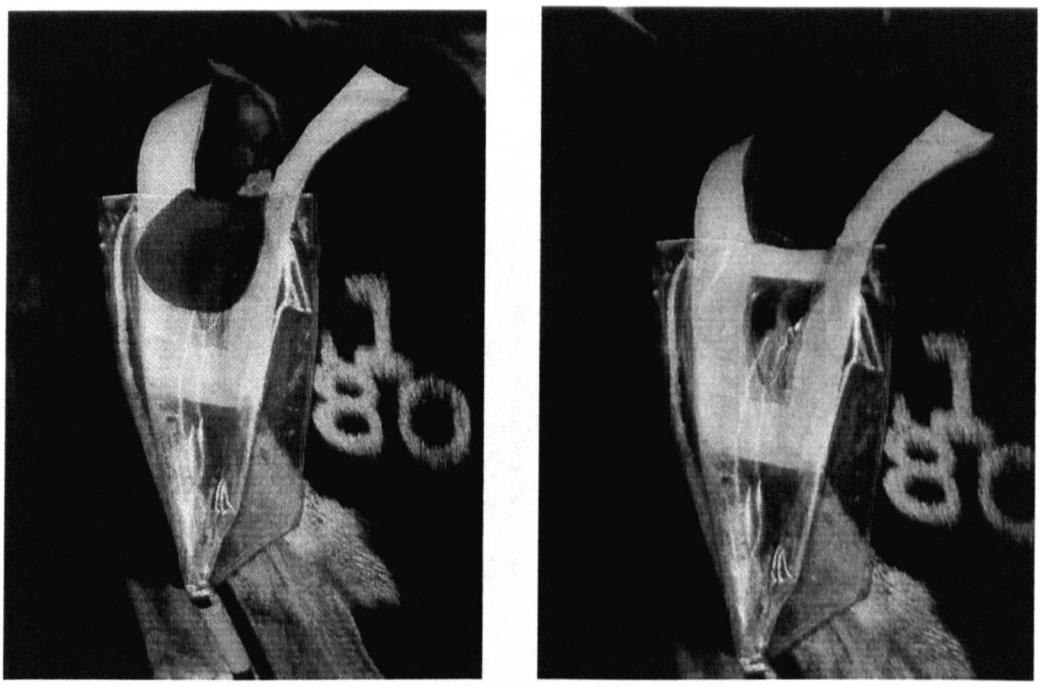


Figure 6.2. Equipment for total urine and faeces collection

Faeces were collected at regular intervals and placed in a weighed plastic dustbin during each 24 h period, one for each cow. The bin was weighed daily to determine faecal output for each cow. Faeces were mixed thoroughly to ensure uniformity and a 100 g sub-sample taken and stored at -18^{0}C until further analysis. The rest of the faeces were discarded and the container cleaned and weighed prior to the following 24 h collection.

Urine was collected in large plastic containers previously primed with 500 ml of 10% H_2SO_4 to acidify the urine. This was used to prevent any microbial activity and

also to prevent losses of nitrogen from the urine as ammonia. Each container was weighed to determine the total urinary output in a given 24 h period and then replaced with another weighed container plus acid.

The urine was thoroughly mixed and a 50 ml sub-sample taken and store at -18^{0}C until further analysis. The rest was discarded and the container was cleaned and weighed for the next 24 h collection.

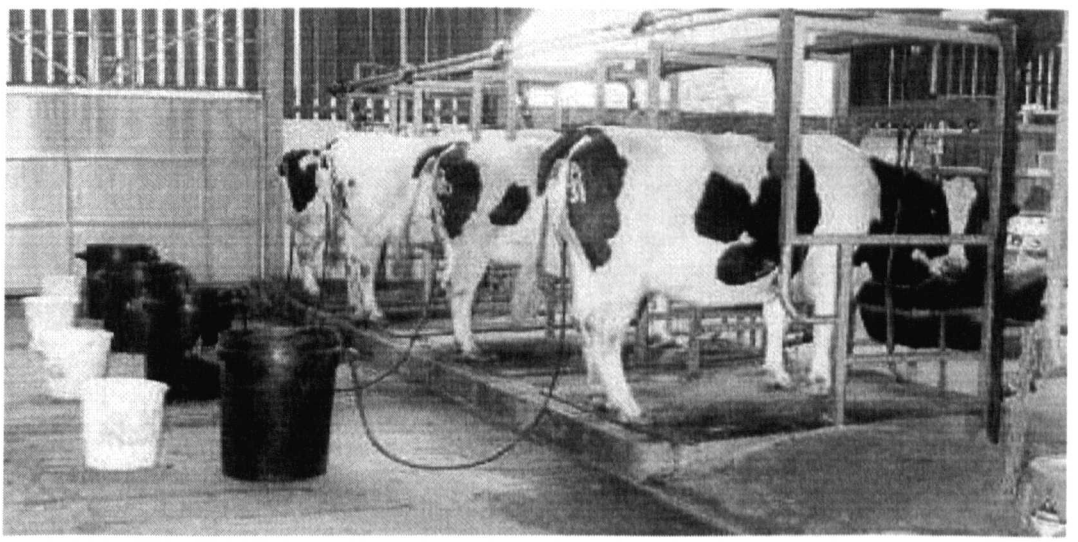


Figure 6.3. Total urine and faeces collection

Analysis

Determination of nitrogen or crude protein (CP) content of solid samples

The nitrogen content of samples was determined using a NA 2000 Nitrogen Analyser (Fison Instrument, Milano, Italy). The reference material used was methionine. Approximately 20 – 50 mg of ground sample was weighed into a 10x10mm tin capsule (EA Scientific, West Sussex, UK).

The analytical conditions were as follows:

Left Furnace Temperature	900^{0}C
Right Furnace Temperature	780^{0}C
GC Column	Activated Carbon
GC Column Temperature	Ambient
Oven Temperature	120^{0}C
Filament Temperature	190^{0}C
Cycle Stop	120 sec
Sample Start	0 sec
Sample Stop	50 sec
Oxygen Injection	90 sec
Air Pressure	350KPa

Helium Flow Rates

Measure	120 ml/min (pressure 150 KPa)
Reference	40-50 ml/min
Purge	50-60 ml/min

Oxygen

Pressure	150 KPa
Flow	100-150 ml/min
Loop	50 ml

The nitrogen content of the sample was determined by comparing the values obtained from the analysis of the reference material (methionine).

A K-factor was calculated according to the equation:

$$K - \text{factor} = \frac{Th\% \times WS}{I - b}$$

where: Th%: theoretical percentage of nitrogen in the standard
WS: weight of the standard sample in mg
I: standard integral (peak area obtained)
b: blank value (obtained by analysing an empty tin capsule)

The nitrogen concentration of the sample was then calculated using the following equation:

$$\%\,\text{Nitrogen} = \frac{K \times (I - b)}{WS}$$

where: K: average factor
I: sample integral (peak area obtained)
WS: weight of sample in mg
B: blank value (obtained by analysing an empty tin capsule).

All samples were measured in duplicate and an average value was calculated for each sample from the duplicate results. Repeats were carried out if the duplicate values were not found to be within 0.25% N of each other.

The CP content of feed or faeces (freeze dried prior to N analysis) was then calculated as the nitrogen content multiplied by the factor 6.25, which is the mean nitrogen content of protein in feedstuffs.

Liquid samples were analysed with the same analytical condition for the solid samples, the liquid samples were also freeze dried prior to analysis. Tin capsules (10x10mm, EA Scientific, West Sussex, UK) were approximately half-filled with the liquid sample (weight recorded to four decimals on electronic balance (Metler Toledo, Leicester, UK)). The samples were then freeze-dried and final weight was recorded. The capsules were then analysed using the Nitrogen Analyser as for the solid samples and the nitrogen content calculated as before.

Ruminal Outflow Rates.

The natural alkane C_{33} was used as a marker for the study of particulate digesta flow, while Co-EDTA was used to study the liquid phase.

Using a n-alkane as a solid-phase marker

Plant alkanes, long-chain hydrocarbons in the cuticular wax, are predominantly odd-chain in the range C_{25} to C_{35}. They are substantially indigestible and therefore have been suggested as indigestible internal markers in nutritional studies (Dove and Mayes, 1996). In this study, the natural alkane C_{33} (tritriacontane), the most predominant alkane in all plant species, was used to quantify flow rate of the solid phase of rumen fluid through the duodenum.

The method used for determination of alkane content of feed and duodenal fluid was that of Unal (1998) and Unal and Garnsworthy (1999), who used some modifications of methods of Mayes *et al.* (1986) and Vulich *et al.* (1995).

Basically, all freeze dried samples were ground using a laboratory grinder (Moulinex Optiblend 2000). 1 g faeces (1.5 g for herbage and concentrates) was accurately weighed and placed into a glass universal bottle. An internal standard (Tetratriacontane, C_{34}, Fluka Chemicals) 0.25 mg in 0.5ml heptane was added, since this alkane is not present, or has very low concentrations, in herbage or faeces (Vulich *et al.*, 1991). Potassium hydroxide (14 ml, 1 M in ethanol) was added for direct saponification of the sample. Saponification is necessary to convert esters to the corresponding alcohol and potassium salts of the acids to facilitate extraction and purification of the hydrocarbon fraction (Unal, 1998). The samples were heated on a

heating block (Hotplate SH2, Stuart Scientific Co., UK) for 8 h at 90^0C. The samples were allowed to cool and extraction was performed by adding heptane (14 ml) and water (2 ml). After mixing, the samples were vortexed for 1 min, and centrifuged (Megafuge 1.0R, Heraeus Instuments) at 2000 rpm for 15 min to obtain a clear top heptane layer, which was then removed using a Pasteur pipette, while the residues were discarded. This extract was passed through a 5 ml silica gel column (Silica gel 60, particle size 0.2-0.5 mm, Fluka Chemicals) to remove plant pigments, lipids and long chain alcohols. The column was preconditioned with 5 ml heptane before the extraction and then washed with 10 ml heptane afterward. The eluent was then dried using N_2 with gentle heating on a heating block in a fume cupboard. The solute was redissolved in 0.5 ml heptane and 0.2 ml of this solution was transferred into a micro vial, crimped and stored frozen at -20^0C prior to analysis. For the analysis, 4 µl of the final solution was injected into a gas chromatograph (Hewlett Packard 5890 Series II Plus Gas Chromatography, Bristol, UK) fitted with an SGE 25 m x 0.53 mm i.d. (0.1µm OV-1 film) column equipped with an auto sampler. The operating conditions are shown in Table 6.1.

Table 6.1. Operating conditions for the determination of alkanes using a Hewlett Packard 5890 Series II Plus Gas Chromatography.

Carrier gas	Helium
Inlet pressure	20psi
Injection temperature	300^0C
Detector temperature	300^0C
Attenuation	0
Offset	5.0 mV
Pre-injection solvent washes	2
Pre-injection sample washes	2
Sample pumps	3
Post-injection solvent washes	2
Injection speed	Fast
Injection volume	4µl
Initial oven temperature	220^0C
Oven temperature programme	220^0C for 2 min
	220^0C to 280^0C at 5^0C/min
	Held for 2 min
Total run time	16 min

The concentrations of C_{33} alkane in duodenal fluid and feed were determined by comparison with the internal standard (C_{34}) using the equations below:

$$\text{Corrected area of } C_{33} = \frac{\text{Mean area of } C_{34} \text{ (standard)}}{\text{Area of } C_{34} \text{ in sample}} \times \text{Area of } C_{33} \text{ in sample}$$

$$\text{Amount of } C_{33} \text{ in } 4\mu l = \frac{\text{Corrected area of } C_{33} \text{ (in sample)}}{\text{Mean area of } C_{33} \text{ (standard)}} \times 0.5$$

$$\text{Amount of } C_{33} \text{ in 1g sample } (\mu g) = \frac{\text{amount of } C_{33} \text{ in } 4\mu l \text{ x500}}{\text{Sample weight (g)}}$$

Using Co-EDTA as a liquid phase marker

Preparation of Cobalt ethylenediaminetetraacetic acid (Co-EDTA)

Co-EDTA was prepared by the method of Uden *et al.* (1980). Na-EDTA (297.2 g), $CoCl_2.6H_2O$ (190.4 g) and NaOH (32.0 g) were dissolved in 1600 ml distilled water in a 5 l beaker while gently heating. More NaOH pellets were added to ensure that all the reagents dissolved. The solution was allowed to cool to room temperature and hydrogen peroxide (160 ml) was added. The mixture was left to stand at room temperature for 4 h, and then 2400 ml of 95% (v/v) ethanol was added. The mixture was stored in a refrigerator overnight. The resulting crystals were filtered and repeatedly washed with 80% (v/v) ethanol and dried overnight at 100°C.

Administration of Co-EDTA and sampling procedure

The liquid phase marker solution (15 g Co-EDTA/l/cow) was injected into the rumen via the rumen fistula before morning feeding, followed by a rinse with 100 ml of water. Samples of rumen fluid (150 ml) were taken 0.5, 2, 4, 6, 8, 12, and 24 h after administration of Co-EDTA.

Determination of Cobalt concentration

The samples of rumen fluid (8 ml) were centrifuged at 11000 g for 20 min. The supernatant solution was then diluted with 5% (v/v) sulphuric acid and analysed by atomic absorption spectroscopy (AAS) using a Unicam Spectrophotometer (model Solaar 969, Unicam Ltd, Cambridge, UK) with air/acetylene as the oxidant and fuel, a wavelength measurement of 240.7 nm with a Co hollow-cathode lamp as the radiation source. The reading of the AAS was standardised with Co standards of 0.625, 1.25, 2.5, 5 and 10 mg Co/ml in 5% (v/v) sulphuric acid.

Rumen volume and rumen outflow rate

The dilution curve of Co-EDTA in the rumen was used to estimate rumen volume and dilution rate of rumen fluid. Liquid rumen outflow rates were determined by fitting a mono exponential equation to the decline of Co concentration in rumen fluid over time for each cow. The equation used to calculate rumen outflow rate was:

$$C = C_0 e^{-Dt}$$

$$D = (\ln 2)/t_{½} = 0.693/ t_{½}$$

Where D is outflow rate, $t_{½}$ is half-life of the marker in the rumen and C is concentration of the Co.

Marker half-life ($t_{½}$) was calculated from a regression of log concentration of Co against time:

$$Y = -b (x) + a$$

$$X = \frac{Y - a}{-b}$$

Half-life ($t_{½}$) was the difference between the value of X when Y was equal to log of 100 and Y equal to log of 50. Then the outflow rate was calculated from the equation:

$$D = 0.693/ t_{½}$$

Rumen fluid volume was then calculated by division of the Co dose (the amount added into the rumen) by the zero intercept (b) of the fitted equation:

$$Y = a \ln(x) + b$$

Dry Matter (DM)

Sub-samples of the pelleted rations, which had been ground in a mill, were dried to constant weight in an oven at 80^0C. Duodenal digesta samples and microbial isolates were frozen and freeze dried until they had reached a constant weight (Edwards, 12K Supermodulyo, Sussex), sealed in airtight containers and stored at room temperature.

Organic Matter (OM)

Approximately 1-2 g of dried sample was accurately weighed into crucibles and heated at 450^0C in a muffle furnace (Cestradent Ltd) for 16 h, cooled in a desiccator and then weight of the residue recorded as the ash content. Organic matter was calculated as 1000 - ash (g/kg) (AOAC, 1980).

Digestibility

Digestibility is defined as the proportion of food that is not excreted in the faeces and, therefore, assumed to be absorbed by the animal (McDonald *et al.*, 2002). Digestibility measured using n-alkane as a natural constituent of the food is stated in terms of dry matter and presented as a coefficient. In equation form:

$$\text{Digestibility of DM} = \frac{\text{g indicator/kg faeces DM} - \text{g indicator/kg food DM}}{\text{g indicator/kg faeces DM}}$$

Rumen Ammonia Nitrogen

Rumen ammonia concentration was determined using a colorimetric method adapted from Whitehead *et al.*, (1967). The principle was based on the reaction between ammonia, phenol and hypochlorite to produce indophenol blue, a complex which

absorbs light at 610 nm. Samples of strained rumen fluid (1 ml) were acidified with an equal volume of 1M HCl, centrifuged (3000g, 15 min at 4^0C) and diluted 1:50 with distilled water. 1 ml of the diluted centrifuged rumen fluid was analysed for ammonia concentration by the phenol hypochlorite reaction using a standard curve of 0-1 μM ammonium ion concentration (ammonium phosphate).

The phenol hypochlorite reaction was carried out by adding 1 ml of test samples (or standard) to 2 ml of phosphate buffer (0.1 M NaH_2PO_4 pH adjusted to 11.7 by addition of NaOH). Then phenol reagent (0.5 ml, 3% v/v), sodium nitroprusside (0.25 ml, 0.05% w/v) and hypochlorite solution (0.25 ml, 2% w/v) were added. Mixing was carried out between each addition. The mixture was incubated (80^0C for 10 min) and cooled. Absorbance was measured at 610 nm using Technicon analyser. Ammonia concentration of the sample was estimated from the standard curve.

Duodenal Digesta Ammonia Nitrogen

Approximately 0.5 g of freeze-dried duodenal digesta was accurately weighed into a centrifuge tube and mixed with 5 ml of 0.2M HCl and left to stand for 30 min at 4^0C. The samples were then centrifuge for 10 min. The supernatant was diluted 1:10 and NH_3-N content determined against standards prepared in 0.02 M HCl (as above).

VFA Analysis

Samples of strained rumen fluid (4 ml) were added to 1 ml of orthophosphoric acid (25%, v/v) containing 20 mM of 2-ethylbutyric acid as internal standard. They were then centrifuged at 24,000 g for 15 min at 4^0C. The supernatant obtained was used for VFA analysis by gas chromatography (GCV Chromatography, PYE Unicam,

Cambridge, UK) using a 1 m x 4 mm glass packed column containing Supelco GP 10% SP-1200/1% H_3PO_4 on 80/100 mesh chromosorb WAW. The temperature of oven, detector and injector were 140 ^{0}C, 180 ^{0}C and 170 ^{0}C respectively. The carrier gas was helium at a flow rate of 40 ml/min. A Spectro Physics 4270 integrator was used. Peaks were identified by comparison with a standard mixture of known composition. The relationship between internal standard and each VFA was established as the response factor and calculated as following: (for example acetic acid concentration)

$$F_{ac} = \frac{C_{ac} \times STDIC_{ac}\ area}{STD_{ac}\ area}$$

Where:

F_{ac} = Acetic acid response factor

C_{ac} = Concentration (μm/ml) of acetic acid in the VFA standard

$STDIC_{ac}$ area = area of isocaproic acid peak in the standard's chromatogram...

STD_{ac} area = area of acetic acid peak in the standard's chromatogram.

Then this factor was used to calculate the acetic acid concentration with the relation follows:

$$[AC] = F_{ac} \times \frac{sample\ ac\ area}{sample\ IC\ area}$$

The sum of all individual VFA would give the total VFA concentration in μm/ml of rumen fluid. The molar proportion of each acid was calculated as a percentage of the total VFA concentration.

Fibre Analysis

Fibre was determined by the detergent system described by Van Soest *et al.* (1991). As there was a comparatively small amount of sample available for analysis, the sequential method of analysis developed at U.C.N.W, Bangor (Catton, 1985) was employed. This method required a maximum sample of 1g and the steps involved are shown in Fig. 6.4. At each stage, there was a progressive removal of each fibre component.

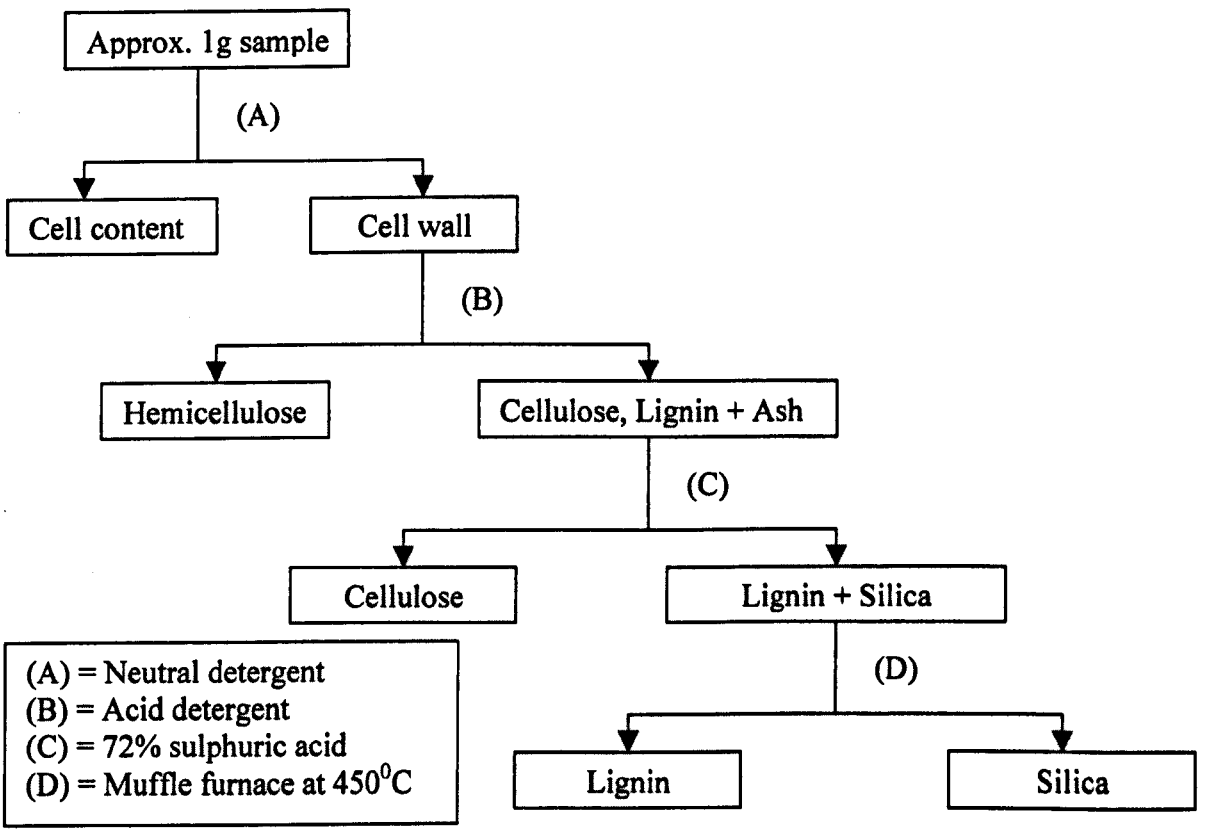


Figure 6.4. Sequential method of fibre analysis (Catton, 1985).

Neutral Detergent Fibre Analysis

The neutral detergent solution was prepared by dissolving 93 g of disodium ethylenediamine tetraacetic acid (EDTA) and 34 g of disodium tetraborate in 2 litres of distilled water. In a separate beaker, 22.8 g of disodium hydrogen phosphate was dissolved in 1 litres of distilled water and 50 ml of 2-ehoxyethanol was added. The two solutions were transferred and mixed in a round tank and made up to 5 l with distilled water. Approximately 1 g of sample (W_1) was weighed into a pre-weighed crucible, then approximately 0.25 g of sodium sulphate was added. The crucibles were then immersed in a tank containing the neutral detergent solution and incubated

at 90^0C for 16 h. The crucibles were then removed and filtered using the Buchner apparatus. Each crucible was washed with four 50 ml portions of hot water, and then twice with 30 ml of acetone to remove traces of the reagent from the crucibles. The crucibles were then dried at 90^0C for 12 h, allowed to cool in a desiccator, and reweighed to give measurement (W_2).

Acid Detergent Fibre Analysis

The acid detergent solution was prepared by dissolving 50 g cetyl trimethylammonium bromide (CTAB) in 5 l of 0.5M sulphuric acid. The crucibles, with the remainder of the sample in them, were immersed in acid detergent solution at 90^0C for 16 h. The crucibles were then removed from the solution, washed, filtered, dried and reweighed as before to give measurement (W_3).

Lignin Analysis

The crucibles were immersed in a beaker containing 72% sulphuric acid and then warmed to 25^0C for 60 min. The residues were filtered in the Buchner apparatus and washed with copious amounts of cold water and then washed twice with 30 ml acetone. The crucibles were then dried at 90^0C for 12 h before reweighed to give measurement (W_4).

Ash Analysis

The crucibles was put in a muffle furnace at 450^0C for 12 h, and then allowed to cool before being reweighed to give measurement (W_5).

The results can be calculated as follows:

$$\% \text{ Neutral Detergent Fibre} = \frac{(W_1 - W_2) - W_5}{W_1} \times 100$$

$$\% \text{ Acid Detergent Fibre} = \frac{(W_2 - W_3) - W_5}{W_1} \times 100$$

$$\% \text{ Detergent Lignin} = \frac{(W_3 - W_4) - W_5}{W_1} \times 100$$

$$\% \text{ Ash} = (W_5 - \text{Weight of crucible (g)}) \times 100$$

Microbial synthesis

DAPA as a natural microbial marker was used for estimating microbial protein synthesis in this experiment:

Determination of 2,6-diaminopimelic acid (DAPA)

DAPA has been used to estimate the magnitude of bacterial protein synthesis (Ørskov, 1982). DAPA, a component of the majority of peptidoglycans in the bacterial cell wall, is present in almost every bacterium and does not occur in animal feeds.

Sample Hydrolysis

Known quantities of microbial isolates and duodenal digesta (see below) were hydrolysed in 6M HCl for 18 h at 115^{0}C under nitrogen. The hydrolysates were cooled and filtered into round bottom flasks, an internal standard (DL-nor-leucine) added, and rotary evaporated to dryness. The dried residue was washed three times with distilled water and made up to volume.

Microbial Isolates

Approximately 0.05 g of freeze-dried material was hydrolysed in 50 ml 6M HCl, cooled and 2.0 μm DL-nor-leucine added. The hydrolysate was dried and made up to a final volume of 3 ml with distilled water and stored at 1^0C prior to analyses.

Duodenal Digesta

Approximately 0.4 g of freeze-dried particulate and whole duodenal digesta were hydrolysed in 100ml 6M HCl, cooled and 20 μm DL-nor-leucine added. The sample was evaporated to dryness, washed three times with distilled water, made up to 10 ml with distilled water and stored at 1^0C.

DAPA Analysis

A simple bench top amino acid analyser was used. The peak was eluted from the column using citrate buffer pH 3.3. Eluted samples of DAPA (Sigma Chemical Co.) produce a linear response within the ranges used (0-2.5 mM). The recovery of DAPA added to duodenal digesta was determined.

Statistical Analysis

Data were subjected to one way ANOVA analysis with treatment, period and time for the main effects and cow as a block. All analyses were carried out using the Genstat 6 computer programme.

6.4. Results and Discussion

Protozoa Numbers

S. rarak was infused directly into the rumen through the rumen fistula and the effects on ciliate protozoa were observed microscopically. Concentration of rumen ciliates, presented as number of cells per ml of rumen fluid, were determined by counting fixed cells in a volumetric chamber deep enough to accommodate the largest of the protozoa. Observation was focused on two types of protozoa based on the basic body forms, i.e. (1) Holotrich and (2) Entodiniomorph.

Despite having been shown to have antiprotozoal activity *in vitro* (Chapter 5), there was no effect ($P>0.05$) of adding *S. rarak* on total or individual species counts of ciliate protozoa in the rumen (Figures 6.5, 6.6 and 6.7). However, *S. rarak* showed a selective effect on Holotrichs (Figure 6.6.) as the number of holotrichs was consistently lower in treated than in control animals, although the pattern was similar. This indicated that holotrichs were more susceptible than entodiniomorphs, which is in agreement with the finding of Burgraaf and Leng (1980) and Navas *et al.* (1992).

The adaptation of the rumen ecosystem to plant secondary compounds could be considered as advantageous or disadvantageous. The advantage is that adaptation enables ruminant to feed on a wide range of plants which otherwise would be toxic. Animals in some regions developed tolerance to *Leucaena leucocephala* due to the presence of the ruminal bacterium, *Synergistes jonesii*, which capable of degrading the ruminal metabolite of mimosine, 3,4-dihydroxy pyridine, which toxic to

ruminants (Jones, 1981). However, in some cases the adaptation of rumen microorganisms to antiprotozoal factors from plants could be disadvantageous as it diminishes the potential exploitation of their selective antimicrobial activity to manipulate rumen function.

In this experiment, there is no sign of adaptation by rumen microbes to the addition of *S. rarak* shown in 4 days observation. (Figures 6.7, 6.8, and 6.9). Wang *et al.* (1998) found that ruminal populations were markedly reduced in sheep fed 5 g day^{-1} of *Yucca shidigera* extract, but they recovered after about 2 weeks of treatment. This indicates some form of adaptation of the mixed ruminal population to *Yucca shidigera*. The effects may not have been expressed here as the observation was only for 4 days in one period instead of 2 weeks or more.

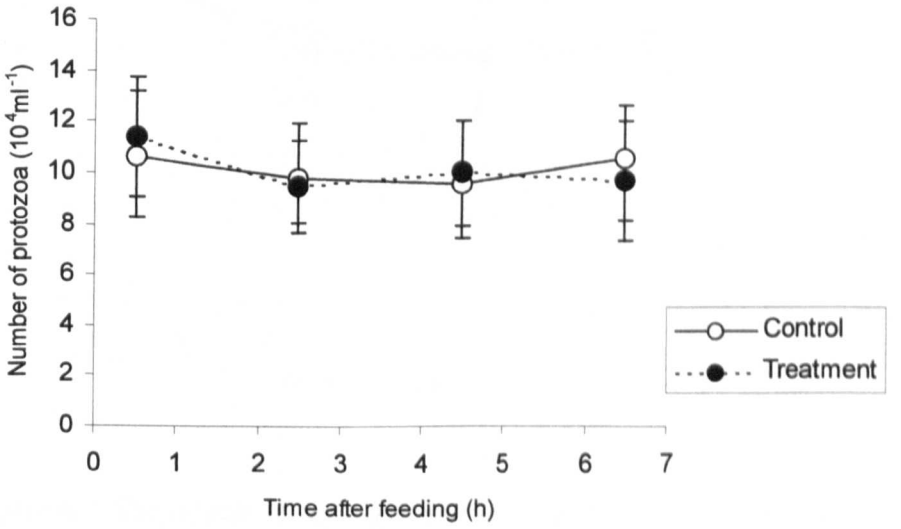


Figure 6.5. The effects of adding *S. rarak* on Entodiniomorphs in the rumen

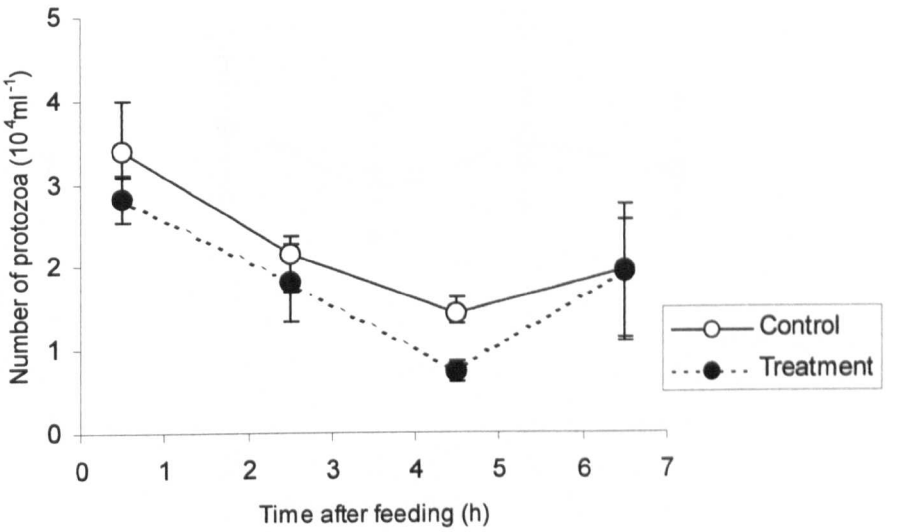


Figure 6.6. The effects of adding *S. rarak* on Holotrichs in the rumen

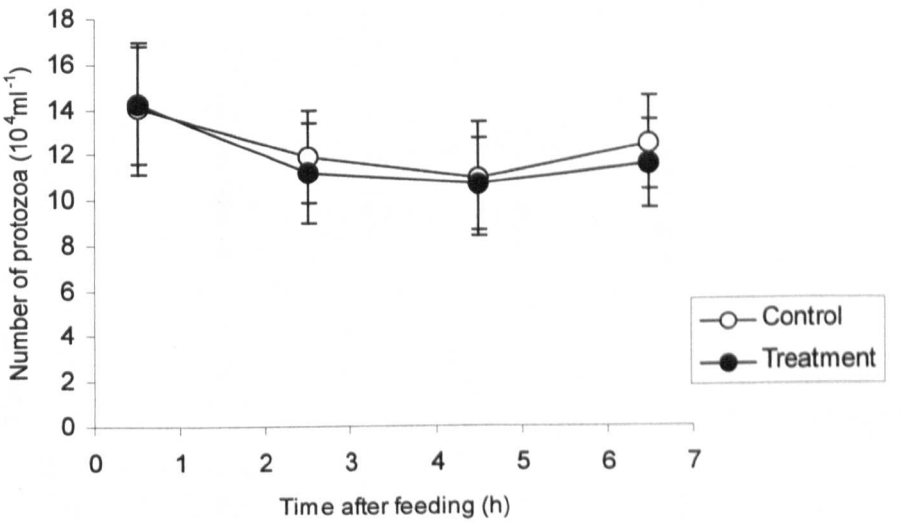


Figure 6.7. The effects of adding *S. rarak* on total ciliate protozoa in the rumen

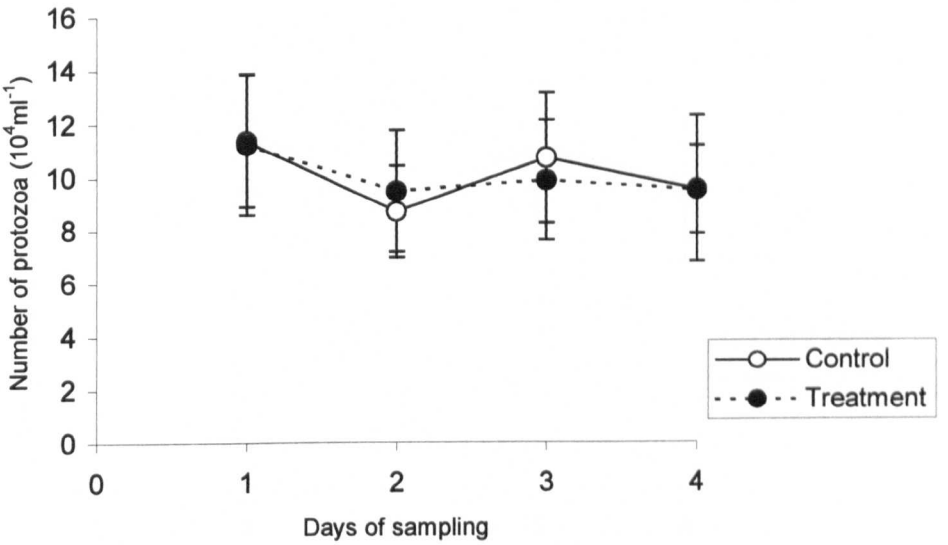


Figure 6.8. The effects of *S. rarak* added directly to the rumen on the number of Entodiniomorphs.

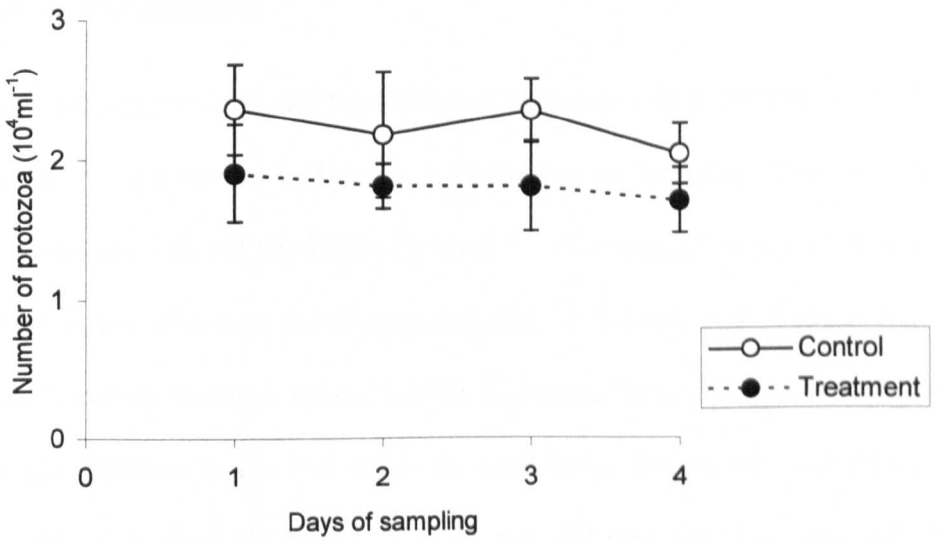


Figure 6.9. The effects of *S. rarak* added directly to the rumen on the number of Holotrichs.

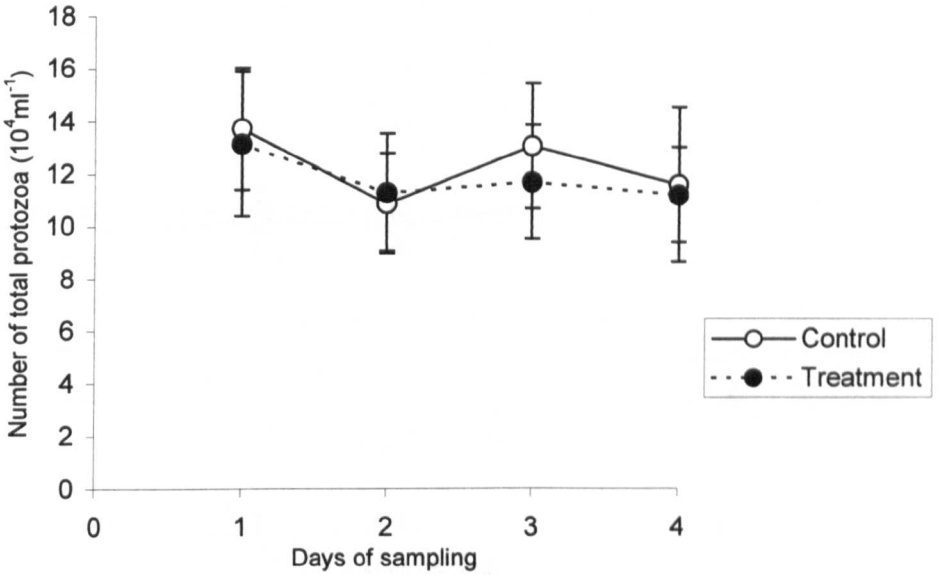


Figure 6.10. The effects of *S. rarak* added directly to the rumen on the total ciliate protozoa in the rumen.

Rumen Fermentation

Mean pH, ammonia N and total concentration and molar proportions of VFA in the rumen are summarised in Table 6.3. There was no treatment effect of addition of *S. rarak* on ruminal pH ($P>0.05$) (Figure 6.11). Rumen pH is one of the most critical determinants of rumen function as cellulolytic bacteria fail to grow below pH 6.0 and the rumen protozoa are completely eliminated at pH 5.5 and below. Meanwhile, a slight increase in ruminal pH is favourable for the growth and activity of these cellulolytic bacteria (Stewart, 1977). Rumen pH was not associated with any effect of diet on protozoa numbers and variation in the proportions of different species of protozoa in the rumen (Navas *et al.*, 1992).

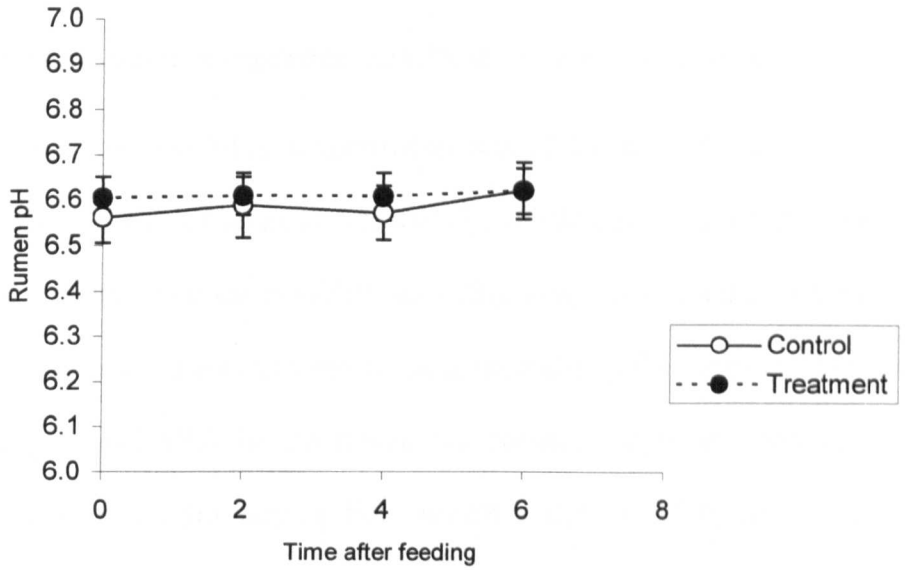


Figure 6.11. The effects of adding *S. rarak* on pH in the rumen

Rumen ammonia concentration was significantly ($P<0.01$) decreased by the addition of *S. rarak* compared with the control treatment (Table 6.2). *S. rarak* caused a 9.7% decrease in ammonia concentration. Similar results have been reported by Wallace *et al.* (1994) and Hussain and Cheeke (1995). The observed ammonia levels represent a balance between the processes of degradation of feed protein and the uptake of ammonia for the synthesis of microbial protein. A lower ammonia level could be due to higher incorporation of ammonia, peptide, or amino acids into microbial protein. The improved efficiency of utilization of nitrogen in the rumen could be due to binding of ammonia to saponin fractions (Cheeke, 1996) as shown by Yucca extract used to reduce the emission of ammonia from animal excreta. Yucca extract has ammonia-binding properties that results in ammonia in animal waste being bound in a non-volatile form. The selective effect of *S. rarak* on rumen protozoa could also

contribute to the lower level of ammonia in the rumen. It is possible that *S. rarak* could have a role in regulating the release of ammonia in the rumen.

The average total VFA concentration was 72.74 mmol l^{-1} and 71.74 mmol l^{-1} for control and treated animals respectively. McDonald *et al.* (2002) reported that the total concentration varies widely according to the animal's diet and the time that has elapsed since the previous meal, but is normally in the range of 70-150 mmol/litre. The principal VFA in the rumen are acetate, propionate, and butyrate and are produced in a ratio varying from approximately 75:15:10 to 40:40:20 (Bergman, 1990). The results showed that there was no difference in molar proportion of rumen acetate, butyrate or total VFA concentrations. However, the molar proportion of propionate was significantly increased ($P<0.01$) and the ratio of lipogenic to glycogenic VFA was significantly decreased ($P<0.001$). Increased molar proportions of propionic acid in the rumen are often found in studies with defaunated sheep (Williams and Coleman, 1992). As the single most important rumen bacterium involved in decarboxylation of succinate, *Selenomonas ruminantium* is apparently responsible for most of the propionate production in the rumen arising from the randomizing pathway (Wolin and Miller, 1988). Wallace *et al.* (1994) found that the growth of *S. ruminantium* was not affected by yucca saponins (YS), whereas growth of some other rumen bacterial species (*Streptococcus bovis* and *Butyrivibrio fibrisolvens*) was strongly inhibited. It is possible that, by inhibiting bacteria and protozoa not involved in propionate production in the rumen, the high levels of YS used in the experiment promoted species such as *S. ruminantium* to fill the niche, thereby increasing the accumulation of propionic acid in the rumen.

Table 6.2. Mean pH, ammonia N and mean total concentration and molar proportions of VFA in rumen fluid.

Parameters	Control	Treatment	SED	Significance
pH	6.63	6.60	0.022	NS
Ammonia (mM)	8.25	7.45	0.301	0.009
Acetate (mmol l^{-1}) (A)	55.86	54.49	0.903	NS
Propionate (mmol l^{-1}) (P)	9.14	9.75	0.194	0.002
Butyrate (mmol l^{-1}) (B)	6.86	6.89	0.386	NS
Total VFA (mmol l^{-1})	72.74	71.74	1.636	NS
Ratio (A+B)/P	7.91	7.08	0.232	<0.001

Microbial Synthesis

Two main sources of amino acids for ruminants are microbial protein synthesised in the rumen and ruminally undegraded dietary proteins (RUDP). Microbial proteins represents between 50 – 90% of the total proteins reaching the duodenum (Jouany *et al.*, 2000). The quantity of protein leaving the rumen is a major factor limiting the productivity of ruminant livestock production. The extent of digestion depends on its rate of digestion and on the time the feed spends in the organ. In this experiment, Cobalt ethylenediaminetetraacetic acid (Co-EDTA) as liquid phase marker was used to estimate both rumen outflow rate and rumen volume (Osuji *et al.*, 1993), while the natural alkane C_{33} was used as a marker for particulate digesta flow (Unal, 1998; Unal and Garnsworthy, 1999). Infusion of *S. rarak* intraruminally decreased the liquid outflow rate significantly ($P<0.05$) (Table 6.3), but not for particulate digesta flow ($P>0.05$). This indicates that 5 per cent of the rumen liquor flows out of the

rumen per hour. McDonald *et al.* (2002) reported that the typical flow rate of liquid from the rumen might be in the range of 0.05- 0.20.

Table 6.3. Effects of *S. rarak* on rumen outflow rate, rumen volume and digestibility

Parameters	Control	Treatment	SED	Significance
Rumen outflow rate				
Liquid outflow rate	0.058	0.051	0.001	0.037
DAPA (mg/g)	1.82	1.56	0.213	NS
Duodenal flow				
DM (kg/d)	6.89	6.93	0.443	NS
DAPA (g/d)	27.8	26.8	3.37	NS
Bacterial N (g/d)	541	534	42.7	NS
Duodenal concentrations				
C_{33} (mg/kg DM)	67.5	67.9	4.96	NS
DAPA (mg/g)	3.71	3.63	0.409	NS
Bacterial N (g/kg)	72.9	73.8	3.93	NS
Rumen volume (l)	82.3	74.6	8.5	NS
Digestibility coefficient	0.507	0.501	0.026	NS

Microbial protein synthesis, estimated using DAPA as a natural microbial marker, was not significantly affected by the addition of *S. rarak*. The absence of any effects might arise from insignificant effects of *S. rarak* towards total rumen ciliate population.

Fibre Degradation

The effect of *S. rarak* on fibre degradation is shown in Table 6.4. The addition of *S. rarak* significantly improved the degradation of NDF, with a tendency toward increased digestibility of ADF. Fibre degradation in the rumen is usually observed to

decrease in defaunated animals (William and Coleman, 1992). Rumen ciliate protozoa play a significant role in fibre digestibility since they secrete various enzymes which are responsible for breakdown of the plant cell wall polysaccharides. Since the addition of *S. rarak* in this experiment did not eliminate ciliate protozoa totally but selectively towards holotrichs, which primarily use soluble carbohydrates, resulted in an improved fibre digestion. Probably both the direct action of cellulase producing ciliates and the synergism with bacteria may contribute to the greater digestion of dietary fibre.

Table 6.4. Effects of *S. rarak* on fibre degradation in the rumen

Parameters	Control	Treatment	SED	Significance
NDF (%)	36.98	46.57	1.90	0.037
ADF (%)	9.75	11.69	1.58	NS

The results of this experiment could imply that for those animals fed high fibre diets, controlling rumen protozoa by partial defaunation not only decrease the negative effect but also increase the positive effect of protozoa. Partial defaunation could overcomes the difficulty of complete defaunation, because the defaunation agent could be easily added to the diet.

6.5. Conclusion

The relative antiprotozoal activity shown by S. rarak *in vitro* (Chapter 4) are not necessarily *in vivo*. Part of this discrepancy might arise from extrapolation of *in vitro* concentration to *in vivo* concentration. With the intention of maintaining the concentration of antiprotozoal factor in the rumen environment throughout the day

of experiment, the daily dose of *S. rarak* was partitioned into two equal meals. Addition of 50 g *S. rarak* in a 100 l rumen volume with an average passage rate 5%/h, the initial 0.5 g/l concentration would be 0.35 g/l after 6 h, just before the next feeding. This is much less that what was demonstrated as an effective concentration *in vitro* 1 g/l. Moreover, the rumen outflow rate cannot be assumed to be constant, so that the concentration of antiprotozoal factor in the rumen content required for the effective activity towards rumen protozoa might not be maintained. It is also possible that significant antiprotozoal activity of the plants might be evident only after the rumen contents are saturated with the antiprotozoal factor. Degradation of antiprotozoal factor by rumen bacteria is another possibility. The microbial adaptation was not determined in the present experiment. Teferedegne (2000) indicated that rumen bacteria could develop a capability in the rumen to degrade the antiprotozoal agent from *Sesbania sesban,* although this microbial adaptation phenomenon was not universal as some other saponin-containing plants had a persistent antiprotozoal effect. It is then suggested that the nature and the rate of degradation of saponins in the rumen is important.

In conclusion, Sapindus rarak, despite showing antiprotozoal activity *in vitro,* did not decrease protozoal numbers *in vivo.* However, there were some indicators of selective activity towards holotrichs. Changes in propionate concentration in the rumen and lack of change in microbial/flow parameters suggest no adverse effects on other fermentation measures.

CHAPTER 7

GENERAL DISCUSSION

7.1. Research Findings

The major objectives of the studies described here were to evaluate if *Sapindus rarak* contained natural factors that were antiprotozoal and therefore potentially able to defaunate the rumen, either partially or completely, without having a detrimental effect on bacterial population and hence enhancing rumen productivity. This project focussed on determination of antiprotozoal activity (Chapter 3 and 4) from some plants containing saponins, antibacterial determination (Chapter 5) and the general effects on rumen fermentation, microbial protein synthesis and fibre digestion (Chapter 6).

7.1.1. Effects on rumen protozoa

Many attempts have been made to alter the patterns of digestion to improve the nutrition of ruminants. The primary approach has been to modify the microbial population in order to suppress undesirable processes or stimulate desirable processes. Changing the bacterial population through the introduction of specific organisms has generally proved difficult to achieve or, if achieved, has failed to yield nutritional benefits. Changing an existing population by adding antibiotics to feeds has proved more effective, although the use of many antibiotics has been prohibited because of their value for treating diseases in animals and man, and the possibility that their wider application would lead to the evolution of resistant strains of disease-causing organisms. Another recent development has been the

use of probiotics, such as live yeast cultures, to stimulate bacterial activity in the rumen. However, the rumen protozoal population has proved to be more susceptible to modification than the bacterial population, principally because protozoa can be totally eliminated from the rumen (McDonald *et al.*, 2002) and defaunation experiments established many years ago that protozoa are not essential to the host animals (Eadie and Gill, 1971; Veira, 1986). The contribution of protozoa to rumen digestion, and hence to the nutrition and productivity of ruminants, has long been a matter of controversy. Although protozoa make a significant contribution to the digestion of starch and soluble sugars that could prevent the alternative rapid bacterial fermentation to lactic acid, and their stimulation of cellulolytic activity by actively metabolising lactate and by stabilising rumen pH, they tend to be retained in the rumen and thus have the undesirable effect of recycling microbial protein in the rumen and preventing its passage to the small intestine. Protozoa are often attached to large food particles, which could prevent them leaving the rumen in the liquid phase. The tendency of ciliate protozoa to be retained in the rumen may be a condition for their survival. Harrison and McAllan (1980) found that while the average division time of protozoal cells may be 24 h, the rumen retention of fluid is considerably less than 24 h and often less than 10 h. If protozoa were leaving at the same rate as rumen fluid, their survival in the rumen would be at high risk. Previous studies on methods and the effects of defaunation on ruminal fermentation and animal performance are discussed in Chapter 2. Scientists have recently become interested in evaluating alternative means for manipulating gastrointestinal microflora, especially in exploiting natural products as feed

additives to solve problems in animal nutrition and livestock production (Wallace *et al.*, 2002).

The quantity of protein leaving the rumen is a major factor limiting the productivity of ruminant livestock production. Microbial protein flow depends on the efficiency of microbial protein synthesis and the extent to which microbial protein breaks down before it leaves the rumen. In the rumen, however, bacterial breakdown is initiated mainly by exogenous factors, principally ciliate protozoa (Wallace and McPherson, 1987). Therefore, avoiding the engulfment and digestion of bacteria cells by protozoa would have the greatest impact on microbial flow from the rumen and hence could improve feed efficiency.

Recently, there has been increased interest in saponin-containing plants as a potential means for achieving the suppression of rumen ciliate populations (Navas-Camacho *et al.*, 1993; Diaz *et al.*, 1993; Newbold *et al.*, 1997; Odenyo *et al.*, 1997). Saponins have pronounced antiprotozoal activity. The activity of saponins toward rumen ciliates was determined by direct counting of protozoal population density microscopically (Chapter 3). The other method employed to determine antiprotozoal activity was based on the use of radioisotopes (Chapter 5). Antiprotozoal activity was measured by the breakdown of ^{14}C-leucine-labelled *Selenomonas ruminantium in vitro* as described by Wallace and McPherson (1987) and Wallace and Newbold (1991). This technique has been proved for detection of bacteriolysis by rumen protozoa.

Results from both techniques confirmed that *S. rarak* has the potential to suppress rumen protozoa. *S. rarak* is well known for the high saponin content of its fruits. The mechanism for the antiprotozoal effects is that saponins form irreversible complexes with cholesterol in the protozoal cell membrane, causing

breakdown of the membrane, cell lysis, and death (Cheeke, 1999). Saponins may interact with the polar heads of membrane phospholipids and the -OH group of cholesterol through OH groups at C3 or C28, which will result in their later ability to form micelle-like aggregates. Moreover, their hydrophobic aglycone backbone could intercalate into the hydrophobic interior of the bilayer. Both of these effects may contribute to the alteration of the lipid environment around membrane proteins (Francis *et al.*, 2002). The antiprotozoal activity requires an intact saponin structure with both the nucleus and side chains present because saponins were found to be toxic to rumen protozoa, but aglycone sapogenins were non-toxic (Teferedgne, 2000). However, the antiprotozoal property of saponins may be lost upon deglycosylation (Wang *et al.*, 2000). Ruminal protozoa are unable to adapt to or detoxify saponins (Newbold *et al.*, 1997), but rumen bacteria may be able to adapt and metabolise saponins. Saponins are hydrolyzed by ruminal bacteria that remove the carbohydrate side chains (Makkar and Becker, 1997; Wang *et al.*, 1998). Different glycosidase enzymes are involved in the hydrolysis of saponins. The kinds of hydrolase enzyme involved in the deglycosylation of saponins are determined by the type and anomeric configuration (α or β) of the last monosaccharides in the sequence of the carbohydrate moiety that attaches to the aglycone of saponins (Hostettmann and Marston, 1995). If cleavage of the sugar moiety causes detoxification of saponins, the rates of detoxification may depend on the configuration of the specific saponins and the abundance and the activity of the corresponding glycosidases with a specificity enabling hydrolysis of the saponins in the rumen. Four monodesmosidic triglycosides, bearing up to two acetyl moieties on the terminal sugar, have been isolated from the pericarp of fruit of *S. rarak*. Acid

hydrolysis of the methanolic extract yielded hederagenin as the triterpene moiety (Hamburger *et al.*, 1992). The persistency of antiprotozoal activity of *S. rarak* has not been examined.

7.1.2. Effects on rumen bacteria

Saponins have been shown to affect ruminal bacteria. Newbold *et al.* (1997) reported that bacterial numbers increased when foliage of *Sesbania sesban* was introduced into the diet, presumably as a consequence of the suppression of protozoal population density which apparently associated with the fraction of the plant containing saponins. A similar trend was found with *Yucca schidighera* extract (Valdez et al., 1986). However, steroidal saponin from *Y. schidighera* had no effect on total or cellulolytic bacteria count in Rusitec (Wang *et al.*, 1998).

Because dietary saponins are poorly absorbed, their biological effects occur in the digestive tract (Cheeke, 1996). Although the most obvious effect of saponins on ruminal microbes is the suppression of protozoa, pure culture studies also indicate possible antibacterial effects of saponins (Chapter 5). The methanolic extract of saponin from *S. rarak* had no effect on growth of non-cellulolytic bacteria. However, in this study it affected cellulolytic bacteria, by prolonging the lag phase following inoculation of *Streptococcus bovis* or abolishing the growth of *Butyrifibrio fibrisolvens*, *Ruminococcos albus*, and *Ruminococcos flavefaciens*. It seems that antibacterial properties were more pronounced with gram-positive bacteria; similar to the action of ionophores (McGuffey *et al.*, 2001). The resistance of gram-negative bacteria appears to be due to the presence of an outer membrane that is impermeable to large molecules (Russell and Strobel, 1989). Therefore, the mode of action of antibacterial effects of saponins

might involve membranolytic properties, rather than simply altering the surface tension of the extracellular medium (Killeen *et al.*, 1998).

7.1.3. Effects on rumen fermentation

Maximising ruminant productivity involves meeting the nutrient requirements for both rumen microbial metabolism and mammalian metabolism in tissues. Any alteration of rumen fermentation will result in a series of interrelated effects, some positive and others negative, because rumen fermentation can be described as an integrated system consisting of an interactive network of reactions (Van Navel and Demeyer, 1988). The main objective in controlling rumen fermentation is to restrict the activity of microbes to the components of the diet that the host animal cannot digest with its own enzymes or cannot utilise without microbial intervention, hence improving the efficiency of feed utilisation and increase ruminant productivity (Nagaraja *et al.*, 1997).

Despite showing antiprotozoal activity *in vitro*, direct administration of *S. rarak* into the rumen did not decrease protozoal numbers *in vivo* (Chapter 6), possibly because the dosage was insufficient. However, there were some indicators of selective activity towards holotrichs. Changes in propionate concentration in the rumen and lack of change in microbial flow parameters suggest no adverse effects on other fermentation measures. Increased production of propionate is beneficial to the animal by affecting the capture of fermentation energy in the rumen.

S. rarak caused a decrease in rumen ammonia concentration. The observed ammonia levels represent a balance between the processes of degradation of feed protein and the uptake of ammonia for the synthesis of microbial protein. The

lower ammonia levels could be due to greater incorporation of ammonia, peptides, or amino acids into microbial protein, which would be expected if defaunation were occurring. The improved efficiency of nitrogen utilization in the rumen could also be due to binding of ammonia to saponin fractions in *S. rarak*. It is possible, therefore, that *S. rarak* could have a role in regulating the release of ammonia in the rumen. This could be a great advantage when ruminants are fed low-protein diets.

7.1.4. Conclusion and implications

In this study *Sapindus rarak* showed a great potential for suppressing rumen ciliate populations, whilst generally exerting negligible effects on other aspects of rumen fermentation.

The incorporation of saponins into ruminant diets, in particular roughage-based diets, might be advantageous, as it would lead to a higher microbial yield and lower emissions of environmental-polluting gases (CO_2 and CH_4).

7.2. Future Research

Further research will be required to identify the chemical nature of saponins found in *S. rarak*. The elucidation of the structural nature of the antiprotozoal factor is equally important for the investigation of its susceptibility to microbial degradation and to design a method of protection. The identification of an antiprotozoal factor that is not degradable in the rumen would be desirable.

Reducing the protozoal population of the rumen could enhance daily gain and improve feed efficiency without adversely affecting organic matter degradation in the rumen. Any benefit in ruminal protein metabolism arising from reduced

protozoal numbers may be negated by a reduction in bacterial growth and nutrient digestion. Consequently, the antiprotozoal properties of these compounds should be studied in conjunction with their effects on ruminal fermentation to ensure that their inclusion in the diet will have a net benefit on rumen metabolism. A dose-response study *in vivo* would be necessary to determine the correct dose to use. The efficacy of the compounds in long-term applications would be required to identify the potential ruminal adaptation which could lead to inactivation of the plant constituents.

Other plant secondary compounds, beside saponins, might be useful in the same way. Essential oils, alkaloids, phenolics, and glycosides, which also provide defensive functions in plants, are potential as natural manipulators of rumen fermentation. Useful effects of essential oils have been demonstrated against pathogenic bacteria, decreased the rate of NH3 production from amino acids in ruminal fluid taken from sheep and cattle, yet proteinase and peptidase activities were unchanged (Wallace *et al.*, 2002). Alkaloids, heterocyclic nitrogen compounds, are an internal part of many medicinal plants and have been found to have antimicrobial properties (Roberts and Strack, 1999). The effects of some polyphenolic compounds on some pathogenic microorganisms found in the gastro intestinal tract may be of benefit to animals. Flavonoids, hydroxylated phenolic substances known to be synthesized by plants in response to microbial infection, have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble protein and to complex with bacterial cell wall (Cowan, 1999). Glycosides are plant secondary metabolites containing component linked by an ether bond to a noncarbohydrate aglycone.

The aglycone is released by enzymatic action when the plant tissue is damaged, as by wilting, freezing, mastication or trampling. Some of the glycosides of most important in human and animal nutrition include glucosinolates, cyanogenic glycosides, cardiac glycosides, glycoalkaloids and saponins (Cheeke, 1998).

Another important area for investigation is the effects of saponins and other plant secondary compounds on animal performance, animal products and the safety of their application to the animals themselves and to the consumers who eat the products.

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