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Studies on the Stability of Rumen Metabolism in Sheep and the Effects of Dietary
Addition of Yeast Culture

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A thesis submitted to the Faculty of Science of the University of Glasgow
to fulfill requirements for the award of the Degree of Doctor of Philosophy

JAMES ARNOLD HUNTINGTON

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And now I will show you the best way of all.

I may speak in tongues of men or of angels, but if I am without love, I am a sounding of gongs or a clanging cymbal. I may have the gift of prophecy, and know every hidden truth; I may have faith strong enough to move mountains; but if I have no love, I am nothing. I may dole out all I possess, or even give my body to be burnt, but if I have no love, I am none the better.

Love is patient; love is kind and envies no one. Love is never boastful, nor conceited, nor rude; never selfish, not quick to take offence. Love keeps no score of wrongs; does not gloat over other men's sins, but delights in the truth. There is nothing love cannot face; there is no limit to its faith, its hope and its endurance.

Love will never come to an end. Are there prophets ? their work will be over. Are there tongues of ecstasy ? they will cease. Is there knowledge ? it will vanish away; for our knowledge and our prophecy alike are partial, and the partial vanishes when wholeness comes. When I was a child, my speech, my outlook, and my thoughts were all childish. When I grew up, I had finished with childish things. Now we see only puzzling reflections in a mirror, but then we shall see face to face. My knowledge now is partial; then it will be whole, like God's knowledge of me. In a word, there are three things that last for ever: faith, hope, and love; but the greatest of them all is love.

(Taken from: The new English Bible; The first letter of Paul to the Corinthians; Chapter 13.)

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LIST OF ABBREVIATIONS

ACW	Absorbent cotton wool
ADF	Acid detergent fibre
Ao	<i>Aspergillus oryzae</i>
A:P	Acetate: propionate ratio
CP	Crude protein
CV%	Coefficient of variation
Dg	Digestibility
DMI	Dry matter intake
DMD	Dry matter digestibility
FCE	Feed conversion efficiency
FPr	Frequency predictor
FW	Fresh weight
EMNS	Efficiency of microbial nitrogen synthesis
Hrs	Hours
ISDMD	<i>in sacco</i> dry matter disappearance
IVOMD	<i>in vitro</i> organic matter digestibility
IYC	Irradiated yeast culture
LDR	Liquid dilution rate
LWG	Live weight gain
ME	Metabolisable energy
MP	Microbial protein
N	Nitrogen
NAN	Non-ammonia nitrogen
OM	Organic matter

OMD	Organic matter digestibility
PAS	Pot ale syrup
r	Coefficient of correlation
RFC	Readily fermented carbohydrates
SD	Standard deviation
SED	Standard error of difference
VFA	Volatile fatty acid
t[VFA]	Total volatile fatty acid concentration
p[VFA]	Proportions of the individual volatile fatty acids
Vit	Vitamin
YC	Yeast culture

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SUMMARY

Literature concerning the use of feed additives for animal production were reviewed. Special emphasis was given to the use of probiotics in animal production and especially to the use of fungal probiotics in ruminants.

The first experiment examined the ruminal responses to additional yeast culture (YC) and irradiated yeast culture (IYC). Six rumen fistulated wethers were randomly allocated to two 3 x 3 Latin squares. Both additives were found to alter the diurnal pattern of molar % propionate and ruminal ethanol concentration in comparison to the control diet. Supplementation with either YC or IYC did not affect diet organic matter digestibility (OMD), 24 hr. *in sacco* dry matter degradability (ISDMD), *in vitro* organic matter degradability (IVOMD), the efficiency of microbial protein synthesis (EMPS), or ruminal ammonia concentration, pH, and total volatile fatty acid concentration (t[VFA]). It was evident from the results of experiment 1 that substantial inherent daily variation existed in the rumen, as shown by the *in sacco* degradability measurements. Recovery of viable yeast from the rumen and faeces suggested that the viable yeast supplement had a limited ability to survive in the rumen and pass into the abomasum and intestines.

A short *in vitro* experiment was then described which showed that the IYC supplement was not metabolically active, which indicated that the YC supplement may not need to be viable and metabolically active in order to induce a ruminal response.

The method of urine collection for the determination of urinary purine derivatives was investigated and the results described in the third experimental chapter suggested that good hygiene and immediate sample freezing (-20°C) was required for satisfactory sample preservation without losses.

The fourth experimental chapter described the results of a time series experiment designed with two main aims. The first was to investigate rumen stability and the second to determine the effects of additional YC on rumen metabolism. Three rumen fistulated wethers were kept in digestibility crates continuously for 8 weeks. Pre-washing the soyahulls used as the *in sacco* substrate had no effect on the daily variation observed for the 24 hr. ISDMD. Long and short-term trends were observed in all rumen parameters. The addition of YC to the control diet was noted to correspond to a change in the trend of urinary allantoin excretion, t[VFA] and the

molar % propionate, suggesting that the additional YC stimulated an increase in the proportion of propionate in the VFA mix. Trend changes were also observed for molar % acetate and ammonia concentration but these did not correspond to the addition of YC. No trend changes were observed for ruminal pH or diet OMD.

The effect of additional YC on two contrasting diets was then investigated using 4 fistulated wethers randomly allocated to a 4 x 4 latin square design. No diet x YC interaction was observed, but supplementary YC was noted to increase ruminal molar % acetate and decrease the concentration and molar proportion of propionate. YC supplementation of the control diet was not found to affect the rate of liquid dilution (LDR) in either the rumen or hindgut. Ruminal pH, ammonia concentration and t[VFA] were also unaffected by supplemental YC. The *in sacco* technique was capable of detecting changes in diet but was unable to detect changes in rumen function due to additional YC. Recovery of viable YC from the rumen indicated that, following an oral dose of YC, yeast numbers declined at a faster rate than rumen LDR, indicating that no significant yeast replication occurs in the rumen. Faecal yeast counts showed that significant numbers of yeast cells survived passage through the gastro-intestinal tract.

Long term rumen stability was examined by recording daily fluctuations in pH, VFA, NH₃ and *in sacco* in sheep fed a constant diet for 18 weeks. Six fistulated wethers were used, three fed an all hay diet and three fed a mixed barley, maize and hay diet. The results showed that a substantial daily variation existed in all rumen parameters. The CV% for ISDMD_{HAY} was found to be greater on the mixed diet when compared with the all hay diet. The mixture of VFA was also found to vary between consecutive days.

The effect of additional YC, IYC and pot ale syrup (PAS) on rumen metabolism was investigated using 4 rumen fistulated wethers in a 4 x 4 latin square design experiment. In contrast to the previous animal experiments ISDMD measurements were determined by incubating bags for different incubation lengths (0, 8, 16 and 24 hrs.). Ten ISDMD degradability slopes were determined in each 21 day experimental period for both hay and fishmeal. Additional YC reduced 24 h. degradability of fishmeal. Supplementary PAS was found to have an opposite effect on the curve constants fitted to the fishmeal degradability curves, but the curve constants estimated for the control treatment were not found to be significantly different from any other treatment. Supplementation of the control diet with YC, IYC or PAS did not effect ISDMD_{HAY}. Consecutive degradability slopes for both hay and fishmeal were found to vary significantly within an experimental period. Additional YC and

IYC increased t[VFA] due to non-significant increases in acetate and propionate. YC supplementation also increased ruminal ammonia concentration. Urinary allantoin excretion, rumen LDR, and VFA mix were unaffected by additional YC, IYC or PAS. The ability of YC to survive in the rumen and pass throughout the gut was also demonstrated.

An *in vitro* experiment examined the effects of YC, IYC and n-acetyl glucosamine on the growth patterns of individual rumen bacteria. YC supplements manufacture in three different years were tested. Responses to supplementation both within and between individual bacteria were found to vary for the different YC, IYC and n-AG supplements. The results suggested that each supplement may have a bacteria-specific effect.

Dietary addition of YC was found to affect some aspects of rumen metabolism in every animal experiment, but the effects were not found to be consistent between experiments. Similar but smaller effects to those observed for the YC treatment were noted when the control diet was supplemented with IYC. It was theorised that the YC supplement was not required to be either metabolically or reproductively active and that its mode of action was bacteria-specific. Hence the effects of supplementation observed *in vivo* are dependant on the nutrition, biochemistry and physiological state of the individual bacterial species at the time of supplementation. Experimental evidence also suggested that the basal medium of the YC supplement was exerting an effect on bacterial growth, since the responses of the individual rumen bacteria supplemented with either IYC (containing the basal medium) or isolated yeast cells were different. It was therefore postulated that the basal medium may be facilitating the greatest effect and the yeast cells a lesser effect.

Substantial variation was observed in *in sacco* dry matter disappearance (ISDMD). The mean coefficient of variation (CV%) was calculated for nylon bags incubated in different sheep on the same day, same sheep different days and between bags incubated in the same rumen on the same day, for two diet types (all hay and mixed hay concentrates) were found to be: Hay: 3.13, 9.19, 6.88; Mixed: 5.53, 12.81, 12.07 respectively. Further calculations showed that one bag incubated in the rumen of three sheep over two days would be sufficient to detect a significant change ($P < 0.05$) of 10% in mean ISDMD when the sheep were fed an all hay diet or a mixed hay concentrate diet.

The CV% for the daily variation in rumen total volatile fatty acid concentration (t[VFA]) (CV%: Hay 27.94; Mixed 24.19), pH (CV%: Hay 2.44; Mixed 4.93), and ammonia concentration (CV% Hay 35.58; Mixed 23.68) were also calculated. Variation was also noted

in the daily molar proportions of the individual VFA and between consecutive ISDMD degradability curves. These results indicated that inherent rumen variation may vary with diet type and it was theorised that the factors influencing rumen fermentation dynamics included psychological stress, environmental temperature and changes in the eating, drinking and ruminative behaviour of the individual animals. The substantial variation that exists between consecutive days emphasises the need for robust experimental designs such as the "Latin square design". It was also recommended that if a "switch-back design" was used that all animals used in the experiment should not follow the same treatment pattern.

CHAPTER 1: Literature review

1.1: Rumen Metabolism

The rumen metabolism has been reviewed extensively by Hungate (1966) and more recently by Ørskov (1982), Hobson (1988) and Ørskov and Ryle (1990). This review summarises rumen metabolism concerning the areas relevant to the research project under-taken.

1.1.1: Microbe-Microbe Interactions

The rumen contents contains approx. 10^{10} - 10^{11} bacteria/ml, 10^5 - 10^6 protozoa/ml and an unknown number of rumen anaerobic fungi that constitute approx. 8% of the rumen microbial biomass (Ørskov and Ryle, 1990). Although the total count of the protozoa is less than that of the bacteria, they have an equivalent biomass in the rumen due to their larger size. Russell and Hespell (1981) in their review of microbial rumen fermentation discuss several types of ecological interrelationships are that possible between the different microbial populations of the rumen including: neutralism, commensalism, mutualistic, competitive, amensalism and predation.

Neutralism:	Neutralism exists where two species have no effect on one another.
Commensalism:	This describes the relationship that exists when the growth of one species is promoted by the presence of a second species, but the growth of the second is unaffected by the presence of the first.
Mutualistic:	Describes the relationship where the growth of both interacting species is enhanced by the presence of the other.
Competitive:	If two species are dependant on the on the same limiting nutrient, a state of competition exists.
Amensal:	When one species produces a product toxic to other species an amensal relationship is possible.
Predation:	If one species can consume another predation can occur.

The dynamics of rumen metabolism are dependant on the interrelationships described above and ultimately these interrelationships are determined by the nutrition, biochemistry and physiology of individual rumen microbial species (Russell and Hespell, 1981).

1.1.2: Host-Microbe Interactions

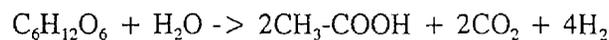
The host animal provides the microbial ecosystem with an environment in which the temperature, anaerobic state and pH levels are maintained within fairly strict limits. The microbiota are also provided with fresh substrate at regular intervals as a result of feeding behaviour and continuous ruminal secretions of recycled urea.

The microbiota provide the host with energy and protein sufficient for growth and production under extensive feeding systems.

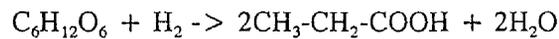
1.1.3: The contribution of rumen microbiota to host energy supply

The production of energy utilisable by the host results from the fermentative degradation of plant fibre and sugars. Plant fibre is a complex of cellulose, hemicellulose and lignin, and contains many β linked polymers of hexoses and pentoses. The enzymes required to break these bonds are not found in mammals or birds. Herbivorous animals are therefore dependent on the microbial degradation of these compounds. Fermentation takes place under strictly anaerobic conditions and results in the production of substances that are not fully oxidised. The major end products of fibre degradation are the volatile fatty acids (VFA) acetic, propionic and butyric acid, plus carbon dioxide and methane. The stoichiometry of the fermentation of hexose to these compounds is shown below.

Acetic acid



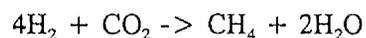
Propionic acid



Butyric acid



Methane



The volatile fatty acids (VFA) are absorbed through the rumen epithelium into the host's portal blood system and may provide a major source of energy while undergoing further oxidative degradation. Since no enzymes have yet been discovered that can degrade methane, the concomitant production of methane results in a major energy loss to the host and rumen ecosystem. The production of propionate is more energetically efficient than the other acids because the production of 2 moles of propionate from 1 mole of hexose requires additional hydrogen that may otherwise have been lost as methane.

1.1.4: Microbial protein production

The microbiota can utilise ammonia, peptides and amino acids for the production of their cellular protein. Under extensive farming practices, microbial turnover in the rumen and the wash-out of microbial cells and debris can provide sufficient protein for animal growth and production. Even under moderately intensive systems, no true protein need be fed to the host so long as the microbial population is not deficient in ammonia. Thus the microbial population provides a way of upgrading the biological value of nitrogen sources that otherwise would not be available to the host. However, the converse is also the case as protein supplements of high biological value may be downgraded as a result of microbial degradation. Much research effort has been aimed at maximising the amount of undegraded protein leaving the rumen.

1.2: Factors affecting microbial growth in the rumen.

1.2.1: Diet type

The chemical composition and physical nature of the diet can have large effects on the microbial ecosystem. The end products of diets containing a high proportion of slowly fermented fibre have been shown to be predominantly acetate (Blaxter, 1962 and Ørskov, 1975). The inclusion of readily fermentable carbohydrate (RFC) into the diet may modify the stoichiometry of fermentation in favour of propionic and butyric acid. The inclusion of lipids at a concentration greater than 7% of the diet may lead to the inhibition of cellulolysis. This may result from a toxic effect of the lipids on the microbiota and/or due to the coating of degradation sites on the fibrous material (Ørskov and Ryle 1990).

The physical nature of the diet has important consequences for ruminal degradation. Diets containing material with a large particle size ferment slowly and require much rumination over long periods and an increased saliva flow. This results in an elevated rumen pH cf. diets with a small particle size and high density which are consumed more rapidly, ferment quickly and require less rumination. Consequently, less saliva is produced whilst VFA production rates are high and the rumen pH is depressed. Although the digestibility of the two diets types may be similar the end products of fermentation would be different.

Frequency of feeding may also affect fermentation processes of the rumen. A diet fed frequently in small quantities would be associated with a more stable fermentation than a diet fed in one large meal.

1.2.2: Rumen pH

Mould and Ørskov (1983) and Mould *et al* (1983) demonstrated that when rumen pH was reduced to 6.0-6.1, cellulolysis was inhibited and ceased when the pH fell below 6.0. The duration for which the rumen pH was depressed was shown to be an important factor in the inhibition of cellulolysis by Istasse and Ørskov (1983). However, some of the amylolytic bacteria are more pH resistant (eg. *Streptococcus bovis*) and may continue to ferment starch at pH levels down to 5.0. At this pH rumenitis may occur, which in cattle may lead to liver abscesses (Fell *et al.*, 1968).

1.2.3: Associative effects.

These effects are observed when two or more feed types are fed in combination. The resulting nutritional value of the diet is different from the sum of the individual components (Ørskov and Ryle, 1990).

a) Negative associative effects.

The addition of RFC to a fibrous diet may depress cellulolysis in two ways. The rapid degradation of the RFC results in a drop in rumen pH which may inhibit microbial action ("pH effect"). The bacteria and protozoa may also show a preferential sequestration of the RFC thus reducing their cellulolytic activity. This has been termed the "carbohydrate effect" (Mould *et al.*, 1983).

b) Positive associative effects.

These effects have been observed on a wide range of diets. Manyuchi *et al.* (1991), noted an increase in untreated straw intake and degradation when the diet was supplemented with either 200 or 400g/d of ammonia-treated straw. The positive effect of including fish meal in the diets of high yielding dairy cows has been discussed by Pike and Esselmont (1991). Thomas *et al.* (1989), observed improvements in milk yield and composition when the protein content of their high-silage diet was increased. A further positive association of dietary protein content and supplemental isoacids was also noted during this experiment. These responses in animal performance may result from increased nutrient availability to both the ruminal microbes and the host animal.

1.3: Manipulation of the rumen ecosystem.

The manipulation of the rumen environment in order to maximise the efficiency of animal production has long been the aim of researchers and producers. A variety of approaches has been employed including the alteration of the chemical and physical nature of the diet and the use of supplements.

1.3.1: Supplemental vitamins

Improved animal performance has been observed in animals supplemented with niacin, thiamine, Vit A and other vitamins. As the rumen microbes are capable both of limited *de novo* synthesis and recycling of vitamins, responses are only noted when the animal's demand for vitamins is greater than that which can be synthesised or recycled in the rumen. For example, during periods of high rates of production such as early lactation.

Supplementation of *in vitro* cultures with niacin or thiamine have been shown to increase the concentration of microbial protein and molar proportion of propionate (Riddell *et al.*, 1981 and Candau and Kone, 1980). Jaster *et al.* (1983), observed increased milk yields in the first 10 weeks of lactation in heifers when they supplemented the control diet with 6g of niacin. They related the increased milk yields to a reduction of subclinical ketosis which agrees with the findings of Fronz and Schultz (1979), who demonstrated that supplemental niacin could alleviate ketosis in dairy cattle. The inconsistency in the results of animal trials and the efficacy of supplemental growth factors may be related to whether the rumen microbial population was sufficient or deficient during the trial. In practise it is unlikely that in a well managed animal production system that these factors would become limiting.

1.3.2: The use of Dietary Buffers

The buffers tetrasodium pyrophosphate (Thomas and Hall 1984), limestone (Ha *et al.* 1983), sodium bentonite and sodium bicarbonate (Esdale and Satter, 1972; Huntington *et al.*, 1977; Ha *et al.*, 1983 and Thomas and Hall 1984), have all been used as in feed additives for ruminant diets.

Buffers are usually added to the diet in order to negate the "negative associative effects" noted when a diet containing a high proportion of RFC is fed. Ha *et al.* (1983), noted that dietary buffers had no effect on either rumen or blood parameters, but a significant increase ($P < 0.05$) in the amount of organic matter, crude protein, N free extract and starch digested in the rumen was observed. Increased feed intake when the diet was supplemented with sodium bicarbonate was indicated by Adams *et al.* (1981). However, Huntington *et al.* (1977) and Thomas and Hall (1984) observed that the effect of the buffers was most prevalent during the first few weeks of supplementation. Prolonged supplementation was shown to increase the incidence of urinary calculi Huntington *et al.* (1977).

The use of dietary buffers would appear to be most useful during periods of dietary stress for the animals and/or the rumen microbes. An example is the newly calved cow which changes rapidly from a diet containing a high proportion of forage to one containing a high proportion of concentrate.

1.3.3: Antibiotic supplements

Antibiotic supplements became popular when antibiotics became readily available post World War II and it was noted that their inclusion in animal rations had growth promoting effects. However, in 1969 the Swann committee limited the antibiotics that could be used as supplements to those that were not used clinically. This was done due to public fear of residues in animal products and the development of antibiotic resistant microbial strains. The antibiotic supplements that are currently available for use are reviewed below.

A) Non-ionophore antibiotics

Avoparcin, has been the most studied antibiotic in this category (Cuthbert and Thickett, 1984). Other non-ionophore antibiotics that have been investigated include virginiamycin (Hedde *et al.* 1980), bacitracin, capreomycin, novobiacin, oxamycin, erythromycin, hygromycin B, and tylosin (Beede and Farlin 1977a).

This class of antibiotics has been shown to inhibit the growth of the rumen Gram [+] bacteria, resulting in a selection for the Gram [-] rumen bacteria (Stewart *et al.*, 1983 and Stewart and Duncan 1985). Associated with these shifts in population are shifts in fermentation

stoichiometry in favour of propionate and a reduction in methane production (Beede and Farlin, 1977b; Froetschel *et al.*, 1983 and MacGregor and Armstrong, 1983).

This class of antibiotic has also been shown to inhibit the growth of *S. bovis* resulting in a reduced incidence of lactic acid acidosis in sheep due to a reduced ruminal lactate concentration (Beede and Farlin 1977b).

Stewart *et al.* (1979), observed that avoparcin was able to shift the balance of the cellulolytic bacteria in favour of those that could degrade the more ordered forms of cellulose such as cotton fibres, and also the cell walls of straws (which contain large quantities of lignin). Inhibition of casein degradation (*in vitro*) by avoparcin has been demonstrated by Lindsey *et al.* (1985), this may explain the decreased rumen ammonia levels noted by Froetschel *et al.*, (1983).

Flavomycin, neomycin and bacitracin have been shown, in addition to these effects on rumen function to alter hindgut digestion (Rowe *et al.*, 1982 and Goetsch and Owens, 1986a,b). A novel finding of the latter workers was that when cattle were fed a high concentrate diet, ileal infusion of neomycin and/or bacitracin increased the outflow of undegraded dietary protein from the rumen. It was suggested that this was as a result of an increased rumen particulate outflow rate. However, the increased particulate outflow following antibiotic administration noted when the cattle were fed a high concentrate diet, was not observed when the diet was changed to one containing a high proportion of forage. The precise mode of action for this observation is not known, but, Goetsch and Owens, 1986a,b suggested that there was feedback from the hindgut that could influence rumen kinetics.

B) Ionophore antibiotics

The word ionophore means "ion bearer" and this group of chemicals was defined by Ovchinnikov (1979) as "substances capable of interacting stoichiometrically with metal ions, thereby serving as a carrier by which these ions can be transported across a bimolecular lipid membrane". More simply the ionophores provide an alternative transport mechanism for metal ions and protons which by its action dissipates the cation and proton gradients across the cell membrane. The cell attempts to correct this dissipation and maintain its primary transport mechanism by expending metabolic energy. Cells that are capable of electron transport coupled with proton extrusion and/or ATP synthesis which is not dependant on substrate level

phosphorylation will exhibit a higher maintenance energy requirement, but will continue to grow and/or survive. However if the cell does not exhibit the afore-mentioned properties it will lyse.

The ionophores are produced by various strains of streptomyces and have been grouped as carboxylic polyether antibiotics and belong to the valinomycin class of antibiotics. Of the 76 different ionophores isolated (Olentine 1982), monensin, lasalocid, salinomycin and narasin are amongst those most commonly used as feed additives. Monensin has been the ionophore that has been most extensively investigated and reviewed (Bergen and Bates, 1984; Schelling, 1984; Chalupa, 1988 and van Nevel and Demeyer, 1988) for its ability to improve animal performance. Lasalocid, salinomycin and narasin have all been shown to induce similar rumen and animal responses to monensin (Schelling, 1984).

The modes of action of the ionophores on the rumen and the host animal may be summarised as:-

- a) Effects on carbohydrate metabolism.
- b) Effects on nitrogen metabolism.
- c) Prophylaxis against feedlot disorders (e.g. lactic acidosis, bloat).
- d) Other effects of supplementary ionophores.

a) Carbohydrate metabolism

Monensin has been consistently shown to alter the stoichiometry of the rumen fermentation pattern. Prange *et al* (1978), observed a significant ($P < 0.05$) increase in the rumen molar proportion of propionic acid (control 19.1% vs. monensin 24.7%). This increase was related to an increase in production rate of 3.44 moles/day of propionate. Similar results were noted by Adams *et al* (1981), Wallace *et al.* (1981) and Yokoyama *et al.* (1985). The change in the fermentation pattern noted in these experiments has been related to changes in the microbial populations as a direct effect of the antibiotic properties of the ionophores.

Chen and Wolin (1979), observed that monensin selected for succinate-forming *Bacteroides* spp. and for *S. ruminantium* which decarboxylate succinate to propionate. They also noted that monensin selected against H_2 and formate producers such as *R. albus*, *R. flaveciens* and *B. fibrisolvens*. The overall consequence was an increase in propionate production at the expense of acetate and butyrate. Bergen and Bates (1984), suggested that the ionophores selected for the bacteria that were resistant to or could rapidly become resistant to an alteration of the

primary transport mechanisms of their cells. In general it is the Gram [+] bacteria that are the most vulnerable to ionophore antibiotics.

van Nevel and Demeyer (1977), found that the methanogenic bacteria are not directly affected by ionophores as monensin supplementation of methanogenic bacteria *in vitro* had no effect on growth. The reduction in methanogenesis often noted when monensin is fed may therefore be related to the inhibition of formate and H₂ producing bacteria (van Nevel and Demeyer, 1977 and Chen and Wolin, 1979).

Potter *et al.* (1984), determined the LD₅₀ of monensin in cattle to be 26.4 mg/Kg body weight. They observed that the toxic effects were induced when steers were force fed greater than 2000mg of monensin. The main symptoms of monensin toxicity include; anorexia, diarrhoea, depression, rapid breathing, ataxia and death. However, their work also showed that when gavage of monensin ceased those animals that survived regained their appetite. Raun *et al* (1976), carried out a series of experiments using various dosage levels (0 - 500 mg/d) of monensin. The results of their work showed that feed intake was depressed as dosage level increased. They also noted that animal performance was greatest at dosage levels less than 500 mg/h/day. Faulkner *et al* (1985), observed that 100mg/d of monensin had a superior effect to 200mg/d when they fed a high fibre diet to steers.

The reduction in feed consumption noted when the diet is supplemented with monensin cannot be explained solely by one mechanism as it relies on the interaction of both physical and chemical parameters as well as the interactions between the rumen microbial ecosystem and the host. Lemenger *et al* (1978), noted a 16% drop in forage consumption when they fed 200mg/d of monensin to steers. They related this to a reduction in both the liquid and solids turn-over rate (per day) of 30.8% and 43.6% respectively as a result of reduced ruminal dry matter digestion (Muntifering *et al.* (1981), Wallace *et al.* (1981) and Owens *et al.* (1978)). Muntifering *et al.* (1981) calculated that for their corn based diet, monensin administration increased by-pass starch outflow by 19%. Raun *et al* (1976), suggested that the reduction in feed intake noted during their work was due to increased ruminal and/or blood propionic acid or some other chemostatic mechanism in the animal (Theur *et al*, 1974; Baile and Mayer, 1970).

Nissen and Tenkle (1976), showed that although feed intake dropped initially in steers supplemented with monensin, feed intake returned to 90% of the control diet intake by day 30. This observation suggests that there is a lag period during which the rumen microbial

ecosystem and/or the host is adapting to the supplement. Dawson and Boling (1983), noticed a lag period of 18 days before a shift in ruminal populations was observed.

Decreased feed intake may not affect animal performance due to the compensatory effects of:

- 1) Increased rumen molar % propionate.
- 2) Decreased methanogenesis.
- 3) Reduced heat loss, (the synthesis of propionate has a lower heat increment than the other volatile fatty acids).
- 4) Decreased energy expenditure for grazing.
- 5) Decreased metabolic faecal energy losses.

(Lemenger *et al.*, 1978)

The ability of monensin to improve the energy metabolism of the rumen and its host has been demonstrated by Raun *et al.* (1976), Lemenger *et al.*, 1978 and Adams *et al.* (1981) who observed an improvement in the feed conversion efficiency. This improvement was achieved through the maintenance of live weight gain even though feed consumption was reduced. Blaxter (1962), suggested that increases in the proportion of propionic acid available to the host increased the efficiency of adipose tissue synthesis. Ørskov (1977), indicated that the increased flow of starch from the rumen as observed following monensin administration by Muntifering *et al.* (1981), resulted in an increase in glucose uptake from starch digestion in the small intestine. This would benefit the host as glucose may be utilised more efficiently than the volatile fatty acids which would otherwise have been produced during ruminal fermentation.

b) N metabolism

The "protein sparing" effect of the ionophores is discussed by van Nevel and Demeyer (1977), Poos *et al.* (1979), Bergen and Bates (1984) and Schelling (1984). However there is some doubt as to whether this effect is due mainly to the sparing of glucogenic amino acids from gluconeogenesis by increased propionate production, or to direct effects on rumen proteolysis and deamination (Wallace *et al.* (1981).

van Nevel and Demeyer (1977), working *in vitro*, showed a reduction in protein degradation, a depression in culture ammonia concentration and a net decrease in microbial growth. An *in vivo* study carried out by Faulkner *et al.* (1985), observed similar effects and suggested that either less protein was being broken down or less bacterial protein was being synthesised. A reduction in the proportion of microbial N contributing to the total abomasal N (52% monensin

supplemented vs. 58% control) and an increase in the proportion of undegraded dietary protein (46% monensin vs. 40% control) was noted by Muntifering *et al.* (1981). Their work also showed that there was no overall effect on the passage of total N or amino acids from the abomasum. Owens and Isaacson, (1977) suggested that the increased flow of dietary protein to the abomasum and small intestines may have resulted from a decreased efficiency of microbial growth, noted when the turnover rate of the rumen fermentation system is reduced.

The increase in by-pass protein leaving the rumen may be expected to improve the efficiency of utilisation of dietary protein sources of high biological value. This is not only of biological benefit to the animal but also of financial benefit to the producer.

c) Feedlot disorders

i) Lactic acidosis

Monensin and lasalocid have been shown to inhibit the lactate producing bacteria but not the lactate utilisers (Dennis *et al.*, 1981a,b). Nagaraja *et al.* (1981,1982) observed that both monensin and lasalocid prevented lactic acidosis in cattle given large quantities of grain or a glucose drench. They related the reduced incidence of lactic acidosis to the inhibition of several strains of the Gram [+] bacterium *Streptococcus bovis*. The *S. bovis* population has been shown to increase markedly during the early stages of lactic acidosis (Hungate *et al.* 1952).

ii) Bloat

Mellman *et al.* (1988), suggested that monensin altered the secretory activity of cells by interfering with the Golgi apparatus found within the cell. Sakauchi and Hostino (1981) related the lower incidence of bloat in their work to a depression in ruminal fluid viscosity, which may have resulted from a reduction in slime production from *S.bovis* (Prins 1977).

d) Other effects of supplementary ionophores

i) Coccidiostatic effects

Fitzgerald and Mansfield (1978), showed that the control of coccidiosis in the hindgut may lead to improved feed utilisation.

ii) Direct effects on the host.

Wahle and Livesey (1985), suggested that monensin may have an effect on host tissue propionate utilisation. Mineral retention by the host may also be affected due to changes in ion transport through cell membranes, created by the presence of the ionophore (Kirk *et al.* (1985).

Summary of the affects of supplementary ionophores

- 1) Increased rumen molar % propionate (Prange *et al.*, 1978; Adams *et al.*, 1981; Wallace *et al.* (1981) and Yokoyama *et al.*, 1985).
- 2) An indirect inhibition of methanogenesis (van Nevel and Demeyer, 1977 and Chen and Wolin 1979).
- 3) Reduced Feed intake with a concomitant reduction in ruminal turnover (Dinius *et al.*, 1976; Raun *et al.*, 1976; Lemenger *et al.*, 1978; Muntifering *et al.*, 1981 and Faulkner *et al.*, 1985).
- 4) Increased flow of non-ammonia nitrogen (NAN) to the small intestine due to increased by-pass protein (Owens and Isaacson, 1977; van Nevel and Demeyer, 1977; Poos *et al.*, 1979; Muntifering *et al.*, 1981; Bergen and Bates, 1984; Schelling, 1984 and Faulkner *et al.*, 1985).
- 5) Reduced incidence of feedlot disorders (Dennis *et al.*, 1981a,b; Nagajara *et al.*, 1981,1982 and Sakauchi and Hostino, 1981).
- 6) Improved feed conversion efficiency (Blaxter, 1962; Raun *et al.*, 1976; Ørskov, 1977 and Lemenger *et al.*, 1978).

The ionophore antibiotics have been shown to have several modes of action that work in concert resulting in the observed animal effects. Whilst improved animal FCE and N retention have been illustrated no effect of monensin on the proportion of fat, lean and bone parameter of the carcass have been shown (Potter *et al.* 1976).

1.4: The use of probiotics in animal production.

1.4.1: Introduction

The word probiotic comes from the Greek meaning "for life" and was first used by Lilley and Stillwell (1965) to describe substances secreted by one micro-organism which stimulate another. Sperti (1971), changed the emphasis of the definition to "tissue extracts that stimulate the microbial growth". This version of the definition implies that a probiotic supplement need not be of microbial origin. Parker (1974), redefined a probiotic as "organisms and substances which contribute to intestinal microbial balance". In the most literal sense of this definition antibiotics could be regarded as having a probiotic effect. This led to Fuller (1989), to define probiotics as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance.

Recent research has indicated that the probiotic supplement need not be viable (Newbold *et al.* 1991a) or be of microbial origin (Pusztai *et al.*, 1990), in order to exert a probiotic effect. There is therefore a need for a broadening of Fuller's definition which will encompass both these types. In this thesis a probiotic will be defined as viable or non-viable microbes and/or chemical compounds which, when provided in small amounts directly favour intestinal microbial balance either by, the colonisation of the intestinal mucosa, or indirectly by the stimulation of indigenous beneficial microflora of the gut which may lead to selection against the undesired gut microflora.

1.4.2: History

There are references to the use of soured milk in the Bible (Genesis 18.8) and wall paintings have indicated that the Sumarians (2500 BC) inoculated milk in order to induce fermentation (Kroger *et al.*, 1989). However, it was the work of Eli Metchnikof and the publication of his book "The prolongation of Life: Opportunistic Studies" (Metchnikof 1907), that widened the interest in probiotics.

The discovery and accessibility of antibiotics after the second world war led to increased interest in gut microflora - host interactions. The ability of the indigenous microflora to stimulate the host immune system was demonstrated by Bohnhoff *et al.*, (1954) and Freter

(1955,1956). They showed that administration *per os* of antibiotics left the animals more susceptible to *Salmonella typhimurium*, *Shigella flexeri* and *Vibrio cholera*. Collins and Carter (1978), observed that gnotobiotic guinea-pigs were killed by only 10 cells of *Sal. enteritidis*, but, in guinea-pigs with a complete microflora 10^9 cells were required to have a pathogenic effect. This demonstrates that not all the micro-organisms of the gut are detrimental to the health of the host animal.

In the wild the neonate would acquire its indigenous microflora from its dam and its surroundings. However, the intervention of man may reduce the transfer of the microflora for example in the case of eggs collected from the hen and hatched in an incubator. Chicks hatched in this way are then dependent on the microbial contamination of the feed and the environment to provide the microflora of its gut. Environmental factors, stress and the administration of both therapeutic and feed supplement antibiotics may also have a dramatic effect on the intestinal microbial ecosystem (Tannock 1983; Fuller 1989). It is under these conditions that a probiotic may be given in order to redress the microbial balance of the gut.

1.4.3: Choice of probiotic supplement

The micro-organisms that are currently available for inclusion in probiotic supplements are shown in Table 1.4.1

Other bacterial genera used include *Leuconostoc*, *Pediococcus*, *Torulopsis*, *Propionibacterium* and *Bacillus* (Ewing and Haresign, 1989 and Fuller, 1992).

Probiotic products in current use may contain one or several different micro-organisms and may be given to the animal in the form of powders (loose or encapsulated), tablets, granules, as a paste or sprayed into the local environment as an aerosol.

The choice of an organism for a probiotic should be based on the desired aim for the probiotic. This may be related to the target site in the host and whether or not the organism needs to be able to colonise the target site or only exist in a sustained transient state. Many of the organisms that are currently used as probiotics were chosen because of their prevalence in healthy animals. However, this selection criterion may not always be a valid as the existence of an organism in large numbers in the gut may simply reflect health of the digestive tract rather than the cause. Lee (1985), suggests that the bacterial probiotic organisms were chosen for historical reasons and because they

Table 1.4.1: Micro-organisms currently used in probiotic products.

Type	Genus	Species	Subspecies	Strain
Bacteria	<i>Lactobacillus</i>	<i>delbreuckii</i> <i>acidophilus</i> <i>casei</i> <i>fermentum</i> <i>plantarum</i> <i>brevis</i> <i>cellobiosus</i> <i>lactis</i> <i>reuteri</i> <i>bifidus</i>	<i>bulgaricus</i>	
	<i>Bifidiobacterium</i>	<i>adolescentis</i> <i>animalis</i> <i>bifidum</i> <i>infantis</i> <i>longum</i> <i>thermophilum</i>		
	<i>Enterococcus</i>	<i>faecium</i>		M74, SF68
	<i>Lactococcus</i>	<i>lactis</i>	<i>lactis</i> <i>cremoris</i> <i>diacetylactis</i> <i>intermedius</i> <i>thermophilus</i>	
Fungi	<i>Saccharomyces</i>	<i>ceriviseae</i>		1026
	<i>Candida</i>	<i>pintolopesii</i>		
	<i>Aspergillus</i>	<i>oryzae</i> <i>niger</i>		

(Source: Ewing and Haresign 1989; Fuller 1992)

were easy to culture. These bacteria have also been considered as harmless intestinal symbionts, but *E. coli* and *Enterococcus* species have been shown to cause a variety of severe gut infections and some lactobacilli have been associated with septicaemia and the hydrolysis of bile salts. Freter (1992) concludes that there is no compelling ecological or other scientific rationale for the current narrow choice of bacterial species utilised in probiotic preparations. The technology to genetically alter bacteria or yeasts for a specific role in the gut exists (Tannock, 1992). This technology may provide the next generation of probiotics for animal health and production.

1.4.4: The principle aims of a probiotic supplement

There are two principle aims of probiotics:

- A) The maintenance of animal health through the provision or re-establishment of indigenous beneficial micro-organisms in the gut.
- B) Improved utilisation of the feed through the stimulation of the indigenous microflora.

A) The use of probiotics in prophylactic health care.

Bacterial probiotics have been shown to reduce coliform counts in the intestines of monogastric and young ruminant animals. High coliform counts have been associated with scouring in calves (Smith 1971; Youanes and Herdt 1987). In severe cases, scouring may result in the death of the animal due to dehydration whilst, in milder forms nutrient absorption and animal performance may be reduced (Youanes and Herdt 1987). There are several ways in which a probiotic organism may act in order to prevent the overgrowth of potential gut pathogens including:-

- i) Formation of a physical barrier.
- ii) Formation of a chemical barrier.
- iii) Stimulation of host immunity.

i) Formation of a physical barrier.

The target site for the probiotic has a major effect on the probiotics ability to adhere to and colonise the gut wall. Freter (1992), suggests that the ability of a probiotic to form a physical barrier against undesired micro-organisms relates to its ability to colonise either the surface of the epithelial cells or the mucous layer that covers the gastro-intestinal tract. In the areas of the gut where the rate of digesta transit is high it is important for the probiotic organism to be able to adhere to the host before it is excluded from the gut. However, in the areas of slow transit a probiotic organism may be able to colonise the lumen if its rate of replication is sufficiently fast to prevent exclusion and if it is metabolically active enough to compete with the existing micro-flora for the available substrate. Fuller (1973), observed that the indigenous lactobacilli population of fowl was able to colonise the crop epithelium, and suggested that this may be one way in which the lactobacilli exert an antagonistic effect against coliform bacteria.

ii) Formation of a chemical barrier.

Lactic acid bacteria have been shown to produce various antibacterial substances (Havenaar *et al.*, 1992). These include lactic acid, hydrogen peroxide and bacteriocins.

Bacteriocins are proteinaceous and are generally only active against organisms that are closely related to the producer (Koninsky, 1982). Table 1.2 shows the various bacteriocins produced by the lactobacilli.

Table 1.4.2: Antimicrobial agents produced by various *Lactobacillus* species

Species	Product	References
<i>L. acidophilus</i>	acidolin acidophilin lactacin B	Hamdam and Mikolajcik (1974) Shahani <i>et al.</i> (1976,1977) Barefoot and Klaenhammer (1983,1984)
<i>L. Bulgaricus</i>	bulgaricin	Shahani <i>et al.</i> (1976); Reddy <i>et al.</i> (1983).
<i>L. helveticus</i>	lactocin 27 helveticin J	Upreti and Hinsdill (1973) Joerger and Klaenhammer (1986)
<i>L. plantarum</i>	plantacin B plantaricin A plantaricin SIK 83*	West and Warner (1988) Daeschel <i>et al.</i> (1990) Andersson (1986); Andersson <i>et al.</i> (1988)
<i>L. reuteri</i>	reuterin	Talarico and Dobrogosz (1989); Talarico, <i>et al.</i> (1988); Axelsson, <i>et al.</i> (1989); Chung <i>et al.</i> (1989)
<i>L. sake</i>	sakacin A lactocin S	Schillinger and Lucke (1989) Mortvedt and Nes (1990)

*NB: The organism used was probably *Lactococcus lactis* producing a compound very similar to nisin (Andersson *et al.*, 1988).

(Taken from Havenaar *et al.*, 1992)

Acidolin (Hamdon and Mikolajcik, 1974), acidophilin (Shahani *et al.*, 1976,1977) and bulgaricin (Shahani *et al.*, 1976; Reddy *et al.*, 1983) have been shown to exert a broad inhibitory spectrum. The organisms that are susceptible to these types of bacteriocins include both Gram [+] and Gram [-] bacteria. However, to date most of this work has been carried out *in vitro* and no intestinal

activity of these substances has yet been shown (Fuller, 1989; Freter, 1992). The principle inhibitory action in the formation of the chemical barrier is thought to be due to a reduced intestinal pH through the production of lactic acid (ten Brink *et al.*, 1987).

iii) Stimulation of the host immunity

McSweegan *et al.* (1987), observed that antibodies secreted into the mucous gel could prevent the adhesion of bacteria to the underlying epithelial cells. Increased mucous flow was noted by Walker and Bioch, (1977) when antibody-antigen complexes were present in the lumen of the intestines. The increase in mucus flow would facilitate the more rapid expulsion of micro-organisms from the gut. It may be assumed that this type of immune response has some degree of specificity because of the fact that the epithelial surface may be colonised by various beneficial indigenous species. Shedlofsky and Freter (1974), working with mono-associated gnotobiotic mice, demonstrated that the local immune response had relatively little effect on large populations of *Vibrio cholerae*. However, the *Vibrio* population density was reduced when the mice were associated with additional indigenous enteric species due to an increase in the activity of the local immune response. The authors concluded that there may be a synergistic link between local immunity and bacterial antagonism.

Perdigon *et al.* (1986,1988) noted that macrophage activity in mice was increased by oral administration of *Lactobacillus* spp., indicating stimulation of the non-specific immune system. This phenomenon may relate to the ability of the lactobacilli to penetrate the circulation system of the host and establish stable populations in the liver, lungs and spleen (Fuller 1973; Bloksma *et al.* 1981).

Summary of the use of probiotics in prophylactic animal health

The mechanisms by which a probiotic organism may mediate good animal health through the maintenance of a beneficial enteric microflora should be viewed holistically, as the modes of action that the organisms employ do not work individually but in concert. Animal performance responses when probiotic organisms are added to the diet may be variable due to variability in the initial enteric populations and may be considered as secondary to the prophylactic role of the probiotic against potential pathogens.

B) The use of probiotics in ruminants

Probiotics given to ruminants with the primary aim of improving feed utilisation and it is this use of probiotics in ruminant animals that this thesis will consider.

Bacterial probiotics have been shown to improve feed utilisation in adult dairy cattle. Jacquette *et al.* 1988; Ware *et al.* 1988, showed that supplementing the ration of dairy cows with *L. acidophilus* resulted in increased milk yield. However, one of the major modes of action of these bacteria is the production of lactic acid which if active in the rumen, would result in a lowering of rumen pH and thus impair rumen function. It is therefore assumed that this type of bacterial probiotic exerts its beneficial effect in the intestines. Specialised bacterial probiotics prepared from rumen bacteria have been shown to have beneficial effects in cattle. Jahn *et al.* (1973) demonstrated that it was possible to increase milk production in dairy cows by supplementing them with bacteria specially adapted *in vitro* for efficient starch fermentation. However, the use of probiotic feed additives of fungal origin have been the most extensively studied for ruminants.

The fungal supplements that are widely available are based either on yeast (*Saccharomyces cerevisiae*), *Aspergillus oryzae* or their fermentation extracts. Other fungal supplements that are used as probiotics include: *Candida pintolopesii* (Fuller 1992), *Aspergillus niger* (Tapia and Herrera-Saldama, 1989; Campos *et al.* 1990) and the indigenous rumen fungus *Neocallimastix* (Theodorou *et al.* 1990). Fungal additives may be used in both meat and milk production, unlike the ionophore antibiotics that modulate the rumen stoichiometry in a disadvantageous manner for milk composition and may give rise to undesired residues in the milk (Wallace and Newbold 1992).

Reports on the efficacy of both yeast and *A. oryzae* supplements have been conflicting, and a possible bias may exist in the literature regarding the nature of the animal response to these probiotics. This is due to the high level of commercial investment in the research into the mode of action of these products. Careful consideration must therefore be taken when appraising the literature.

Since yeast and *A. oryzae* appear to exert different effects in the rumen they will be discussed separately.

1.5: Products containing Yeast or Yeast extract

Yeast cells have been shown to utilise both pentose (hemicellulosic) and hexose (cellulosic) sugars anaerobically (Sols *et al.* 1971 and Jefferies 1983). The optimum pH for yeast metabolism has been shown to be in the range of 3.5-5. However, the fermentation of sugars in the higher pH ranges has been observed although yeast autolysis has been shown to increase with increasing pH levels (Rose 1987a).

Yeast products usually contain a mixture of live and dead cells in the basal medium on which they were grown. Together these components comprise yeast culture. The association of American feed control officials (1986), defined yeast culture as "... a dry product composed of yeast and the media on which it is grown, dried in such a manner as to preserve the fermenting capacity of the yeast. The media used must be stated on the label."

The rumen has a much slower rate of turnover as compared to the intestinal tract increasing the possibility for a probiotic organism to colonise the rumen wall and/or the liquid and solid fractions of the rumen. Rumen yeast numbers were shown to increase by Dawson (1987) and Newbold *et al* (1990). In the trial carried out by Newbold *et al* (1990), viable yeast numbers following the oral administration of yeast culture declined at a rate similar to the likely rate of liquid outflow from the rumen, but levels two orders of magnitude above the controls (which received no yeast supplement were maintained. This indicates that the yeast has a limited ability to colonise the rumen, but probably exists predominantly in a sustained transient state.

1.5.1: The influence of yeast culture on rumen function

The influence of supplemental yeast culture on rumen function has been extensively reviewed by Chase (1989; Günther (1990); Williams and Newbold (1990); Erasmus (1991; Higginbotham (1991); Newbold (1991); Martin and Nesbit (1992) and Wallace and Newbold (1992). Table 1.3 demonstrates the highly variable *in vivo* and *in vitro* responses to the daily dietary inclusion of a few grams of yeast culture.

Table 1.5.1: Summary of the effects of supplemental yeast culture on rumen parameters.

Reference	Species	Dose g/d	pH	t[VFA]	p[VFA]	NH ₃	LDR ¹	Dg ²	Microbial Effects.	Other
Adams <i>et al.</i> 1981	sheep cattle	1.85 %	=	=	=	-	+	=		= N Bal.
Arambel and Wiedmier 1986	Cattle	90	=	=			=	+	> Cellulolytics	
Arambel <i>et al.</i> 1987	<i>in vitro</i>	90		+	> A:P	+				
Chadamana and Offer 1990	Sheep	4	+	=	< A:P	-	+	> 24Hr.		= overall Dg
Dawson and Newman 1988	<i>in vitro</i>	1g/kg feed	-	+	< A:P + But				No effect	
Dawson <i>et al.</i> 1990	<i>in vitro</i>	1g/kg feed	+	-	< A:P	-	+		> Cellulolytics	
Edwards <i>et al.</i> 1991a	Cattle	1.5g/kg feed		+					> Urinary allantoin	
Edwards <i>et al.</i> 1991b	Cattle	10	=	+		=				
Gomez-Alarcon <i>et al.</i> 1987	Cattle							+	> Microbial Protein	
Gray 1989	Sheep/ <i>in vitro</i>	0.2g/l 0.5g/l		= +	= > A:P					
Gray and Ryan 1990a	Sheep/ <i>in vitro</i>	2.5		+						
Gray and Ryan 1990b	Sheep/ <i>in vitro</i>	2.5	+	+						

Characters in SHADED boxes indicate responses to yeast culture significant at $P \leq 0.05$

Reference	Species	Dose g/d	pH	t[VFA]	p[VFA]	NH ₃	LDR ¹	Dg ²	Microbial Effects.	Other
Harrison <i>et al.</i> 1987a	Cattle	114	-	=	<A:P				> Total Anaerobes > Cellulolytics	
Harrison <i>et al.</i> 1987b	Cattle	114				+	+ Apparent			> <i>in vitro</i> cellulose Dg.
Harrison <i>et al.</i> 1988	Cattle	114	-	=	<A:P	-	=	=	> Total anaerobes > Cellulolytics > Yeast (x2)	
Huhtanen 1991	Cattle	10	=	=	=	=		= Overall > Rate > Lag	> Protozoa	> D-,L+ Lactic acid = N flow No hindgut effects
Malcom and Kiesling 1986	Cattle	1.6%	+		<A:P -But					
Malcom and Kiesling 1990	Cattle	1.6%	=	=	=	=	+			
Martin <i>et al.</i> 1989	<i>in vitro</i>	1g/l	-		<A:P			=		
Moloney 1989	Cattle	10			>A:P -But & Val	=	+			
Newbold <i>et al.</i> 1990	Sheep	4	+	=	<A:P >P	-			< Yeast (= LDR)	< L-Lactate
Newbold and Wallace 1991	<i>in vitro</i>	4		+						Interaction with ionophores
Ryan and Gray 1989	Sheep/ <i>in vitro</i>	0.2-0.5g/l		+						

Characters in SHADED boxes indicate responses to yeast culture significant at $P \leq 0.05$

Reference	Species	Dose g/d	pH	t[VFA]	p[VFA]	NH ₃	LDR ¹	Dg ²	Microbial Effects.	Other
Wagner <i>et al.</i> 1990	Cattle	1g/kg	=	=	=					
Wiedmeir <i>et al.</i> 1987	Cattle	90	=	=	=	=	-	+	> Anaerobes	
Williams 1988	Cattle	10	+	=	<A:P			+		
Williams <i>et al.</i> 1990	Sheep	4								> DM & NAN to S/I
Williams <i>et al.</i> 1991	Cattle	10	+	=	<A:P			> 12Hr		< L-Lactate

¹LDR Liquid dilution rate (%/h)

²Dg Ruminal degradability

Characters in SHADED boxes indicate responses to yeast culture significant at P ≤ 0.05

a) The effect of yeast culture on carbohydrate metabolism

i) Rumen pH

Rumen pH has a marked effect on the degradation of carbohydrate in the rumen (Mould and Ørskov, 1983 and Mould *et al.* 1983). Dawson and Newman (1988) and Harrison *et al.* (1987a, 1988) observed a depression in rumen pH, following dietary inclusion of yeast culture, but were unable to show a concomitant reduction in digestion (Harrison *et al.* 1988). Whereas, Chademana and Offer (1990), Dawson *et al.* (1990), Gray and Ryan (1990b), Malcom and Kiesling (1986), Williams (1988) and Williams *et al.* (1991) noted an increase in ruminal pH in response to yeast culture, and increased degradation in the short term incubation of fibre (Chademana and Offer 1990 and Williams *et al.* 1991).

The relationship between rumen pH and rumen VFA metabolism has been shown to be complex (Ørskov and Ryle 1990). Absorption of the VFA was shown to be greater at lower rumen pH levels. The increased production of VFA following feeding would result in a depression of rumen pH and increased absorption of the VFA. The net result would be a more rapid pH recovery than would be observed if the rate of absorption remained constant. Figure 1.1 shows the relationship between rumen pH and t[VFA] obtained from experiments listed in Table 1.5.1. A highly significant ($P < 0.001$) negative correlation (Coefficient = -0.923) between pH and t[VFA] exists (Figure 1.1) indicating that the total concentration of the volatile fatty acids (t[VFA]) has a large influence on rumen pH. However, the change in t[VFA] observed when yeast was added to the diet was not always associated with an inverse alteration in rumen pH (correlation coefficient (r) = -0.117). This suggests that some other factor may also be influencing rumen pH in yeast supplemented diets.

During the periods of rapid bacterial growth, lactic acid has been shown to be the major fermentation end product of *Selenomonas ruminantium* and *Streptococcus bovis* (Dawson and Allison 1988). At slower rates of growth, *Selenomonas ruminantium* can utilise lactate as a substrate. Nisbet and Martin (1990b), demonstrated that the uptake of lactate could be stimulated by the inclusion of yeast culture or the filtrate from a filter sterilised suspension of yeast culture. They suggested that this response was due to the provision by yeast culture of L-malate which had previously been shown to stimulate lactate uptake by *Selenomonas ruminantium*.

The elevation of the minimal rumen pH ($P < 0.05$) by yeast culture was suggested by Williams *et al.* (1991), to be related to the reduction in the lactic acid peak post-feeding (also noted by

Newbold *et al.* (1990)). Williams *et al.* (1991) went on to suggest that this reduced peak could result from either the utilisation by yeast of lactate precursors, inhibition of lactate production or increased lactate utilisation. Gray and Ryan (1990a), suggested that yeast supplements may act by fermenting the oligosaccharides in the rumen, thus reducing the supply of lactate precursors. However, the responses of rumen lactate to yeast culture is inconsistent as shown by the work of Huhtanen (1991), who showed an increase in the concentration of D- and L+ lactic acid in the rumen of bulls supplemented with yeast culture.

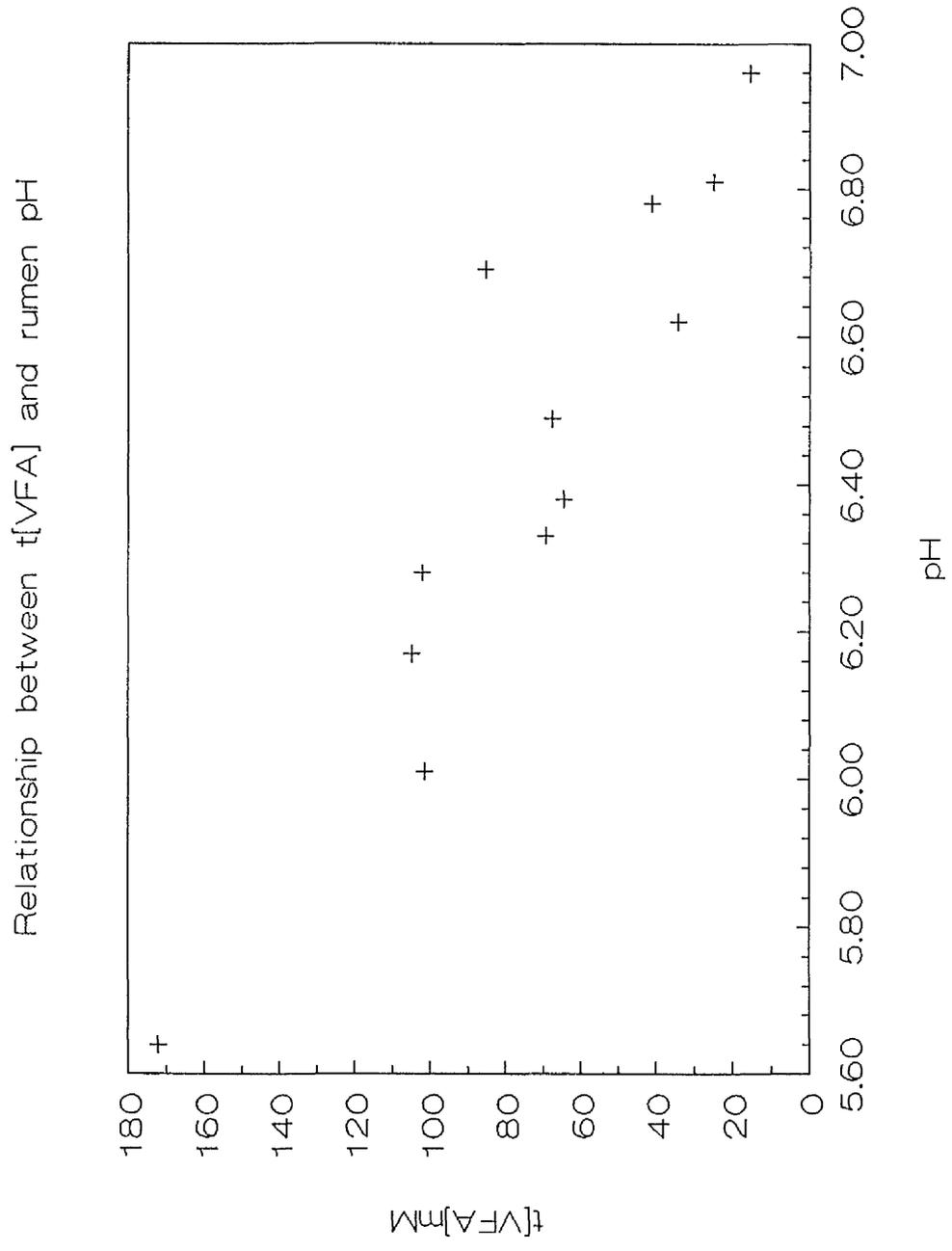
The inconsistency of the pH response to yeast culture may result from the complexity of the interacting factors which determine rumen pH, such as the rate and pattern of fermentation, the buffering capacity of the feed, the rate of VFA absorption, the rate of saliva secretion and the rate of liquid and solid outflow. Lactic acid has a pK of 3.08 which makes it a stronger acid than the VFA which have pK values of 4.75, 4.81 and 4.87 for acetic, propionic and butyric acid respectively. Williams *et al.* (1991), observed a peak rumen lactate concentration of 7.75 mM as compared to a t[VFA] of 88 mM. A comparison of the proportion of H⁺ ions contributed by lactic acid cf. t[VFA] at the observed rumen pH values with or without additional YC indicates a four fold shift in the relative H⁺ ion concentration (1:38 cf. 1:10.4 respectively) due to yeast supplementation. The concentration of lactic acid in the rumen would therefore be expected to have an effect on rumen pH even at relatively low concentrations.

ii) The effect of supplemental yeast culture on rumen degradation

Chademana and Offer (1990), observed an increase in the 24hr *in sacco* degradability of hay following yeast supplementation. This response occurred at each of the forage : concentrate ratios (90:10, 65:35 and 40:60) fed to experimental animals and contrasts with the findings of Williams *et al.* (1991) who demonstrated that yeast culture increased the initial rate of *in sacco* degradation of hay with increasing levels of dietary concentrate. The work of Williams *et al.* (1991), suggests that yeast culture may be most effective when cellulolysis is most likely to be compromised, through the alleviation of the pH and carbohydrate effects that contribute to the "negative associative effects" described by Stewart (1977), Istasse and Ørskov (1983), Mould and Ørskov (1983) and Mould *et al.* (1983).

Huhtanen (1991), observed a non-significant increase in the 48 hr *in sacco* degradability of ground hay following yeast culture supplementation. This work also indicated an increase in the rate of degradation after a prolonged lag time. However, no effect on polysaccharide

Figure 1.1



enzyme activity was observed in bulls supplemented with 10g/d of yeast culture. Chademana and Offer (1990), demonstrated that the 24hr. feed degradability in the rumen could be increased with supplemental yeast culture, but unlike Huhtanen (1991) did not observe an effect at 48 hrs. The findings of Williams *et al.* (1991), showed a significant increase ($P < 0.05$) in 12hr. *in sacco* degradability measurements and non-significant decreases in the rate of degradation and lag time.

Where whole gut digestibility work has been carried out, the majority of results have shown no significant effect ($P > 0.05$) on digestibility (Adams *et al.* 1981; Arambel and Wiedmeir 1986; Gomez-Alarcon *et al.* 1987; Harrison *et al.* 1987b, 1988; Martin *et al.* 1989; Chademana and Offer 1990; Huhtanen 1991 and Williams *et al.* 1991). However, Wiedmeir *et al.* 1987 showed a significant increase in crude protein and hemicellulose digestibility. Changes in rate or extent of digestion when yeast is added have been associated in some experiments with non significant increases in rumen outflow rate (Dawson 1987 and Chademana and Offer 1990 and Malcom and Kiesling 1990). An improvement in the rate of forage degradability, increased rumen outflow rate and an overall improvement in digestibility may be expected to lead to an increased dry matter intake and a possible improvement in efficiency of feed utilisation, leading to an improvement in production parameters such as milk yield, liveweight gain or feed conversion efficiency.

iii) The effect of yeast culture on rumen fermentation

Dawson *et al.* (1990) found that yeast supplementation caused a significant ($P < 0.05$) reduction in t[VFA] *in vitro*. However, Table 1.5.1 demonstrates that most studies have either shown an increase in the concentration of tVFA or no response at all.

The t[VFA] and proportions of the VFA are a good indicator of rumen function. High values for molar % acetate ($> 70\%$) are usually associated with a gradual degradation of the fibrous material and an improved milk composition. Whereas, for improved meat production an increase in molar % propionate is desirable as an increase in propionate concentration at the liver stimulates insulin release and deposition of body fat (Lees *et al.*, 1990). The evidence for the effect of yeast culture on rumen fermentation stoichiometry is very conflicting. Dawson and Newman (1988), observed no changes in the total anaerobic or cellulolytic microbial counts in their fermenters, but observed an increase in t[VFA] and a shift in the fermentation pattern towards propionate suggesting that the activity of the microbial population was stimulated. In

contrast Gray and Ryan (1990a), suggested that the *ad finem* increase in t[VFA] noted in their work resulted from an increase in the microbial numbers. However, an increase in microbial numbers is not always associated with an increase in t[VFA] (Harrison *et al.* 1988).

Table 1.5.1 shows that 4 authors (identified in Table 1.5.1) observed a non-significant increase in the acetate : propionate ratio (A:P), whilst 10 authors showed a reduction (3 significant ($P < 0.05$)) and 6 authors reported no effect. The relationship between the % change in the A:P ratio following yeast supplementation and the A:P ratio for the unsupplemented control diet is shown in Figure 1.2. The coefficient of correlation (r) for this relationship was -0.329 ($P > 0.05$). However, there was a tendency for yeast culture to stabilise rumen fermentation, ie. YC decreased the A:P ratio when control levels were high and *vice versa*. The % change in the A:P was not related to the pH of the rumen ($r = -0.048$). Whereas, a strong positive correlation between rumen pH and A:P was observed ($r = 0.877$). The high pH values recorded by Edwards *et al.* (1991a) were not included in the calculation as they were found to be anomalous, probably due to salivary contamination of the rumen liquor which was obtained by stomach tube.

iv) The response of the rumen microbial population to yeast culture

Supplemental yeast culture has been shown to increase rumen bacterial counts (Arambel and Wiedmeir 1986; Gomez-Alarcon *et al.* 1987; Harrison *et al.* 1987a; Wiedmier *et al.* 1987; Harrison *et al.* 1988; Dawson *et al.* 1990 and Edwards *et al.* 1991a) and protozoal counts (Huhtanen 1991). No reports of the effects of yeast culture on the biomass of the rumen fungi have yet been published.

The observed increases in anaerobic bacteria are not specific, since both increases in cellulolytic (Harrison *et al.* 1987a) and proteolytic (Arambel *et al.* 1987 and Weidmeir and Arambel 1985) bacterial numbers have been noted.

Weidmeir and Arambel (1985), suggested that the proliferation of the cellulolytic bacteria may be due to the yeast providing stimulatory factors, such as, B vitamins or branched chain fatty acids. They then went on to propose that the increase in crude protein digestibility was as a result of stimulation of the proteolytic bacteria, whilst the increased hemicellulose and structural carbohydrate degradability was concurrent with an increased number and proportion of cellulolytic organisms.

Lyons (1986), cited similar findings including the bacterial growth factor para amino benzoic acid as one of ten water soluble vitamins, partly responsible for increased anaerobic bacteria numbers. Ruf *et al* (1953), demonstrated that ashing destroyed the stimulatory factor suggesting that the factor is organic. However, Ruf *et al* (1953) went on to try various B vitamin and amino acid supplements but failed to show a response and concluded that the factor did not appear to be either of these.

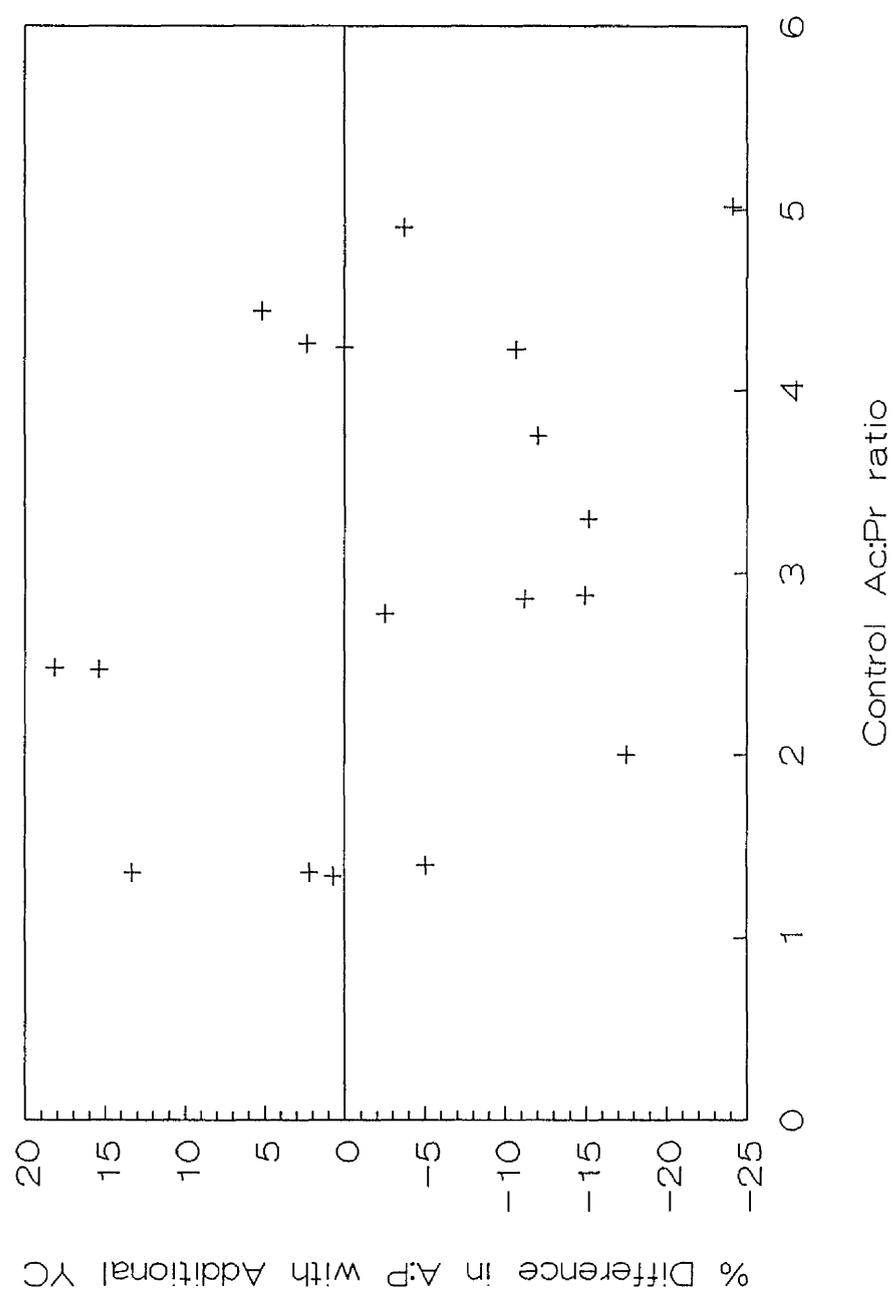
b) The effect of yeast culture on nitrogen metabolism

Harrison *et al* (1987a) observed that supplemental yeast culture resulted in an increase in the molar concentration of the total isoacids. Higher molar proportions of valerate ($P < 0.05$) in the steers fed yeast culture accounted for this increase although the total VFA concentration did not differ between the treatment groups. Arambel *et al.* (1987), observed a significant increase in the *in vitro* concentration of branched chain fatty acids following yeast supplementation. They related this to an increase in proteolytic activity. This conclusion is supported by the findings of Allison and Bryant (1963), who found that elevated rumen branched chain VFA concentration was a result of deamination and decarboxylation of branched chain amino acids. A corresponding increase in $\text{NH}_3\text{-N}$ was observed *in vitro* by Arambel *et al.* (1987) which, in the absence of absorption which would occur *in vivo*, suggests that yeast increased the rate of ammonia production more than it increased the rate of ammonia uptake by the microorganisms.

The decrease in NH_3 concentration observed *in vitro* by Dawson (1987), was related to an elevation in the microbial population, suggesting that increased incorporation of nitrogen into microbial protein was the predominant effect. Reduced rumen ammonia concentration in response to dietary yeast inclusion *in vivo*, was found by Harrison *et al.* (1987a), Chademana and Offer (1990), Molony (1989) and Newbold *et al* (1990) but the effects were not statistically significant ($P > 0.05$).

Williams *et al* (1990), demonstrated that the presence of *S. cerevisiae* tended to increase the duodenal flow of both dry matter (DM) and non ammonia nitrogen (NAN) in sheep. Calculation of the apparent daily absorption of DM and NAN showed that yeast culture increased ($P < 0.05$) the absorption of DM by 32% and NAN by 20%. The increased NAN flow was related to an increased microbial protein production, which is consistent with the

Figure 1.2: The relationship found to exist between the observed % change in the acetate : propionate ratio (Ac:Pr) noted when a diet was supplemented with YC as compared to the Ac:Pr ratio on the unsupplemented diet



reduced ruminal ammonia-N concentration already cited. The increased protein absorption from the small intestine could partly explain the production responses reported in dairy and beef cattle.

1.5.2: The Effects of supplemental yeast culture on animal performance.

The effects of supplemental yeast culture on animal performance traits are summarised in the Table 1.5.2.

Statistically significant effects ($P < 0.05$) are rare ($< 25\%$ of all reported responses) but, it may be observed that supplemental yeast culture has a tendency to increase dry matter intake, milk yield and milk butterfat. However, as these experiments are funded predominantly by commercial companies it is likely that experiments showing no response or a negative response may not always be published. This would result in a bias of the information published in favour of yeast culture supplementation.

Williams *et al* (1991), demonstrated that the additional ME supplied by the increased intake of complete diets of cows given yeast culture, equates well with the additional energy required for the increased milk and live weight gain observed. This suggests that no other physiological effect other than an increase in intake need be proposed to explain the performance effects of yeast culture.

The results of Wholt *et al.* (1991) agree with the findings of Williams *et al.* (1991). The former observed an increase in milk yield during early lactation that was related to an increase in dry matter intake (DMI) and improved digestibility of the cellulose and protein fraction of the diet. Bax (1988), found similar results when the yeast culture was fed to dairy cows on either a complete ration or when the concentrate portion was fed separately. He observed that responses were greatest when the concentrate was fed separately ie. when cellulolysis was most likely to be compromised.

Increased milk butterfat levels are frequently cited in the literature. This would appear to be in conflict with the rumen effects noted by Harrison *et al* (1987a), Newbold *et al* (1990) and Williams *et al.* (1991) who observed an increase in molar % propionate when yeast culture was included in the diet. This apparent contradiction may be related to the fact that the animals used to investigate the rumen effects were fed a fixed ration. Whereas the animals used in the

performance trials were allowed access to *ad lib* forage. An increased forage intake in *ad lib* fed animals following yeast supplementation would result in increased acetate production leading to improved butterfat levels. However this hypothesis has yet to be confirmed.

Hughes (1988), demonstrated a significant ($P < 0.005$) increase in intake and live weight gain of calves aged 0-12 weeks following yeast supplementation. Phillips and von Tungeln (1985), also observed improved animal performance in calves fed yeast but, showed no increase in response when the inclusion rate was doubled. These results indicate that yeast culture may have beneficial effects in preruminants. Newbold *et al* (1990), demonstrated that *S. cerevisiae* is capable of proliferating in the intestines and therefore intestinal probiosis effects cannot be ruled out.

The animal performance responses to additional yeast culture were suggested by Wallace and Newbold (1992) to be related to the diet and nutritional demands of the animal. Thus cows in early lactation respond better than those in the latter stages (Harris and Lobo 1988; Günther 1990).

Table 1.5.2: Summary of the effects of supplemental yeast culture and yeast culture extracts on animal performance parameters.

Reference	Species	Dose g/d	Dry matter intake	Milk Yield	Milk Comp.	Live weight gain	Feed conversion efficiency	Other
Arambel and Kent 1988	Cattle	90		=	=			= Overall Dg.
Arambel <i>et al.</i> 1990	Cattle - Early Lactation - Mid Lactation	90	=	=	=			
Boland 1986	Cattle	?		+	> Bfat			
Deaville and Galbraith 1990	Goats	4	=			=		= Fibre Growth
Dilley 1987	Cattle	?		+	=			
Edwards <i>et al.</i> 1991a	Cattle	1.5g/kg	+			+	+	
Edwards <i>et al.</i> 1991b	Cattle	10	+			+	+	
Erdman and Sharma 1989	Cattle - Mid Lactation	1%	=	=	> Protein		+	
Fallon and Harte 1987	Cattle	?	+			+	+	
Harris and Lobo 1988	Cattle - Early Lactation - Late Lactation	4oz	+	+	> Bfat < Protein			
Harris and Webb 1990	Cattle	?	-	+	> Bfat > Protein			

Characters in SHADED boxes indicate responses to yeast culture significant at $P \leq 0.05$

Reference	Species	Dose g/d	Dry matter intake	Milk Yield	Milk Comp.	Live weight gain	Feed conversion efficiency	Other
Hoyos <i>et al.</i> 1987	Cattle	?		+	> Bfat			
Hughes 1987	Cattle	2→1g/kg	+			+		
Jordan and Johnston 1990	Sheep	5	+			+		Interaction with diet > on Hay cf. Silage
Kumar <i>et al.</i> 1992	Buffaloe	10		+	> Bfat > Protein > Lactose			
Malcom and Kiesling 1986	Cattle	1.6 %	+	=	< Bfat			
McEnroe 1986	Cattle	?		+	> Bfat			
Phillips and von Tungen 1985	Cattle	1-2 %	+			+	+	
Quinoez <i>et al.</i> 1988	Cattle	1.36kg/ton	=	=	=			
Quinoez <i>et al.</i> 1989a	Cattle	11b/ton	=			=		
Quinoez <i>et al.</i> 1989b	Cattle	31b/ton	=	=	=			
Wagner <i>et al.</i> 1990	Cattle	1g/kg	=			=	=	
Wells and Mason 1976	Sheep	1.25 %				=		= Carcase grade No deaths due to over-eating
Williams <i>et al.</i> 1991	Cattle	10	+	+	=			Interaction with diet > on high concentrate
Wholt <i>et al.</i> 1991	Cattle - Early Lactation	10	+	+				

Characters in SHADED boxes indicate responses to yeast culture significant at $P \leq 0.05$

1.6: Products containing *Aspergillus oryzae* fermentation extracts.

A. oryzae (Ao) is utilised in the soy and sake fermentations but unlike *S. cerevisiae*, Ao is a multicellular, filamentous organism that forms a vegetative mycelium consisting of septate branching hyphae.

The optimum temperature for its growth is in the 30-35°C range. Good growth up to 42°C has been cited by Thom and Church (1926). The Aspergilli tend to show a preference for more acid conditions but *A. niger* has been successfully cultured at pH 8.8 indicating that these organisms are very adaptable and are capable of growth under a wide range of conditions.

1.6.1: The influence of supplemental *Aspergillus oryzae* on rumen function.

The effects of supplemental *A. oryzae* on rumen parameters are summarised in Table 1.6.1.

a) The effect of *A. oryzae* on carbohydrate metabolism.

i) Effects on Rumen pH

From the Table 1.6.1 it may be seen that *A. oryzae* has no overall effect on ruminal pH. However, Frumholtz *et al* (1989), demonstrated that the characteristic dip in rumen pH usually observed post feeding was abolished. This effect was shown not to be due to *A. oryzae* directly improving the buffering capacity of the ruminal fluid and the mechanism remains unclear. However these results suggest that the fungal supplement may be acting in a similar manner to that suggested of *S. cerevisiae* ie. through a reduction of the rumen lactic acid concentration (Martin and Nesbit, 1992). Fondevila *et al.* (1990), also observed a non-significant reduction in lactic acid concentration, however pH levels tended to be depressed probably due to the increased t[VFA] recorded during their work.

ii) The effects of *A. oryzae* on rumen degradation.

Fogarty and Kelly (1979) showed that Aspergillus species produce a wide range of polysaccharidase enzymes and *A. oryzae* in particular has been shown to exhibit *in vitro* and *in vivo* cellulolytic activity (Walsh and Stewart, 1969; Wiedmier *et al.*, 1987). This observation, along with increased anaerobic bacteria numbers, may explain the improved

ruminal degradability (Gomez-Alarcon *et al.* 1987,1988b,1990; Campos *et al.* 1990), increased VFA concentrations (Arambel *et al* 1987) and improved overall digestibility (Weidmeir *et al* 1987) when the fungus and/or its extract is incorporated in the diet. Gomez-Alarcon (1988b) and Fondevila *et al.* (1990) indicated that total digestibility was not altered but the rate of straw degradability was increased with supplements of *A. oryzae*. Similarly Arambel *et al* (1990), observed no effect of *A. oryzae* on the digestibility of ADF or CP even though cellulolytic numbers tended to be increased. The role of *A. oryzae* on fibre digestion is inconsistent as Martin and Nisbet (1990), found that *A. oryzae* depressed the digestion of the fibre fractions *in vitro*.

iii) The effect of *A. oryzae* on rumen fermentation.

An increase in the A:P ratio brought about by increased acetate concentration has been the most commonly recorded occurrence. However, Martin and Nisbet (1990), noted increased propionate levels causing a significant ($P < 0.05$) reduction in the A:P ratio.

Methane production (*in vitro*) tended to be increased in the work of Martin and Nisbet (1990). This was in contrast to the previous (*in vitro*) work carried out by Frumholtz *et al.* (1989). The work of Frumholtz *et al.* (1989) noted a decrease in methane production and protozoal numbers. They related the reduction in methane production with the increased production of alternative fermentation end products such as butyrate and valerate. If confirmed *in vivo* this effect would imply that *A. oryzae* has a major effect on the intracellular hydrogen pathways in the rumen. Similar decreases in methane have been observed by Williams (1988), using yeast culture *in vivo*.

iv) The response of the rumen microbial population to additional *A. oryzae*.

Increased total anaerobic bacteria numbers have been observed both *in vitro* (Frumholtz *et al* 1989, Arambel *et al* 1987) and *in vivo* (Weidmeir *et al* 1987), but this may not be considered a consistent effect since, Oellermann *et al.* (1990) reported lower proteolytic bacteria numbers in cows fed different concentrations of *A. oryzae*. The ruminal protozoa, total viable bacteria, amylolytic bacteria, cellulolytic bacteria and anaerobic fungi were not significantly altered in

Table 1.6.1: Summary of the effects of supplemental *Aspergillus oryzae* on rumen parameters.

Reference	Species	Dose g/d	pH	t[VFA]	p[VFA]	CH ₄	NH ₃	¹ LDR	² Dg	Microbial effects.
Arambel <i>et al.</i> 1987	<i>in vitro</i>	90		+ Branched	> A:P		+		+ CP	
Arambel <i>et al.</i> 1990	Cattle	80	=						= CP = ADF	= Total bacteria > Cellulolytics > Anaerobes
Beharka <i>et al.</i> 1990	Cattle	3	=	+						
Firkins <i>et al.</i> 1990	Cattle	7.5		=	> A:P		=		=	No effect on MP
Fondevila <i>et al.</i> 1990	Sheep	2	=	=	=		=	=	+ Initial rate	> Anaerobes
Frumholtz <i>et al.</i> 1989	<i>in vitro</i>		=	+	> A:P + But + Val	-	+		=	
Gomez-Alarcon <i>et al.</i> 1987	Cattle								+	> Anaerobes
Gomez-Alarcon <i>et al.</i> 1988b	Cattle <i>in vitro</i>	3							= Fibre > Rate > Fibre	
Gomez-Alarcon <i>et al.</i> 1990	Cattle	3		=	=				> Fibre	
Martin and Nisbet 1990	<i>in vitro</i>	1g/l	=	+	< A:P	+	+		< NDF, ADF	
McKain <i>et al.</i> 1991	<i>in vitro</i>	0.5		=	=		+			> Anaerobes

Characters in SHADED boxes indicate responses to *Aspergillus oryzae* significant at $P \leq 0.05$

Reference	Species	Dose g/d	pH	t[VFA]	p[VFA]	CH ₄	NH ₃	¹ LDR	² Dg	Microbial effects.
Newbold <i>et al.</i> 1991b	<i>in vitro</i>	2		+	> A:P					
Tapia and Heira-Saldana 1989	<i>in vitro</i>	3%							> DMD	
Weidmeir <i>et al.</i> 1987	Cattle	90	=	=			=	=		> Anaerobes

¹LDR Liquid dilution rate (%/H)

²Dg Rumens degradability

Characters in SHADED boxes indicate responses to *Aspergillus oryzae* significant at $P \leq 0.05$

this study. The reduction in protozoal numbers observed by Frumholtz *et al.* (1989) in response to *A. oryzae* might partly explain the increased bacterial counts because of, reduced predation by protozoa. However, the protozoal population in rumen simulators is less stable than that found *in vivo* (Hillman 1987), and the same depression did not occur during the *in vivo* trial carried out by Fondevila *et al.* (1990). Wallace and Newbold (1992), indicated that supplemental *A. oryzae* had no effect on growth in pure culture of the indigenous rumen fungi *Neocallimastix frontalis*, *N. patriciarum* and *Sphaeromonas cumminus*.

b) The effects of *A. oryzae* on rumen N metabolism.

Arambel *et al* (1987), Frumholtz *et al* (1989) and McKain *et al.*(1991) observed increased proteolysis and rumen ammonia concentration in response to supplemental *A. oryzae*. They suggested that this was due to the provision of additional nutrients to the ruminal micro-organisms or possibly via the endogenous proteolytic activity of *A. oryzae* (Boing, 1983). Although ammonia concentration was not significantly affected by supplemental *A. oryzae* in the trial of Weidmeir *et al* (1987), there was a tendency for increased ammonia levels and a significant increase in crude protein digestibility. Firkins *et al.* (1990), was not able to confirm any of these results, observing no increase in ammonia, branched chain VFA of total N digestibility. They went on to suggest that more research was required to elucidate the possible mode of action of *A. oryzae* and its extracts.

1.6.2: The effects of supplemental *Aspergillus oryzae* on animal performance

The observed effects of supplemental *A. oryzae* on animal performance traits are summarised in Table 1.6.2.

Increased milk yield (Huber *et al.* 1986; Wallentine *et al.* 1986; Kellems *et al.* 1987,1988,1990; Gomez-Alarcon *et al.* 1988c and Sievert and Shaver 1990) and increased DMI (Gomez-Alarcon *et al.* 1986; Huber *et al.* 1986 and Sievert and Shaver 1990) are the most commonly observed effects of supplemental *A. oryzae*. This evidence supports the theory proposed by Williams *et al.* (1991) that performance responses result from increased intake, and explains the increased milk yield was observed with additional yeast culture. Gomez-Alarcon *et al.* (1987) carried out two experimental trials and observed no significant ($P > 0.05$) differences in milk yield or feed intake in the first trial when the basal diet of cows was

supplemented with either yeast culture or *A. oryzae*. However, they did show an increase in the rumen DM degradability due to supplementation. In a second trial they looked at the ruminal responses to supplemental *A. oryzae*. The results of this work showed an increase in rumen cellulose digestibility, and efficiency of microbial protein (MP) production (gMP/100g fermented OM) resulting in an increase in MP reaching the duodenum. The increase in MP reaching the duodenum and the improved nutrient digestibility noted by Gomez-Alarcon *et al.* (1988b) and Campos *et al.* (1990) also add support to the theory proposed by Williams *et al.* (1991). However, van Horn *et al.* (1984), observed similar results when they supplemented the diet with 56.7g/d *A. oryzae* but when they doubled the inclusion rate both DMI and Milk yield were reduced.

A reduction in the rate of respiration and in body temperature in response to supplemental *A. oryzae* was demonstrated by Huber *et al.* 1986 and Gomez-Alarcon *et al.* 1988b. The evidence for this effect was not confirmed by Wallentine *et al.* 1986 and Kellems *et al.* 1990, as they observed that supplemental *A. oryzae* elevated rectal temperatures when the ambient temperature was less than 0°C or greater than 32°C.

Table 1.6.2: Summary of the effects of Supplemental *Aspergillus oryzae* on animal performance parameters.

Reference	Species	Dose g/d	Dry matter intake	Milk Yield	Milk Comp.	Live weight gain	Feed conversion efficiency	Other
Campos <i>et al.</i> 1990	Cattle	3						> CP & DM digestibility
Gomez-Alarcon <i>et al.</i> 1986	Cattle	3	+					= DM Digestibility
Gomez-Alarcon <i>et al.</i> 1987	Cattle	?	=	=			=	> MP to hindgut
Gomez-Alarcon <i>et al.</i> 1988c	Cattle - Early Lactation	?	=	+	=		=	> DM digestibility < Anim. Temperature
Huber <i>et al.</i> 1986	Cattle - Mid-Lactation	3	+	+	=	=		< Anim. Temperature < Rate of respiration
Kellems <i>et al.</i> 1987	Cattle - Early Lactation	3		+		=		No effect on fertility
Kellems <i>et al.</i> 1988	Cattle	3	=	+	=	=		No effect on fertility
Kellems <i>et al.</i> 1990	Cattle	3	=	+	=	=		> Rectal temp. No effect on fertility
Sievert and Shaver 1990	Cattle	3	+	+	=			
van Horn <i>et al.</i> 1984	Cattle	3						> Microbial protein flow
Wanderly <i>et al.</i> 1987	Cattle	56.7 113	= -	= -				> ADF digestibility
Wallentine <i>et al.</i> 1986	Cattle	3		+	=			> Rectal Temp. > Rate of respiration

Characters in SHADED boxes represent significant results ($P < 0.05$).

1.7: Possible modes of action of fungal probiotics

1.7.1: A possible sequential model for the mode of action of fungal probiotics.

Figure 1.3 illustrates a possible sequential model for the modes of action of the fungal additives. This model is based on those proposed by Offer (1990) and Wallace and Newbold (1992). In the model suggested by Offer (1990) there were 3 main pathways to increased microbial numbers. The first path considered the direct stimulation of the rumen ecosystem due to the provision of microbial growth promoters, the second pathway concerned the benefits to both the ruminal microflora and the animal due to the supplement providing an alternative "hydrogen sink". This may result in a reduced methane production, and the reduced metabolic hydrogen concentration in the rumen may stimulate microbial activity. The last route suggests that pH stabilisation of the rumen as a result of reduced lactic acid production and a direct buffering effect may result in stimulated microbial growth.

In the model described by Wallace and Newbold (1992), the effects on rumen pH were regarded as a symptom rather than a cause of increased bacterial numbers. Yeast has previously been shown to enhance lactic acid uptake by the rumen bacterium *Selenomonas ruminantium* (Nisbet and Martin 1990a,b), and this was related to reduction in rumen lactic acid concentrations noted in some experiments. Cartwright *et al* (1986), showed that the yeast cell wall has a large buffering capacity. However, the possibility of direct mediation of rumen pH by the yeast cell wall was demonstrated to be untenable by Ryan (1990) due to its low concentration in the rumen. The ruminal pH response to the fungal additives has been very small and often not significant indicating that this may not be the primary mode of action. The increased short term degradation of fibrous material noted by Williams *et al.* (1991) and Chademana and Offer (1990) would suggest that the supplemental yeast culture may be having a direct effect on cellulolysis, that is independent of an interaction with the readily fermentable carbohydrates. Dawson *et al.* (1990) demonstrated that yeast could stimulate the growth *in vitro* of the rumen bacterium *Fibrobacter succinogenes*. The scale of this response was much greater than could be explained by a change in culture pH. It may therefore be assumed that the mediation of the rumen pH was a secondary consequence of the additional yeast culture arising from, but not responsible for, improved microbial numbers and/or activity. However, it is possible that the elevated pH levels may stimulate a further increase in microbial numbers.

The model considered in this work is discussed below:

Increased feed intake (Step 1) and hence energy was shown by Williams *et al.* (1991) to be responsible for the increased milk yield noted in their work. This observation would appear to hold true for the majority of other studies (Tables 1.4 and 1.6).

Increased cellulolysis and flow of microbial protein (Steps 2a,b) provide the most plausible way in which feed intake may be stimulated. These two factors are unlikely to be independent since faster fibre degradation would lead to more rapid bacterial growth and increased microbial protein synthesis because of increased fermentable energy supply. Furthermore, the energetic efficiency of microbial protein may be improved and increased microbial growth rates would decrease bacterial maintenance requirements (Hobson and Wallace, 1982).

In the majority of studies where an assessment of microbial numbers has been made, an increase in the total bacterial count and occasionally the cellulolytic count have been observed. These data help explain steps 3a and b.

The influence of pH and Lactic acid on Steps 4b and d have previously been discussed overleaf. A change in the total concentration and proportions of the VFA (Step 4a) may be as a result of the increased microbial metabolic activity and/or population density in the rumen. These responses have not been shown to be consistent and may be dependent on the initial composition of the microbial populations. A shift in the fermentation pattern away from methane was observed by Williams (1988) and Frumholtz *et al.* (1989). This represents a possible increase in fermentation efficiency as discussed for the ionophore antibiotics. However, this effect is not consistent as Martin *et al.* (1989) and Martin and Nisbet (1990b) observed no increase in methane production when yeast was added to a batch culture system.

The stimulation of the microbial ecosystem (Step 5) may be a response to the provision of a heat labile yeast constituent or metabolite (Step 5a). Newbold *et al.* (1991a), demonstrated that both live and irradiated *A. oryzae* stimulated both cellulolytic and total bacterial numbers, but this effect was lost when the supplement was autoclaved before administration. It is possible that the heat labile substance is an amino acid. Blood and milk plasma concentrations of arginine, glutamine, lysine, valine and methionine in newly foaled mares and their offspring were shown to be significantly ($P < 0.05$) elevated in response to supplemental yeast culture

(Glade and Sist, 1990). Ayoade, Buttery and Lewis (1987), demonstrated that the inclusion of methionine in the diet fed to sheep resulted in an increased rumen VFA concentration and a shift towards propionate at the expense of acetate. Rumen microbial numbers were shown to be elevated with the oral administration of methionine by Huisman *et al.* (1988). Clark and Petersen (1988), in contrast to Ruf *et al.* (1953), indicated that addition of amino acids improved microbial growth rate.

The production of non-heat labile metabolites such as the dicarboxylic acids formate and malate (Step 5c) may provide another method by which specific bacterial populations may be stimulated. Yeast has been shown to hyper-accumulate vitamins and trace elements when it is cultured at pH levels above its optimum (Lyons 1986). These substances would become available to the rumen when the yeast cell finally autolyses (Hough and Maddox 1970; Pepler 1982; Sniffen 1986).

The highly ionic yeast cell wall (Rose, 1987b) may enable it to remove toxic metal ions and oxygen molecules from the rumen liquor (Step 5b). Oxygen was shown by Marounek and Wallace (1984) to be more toxic to rumen bacteria than an increase in rumen Eh. The removal of oxygen from the rumen liquor would therefore benefit the rumen microbial populations.

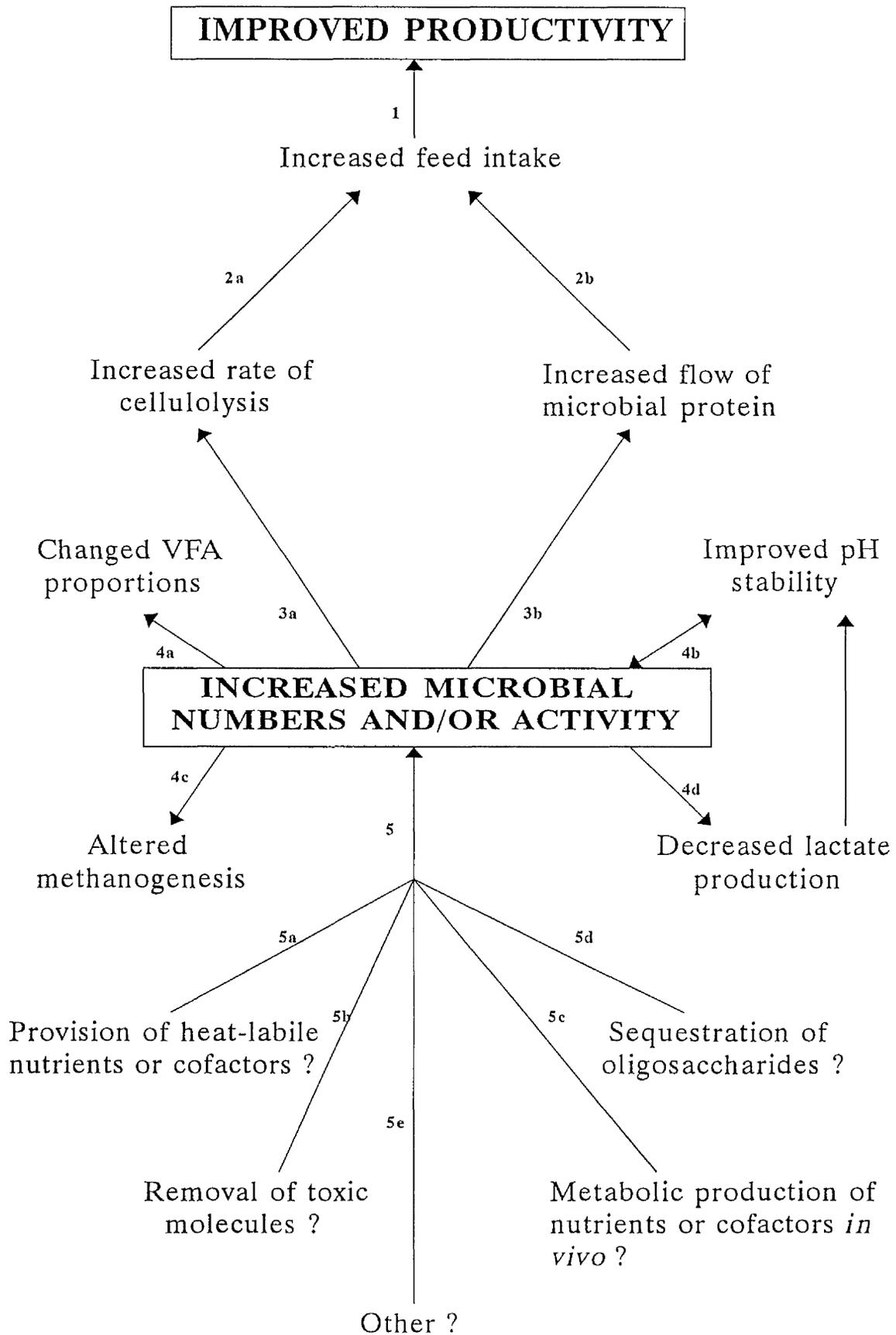
The sequestration of the readily fermentable carbohydrates by the fungal additives (Step 5c) would alleviate the negative associative effects described by Mould and Ørskov (1983) and Mould *et al.* (1983). This would lead to an increase in cellulolysis and a more stable rumen environment for microbial growth.

The other possible modes of action (Step 5e) that have been cited in the literature include, improved palatability of the feed due to the production of glutamic acid by yeast. Lyons (1987) and Newman *et al.* (1990) suggested that yeast culture may produce a selective anti-bacterial substance. It is possible that yeast culture and *A. oryzae* exhibit these two modes of action however it is unlikely that they are the major effects.

1.7.2: Possible interaction with the rumen anaerobic fungi.

Orpin (1975,1977a,b), discovered a stable population of anaerobic fungi within the rumen, previously thought to be transient. Further work revealed that these organisms had the ability to degrade cellulose and metabolise carbohydrates (Orpin and Letcher 1979 and Mountford and

Figure 1.3: A model for the mode of action of fungal feed additives
 (Based on: Offer (1990) and Wallace and Newbold, 1992)



Asher 1983). The end products produced from this metabolism include formate, acetate, ethanol and lactate (Lowe *et al* 1987a,b).

Bauchop (1981) observed that the more fibrous the diet, the greater the fungal population. Orpin (1974) observed that the zoospores (whose release is stimulated by the ingestion of fresh material to the rumen) show an initial chemotactic response to the RFC proportion of the ingested material before they colonise the fibre portion of the ingesta. A preferential colonisation of the structural lignocelluloses, celluloses and xylans was demonstrated by Akin *et al.* (1983). The mycelium they produce penetrating deep into areas not normally accessible to the rumen bacteria.

These effects would provide a method of mopping up the potential lactate precursors and provide a method of making normally inaccessible structural carbohydrates available to the rumen bacteria (Bauchop, 1981). Any synergistic interaction between yeast culture and the anaerobic rumen fungi may result in increased fibre degradation, increased acetate levels and possible pH stabilisation. The possibility of this mechanism should not be ruled out.

1.7.3: Potential hind gut effects

The mechanisms of production and absorption of VFA in the large intestine are assumed to be similar to those in the rumen. Sakata (1987), showed that the mucous epithelium of the caecum wall could rapidly absorb VFA. The capacity for caecal starch fermentation in sheep was demonstrated by Ørskov *et al.* (1970) to be 138g/d. When greater quantities were infused excess starch appeared in the faeces and the faeces became increasingly soft and unpeletted.

Glade and Sist (1987), demonstrated that yeast cultures may enhance feed utilisation by yearling horses. In their trial dietary supplementation with yeast culture resulted in greater fermentative activity by the hemicellulosic and cellulosic microbes and a decrease in endogenous faecal nitrogen excretion in the lower digestive tract. More recently Glade and Sist (1990) and Glade (1991) have demonstrated that supplemental yeast culture can influence the plasma and milk amino acid profiles of mares and resulted in the improved foal performance. Milk yield, energy composition, and digestibility was also shown to be enhanced (Glade 1991).

Newbold *et al* (1990), showed that yeasts can persist in the duodenum and ileum of animals receiving yeast supplements. Viable yeast numbers were shown to be 6.5 times greater than the controls. No estimation of lower tract numbers were made but it is possible that yeast may proliferate in the hind gut of ruminants, exerting similar effects to those noted by Glade and Sist (1987, 1990) and Glade (1991) above. However, Ørskov *et al.* (1970), showed that the microbial protein formed in the hindgut cannot be absorbed in the caecum and is lost in the faeces.

It is unlikely that an enhanced hind gut fermentation could be the sole explanation for the improved animal performance in ruminants discussed earlier. Not only is the efficiency of hindgut fermentation poor (Ørskov *et al.* 1970) but comparatively the large intestine of the cow comprises only 11% of its total digestive tract compared to 60% in the horse (Pagan 1991). The possible role of yeast culture on metabolism in the hindgut is thus diminished but cannot be ignored.

1.8: Summary of the role of probiotics for the improvement of animal performance

With the exception of the experiment of Theodorou (1990) the fungi that are used for the probiosis of the rumen are non-indigenous *Saccharomyces* and *Aspergillus* species. It has been assumed that these organisms exist in the rumen in a transient but metabolically active state. The ruminal responses to supplementation have been shown to be very variable and may depend on the diet type and the initial conditions of the rumen bacterial, protozoal and fungal populations. The improvements in animal performance that have been observed in the majority of published experiments suggest that fungal supplements are exerting some effect on the rumen/host metabolism, even if these responses have not yet been fully described and quantified. However, the possibility of a bias in the experimental data published must be remembered.

There are distinct differences in the modes of action for the probiosis of the gastro-intestinal tract and the rumen. Products aimed at the probiosis of the gastric stomach and intestines are required to either colonise the epithelial wall and mucosa in order to provide a physical barrier to the undesirable enteric micro-organisms, (without the products themselves proliferating to such an extent that they become detrimental) or, provide substances that have anti-microbial properties. These products may also exert their effects by stimulating the specific and non-specific immune system of the host animal. Probiosis of the rumen by contrast is not concerned with the control of enteric pathogens but with improving the efficiency of digestion. In order to achieve this aim it is less important for the product to colonise the target site due to the slower transit of the digesta in the rumen. Therefore, a given probiotic may exert its effects in a sustained transient state, by providing microbial stimulants as part of its metabolism or from within its own cell constituents.

Other criteria for probiotic products include: non-toxicity even at high levels of supplementation, genetic stability and the capability to retain their effects after industrial production and over long periods.

1.9: Introduction to experimental studies

Studies reviewed in the previous sections suggest that supplementation of the diet with small quantities of viable yeast, *Aspergillus oryzae* or their culture extracts may alter the rumen metabolism. However, consistent effects within and between authors are rare. The need for a robust *in vivo* experimental model exists.

The primary aim of this work was to develop an *in vivo* methodology that could show consistent responses to the inclusion of a dietary supplement which would form the basis of attempts to determine the precise mode of action of supplemental yeast culture in the rumen.

The programme of work involves a detailed evaluation of the ruminal responses to supplemental yeast culture using a variety of experimental approaches which are critically assessed.

1. Ruminal responses to treatment in animals fed a semipurified diet twice daily.
2. Gas production *in vitro* of viable and irradiated yeast culture.
3. Ruminal responses to treatment in animals fed a semipurified 3 hourly.
4. Evaluation of contrasting diets on the ruminal response to yeast culture.
5. Evaluation of the daily rumen *in sacco* technique.
6. The effect of supplemental yeast culture on N degradability and rumen metabolism in animals given a conventional diet.
7. The growth response of isolated rumen bacteria to the inclusion of a given treatment in the medium.

Initially the development of the *in vivo* model was the main focus of the work. However, as the work progressed it became apparent that the rumen metabolism exhibited a large day-to-day variation. This discovery led to the inclusion of procedures within progressive experiments designed to investigate the source of the observed rumen variations.

CHAPTER 2: General materials and methods

2.1: Measurement of rumen *in sacco* degradability

The dacron bags used (as supplied by Henry Simons, Box 31, Stockport, Cheshire.) were made with double stitching and rounded shoulders to prevent any lodging of food residues post incubation. The bags were approx. 150mm x 60mm with a pore size of 40 μ m and open area of approximately 25%. The seams were not sealed with a silicon sealant.

Approximately 4-8g (fresh weight) of samples obtained from a well mixed, air dried bulk of substrate was placed into each weighed bag. The bags were reweighed and were then securely tied with 25cm of nylon cord, after twisting the neck of the bag. Sets of three bags were then tied to an appropriate bung with the individual bag strings sheathed in polythene tubing. This system prevented entanglement of the bags within the rumen yet facilitated free movement and mixing within the rumen liquor.

After a given incubation period, the bags were removed and replaced by a new set of bags. The incubated bags were then washed in an automatic washing machine (Zanussi model Z915T). The washing programme "B" was used to wash all bags, this programme consisted of a main wash of approximately 45 minutes followed by 4 cold rinses, each lasting approximately 10 minutes and finally a fast spin, also approximately 10 minutes. A total of approximately 100l of cold water was used. All bags were subsequently oven dried at 60°C for at least 48 hours. Once the bags were dry, they were reweighed in order to establish the dry matter disappearance.

As the bags were prepared, representative samples of substrate was taken in triplicate for dry matter determination (48hrs at 60°C).

2.2: Analysis of rumen liquor

2.2.1: Removal and preservation of rumen liquor

Aliquots of rumen liquor were aspirated from each sheep (mature Suffolk cross wethers) via the cannula using a flexible polythene tube fitted to suction equipment as described by Alexander and McGowan (1969). The pH of each sample was measured immediately using

a portable pH meter. An 8ml liquor sample was taken using a wide bore pipette and placed in individual centrifuge tubes. A 2cm³ aliquot of preservative (25% w/w metaphosphoric acid) was then added to the samples. The centrifuge tubes were then stoppered and mixed by shaking. The preserved samples were then centrifugated for 10 minutes at 3000 rpm. in a pre-cooled centrifuge (4°C). The supernatant was then removed using a disposable pasteur pipette and frozen at -20°C in 7ml bijoux bottles and 1.5ml microtubes awaiting analysis.

2.2.2: Analysis of volatile fatty acid content of rumen liquor

Reagents

Preservative solution

The preservative solution was prepared by dissolving 15g of Metaphosphoric acid (flake - approx. 60% HPO₃) in 100ml of distilled water.

Internal standard

The internal standard was prepared by dissolving 0.22g of Pivalic acid in 100ml of distilled water.

VFA standard solution

Stock solutions

The stock solutions of the individual volatile fatty acids were prepared by making the following quantities up to 50ml in volumetric flasks.

Acetic acid	3.0g to produce a 1M solution
Propionic acid	3.7g to produce a 1M solution
Butyric acid	4.4g to produce a 1M solution
Isobutyric acid	0.44g to produce a 0.1M solution
Valeric acid	0.51g to produce a 0.1M solution
Isovaleric acid	0.51g to produce a 0.1M solution

Working standard

The working standard was prepared by mixing 5ml of acetic and 2ml of each of the other stock solutions in a 100ml volumetric flask and making it up to the 100ml mark with distilled water.

Oxalic acid solution

Oxalic acid (4.725g) was dissolved in 500ml of distilled water.

Sodium hydroxide solution

Aliquots of 1M Analar NaOH were taken from a proprietary solution (BDH Ltd., Poole, UK.) as required.

Procedure

Preserved samples were defrosted and, if necessary, recentrifuged to remove any particulate matter at 1000 rpm. 2.5ml of liquor, 1.1ml NaOH, 0.5ml of internal standard and 2.0ml of oxalic acid were pipetted into a 10ml centrifuge tube and mixed by shaking. The working standard solution was prepared in a similar manner except distilled water was substituted for the 1M NaOH solution. The samples were then centrifuged at 3000 rpm. for 10 mins. in a centrifuge pre-cooled to 4°C. An aliquot of the supernatant was taken and placed in an auto sampler vial ready for GC analysis. 1µl of sample was injected into a glass GC column (2m x 2mm ID packed with 4% Carbowax on Carbowax B-DA 80/120 mesh (Supelchem UK Ltd., Essex, UK). The measurement was carried out isothermally at 175°C using N₂ as the carrier gas at a flow rate of 24 ml/min. An electronic integrator was used to quantify peak areas.

2.2.3: Analysis of the rumen liquor ammonium content

Reagents

Blank solution

The blank solution was prepared by dissolving 30g of Metaphosphoric acid (Flake - Approx. 60% HPO₃) in 100ml of distilled water.

Caustic phenol solution

2.4g of sodium hydroxide (AR), 20g of phenol (AR) and 0.1g of sodium nitroprusside were added to a 2l beaker and the volume made up to 1600ml with distilled water.

Buffer solution

The buffer solution was prepared by mixing 10g of sodium hydroxide (AR), 7.48g of anhydrous Na₂HPO₄ (AR), 63.6g of Na₃PO₄.12H₂O and 20ml of sodium hypochlorite. The mixture was then made up to a volume of 4l using distilled water.

Both the caustic phenol and buffer solutions were stored in dark Winchester at 4°C, and were allowed to warm to room temperature prior to use.

Stock standard solution

7.5892g of pre-dried $(\text{NH}_4)_2\text{SO}_4$ was accurately weighed out and placed in a 500ml volumetric flask. The flask was then made up to the mark using the blank solution.

Working standards

The working standards were prepared by adding the quantities listed below to 200ml volumetric flasks and making them up to the mark with blank solution.

mg/l Ammonia	ml stock standard
0	0
60	5
120	10
180	15
240	20
300	25
360	30

Procedure

The preserved microtube samples were defrosted and diluted 1 to 10 into labelled microtubes using a "Hook and Tucker Diluter III" (Hook and Tucker Instruments Ltd., Croydon, UK.). The diluted samples were then mixed by vortex. The working standard solutions should be treated in the same way. 20 μ l of the samples and standards was then transferred by positive displacement pipette into a 96 well microplate. 80 μ l of caustic phenol reagent was then added to each well using an 8-way pipettor. The microplate was mixed using the microplate reader (Dynatech MR5000; Dynatech Laboratories Ltd., Sussex, UK.) and 200 μ l of buffer added to each well before a second mixing stage. The plate was then left to incubate at room temperature for 1 hour. The absorbance of the plate was then read at 570nm and the ammonia concentration of the liquor calculated using the microplate reader (Dynatech MR5000).

2.3: Assessment of rumen liquid dilution rate

Reagents

Cr-EDTA solution

The chromium ethylenediaminetetraacetic acid complex (Cr-EDTA) was prepared according to the method described by Binnerts et al. (1968).

Acid mixture

The acid mixture was prepared by mixing slowly, with cooling, the following:

- a. 250ml concentrated sulphuric acid
- b. 250ml concentrated phosphoric acid (SG 1.75)
- c. 500ml distilled water
- d. 50ml 15% manganese sulphate

Colour reagent

The colour reagent was prepared by filtering a 0.25% solution of 1.5 diphenyl-carbazide in ethanol into 10 volumes of 0.25M sulphuric acid, using a Whatman number 42 filter paper.

Other reagents

- 1) A 4.5% solution potassium bromate was made up by dissolving 4.5g of KBrO_3 in 100ml of distilled water.
- 2) A 0.45% solution potassium bromate was prepared by further diluting the above solution with distilled water.

Standards

Potassium dichromate (1.936g) was dissolved into 1l of distilled water. This solution was then further diluted to give the following concentrations: 2.5, 5, 10, 20 and 30mg/l. Distilled water was used to provide a blank solution.

Procedure

100ml of Cr-EDTA solution was infused into the rumen via the cannula at 1700hrs. on the day preceding faecal collection. The internal area around the cannula was then stirred using a small length of flexible polythene tubing. This was carried out in order to ensure good incorporation of the marker. Faecal collection commenced at 0900hrs. the following morning and ceased after 88 hours. The faeces were sampled at regular intervals over the intervening period. After collection the faeces were dried at 60°C for at least 48hrs. The dry samples were subsequently hammer milled through a 1mm screen.

Approximately 2g of the milled sample was weighed into a crucible and heated to 550°C for 48hrs. The resulting ash was quantitatively brushed into a dry 50ml wide necked conical flask and the crucible washed with two 3ml portions of the acid mixture, which were also added to

the conical flask. The flask was then heated until the mixture boiled at which point 3ml aliquot of 4.5% KBrO_3 was added to the mixture. Boiling was continued until bromine production ceased. The flask and contents were then allowed to cool to room temperature for 10 mins. 20ml of 0.45% KBrO_3 was then added and the mixture boiled for a further 3-4 mins to complete the oxidation. After cooling, the solution was made up to 100ml with distilled water using a 100ml volumetric flask. An aliquot of the ultimate solution was stored in a 7ml Bijoux bottle awaiting analysis.

250 μl of colour reagent was added to 50 μl of the samples and standards that had previously been pipetted into a 96 well microplate. The resulting mixture was shaken for 30 secs and read at 570nm using the option available on the "Dynatec MR5000" microplate reader. The calculation of chromium concentration was also carried by the microplate reader which used a regression analysis of the standards to compute the results.

2.4: Assessment of urinary allantoin

Reagents

Stock buffer solution

The stock buffer solution was prepared by dissolving 11.503g of $\text{NH}_4\text{H}_2\text{PO}_4$ in 1l of deionised water.

Dilution solution

The solution used to dilute the urine was prepared from the stock buffer solution with the addition of 0.1483g/l of allopurinol.

Eluent A

Eluent A was prepared by diluting the stock buffer solution 1 in 10. The new solution was then adjusted to pH 4 using 0.1M phosphoric acid.

Eluent B

Eluent B was prepared by adding 50ml of stock buffer solution to 350ml of deionised water. The pH was then adjusted to pH 4 using a 0.1M phosphoric acid and ultimately 100ml HPLC uv grade acetonitrile added.

Before and during use, both eluents were degassed using a constant stream of helium.

Standard solution

Uric acid (0.1681g) and xanthine (0.1521g) were dissolved in hot alkalinised (pH > 10) 0.1M NaOH solution. This solution was then allowed to cool and the pH adjusted to pH 6.0 using 0.1M phosphoric acid, before being made up to 500ml in a volumetric flask with deionised water.

Allantoin (0.1581g), hypoxanthine (0.1361g) and creatinine (0.1131g) were dissolved in 500ml of deionised water using a volumetric flask.

The final solution was prepared by adding together the two 500ml solutions. 1.5ml aliquots of this final solution were taken and frozen immediately to await analysis.

Procedure

Total daily urine collections were made using digestibility crates. No preservative was added to the urine but the surfaces of the urine collector were thoroughly washed each day with a 0.1% hypochlorite solution (Deosan - 11% w/w available chlorine) and then water in order to minimise microbial contamination of the urine which may lead to the rapid degradation of the sample. The total weight of urine collected over the 24 hour period was recorded and a 0.075ml aliquot diluted with 1.475ml of buffer solution. The diluted samples were immediately frozen at -20°C before analysis.

Samples and standards were defrosted and centrifuged at 3000 rpm. for 10 mins. to remove particulate matter. The supernatant was transferred to an autosampler vial. 20µl of sample or standard was injected on to the columns using a Gilson 232-401 Bio autosampler (Anachem Ltd., Bedfordshire, UK.). The sample was analysed using two 25cm x 4.6mm C18 reverse phase columns packed spherosorb S5 ODS2 with a 5µm particle size (Phase Separations Ltd., Clwyd, UK.). The measurement was carried out using the gradient programme with a flow rate of 1ml/min shown below.

Table 2.1: Eluent gradients programmed into the pump used for the HPLC analysis of urinary allantoin.

Time (min.)	Eluent A (%)	Eluent B (%)
0	100	0
6	100	0
20	50	50
25	0	100
35	0	100
40	100	0
55	100	0

The purine bases were detected using a UV detector (Spectra Physics 200 programme wavelength detector; Sepetra-Physics Inc., San Jose, CA, U.S.A.) set to measure at a wavelength of 205nm. An electronic integrator (Spectra Physics, Chromjet) was used to quantify peak heights.

2.5: Animals

Mature Suffolk cross, rumen cannulated wethers were used in all animal experiments. All animals were allowed access to water ad libitum throughout all experiments. The numbers of animals and the experiment diets they received have been described within each relevant Chapter.

CHAPTER 3: Determination of the optimum *in vivo* techniques that may be used for the assessment of potential rumen probiotics

3.1: Introduction

The effects of yeast culture on rumen metabolism have been investigated in many ways. Table 1.5.1 illustrates the types of measurements that have been made and the variability in ruminal response to treatment.

Williams (1988) and Williams *et al* (1991), observed a significant ($P < 0.05$) increase in the ruminal pH of steers, whilst Huhtanen (1991), observed no effect on the ruminal pH of monozygote bulls and Harrison *et al* (1987a) noted a significant decrease in ruminal pH of dairy cattle. A similar variation exists in the results for other rumen parameters, such as ruminal ammonia or the proportions of the individual volatile fatty acid (pVFA). However, the most common responses in the cited experiments were either an increase in total VFA, Liquid dilution rate (LDR) and microbial population density or have shown no effect due to supplementary yeast culture (YC). Nevertheless, a singular incidence of depressed tVFA and LDR have been demonstrated by Dawson *et al.* (1990) and Wiedmeir *et al.* (1987) respectively, and this may be a further indication of the possible bias in the data made available for publication. Other measurements that have been made include rumen lactic acid concentration and the nitrogen balance of the animal, but these were not measured in this experiment.

Williams *et al.* (1991), found that the greatest response to additional yeast culture was noted when the diet was high in readily fermentable carbohydrates (RFC). In contrast to this, Chademana and Offer (1990) observed no interaction of yeast with concentrate level. However, with the aim of maximising the possible responses to the yeast supplement, a semi-purified diet containing very little true protein and approx. 60% RFC was formulated.

The aim of this experiment was to develop a sensitive, reproducible method of measuring small changes in rumen function in response to yeast supplementation. To achieve this a wide variety of experimental procedures were assessed for their sensitivity and robustness.

3.2: Materials and methods

3.2.1: Animals and design

Six rumen-cannulated mature wethers averaging 55 kg were assigned to two 3x3 latin square designs (Appendix 4). Each experimental period was 4 weeks long and the first 14 days of each period was an adaptation phase to allow the animals to adjust to the new regimen.

3.2.2: Diet

A semi-purified diet was offered to all sheep. This consisted of 800 g/d of concentrate plus 200 g/d of chopped straw fed in two equal meals at 0900 and 1700 hrs each day. The ingredient composition of the concentrate is given in Table 3.2.1 and chemical composition in Table 3.2.2. The diet was supplemented with either 20 g/d live yeast culture (Alltech Plc. Kentucky, U.S.A.) or 20 g/d irradiated yeast culture or fed unsupplemented according to the experimental design. Water was available *ad libitum*.

Table 3.2.1: Ingredient composition of the semi-purified concentrate

Name	Level (g/kgFW)
Straw NIS	320
Starch	483
Urea	50
Min/Vit mix	2.5
Molasses (cane)	80
Fat 50%	45
Dicalcium phosphate	15
Calcium magnesite	2
Sodium sulphate	2

Table 3.2.2: Chemical composition of the semi-purified concentrate

Dry matter (g/kg)	891
Crude protein (g/kgDM)	152
Metabolisable energy (MJ/kgDM)	12.5

3.2.3: Measurements

Twenty-four hour *in sacco* degradability measurements were made on consecutive days throughout the experiment and the following measurements were made during the last 14 days of each experimental period:

- a) Overall digestibility of OM.
- b) *in vitro* degradability measurements.
- c) Rumen liquor analysis for: pH, total and individual volatile fatty acids (VFA), ethanol and ammonia content.
- d) Microbial analysis of the rumen liquor to determine cellulolytic activity and yeast culture viability.
- e) Microbial analysis of the faeces to determine yeast viability through the alimentary tract.
- f) Urinary excretion of allantoin.

3.2.4: *in sacco* rumen degradability measurements

The rumen degradability of unwashed soya hulls was measured by the *in sacco* technique using 24 hour incubation periods. Soya hulls were chosen for their high cellulose content and because of the homogeneous nature of the bulk sample. Work carried out by R. Dewhurst (personal communication) showed that the 24 hr. degradability of soya hulls was in the exponential region of its degradability curve. It is in this region that responses to small changes in rumen function would be the greatest.

3.2.5: Measurement of in vitro organic matter disappearance (IVOMD)

The analysis for IVOMD was carried out the last day of each period. The technique was based on the method described by Tilley and Terry (1963). The digestion tubes contained a standard feedstuff (soya hulls) and each set of tubes used rumen liquor from different sheep. The digestion tubes were incubated at 37°C for 36 Hrs. Further microbial digestion was stopped by the addition of 6 mls of molar HCl. The tube contents were then filtered, dried and ashed to determine the organic matter disappearance.

3.2.6: Measurement of the overall digestibility of the diet.

The assessment of the overall digestibility of the diet OM was carried out between days 14-20 of each trial period. The total faecal collection for each 3 day period were bulked. All feed refusals were collected daily. The refusals and bulked faeces samples were dried to a constant weight at 60°C to determine their dry matter content. The OM content of the samples was measured by ashing at 450°C for 24 Hrs.

3.2.7: Measurement of urinary allantoin excretion

The total urine excreted over a 24h period during days 14-20 of each experimental period was collected and immediately frozen. The urine sample was defrosted and diluted 1 in 20 prior to injection on to the HPLC column as described in Chapter 2.

3.2.8: Analysis of rumen liquor for VFA and ammonia

Samples of rumen liquor were taken from each sheep on the last 2 days of each 4 week experimental period. A series of samples were taken during the day with the first being extracted just prior to morning feeding and subsequently at 120 minute intervals until 6 hrs. post-feeding. Samples were preserved by acidification and stored frozen (-20°C) until the end of the experiment when they were analyzed for VFA and ammonia concentration according to the methods described in Chapter 2.

3.2.9 Preparation of irradiated yeast culture (IYC)

Approx. 3 kg of viable yeast culture was irradiated near a ⁶⁰cobalt source for 16 hrs. and received a dose of approx. 3.2 MRad.

3.2.10: Rumen yeast culture analysis

Rumen liquor was collected 120 minutes post-feeding on day 24 of each trial period. 10 ml aliquots of rumen liquor were diluted in 90 mls of quarter strength Ringer's solution (Unipath Ltd., Basingstoke, UK.) and "Stomacher" blended for 60 seconds. This dilution was then serially diluted to 10⁻² using Ringer's solution. Oxytetracycline Glucose Yeast extract Agar (OGYA) (Unipath Ltd., Basingstoke, UK.) duplicate plates were then inoculated with these dilutions using a 0.1ml spread plate technique. These plates were then incubated at 15°C for 5 days before the yeast colonies were counted.

3.2.11: Faecal Yeast Culture Analysis

10 g of faeces was collected from each sheep at the end of the experiment. Each 10 g sample was broken up in 90 mls Ringer's solution and then "Stomacher" blended for 60 seconds. This solution was then diluted further to give a 10⁻² dilution. Both dilutions were then plated onto OGYA plates and incubated at 15°C for 5 days. After the 5 day incubation period the number of yeast colonies was counted.

3.2.12: Assessment of Rumen Cellulolytic Activity

Reagents

The Hungate media and diluent tubes were prepared as described in Appendices 1 and 3.

Procedure

A rumen liquor sample was aspirated from each sheep 12 hours post feeding during the last week of each experimental period. From this initial sample, 10 mls was taken and added to

90 mls of anaerobic diluent. This solution was blended for 60 seconds in a "Stomacher" blender.

After blending, a 1ml sample was added directly to the first Hungate diluent tube by removing the seal on the tube. This gave a 10^{-2} dilution. This should be carried out in an anaerobic chamber, however if the operator is quick and works adjacent to a carbon dioxide gas line this operation may be accomplished "on bench" without the inclusion of toxic levels of oxygen. The serial dilution series may then be completed "on bench" using a hypodermic syringe to transfer the tube contents, thus ensuring anaerobiosis is maintained in the tubes. The Hungate media tubes could then be inoculated (0.1ml) in triplicate with the desired dilutions.

The results are obtained after the tubes were incubated for three weeks at 37°C and cellulolytic activity was assessed by observing the filter paper strips for pitting and/or disintegration. The criterion for a positive test at any dilution was that at least two of the triplicate cultures showed cellulolysis.

3.2.13: Interpretation, presentation and analysis of the results

The daily 24 hour *in sacco* degradabilities were analyzed by cusum analysis. This method may be used to detect significant changes in average levels in a time series (that is changes in an observed trend), (Offer 1973).

The other measured parameters were all analyzed using analysis of variance option in Genstat 5.1. The block - treatment structures that determine the degrees of freedom used in the statistical analysis are shown below (Table 3.2.3).

Table 3.2.3: Block - treatment structure used for the analysis of variance.

BLOCK ((SQUARE/SHEEP)*PERIOD)/DAY/SAMPTIME
 TREATMENT SHEEP+(TREAT*DAY*SAMPTIME)

Analysis of Variance table

Source of variation	d.f.
square stratum	1
period stratum	2
square.sheep stratum	
sheep	2
Residual	2
square.period stratum	2
square.sheep.period stratum	
treat	2
Residual	6
square.sheep.period.day stratum	
day	1
day.treat	2
Residual	15
square.sheep.period.day.samptime stratum	
samptime	3
day.samptime	3
samptime.treat	6
day.samptime.treat	6
Residual	90
Total	143

In analyses containing less replication the block structure contained fewer nesting components resulting in a reduced number of strata in the analysis of variance table.

3.3: Results and Discussion

3.3.1: Measurement of the overall digestibility of the diet OM

The overall digestibility of the diet OM was not significantly ($P > 0.05$) affected by treatment or period. The treatment means were found to be 0.739, 0.736 and 0.733 (S.E.D. = 0.010) for control, IYC and YC respectively.

A comparison of the replicate 3 day collection periods in each experimental period indicated that there was a tendency for OMD to be greater in the second set of 3 days. This may have resulted from a depression in OMD during the first three days due to an alteration in the drinking and rumination behaviour of the animals as they adapted to the new environment of the digestibility crate.

3.3.2: *in sacco* degradability of soya hulls

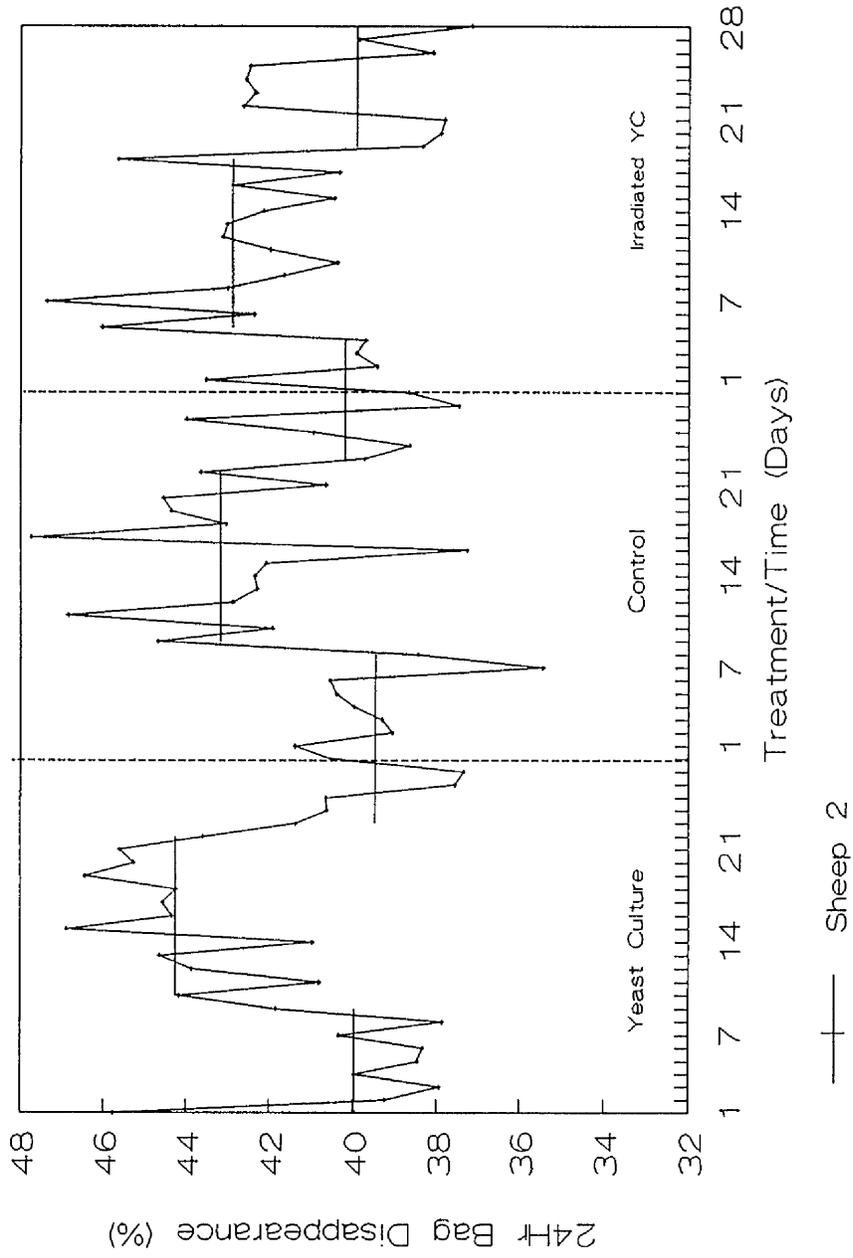
The 24 Hr dry matter disappearance (ISDMD) was not significantly ($P > 0.05$) affected by supplementation with either viable yeast culture (YC) or irradiated yeast culture (IYC).

The individual sheep Cusum Statistical analysis (Figure 3.1 shows data for sheep 2) indicated unstable rumen conditions and appeared unrelated to the supplements given. The cusum means of the individual sheep may be found in appendix 5. The average rumen pH was found to be in the range of 6.07-6.83, however the actual minimum and maximum values recorded were 5.25 and 7.23 respectively. It is therefore likely that the very low rumen pH values may have caused the cellulolytic microflora to have undergone partial washout from the rumen (Mould and Ørskov 1983) and hence with a consequent temporary reduction in cellulolysis during an unspecified period of the day. (Section 3.3.8). As the depression of ruminal pH in a particular sheep on a given day is likely to be highly variable due to variable eating and drinking behaviour, the overall effect on rumen degradation of cellulose is also likely to be highly variable both between sheep and from day-to-day.

Subsequent work attempted to minimise the variations in rumen pH and possible variations caused by the *in sacco* substrate in an attempt to establish the cause of the observed variations in ISDMD.

Figure 3.1

in sacco Degradability of soyahulls



L

The correlation table below demonstrates that the peaks and troughs of the oscillations did not occur at the same time in the different sheep. The *in sacco* bags were prepared and processed in batches, but the results were unaffected by changes from one batch to the next suggesting that variations in ISDMD were not the result of technical error in bag preparation.

Table 3.3.1: Correlation coefficients between daily mean ISDMD values identified by Cusum analysis.

	S1	S2	S3	S4	S5
S2	0.225				
S3	-0.186	0.370*			
S4	-0.186	-0.029	0.050		
S5	-0.005	0.124	0.368*	0.065	
S6	-0.041	-0.113	0.129	0.176	0.260

Values with an asterisk were found to be significant at the $P < 0.1$ significance level.

The analysis of variance was based on the values for the last 7 days of each period. Statistical analysis of this data showed no significant effect of treatment ($P > 0.05$). Treatment means were 39.73, 39.81 and 39.47 for control YC and IYC respectively (S.E.D. = 1.163). However, Sheep 5 was noted to have a significantly lower mean ISDMD than the other sheep ($P = 0.038$). These results are shown in the table below.

Table 3.3.2 Sheep ISDMD (%) means for the last seven days of each experimental period

Sheep 1	Sheep 2	Sheep 3	Sheep 4	Sheep 5	Sheep 6
39.35 ^a	40.41 ^a	41.97 ^a	41.7 ^a	35.47 ^b	39.14 ^a

Means not sharing a common superscripts are significantly different ($P < 0.05$)

3.3.3: *in vitro* OM digestibility (IVOMD)

IVOMD was not significantly ($P > 0.05$) affected by treatment. The treatment means (%) were found to be 51.82, 51.73 and 51.71 for control, IYC and YC respectively. A statistical analysis that compared the individual sheep showed a significant difference between periods. It was assumed that these period differences resulted from experimental error, and was most likely to be due to the variable efficiency of the filtering procedure involved in this technique.

3.3.4: Measurement of urinary allantoin excretion.

Urinary allantoin concentration was measured. Allantoin excretion was expressed as mmol allantoin per kg body weight^{0.75}, as g/day allantoin nitrogen per kg digestible DOM (g/dAN/kgDOM) and as g/day microbial N per kg DOM (gMN/kgDOM) for an average sheep weight of 65 kg. The assumptions used for these calculations are discussed on page 70. Table 3.3.3 shows these results.

Table 3.3.3 (overleaf) indicates that the irradiated yeast culture (IYC) treatment significantly ($P = 0.012$) reduced the mmolA/kgW^{0.75}. However, a statistically significant difference between the periods ($P = 0.096$), and the period x treatment interaction ($P = 0.008$) were also found to exist (Table 3.3.4).

Table 3.3.4: Treatment means of allantoin excretion (mmol/kgW^{0.75}) in each period.

Period	Treatment		
	Control	IYC	YC
1	1.034 ^a	0.711 ^a	0.937 ^a
2	0.564 ^b	0.469 ^b	0.532 ^a
3	0.461 ^c	0.643 ^a	0.553 ^b

Means not sharing a common superscripts in the same column are significantly different ($P < 0.05$).

Table 3.3.3: Microbial yield estimated by allantoin excretion and expressed as g of microbial N per kg OM digested.

Treatment	¹ mmol/A/kg W ^{0.75})	OMD	OMI(g/d)	A (mmol/d)	² g of AN/d	³ gMN/kg DOM
Control	0.686 ^a	0.739	804	15.7 ^a	0.880 ^a	24.7
Irradiated YC	0.608 ^b	0.736	826	13.9 ^b	0.779 ^{ab}	23.3
Viable YC	0.674 ^a	0.733	823	15.4 ^a	0.709 ^b	24.8
S.E.D.	0.0066	10.37	17.45	0.15	0.0084	1.38
F pr.	0.012	0.857	0.504	0.012	0.012	0.591

Means not sharing a common superscripts in the same column are significantly different (P = 0.012).

¹ A = allantoin

² gAN/d was calculated by multiplying the urinary excretion of allantoin (mmol/d) by the molecular weight of allantoin (158.12) and then by the proportion of N in allantoin (34.5%).

³ gMN/kgDOM was derived using the assumptions discussed on page 70

The statistically significant effect of treatment on urinary allantoin levels was assumed to be an artifact as the response in different periods was inconsistent. This inconsistency may relate to the unstable rumen conditions that were demonstrated *in sacco*.

Chen *et al* (1989), proposed that the endogenous purine derivatives excreted in urine originate from the turnover of tissue nucleic acids. Most of the exogenous purine nucleosides and free bases released from the degradation of the microbial nucleic acids are salvaged for re-synthesis of new nucleotides in sheep. Uric acid and allantoin are not salvageable (Gots 1971) making them useful as estimators of rumen microbial biomass. Chen *et al* (1989), demonstrated that a threshold level of urinary allantoin excretion of $0.6\text{mmol/kgW}^{0.75}$ existed which the amount of allantoin excreted was in direct proportion to the amount of microbial nucleic acid leaving the rumen. Below the threshold level the net contribution of endogenous purine resulted in the curvilinear relationship.

The daily allantoin measurements were grouped to form 3 day means that corresponded to the *in vivo* OMD measurements. From this analysis the first 3d mean was noted to be significantly higher than the second mean ($P= 0.004$). The calculated values were 0.774 and 0.538 $\text{mmol/KgW}^{0.75}$ for the first and second 3d collection periods respectively. This difference was probably due to microbial degradation of allantoin in the urine. This was assumed to be the case because the washing procedure that was developed for subsequent experiments was not used during this experiment, and the collection trays were clearly more dirty at the end of each 6d collection period than at the start. As a consequence of these findings a urine degradability study was carried out in order to improve the collection procedure (see Chapter 5).

The poor recovery of allantoin during the last 3 days of collection lowered the grand mean for urinary allantoin excretion ($\text{mmolA/kgW}^{0.75}$) to 0.656 . This would place the amount of allantoin excretion on the border line between a linear and nonlinear response to increased microbial outflow from the rumen. However, when the statistical analysis was restricted to the first 3 days of collection, the $\text{mmolA/kgW}^{0.75}$ fell below the threshold level of $0.6\text{mmolA/kgW}^{0.75}$ in period 2 only (mean value = $0.578\text{mmolA/kgW}^{0.75}$). This suggests that the microbial outflow was sufficient to ensure that the allantoin excretion was in the linear region for the majority of the time. No allowance for the extra DNA added to the rumen via the supplemental YC or IYC was made. Although it is presumed that this amount of extra DNA would only make a minute difference to the total amount of DNA passing out of the rumen.

The efficiency of microbial protein synthesis per kg DOM (gMN/kgDOM) (EMPS) was calculated by making the following assumptions (Dewhurst *et al.* 1987):

- (1) Purine N : Microbial N ratio = 0.10
- (2) True digestibility of purine N = 0.85
- (3) Deamination of purines [1 in 5 lost] = 0.80
- (4) Allantoin N : Total purine derivative N = 0.86
- (5) Allantoin = 35.42% N

Chen *et al* (1992), observed that excretion of purine derivatives per kgDOM increased with with intake. During the present experiment OM Intake was found to increase in the last 3 days of each collection period of this experiment (mean values= 810 and 826 g/d respectively, S.E.D.= 14.22). Table 3.3.3 indicates that whilst there was no significant difference in gMN/kgDOM between treatments, a significant difference between the replicate 3d collection periods may be seen (mean values= 29.2 and 19.4 gMN/kgDOM respectively). This contrasts with the findings of Chen *et al* (1992) but this difference in the results was assumed to result from the poor sampling technique used in the current experiment.

3.3.5: Microbial analysis

3.3.5.1: Assessment of the viable yeasts in the rumen

The number of viable yeasts measured in the rumen (colony forming units (CFU)) rose significantly ($P=0.008$) with the supplementation of 20 g/d of YC. The treatment means were found to be 2.52, 2.26, 5.11 $\text{Log}_{10}\text{CFU/ml}$, for control, IYC and YC diets respectively (S.E.D.= 0.2023). The yeasts observed in the rumen in the during the control and IYC periods were assumed to be due to carry over from a preceding YC period and or due to yeast contamination of the diet mix.

3.3.5.2: Assessment of faecal yeast output

Sheep that were supplemented with yeast culture had a significantly ($P=0.034$) higher viable yeast count per g of faeces than those not being supplemented. The treatment means were

found to be zero for both control and IYC, whereas the YC treatment had a mean of 2.71 $\text{Log}_{10}\text{CFU/ml}$ (S.E.D. = 0.616).

These results suggest that YC has an ability to survive the acid conditions of the abomasum and the action of the bile salts in the small intestines. However, it may be the case that because of the high numbers of yeast cells leaving the rumen, some will survive the transit to the hindgut. Measurements of the ability of YC to survive in the large intestine have not been made, but it is assumed that due to the relatively rapid transit rate of digesta through this region as compared to the rumen, that the YC will exist only in transitory manner. However, hindgut effects cannot be ruled out. Newbold *et al* (1990) also demonstrated that YC could survive passage through the gastro-intestinal tract and suggested that yeast may be having some effects in the hindgut.

3.3.5.3: Assessment of rumen cellulolytic activity

Rumen cellulolytic activity was not significantly ($P > 0.05$) affected by treatment. Treatment means were 8.33, 9.00 and 8.33 for control YC and IYC respectively (S.E.D = 0.425). Figure 3.2 illustrates the varied responses of the sheep to treatment.

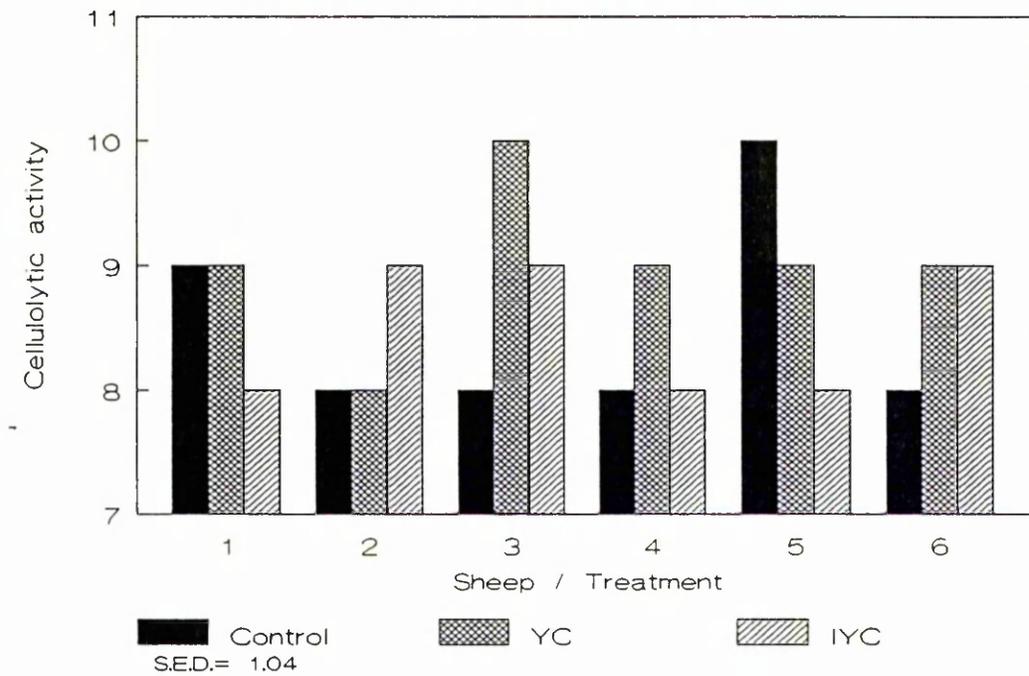
3.3.6: Rumen ethanol concentration (mM)

Rumen ethanol concentration for all sheep times was not significantly ($P > 0.05$) affected by treatment (Tables 3.3.5), but significant effects of time of sampling ($P < 0.001$) and the interaction of time of sampling x treatment ($P = 0.009$) were observed (Figure 3.3).

An increase in the rumen ethanol concentration could be beneficial to the host animal because of the high calorific value of ethanol. However, since ethanol is readily metabolised by the rumen bacteria giving rise to acetate and methane (Czerkawski and Breckenridge 1972), it is unlikely that significant levels of ethanol would build up. The slight ($P > 0.05$) increase in the molar % acetate and decrease in propionate was noted due to yeast supplementation (see 3.3.9) may have resulted from increased flux of metabolic hydrogen into ethanol instead of propionate.

Figure 3.2

Rumen cellulolytic activity response to treatment for each sheep.



¹ Cellulolytic activity: Represents the maximum dilution which was able to degrade a filter paper strip

Figure 3.3

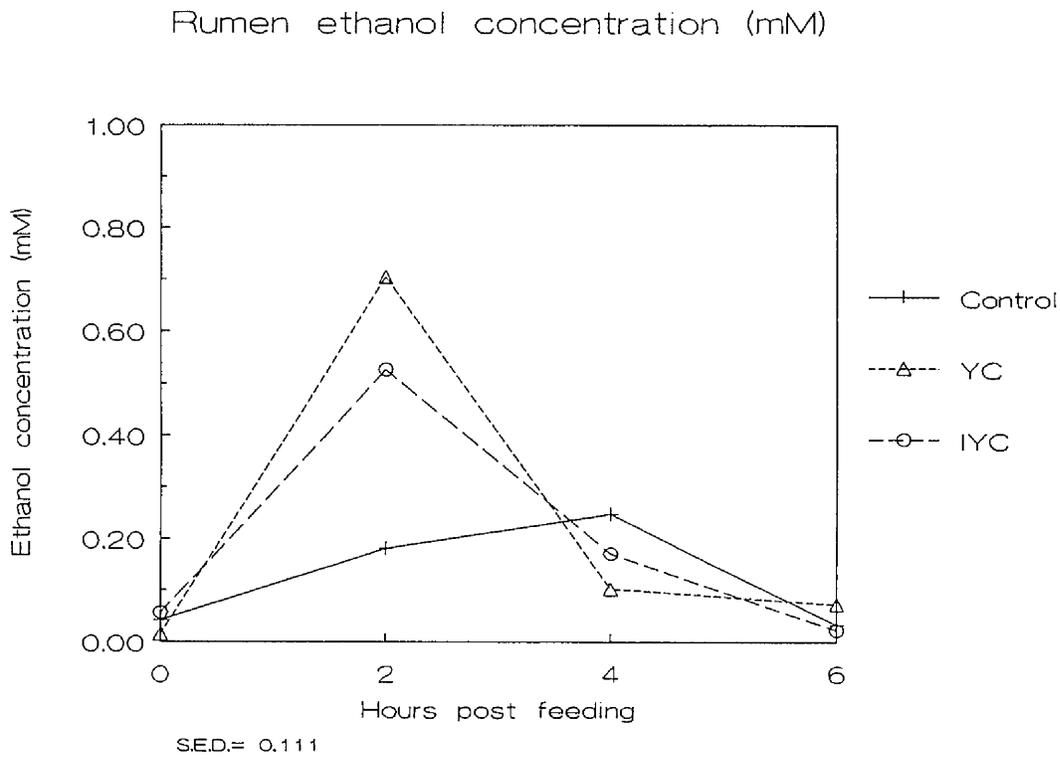


Figure 3.4

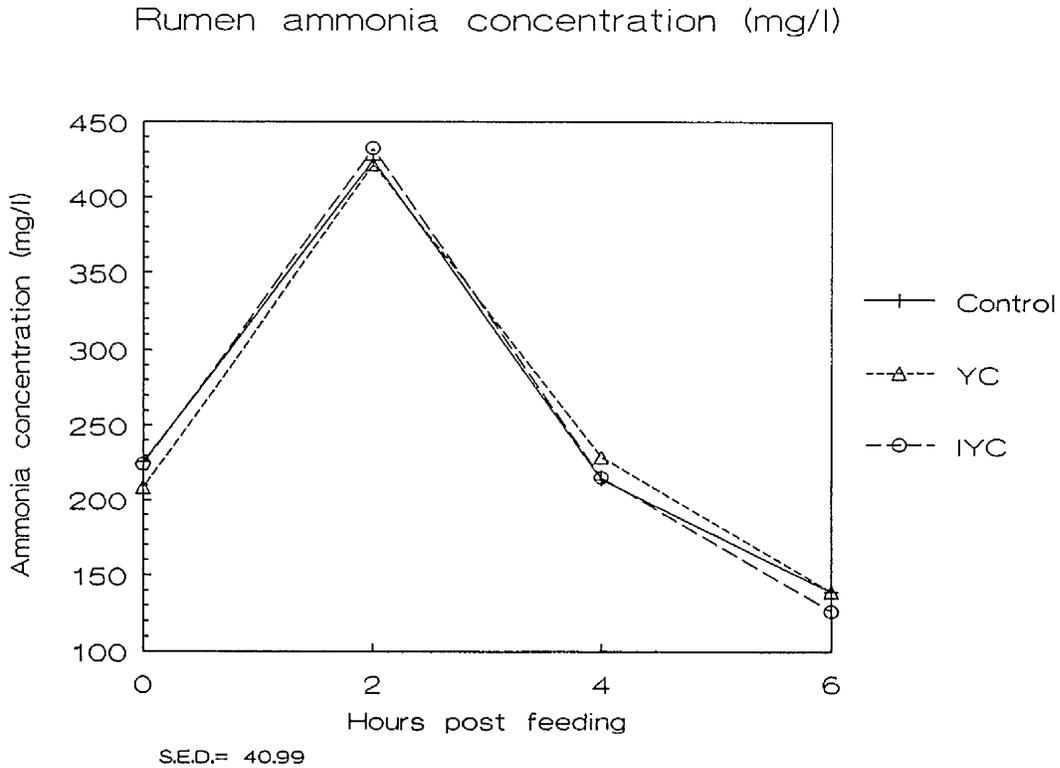


Table 3.3.5: Treatment means for rumen fermentation end-products.

Variate	Treatment			S.E.D.	F pr.
	Control	YC	IYC		
pH	6.40	6.39	6.41	0.052	0.904
NH ₃ (mg/l)	251	250	250	15.7	0.996
Ethanol (mM)	0.2	0.3	0.3	0.070	0.137
tVFA (mM)	64.2	65.6	64.0	4.19	0.918
Acetate (mM)	46.8	47.8	46.8	2.64	0.918
Propionate (mM)	10.8	9.8	10.1	1.23	0.711
Butyrate (mM)	5.4	6.8	6.0	0.99	0.424
isoButyrate (mM)	0.4	0.4	0.4	0.032	0.501
Valerate (mM)	0.5	0.5	0.4	0.099	0.934
isoValerate (mM)	0.2	0.2	0.2	0.023	0.285
Acetate (molar %)	72.8	73.9	73.5	1.16	0.628
Propionate (molar %)	16.9	15.0	15.2	1.27	0.327
Butyrate (molar %)	8.5	9.2	9.3	0.72	0.465
isoButyrate (molar %)	0.7	0.6	0.6	0.036	0.268
Valerate (molar %)	0.7	0.7	0.7	0.107	0.964
iosValerate (molar %)	0.3	0.3	0.3	0.032	0.259
Ac:Pr Ratio	4.6	5.0	5.0	0.460	0.584

Significant differences due to the time of sampling were observed ($P < 0.001$) and these results are expressed graphically in Figures 3.3 - 3.13.

3.3.7: Rumen ammonia concentration

Rumen ammonia concentration was not significantly affected by treatment (Table 3.3.5). However, the ammonia concentration of the rumen was observed to vary significantly with time of sampling ($P < 0.001$). Figure 3.4 illustrates that a peak ammonia concentration of approx. 425 mg/l was observed 2 hours post feeding. This very high peak concentration was likely to have resulted from the rapid degradation of the urea portion of the diet and may have exerted

a buffering affect on the rumen, which may have offset the pH depressing effect of the t[VFA]. The rapid fall of rumen pH post-feeding probably contributed to large increases in rumen ammonia concentration as absorption of ammonia is greatest at pH values nearer to its pK (9.02). The reduction in rumen ammonia after 2 hrs. resulted from both absorption through the rumen wall and assimilation by the rumen micro-flora. Meang and Baldwin (1976), working with cattle fed a more fibrous diet than the present study and containing urea as the sole source of N, observed a peak concentration of 500 mg/l at 4 hours post feeding. This corresponded to peak VFA production in these animals, suggesting that the rate of fermentation in these animals was slower due to the increased fibre content of their diet.

No significant interaction between the time of sampling and treatment ($P > 0.05$) was observed as the diurnal pattern of rumen $[\text{NH}_3]$ was unaffected by treatment (Figure 3.4).

3.3.8: Rumen pH

Table 3.3.5 shows that mean daily rumen pH was not significantly ($P > 0.05$) affected by treatment. However, rumen pH was found to vary significantly ($P < 0.001$) with time of sampling (Figure 3.5).

It is assumed that rapid fermentation of the starch content of the diet was responsible for the low pH levels induced post feeding. Although the treatment means indicate that rumen pH was maintained above pH 6.1 values below 6.0 were recorded in some sheep 4 hours post feeding. These low pH levels are likely to be the cause of the unstable rumen conditions due to the negative associative effect described earlier and may have been a factor in the instability noted in the *in sacco* measurements.

The varied responses noted in the cellulolytic activity may also be as a result of the low pH levels. This is supported by the findings of Mould and Ørskov (1983) who showed a partial washout of the rumen microflora when the ruminal pH fell below 6.0.

Ørskov and Ryle (1990), discussed the effect of rumen pH on the relative absorption rates of the individual VFA. They concluded that ruminal pH, particularly at low levels has a marked effect on the relative absorption of the individual acids. They cited the results of MacLeod *et al* (1984) who, working with lambs wholly maintained by VFA infusion, found that the A:P ratio increased as rumen pH decreased. Previously it has been assumed that the VFA

Figure 3.5

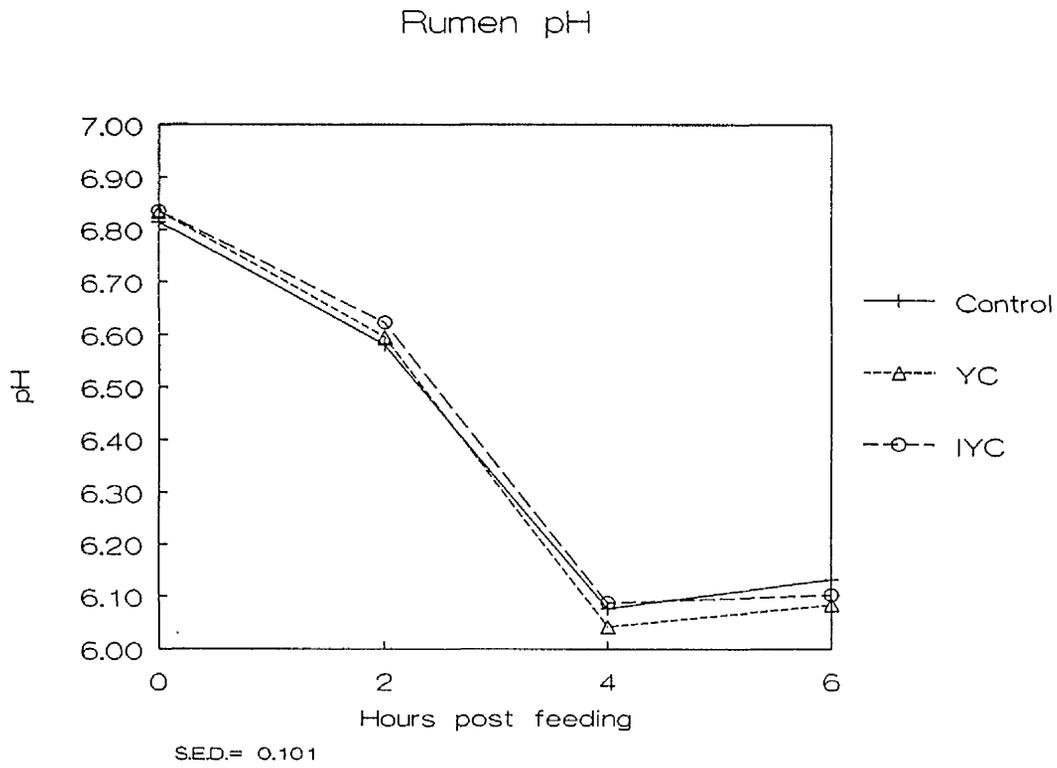
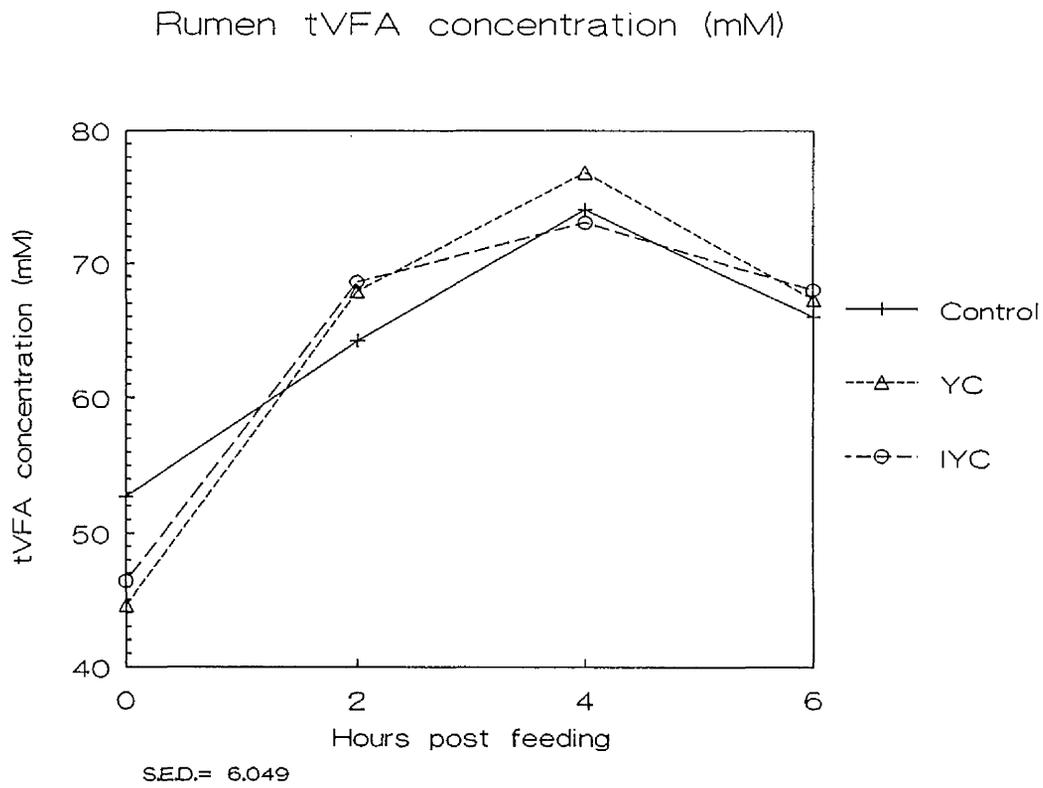


Figure 3.6



proportions in the rumen represent the proportions in which they were produced, but allowance for the differential rates of VFA absorption must be made when comparing the relative proportions of the individual VFA particularly at low rumen pH levels. For the purposes of this experiment the effect of rumen pH on the absorption of the individual VFA was assumed to be the same for all treatments as rumen pH was not effected by treatment.

3.3.9: Volatile fatty acids

3.3.9.1: t[VFA]

The total VFA concentration and the VFA mix was not significantly ($P > 0.05$) affected by treatment (Tables 3.3:5). However, the concentration of the tVFA was found to alter significantly with time of sampling ($P < 0.001$). Figure 3.6 illustrates the diurnal rumen tVFA concentration. From this figure it may be noted that t[VFA] increased steadily until 4 hours post feeding after which the ruminal concentration of tVFA began to fall. Rumen pH (Figure 3.5) showed an inverse relationship to tVFA ($r = -0.869$; $P < 0.05$) in accordance with the observations on the relationship between rumen tVFA and pH reported and discussed in Chapter one (Figure 1.1)

Figure 3.6 shows that t[VFA] was increased by supplementary viable yeast culture 2, 4 and 6 hrs. post-feeding. This increase due to YC was not found to be significant ($P > 0.05$) and is assumed to arise from non-significant increases in rumen acetate and butyrate absolute concentrations (Figure 3.7 and Figure 3.11 respectively) which offset the decrease relative to that of the control in the rumen mM concentration of propionate found 4 and 6 hrs. post-feeding (Figure 3.9). The IYC treatment was found to have a peak t[VFA] lower than the control diet. This resulted primarily from lower concentrations of acetate as the decrease in the rumen absolute concentration of propionate compared to that of the control was no greater than that of the YC treatment at this time. However this effect was partly offset by an increased concentration of butyrate due to supplementary IYC, resulting in only a small reduction in the observed t[VFA].

3.3.9.2: Major volatile fatty acids

The mean daily concentration (mM) and the proportions (molar %) of t[VFA] of the individual VFA were not significantly ($P > 0.05$) affected by treatment (Table 3.3.5). However, both the rumen concentration and the molar % were found to alter significantly ($P < 0.001$) with time of sampling (Table 3.3.6).

Table 3.3.6: Probability values for the effect of time of sampling on the major VFA.

	mM	Molar %
Acetate	$P < 0.001$	$P < 0.001$
Propionate	$P < 0.001$	$P = 0.122$
Butyrate	$P < 0.001$	$P = 0.015$

The interactions of treatment with time of sampling and treatment with day were also found to be significant ($P < 0.05$) for molar % propionate (Figure 3.10) and for acetate and propionate concentrations respectively (Table 3.3.7).

The combination of increased post-prandial rumen acetate and butyrate and decreased propionate concentrations due to treatment resulted in a shift in the molar % proportions of the individual VFA. However, only the interaction of sampling time and treatment for propionate (molar %) was found to approach significance ($P = 0.055$). It may be seen from figure 3.10 that both YC and IYC reduced the molar proportion of propionate post-feeding. The probability value for the interactions for acetate and butyrate with time of sampling were found to be $P = 0.155$ and $P = 0.748$ respectively. There was also a tendency for the YC and IYC treatments to increase the molar % acetate. However the mean daily acetate : propionate (A:P) ratio was not found to be significantly affected by treatment ($P = 0.584$). Figure 3.13 shows the diurnal variation of the A:P ratio. The interaction of time of sampling and treatment was found to be significant only at $P = 0.112$. It may be seen from Figure 3.13 that the three treatments have different diurnal patterns, but also that A:P ratio for both the YC and IYC treatments increases 2 hrs. post-feeding.

Figure 3.7

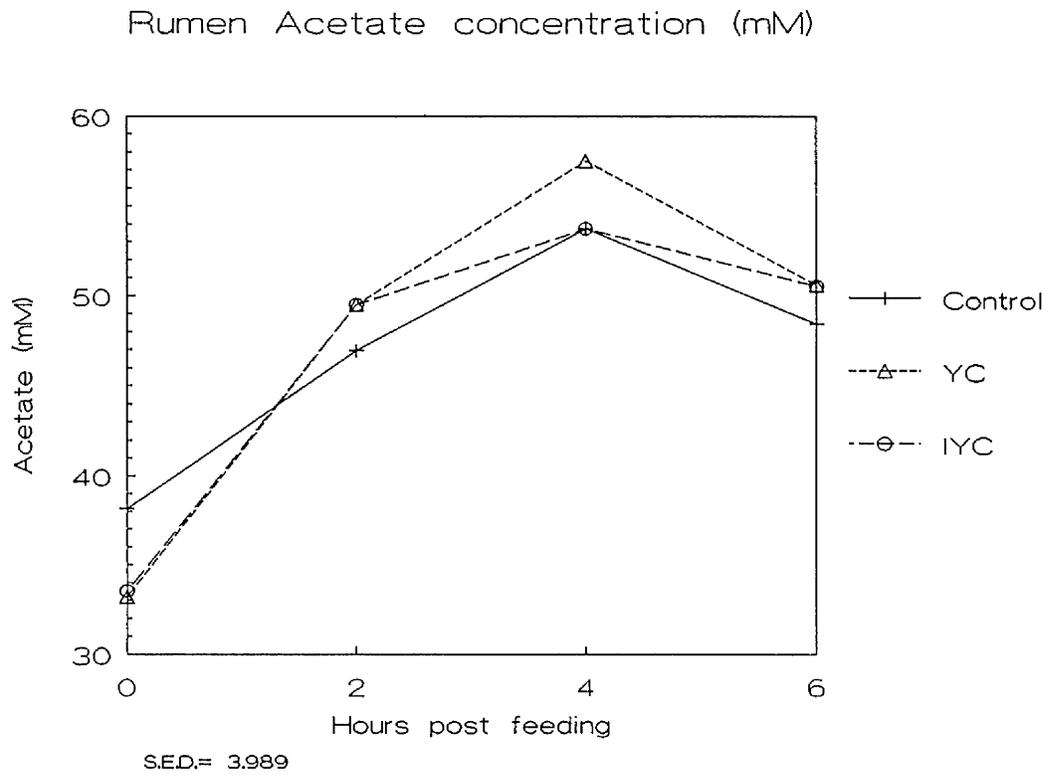


Figure 3.8

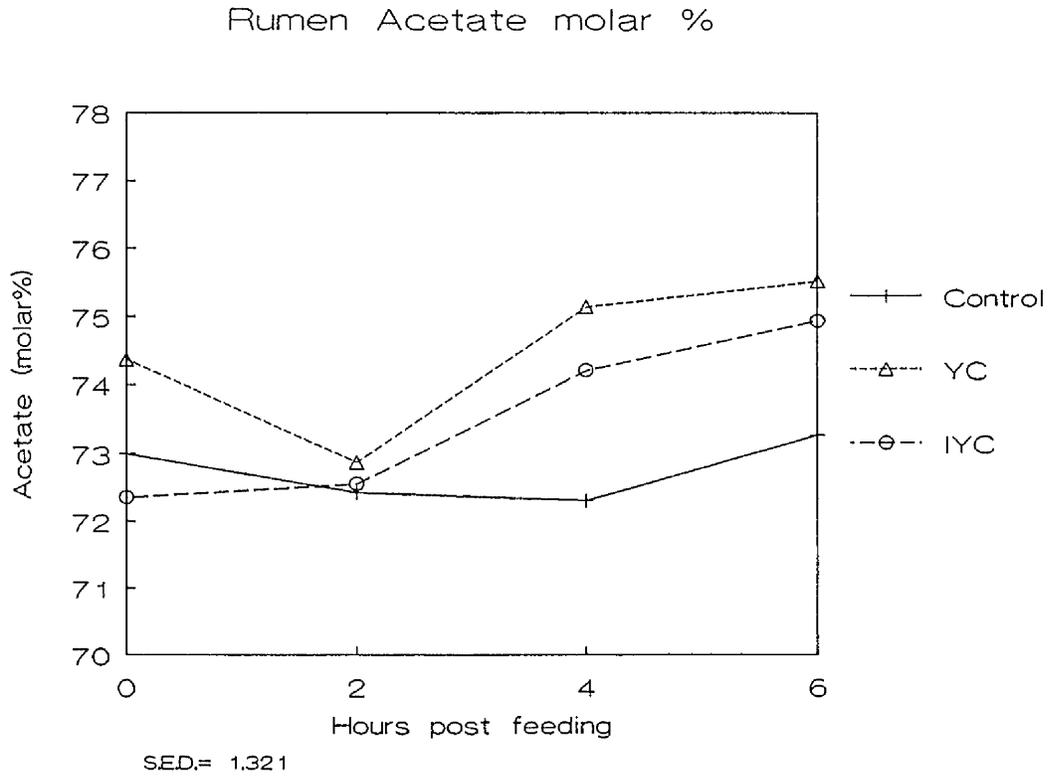


Figure 3.9

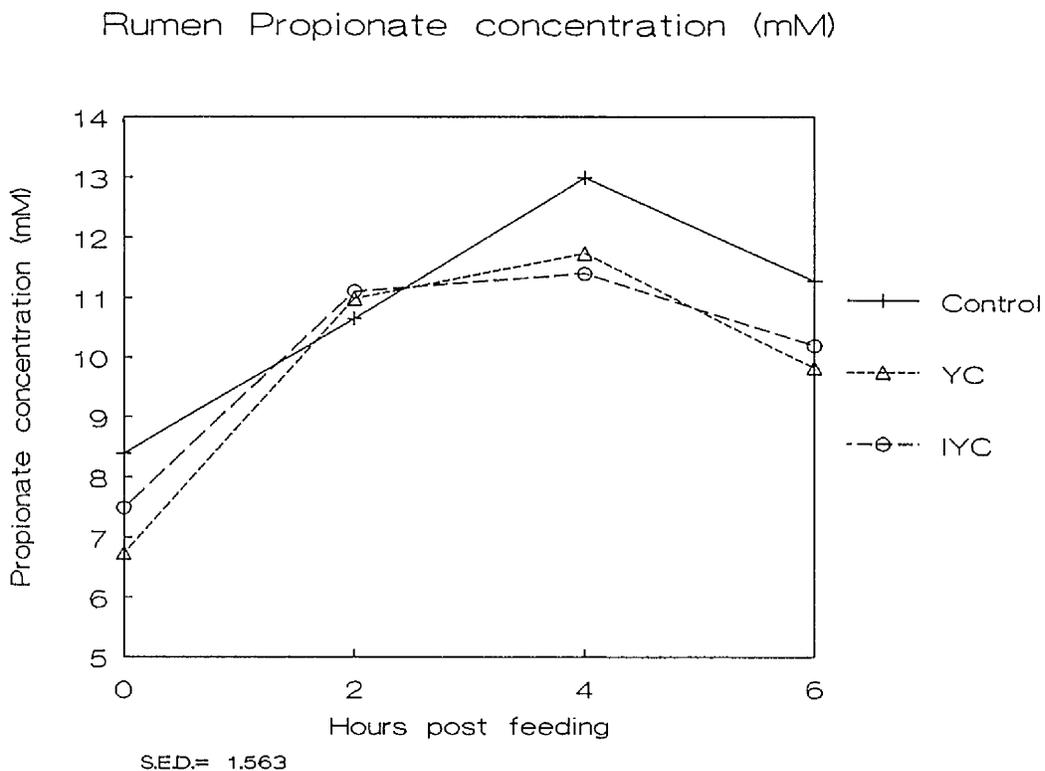


Figure 3.10

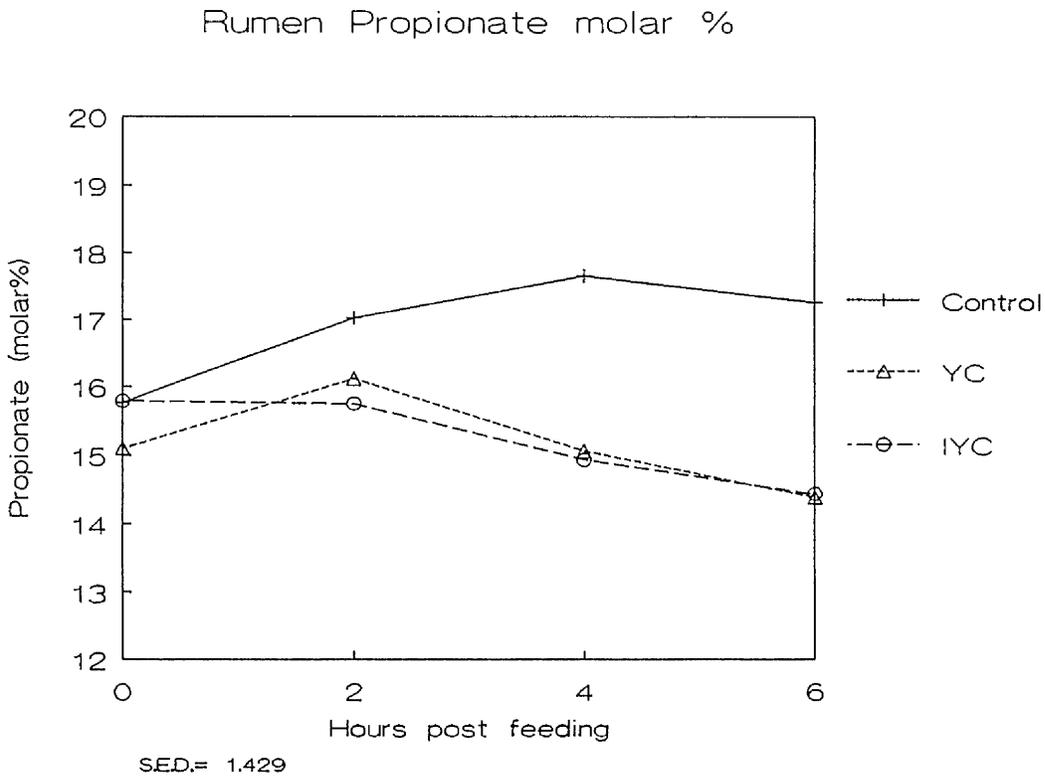


Figure 3.11

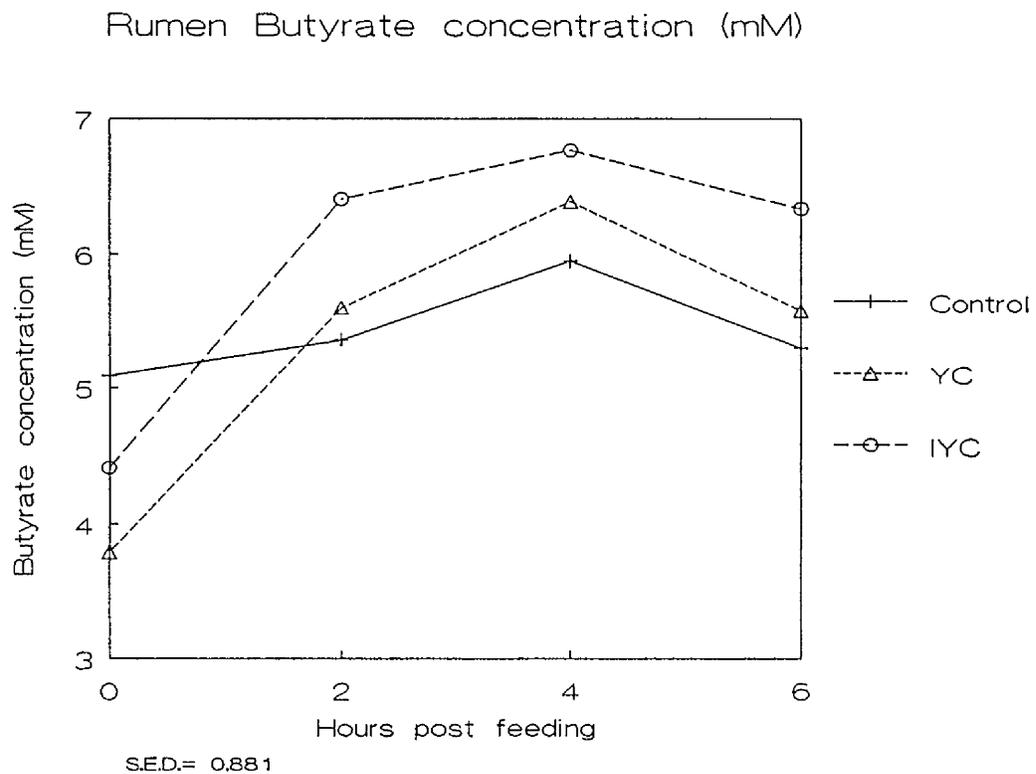


Figure 3.12

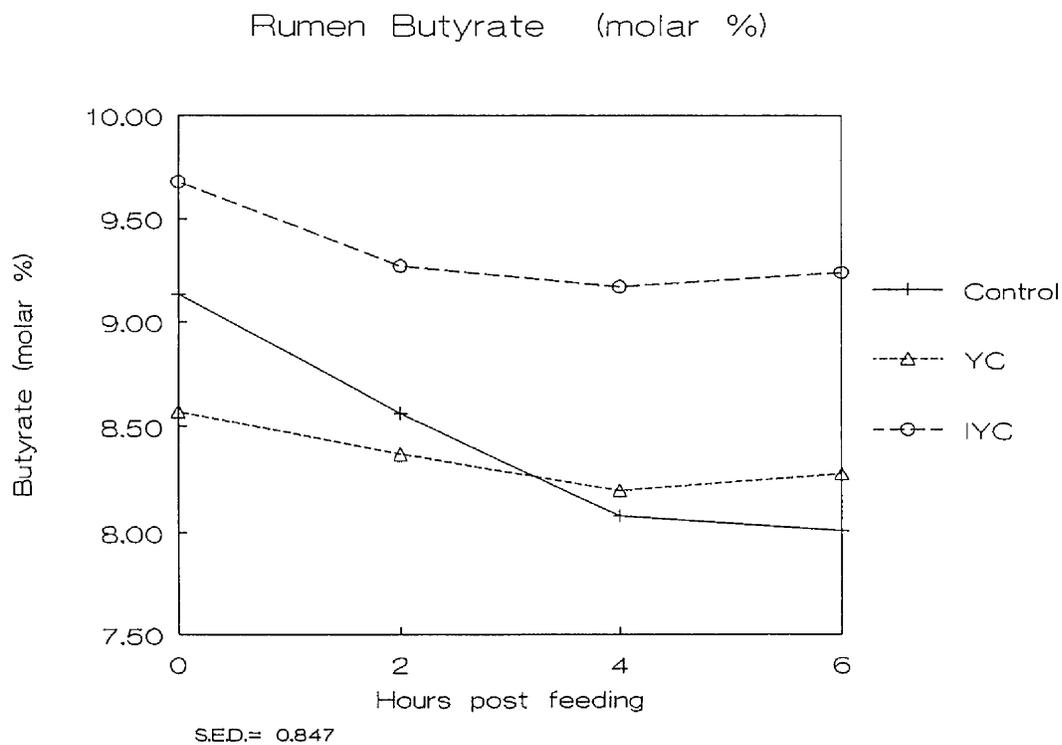
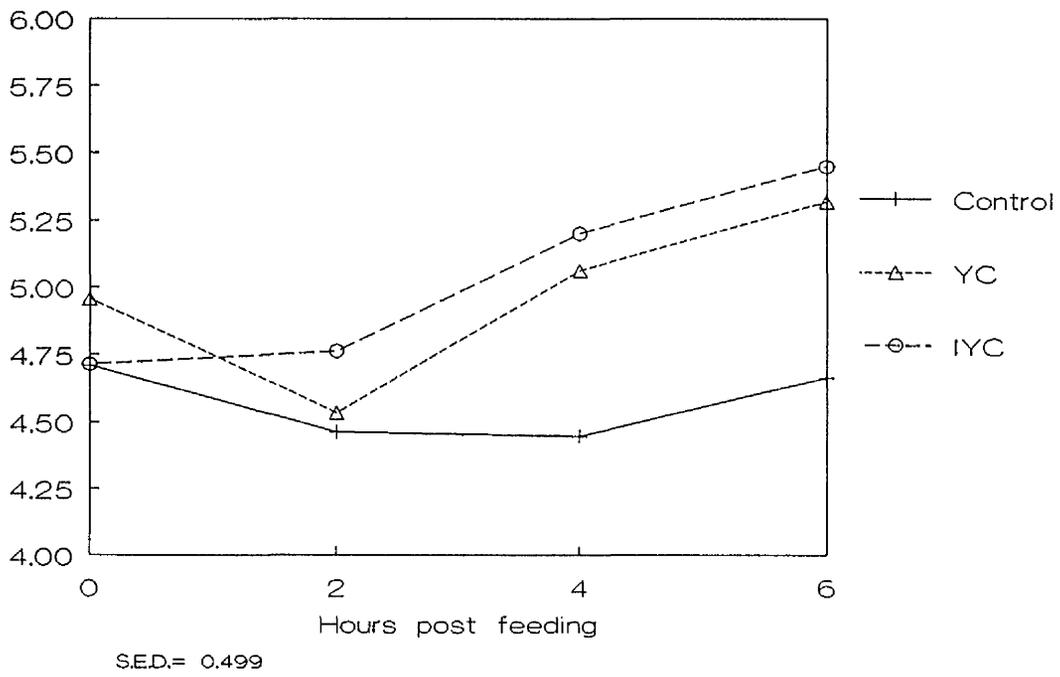


Figure 3.13

Rumen Acetate : Propionate ratio



No significant difference ($P > .05$) between the two days of sampling was found. However, the interaction between treatment and sampling day was found to be significant for acetate and propionate (Table 3.3.7).

Table 3.3.7: The effect of treatment on the mean VFA concentration (mM) of the major acids collected on each day of sampling

VFA	Day	Treatment			Interaction FPr.
		Control	YC	IYC	
Acetate	1	51.2	45.08	47.84	0.005
	2	42.4	50.33	45.79	
Propionate	1	11.67	9.21	9.94	0.02
	2	9.99	10.43	10.16	
Butyrate	1	6.17	5.09	5.95	0.19
	2	4.68	5.59	6.01	

Table 3.3.7 shows that the interaction between the YC and IYC treatments and the day of sampling induced lower acetate, propionate and butyrate levels as compared to the controls on the first day of sampling and higher levels as compared to the control on the second day of sampling. The reasons for this interaction were not clear but were assumed to relate to the inherent rumen instability observed *in sacco* since it is not credible that the YC and IYC supplements induced a different effect on different days.

3.3.9.3: Minor volatile fatty acids

The molar % of the minor acids isobutyrate, isovalerate and valerate were not significantly affected by treatment ($P > 0.05$). The treatment x sampling time and treatment x day interactions for these acids were also found to be non-significant ($P > 0.05$), but the molar % values were found to alter significantly ($P < 0.001$) with time of sampling.

The effect of treatment on the absolute concentrations of these acids was also found to be non-significant. However, time of sampling had a significant effect on all the acids. Isovalerate and valerate were found to vary significantly ($P= 0.095$ and $P=0.035$ respectively) between day of sampling. The day x treatment interaction of valerate was also found to be significant ($P=0.007$).

The branched chain VFA (C4 and C5) are normally produced by the deamination of amino acids, however they may also arise from methylmalonyl-CoA, an intermediate in the production of propionic acid (Czerkawski 1986). Felix *et al* (1980) suggested that when feeds are low in protein or contain low quality proteins, the branch chain fatty acids may be a limiting factor on the growth of ruminal organisms. Dehority *et al* (1967) working with pure cultures *in vitro* found that the minimum concentration of the branch chain fatty acids required for growth were:-

Species	sub species	Valeric mg/l	2-methylbutyric acid (mg/l)	Isobutyric acid (mg/l)
<i>B. succinogenes</i>	A30	0.1	0.05	0.1
	B21	0.1	0.05	0.1
	S-85	0.1	0.05	0.05

The levels of branch chain VFA found during this experiment were 33.22, 17.02 and 46.35 mg/l for isobutyrate, isovalerate and valerate respectively, therefore these microbial growth factors were not likely to be limiting microbial growth.

3.4: Conclusions

The semi-purified diet was designed to induce stressful conditions for the rumen microflora in two ways. Firstly the high levels of starch in the diet were consistent with a low rumen pH, and secondly little true protein was involved to limit the supply amino acids and peptides for microbial assimilation. However, due to the diet being fed as two relatively large meals per day, the wide diurnal variation in rumen pH achieved appeared to have induced unstable rumen conditions. Future experiments designed to overcome this problem by increasing the frequency of feeding, or by decreasing the level of starch in the diet.

The unstable rumen conditions induced by the diet made the detection of treatment responses very difficult. However, whilst no evidence of an increased ruminal degradation or microbial protein production was observed, the inclusion of yeast culture (viable or irradiated) appeared to alter the diurnal VFA fermentation pattern. The total VFA concentration and mean daily concentrations of the individual VFA were not affected by treatment, although treatment significantly affected ($P=0.055$) the diurnal pattern of molar % propionate. Rumen ethanol levels in the 2 hrs immediately following each meal were increased as a result of the inclusion of yeast in the diet. However, the levels detected were very small and unlikely to have any significant effect on the host animal metabolism, but may be linked to the altered diurnal production of acetate.

The analysis of cellulolytic activity in the rumen (liquid phase) also indicated that unstable rumen conditions existed. The microbial analysis of yeast viability in the rumen and hind gut suggested that the live yeast culture was capable of surviving both ruminal and hind gut conditions. However the results also indicated that the yeast culture was unable to form a stable population in the rumen since rumen yeast counts observed on the IYC and Control treatments in the experimental periods post YC supplementation were lower than the viable yeast counts observed when the rumen was supplemented with the viable YC supplement.

Summary of results

- 1) Overall digestibility of diet OM was not significantly ($P > 0.1$) affected by supplemental YC or IYC.

- 2) Supplementation with YC or IYC had no statistically significant effect ($P > 0.05$) on the *in sacco* degradability of soya hulls. However the results showed that the rumen was inherently variable on a day-to-day basis.
- 3) IVOMD was not significantly ($P > 0.05$) affected by either supplemental YC or IYC.
- 4) The IYC treatment significantly ($P < 0.05$) reduced the concentration of allantoin excreted per day, but the efficiency of microbial protein production was not significantly affected by treatment ($P > 0.05$).
- 5) The viable yeast culture was shown to be able to survive in the rumen and during passage through the whole digestive tract.
- 6) Rumen cellulolytic activity was not significantly affected ($P > 0.05$) by treatment.
- 7) The mean daily rumen liquor ethanol concentration was not significantly affected ($P > 0.05$) by treatment, but a significant interaction between treatment and time of sampling was observed ($P < 0.01$).
- 8) Rumen supplementation with either IYC or YC had no statistically significant effect ($P > 0.05$) on rumen ammonia concentration, but the diurnal variation in ammonia concentration was found to be significant ($P < 0.001$).
- 9) Rumen pH was not significantly affected by treatment ($P > 0.1$). However the diurnal variation in rumen pH was found to be significant ($P < 0.001$).
- 10) The t[VFA] was not significantly ($P > 0.1$) affected by treatment, but the variation with time of sampling was found to be significant ($P < 0.001$).
- 11) Treatment had no significant ($P > 0.1$) effect on the mean daily concentrations of the major VFA. However the diurnal variation of these acids was found to be significant ($P < 0.001$) for the molar % of propionate the treatment x time of sampling interaction was found to be significant ($P = 0.055$) for both the YC and IYC treatments. It was noted that the inclusion of YC or IYC led to a reduction in propionate (molar %) post feeding when values increased for the control treatment. The day x treatment interaction was also found to be significant for the molar concentration of acetate and propionate ($P < 0.01$ and $P < 0.05$ respectively).
- 12) The minor VFA were not found to be significantly affected by treatment ($P > 0.05$). However, the diurnal variation of these acids was significant ($P < 0.001$).

The overall instability of rumen conditions in this experiment made it difficult to detect small changes in rumen function due to addition of supplementary YC or IYC. However, the experiment did show that the diurnal pattern of VFA and ethanol production was affected by the addition of the yeast culture supplement (viable or irradiated) to the diet, although the significance of the effect of the YC and IYC supplements on VFA production was weakened

by the significant interaction between treatment and day of sampling ($P < 0.05$). The shift in the VFA mix away from propionate could have important practical implications for the feeding of dairy cattle on high concentrate diets. Lees *et al* (1990), found that the response of dairy cows to the protein level in the concentrate varied with the type of fibre used in the concentrate (fibrous or starchy). Their work showed that the starchy concentrate induced a shift in the fermentation mix towards propionate production and this increase in the production of propionate was associated with increased plasma insulin levels. The concentration (g/kg) of fat in the milk was noted to be lower on the starchy concentrate than on the fibrous concentrate ($P < 0.05$), and the live weight of the cows receiving the starchy concentrate was found to recover more quickly. These effects were also associated with the high propionate levels observed on the high starch concentrate diet. They concluded that the higher levels of propionate noted when the high starch concentrate was fed stimulated plasma insulin secretion which created a shift in the partition of nutrients away from milk synthesis and towards body reserves. The deposition of milk protein and milk yield were not significantly ($P > 0.05$) affected by concentrate type.

CHAPTER 4: Determination of the metabolic activity of IYC by measuring the evolution of gas (*in vitro*).

4.1: Introduction

The results of the first experiment suggested that irradiated yeast culture affected the rumen in a similar manner to the viable yeast supplement. Newbold *et al* (1991a) noted similar results during their work with irradiated, autoclaved and viable *Aspergillus oryzae* (Ao). They observed that autoclaving the Ao supplement destroyed its capacity to affect rumen microbes *in vitro* whereas the response *in vitro* to irradiated supplement was reduced, but similar to that for the viable Ao supplement. They concluded that the supplement need not be reproductively active and that the causal agent is heat labile.

It may be theorised that irradiating the fungal supplement results in it being unable to reproduce, but that its enzymes remain metabolically active. The aim of this experiment was to determine the metabolic activity of irradiated yeast culture (IYC). To achieve this an aliquot of nutrient medium was placed in an air tight gas syringe either with, or without inoculation of the viable yeast culture (YC) or IYC supplement. The cumulative amount of gas produced was recorded and statistically analyzed.

4.2: Materials and Methods

4.2.1: Preparation and analysis

Malt extract broth (Unipath Ltd., Basingstoke, UK.) was prepared and the pH adjusted to 6.4. The medium was then saturated with carbon dioxide gas by bubbling the gas through the solution for 30 mins. A 50 ml aliquot of the medium was then dispensed into a 100 ml ground glass gas tight syringe (Rockett, London), each syringe was simultaneously inoculated (0.5g) with either IYC, YC or nothing. Syringes were prepared in quadruplet. The syringes were then incubated at 37°C in a water bath. The amount of gas evolved was recorded at regular intervals.

4.2.2: Interpretation, presentation and analysis of the results

The results were statistically analyzed using the analysis of variance option in Genstat 5.12 (Table 4.2.1) and presented graphically (Figure 4.1). Further to this the results were fitted to the Gompertz growth curve (using the fitcurve option in Genstat 5.12

Table 4.2.1: Block - treatment structure for the analysis of variance

```
BLOCK SAMPTIME*REPS
TREATMENT TREATMENT*SAMPTIME
```

Analysis of variance table

source of variation	d.f.
reps stratum	3
samptime.reps stratum	
samptime	13
Residual	39
samptime.reps.*Units* stratum	
treat	2
samptime.treat	26
Residual	84
Total	167

4.3: Results

The mean evolution of gas for the entire time course was significantly ($P < 0.001$) affected by treatment (Table 4.3.1). The time of sampling and the treatment x sample time interaction were also found to be statistically significant ($P < 0.001$). These results are presented graphically in Figure 4.1.

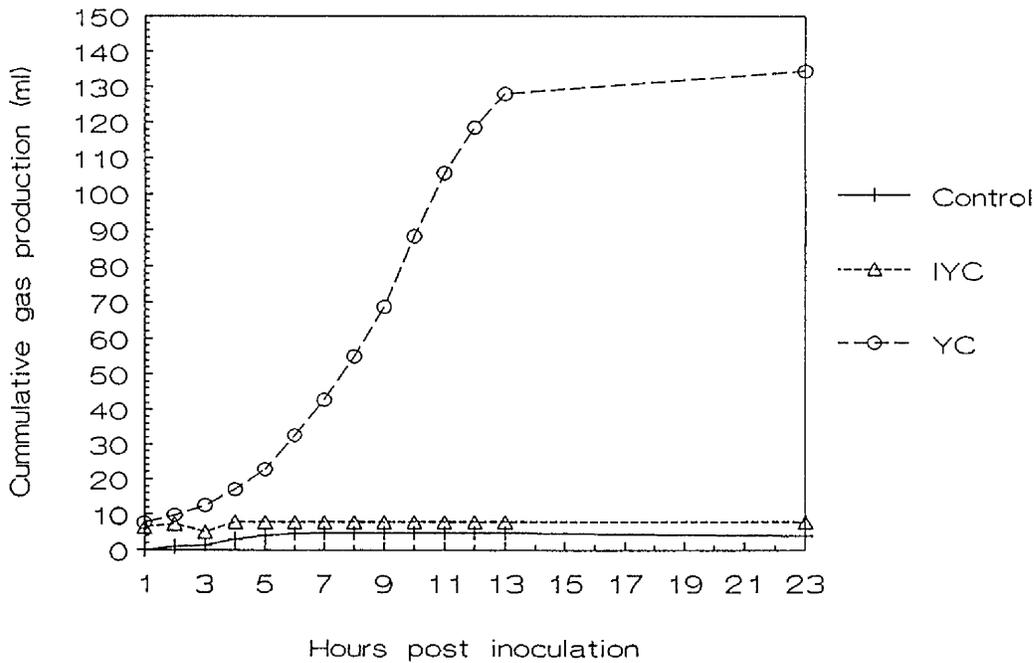
Table 4.3.1: Mean volume of gas produced (ml) by YC and IYC when grown *in vitro*

Treatment		
Control	IYC	YC
3.65 ^a	7.44 ^a	60.26 ^b

Means not sharing a common superscripts are significantly different ($P < 0.001$)

Figure 4.1

Metabolic activity of YC and IYC



4.4: Discussion

The culture supplemented with YC was found to produce more gas than with either of the other two treatments. Figure 4.1 illustrates that the pattern of gas production by the YC supplement corresponds to a microbial growth curve. When the YC data was fitted to the Gompertz growth curve (equation given below) the curve parameters were found to be:-

Gompertz growth curve equation:- $Y = A + C * \text{EXP}(-\text{EXP}(-B * (X - M)))$

Fitted curve parameters:

Zero time amount of gas produced (A)	12.46 ml
Final gas amount of gas produced (A + C)	140.54 ml
Rate of gas production (B * C)	47.06 ml/hr
Mid time (point of inflexion) (M)	8.05 hrs
R ²	98.8%

The volume of gas observed at zero time for the YC treatment and at 1 hour post inoculation for the other treatments was assumed to have resulted from the evolution of carbon dioxide from the CO₂ saturated medium rather than as a result of metabolic activity. The increased gas production noted on the YC treatment was assumed to have resulted from both the metabolic activity of the viable yeast supplement and reproduction of yeast cells resulting in a larger population producing more gas. Figure 4.1 shows that the rate of gas production increased until 8 hrs post-inoculation (point of inflexion). Subsequently the rate of gas production slowed until the curve reached its upper asymptote where there was no further population growth or metabolism by the yeast. This was probably due to limited substrate availability.

It was assumed that the gas produced by an organism, presumed to be metabolically active but unable to reproduce (such as the IYC treatment) would result in a linear increase in the cumulative evolution of gas. However, the IYC treatment failed to show any such increase in gas production. It was therefore assumed that the metabolic activity of IYC was either nonexistent or too low to be detected by this technique.

This experiment showed that YC and IYC are metabolically very different yet both supplements produced similar rumen responses *in vivo* (Chapter 3). A possible explanation is that the mode of action *in vivo* involves the provision of a microbial growth factor from the yeast cell constituents made available when the viable yeast cells autolyse or when the IYC supplement degrades in the rumen. However this hypothesis has yet to be proved.

CHAPTER 5: An investigation into some factors influencing the loss of allantoin during collection.

5.1: Introduction

Statistical analysis of urinary allantoin excretion (Chapter 3) suggested that the collection procedure used during this experiment resulted in a reduction of urinary allantoin output over the 6 day collection period. A possible explanation was a technical error in the collection procedure rather than an a real drop in allantoin excretion. It was assumed that either microbial degradation of the urine during each collection day and/or degradation of the purine during storage at -20°C was the cause of the observed reduction in urinary allantoin output. The storage of the unpreserved urine in the freezer at -20°C was assumed not to have contributed to the observed drop in allantoin (g/d) since Rocks (1977) found that unpreserved urine refrigerated at 5°C maintained the levels of total nitrogen at the initial values for 14 days.

The aim of this experiment was to determine whether microbial contamination of the urine trays had a significant effect on the recovery of urinary allantoin.

5.2: Materials and Methods

5.2.1: Experimental procedure

5 mature wethers were kept in digestibility crates for 5 days. The urine collection trays were left unwashed for the first 4 days. On experimental day 4, the collection trays were thoroughly washed and sterilised with a 0.1% hypochlorite solution (Deosan 11% w/w available chlorine), and subsequently the trays were rinsed with copious quantities of fresh water (the fifth collection tray was left unwashed). Urine was collected, bulked and immediately frozen (-20°C) at regular intervals from the cleaned trays during day 5 until a 2 litre stock urine solution had been attained, after which all the sheep were removed from the digestibility crates.

The following day the stock urine solution was defrosted and split into 2 equal sized aliquots. A sample was taken from each aliquot and immediately refrozen awaiting HPLC analysis. One of the four cleaned trays was rewashed as before and allowed to drain. The remainder of each urine aliquot was then poured over either the clean tray or the unwashed tray and collected in a bowl. A 20 ml sample was taken from each collection bowl every hour prior to the repetition of the pouring procedure. The urine aliquots were recirculated in this manner for 15 hrs. Each sample was labelled and stored by freezing at -20°C to await HPLC analysis.

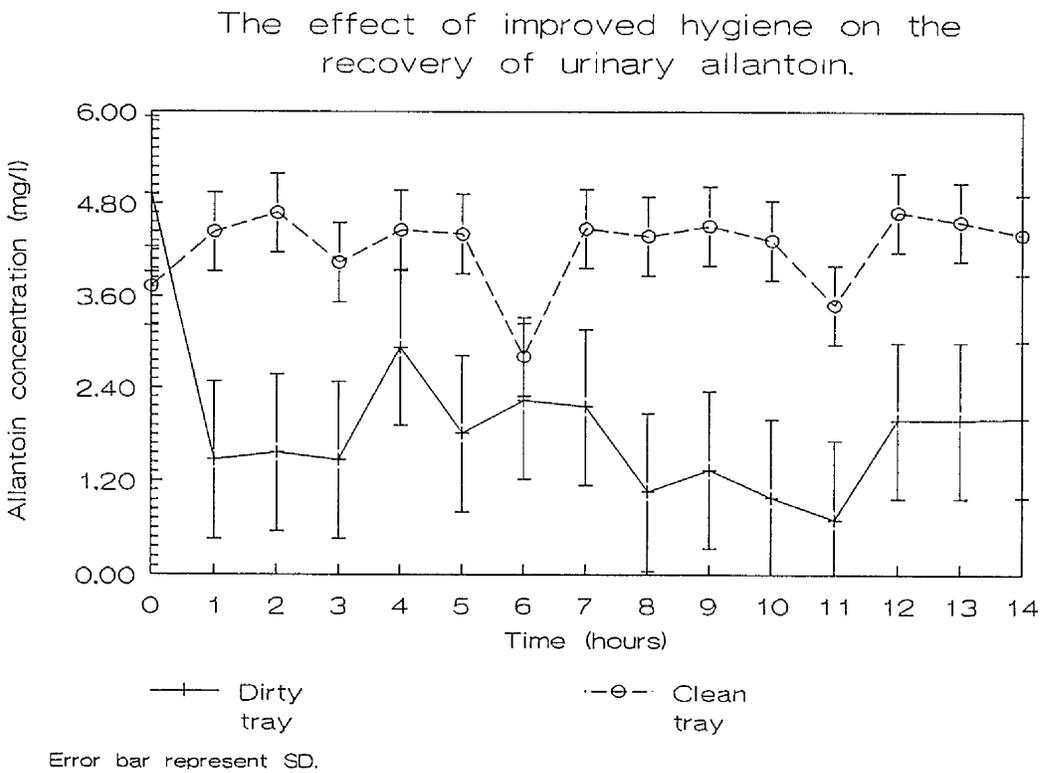
5.2.2: Interpretation, presentation and statistical analysis of the results

The results were statistically analyzed using the twosample test option in Minitab 7.1.

5.3: Results

The recovery of allantoin was significantly increased ($P < 0.001$) following the washing procedure. Treatment means were 1.9 and 4.22 mg/l allantoin (S.E.M. = 0.26 and 0.13) for the dirty and clean tray respectively. Figure 5.1 shows the effect of time on the recovery of urinary allantoin.

Figure 5.1



5.4: Discussion

Figure 5.1 shows that the urinary allantoin concentration dropped from its pre-contamination level within an hour of being poured over the dirty urine collection tray. In contrast the allantoin concentration of the urine poured over the clean collection tray remained at the initial levels. The fluctuations of urinary allantoin observed in the measurements made from the clean collection tray were assumed to have resulted from technical error in the preparation of the urine sample for HPLC analysis and the inherent error of the HPLC technique.

These results indicate that as long as the urine trays are kept clean by a daily washing procedure and the urine samples are stored frozen (-20°C) no further preservation of the urine is necessary.

CHAPTER 6: Ruminant responses to dietary supplements of yeast culture in animals fed a semi-purified diet 3 hourly

6.1: Introduction

The results of the first animal experiment suggested that the semi-purified diet fed twice daily induced stressful conditions on the microflora of the rumen. The rumen microbes may have been over-stressed for the purposes of the experiment, resulting in the observed instability in the rumen. It was postulated that the extreme pH ranges noted in the first experiment were due to the twice a day feeding regimen and the composition of the diet, resulting in the wide inherent variation observed in the *in sacco* results. It was therefore proposed that the same semi-purified diet be fed in eight equal meals instead of two. This would be expected to reduce the diurnal variation in pH and the rumen VFA parameters and hence reduce the stress on the rumen microbial ecosystem.

Measurements on the diurnal variation of rumen pH, ammonia, tVFA and the individual VFA proportions were carried out in order to assess the within-day rumen stability. The day-to-day stability of the rumen was assessed by measuring the overall digestibility of the diet throughout the trial, 24 hr. *in sacco* degradability of soya hulls and the daily excretion of purines for the estimation of the efficiency of microbial protein production.

6.2: Materials and methods

6.2.1 Animals and design

Three rumen-cannulated mature wethers averaging 65 kg were housed in digestibility crates continuously for the 8 week experiment. The diet was supplemented with 20g/d of viable yeast culture (*S. cerevisiae* 1026) fed as 2 equal doses of 10g at 0900 and 1700 during weeks 3-6 inclusive. The sheep were allowed 10 days to become accustomed to the digestibility crates, diet and feeding regimen prior to the start of the experiment.

6.2.2 Diet

The same semi-purified diet that was fed in the first experiment (Chapter 3) was fed every 3 hours by an automatic feeder. Nutrient improved straw (NIS) nuts were substituted for chopped straw because of the nature of the automatic feeder. In total 800gFW of concentrate and 200gFW of NIS nuts were fed daily.

6.2.3 Measurements

Daily

Overall digestibility of diet OM (3 day collection)

in sacco degradability of soyahulls:

Days 1-33 Unwashed Soyahulls

Days 29-54 Prewashed Soyahulls

Urinary purine derivatives

Weekly

Diurnal rumen liquor analysis of:

pH

tVFA

Individual VFA

Ammonia content

6.2.4: *in sacco* rumen degradability measurements

The rumen degradability of soyahulls (unwashed and prewashed) were measured by the *in sacco* technique using 24 hour incubation periods in the rumen.

6.2.5: Assessment of the overall digestibility of the diet OM.

The overall digestibility of the diet OM (OMD) was assessed continually throughout the experiment. The total faecal collection for each 3d period was bulked. All refusals were collected daily. The refusals and bulked samples were dried to a constant weight at 60°C to determine their dry matter content. The OM content was measured by heating at 450°C for 24 hours.

6.2.6: Assessment of urinary allantoin

The total urine excreted over a 24h period was collected and stored frozen until the end of the experiment when the urine samples were defrosted and diluted 1 in 20 prior to injection on to the HPLC column as described in Chapter 2.

6.2.7: VFA analysis of rumen liquor

A series of rumen liquor samples were taken from each sheep twice a week throughout the experiment. The first sample of each series was collected just prior to morning feeding and subsequently at hourly intervals until just prior to the evening feed.

6.2.8: Interpretation, presentation and analysis of the results

The daily 24h *in sacco* degradabilities were analyzed by cusum analysis. The other parameters were statistically analyzed using the analysis of variance option in Genstat 5.13. The BLOCK TREATMENT structures that determine the degrees of freedom used in the statistical analysis are shown below (Tables 6.2.1-6.2.2). Trend analysis (bent stick) was also carried out on the measurements of diet OMD, urinary allantoin excretion and the rumen parameters.

In order to simplify the statistical analysis, the experimental period during which the diet was supplemented with viable yeast culture (YC) was divided into two to give 4 experimental periods with an equal number of observations. The period means equate to the treatment effects as yeast culture was only fed during the second and third periods.

The term PDAY (Table 6.2.1) and BATCHDAY (Table: 6.2.2) refer the sampling day and 3 day faecal collection period in each experimental period respectively.

Table 6.2.1: Block - treatment structure used for the analysis of variance of the VFA, pH and NH₃ measurements

BLOCK WEEK/PDAY

TREATMENT SHEEP*PERIOD*SAMPTIME*DAY

Analysis of Variance table

Source of variation	d.f.
week stratum	
period	2
Residual	5
week.pdays stratum	
day	1
period.day	3
Residual	4
week.pdays *UNITS* stratum	
sheep	2
samptime	8
sheep.period	6
sheep.samptime	16
period.samptime	24
sheep.day	2
samptime.day	8
sheep.period.samptime	48
sheep.period.day	4
sheep.samptime.day	16
period.samptime.day	24
Residual	256
Total	431

Table 6.2.2: Block - treatment structures used for the analysis of variance of urinary allantoin and the measurement of overall diet OM digestibility.

a) BLOCK PERIOD/BATCHDAY
TREATMENT (SHEEP*PERIOD)*SHEEP.PERIOD

Analysis of variance table

Source of variation	d.f
period.batchday stratum	
period	3
Residual	12
period.batchday.*UNITS* stratum	
sheep	2
period	6
Residual	24
Total	47

b) BLOCK SHEEP*PERIOD
TREATMENT BATCHDAY+PERIOD.BATCHDAY

Analysis of variance table

Source of variation	d.f.
Sheep stratum	2
Period stratum	3
Sheep.Period stratum	6
Sheep.Period.*UNITS* stratum	
Batchday	3
Period.Batchday	9
Residual	24
Total	47

6.3: Results and discussion

6.3.1: Assessment of the overall digestibility of the diet OM throughout the trial.

The overall digestibility of the diet OM (OMD) was not significantly ($P=0.915$) affected by period, but the sheep (Table 6.3.1) and batchdays (Table 6.3.2) were in some cases found to be significantly different ($P\leq 0.001$). The period means were 0.819, 0.815, 0.824 and 0.826 (S.E.D. = 0.017) for periods 1 to 4 respectively.

Table 6.3.1: Mean Sheep OM digestibility

Sheep A	Sheep B	Sheep C	S.E.D.
0.826 ^a	0.832 ^a	0.804 ^b	0.006

Means not sharing a common superscript are significantly different ($P < 0.001$)

Table 6.3.2: Mean OM digestibility for the batchdays

Batchday 1	Batchday 2	Batchday 3	Batchday 4	S.E.D.
0.814 ^{ab}	0.838 ^a	0.810 ^b	0.822 ^{ab}	0.007

Means not sharing a common superscript are significantly different ($P=0.001$)

Statistically significant differences between the batchdays in all periods as indicated above would tend to suggest that OMD varied periodically. However, the batchday x period interaction was also found to be significant ($P < 0.001$). This was mainly due to a marked change in the OMD pattern during the fourth period. This interaction is illustrated in Figure 6.1.

Period 2 exhibited the lowest OMD ($P > 0.05$). However, Figure 6.1 demonstrates that the mean OMD for all 3 sheep showed a wide inherent variation between the 3 day faecal collections periods. The correlation table (Table 6.3.3) below shows that the 3-daily OMD values for sheep A & B, and A & C correlated at the $P < 0.05$ significance level and suggests

that the sheep were synchronised in their ability to digest the diet (Individual sheep means are shown in Appendix 6). However, bent stick trend analysis (Table 6.3.14) did not show a common trend for all sheep. A significant ($P < 0.001$), alteration in the OMD trend of sheep B was observed and the point of inflexion occurred on day 17 of the experiment, which coincided with the commencement of YC supplementation of the diet.

Table 6.3.3: Correlation coefficients of mean OM digestibility between sheep

	OMD A	OMD B
OMD B	0.615*	
OMD C	0.498*	0.388

Values with an asterisk correlate at the $P < 0.05$ significance level.

Figure 6.1 indicates that the variation in OMD relates to the amount of faecal fresh weight voided over each three day collection period. A significant ($P < 0.001$) negative correlation ($r = -0.916$) between OMD and faeces FW was found to exist.

Changes in faecal fresh weight voided were only partially compensated for by changes in the dry matter content of the faeces (Table 6.3.4). Thus the main cause of the fluctuations in diet digestibility was variation in the faecal fresh weight excreted.

Table 6.3.4: Correlation coefficients of individual sheep faecal fresh weight (g/d) and faecal DM content (g/kg)

	FW A	FW B	FW C
g/kg A	-0.438		
g/kg B		-0.195	
g/kg C			-0.760*

Values with an asterisk correlate at the $P < 0.05$ significance level.

The reasons for the observed synchronisation of faecal output are not clear. However, urine excretion (g/d) was observed to vary between consecutive days. Figure 6.3 illustrates the daily

excretion of urine for sheep A, B and C. The daily variation in urine excretion was found to vary in a periodic way for sheep B and C, but the periodism was less marked in sheep A. Figure 6.4 shows the correlogram from the auto-correlation function analysis for the correlation of daily urine excretion at different time lags within an individual sheep. The sheep were also found to be synchronised in the amount of urine they excreted on each day (Table 6.3.5) and urine excretion (g/d) was found to be significantly ($P < 0.05$) correlated to OMD and faecal fresh weight (Table 6.3.6). It was assumed that the periodic increase in the excretion of urine (g/d) related to altered drinking behaviour in all animals. An increase in water consumed by an animal fed at a fixed OMI may be expected to result in a concomitant increased ruminal outflow and decreased OMD. This theory is supported by the findings of Waybright and Varga (1991), who observed a decreased OMD when they increased ruminal outflow rate by placing water filled bags of various sizes in the rumen of wethers and was further supported by the observations of Dewhurst and Webster (1992a), who also observed depressed OMD values when ruminal outflow was increased. The reasons for the sheep to periodically increase water consumption are not clear but may relate to all three sheep experiencing an external factor such as daily temperature.

The daily variation in the urine excretion of sheep A was found to be significantly ($P < 0.01$) correlated to the daily minimum temperature over the time for which the experiment was run ($r = 0.399$). It may be postulated that low daily minima induced increased ruminal contractions with a concomitant increase in ruminal outflow (Kennedy *et al.*, 1976) resulting in an increased water loss from the rumen and increased excretion of urine (g/d). It is not clear why Sheep B and C did not show a similar correlation ($r = -0.004$ and 0.072 respectively), nor why these two sheep should be so closely synchronised in their excretion of urine (g/d).

Table 6.3.5: Correlation coefficients of daily urine excretion (g/d) between sheep

	Sheep A	Sheep B
Sheep B	0.474***	
Sheep C	0.365**	0.819***

Coefficients with a series of asterisks relate to the level of significance, *** = $P < 0.001$, ** = 0.01, * = 0.05.

Figure 6.1: Variation in diet organic matter digestibility (OMD) and faecal excretion (FW/d).

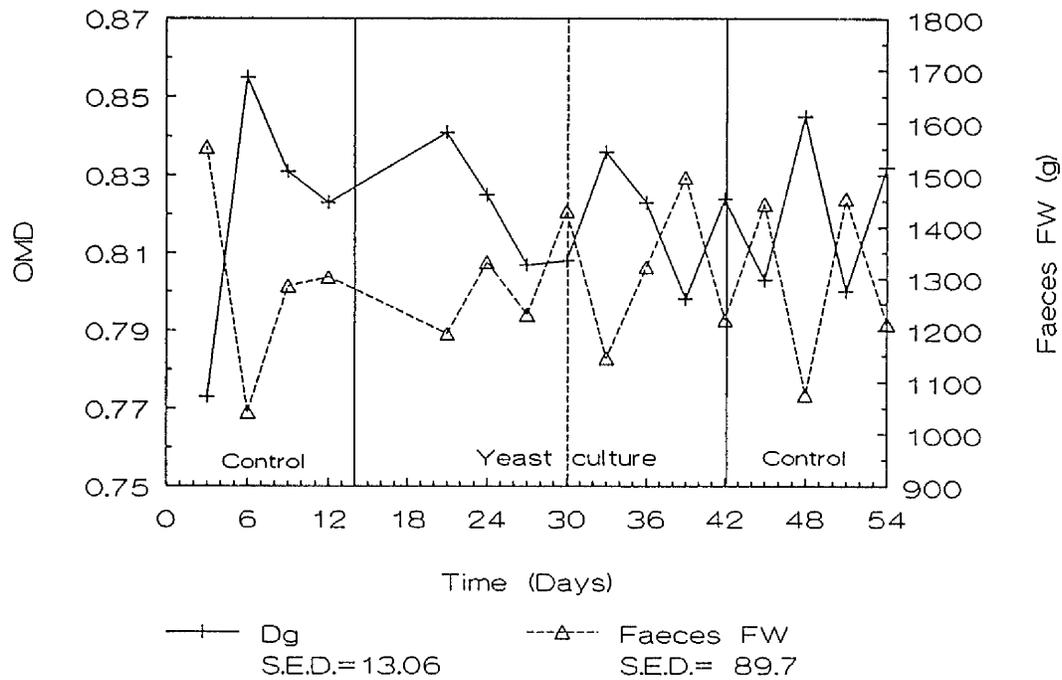


Figure 6.2

Daily urinary allantoin excretion (g/d)

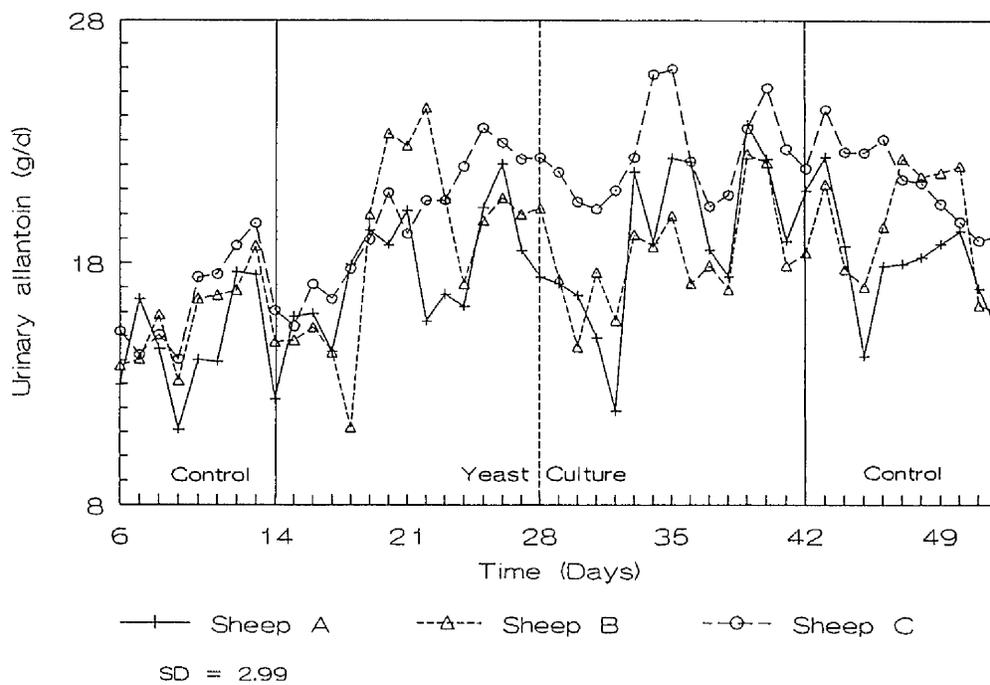


Figure 6.3

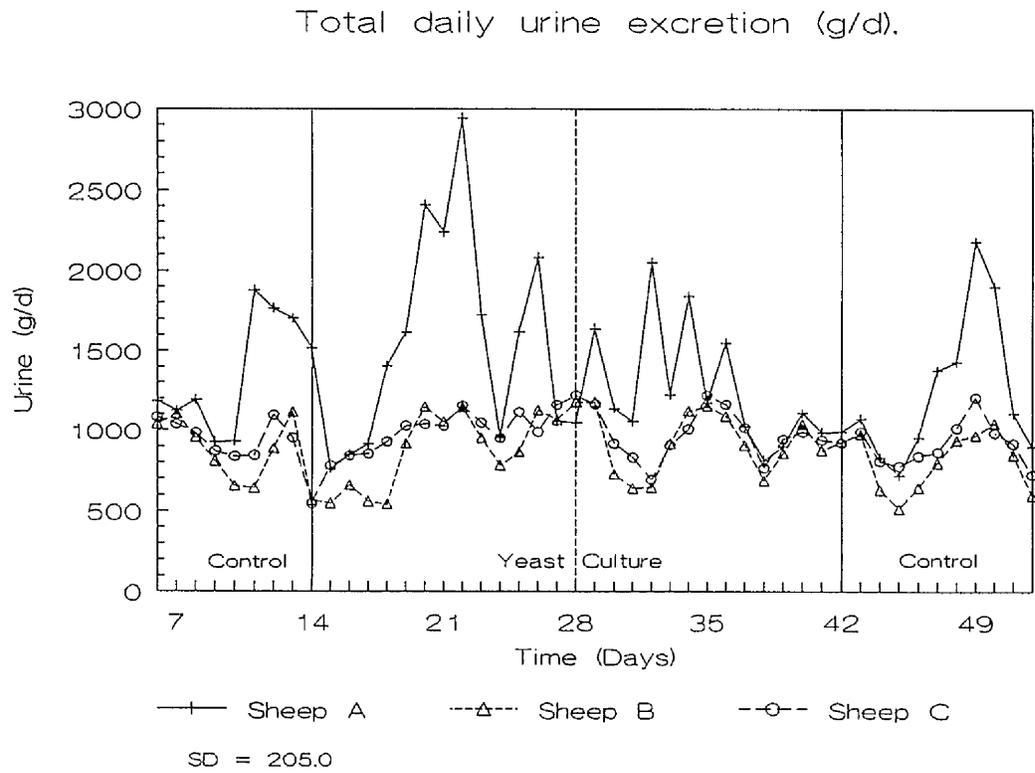


Figure 6.4: Correlogram for the auto-correlation function analysis of daily urine excretion (g/d).

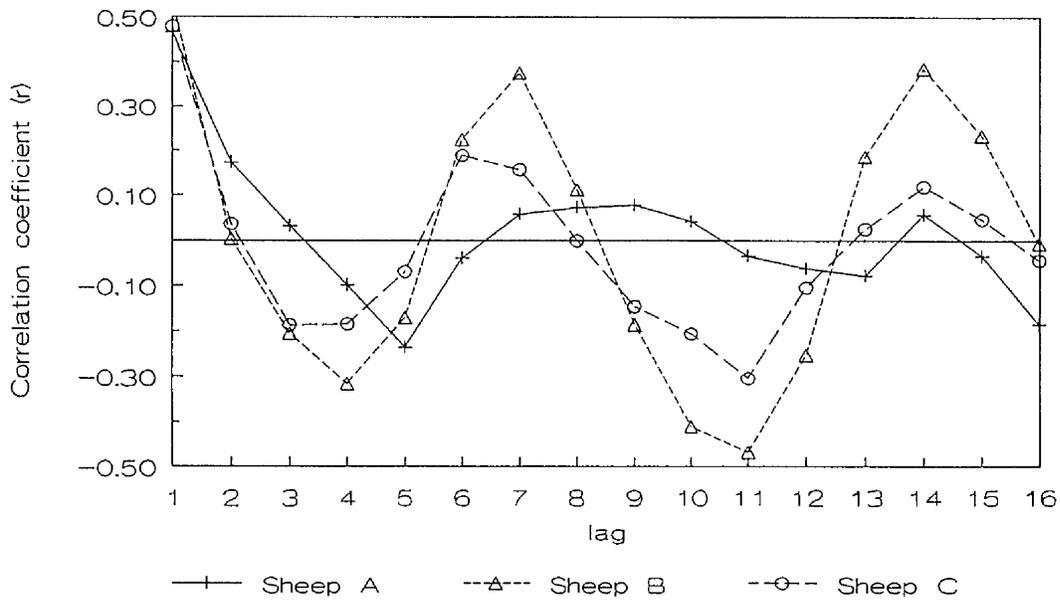


Table 6.3.6: Correlation coefficients for the comparison of daily urine excretion (g/d), OM digestibility and faecal excretion (FW/d).

	¹ Urine excretion (g/d)	OMD
OMD	-0.262*	
Faecal excretion (FW/d)	0.661**	-0.919***

Coefficients with a series of asterisks relate to the level of significance, *** = $P < 0.001$, ** = 0.01, * = 0.05.

¹ 3-day means of urine excretion corresponding to the OMD 3-day collection periods were calculated.

6.3.2: Assessment of microbial protein yield expressed as gMN/kgDOM

Urine was collected daily and weighed and analysed to determine the concentration of allantoin. A 3-day mean corresponding to the OMD measurements was then calculated. Further calculations to estimate the efficiency of microbial nitrogen synthesis (EMNS) and the concentration of allantoin excreted per kg live weight of the animal were also made and statistically analyzed in the same way as the digestibility results. These results are shown in Table 6.3.7. The batchdays were not found to be significantly different ($P > 0.05$), however the gMN/kgDOM period x batchday interaction was found to be significant ($P = 0.032$).

OMD was found to be inversely related (Table 6.3.6) to urine excretion (g/d) and this was assumed to relate to an increase in ruminal outflow. Harrison *et al* (1975) found that by increasing the fractional outflow rate of the rumen by supplementing the diet with NaCl, the yield of microbial protein to the abomasum was increased by 24%. However, the 3d mean urinary allantoin excretion was not found to be significantly negatively correlated to OMD ($r = -0.268$; $P = 0.354$). A possible explanation for this observation may be that the reduced OMD reduced the fermentable energy available for microbial protein synthesis and hence compensated for the increased microbial yield which may be expected due to an increased

rumen outflow rate. Microbial growth in the rumen has been related to the amount of energy which is produced during fermentation, providing that N and other essential nutrients are not limiting (Hvelplund and Madsen, 1985; ARC, 1984).

From Table 6.3.7 it may be observed that the excretion of allantoin (mmol/d) in the first period was significantly lower than the other periods. This reduction in allantoin excretion may relate to the depressed ruminal OMD during the first 6 days of the experiment during which urine was not collected for analysis. It was therefore assumed that the addition of yeast culture to the diet had no effect on EMNS.

Bent stick trend analysis (Table 6.3.14) indicated that urinary allantoin excretion (Figure 6.2) increased during the first 8 experimental observations but either declined (sheep A and C) or was greatly reduced (sheep B) thereafter. The point of inflexion were similar for all sheep and occurred after the supplementation of the diet with YC had commenced. This suggests that the YC supplement may reduce urinary allantoin excretion and concurs with the trend observed for the OMD results of sheep B, but the bent stick analysis did not indicate significant trends in OMD for the other two sheep. However, the OMD value does not represent the true OM digestion in the rumen and these values may not have shown consistent trends (Table 6.3.14) due to a shift (post rumen) in OM digestion since the diet contained approximately 40% starch.

The average concentration of allantoin excreted per kg live weight (for an average animal weight of 65 kg) was found to be 0.802 mmol. It was therefore assumed that the contribution of endogenous allantoin to the urine was not significant and the concentration of allantoin in the urine directly corresponded to the outflow of microbial biomass in accordance with the findings of Chen *et al* (1990a).

Table 6.3.7: Microbial yield estimated by allantoin excretion and expressed as g of MN per kg of OM digested.

Period	Treatment	¹ mmolA/kgW ^{0.75}	OMD	² DOMI (g/d)	A (mmol/d)	³ gAN/d	⁴ gMN/kgDOM
1	Control	0.64 ^a	0.819	681	14.6 ^a	0.82 ^a	20.3 ^a
2	Yeast	0.86 ^b	0.815	678	19.8 ^b	1.11 ^b	28.0 ^b
3	Yeast	0.87 ^b	0.824	685	19.9 ^b	1.11 ^b	27.9 ^b
4	Control	0.85 ^b	0.826	687	19.4 ^b	1.09 ^b	27.2 ^b
S.E.D.		0.039	0.002	10.3	0.90	0.0502	1.52
F. Pr.		<0.001	0.915	0.816	<0.001	<0.001	0.001

Means with different superscripts in the same column are significantly different at the given significance level.

¹ A = allantoin

² DOMI was calculated by assuming a constant OMI (832 g/d) throughout the experiment and multiplying it by the OMD estimation.

³ gAN/d was calculated by multiplying the urinary excretion of allantoin (mmol/d) by the molecular weight of allantoin (158.12) and the proportion of N in allantoin (34.5%).

⁴ gMN/kgDOM was derived using the assumptions discussed in Chapter 3.

6.3.3 24h *in sacco* degradability of soyahulls

Analysis of variance of the 6 days of *in sacco* measurements (Days 7-12, 22-27, 37-42 and 49-54) relating to the equivalent period of OM digestibility was carried out. Sheep B was significantly different from sheep C but not sheep A ($P < 0.001$) (Table 6.3.8).

Table 6.3.8: Sheep *in sacco* degradability means (%).

Sheep A	Sheep B	Sheep C	S.E.D.
31.5 ^{ab}	34.1 ^a	30.8 ^b	0.82

Means not sharing a common superscripts are significantly different ($P < 0.001$)

The control periods were found to be significantly different ($P = 0.026$) (Table 6.3.9). This meant that it was not possible to attribute any effects on rumen function in periods 2 and 3 to supplemental YC.

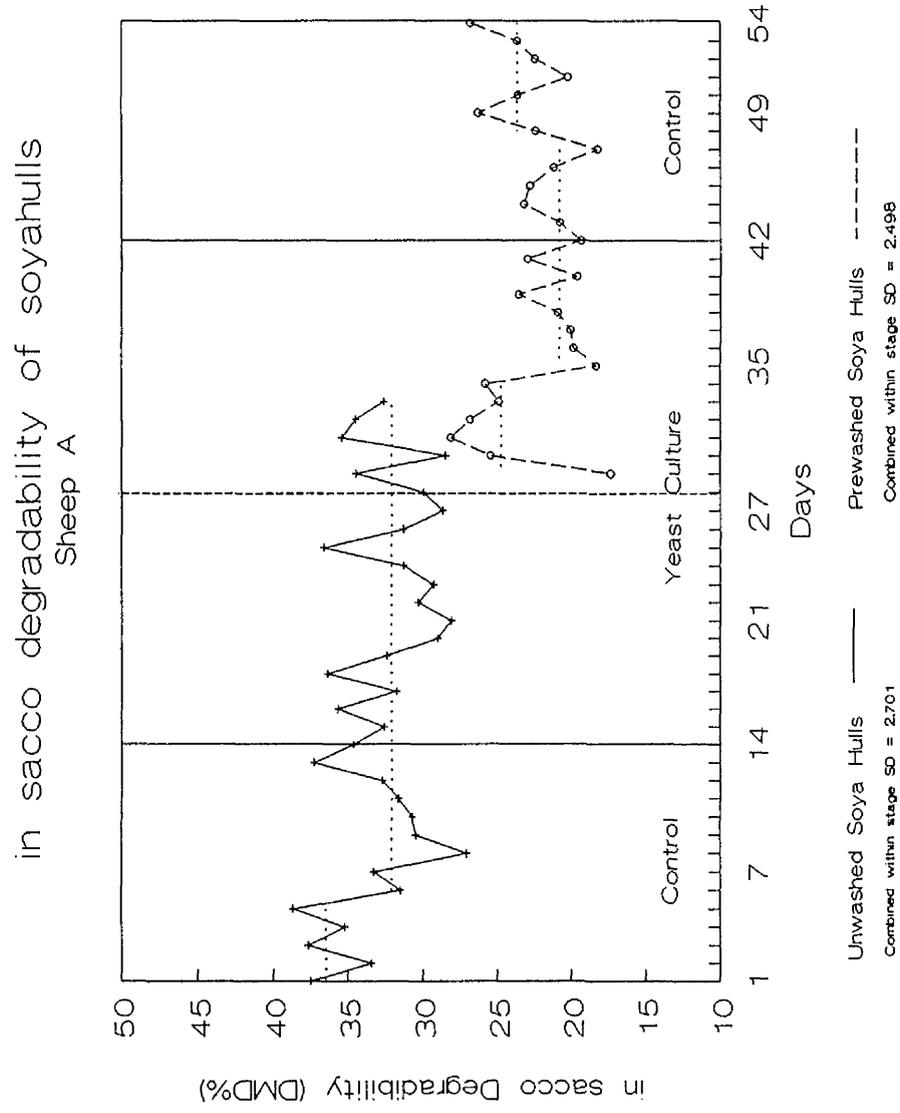
Table 6.3.9: Period *in sacco* degradability means (%).

Period 1 Control	Period 2 YC	Period 3 YC	Period 4 Control	S.E.D.
31.1 ^a	32.0 ^{ab}	31.5 ^a	33.9 ^b	0.94

Means not sharing a common superscripts are significantly different ($P = 0.026$).

Day 2 of each 6 day period exhibited a much lower *in sacco* degradability than any other day ($P < 0.001$) (Table 6.3.10). Day 3 was found to be lower than days 1, 4, 5 and 6 ($P < 0.05$), but this may be attributed to the recovery from the sharp drop noted on day 2. This drop was observed in all sheep in all periods and may relate to cyclical changes in rumen function. However, the cyclical variation noted from the ISDMD statistical analysis was assumed to be coincidental as Figure 6.5 fails to demonstrate periodic ISDMD.

Figure 6.5



Consecutive horizontal lines show significantly different ($P < 0.05$) stages in the time series.

Table 6.3.10: Day *in sacco* degradability means.

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	S.E.D
34.0 ^a	28.5 ^b	30.7 ^{ab}	32.3 ^{ab}	33.9 ^a	33.3 ^a	1.15

Means not sharing a common superscripts are significantly different ($P = < 0.001$).

A wide inherent day-to-day variation was noted across all sheep in all periods. The individual sheep Cusum statistical analysis, show a wide day to day variation in soyahull degradation. Figures 6.5 illustrates the *in sacco* degradability noted in sheep A as an example (cusum, means for all sheep are given in Appendix 7. These results are similar to those found in experiment 1. It was observed that the zero time losses (Table 6.3.11) of the soyahulls may be a source of error contributing to the day to day variation. In order to exclude this error the soyahulls were prewashed in large Dacron bags (40 μ m pore size) and then dried (60°C). These prewashed soyahulls were used from Day 29 onwards as the *in sacco* substrate. A five-day overlap period, where 3 unwashed plus 3 prewashed soyahull bags were incubated in the rumen at the same time was carried out. This was done in order to allow a continuous assessment of rumen degradability. Figure 6.5 shows that the prewashed soyahulls are less degradable than the unwashed soyahulls. However, the 10% difference between the figures is due to the removal of the soluble and small particle fractions of the unwashed soyahulls. The prewashed soyahulls therefore represent a more accurate estimate of the rumen degradability of soyahulls. The day-to-day variation in rumen degradability of soyahulls was however not reduced by the prewashing procedure.

Table 6.3.11: Zero time losses of unwashed and prewashed soyahulls

	Av. DMD%
Unwashed soyahulls	14.67
Prewashed soyahulls	5.77

Cusum analysis indicated that the supplemental YC had no consistent effect on the rumen degradability of soyahulls. The drop in rumen degradability noted in week 6 by all sheep is not thought to be a result of the supplemental YC, but as part of the downward trend in rumen

function observed over the course of the whole experiment. This downward trend is also noted in the ruminal production of tVFA (Figures 6.8 and 6.9) and ammonia (Figures 6.16 and 6.17).

The correlation coefficient table (Table 6.3.12) shows that a significant correlation between the sheep A and C, but not B and C or A and B exists in the first half of the trial. However, during the second half of the trial all the sheep showed a significant correlation. No obvious change in the technical procedure was noted, but technical error in substrate sampling, bag tying and processing post-incubation was not thought to be the cause of the wide inherent variation observed during this trial.

Since the *in sacco* bags were prepared in batches and ISDMD variation was not found to correspond to the batch preparation, technical error was been ruled out as a possible explanation for the observed day-to-day variation.

Table 6.3.12: Correlation coefficients between daily mean values identified by Cusum analysis.

	Unwashed soyahull		Prewashed soyahulls		
	A	B	A	B	
B	0.023		B	0.478*	
C	0.534*	0.217	C	0.656*	0.396*

Values with an asterisk correlate at the $P < 0.05$ significance level.

6.3.4: Rumen pH

Statistically significant differences between periods were found at the $P = 0.065$ level. The period means are given in Table 6.3.13. The mean diurnal variation and the rumen pH for each sheep through the experiment are illustrated in Figures 6.6 and 6.7 respectively. The sheep and time of sampling means were found to be significantly different ($P < 0.001$) as were the sheep x period and sheep sample-time interactions ($P < 0.05$). Although the time of sampling means were found to be significantly different, data in Figure 6.6 shows that by feeding the diet 8 times a day the diurnal variation in rumen pH was much less than that observed when the diet was fed twice daily (Chapter 3). The diurnal variation noted in the

Figure 6.6

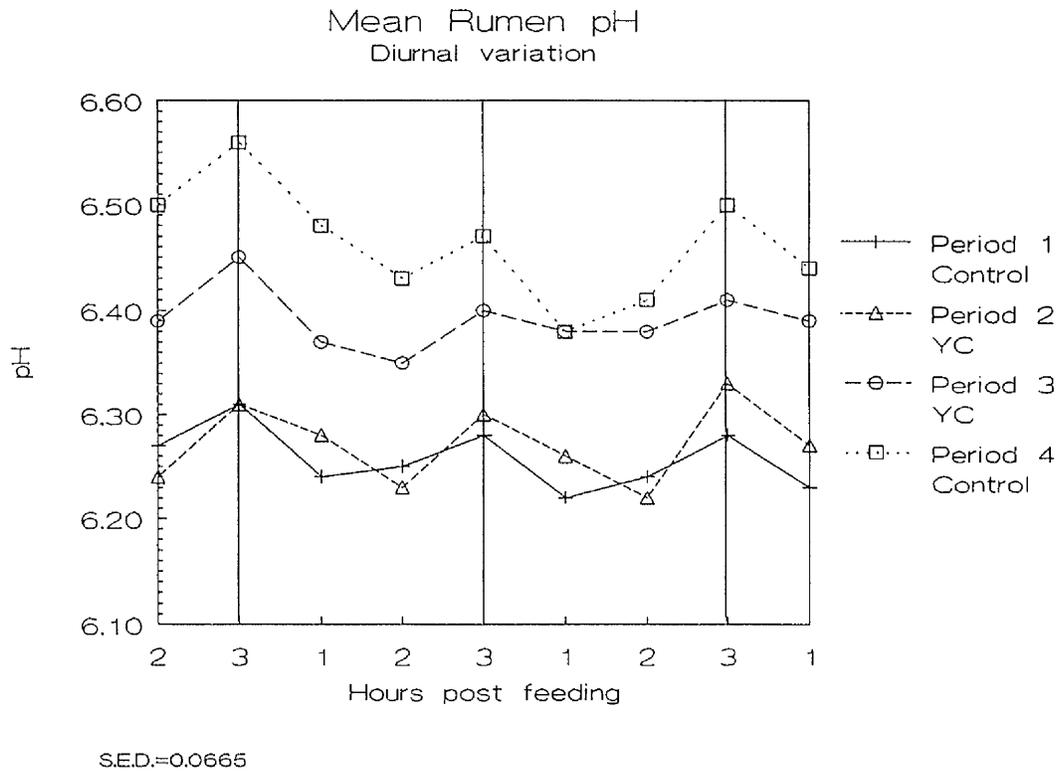
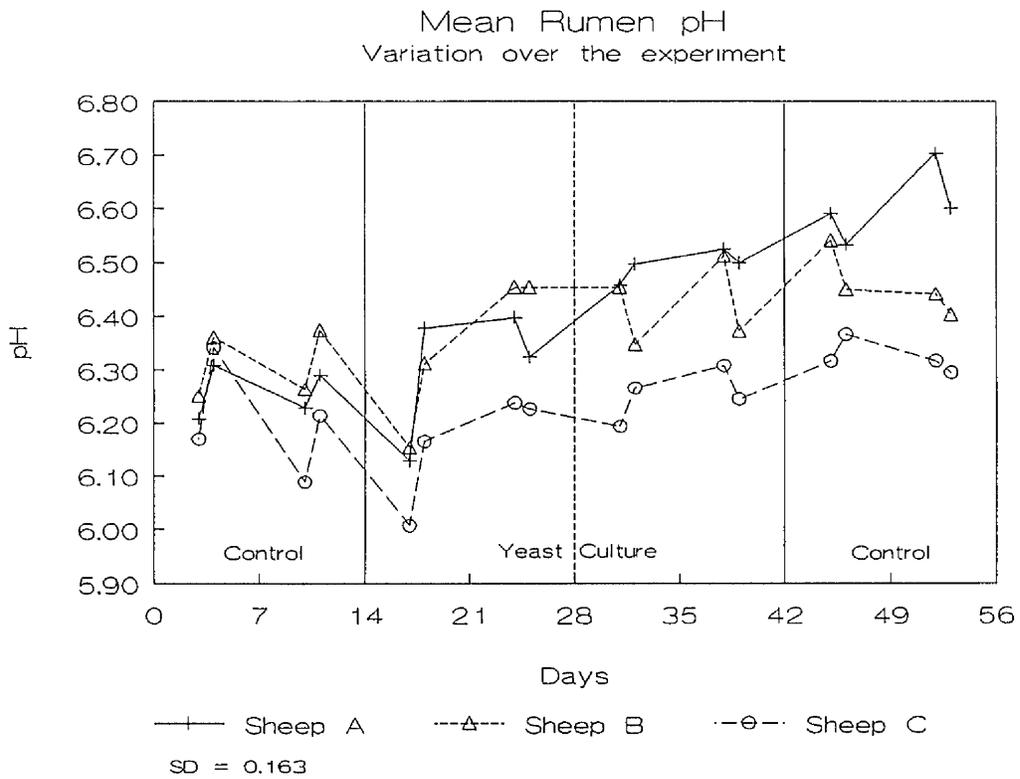


Figure 6.7



current experiment was likely to have resulted from the rapid degradation of the starch portion of the diet giving rise to an increased concentration in t[VFA]. This is in accordance with the findings of Barry *et al* (1977a,b). However, it may also be observed from Figure 6.6 that pH levels remained above pH 6.0, therefore the negative associative effects of pH on cellulolysis can not account for the day-to-day variation noted in the *in sacco* degradability of soyahulls (unwashed or prewashed).

The increase in rumen pH noted in Figure 6.7 after day 17 of the experiment was assumed to be associated with a gradual decline in the t[VFA]. A significant ($P < 0.001$) negative correlation ($r = -0.986$) was found to exist between rumen t[VFA] and pH. The gradients of the slopes determined by bent stick trend analysis (Table 6.3.14) were not found to be significantly different ($P > 0.05$). However, the point of inflexion was observed to occur at the same time in all sheep. The regression analysis for the overall trend in rumen pH showed that it tended to increase throughout the current experiment.

6.3.5: Rumen volatile fatty acids

6.3.5.1: t[VFA] (mM)

Analysis of variance demonstrated that significant differences ($P < 0.1$) between periods 1 and 4 existed (Table 6.3.13) but not between periods 1 and 2, 2 and 3 or 3 and 4 indicating a gradual decrease in rumen tVFA concentration during successive periods (Figures 6.8 and 6.9). The differences between sheep means ($P < 0.001$) and the sheep x period interaction ($P = 0.066$) were found to be significant.

6.3.5.2: Major VFA

The decline in t[VFA] was found to be associated with a decline in ruminal acetate concentration (mM) over consecutive periods ($P < 0.1$) (Table 6.3.13). The ruminal concentration of propionate and butyrate did not significantly alter between periods ($P > 0.1$). As a result a reduction in the molar % proportion of acetate ($P > 0.1$) (Figure 6.10 and 6.11) and a significant increase in the molar % proportion of butyrate (Figures 6.14 and 6.15) was observed. The molar % of propionate did not alter between periods ($P = 0.535$). The sheep x

Figure 6.8

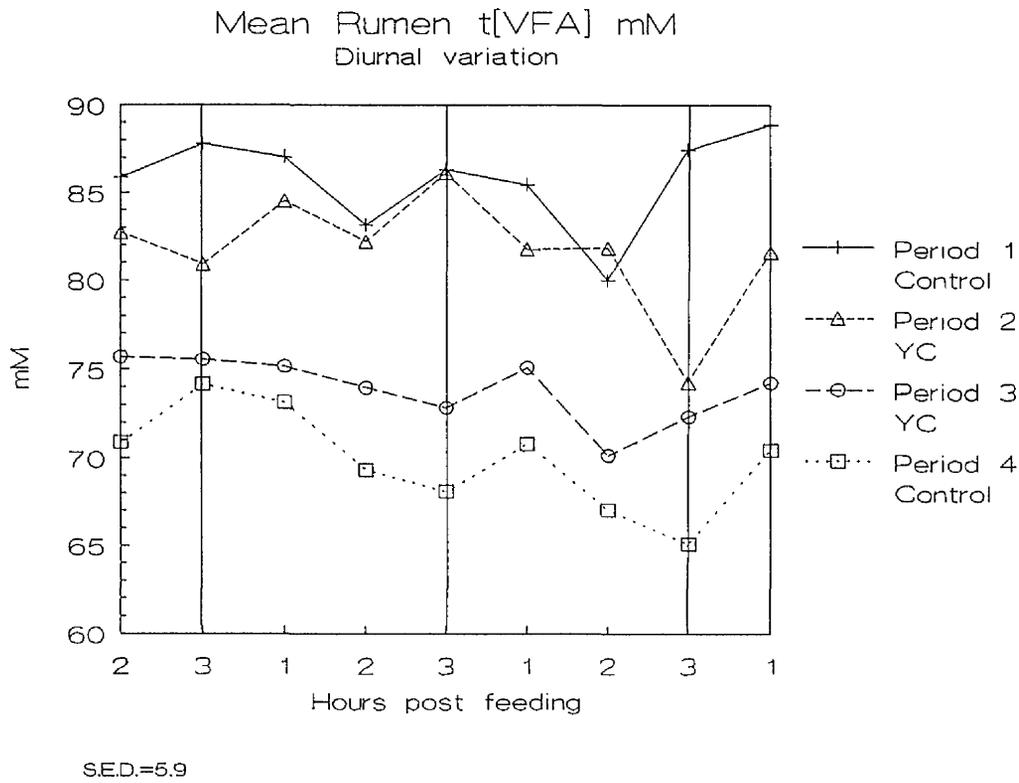
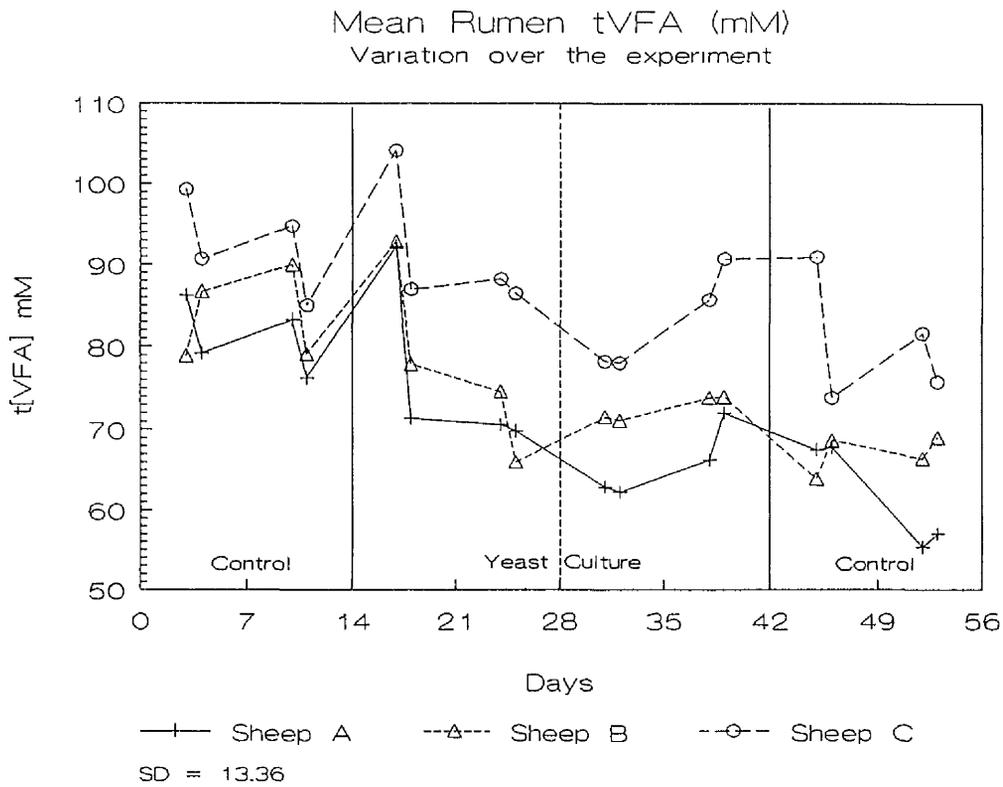


Figure 6.9



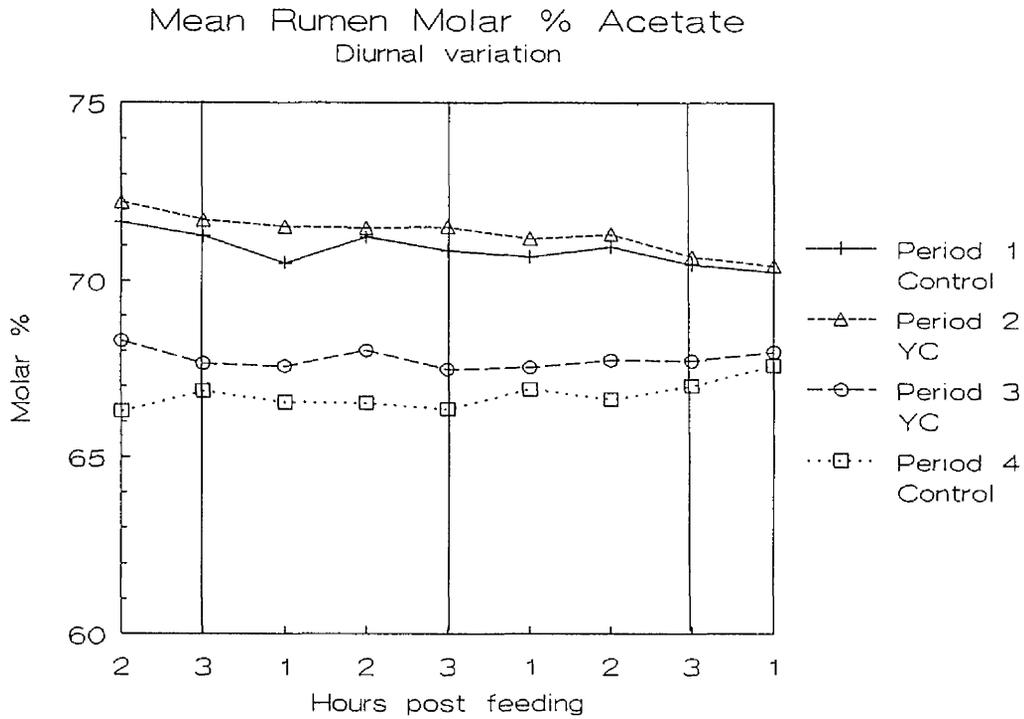
period interaction for all three VFA was found to be significant ($P < 0.001$). The combination of these effect on the rumen VFA mix did not alter the A:P ratio significantly ($P > 0.1$). However it may be noted from Table 6.3.13 that the ratio was lowest in period 4.

Since the analysis of variance demonstrated that significant differences ($P < 0.1$) between the control periods exist (Table 6.3.11) for t[VFA] and the ruminal concentration of acetate (mM), an analysis of the data to determine the effect of supplemental yeast culture is not possible. However, significant trends in ruminal t[VFA] (mM), acetate (molar %), propionate (molar %) and butyrate (molar %) were determined by bent stick trend analysis (Table 6.3.14).

The trend in ruminal t[VFA] production over the experiment was found to be similar for each sheep (Table 6.3.14; Figure 6.9), the ruminal t[VFA] was found to increase up until to day 17 (observation 5) of the experiment and thereafter tVFA production declined for all sheep. Ruminal molar % acetate was found to vary in a similar way to t[VFA] for sheep, as was shown by the statistical analysis by anova. However, the bent stick trend analysis indicated two possible points of inflexion for sheep B (Table 6.3.14), but both bent stick models indicate that acetate production was reduced in the later part of the experiment. Ruminal propionate production was found to be similar for all sheep, but the point of inflexion was found to be earlier in sheep C than A or B and earlier in sheep A than B ($C < A < B$) (Table 6.4.14). The bent stick trend analysis found different trends in molar % butyrate in all sheep. The molar proportion of butyrate in sheep A was observed to increase throughout the experiment, but after the third observation the rate reduced. The molar proportion of butyrate in sheep B was found to decrease during the experiment, but the rate of decline was reduced after the fifth observation. The trend of butyrate production in sheep C indicated that the molar proportion of butyrate increased up until day 17 of the experiment (observation 5) after which it declined. Overall the trends of the VFA mix (molar %) were different for each sheep. The shift in the VFA mix in sheep A was noted to be from acetate production to butyrate and propionate production. The shift in VFA mix in sheep B was less marked than that observed in sheep A or C, but acetate and butyrate were found to decline through the experiment with an increase in molar % propionate. Production of VFA in sheep C showed a shift in VFA production from the production of acetate and butyrate in the first 5 observations to increased propionate production for the remainder of the experiment. However, all sheep were found to show similar points of inflexion at approximately day 17 of the experiment.

Supplementation of the diet with 20g/d of YC commenced on day 14 of the experiment, it may therefore be postulated that the addition of YC to the diet induced a shift in the ruminal

Figure 6.10



SED.=2.358

Figure 6.11

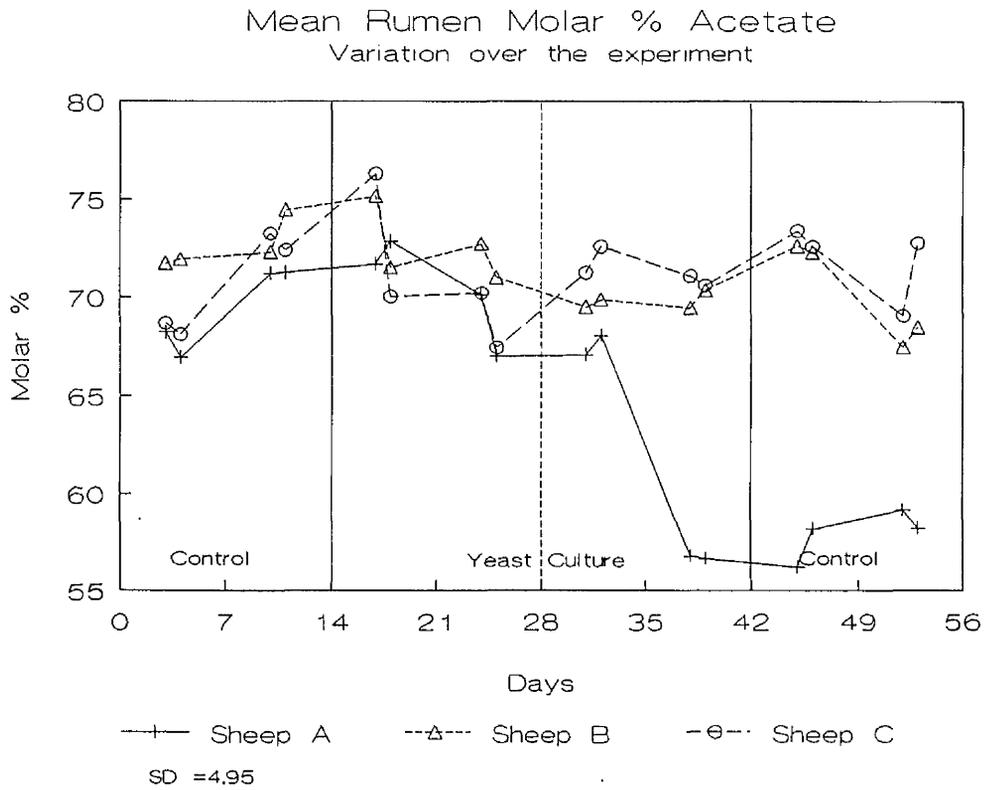
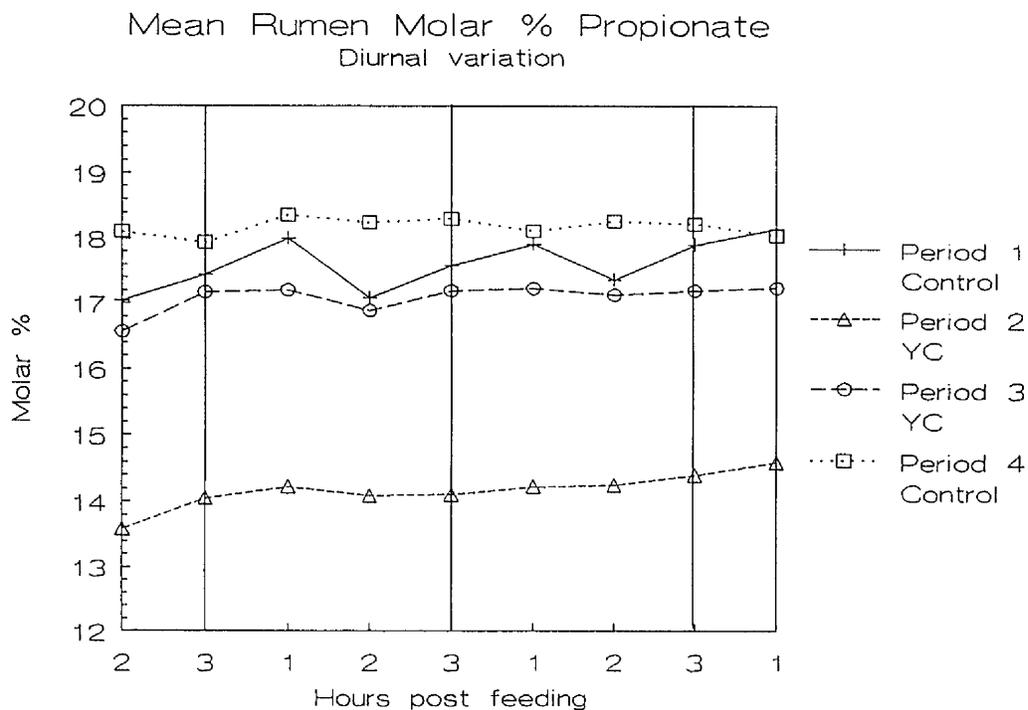
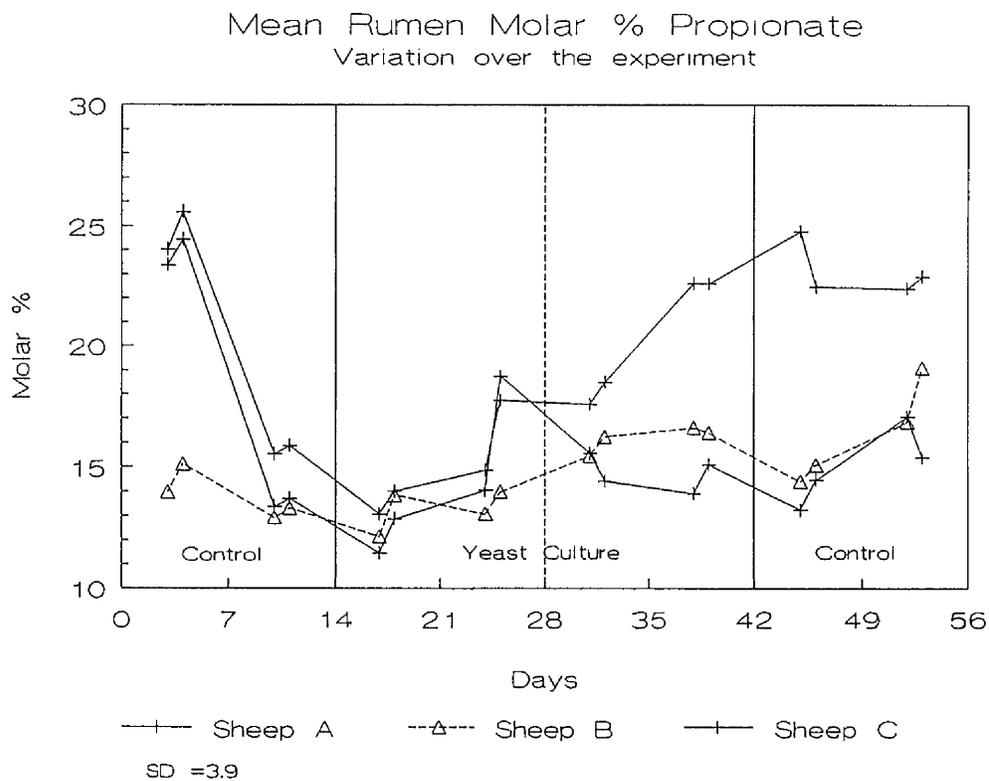


Figure 6.12



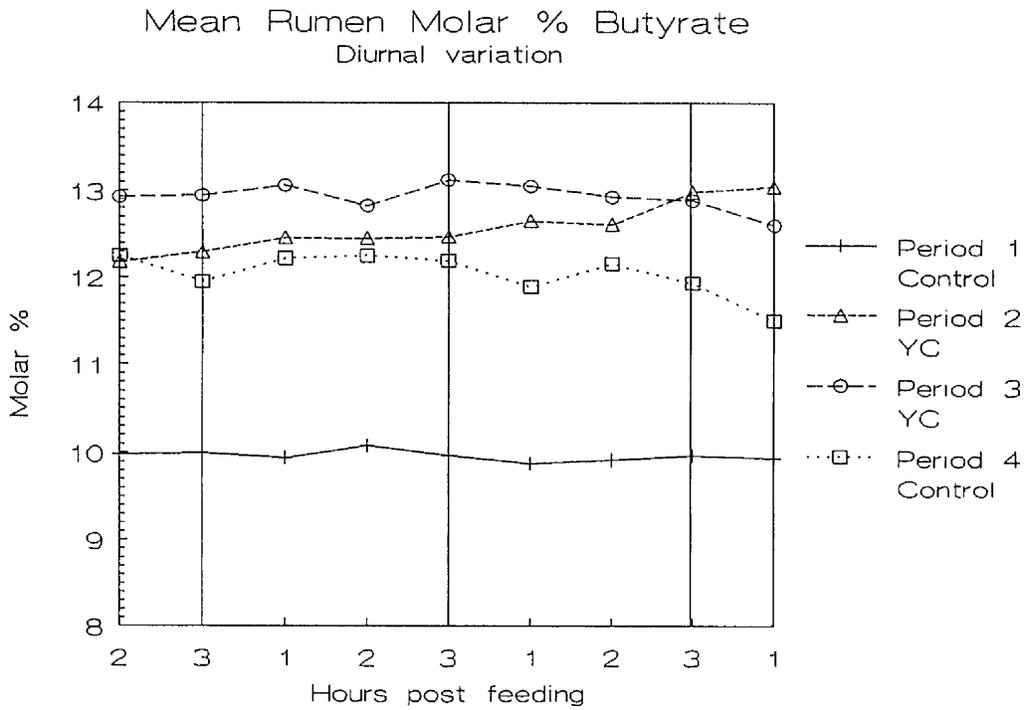
S.E.D.=2.829

Figure 6.13



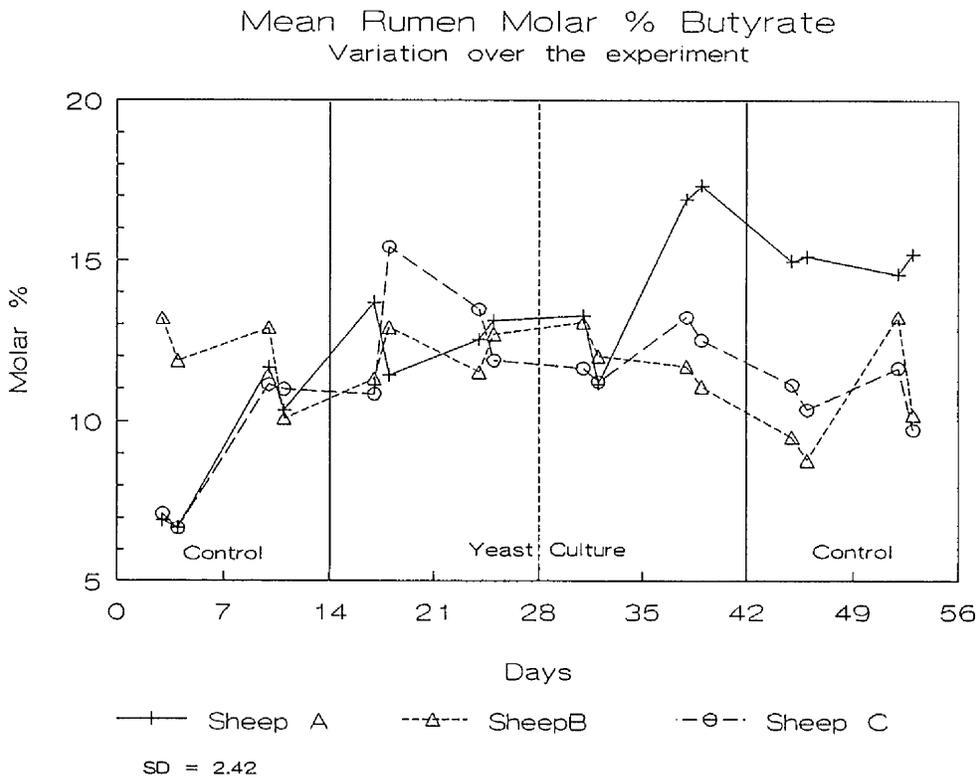
SD =3.9

Figure 6.14



S.E.D.=1.243

Figure 6.15



fermentation pattern of each sheep. Since all but one trend (molar % acetate, sheep B) did not show a point of inflexion after supplementation of the diet with YC had stopped it may be theorised that the YC supplement induced long term carry over effects. However, sheep B showed a significant trend (Table 6.3.14) in OMD suggesting that for this sheep OMD was reduced post observation 6 (day 18). This may partly explain the drop in ruminal t[VFA] for this sheep but similar trends in OMD were not observed for the other sheep.

The experimental design used for the current experiment was confounded with time making the definite conclusions about the mode of action of supplemental YC difficult. It may be theorised that the trends observed in the absolute concentration of tVFA and the VFA mix may have occurred due to natural inherent variation indicated in the ISDMD measurements.

6.5.3.3: Minor VFA

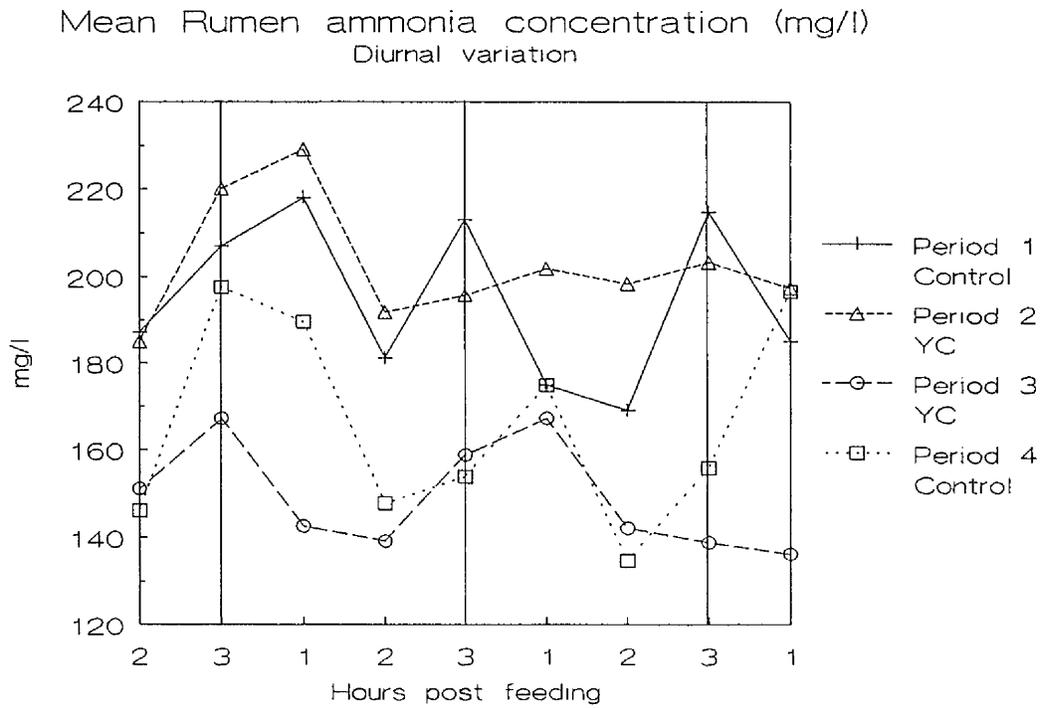
Table 6.3.11 shows that the ruminal concentration of isovalerate was found to increase significantly ($P < 0.1$) in period 4 and the molar % of isobutyrate and isovalerate were found to increase progressively with each period. However the concentration of these acids whilst being assumed to be above the threshold level discussed in Chapter 3 and were considered not to be of sufficient concentration to have a significant effect on the rumen or host metabolism.

6.3.6: Rumen ammonia concentration (mg/l)

A significant difference between the sampling times was observed ($P < 0.001$). Figure 6.16 demonstrates that the concentration of ammonia in the rumen showed short-term increases. These increases were assumed to relate to the rapid degradation of the urea content of the diet which was fed every three hours. Although the sampling times were found to significantly different ($P < 0.001$) the diurnal variation was noted to much less than that when the diet was fed twice a day (Chapter 3)

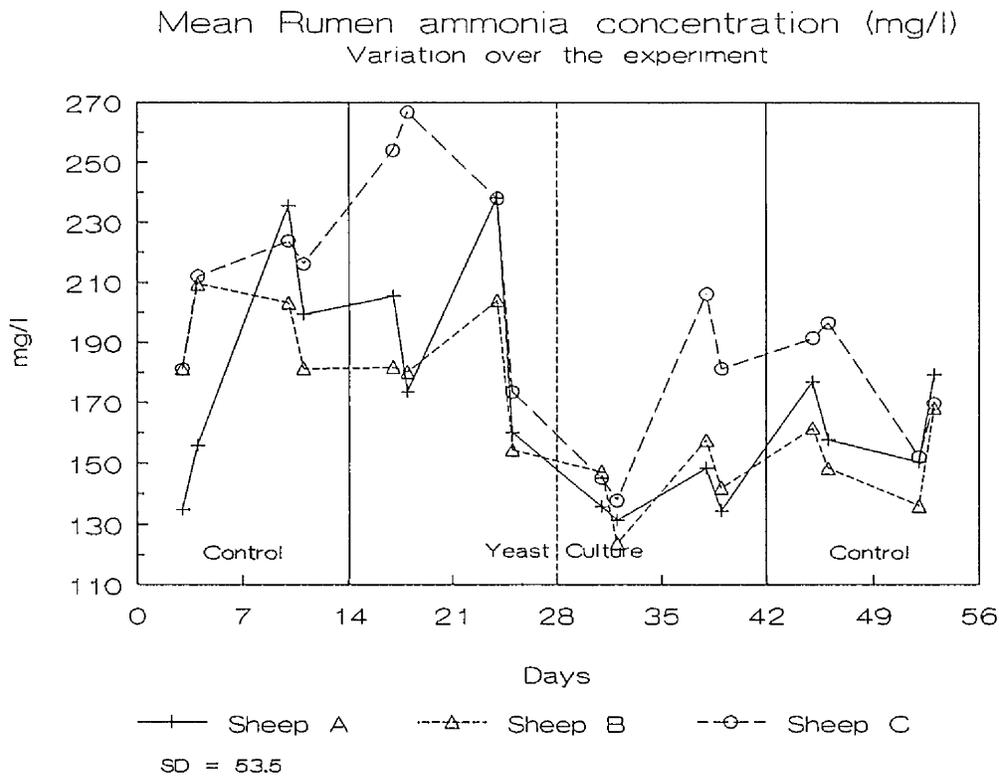
Rumen ammonia concentration was also found to be significantly different ($P < 0.1$) between periods (Table 6.3.13). Figure 6.17 shows that the rumen ammonia concentration tended to be lower during the second half of the experiment is in accordance with the VFA results. Bent stick trend analysis indicated that each sheep showed a different trend. The ruminal NH_3 concentration in sheep A was found to increase up until experimental observation 7 whereafter

Figure 6.16



SED.=23.12

Figure 6.17



the rate of increase in ammonia concentration was reduced. Ruminal ammonia concentration in sheep B was observed to decline during the first 9 observations of the experiment , after which the ruminal concentration of ammonia increased. The ammonia concentration of sheep C was found to increase up until experimental observation 5 after which the concentration of ruminal ammonia declined. Since all sheep showed different trends and the points of inflexion were not consistent the observed trends were not assumed to relate to the addition of YC to the diet. The trends observed in ruminal ammonia may therefore have occur due to natural variation

Table 6.3.13: Mean period effect on rumen fermentation end-products

Variate	Period/Treatment				S.E.D.	F.Pr.
	Period 1 Control	Period 2 YC	Period 3 YC	Period 4 Control		
pH	6.26 ^a	6.27 ^{ab}	6.39 ^{bc}	6.46 ^c	0.0589	≤0.1
NH ₃ (mg/l)	194.5 ^{ac}	202.5 ^a	149.3 ^b	166.3 ^{bc}	15.78	≤0.1
tVFA (mM)	85.76 ^a	81.76 ^{ab}	73.88 ^{bc}	69.87 ^c	5.028	≤0.1
Acetate mM	60.76 ^a	58.47 ^{ab}	50.26 ^{bc}	47.04 ^c	4.243	≤0.1
Propionate mM	15.17	11.49	12.49	12.46	2.435	0.538
Butyrate mM	8.507	10.257	9.516	8.297	1.141	0.395
isobutyrate mM	0.347	0.364	0.407	0.454	0.047	0.247
Valerate mM	0.823	1.033	1.039	1.37	0.446	0.694
isoValerate mM	0.146 ^a	0.143 ^a	0.162 ^a	0.242 ^b	0.029	≤0.1
Acetate molar %	70.87	71.34	67.77	66.75	2.199	0.24
Propionate molar %	17.606	14.163	17.087	18.173	2.74	0.535
Butyrate molar %	9.962	12.569	12.926	12.038	1.099	0.164
isoButyrate molar %	0.407 ^a	0.459 ^a	0.561 ^{ab}	0.668 ^b	0.07	≤0.1
Valerate molar %	0.98	1.289	1.424	2.008	0.557	0.417
isoValerate molar %	0.17 ^a	0.183 ^a	0.224 ^a	0.354 ^b	0.039	<0.05
A:P ratio	4.348	5.157	4.099	3.903	0.75	0.454

Means not sharing a common superscripts in the same row are significantly different (P < 0.1)

Table 6.3.14: Bent stick analysis of diet OMD, urinary allantoin excretion (g/d) and the rumen parameters NH₃, pH, tVFA plus the individual VFA

Parameter	Sheep	Overall			Slope 1				Slope 2				t value ¹
		a	b	r	Obs.	a	b	r	Obs.	a	b	r	
Mean (3d) OMD	A			0.073	1-5	821	1.289	0.332	6-16	768	1.394	0.554	0.399 ^{NS}
	B			0.288	1-6	801	2.379	0.756	7-16	806	0.655	0.306	7.336 ^{***}
	C			0.046	1-4			0.516	5-16			0.053	
Mean (3d) Urinary allantoin excretion (g/d)	A	15.2	0.071	0.423	1-8	13.31	0.144	0.704	9-14	36.6	-0.385	-0.849	6.780 ^{***}
	B	15.3	0.092	0.485	1-6	10.94	0.338	0.760	7-14	13.7	0.115	0.505	2.53 [*]
	C	17.0	0.118	0.603	1-8	13.04	0.295	0.880	9-14	32.7	-0.232	-0.847	8.932 ^{***}
Mean [NH ₃] (mg/l)	A			-0.272	1-7	148.8	3.457	0.678	8-16	114.6	0.949	0.524	9.76 ^{***}
	B	197.4	-1.068	-0.695	1-9	204.5	-1.387	-0.627	10-16	102.9	1.042	0.513	11.57 ^{***}
	C	227.3	-1.097	-0.484	1-5	179.9	4.163	0.909	6-16	245.8	-1.602	-0.478	22.881 ^{***}
Mean pH	A	6.2	0.009	0.904	1-5	6.3	-0.007	-0.591	6-16	6.2	0.009	0.909	1.247 ^{NS}
	B	6.3	0.005	0.682	1-5	6.4	-0.009	-0.534	6-16	6.3	0.003	0.484	0.77 ^{NS}
	C	6.1	0.003	0.577	1-5	6.3	-0.016	-0.743	6-16	6.1	0.004	0.816	1.481 ^{NS}

Parameter	Sheep	Overall			Slope 1				Slope 2				t value ¹
		a	b	r	Obs.	a	b	r	Obs.	a	b	r	
Mean t[VFA] (mM)	A	85.2	-0.498	-0.810	1-5	79.2	0.468	0.427	6-16	77.3	-0.315	-0.650	6.264***
	B	86.3	-0.392	-0.759	1-5	79.7	0.647	0.590	6-16	78.4	-0.211	-0.581	7.33***
	C	95.8	-0.316	-0.630	1-5	91.4	0.376	0.289	6-16	90.5	-0.195	-0.375	4.05***
Mean acetate (molar %)	A	73.7	-0.310	-0.816	1-5	65.7	0.561	0.918	6-16	79.6	-0.460	-0.884	12.655***
	B	73.4	-0.075	-0.602	1-5	70.9	0.250	0.901	6-16	72.9	-0.067	-0.452	5.556***
	C			0.174	1-12	73.6	-0.090	-0.621	13-16	100.6	-0.621	-0.965	9.15***
Mean propionate (molar %)	A	17.1	0.092	0.364	1-4	29.1	-1.262	-0.967	5-16	9.14	0.295	0.919	21.93***
	B	12.8	0.077	0.709	1-5	15.1	-0.174	-0.886	6-16	11.6	0.107	0.728	5.688***
	C	17.9	-0.078	-0.357	1-3	29.3	-1.572	-0.976	4-16	13.0	0.049	0.364	22.514***
Mean butyrate (molar %)	A	8.54	0.153	0.822	1-3	4.28	0.731	0.985	4-16	10.20	0.109	0.698	11.48***
	B	12.56	-0.033	-0.393	1-5	12.89	-0.114	-0.520	6-16	13.90	-0.065	-0.507	0.821 ^{NS}
	C			0.299	1-5	6.33	0.336	0.853	6-16	15.71	-0.101	-0.742	7.404***

¹ students t value represents comparison between gradients of slopes 1 and 2.

t values with a series of asterisks relate to the level of significance, *** = P < 0.001, ** = 0.01, * = 0.05.

6.4: Conclusions

Feeding the semi-purified diet 8 times a day successfully placed the rumen microflora under stress by inducing a low rumen pH whilst minimising the rumen diurnal variation of pH, ammonia, tVFA and the individual VFA proportions. Therefore EMNS may have been expected to be greater in this experiment than the first as balance in the supply of carbohydrate and N for microbial growth was improved. However, the average EMNS for this experiment was 26 gMN/kgDOM as compared to 24 gMN/kgDOM for the first animal experiment, which was assumed to be an under-estimate due to the poor urine collection technique used during the experiment. Therefore these results suggest that some other factor was inhibiting EMNS.

The trend analysis (bent stick) indicated that the YC supplement may have caused a shift in the fermentation pattern of each sheep to one which favoured the production of propionate. This is in contrast to the results discussed in Chapter 3 where supplemental YC was found to reduce the peak propionate production, but is in accordance with the findings of Harrison *et al* (1987a, 1988).

A shift in the absolute concentration (mM) of VFA, urinary excretion of allantoin and OMD (sheep B) was also found to coincide with the supplementation of YC. It may be postulated that the YC supplement induced an increase in saliva flow which due to its inherent buffering capacity increased rumen pH ($P < 0.1$). The increased saliva flow to the rumen may also have increased rumen outflow rate resulting in reduced rumen OM digestibility, ISDMD, tVFA production and increased EMNS. The compensatory effects of water reabsorption and recycling would explain why no trends in urinary excretion (g/d) consistent with YC supplementation were observed (Figure 6.3) and as the diet contained approx. 40% starch which may be digested post-ruminally, reduced OM digestion in the rumen may be compensated for by increased post-ruminal digestion of the diet and hence the lack of a consistent effect on the overall diet OMD.

The results of the current experiment also indicate that there may be a lag time before the effects of supplemental YC were observed and that the YC supplement may induce long term carry over effects. This is in accordance with the findings of Offer (1990), who measured rumen ISDMD daily and observed a 4-5 day lag after supplementation of the diet with YC before ISDMD increased, and a 7-8 day lag post-supplementation before ISDMD levels return to pre-treatment levels. However, the effect of additional YC in the present experiment was

confounded with period and makes it difficult to draw definite conclusions about the mode of action of the YC supplement from these results. Subsequent work utilised the latin square design which is not confounded by period to investigate the mode of action of additional YC on rumen function. The results of the current experiment also indicate that the experimental period length for a change over design experiment should be sufficiently long to avoid possible carry over effects.

In contrast to the above theory concerning the mode of action of supplemental YC, the variations observed in the rumen parameters may have resulted from natural inherent variation in rumen function. Subsequent work compared the biological variation observed in the rumen of sheep fed a fixed diet for 18 weeks (Chapter 8) in order to establish the magnitude of the inherent variation of rumen function.

CHAPTER 7: Evaluation of the effect of inclusion of yeast culture in the diets of sheep in two feeding regimens.

7.1: Introduction

The aim of the current experiment was to determine the effects of supplementary yeast culture on rumen function. The results of the first two animal experiments suggested that the semi-purified diet induced stressful conditions for the microflora in the rumen. However, the results of these experiments also suggested that the microflora may have been over-stressed resulting in unstable rumen conditions, and making the detection of possible supplementary yeast culture affects difficult. Therefore, a second aim of the experiment was to examine the effects of diet type. Williams *et al* (1991), found a significant interaction between the concentrate content of a diet and the ruminal response to supplementary YC. Their work showed that the response to supplementary YC was more pronounced with increasing levels of concentrate. This is in contrast to the findings of Chademana and Offer (1990), who found no such interaction when they compared the ruminal effects of supplementary YC at 3 forage : concentrate ratios. In order to achieve the aims of the current experiment, 4 mature fistulated sheep were fed either the semi-purified diet used in Chapters 3 and 6, 8 times a day or a more conventional hay-concentrate diet fed twice daily (Chademana and Offer (1990) medium diet). Both diets were fed with or without yeast culture, to mature fistulated wethers.

A possible cause of the variation noted in the 24h *in sacco* degradability is the type of substrate used in the bag. Previous experiments have used soya hulls and washed soya hulls. The current experiment compared the degradation patterns of absorbent cotton wool, (used because of its homogeneity and purity of cellulose type) and washed hay, (used because of its diversity of cellulose types). The hay was chopped in order to improve its homogeneity and washed to remove any fines and soluble material.

7.2: Materials and Methods

7.2.1: Animals and design

Four rumen-cannulated, mature wethers averaging 65kg live weight (LW.) were assigned to a 4x4 latin square design. Each experimental period was 3 weeks long and the first 14 days of each period was an adaptation phase to allow the rumen to adjust to the new diet. The design of the latin square used for this experiment is shown in Appendix 8

7.2.2: Diet

- a) Diet 1: The same semi-purified diet that was fed in both Chapters 3 and 6 was fed eight times-a-day by an automatic feeding system (Chapter 6). In total, 800g/dFW of concentrate and 200g/dFW of NIS nuts was fed/day.
- b) Diet 2: The ingredient and chemical composition of diet 2 is given below. This diet was fed in two equal feeds per day at 0900 and 1700.

Both diets were fed with or without 20 g/d of live yeast culture as specified by the experimental design fed in two equal doses at 0900 and 1700. The rolled barley, flaked maize, urea and mineral components of the diet were weighed out accurately (Table 7.2.2) and then mixed in a plastic bag. The hay was also accurately weighed into plastic bags. A plastic bag containing the mixed components (barley, maize, urea, minerals) was fed to each animal and was completely eaten before the hay was placed in front of the animals.

Table 7.2.1: Chemical composition of the ingredients of Diet 2

	Chopped hay	Rolled barley	Flaked maize	Urea
Dry matter (g/kg)	820	821	852	1000
Organic matter (g/kgDM)	930	973	980	1000
Crude protein (g/kg DM)	82	90	90	2910
Metabolisable energy (MJ/kg DM)	8.6	12.8	14.2	0

Table 7.2.2: Fresh weight of each ingredient consumed (g/day).

Feedstuff	g/day
Hay	766
Rolled barley	244
Flaked maize	244
Urea	14
Minerals	14

7.2.3: Measurements

- Daily: Rumen degradability of absorbent cotton wool (ACW)
Rumen degradability of washed hay
Rumen pH
- Days 10-14: Assessment of rumen and hindgut liquid dilution rate (LDR)
- Days 15 and 16: Diurnal rumen sampling for pH, NH₃ and VFA analysis
- Days 1,3,7 and 21: Assessment of rumen yeast viability sampling times 0900, 1100 and 1500
- Days 2,7 and 21: Faecal yeast counts

7.2.4: *in sacco* rumen degradability

The rumen degradability of absorbent cotton wool (ACW) and washed hay were measured simultaneously by the *in sacco* technique using 24h incubation periods in the rumen using the technique described in Chapter 2.

7.2.5: Analysis of rumen liquor for VFA and ammonia concentration

A series of rumen samples were taken from each sheep on days 15 and 16 of each experimental period. The first sample of each series was taken just prior to the morning feed and subsequently at 90 minute intervals. Each sample was preserved as described in Chapter 2 awaiting analysis at the end of the experiment.

7.2.6: Daily rumen pH

Daily rumen pH was measured directly through the rumen cannula just prior to morning feeding using a 30 cm pH probe connected to a portable pH meter. Prior to the insertion of the probe the *in sacco* bags were removed and the rumen contents around the cannula opening mixed with a short length of stiff polythene piping in order to allow access to the rumen solids phase.

7.2.7: Assessment of the rate of liquid outflow from the rumen.

Rumen LDR was estimated by faecal collection between days 10 and 14 of each experimental period, as described in Chapter 2.

7.2.8: Assessment of yeast viability in the rumen

Rumen liquor was aspirated from the rumen at 0900, 1100 and 1500 on days 1, 3, 7 and 21 of each experimental period. Yeast numbers were estimated by culturing the serial dilutions of 10^{-2} and 10^{-1} on potato dextrose agar, using the technique described in Chapter 3.

7.2.9: Faecal yeast culture analysis

Faeces (10g) was collected from each sheep at 1500 on days 2, 6 and 21 of each experimental period. The yeast was cultured on potato dextrose agar using the technique described in Chapter 3.

7.2.10: Interpretation, presentation and analysis of the results.

The daily 24h *in sacco* degradabilities and rumen pH levels were statistically analysed using cusum analysis. The other parameters were analysed using the analysis of variance option in Genstat 5.13. The BLOCK and TREATMENT structures used to determine the degrees of freedom used in the statistical analysis are shown in table 7.2.1.

Table 7.2.3: Block - treatment structure used for the analysis of variance.

BLOCK SHEEP*PERIOD/DAY/SAMPTIME

TREATMENT (SHEEP*DAY)+(DIET*YEAST*SAMPTIME)

Analysis of Variance table

Source of variation	d.f.
period stratum	3
sheep.period stratum	
sheep	3
diet	1
yeast	1
diet.yeast	1
Residual	6
period.day stratum	
day	1
Residual	3
sheep.period.day stratum	3
Residual	9
period.day.samptime stratum	
samptime.diet	5
samptime.yeast	5
samptime.diet.yeast	5
Residual	105
Total	191

7.3: Results and discussion

7.3.1: Rumen *in sacco* degradability (ISDMD)

The individual sheep CUSUM statistical analysis (Figures 7.1-7.8) show a wide inherent day-to-day variation in the degradation of both the washed hay and absorbent cotton wool (ACW).

The degradability results of both the ACW and washed hay are directly comparable since two bags of each type were incubated simultaneously in the rumen of each sheep on each day. This was carried out in order to evaluate the effect of substrate type on the day-to-day variation noted in the degradability measurements made in the rumen. The data shown in Figures 7.1-7.8 indicated that the *in sacco* substrate type did not have an effect on the inherent day-to-day variation.

Analysis of variance, based on the last seven days of each period showed that supplementation of either diet with yeast culture did not have a significant ($P > 0.05$) effect on the ISDMD of either substrate. The diet type and substrate used in the *in sacco* bags, were found to have a significant effect on ISDMD at the $P = 0.002$ and $P < 0.001$ level respectively (Table 7.3.1).

Table 7.3.1: The effect of diet and additional YC on rumen *in sacco* degradability (%)

Substrate	Viable yeast culture		Control	
	Diet 1	Diet 2	Diet 1	Diet 2
Hay	21.7 ^a	27.0 ^b	21.8 ^a	26.9 ^b
ACW	8.4 ^a	14.1 ^b	9.3 ^a	15.7 ^b

S.E.D. = 1.724

Means not sharing a common superscript, in the same row are significantly different ($P < 0.05$).

On average the washed hay was found to be more degradable (24.33%) than the absorbent cotton wool (11.89%) ($P < 0.001$). A preliminary experiment suggested that ACW would make

a suitable *in sacco* substrate. A possible explanation for the low ISDMD of the cotton wool may be because of its tendency to form mats in the *in sacco* bags which may reduce the effective surface area available for microbial degradation and inhibit thorough mixing of the *in sacco* bag contents with the rumen liquor, which has been shown to be an important factor for the correct assessment of ISDMD (Mehrez and Ørskov, 1977; Marinucci *et al.*, 1992).

Diet 2 was found to induce a higher rumen degradability (mean for ACW and hay 20.94%) as compared to Diet 1 (mean for ACW and hay 15.28%). This may be related to the effects of diet and the feeding regimen on conditions in the rumen (Table 7.3.2a,b,c,d).

The correlation tables below (Tables 7.3.2a,b,c,d) show the relationship between daily values for degradation of ACW and washed hay, and for daily pre-feeding rumen pH for each sheep:

Tables 7.3.2a,b,c,d: Correlation coefficients between daily mean ISDMD values of washed hay, ACW and rumen pH for each sheep.

a) Sheep A			b) Sheep B		
	ACW	Hay		ACW	Hay
Hay	0.453***		Hay	0.393**	
pH	0.318**	0.544***	pH	0.292**	0.346**
c) Sheep C			d) Sheep D		
	ACW	Hay		ACW	Hay
Hay	0.468***		Hay	0.259**	
pH	0.782***	0.361***	pH	0.088	0.368***

Asterisks show the significance of the correlation, (*** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$).

The above correlation coefficients suggest that ISDMD (for hay or ACW) was partially correlated with the pre-feeding rumen pH..

Table 7.3.3 shows the correlation coefficients between sheep for degradation *in sacco* of ACW or hay. Significant correlations ($P < 0.001$) were observed for some but not all sheep, but the correlations between sheep were found to be different for each *in sacco* substrate. This suggests that the significant correlations between sheep related to the order in which each animal received each diet type, the sensitivity of the *in sacco* substrate to detect changes in treatment and the degree of synchronisation between the ISDMD of the animals. Thus, for the $ISDMD_{ACW}$ (Figures 7.3 and 7.5) a positive ($P < 0.01$) correlation ($r = 0.354$) between sheep B and C was observed. This significant correlation probably reflects the change of diet from diet 1 to diet 2 in both animals (irrespective of YC supplementation) in the middle of the experiment. The significant ($P < 0.001$) negative correlation ($r = -0.295$) between sheep B and D ($ISDMD_{ACW}$) may also be related to the treatment structure of the Latin square in which these sheep received opposite diets in each experimental period (irrespective of YC supplementation).

The significant ($P < 0.001$) correlation ($r = 0.635$) between $ISDMD_{HAY}$ for sheep A and B was assumed to relate to the increased $ISDMD_{HAY}$ during the last two periods for these sheep. In sheep A the elevated $ISDMD_{HAY}$ values during periods 3 and 4 was assumed to relate to the change in diet type (from diet 1 to diet 2), but $ISDMD_{HAY}$ values were found to increase within experimental period 2 having steadily fallen through experimental period 1. The reason for this abrupt change in $ISDMD_{HAY}$ values was not clear but similar changes in trend were observed in sheep B and C (Figures 7.4 and 7.6) but not sheep D (Figure 7.8). The trend in $ISDMD_{HAY}$ did not occur in all sheep which suggests it was not related to either a variation in the hay substrate used *in sacco*, the hay substrate sampling procedure or the processing of the *in sacco* bags post-incubation. The trend in $ISDMD_{HAY}$ in sheep A fitted the bent stick trend model used in Chapter 6 but may have been due to inherent fluctuations in the ability of the rumen to degrade washed hay rather than YC inclusion.

The significant ($P < 0.001$) correlation ($r = 0.552$) between sheep A and D was assumed to relate to a decline in $ISDMD_{HAY}$ in experimental period 1 and increased $ISDMD_{HAY}$ during experimental period 3. Similar daily fluctuations in both sheep, not related to diet or YC supplementation were also observed such as those observed between experimental days 1-14, 22-25, and 43-46.

Sheep B and C were assumed to be significantly ($P < 0.01$) correlated ($r = 0.252$) due to an increased $ISDMD_{HAY}$ during experimental period 2 and the subsequent drop in $ISDMD_{HAY}$ observed in both sheep when the diet changed from diet 2 to diet 1. The increased $ISDMD_{HAY}$

Figure 7.1

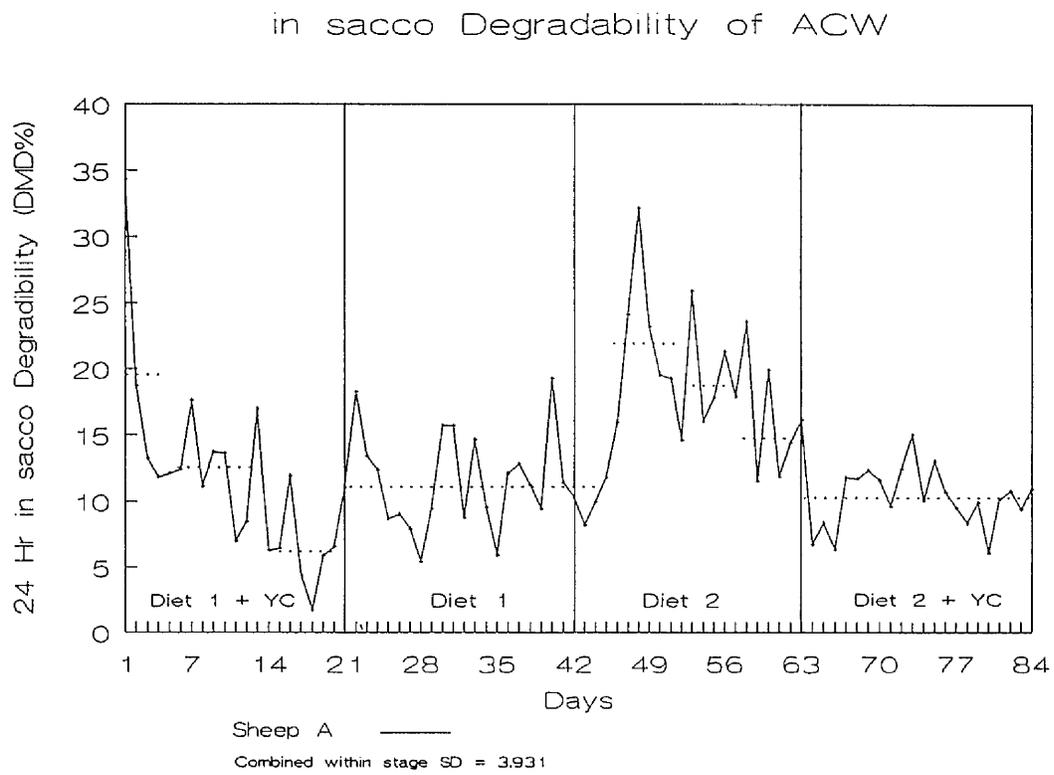


Figure 7.2

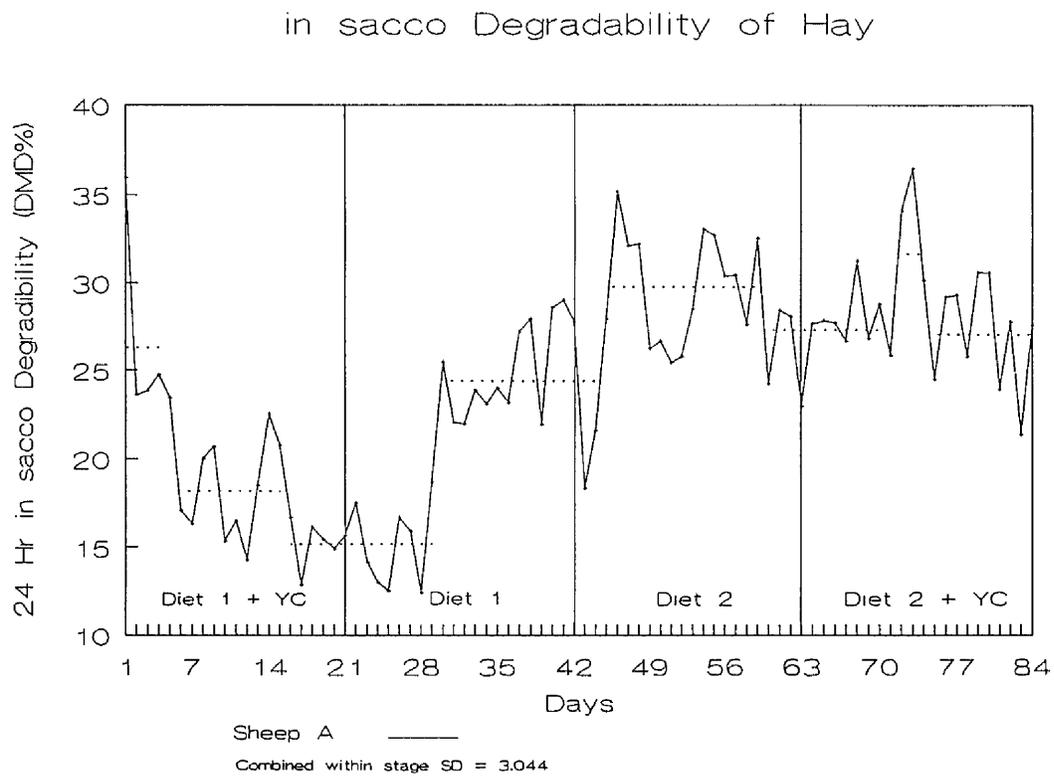


Figure 7.3

in sacco Degradability of ACW

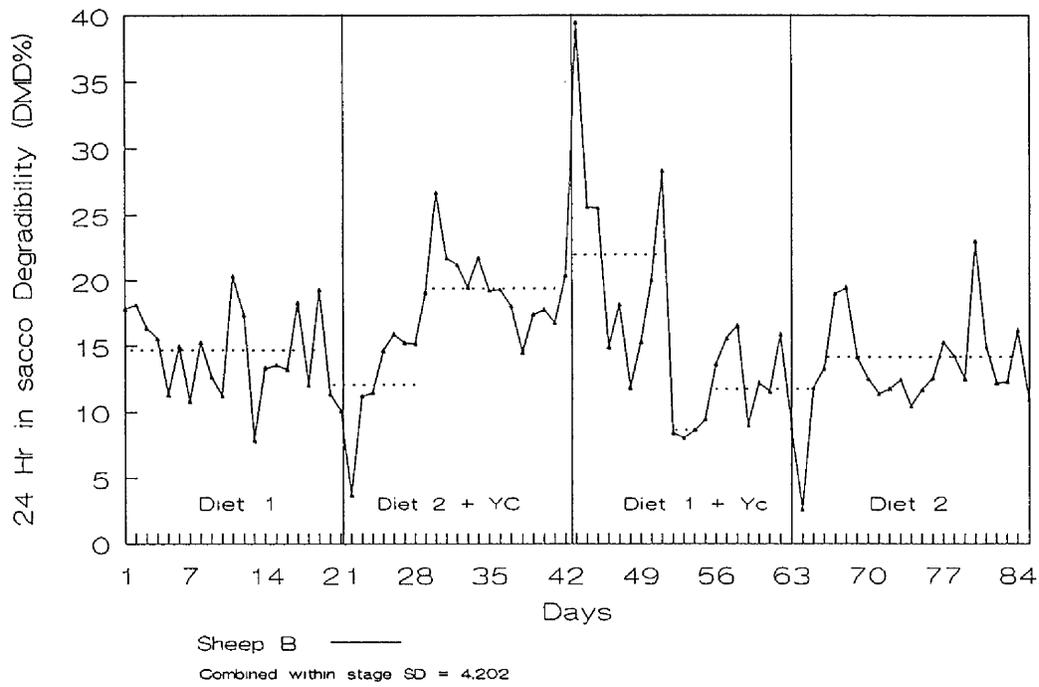


Figure 7.4

in sacco Degradability of Hay

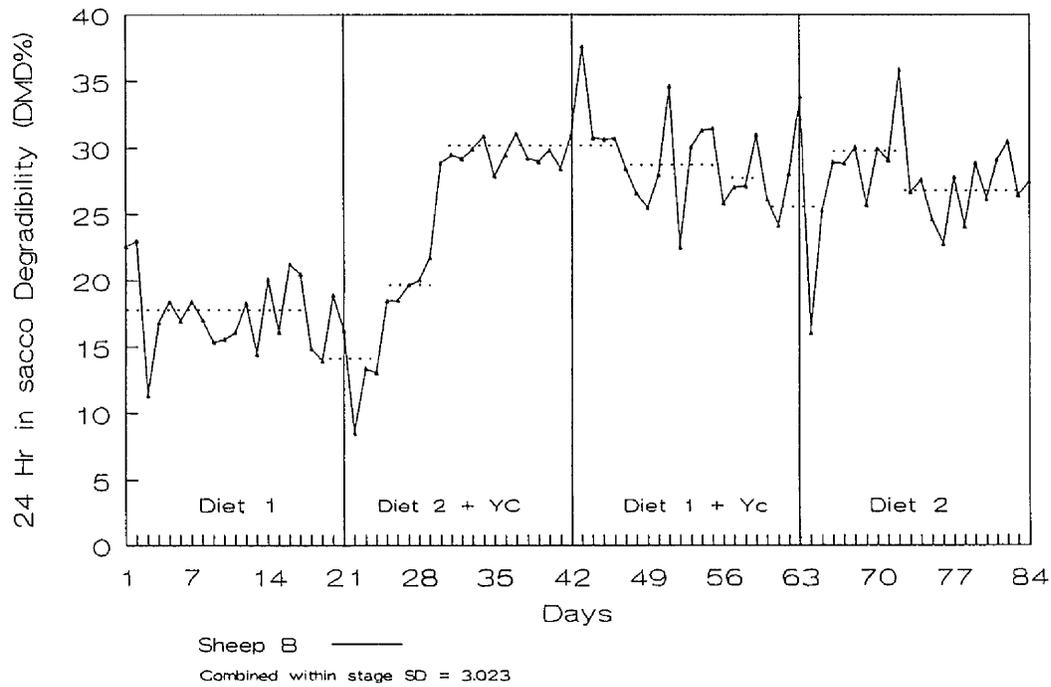


Figure 7.5

in sacco Degradability of ACW

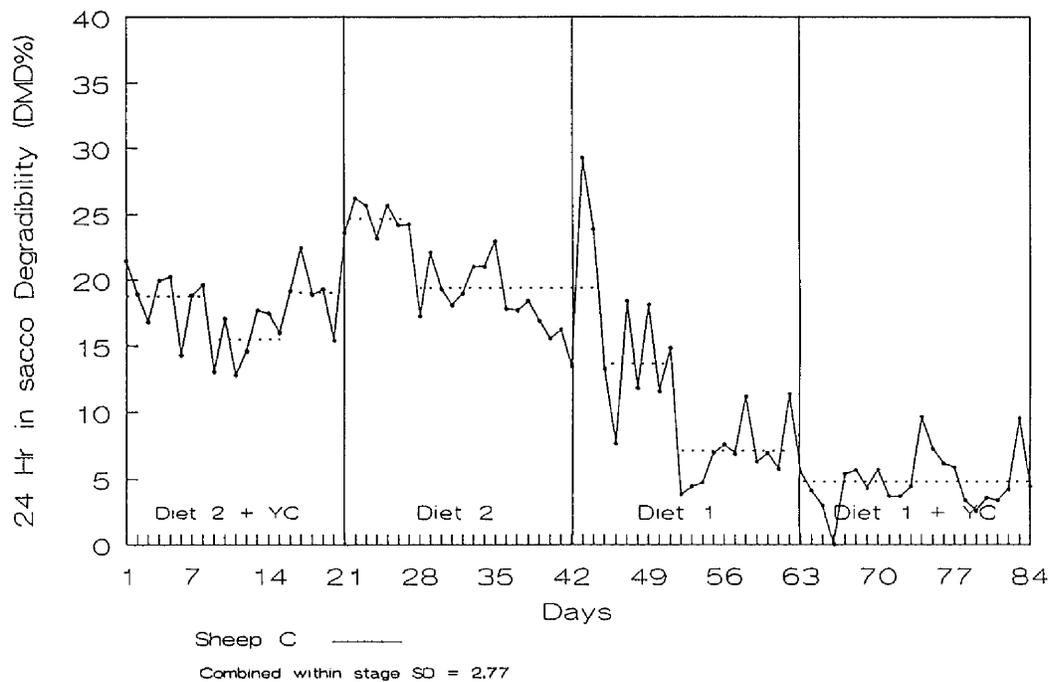


Figure 7.6

in sacco Degradability of Hay

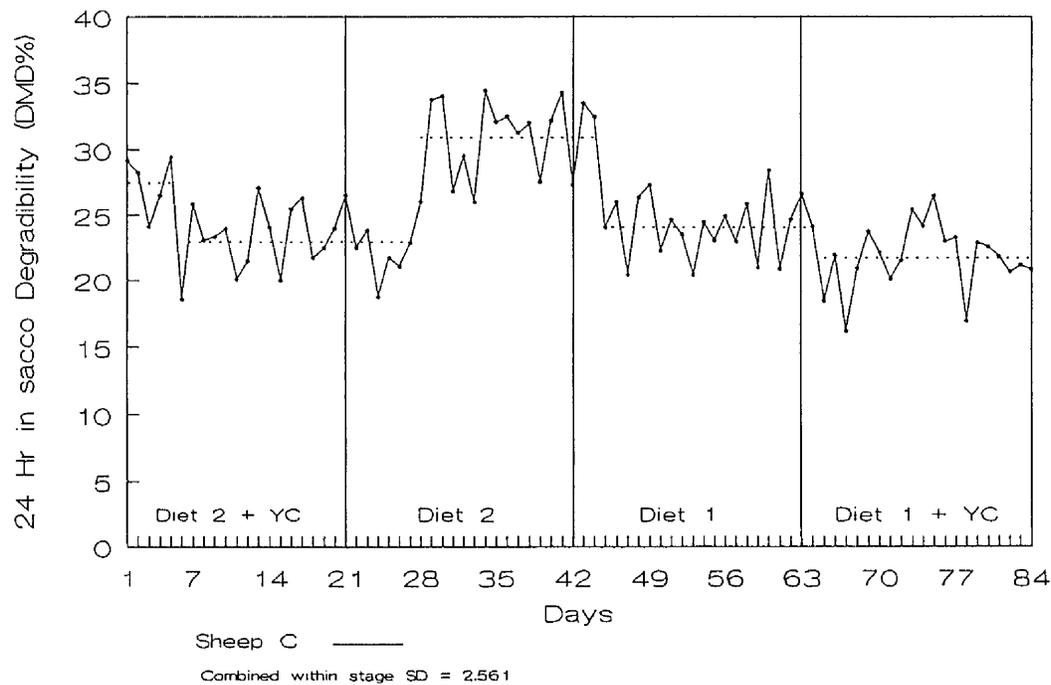


Figure 7.7

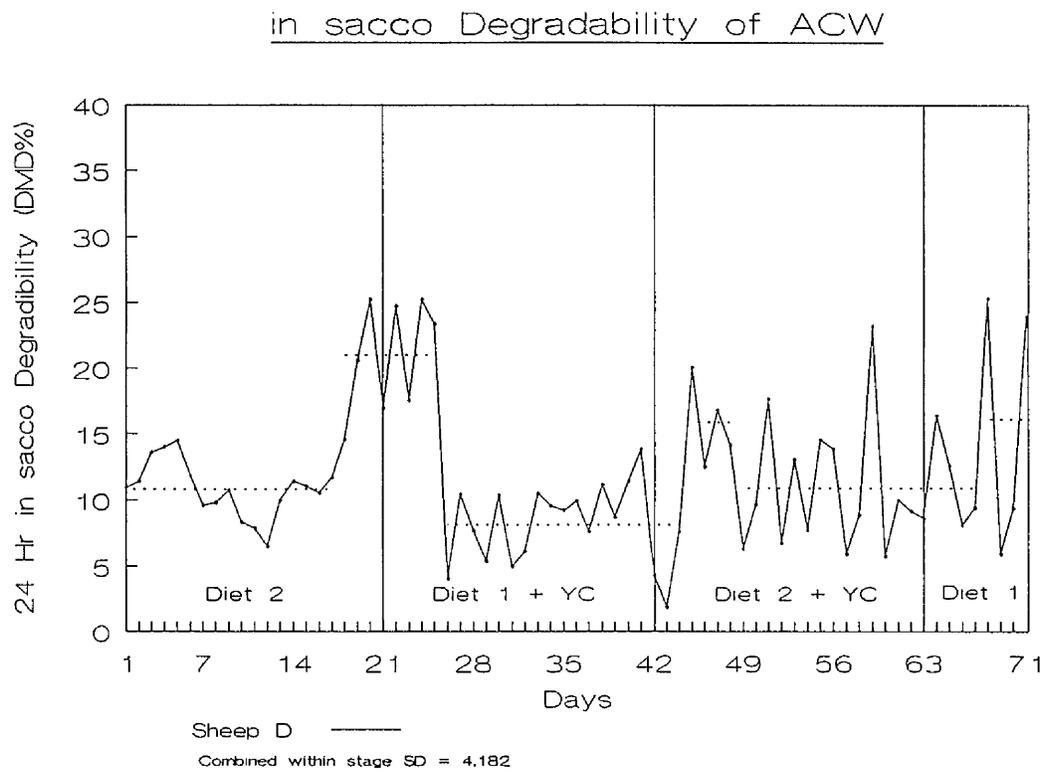
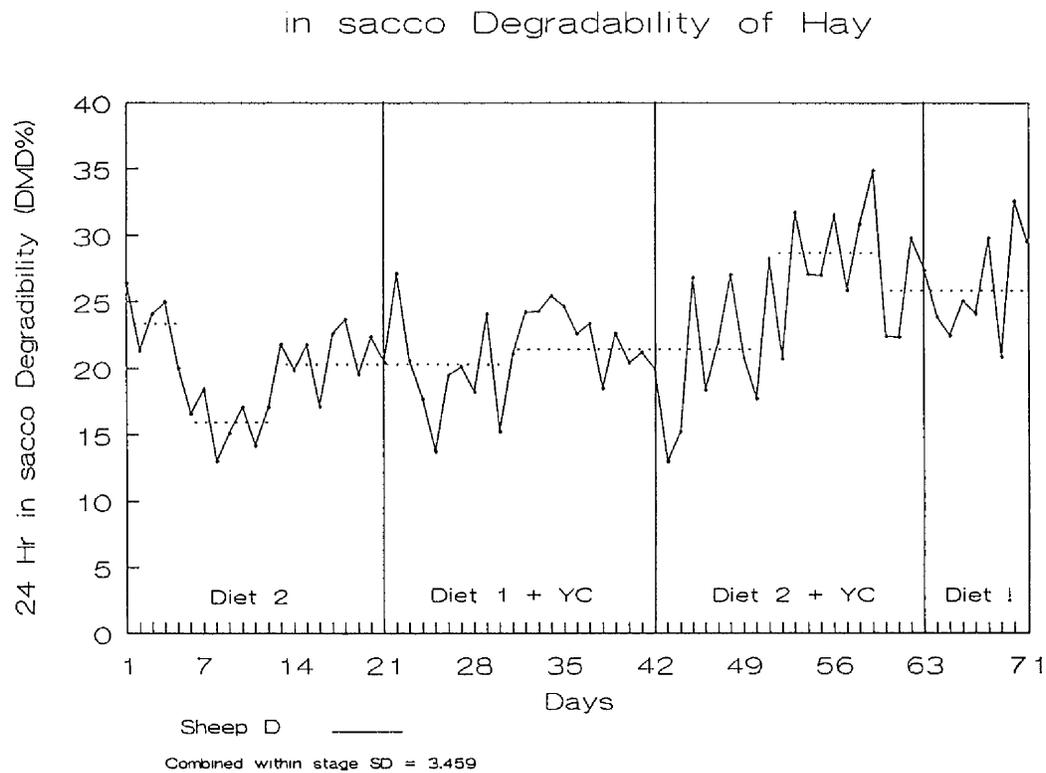


Figure 7.8



during experimental period 2 for both sheep was assumed to relate to the change in diet from diet 1 to diet 2 in sheep B. However, it was not clear why sheep C should follow a similar trend at this time in the experiment, since this sheep (C) did not experience the same change in diet. The corresponding trend in sheep C was assumed to relate to the inherent fluctuation in rumen function which may have been modulated by an external factor such as temperature which affected all sheep similarly, but inherent fluctuations were not found to be exactly replicated in the individual sheep (Figures 7.1-7.8).

The statistically significant correlation ($P < 0.01$; $r = 0.348$) between changes in $ISDMD_{HAY}$ for sheep B and D were assumed to similar daily fluctuations in $ISDMD_{HAY}$ which were not related to diet or supplemental YC. However, as the *in sacco* bags were prepared and processed in batches and the degradation patterns do not correspond with these batches, technical error has been ruled out as a possible explanation for the day-to-day variation.

Table 7.3.3: Correlation coefficients of *in sacco* degradation between sheep.

	Absorbent cotton wool			Washed hay			
	A	B	C	A	B	C	
B	-0.081			B	0.635***		
C	-0.036	0.354**		C	0.008	0.252**	
D	0.029	-0.295**	0.015	D	0.552***	0.348**	-0.129

Asterisks show the significance of the correlation (*** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$).

7.3.2: Daily rumen pH (taken pre-feeding).

Analysis of variance showed that diet type had a significant ($P = 0.047$) effect on rumen pH, but supplementation of either diet with yeast culture did not affect pH significantly ($P > 0.05$) (Table 7.3.2).

Table 7.3.4: Daily rumen pH treatment means

	YC	Control
Diet 1	6.306 ^a	6.428 ^a
Diet 2	6.601 ^b	6.621 ^b

S.E.D. = 0.0979

Means not sharing a common superscript, in the same column are significantly different ($P < 0.05$).

The individual sheep CUSUM statistical analysis shows an inherent day- to-day variation. The correlation between the daily rumen pH and *in sacco* measurements are given in Table 7.3.1. These correlations demonstrate that the degradability pattern of both the washed hay, and ACW was positively correlated to daily rumen pH variations within each sheep.

It may also be noted from Figures 7.9-7.12 that the rumen pH of diet 2 was significantly ($P=0.047$) higher than that of diet 1. The lower pH noted with Diet 1 was likely to have resulted from its high starch content. This finding agrees with the results of Chapters 3 and 6 in which the semi-purified induced low rumen pH values and is in accordance with the findings of Barry *et al* (1977a). The higher fibre content of Diet 2 would be expected to lead to a slower degradation of the feed in the rumen and hence result in a higher rumen pH. The higher pH noted on Diet 2 was thought to be responsible for the higher *in sacco* degradability as the rumen pH was maintained closer to the optimum pH for microbial activity (Mould and Ørskov 1983).

Similar long-term trends in daily rumen pH to those observed in Chapter 6 (Figures 6.7) were observed within a diet type. However, in the current experiment, trends in rumen pH were not consistent in all sheep (Figures 7.9-7.12). It was therefore assumed that the observed trends in daily pre-feeding rumen pH in the present experiment related to natural variation, possibly due to daily changes in tvFA production due to altered microbial activity on each day, daily fluctuations in drinking behaviour causing fluctuations in rumen outflow rates or changes in ruminal buffering due altered saliva secretion on a day-to-day basis by the animals.

Figure 7.9

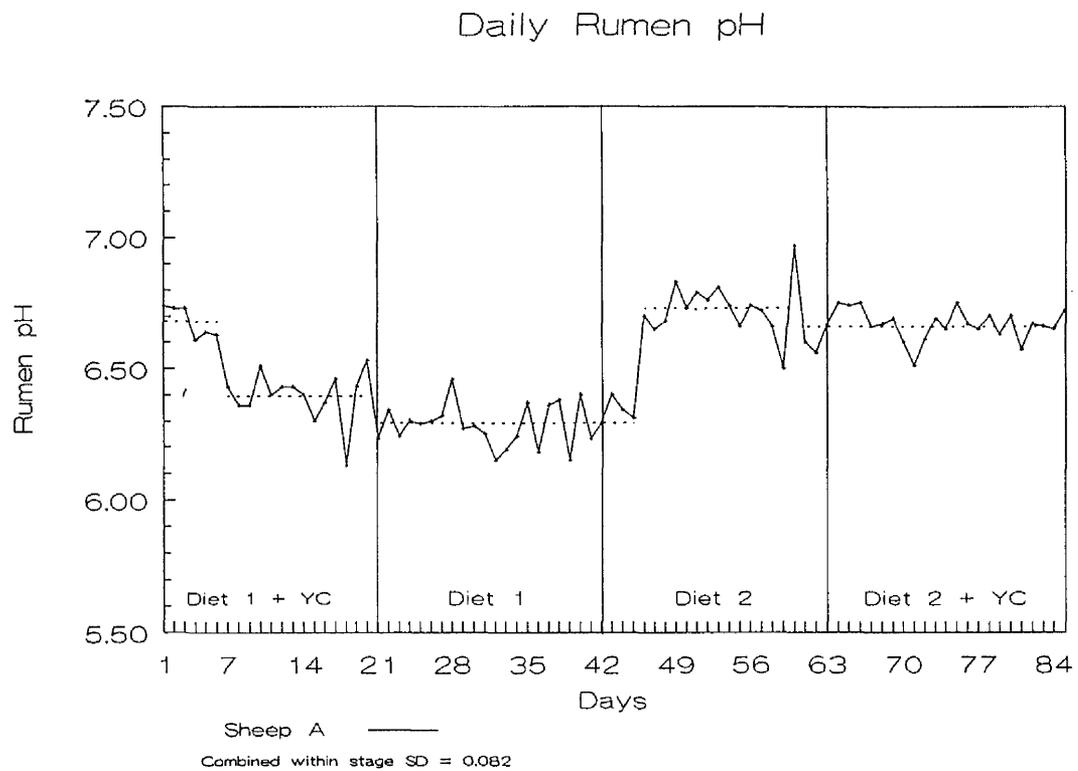


Figure 7.10

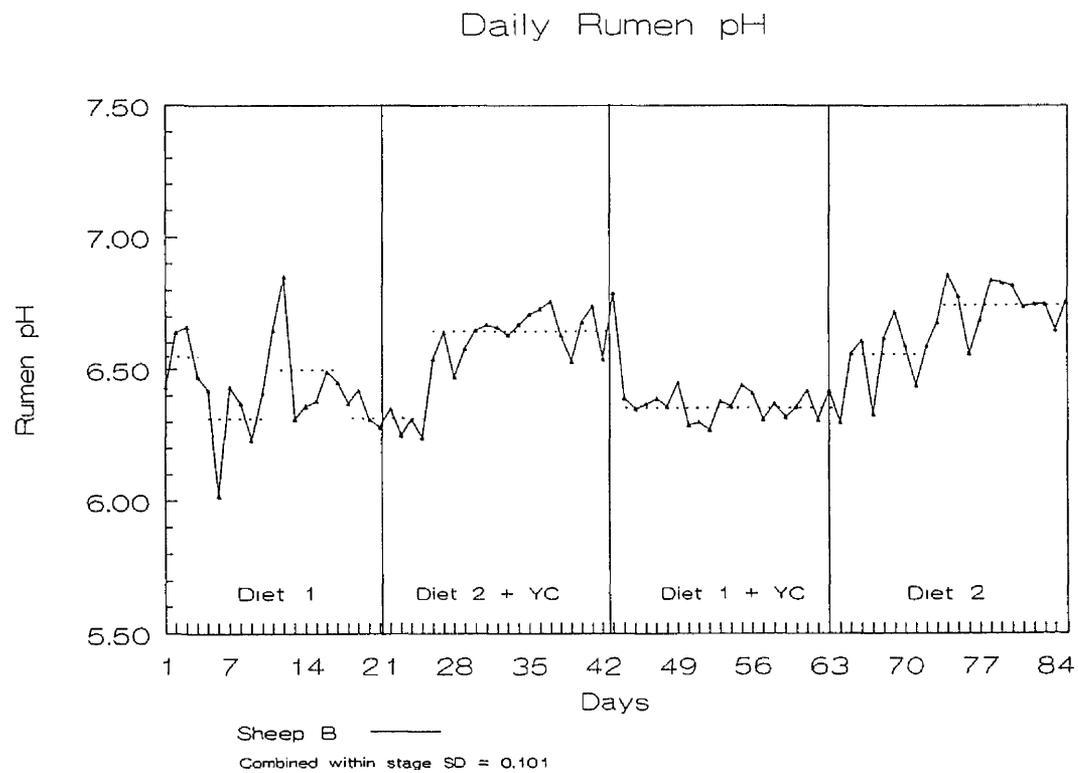


Figure 7.11

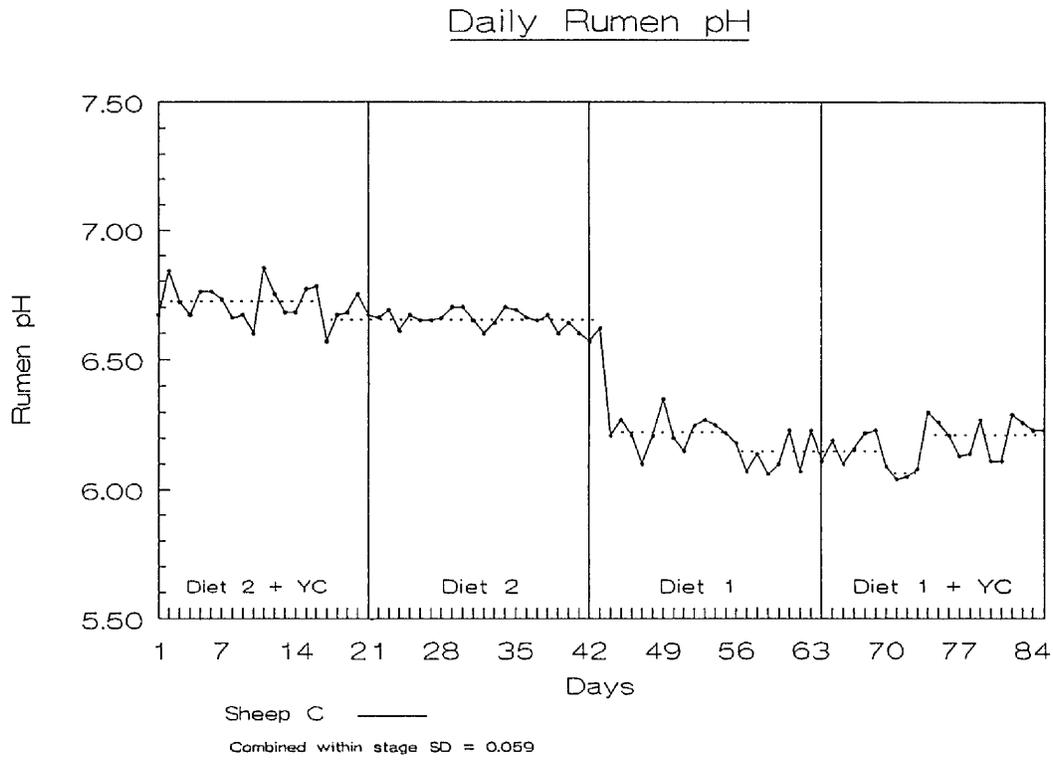
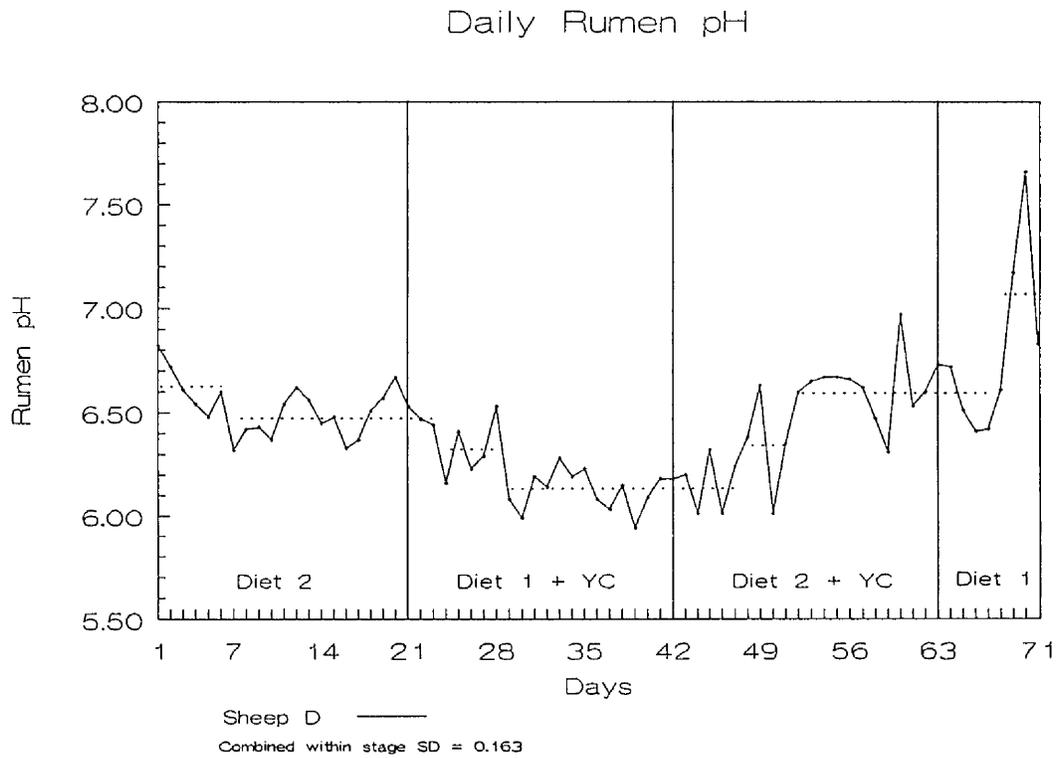


Figure 7.12



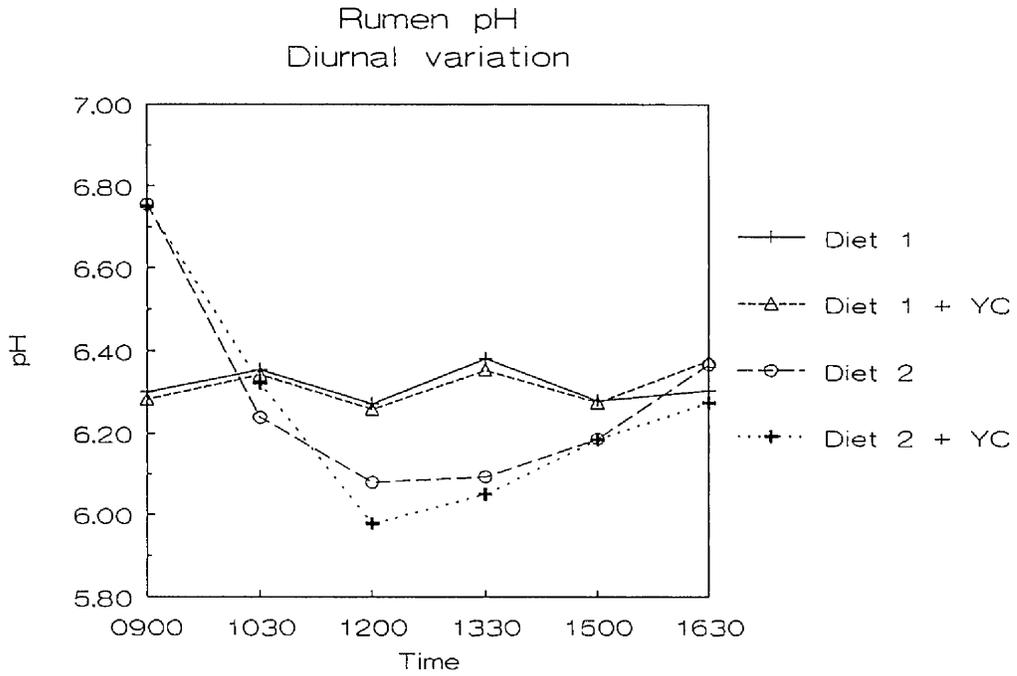
7.3.3: Rumen pH - Diurnal variation

Analysis of variance showed that diet type and supplemental YC did not significantly ($P > 0.05$) affect mean diurnal rumen pH. The effect of diet type on mean diurnal rumen pH was much less when measured throughout the day, than for the pre-feeding daily measurements (Table 7.3.4) Sampling time and the sampling time x diet interaction were however significant ($P < 0.001$), showing that the different diets led to significantly different diurnal patterns of rumen pH.

Figure 7.13 shows that by feeding Diet 1 eight times a day, the diurnal variation in rumen pH was eliminated. This agrees with the findings of Chapter 6. The diurnal pH pattern observed as a result of feeding Diet 2 twice a day was typical of that normally seen on a two times a day feeding regime and concurs with the results of Chapter 3.

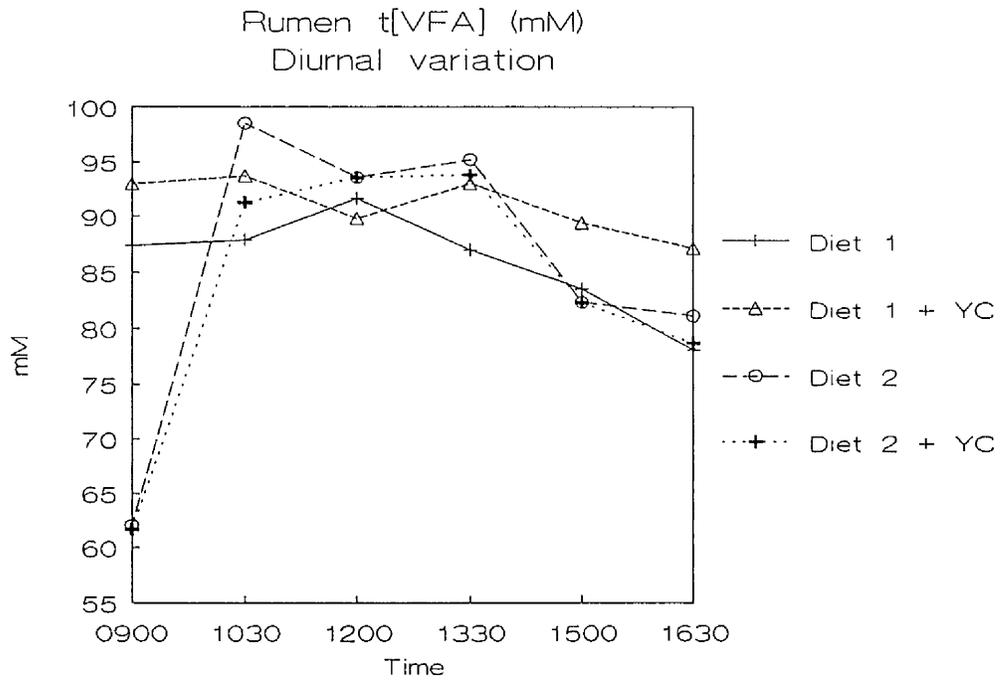
The greater effect of diet type on pre-feeding rumen pH compared to samples taken throughout the day may have resulted from the unequal feeding periods carried out on the twice a day feeding regimen (0900 and 1700). This would lead to an over-night recovery of the rumen to give a morning pre-feeding pH greater than that observed at 1630. It may also be suggested that as the rumen pH of diet 2 was not found to recover by 1630 to AM pre-feeding levels, the subsequent PM feeding may be expected to induce rumen pH levels below 6.0, the threshold level for the negative associative effects (Mould and Ørskov 1983; Mould *et al.* 1983). However, the *in sacco* results show that the degradability of both hay and ACW was greatest on diet 2, therefore either the absolute drop and the duration of the depressed rumen pH levels was not substantial, or some other factor in diet 1 was inhibiting cellulolysis. For example reduced rumination due to the small particle size of diet 1 (Eliman and Ørskov, 1984). The activity of rumination has been shown to be an important factor in rumen function (Czerkawski 1986), as this activity not only facilitates the mechanical breakdown of fibre but also aids the mixing of the rumen contents and stimulates salivary secretion (Woodford *et al* 1986). It may therefore be postulated that increased rumination due to the higher content of long fibre fed on diet 2 facilitated an improved rumen fermentation and improved infusion of the *in sacco* bags with the liquid phase of the rumen contents and hence show increased ISDMD on diet 2.

Figure 7.13



S.E.D.= 0.0897

Figure 7.14



S.E.D.= 7.561

7.3.4: Liquid outflow rate (LDR)

7.3.4.1: Rumen

Analysis of variance showed that the rumen LDR was not significantly affected by supplemental yeast culture ($P > 0.05$) for both diets. Sheep ($P = 0.004$) and diet type ($P = 0.003$) had a significant effect.

Table 7.3.5: Mean LDR for each sheep (mgCr-EDTA/h)

Sheep A	Sheep B	Sheep C	Sheep D	S.E.D.
0.0229 ^a	0.06 ^b	0.0481 ^b	0.046 ^b	0.00524

Means not sharing a common superscript are significantly different ($P < 0.01$).

It can be seen from table 7.3.5 that sheep A had a much slower LDR than the other sheep. It was assumed that the LDR for this sheep was slower throughout the experiment and responded to treatment in the same way as the other sheep. Biological variation was assumed to be responsible for this difference between sheep.

Eliman and Ørskov (1984), found that an increased concentrate : forage ratio and shortening the length of the fibre in the diet reduced rumen outflow rate. Table 7.3.6 shows that Diet 1 induced a much slower LDR than Diet 2. This was assumed to be as a result of the pelleted nature of Diet 1 reducing salivation and rumination, these results concur with the theory postulated for the effect of diet on ISDMD and daily rumen pH levels.

Table 7.3.6: The effect of diet and supplementary YC on rumen LDR (mgCr-EDTA/h)

Diet	YC	Control
1	0.0323 ^a	0.0368 ^a
2	0.0572 ^b	0.0509 ^b

S.E.D. = 0.00371

Means not sharing a common superscript, in the same column are significantly different ($P < 0.01$)

7.3.4.2: Hindgut

Analysis of variance showed that hindgut outflow rate was not significantly ($P > 0.05$) affected by sheep, diet or yeast (Tables 7.3.7 and 7.3.8). The accuracy of these results was reduced due to a reduced numbers of samples taken before the point of inflexion in the Grovum and Williams (1973) model.

Table 7.3.7: Mean hindgut outflow rate for each sheep (mgCr-EDTA/h)

Sheep A	Sheep B	Sheep C	Sheep D	S.E.D.
0.16	0.19	0.135	0.175	0.0518

Table 7.3.8: The effect of treatment on hindgut outflow rate (mgCr-EDTA/h)

Diet	YC	Control
1	0.134	0.149
2	0.150	0.183

S.E.D. = 0.0518

7.3.5: Microbial analysis

7.3.5.1: Rumen yeast viability

Analysis of variance showed that diet type did not have a significant ($P > 0.05$) affect on yeast viability in the rumen. The numbers of yeast recovered ($P < 0.001$) and time of sampling ($P = 0.017$) were found to be significant for diets supplemented as apposed to unsupplemented diets (Table 7.3.9).

Table 7.3.9: Mean yeast recovered ($\text{Log}_{10}\text{CFU/ml}$)

Diet	YC	Control
1	3.63 ^a	1.50 ^b
2	3.45 ^a	1.39 ^b

S.E.D. = 0.267

Means not sharing a common superscript, in the same row are significantly different ($P < 0.01$)

Table 7.3.10 shows that there was an increase in viable yeast recovered from the rumen 2 hours post supplementation. The regression of the viable counts post supplementation against time gave a rate loss constant of 0.149 CFU/ml/h. The average rumen liquid outflow (rate constant) was found to be 0.0443. It was assumed that the viable yeast count showed a faster outflow due to sampling error, death of yeast cells due to nutrient shock during the culturing techniques, and autolysis of the yeast in the rumen. The yeast was assumed to be able to survive in the rumen environment, but unable to thrive there making daily supplementation of the probiotic necessary. These results are in accordance with those of Chapter 3 and agree with the findings of Newbold *et al* (1990).

Table 7.3.10: Diurnal mean yeast culture recovery ($\text{Log}_{10}\text{CFU/ml}$) from the rumen

Time of sampling			S.E.D.
09.00	11.00	15.00	
2.03 ^a	2.74 ^b	2.71 ^b	0.274

Means not sharing a common superscript, are significantly different ($P < 0.05$).

Table 7.3.11: Variation in viable rumen yeast count ($\text{Log}_{10}\text{CFU/ml}$) within each experimental period.

Period day	Diet 1		Diet 2	
	YC	Control	YC	Control
1	3.95 ^a	2.04 ^b	3.59 ^a	1.88 ^b
3	3.23 ^{ab}	2.24 ^b	3.84 ^a	0.2 ^c
7	3.82 ^a	1.48 ^b	3.02 ^a	2.07 ^b
21	3.42 ^a	0.41 ^b	3.48 ^a	1.05 ^b

S.E.D. = 0.59

Means not sharing a common superscript, in the same row are significantly different ($P < 0.05$).

The high viable yeast count ($\text{Log}_{10}\text{CFU/ml}$) on day 1 of each experimental period (Table 7.3.11) was assumed to relate to carry over effects from previous experimental periods. However, rumen viable yeast counts were found to be significantly higher in supplemented animals than control animals by the end of the experiment period.

7.3.5.2: Faecal viable yeast counts

Analysis of variance showed that significant levels of yeast were recovered in the faeces ($P < 0.001$) (Table 7.3.12). The sampling day ($P = 0.049$) and the sampling day x yeast ($P = 0.007$) interaction were also found to be significant.

As in the first animal experiment, YC was shown to be able to survive the rumen, abomasum and hindgut. The potential for yeast culture to exert an effect in the hindgut must therefore be considered.

Table 7.3.12: Faecal yeast culture recovery on different experimental period days
(Log₁₀CFU/ml)

Period Day	Diet 1		Diet 2	
	YC	Control	YC	Control
2	4.138 ^a	1.927 ^a	3.291 ^a	1.774 ^a
7	2.237 ^b	1.885 ^a	2.307 ^b	1.674 ^a
21	2.471 ^b	1.618 ^a	2.459 ^b	1.774 ^a

S.E.D. = 0.306

Means not sharing a common superscript, in the same column are significantly different (P < 0.05)

The viable yeast numbers recovered from the faeces on day 2 of each experimental period was significantly higher than the levels recovered on days 7 and 21. This may be explained by postulating that autolysed yeast cells stimulate the autolysis of other yeast cells, or that the fresh addition of the yeast supplement stimulated the autolysis of the yeast cells already present in the rumen, and that on day 2 of each experimental period an equilibrium between the fresh addition of viable yeast cells and autolysed cells in the rumen had yet to be achieved. Therefore, more viable cells were passing out of the rumen on day 2. By day 6 of each experimental period an equilibrium had been reached resulting in less viable yeast cells being washed out of the rumen, and hence reducing the faecal viable yeast count on days 7 and 21.

Tables 7.3.11 and 7.3.12 both indicate that the yeast supplement may survive in the rumen and hindgut for long periods after supplementation has ceased, and therefore confound the experimental periods. However, the rumen viable yeast counts (Table 7.3.11) indicated that significantly higher yeast counts were observed in the rumen by the end of the experiment and control levels had dropped to those which may be expected due to microbial contamination of the feedstuff (Lund 1974). Thus, since the effects of the supplemental YC on the ruminal parameters were assessed during the last week of each experimental period it was assumed that the carry over effects would be minimal.

7.3.6: Rumen volatile fatty acids

7.3.6.1: t[VFA] (mM)

Analysis of variance showed that tVFA concentration (mM) was not significantly ($P > 0.05$) affected by diet or yeast supplementation (Table 7.3.15). However, sheep ($P = 0.045$), sampling time ($P < 0.001$) and sampling time x diet interaction ($P < 0.001$) were found to be significant.

Table 7.3.13: Mean sheep t[VFA] mM

Sheep A	Sheep B	Sheep C	Sheep D	S.E.D.
79.54 ^a	86.38 ^{abc}	82.70 ^{ac}	97.32 ^b	4.569

Means not sharing a common superscript, are significantly different ($P < 0.05$).

Table 7.3.14: The effect of diet and supplemental YC on t[VFA] mM

Diet	YC	Control
1	91.00	85.91
2	83.55	85.47

S.E.D. = 4.569

Figure 7.14 demonstrates the relationship between the diets, yeast supplementation and the sampling time. It was observed that tVFA concentrations for Diet 1 remained fairly constant during the day as compared to those for Diet 2 which shows a typical pattern for a feedstuff fed twice a day. The effect of rumen pH on the absorption of the individual VFA was not assumed to have a significant effect within a diet as the ruminal pH response to YC treatment was not significant ($P > 0.05$). However, when comparing between diets the lower ruminal pH observed on diet 1 may have favoured the absorption of propionate leading to an increase in the A:P ratio. The inverse diurnal variation between rumen t[VFA] and pH observed in Chapter 3 was not found when diet 1 was fed ($r = -0.094$) but was found to exist when diet

2 was fed ($r = -0.896$; $P < 0.05$). The difference due to diet was assumed to be related to a complex interaction between the rate of fermentation, utilisation and absorption of the VFA and ammonia (Table 7.3.16).

Table 7.3.15 shows that although $t[\text{VFA}]$ was not affected by either diet or YC ($P > 0.05$) the ruminal concentrations of acetate and propionate contributing to the total VFA concentration were significantly affected by diet ($P < 0.01$). It was assumed that the difference in the ruminal concentrations of these acids were due to both a combination of the different RFC content of the diets and the affect of pH on the absorption of these VFA Barry *et al* (1977a, Ørskov and Ryle (1990). Table 7.3.15 shows that the ruminal concentration of acetate was higher and propionate concentration lower on diet 1 as compared to diet 2. The mM butyrate concentration was not affected by diet type. The overall effect resulted in only a small differences in $t[\text{VFA}]$ between the two diets. Table 7.3.15 also shows that the ruminal concentration of acetate increased non-significantly ($P = 0.306$) and propionate decreased ($P = 0.055$) when either diet was supplemented with YC. These changes were assumed to relate to changes in the fermentation activity of the ruminal microflora as the within diet rumen pH was not affected by YC supplementation ($P > 0.05$). However, the combination of these effects did not lead to a significant change in the A:P ratio ($P = 0.151$).

The relationship between the ruminal concentration of the individual major VFA as a proportion of $t[\text{VFA}]$ are discussed below.

7.6.2: Molar % acetate

Analysis of variance showed that molar % acetate was significantly affected by sheep ($P = 0.03$), diet ($P < 0.001$), yeast supplementation ($P = 0.021$) and sampling time ($P < 0.001$). The sampling time x diet interaction and the sampling time x diet x yeast interactions were also found to be significant ($P < 0.001$ and $P = 0.029$ respectively). The means of diet and YC are given in Table 7.3.15.

Figure 3.15 shows the relationship between the diets, yeast supplementation and the time of sampling. It may be noted from this figure that supplementation of either diet with yeast culture elevated molar % acetate (on average) by 2.4% (1.6% absolute). This increase was statistically significant ($P = 0.021$) but not likely to have a major physiological effect on the animal.

Table 7.3.15: The effect of diet and supplemental YC on rumen fermentation end-products

Parameter	Treatment				S.E.D.	F.Pr.
	Diet 1	Diet 1 + YC	Diet 2	Diet 2 + YC		
pH	6.3	6.3	6.3	6.3	0.05	0.804
NH ₃	179.7	173.0	98.3	112.4	7.47	0.644
t[VFA]	85.9	91.0	85.4	83.6	3.23	0.641
Acetate (mM)	60.8	66.0	53.4	53.5	2.33	0.306
Propionate (mM)	14.0 ^a	12.6 ^b	20.1 ^a	17.9 ^b	0.74	0.055
Butyrate (mM)	9.3	11.0	9.9	9.6	0.59	0.255
isoButyrate (mM)	0.4	0.5	0.6	0.6	0.03	0.44
Valerate (mM)	1.2	0.8	1.2	1.5	0.15	0.761
isoValerate (mM)	0.2	0.2	0.4	0.4	0.02	0.327
Acetate molar %	70.8 ^a	72.6 ^b	63.2 ^a	64.5 ^b	0.49	0.021
Propionate molar %	16.5 ^a	13.8 ^b	22.8 ^a	21.0 ^b	0.58	0.011
Butyrate molar %	10.6	12.0	11.6	11.5	0.61	0.34
isoButyrate molar %	0.5	0.5	0.7	0.8	0.04	0.393
Valerate molar %	1.4	0.9	1.3	1.8	0.14	0.77
isoValerate molar %	0.2	0.2	0.5	0.5	0.03	0.28
A:P ratio	4.8	5.3	2.9	3.2	0.25	0.151

Means not sharing a common superscript, for the individual diets, in the same row are significantly different at the stated level of significance.

It can be seen from table 7.3.15 that rumen fluid from sheep fed Diet 1 had a significantly higher proportion of acetate than that of Diet 2 (71.7% cf. 63.8% respectively). Acetate production is generally considered to be favoured in the rumen when a diet of a high fibre content is fed. Under these circumstances, rumen pH values do not fall below 6.2 and show little diurnal variation. In the current experiment by contrast, the highest acetate % was shown by the diet with the smallest fibre content. This discrepancy may relate to the different feeding regimens used for the two diets. Diet 1 (which was fed 8 x per day) led to stable rumen pH values above 6.2, whilst diet 2 (fed 2 x per day) caused a marked diurnal variation with pH values falling below 6.0 and a slightly lower mean pH (Figures 7.9-7.13). Diet 2 however, supported higher rates of cellulose digestion in the rumen (as shown by ISDMD values for hay and ACW). This indicates that mechanisms other than rumen pH may be important in the

regulation of cellulose digestion in the rumen. For example, the high starch content of Diet 1 may have inhibited cellulolytic bacteria (Mould and Ørskov 1983). These findings are in accordance with those of Huhtanen and Khalili (1992). In their study on the effect of sucrose supplements on particle-associated carboxymethylcellulase (CMCase) and xylanase activity in cattle fed a grass-silage-based diets, they observed higher CMCase activity in the microbial population of the rumen liquid phase, when the diet was supplemented with sucrose. They suggested that this may indicate that cellulolytic microbes may preferentially utilise soluble sugars before starting to degrade cell-wall carbohydrates.

7.3.6.3: Molar % propionate

Analysis of variance showed that molar % propionate was significantly affected by sheep ($P=0.003$), diet ($P<0.001$), yeast ($P=0.011$) and sampling time ($P<0.001$). The sheep x day and sampling time x diet interactions were also significant $P=0.025$ and $P<0.001$ respectively. The means of diet and YC are given in Table 7.3.15.

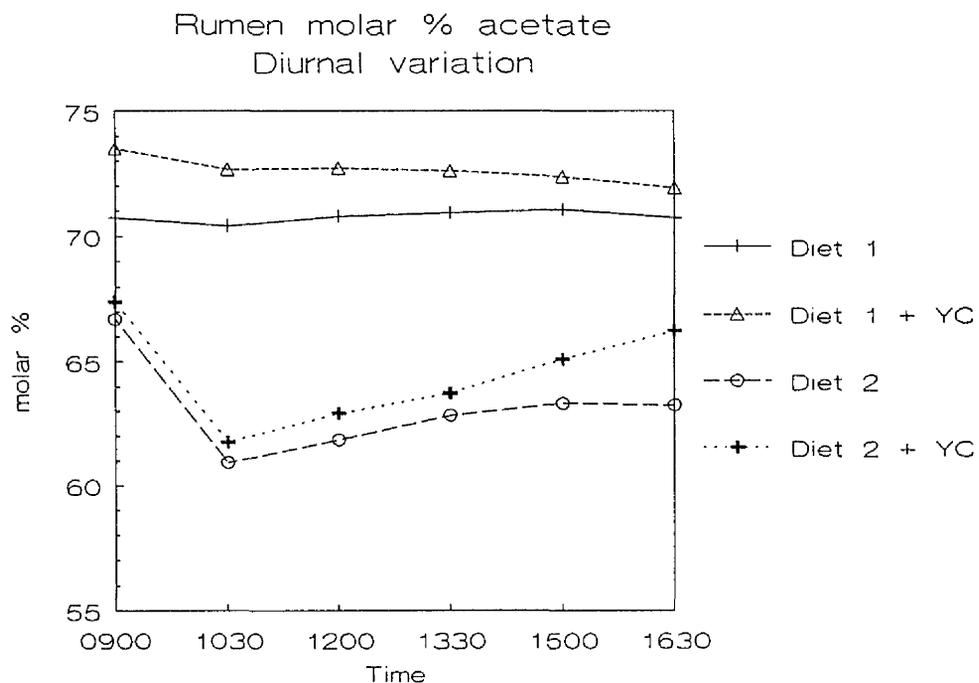
The effect of diet, yeast and time of sampling is shown in Figure 7.16. It may be seen from this figure that diet 1 induced a lower rumen molar % propionate than diet 2, and that the supplementation of either diet with yeast culture significantly ($P=0.011$) lowered molar % propionate levels by 13% (2.3% absolute). The interaction of time and diet is also demonstrated in this figure. It can be seen that diet 1 induced very little diurnal variation whereas diet 2 induced the type of diurnal variation normally observed on a twice a day feeding regime.

The significant ($P=0.025$) interaction between sheep and day was assumed to be relate to inherent fluctuations in daily rumen function as indicated by daily ISDMD and rumen pH measurements. However, future work measuring daily rumen VFA will have to be carried out in order to verify this.

7.3.6.4: Molar % butyrate

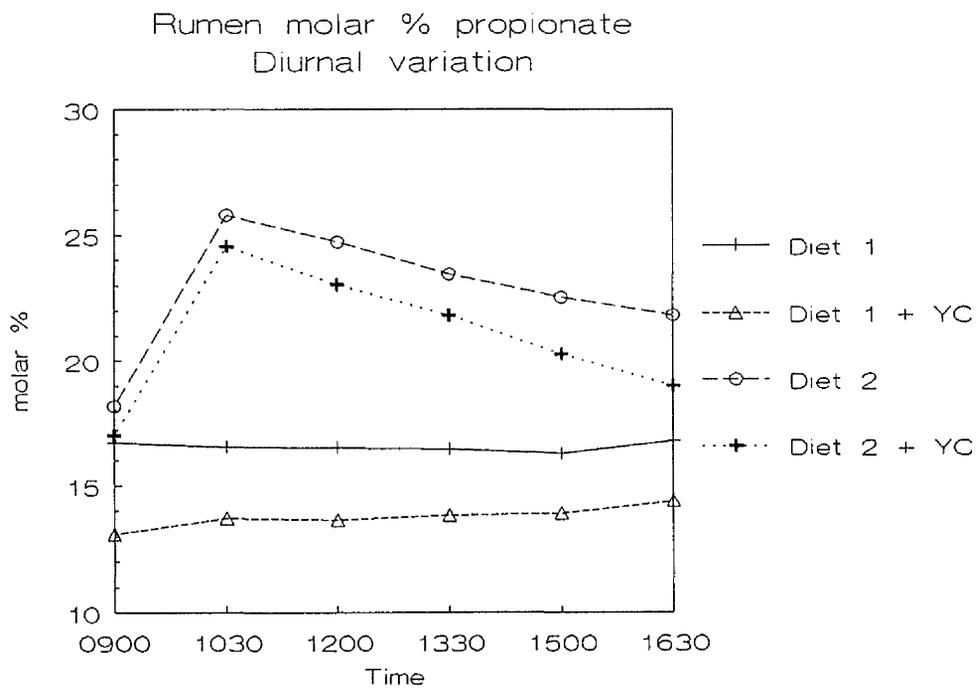
Analysis of variance showed that diet and supplemental yeast culture did not have a significant effect on rumen molar % butyrate. However, the time of sampling and its interaction with diet were shown to be significant ($P<0.001$). The means of values as affected by diet and YC are given in Table 7.3.15.

Figure 7.15



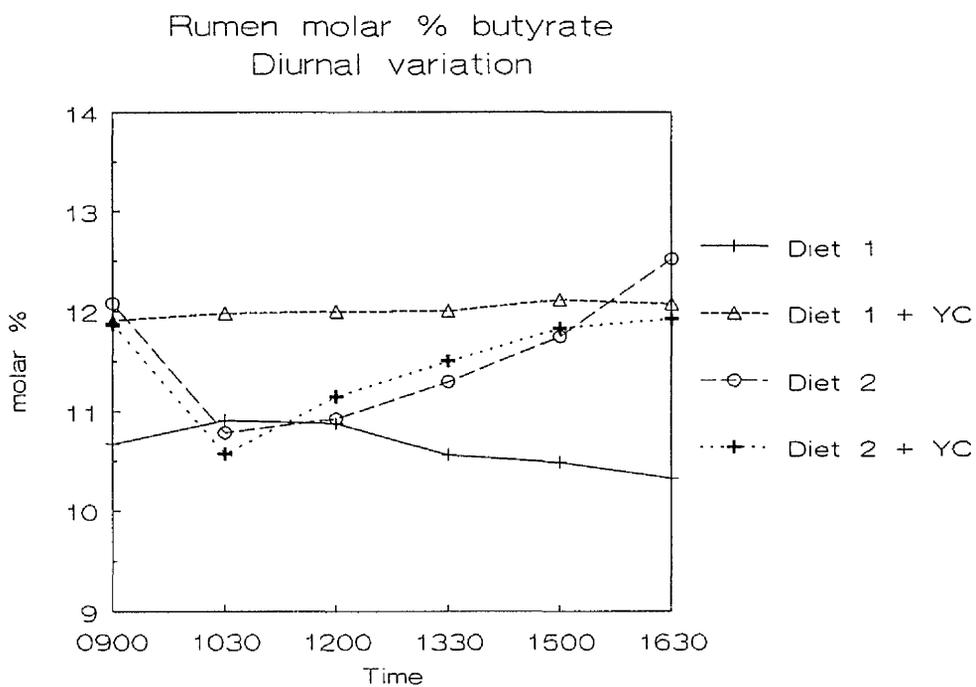
SED.= 0.874

Figure 7.16



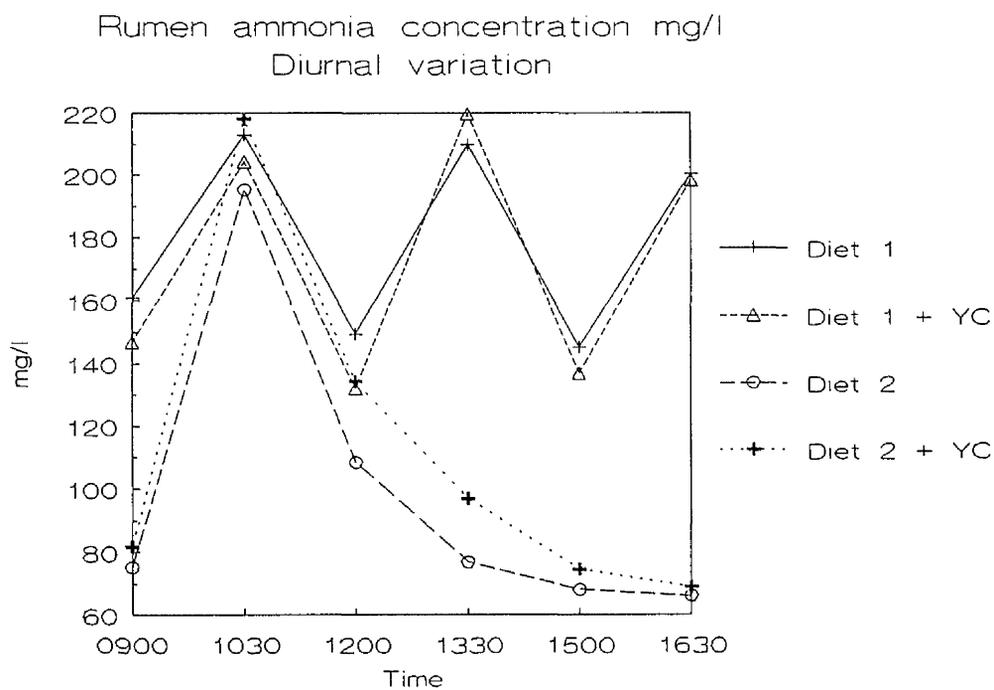
SED.= 0.951

Figure 7.17



S.E.D.= 0.904

Figure 7.18



S.E.D.= 16.96

Figure 7.17 shows the relationship between diet, yeast supplementation and time of sampling. From this figure it was observed that Diet 1 showed very little diurnal variation whereas Diet 2 demonstrated the characteristic drop in Butyrate levels noted on a twice daily feeding regime (Chapter 3).

7.3.6.5: Minor acids

Analysis of variance of isobutyrate and isovalerate showed that diet, sampling time and the sampling time x diet interaction had a significant ($P < 0.001$) effect on the molar % of these acids. The means for diet and YC are given in Table 7.3.15.

Molar % isobutyrate also showed a significant interaction between sheep and day. This as with the same interaction noted in molar % propionate levels is likely to be due to inherent daily fluctuations in rumen function.

Analysis of variance showed that molar % valerate was significantly affected by diet ($P = 0.041$) and its interaction with supplemental yeast culture ($P = 0.018$). Table 5.14.4 shows that on Diet 1, supplemental yeast lowered rumen molar % valerate in the rumen, whereas it stimulated a higher molar % valerate on Diet 2. The reason and the importance for this diet dependent effect is unclear. However, the levels at which this acid and the other minor acids are found in the rumen makes them relatively unimportant as regards their physiological effects on the animal, so long as their concentration in the rumen is sufficient to supply the needs of the rumen bacteria.

7.3.7: Ruminal ammonia concentration (mg/l)

Analysis of variance showed that the ruminal ammonia concentration was significantly affected by sheep ($P = 0.013$), diet ($P < 0.001$) and time of sampling ($P < 0.001$). Supplementation of either diet with YC did not have a significant effect ($P = 0.614$) on rumen ammonia concentration. The sampling time x diet interaction was also found to be statistically significant at the $P < 0.001$ level.

Figure 7.18 shows the relationship between the diets, YC supplementation and time of sampling. This figure demonstrates that the 3 hourly feeding of diet 1 maintained the diurnal variation of rumen NH_3 concentration within a smaller range as compared to the variation observed on the twice-a-day feeding regimen of diet 2. The periodic peaks noted on diet 1 coincided with the 3 hourly feeds due to the rapid degradation of feed urea to ammonia. These peaks were not found to correlate ($r = -0.089$) with the t[VFA] peaks for this diet but a strong positive correlation with rumen pH ($r = 0.991$) was observed suggesting that the increased ammonia concentration caused rumen pH to increase. The peak ammonia concentration of diet 2 at 1.5 hours post feeding corresponds to the t[VFA] peak of this diet. However, the patterns production of ammonia and t[VFA] for Diet 2 were not found to correlate significantly ($P > 0.05$) ($r = 0.545$), presumably because the degradation of starch is slower than that of urea.

The mean ruminal ammonia concentration of diet 1 was found to be significantly higher ($P < 0.001$) than that of diet 2. It was assumed that this difference related to the different urea contents of each diet (diet 1 = 50 g urea/kg and diet 2 = 11 g urea/kg). As the urea concentration of diet 1 was 5 times that of diet 2 the difference in the ruminal ammonia concentration may have been expected to be greater. Other factors that may have affected the ruminal ammonia concentration include microbial utilisation as reflected by different fermentable metabolisable energy (FME) supply from the two diets, absorption through the rumen wall and the recycling of urea via the secretion of urea through the rumen wall. No estimate of EMNS was made during this experiment therefore the effects of different rates of microbial uptake of ammonia on ruminal ammonia concentration cannot be assessed. Maeng and Baldwin (1976) showed that the absorption of ammonia increased as rumen pH increased. Therefore, as diet 2 was found to have a higher mean pH the absorption of ammonia may have been expected to be greater on this diet which would have minimised the difference between the rumen ammonia concentrations for the two diets.

Table 7.3.16: Correlation coefficients for the comparison between diurnal rumen pH, tVFA and ammonia

	Diet 1		Diet 2		
	pH	tVFA		pH	tVFA
tVFA	0.094		tVFA	-0.896*	
NH ₃	0.991***	-0.089	NH ₃	-0.160	0.545

Asterisks show the significance of the correlation, *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$.

7.4: Conclusions

The *in sacco* technique was shown not to have the required sensitivity to detect possible small changes in degradability of fibrous materials in the rumen due to the addition of yeast culture to the diet. However, the statistical analysis of the results demonstrated that both CUSUM and analysis of variance were able to show that the *in sacco* technique was able to detect differences in ISDMD caused by diet type. A proportionate difference of approx. 10 % in ISDMD due to YC inclusion would have been sufficient for their detection at the $P < 0.05$ level of statistical significance.

Within each sheep, the degradation of ACW and hay were highly correlated, but analysis of variance did show differences ($P < 0.001$) in extent of degradation for the two materials. Correlation coefficients for the comparison of ISDMD between individual sheep were found to be different for each *in sacco* substrate. This was assumed to be related to differences in the sensitivity to the individual *in sacco* substrate.

The daily degradation values for both substrates were found to correlate with the daily pre-feeding rumen pH ($P < 0.01$), for all sheep except for the ACW values in sheep D. However, the rumen fistula in this sheep became infected and it was necessary to remove the animal from the trial. The rumen of this sheep may therefore have been responding "abnormally". Hence the measurement of daily rumen pH, taken prior to feeding, may provide a good indication of rumen function in an individual sheep although, as discussed in 7.3.6, it is not a reliable index for comparisons between different diets and feeding regimens.

The ISDMD data showed both short and long term variability similar to that observed in Chapters 3 and 6. It may be postulated from these observations that the rumen exists in a dynamic state, in which the predominance of individual microbial species is only transient (Wilson and Briggs 1955). Subsequent work attempted to quantify the magnitude of the inherent ruminal variations in ISDMD, pH, t[VFA] and ammonia concentration.

Significant ($P < 0.001$) increases in the viable yeast count during the periods of yeast culture supplementation were detected. This was possible even though there was a certain amount of carry over of the yeast into the control periods. This suggests that the yeast was able to survive in the rumen for long periods. However, the viable yeast counts obtained at various sampling times from the rumen declined at a rate faster than that of the liquid outflow. This may be as a result of yeast autolysis in the rumen. The viable yeast count of the faeces showed that the

yeast was capable of surviving transit through the abomasum and hindgut. When yeast was given a higher count was observed on day 2 of each experimental period, than on day 6 or day 21. This was postulated to be as a result of yeast autolysis in the rumen, promoting the further autolysis of freshly fed yeast culture. This was assumed to continue until an equilibrium between yeast autolysis and wash-out had been established. By day 2 the equilibrium was assumed not to exist and therefore the viable yeast count in the faeces may be expected to be higher. An alternative theory may be that the fresh addition of YC may promote the autolysis of the yeast cells already present in the rumen. This too would have resulted in a greater outflow of live yeast cells from the rumen at the beginning of supplementation.

Supplemental yeast culture significantly ($P < 0.05$) altered the rumen fermentation pattern, but not the total VFA production. Rumen molar % propionate was significantly ($P = 0.011$) reduced by the addition of yeast culture to the diet. A corresponding rise in molar % acetate ($P = 0.021$) and butyrate (not significant) was found to account for 99% of the drop in molar % propionate. The affect of YC inclusion on the VFA mix in the current experiment is in contrast to corresponding values in Chapter 6 but are similar to the affects due to supplementary YC observed in Chapter 3. The reasons for the shift in VFA mix in the current experiment are not clear but may relate to modulation of specific microbe activity which favoured the production of acetate and butyrate at the expense of propionate production. The reduction in propionate proportion when yeast was given was substantial (13%) and could have practical implications for the feeding of dairy cattle on high concentrate diets (Lees *et al.*, 1990), as discussed in Chapter 3.

No significant interaction between the response to YC treatment and diet type was observed for any of the parameters measured, in accordance with the findings of Chademana and Offer (1990), but in contrast to the results of Williams *et al* (1991). It was therefore assumed that semi-purified diet successfully induced stressful conditions for the microbes in the rumen which reduced the cellulose degradation which was not alleviated by the YC treatment.

Summary of the results

- 1) *in sacco* rumen degradability of washed hay or ACW was not significantly affected ($P > 0.05$) by supplemental YC but significant differences ($P < 0.05$) between diets were noted.

- 2) Rumen pH measured daily was not significantly ($P > 0.05$) affected by YC treatment but was significantly ($P < 0.05$) affected by diet type.
- 3) Rumen pH measured diurnally was significantly affected by diet type and feeding regimen ($P < 0.05$) but not by the YC treatment.
- 4) The supplementation of the diet with YC did not significantly ($P > 0.05$) affect LDR of either the rumen or hindgut. However significant difference due to diet type were observed for the LDR of the rumen but not the hindgut ($P < 0.05$).
- 5) Yeast recovery from the rumen and faeces suggested that the yeast supplement could survive in the rumen and pass through the abomasum and hindgut.
- 6) The total rumen VFA concentration was not significantly affected by diet or YC ($P > 0.05$).
- 7) The VFA mix was significantly affected by both diet and supplementary YC,
 - a) Diet 1 was found to have significantly higher acetate and lower propionate molar % than diet 2. Diet type had no effect on molar % butyrate ($P > 0.05$).
 - b) Supplemental YC was observed to decrease the concentration (mM) and molar % of propionate ($P = 0.055$) and increase molar % acetate ($P = 0.306$) but supplemental YC had no effect on molar % butyrate ($P > 0.05$).No diet x YC interaction was found for the major VFA suggesting that the YC treatment had the same effect on both diets.
- 8) Ruminal ammonia concentration was significantly affected by diet type ($P < 0.001$) but not significantly affected by supplemental YC ($P > 0.05$).

CHAPTER 8 Assessment of the day-to-day variation in rumen fermentation and *in sacco* degradability of washed hay.

8.1: Introduction

Previous animal experiments (Chapters 3, 6 and 7) have shown that the day-to-day *in sacco* degradation of soyahulls, washed hay and absorbent cotton wool is very variable and makes the detection of small changes in rumen function difficult. This variation was not reduced by improving the purity of the cellulose source nor by altering the feeding regimen or diet type. A possible theory for the observed variation in *in sacco* degradation in previous experiments may include the non quantifiable "negative associative effects" which can affect cellulolysis when the rumen pH is depressed below 6.1.

In a recent review of the *in sacco* technique, Kandylis and Nikokyris (1991) suggested that the main factors influencing the degradability estimates of ruminant feedstuffs were: (a) the basal diet of the cannulated animal (Ganev *et al.* 1979 and Kristensen *et al.* 1982) and (b) the rate of outflow from the rumen of unfermented feed particles (Mehrez and Ørskov 1977; Ørskov *et al.* 1980 and Ørskov *et al.* 1983). Kandylis and Nikokyris (1991) also cited several other factors that can influence the accuracy of the nylon bag technique, these include: (a) sample particle size (Erwin and Elliston 1959; Weakley *et al.* 1983); (b) pore size of the bag material (Mathers *et al.* 1977; Weakley *et al.* 1983); (c) preparation of the *in sacco* substrate (Chapman and Norton 1982; Freer and Dove 1984) and (d) incubation time in the rumen (Ørskov *et al.* 1980). The ratio of sample size to bag size was shown to be an important factor by Mehrez and Ørskov (1977), who observed that the most important factor determining the variability in disappearance from bags incubated simultaneously in a rumen was the sample size in relation to bag size. They suggested that the bag should be big enough to allow complete mixing with, and removal by, the rumen liquor. Other factors that have been shown to affect ISDMD include, nylon bag placement in the rumen (Balch and Johnston, 1950; Mehrez and Ørskov 1977). Oldham (1987), reported the results of a ring test carried out to investigate the sources of variation in ISDMD values obtained at different scientific centres. He concluded that it was essential to standardise the procedures carried out at each centre. Therefore, the ISDMD measurements of the current experiment as in previous experiments were carried out in accordance with the standard procedure outlined in AFRC (1992), but only for one incubation time (24h).

A second experiment carried out by Mehrez and Ørskov (1977) found that the variation due to bags incubated in different sheep (V_s) was greater than that caused by bags incubated in the same sheep on different days (V_D) and the least source of variation was between individual bags incubated in the same sheep on the same day (V_B). The coefficients of variation for DMD disappearance were found to be 6.2%, 4.9% and 3.3% respectively. Lindberg (1985), in his review concerning the estimation of rumen degradability of feed proteins cites values for V_s , V_D and V_B of 7.9, 1.1 and 4.5% respectively. However, the variation between different days in the experiment of Mehrez and Ørskov (1977) and that cited by Lindberg (1985) was estimated by comparing the results from 24 hour *in sacco* incubations of barley on 2 consecutive days per period. The results of the experiments previously discussed in this thesis (Chapters 3, 6 and 7) suggest that day-to-day variation of *in sacco* DM disappearance is much greater than that observed by Mehrez and Ørskov (1977) and Lindberg (1985).

The aim of the present experiment was to investigate long-term variation in rumen function by measuring the day-to-day variation of *in sacco* DM disappearance of washed hay. A diet was used which would not compromise cellulolysis with the inherent variation which may be observed in sheep fed a conventional diet containing 40 % concentrate due short term rumen pH fluctuations below pH 6.0. It was hoped that this study would quantify the long and short term variation in rumen activity and thus provide a basis for the design of future experiments to investigate changes in rumen function following the administration of yeast culture.

8.2: Materials and methods

8.2.1: Animals and design

6 rumen-cannulated mature wethers averaging 65 kg were penned and fed individually for the 18 weeks of the experiment. 3 sheep were chosen at random and fed an all hay diet and the remaining 3 sheep were offered the mixed conventional diet fed in Chapter 7.

8.2.2: Diet

- a) Diet 1: 1kg FW of medium quality hay plus 14g of urea and 14g of a proprietary sheep vit/min mix was fed twice a-day.
- b) Diet 2: The same mixed conventional diet (Diet 2) that was fed in Chapter 7 was fed in 2 equal feeds at 0900 and 1700 daily. See Table 7.2.1 for the diet composition.

8.2.3: Measurements

Daily: Rumen *in sacco* degradability of washed hay.
Rumen pH
Rumen total VFA plus the concentrations of individual VFA and their molar % proportion of the t[VFA].

Weekly: Diurnal rumen pH.

8.2.4: *in sacco* rumen degradability

A large quantity of hay was chopped and washed prior to the start of the experiment. However due to the length of the experiment it was not possible to prepare a large enough substrate batch for the whole experiment. Therefore a second batch of washed hay from the same source was prepared in the same way as the first approximately half way through the experiment. The dacron bags were prepared as described in Chapter 2 and incubated in the rumen for 24h before being removed and processed.

8.2.5: Daily rumen VFA, NH₃ and pH

A rumen liquor sample was aspirated from the rumen each morning as the *in sacco* bags were removed. The pH of the sample was immediately measured and recorded. The rumen sample was then preserved and stored awaiting VFA analysis at the end of the experiment.

8.2.6: Weekly diurnal pH

A series of rumen liquor samples were taken on the same day of each week. The first sample was taken just prior to morning feeding and subsequently at 90 min intervals. Rumen liquor pH was measured using a portable pH meter.

8.2.7: Interpretation, presentation and analysis of the results

The daily *in sacco*, rumen t[VFA] and pH results were statistically analyzed using cusum analysis (see Chapter 3). Calculation of the coefficient of variation were carried out using the DESCRIBE option in Minitab 7.1. The analysis of the variation in diurnal rumen pH was carried out using the analysis of variance option in Genstat 5.13. Table 8.2.1 shows the BLOCK TREATMENT structure which determines the degrees of freedom used in the statistical analysis.

Table 8.2.1: Block - treatment structure used for the analysis of variance of rumen pH (diurnal variation) restricted to the individual diets.

BLOCK SHEEP/WEEK/SAMPTIME	
TREATMENT SHEEP*WEEK*SAMPTIME	
Source of variation	d.f.
sheep	2
week	17
samptime	5
sheep.week	34
sheep.samptime	10
week.samptime	85
Residual	170
Total	323

8.3: Results and discussion

8.3.1: *in sacco* degradability

The individual sheep cusum statistical analysis shows a wide inherent day-to-day variation in the degradation of washed hay on either diet. Figures 8.1 and 8.2 illustrate this variation for sheep A and D as an example for each diet.

The coefficient of variation expressed as: $(SD/mean) \times 100$ between the bags incubated in the same rumen on the same day was found to be different for the two diets (Table 8.3.1). In an attempt to reduce the variation due to substrate solubilisation and small particle washout zero time losses for the washed hay were measured corresponding to the batch of bags prepared at the same time. The final ISDMD was then corrected for these losses according to the equation shown below.

$$\text{Corrected ISDMD} = \text{ISDMD} - \left(\begin{array}{l} \text{Batch zero} \\ \text{time losses} \end{array} - \begin{array}{l} \text{Average zero time losses} \\ \text{for the whole experiment} \end{array} \right)$$

Table 8.3.1 shows that correcting the ISDMD mean for zero time losses results in a small reduction of the mean but has no effect on the standard deviation and as a result the coefficient of variation (CV%) was found to show a corresponding increase.

Table 8.3.1: Coefficient of variation between bags incubated in a rumen on the same day

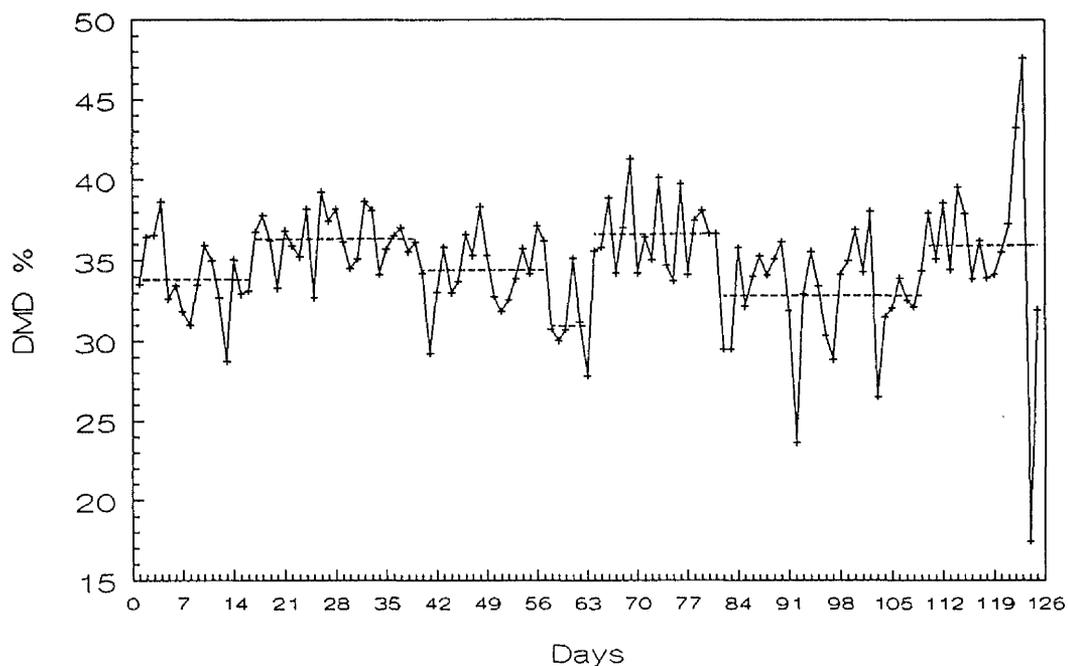
Diet	Mean Diet ISDMD	SD	CV%
Hay mean uncorrected	33.586	2.3075	6.87
Mixed mean uncorrected	28.417	3.419	12.03
Hay mean corrected	33.496	2.3075	6.88
Mixed mean corrected	28.318	3.419	12.07

Table 8.3.1 also shows that the variation between bags incubated at the same in the same animal was different for the two diets. However, comparisons between the diets are confounded by sheep since all the sheep did not eat both diets. In a further attempt to reduce the day-to-day variation associated with the replication between the three bags incubated in the rumen at the same time, the ISDMD values for each bag were calculated and compared with the values of the other two bags. If the ISDMD value of one bag out of a set of three was found to be different by more than 7% (absolute) (ie. a freak) from the mean of the other two bags then this bag was excluded and the mean of the comparable bags used as the estimate for that day. If all three bags were found to be markedly different then the mean of all three bags was used. A freak bag may occur as a due to one of the bags of a set of three not becoming properly incorporated into the rumen matrix resulting in an artificially low ISDMD value. A high value may be observed if the bag was leaking, however all bags were inspected prior to use and dubious bags such as those punctured by scissors whilst removing the connecting cord, discarded. A freak may also occur as a result of poor sampling of the washed hay leading to either an excess of "stalky" material being placed in the dacron bag facilitating a low ISDMD value or by placing a high proportion of fine leaf material in the bag resulting in an artificially high ISDMD value. Good mixing and sampling of the substrate was therefore essential.

The amount of variation in ISDMD between bags incubated in the rumen on consecutive days was also found to be different for the different diets (Table 8.3.2). The reason for the variation caused by diet type is not clear. However, the basal diet of the cannulated animal has been shown to be an important factor relating to the accuracy of the *in sacco* technique (Ganev *et al.* 1979; Lindberg 1985). Thus, since chopped hay was used as the test *in sacco* material for both experimental diets, it may be argued that ISDMD of the chopped hay may be less variable in the rumen of sheep adapted to the all hay diet in comparison to those sheep fed the mixed diet. Due to the variation in ISDMD that exists between diets further calculations will be confined to the individual diets. Table 8.3.2 also indicates that ISDMD was found to be vary with the individual sheep. Table 8.3.3 shows the CV% of ISDMD for the individual sheep on a given diet.

Figure 8.1

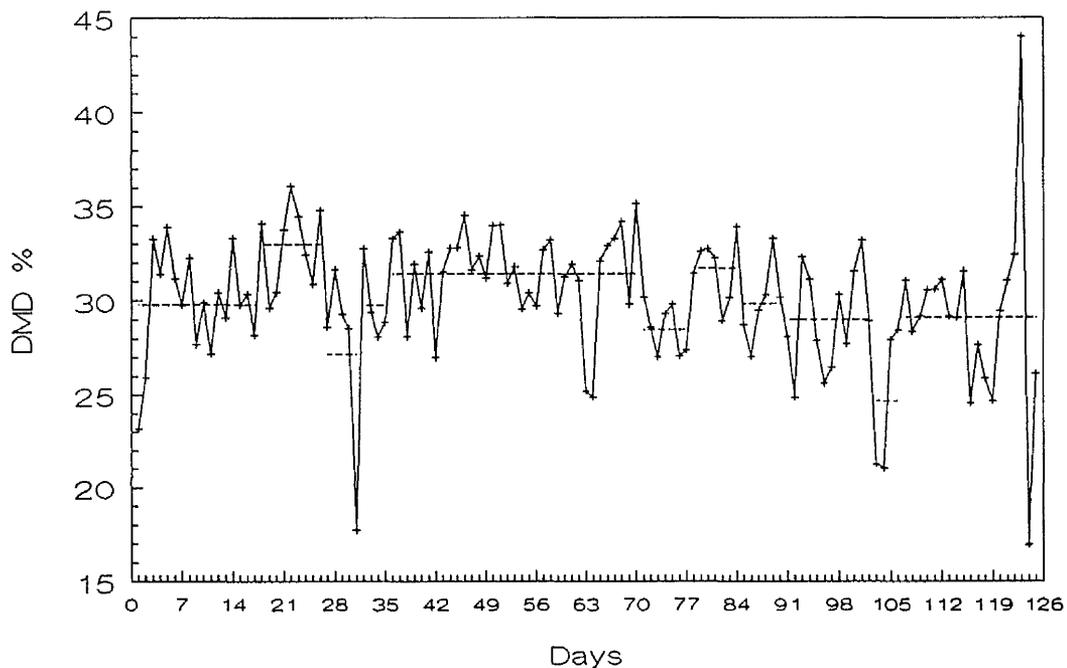
Rumen in sacco degradability of washed hay. Day-to day variation Sheep A



combined within stage SD = 3.201

Figure 8.2

Rumen in sacco degradability of washed hay. Day-to day variation Sheep D



combined within stage SD = 3.165

Table 8.3.2: Coefficient of variation % between bags incubated on consecutive days in different sheep

Diet	Sheep	Mean	SD	CV%
Hay	A mean uncorrected	34.7	2.94	8.45
	B mean uncorrected	33.9	3.20	9.44
	C mean uncorrected	32.5	3.13	9.62
Mixed	D mean uncorrected	29.8	3.47	11.67
	E mean uncorrected	27.1	3.45	12.72
	F mean uncorrected	28.6	3.35	11.71
Hay	A mean corrected	34.6	2.81	8.10
	B mean corrected	33.7	2.93	8.69
	C mean corrected	32.4	3.05	9.43
Mixed	D mean corrected	29.6	3.16	10.66
	E mean corrected	26.9	3.22	11.94
	F mean corrected	28.5	3.18	11.17
Hay	A mean corrected and defreaked	34.6	2.99	8.63
	B mean corrected and defreaked	33.7	3.00	8.89
	C mean corrected and defreaked	32.5	2.94	9.04
Mixed	D mean corrected and defreaked	30.2	3.07	10.18
	E mean corrected and defreaked	27.0	3.76	13.95
	F mean corrected and defreaked	28.7	3.43	11.96

Table 8.3.3: Coefficient of variation % between the individual sheep and consecutive days on different diets.

Diet	Mean	Sheep		Days	
		SD	CV%	SD	CV%
Hay mean uncorrected	33.7	1.14	3.37	3.22	9.55
Mixed mean uncorrected	28.5	1.34	4.71	3.59	12.59
Hay mean corrected	33.6	1.14	3.39	3.07	9.14
Mixed mean corrected	28.4	1.35	4.77	3.37	11.87
Hay mean corrected and defreaked	33.6	1.05	3.13	3.09	9.19
Mixed mean corrected and defreaked	28.6	1.59	5.53	3.66	12.81

Table 8.3.3 shows that diet also has an effect on the variation of bags incubated on consecutive days. The effect of diet on the variation noted between sheep was less marked but was still evident.

The CV% for bags incubated on consecutive days observed by Mehrez and Ørskov (1977) was 4.9%. The results of the present experiment suggest that this may be an under-estimate of the day-to-day variation within a sheep, as the afore-mentioned authors based their estimate on two consecutive days. It may be theorised that, over a given two period days that the *in sacco* degradability observed in the individual sheep may be markedly different, but that variation between days may only be small. However, if the *in sacco* degradability of a substrate is measured on consecutive days for a longer period of time, the differences between sheep may be seen to reduce and the differences between consecutive days increase.

The above tables indicate that correcting the ISDMD mean for zero time losses reduces the observed variation and, as this is a simple procedure, future work will incorporate this correction factor in order to minimise the variation between sheep and consecutive days. Whilst correcting the mean for zero time losses and then removing freak ISDMD values for individual bags appears to increase the variation between consecutive days it was theorised that the defreaked mean was closer to the true mean for a given day as the removal of a freak effectively reduced the between bag variation for a given set of bags incubated on the same day in the same animal. Therefore, the zero time losses corrected and defreaked means that will be used for further discussion. A summary of the variation due to sheep, day and individual bags for the two diets is given below.

Table 8.3.4: Summary of the CV% between bags, sheep and consecutive days.

	Diet 1: Hay	Diet 2: Mixed
Bags	6.88 %	12.07 %
Sheep	3.13 %	5.53 %
Days	9.19 %	12.81 %

The correlation coefficients (Table 8.3.5) for the comparison of the daily ISDMD values for the sheep shows that the sheep were synchronised significantly ($P < 0.01$) in their ability to degrade hay *in sacco*. The reasons for this synchronisation between sheep both within and

between diets are not clear. It may be postulated that the synchronisation between sheep is related to the effects of an external factor such as environmental temperature, affecting rumen function similarly in all sheep. However, the *in sacco* cusum means were not found to vary according to preparation batch and therefore technical error in the preparation and analysis was ruled out as a possible explanation for the observed variation.

Table 8.3.5: Correlation coefficients for the day-to-day variation between sheep ISDMD

	Sheep A	Sheep B	Sheep C	Sheep D	Sheep E
Sheep B	0.456***				
Sheep C	0.447***	0.583***			
Sheep D	0.498***	0.494***	0.465***		
Sheep E	0.429***	0.416***	0.321**	0.529***	
Sheep F	0.370***	0.432***	0.437***	0.379***	0.392***

Coefficients with a series of asterisks relate to the significance of correlation, (***) = $P < 0.001$, (**) = $P < 0.01$, (*) = $P < 0.05$).

8.3.2: Day-to-day variation in rumen parameters

The individual sheep cusum statistical analysis showed a wide inherent day-to-day variation in the t[VFA], pH and rumen ammonia concentration on either diet. Figures 8.3-8.8 illustrate this variation for sheep A and D as an example for each diet. The cusum means for each sheep are given in Appendices 13-15.

8.3.2.1: t[VFA]

Analysis of the cusum means reveals that the sheep on the all hay diet show no overall trend in t[VFA] with time, although the t[VFA] in sheep A showed an increase and subsequent decline from approximately days 93 to 126, peaking at day 108. The total concentration of tVFA in sheep B appeared to become more variable after day 78. Sheep on the mixed diet type also showed no overall trend in the variation but sheep D tended to show an increased t[VFA] after approx. day 39 and the t[VFA] of sheep F became more variable post day 69.

Figure 8.3

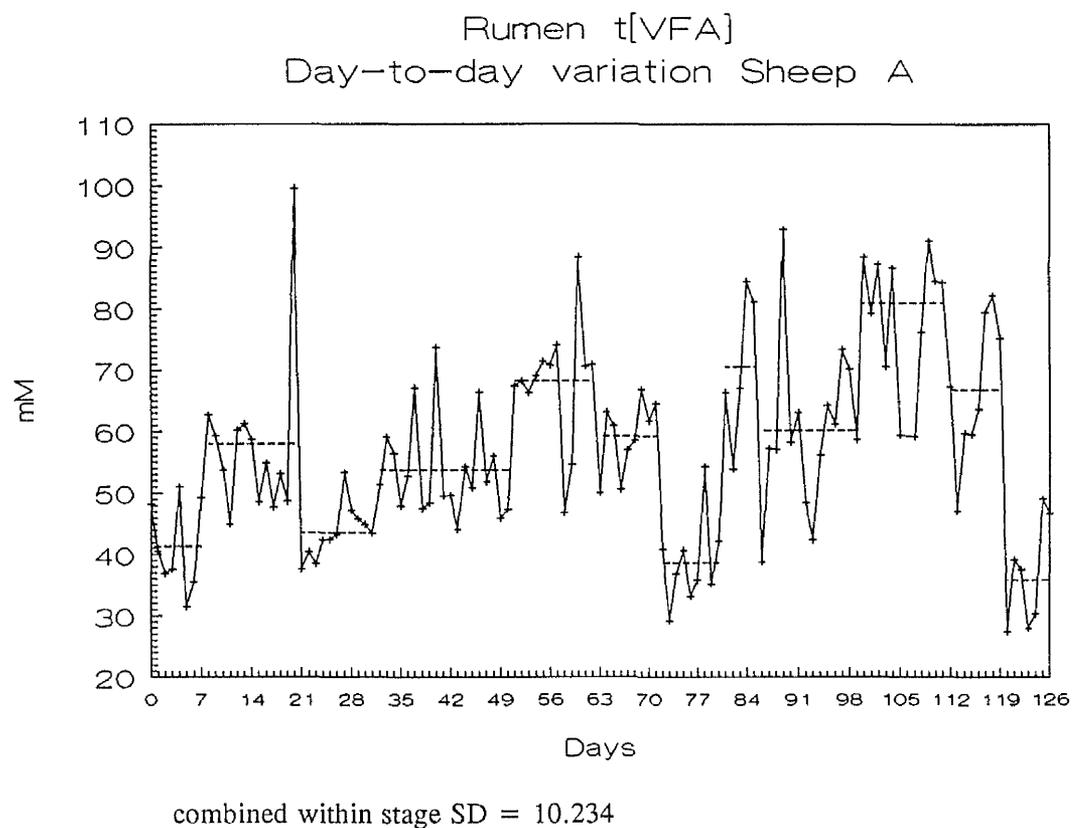


Figure 8.4

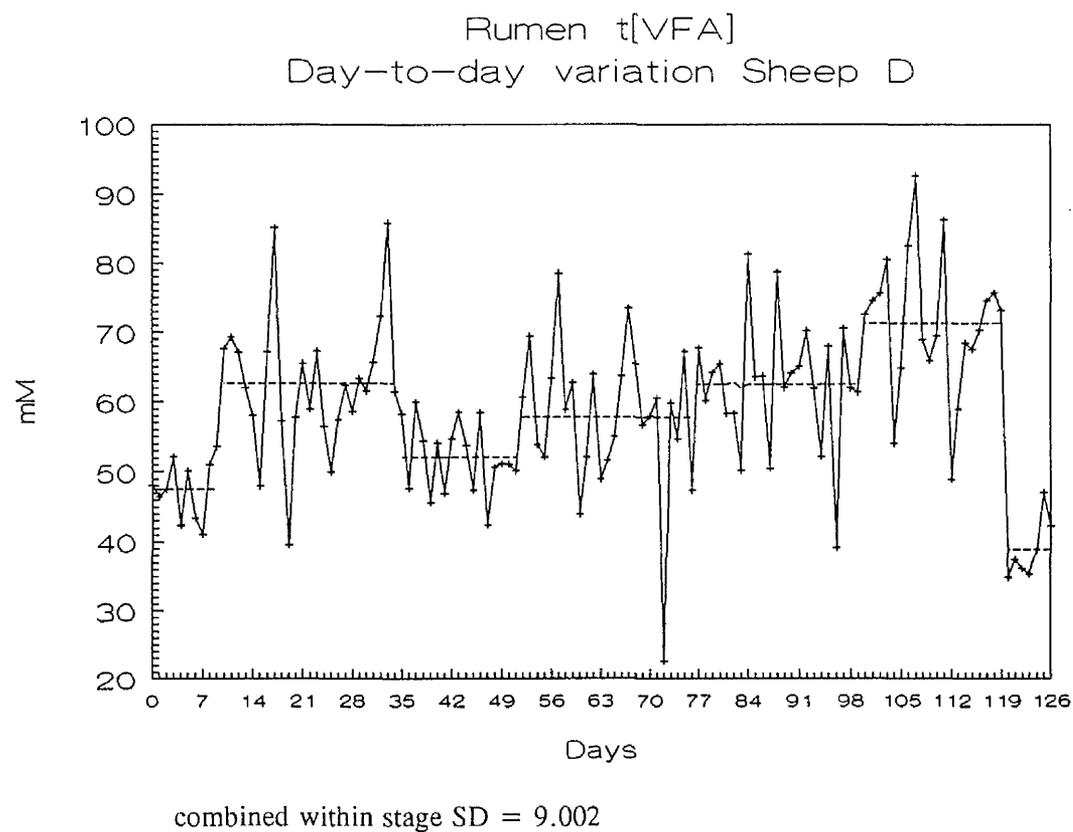


Table 8.3.6: Day-to-day variation in rumen fermentation end-products

Parameter		Sheep (Diet 1)			Diet mean (Hay)	Sheep (Diet 2)			Diet mean (Mixed)
		A	B	C		D	E	F	
t[VFA]	Mean	45.6	48.2	44.9	46.3	47.3	49.7	42.3	46.4
	SD	12.8	13.4	12.4	12.9	9.7	11.8	11.0	11.2
	CV %	28.1	27.7	27.6	27.9	20.4	23.7	23.9	24.2
Acetate (mM)	Mean	35.7	36.9	34.6	35.7	34.9	36.4	30.9	34.1
	SD	10.7	11.5	10.4	10.9	7.4	9.2	8.4	8.6
	CV %	30.1	31.0	29.9	30.4	21.3	25.2	27.1	25.4
Propionate (mM)	Mean	6.9	7.8	7.2	7.3	6.6	7.1	6.4	6.7
	SD	1.8	1.9	1.8	1.9	1.3	1.6	1.7	1.5
	CV %	26.3	24.8	24.9	25.7	19.2	21.8	25.8	22.7
Butyrate (mM)	Mean	2.4	2.6	2.5	2.5	4.9	5.1	4.2	4.7
	SD	0.7	0.8	0.8	0.8	1.1	1.3	1.1	1.2
	CV %	31.0	28.9	33.3	31.1	22.7	24.5	25.5	25.5
Acetate (molar %)	Mean	78.0	76.2	76.6	76.9	73.8	73.1	73.0	73.3
	SD	2.9	5.7	2.7	4.1	2.0	3.1	4.2	3.3
	CV %	3.7	7.5	3.6	5.3	2.7	4.3	5.8	4.4
Propionate (molar %)	Mean	15.2	16.2	16.3	15.9	14.0	14.4	15.1	14.5
	SD	1.3	1.2	1.6	1.5	1.1	1.6	1.1	1.4
	CV %	8.7	7.5	10.1	9.4	7.6	11.0	7.1	9.3
Butyrate (molar %)	Mean	5.4	5.6	5.7	5.6	10.4	10.3	10.0	10.2
	SD	1.5	1.5	1.6	1.6	1.3	1.3	1.4	1.3
	CV %	27.9	27.6	27.7	27.8	12.2	12.6	13.7	12.9
pH	Mean	6.7	6.8	6.8	6.8	6.6	6.5	6.6	6.6
	SD	0.2	0.1	0.2	0.2	0.1	0.1	0.5	0.3
	CV %	2.2	2.1	2.5	2.4	1.2	2.1	8.1	4.9
NH ₃ (mg/l)	Mean	74.1	77.9	63.7	71.9	102.1	109.0	105.7	105.6
	SD	21.7	31.7	19.8	25.6	24.3	27.6	22.5	25.0
	CV %	29.3	40.7	31.0	35.6	23.8	25.4	21.3	23.7

It may be noted from Appendix 13 that during the last few days of the experiment all sheep had reduced tVFA concentrations. Rumen liquor samples were prepared and analyzed by GC in batches. The last batch of samples analyzed contained 42 samples which coincided with the last week of the experiment. The last cusum mean for sheep A, B and C all contain 7 values equating to the last batch of samples analyzed. However, closer analysis of the results indicates that in Sheep C the rumen t[VFA] had peaked on day 118 and then started to decline during the next 4 consecutive days until reaching a nadir on day 121. This decline overlaps with the last cusum mean and indicates that the decline commenced prior to the last week and the last batch of samples, and indicating that it may not be attributed to analytical error.

The sheep fed the mixed diet were also found to have low rumen t[VFA] concentrations during the last few days of the experiment. However, the last cusum mean of sheep D contained only 6 values, and sheep E 5 values, indicating that the observed reduction in t[VFA] occurred within the last batch of rumen liquor prepared for these sheep. The last cusum mean for sheep F was found to contain 8 values suggesting that the drop in t[VFA] for this sheep commenced before the analysis of t[VFA] for the ultimate week of the experiment.

This closer inspection of the results suggests that technical error in the preparation of the last batch of samples was not solely to fault for the low cusum means observed in all sheep during the last week of the experiment, but some external factor had a similar effect on all sheep and that response of the individual sheep varied.

The correlation table (Table 8.3.7) below shows that the daily tVFA concentrations between sheep correlate significantly ($P < 0.001$). The reasons for the high degree of correlation between the daily tVFA samples are not clear. However, it appears that, as for the synchronisation noted in the *in sacco* results, that an external factor such environmental temperature may be mediating a similar effect on the rumen function of all sheep. Lirette *et al.* (1988), observed an increase in both ruminal and heart muscle contractions when they subjected steers to acute cold and/or psychological stress. Increased fore-stomach motility may be expected to increase the abrasion of the nylon bags and improve the flux of rumen liquid through them, thus facilitating an increased ISDMD value. This theory is supported by the findings of Marinucci *et al.* (1992) working with cannulated steers who noted that DMD and cellulose digestibility was reduced when alfalfa hay was incubated inside a rigid plastic container. They concluded that the physical effect of pressure and massaging by the ruminal contents is critical in movement of fluid and gases in and out of bags. Further, it may be theorised that enhanced VFA absorption may be facilitated as a result of increased blood flow to the reticulo-rumen

stimulated by its increased muscular activity and an increase in the rate of cardiac contractions. Coefficients for the correlation of daily tVFA and ISDMD (Table 8.4.1) show that these parameters tended to be negatively related (1 sheep out of 6 at $P < 0.01$ and 4 out of 6 sheep at the $P < 0.1$ level of statistical significance). It may be postulated that the increase in rumen motility as a result of stressing the animals increased ISDMD and decreased rumen VFA concentration due to improved VFA absorption. Hence, a negative relationship between tVFA concentration in the rumen and ISDMD may be observed.

The psychological stress of steers induced by Lirette *et al.* (1988), was achieved by subjecting the steers to the presence of a German Shepherd dog for 10-15 minutes trained to sniff, growl and bark but not touch the experimental animals. The steers were therefore subjected to visual, auditory and olfactory stimuli. The sheep used in the current experiment were not housed in isolation, but along-side animals taking part in other experiments. It may be theorised that the day-to-day variations resulted from stress induced during the handling of the sheep involved in the current experiment, and also as a result of experimental procedures involving other animals in the building. The stressing effect of both direct and indirect handling may not be apparent. Syme and Elphick (1982/83), in their study of heart rate and the behaviour of sheep in yards observed that, certain individuals readily become extremely agitated with a concurrent increase in heart rate, whilst some sheep respond with only low levels of excitement, but show the same elevated heart rate as the agitated animals. Only completely inexcitable sheep failed to show the heart rate response. The choice of animals and the habituation of experimental animals to the experimental techniques which they are subject to may be seen as important factors in experimental procedures.

Table 8.3.7: Correlation coefficients between the individual sheep on both diets.

	t[VFA]				
	Sheep A	Sheep B	Sheep C	Sheep D	Sheep E
Sheep B	0.607***				
Sheep C	0.536***	0.544***			
Sheep D	0.436***	0.464***	0.540***		
Sheep E	0.439***	0.527***	0.515***	0.543***	
Sheep F	0.367***	0.429***	0.399***	0.413***	0.627***

Coefficients with *** correlate significantly at the $P < 0.001$ level of significance.

The coefficient of variation for the day-to-day ruminal tVFA concentration was found to be significantly correlated with increases in the individual VFA concentrations, mainly due to increase in the mM concentration of acetate, propionate and butyrate. Table 8.3.8 below shows the relationship between t[VFA] and the ruminal concentration of the individual VFA.

Table 8.3.8: Correlation coefficients between the daily variation of the individual VFA and tVFA concentrations for both diets.

	Acetate	Propionate	isoButyrate	Butyrate	isoValerate	Valerate
Propionate	0.886***					
isoButyrate	0.081	0.171				
Butyrate	0.355***	0.315**	0.539***			
isoValerate	0.150	0.233*	0.720***	0.818***		
Valerate	0.199	0.245*	0.475***	0.644***	0.639***	
T[VFA]	0.979***	0.917***	0.177	0.476***	0.276*	0.294*

Coefficients with a series of asterisks relate to the significance of correlation, (***) = $P < 0.001$, (**) = $P < 0.01$, (*) = $P < 0.05$).

Table 8.3.6 show that the day-to-day variation of the individual major acids was found to be similar for both diets. This table also shows that diet 2 favoured the production of butyrate at the expense of propionate. Not only was there substantial day-to-day variation in the concentration of individual VFA, but the relative concentrations of the acids expressed as a molar % of the total VFA concentration was also found to vary on a day-to-day basis. This suggests that not only do the absolute concentrations of the individual VFA's contributing to t[VFA] vary daily but the pattern of fermentation of a constantly fed diet also varies on different days.

The sheep on a given diet were found to vary more on the mixed diet than those on the all hay diet. The coefficient of variation between sheep on the mixed diet was 8.21 % and on the hay diet 3.82 %. It must however be remembered that diet comparisons are confounded by sheep because all sheep did not eat both diets. It was therefore assumed that diet may have had an effect on the observed variation within and between sheep.

8.3.2.2: Rumen [NH₃]

Analysis of the cusum means for the daily variation of pre-feeding rumen NH₃ concentration showed no overall trends in any of the sheep on either diet.

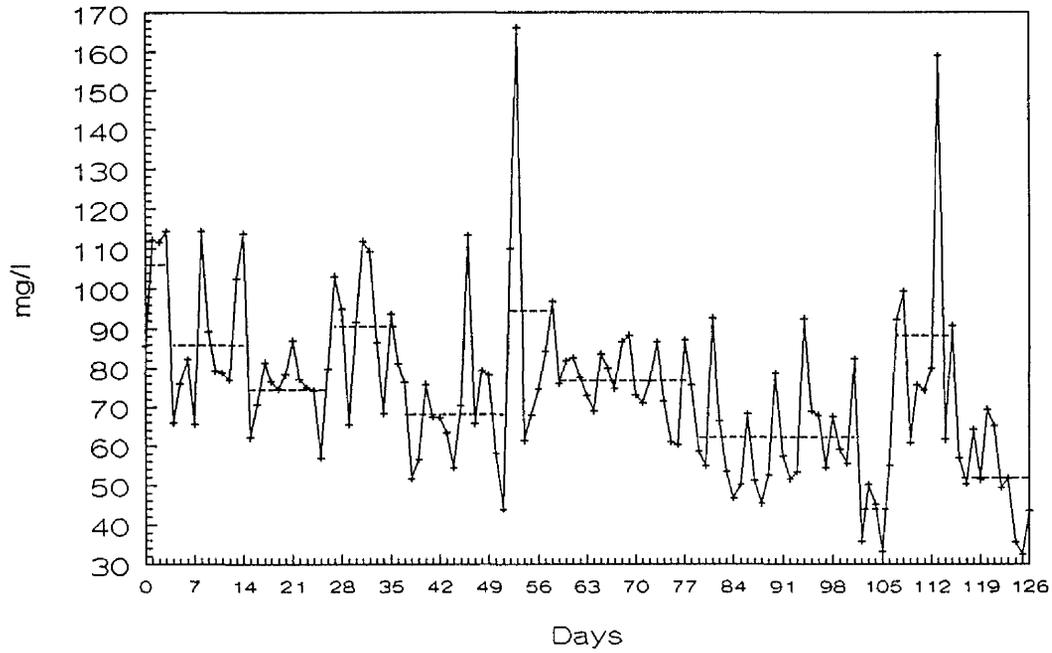
Table 8.3.6 shows that the mixed diet (diet 2) resulted in a higher ruminal concentration of ammonia. This was assumed to relate to a higher crude protein content of this diet and the increased microbial turn over that would be expected on a higher ME diet (gMN/MJME). This table also shows that the day-to-day variation in ruminal ammonia concentration was in the same range as that of t[VFA].

8.3.2.3: pH

Analysis of the cusum means for the daily variation in pre-feeding rumen pH shows that the ruminal pH in sheep A increased during the first two weeks of the experiment and then declined gradually until approximately day 95 when the rumen pH remained relatively constant. Sheep B showed no obvious trends until day 80 when rumen pH declined to a nadir at approximately day 116 when rumen pH began to increase again. For Sheep C rumen pH was found to decrease gradually in an exponential manner with the rate of decline becoming slower after the third week. No obvious trends were noted in sheep D except for a consecutive decline in cusum means from day 57 to day 99 after which the rumen pH began to increase again. The rumen pH of sheep E was found to show greater variation than sheep D and its rumen pH was observed to decline from day 23 to day 100. Sheep F was found to show the greatest variation in day-to-day rumen pH and a gradual decline in pH was noted from the start of the experiment until day 100 when rumen pH started to increase again (Table 8.3.6). The reason for these variations is not clear and although the daily variation within a sheep on diet 2 was found to be greatest (4.93 % cf. 2.44 % diets 2 and 1 respectively) the between sheep variation was much the same (1.12 % and 1.01 % for diets 1 and 2 respectively). However, the within sheep CV % for diet 2 may be artificially high due to the large % variance in one sheep (F). Analysis of the cusum means and the CV % observed within the other 2 sheep suggests that both diets were equally variable day-to-day. Correlations between the daily rumen pH values within sheep (Table 8.3.9) were found to be significant for all sheep with the exception of sheep F, which was not found to correlate significantly with any other sheep ($P > 0.05$). The high degree of similarity in the day-to-day variations of the individual sheep further supports the theory of an external factor such as temperature or psychological stress having a global effect on all sheep.

Figure 8.5

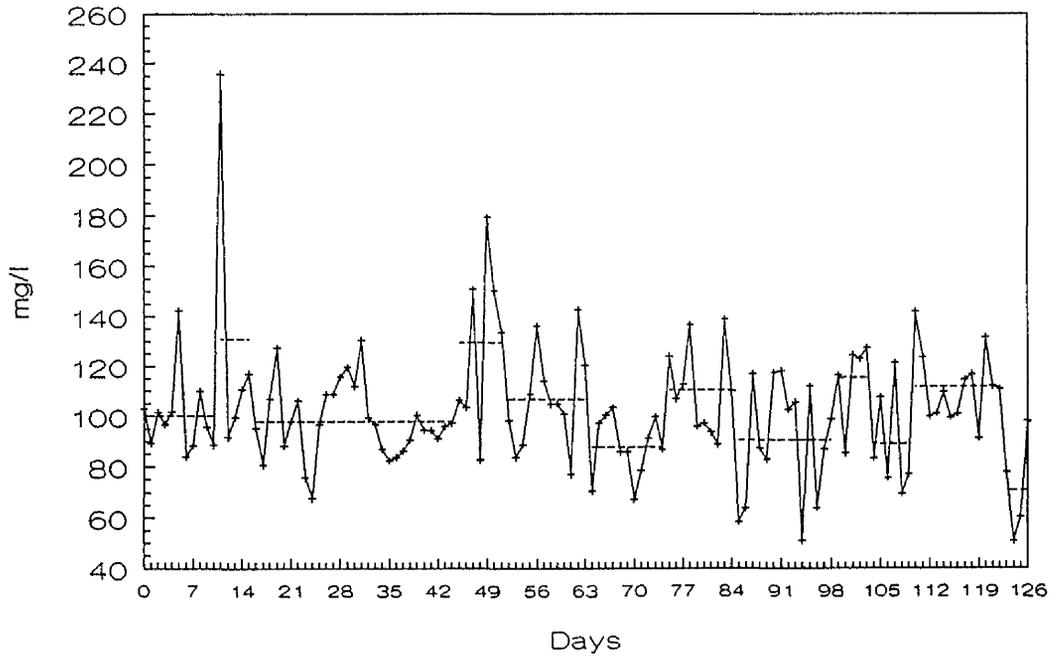
Rumen ammonia concentration
Day-to-day variation Sheep A



combined within stage SD = 16.584

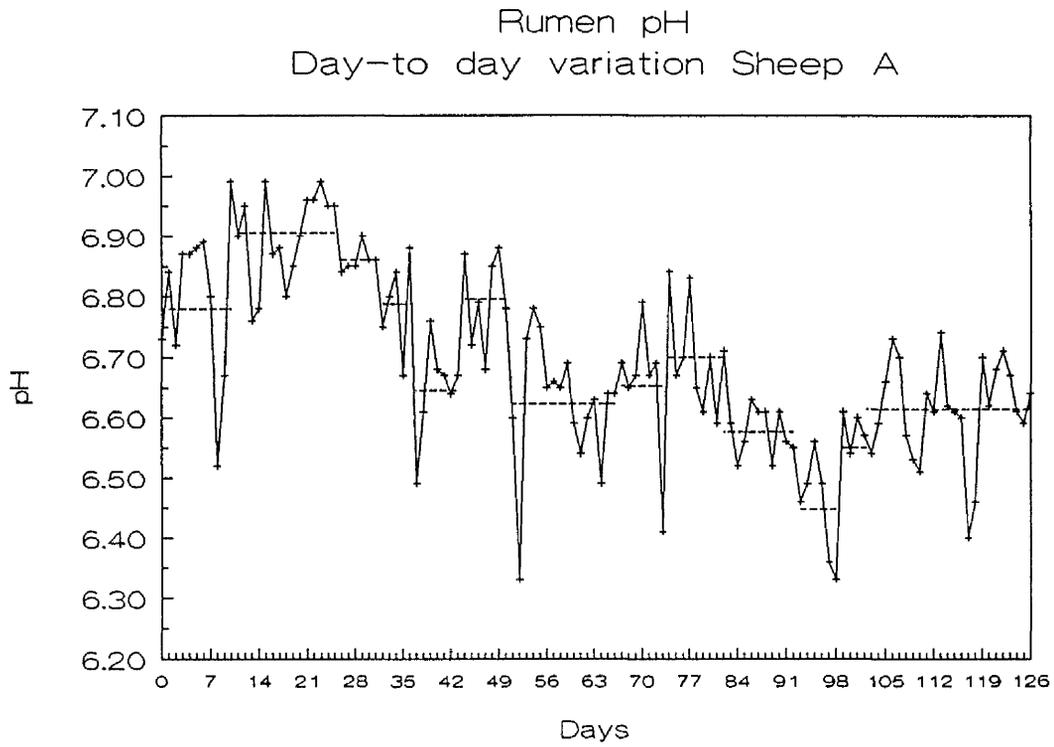
Figure 8.6

Rumen ammonia concentration
Day-to-day variation Sheep D



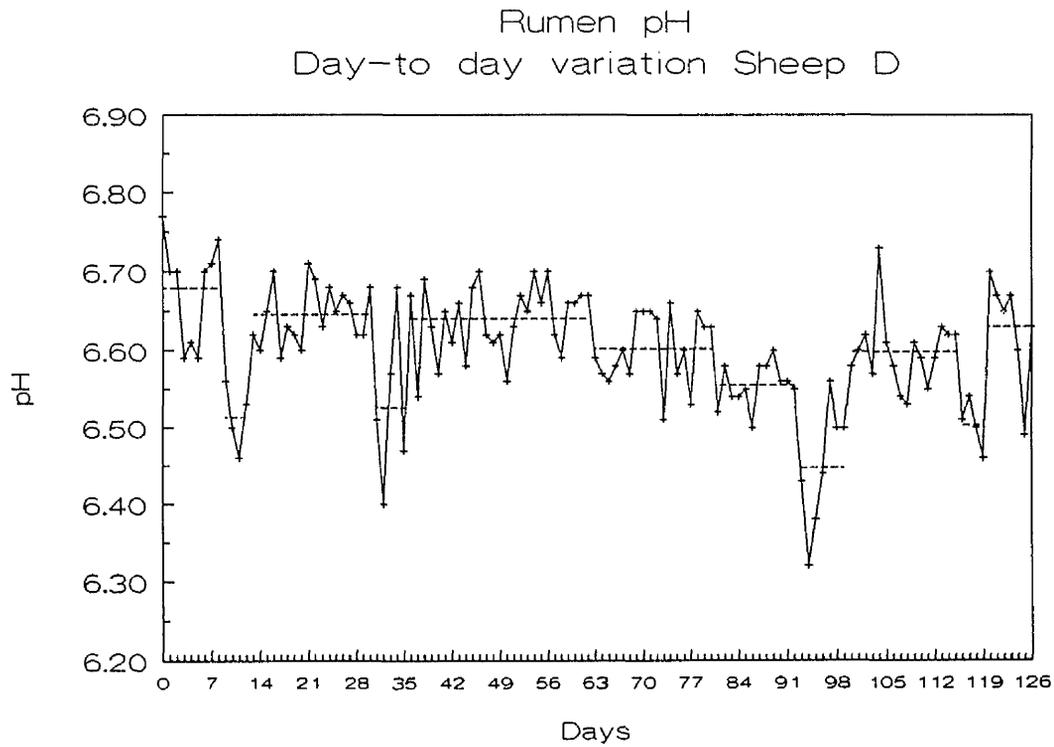
combined within stage SD = 21.32

Figure 8.7



combined within stage SD = 0.086

Figure 8.8



combined within stage SD = 0.051

Table 8.3.9: Correlation coefficients between the individual sheep on both diets

	pH				
	Sheep A	Sheep B	Sheep C	Sheep D	Sheep E
Sheep B	0.543***				
Sheep C	0.425***	0.535***			
Sheep D	0.321**	0.275*	0.410***		
Sheep E	0.581***	0.464***	0.456***	0.493***	
Sheep F	0.191	-0.004	0.078	0.146	0.094

Coefficients with a series of asterisks relate to the significance of correlation, (*** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$).

The mean rumen pH taken before feeding was found to be lower on the mixed diet. This was assumed to relate to the higher RFC content of this diet. Mean rumen degradability measured by *in sacco* was also noted to be lower on diet 2 and is in accordance with the findings of animal experiment 3 (Chapter 7). The different rumen pH observed on the different diets may affect the absorption of the individual VFA (Ørskov and Ryle, 1990) and may account for some of the variance observed in the daily fermentation pattern. However, the daily pre-feeding pH may not have the required sensitivity to pick up these effects. The effect of pH on the absorption of the individual VFA may be more evident in an analysis of the diurnal pattern of VFA fermentation as discussed in Chapter 7.

8.3.3: Diurnal variation of rumen pH

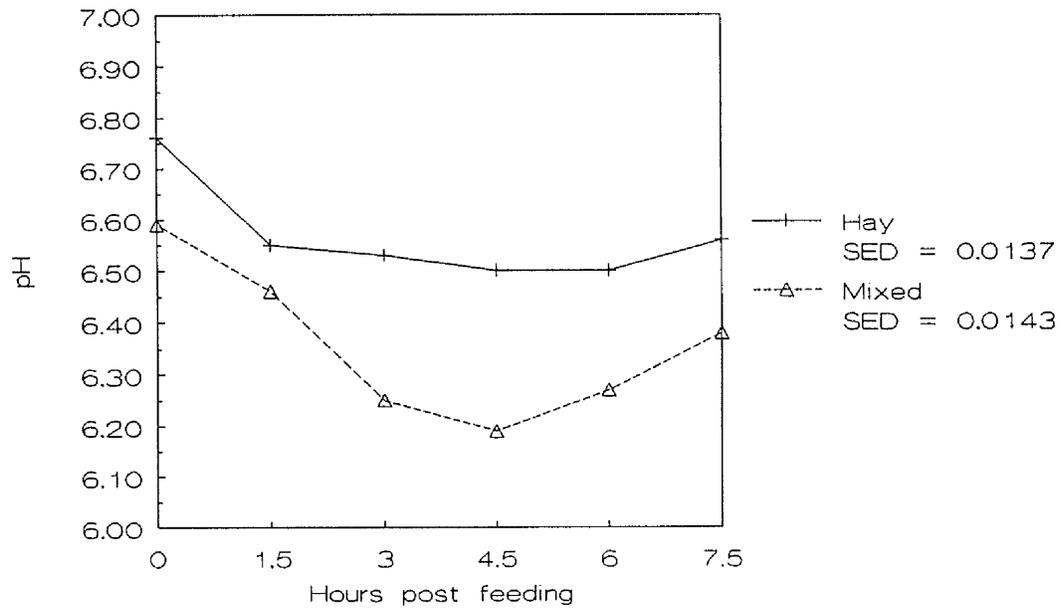
Analysis of variance showed that the diet means of the diurnal variation were significantly different ($P < 0.001$). The individual sheep means on a specific diet were also found to be significantly different as were the week means ($P < 0.001$). The interaction of sheep and week and week and sampling time were found to be significant for both diets at the ($P < 0.001$) level of significance. These significant interactions indicate that both the within day ruminal pH pattern and the sheep means in different weeks were similar.

The diurnal ruminal pH pattern between individual sheep were found to be significantly different ($P= 0.002$) for the sheep on the hay diet whereas, those sheep on the mixed diet showed similar diurnal patterns ($P=0.346$). It was assumed that the different dietary effects noted related to the eating behaviour of the animals on a given diet resulting in different diurnal patterns. It was noted during feeding that the sheep on the hay diet tended not to eat all the hay that was placed in front of them as soon as it was placed in the feed box, whereas when the barley, maize mix was placed in front of animals D to F it was always eaten immediately whereas, the hay portion of their diet was consumed more gradually. Therefore, in the case of the sheep on the hay diet although there was relatively less diurnal variation than on the mixed diet, a small change in eating behaviour in a specific animal on the day of recording would have greater effect on the observed diurnal variation for that day. Whereas, for the sheep fed the mixed diet a small change in the eating behaviour of the hay would have less effect on the observed between sheep variation as hay only made up a small proportion of this diet. Figure 8.11 shows the mean diurnal variation for the hay and mixed diets.

Figure 8.11 also shows that the diurnal variation of the mixed diet was similar to that observed in animal experiment 3 (Chapter 7). These results suggest that the minimal rumen pH that observed following the 17:00 feed would result in pH levels below the threshold level (6.0-6.1) at which cellulolysis is inhibited and may explain the lower rumen *in sacco* degradability results observed on the mixed diet. The greater inherent diurnal variation observed on the mixed diet may also explain the greater variation of rumen pre-feeding pH between consecutive days as the rumen may not always recover to pre-feeding levels at the same rate on ever day due to the variable dynamics of the ecological system.

Figure 8.9

Rumen diurnal pH variation of the hay and mixed diets



8.4: Conclusions

The results of the current experiment indicated that there was substantial variation in rumen function on consecutive days, even though the animals were consistently fed the same diet in the same way and at the same time on each day. The general nature of the variability occurred in 3 forms: (1) Short-term noise, (2) temporary short-term stable states and (3) long term trends.

8.4.1: Short term noise

Short-term noise was observed in all parameters and was considered to relate to dynamic ruminal microbial activity over the 24h period since the last recording. It may be postulated that the interactions between the host and changes in rumen microflora population densities (Wolin and Miller 1988) and/or activity over the 24h period culminated in the recorded value. These interactions may be modulated by short term (≤ 1 day) external factors such as environmental temperature or psychological stress. It may also be suggested that the activity of the subsequent 24h period will depend on the ruminal conditions prevalent at the end of the previous 24h period i.e., ruminal activity may exhibit sensitivity dependence on initial conditions ("The butterfly effect"; Gleik (1987); Appendix 17). This theory is supported by the findings of Wilson and Briggs (1955), who carried out a quantitative bacteriological study of the normal bovine flora. Their work showed that the rumen bacterial count varied markedly within a day and between days. The results of Leedle *et al* (1982), are in accordance with those of Wilson and Briggs (1955) but Leedle *et al* (1982) observed similar variations in bacterial numbers of heifers fed either a high-forage or high-concentrate diet. However, the high-concentrate diet was not found to depress rumen pH to levels at which the rumen bacteria may be inhibited (pH 6.0), and they speculated that if a diet fed to an animal did induce rumen pH levels below pH 6.0, under these conditions the ruminal ecosystem would be unstable and subject to large fluctuations in bacterial, protozoal and fungal numbers, and species diversity.

Experimental error may also have contributed to the observed short-term noise. Figures 8.1-8.8 and Tables 8.3.4 and 8.3.7 indicate that all the rumen parameters measured showed a high degree of day-to-day variation. However, different parameters were not found to be synchronised in their fluctuations (Table 8.4.1). This suggests that the short-term noise cannot be solely be attributed to error in rumen sampling but does not exclude possible error in individual parameter analysis.

8.4.2: Temporary short-term stable states

Periods of semi-steady states were highlighted by the cusum statistical analysis (Figures 8.1 and 8.2). This statistical technique determined significantly different ($P < 0.05$) plateaus of semi-steady state (short-term noise was prevalent in these plateaus). The number of observations in a plateau was not constant ranging from 4 to 65 observations (Appendix 12) and different sheep were not noted to be synchronised in their cusum means (Appendices 12-15). Czerkawski (1980), working *in vitro*, noted prolonged periods when incubation of rumen contents of sheep resulted in an unstable fermentation. He observed that instability affected all sheep and lasted 6-8 weeks. It is possible that Czerkawski was detecting the semi-steady states similar to those observed in the current experiment. As *in vitro* techniques involve dilution of the collected rumen liquor with a buffer solution it is possible that the variation in rumen contents due to short-term noise may be lost during this process and hence not detected by Czerkawski (1980). The reasons for these shifts in rumen function are not clear but may relate to the affects of medium term (2- 14 days) changes in an external factor such as those discussed previously.

8.4.3: Long-term trends

Long-term trends were observed in some but not all sheep. Figure 8.7 shows that the pre-feeding rumen pH of sheep A tended to decline progressively from day 14 to day 98 of the experiment. Long-term trends were also noted in the rumen parameter measured in Chapter 4. Shifts in rumen function were tentatively associated with changes in diet supplementation during the course of the experiment described in Chapter 4. However, the current experiment suggests that the trends observed in the previous experiment may have occurred due to chance and were not related to supplementation of the rumen with 20g/d of YC. Long-term trends in rumen function may be caused by animal changes in the behaviour of the host, i.e., eating and drinking behaviour. Adaptation to a new diet may also cause longer term trends in rumen function to be observed, but in the current experiment all animals were allow a 10 day adaptation period prior to the start of the experiment. It must also be remembered that long-term trends commenced after the experiment had been running for 2 weeks (Figure 8.7) and it unlikely that the animal had not become adapted to the new diet by this time. It may be postulated that the psychological stress of boredom causes shifts in the eating and drinking behaviour of animals constantly housed. It has been suggested that animals try to maintain an optimal level of sensory input (Hebb, 1955 and Leuba, 1955) and that sensory input can be increased by an animal exploring its environment (Johnson, 1975). In a confined and barren environment an

animal is restricted in the amount of exploration that it may do and this may lead to boredom (Duncan, 1981). Stereotypic behaviour in pigs and poultry associated with boredom has been reviewed widely (Dantzer and Mormedè, 1979; Duncan, 1981 and Hart, 1985). These reviews cite abnormal behaviour including "bar chewing" and tail biting in pigs and "head shaking" in battery hens. In the current and previous experiments the sheep involved in the experiments have been noted to exhibit behaviour which may relate to the stress induced by continual housing. These manifestations included rocking from side-to-side on their feet, head butting of neighbouring individuals through the wooden partitions and chewing of the wooden partitions. The individual sheep pens used in the current experiment and in particular the digestibility crates used in previous experiments (Chapters 3 and 6) may be considered as barren environments and hence lead to boredom and shifts in behaviour patterns.

The correlation coefficients between the daily variations of rumen pH, t[VFA], ammonia and *in sacco* washed hay degradability are shown in Table 8.4.1. This table shows that all the sheep on the hay diet, and 2 out of 3 on the mixed, diet showed a significant ($P < 0.001$) negative correlation between rumen pH and t[VFA]. The correlation coefficients were not as strong as those previously observed for this relationship, however it was theorised that at the time of sampling the relationship between these parameters would be at its weakest point. Whereas if the samples had been taken at peak t[VFA] the inverse relationship with rumen pH would have been more evident.

Rumen *in sacco* degradability of washed hay was found to show a significant positive correlation with rumen pH in 2 sheep on the hay diet ($P < 0.001$) but only in 1 sheep on the mixed diet ($P < 0.05$). The differences between diets was assumed to relate to the lack of sensitivity in the *in sacco* technique to respond to short term changes in rumen fermentation, which were more evident on diet 2.

Table 8.4.1 also shows that rumen pH was found to correlate positively with rumen ammonia concentration ($P < 0.05$) for all the sheep on the hay diet but, for the sheep on the mixed diet, was either not related or significantly negatively correlated ($P < 0.001$) to rumen ammonia concentration. This was assumed to relate to the different fermentation patterns of the individual diets. The fermentation pattern of the hay diet resulted in less day-to-day and diurnal variation of rumen pH compared to that observed on the mixed diet. Therefore the consumption and rapid degradation of the urea content of this diet might be expected to increase both the rumen ammonia concentration and rumen pH. For the sheep on the mixed diet, the rapid fermentation

of the RFC which caused the peak t[VFA] was assumed to coincide with the degradation of the urea portion of the diet ($r = 0.284^{***}$), thus the peak rumen ammonia concentration would occur at peak t[VFA] and consequently minimum rumen pH. However, as with the relationship between rumen pH and t[VFA], the pre-feeding relationship between rumen pH and ammonia concentration measured pre-feeding may be expected to be at its weakest at this time and hence 2 of the sheep failed to show any correlation.

Table 8.4.1: Correlation coefficients between the daily variations of pH, t[VFA], NH₃ and *in sacco* degradability for the individual sheep

	Diet 1: Hay			Diet 2: Mixed		
	pH	<i>in sacco</i>	t[VFA]	pH	<i>in sacco</i>	t[VFA]
Sheep A				Sheep D		
<i>in sacco</i>	0.159			<i>in sacco</i>	0.225*	
t[VFA]	-0.414 ^{***}	-0.118		t[VFA]	-0.325 ^{***}	-0.082
NH ₃	0.229*	0.147	-0.119	NH ₃	0.005	0.040
						0.090
Sheep B				Sheep E		
<i>in sacco</i>	0.525 ^{***}			<i>in sacco</i>	0.131	
t[VFA]	-0.532 ^{***}	-0.274 ^{**}		t[VFA]	-0.448 ^{***}	-0.180
NH ₃	0.291 ^{**}	0.140	-0.045	NH ₃	-0.372 ^{***}	-0.100
						0.284 ^{**}
Sheep C				Sheep F		
<i>in sacco</i>	0.352 ^{***}			<i>in sacco</i>	-0.110	
t[VFA]	-0.526 ^{***}	-0.180		t[VFA]	0.000	-0.122
NH ₃	0.284 ^{**}	0.174	-0.046	NH ₃	0.055	0.026
						-0.032

Asterisks relate to the significance of correlation, (***) = $P < 0.001$, (**) = $P < 0.01$, (*) = $P < 0.05$).

Summary of observed variations

- 1) Day-to-day variations could be classified into 3 groups:
 - a) Short-term noise.
 - b) Temporary short-term stable states.
 - c) Long-term trends.
- 2) Diet was found to have a big effect on the variance between *in sacco* bags incubated in the rumen of a sheep on specific day, between consecutive days in a given sheep and between sheep.
- 3) The day-to-day variation in t[VFA] in an individual sheep was found to be very large (CV approx. 25 %).
- 4) The variation in t[VFA] was primarily due to increases and decreases in the ruminal concentrations of acetate, propionate and butyrate.
- 5) The stoichiometry of fermentation was also found to alter on a daily basis within a sheep.
- 6) Mean rumen pH for all sheep was observed to be lower on the mixed diet as compared to the hay diet.
- 7) Rumen pH was found to vary more on the mixed diet than on the hay diet, but the coefficient of variation for the daily rumen pH within a sheep was found to be relatively low at approx. 3.75 % for both diets.
- 8) Rumen ammonia concentration was noted to be lower on the hay cf. the mixed diet and the daily variation within a sheep was found to be greater on diet 1 than diet 2.

Overall these results show that not only do the absolute concentrations of the fermentation end-products vary substantially between consecutive days but, the stoichiometry of rumen fermentation also varies on a day-to-day basis and this daily variation appears to increase with increased diurnal variation within the rumen of a sheep. Therefore, when investigating an effect of a rumen treatment by its effects on the end-products of fermentation, the experimental design should be such that the treatment effects can be distinguished from the natural variation of the rumen.

The results of this experiment give further credence to the use of the semi-purified diet as a research tool, since the day-to-day variation in the *in sacco* ISDMD values of soyahulls (Chapter 1, sheep fed the semi-purified diet) was no greater than that observed on the hay diet where cellulolysis was not assumed to be compromised by rumen pH fluctuations. However,

the degree of variability observed means the use of repeated 24 hour rumen incubations and has the potential only to detect large changes in rumen function. The conventional approach to *in sacco* degradability studies involves the use of a series of bags incubated in the rumen for different lengths of time. A degradability curve is then fitted to the results and the effect of treatment determined by analyzing changes the slope, initial losses and upper asymptote. Subsequent work assessed the variation between consecutive degradability curves fitted to the Ørskov, McDonald (1979) model.

The results of the current experiment also highlight the need for a robust experimental design capable of distinguishing the effects of a given ruminal treatment from the inherent noise of the ruminal ecosystem. In order to accomplish this repeated measurements in a change over design may be necessary in order to determine an accurate estimate of the standard error.

CHAPTER 9: The effect of supplemental YC on rumen protein and cellulose degradation in the rumen.

9.1: Introduction

Williams *et al* (1990), showed that supplemental YC increased the flow of non-ammonia nitrogen (NAN) to the small intestines ($P > 0.05$), accompanied by a reduction in ruminal ammonia-nitrogen concentration. The afore-mentioned authors concluded that the efficiency of microbial protein production was increased resulting in a lower rumen ammonia concentration and increased NAN flow to the duodenum. However, as discussed in Chapter 1 other authors have observed a variety responses of rumen ammonia concentration to supplemental YC, including significant ($P < 0.05$) increases (Arambel *et al* 1987) and decreases (Dawson *et al* 1990; Newbold *et al* 1990) in rumen ammonia concentration or no significant effect (Molony 1989, Malcom and Kiesling 1990, Edwards *et al* 1991a and Huhtanen 1991). The experiments described in Chapters 3, 6, and 7 failed to show a consistent response to the supplementation of YC on ruminal ammonia concentration. Therefore, as the effects of supplemental YC on rumen proteolysis has not been thoroughly investigated the main aim of the present experiment was to investigate the possible mode of action of supplemental YC on this process.

The results from the previous experiments discussed in Chapters 3, 6, 7 and 8 showed that the rumen degradability of washed hay measured using 24 hour incubations in the rumen varied substantially between consecutive days. Consequently a different approach was used for this experiment. Dacron bags were prepared using the previously described method containing either washed hay or washed fishmeal and then incubated in the rumen for either 0, 8, 16 or 24 hours and a degradation curve fitted according to Ørskov and McDonald (1979). This series of incubations was repeated 10 times within each experimental period in order to maximise the possibility of detecting small changes in rumen DM and CP degradability due to supplemental YC. Two other treatments were assessed, these included supplemental irradiated YC (IYC) and pot ale syrup (PAS) from the distillery industry. Supplementary PAS was included as a treatment as previous experiments have indicated that the viability of the YC supplement was not necessary for it to exert an effect on the rumen. Pot ale syrup was used as it contains large quantities of yeast cell debris and its use allowed a comparison between intact non viable yeast cells (IYC) and non viable, non intact yeast cells (PAS).

The diurnal variation in rumen fermentation noted on the semi-purified and the more conventional mixed diet used in Chapters 3, 7 and 8 respectively, indicated that after the evening feed rumen pH levels may drop lower than was observed after the morning feed. The current experiment also investigates this observation.

9.2: Materials and methods

9.2.1: Animals and design

4 rumen-cannulated mature wethers averaging approx. 65 kg were housed in digestibility crates for the 12 week experiment. After a 2 week adaptation period the animals were assigned to a 4x4 latin square described in Appendix 16. Each experimental period lasted 3 weeks.

9.2.2: Diet

The same conventional diet (diet 2) that was used in Chapters 7 and 8 was offered to all sheep for the entire period of the experiment. The diet composition physical and chemical analysis is given in (Table 7.2.1) Chapter 7. The diet was either supplemented with 15g/d of viable (YC) or irradiated (IYC) yeast culture (*Saccharomyces cerevisiae* 1026) or 22g/d of pot ale syrup (PAS).

9.2.3: Measurements

The following measurements were made during each experimental period:

- a) Rumen *in sacco* degradability of washed hay and washed fishmeal using repeated 0, 8, 16 and 24 hour incubation periods.
- b) Rumen VFA (mM; molar %), pH and ammonia (mg/l) diurnal pattern over a 24 hour period.
- c) Yeast viability in the rumen ($\text{Log}_{10}\text{CFU/ml}$).
- d) Viable yeast count of the faeces ($\text{Log}_{10}\text{CFU/ml}$).
- e) Excretion of urinary allantoin (g/d).
- f) Liquid dilution rate from the rumen and hindgut.

9.2.4: *in sacco* rumen degradability of washed hay and washed fishmeal

Sufficient hay for the whole experiment was chopped (to approx. 1 cm in length), washed in large dacron bags with a pore size of $40\mu\text{m}$ and subsequently dried in an oven at 60°C for 48h.

The hay was then thoroughly mixed and left to air dry before being sub-sampled and placed into bags. The washing and drying process was repeated for the fishmeal substrate until sufficient substrate (hay and fishmeal) had been prepared for the entire experiment. Approximately 4g of air dry hay and 3g of air dry fishmeal were placed in individual bags and batched according to the incubation regimen. Bags were incubated in the rumen for 0, 8, 16 and 24 hours over 2 consecutive days. This process was repeated 10 times in each 21 day experimental period. After incubation in the rumen the dacron bags were processed as described in Chapter 2.

9.2.5: Diurnal analysis of rumen VFA, pH and ammonia concentration

Rumen liquor was aspirated from the rumen at 2 hourly intervals over a 24 hour period starting on day 16 of each experimental period. The liquor was immediately preserved and stored as described in Chapter 2, to await analysis at the end of the experiment using the methods described earlier.

9.2.6: Yeast viability in the rumen

Yeast viability in the rumen was assessed by aspirating a rumen sample at 09:00, 11:00 and 15:00. on days 1, 7 and 21 of each experimental period. The samples were then serially diluted and 0.1ml inoculated onto duplicate spread plates containing dicloron rose bengal chloramphenicol agar (d-RBC) (Unipath Ltd., Basingstoke, UK.). The d-RBC agar plates were subsequently incubated at 15°C for 5 days, and the number of yeast colonies counted.

9.2.7: Viable yeast count of the faeces

Faecal samples were collected at 15:00 on days 2, 8 and 21 of each experimental period. The faeces was processed and plated onto d-RBC agar plates using the method described in Chapter 3 (section 3.2.11).

9.2.8: Measurement of urinary allantoin.

Urine was collected daily and processed using the method described in Chapter 2 (section 2.4).

9.2.9: Measurement of the rumen and hindgut liquid dilution rate

Rumen and hindgut LDR was measured using the method described in Chapter 2 (section 2.3).

9.2.10: Interpretation, presentation and analysis of results

The fishmeal degradability curves were fitted to the model of Ørskov and McDonald (1979) using a nonlinear regression analysis programme (NONLIN, Sherrod 1992). A linear model was fitted to the hay degradation data and slope constants for substrates were then statistically analyzed using the analysis of variance option in Genstat 5.21. Yeast viability in the rumen, faecal viable yeast counts and the rumen parameters of VFA, pH and ammonia plus the slope constants for the rumen and hindgut LDR were analysed using the analysis of variance option in Genstat 5.13. The BLOCK and TREATMENT commands that were used to determine the degrees of freedom are shown in tables 9.2.1-9.2.

Table 9.2.1: Block - treatment structure used for the analysis of the *in sacco* degradability of washed hay and fishmeal

BLOCK	(SHEEP*PERIOD)/INCUBATIONREPS/HOUR
TREATMENT	SHEEP + PERIOD + (PERIOD.INCUBATIONREPS) + TREATMENT*HOUR *INCUBATIONREPS

Analysis of variance table	
Source of variation	d.f.
sheep.period stratum	
sheep	3
period	3
treatment	3
Residual	6
sheep.period.incubationreps stratum	
period.incubationreps	36
incubationrep.treatment	36
Residual	72
sheep.period.incubationreps.hour stratum	
hour	3
incubationreps.hour	36
hour.treatment	9
incubationreps.hour.treatment	72
Residual	360
Total	639

Table 9.2.2: Block - treatment structure used for the analysis of rumen VFA, pH and ammonia.

BLOCK (SHEEP*PERIOD)/SAMPTIME
TREATMENT SHEEP + (TREATMENT*SAMPTIME)

Analysis of variance table	
Source of variation	d.f.
period stratum	3
sheep.period stratum	
sheep	3
treatment	3
Residual	6
sheep.period.samptime stratum	
samptime	11
samptime.treatment	33
Residual	132
Total	191

9.3: Results and Discussion

9.3.1: *in sacco* degradability of washed hay.

Analysis of variance showed that supplementation of the diet with either YC, IYC or PAS did not significantly affect the mean ISDMD_{HAY} ($P=0.476$) nor the interaction between the hour and treatment ($P=0.826$) indicating that the supplements did not have a significant ($P>0.1$) effect on the pattern of degradation over the 0, 8, 16 and 24 hour incubation series. However, the incubation times (HOUR) ($P<0.001$) and periods ($P=0.022$) were found to be significantly different. The interaction between the incubation replicates (INCUBATIONREPS) and period was also found to be statistically significant ($P<0.001$). Table 9.3.1 shows that the mean ISDMD of washed hay changed significantly ($P<0.001$) within a period and that these variations were not consistent between periods. This may suggest that the mean ISDMD_{HAY} of the dacron bags incubated for a specific time varied, leading to variation in the rate of degradation of washed hay within a period. This was assumed to relate to the inherent biological variation within a sheep and between sheep. The different sheep were also found to be significantly different ($P=0.006$).

Table 9.3.1: Mean ISDMD^{Hay} (%) incubation replicates in each experimental period

Period	Incubation replicate									
	1	2	3	4	5	6	7	8	9	10
1	16.9 ^a	16.6 ^a	15.0 ^{ac}	15.6 ^{ac}	16.8 ^a	15.2 ^{ac}	14.2 ^{abc}	16.6 ^{ac}	13.7 ^{bc}	12.0 ^b
2	14.8 ^a	13.6 ^{ab}	15.0 ^a	14.0 ^{ab}	13.8 ^{ab}	14.0 ^{ab}	12.4 ^b	11.9 ^b	11.7 ^b	11.4 ^b
3	13.3 ^a	13.1 ^a	14.2 ^a	15.1 ^a	14.2 ^a	13.5 ^a	12.5 ^a	13.5 ^a	13.5 ^a	14.4 ^a
4	13.0 ^a	12.7 ^a	13.8 ^a	14.6 ^a	12.3 ^a	13.7 ^a	13.7 ^a	13.5 ^a	13.7 ^a	13.9 ^a

S.E.D. = 0.82

Means in the same row not sharing a common superscript are significantly different ($P<0.001$).

Sheep were housed and fed in digestibility crates for 2 weeks prior to the start of the experiment in order to allow the animals to adapt to the diet and their new housing. However, Table 9.3.1 indicates that variation in mean $ISDMD_{HAY}$ between consecutive incubation series replicates lessened in the second two periods and may relate to adaptation of the sheep to the diet. Previous experiments have shown that individual sheep show short, medium and long term stability in the rumen between consecutive days. The reduced variation observed in the incubation series replicates for $ISDMD_{HAY}$ in the second two periods may therefore relate to such conditions prevailing in more than one sheep and thus affecting the means accordingly.

Figure 9.1 shows the effect of incubation time and treatment on $ISDMD_{HAY}$. This figure indicates that $ISDMD_{HAY}$ increases linearly up to a rumen incubation period of 24 hours. Linear regression and analysis of variance of the period means for the individual sheep slope constants showed that treatment had no effect on the rate of degradation of washed hay in the rumen. Period means were used for the analysis of variance, due to the wide variation noted between the individual replicate incubation series slopes within a period for a given sheep. The regression was restricted to the 8, 16 and 24 hour incubation bag means in order to reduce the effects of variable lag times in the degradation response created by the time taken for the rumen liquor to infiltrate the dacron bags and hence commence the microbial degradation of the *in sacco* substrate. Table 9.3.2 shows the slope constants from the linear regression equation:

$$Y = A + B * T$$

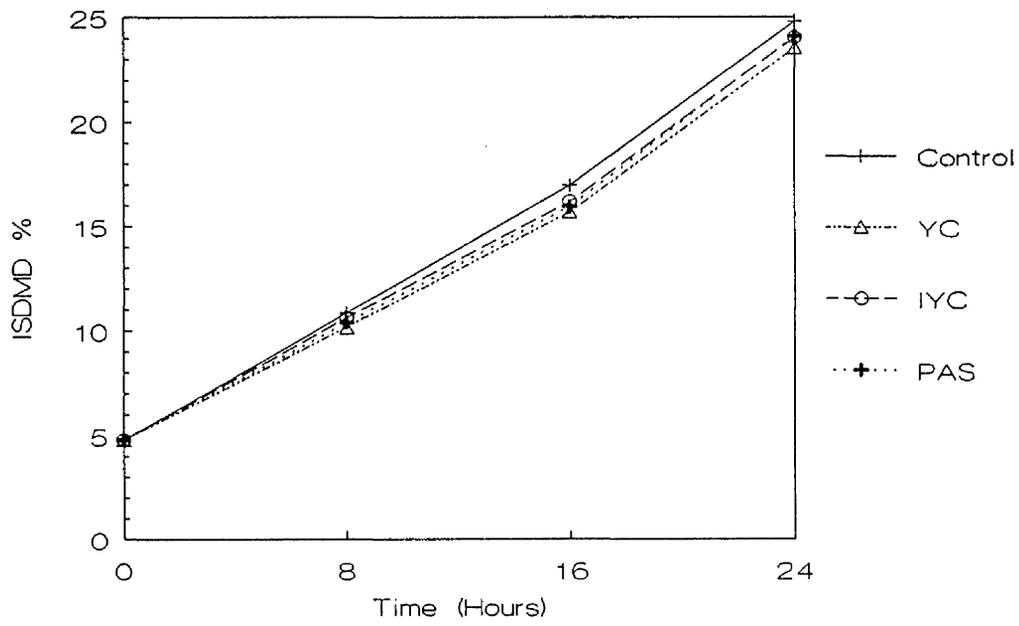
Where: Y = the observed degradation
T = incubation time
A = initial losses due to solubilisation and washout of small particles
B = the rate of degradation

The mean R^2 (% variance accounted for by regression) for this model was found to be 98.8 %.

Analysis of variance showed that treatment had no statistically significant effect on the initial losses or the rate constant of $ISDMD_{HAY}$ ($P > 0.05$) (Table 9.3.2).

Figure 9.1

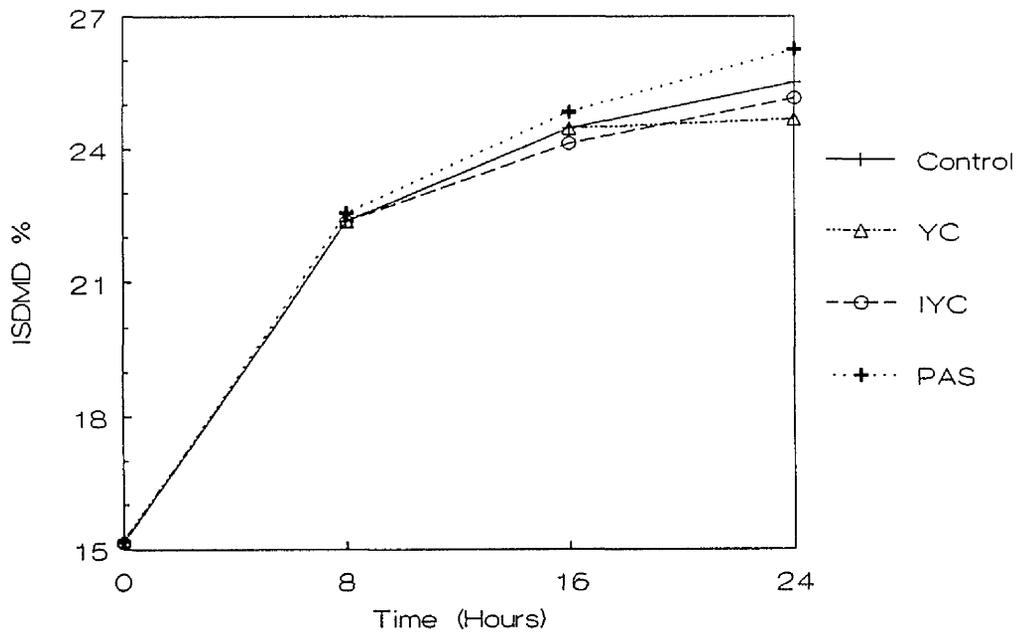
in sacco degradability of washed hay



S.E.D.= 0.824

Figure 9.2

in sacco degradability of fish meal



S.E.D.= 0.428

Table 9.3.2: The effect of additional YC, IYC or PAS on the initial losses and rate of degradation of washed hay

	Treatment				S.E.D.	F.Pr.
	Control	YC	IYC	PAS		
Constant (a) %	1.29	-0.01	0.49	-0.34	2.52	0.921
Slope %/h	0.98	0.98	0.98	1.02	0.128	0.988

9.3.2: *in sacco* degradation of washed fish meal

Analysis of variance showed that supplementation of the basal diet with either YC, IYC, or PAS did not significantly affect the overall mean degradation of fishmeal ($P=0.211$). However, the interaction between treatment and hour was found to be significant ($P=0.031$). This indicates that treatment had a significant effect on the degradability curve of fishmeal.

The interaction between period and replicate incubation series was also found to be significant and was in accordance with the hay degradability results which suggests that the replicate incubation series varied within a period but not in a uniform manner. The mean $ISDMD_{FISHMEAL}$ for the individual replicate series within a period are given in Table 9.3.3

Table 9.3.3: Mean $ISDMD_{FISHMEAL}$ (%) incubation replicates in each experimental period

Period	Incubation replicate									
	1	2	3	4	5	6	7	8	9	10
1	23.3 ^{abc}	23.6 ^a	22.9 ^{ac}	22.6 ^a	22.4 ^{ac}	22.0 ^{abc}	21.4 ^{bc}	21.4 ^{bc}	20.6 ^{bc}	20.6 ^{bc}
2	22.2 ^{acd}	22.3 ^{acd}	22.9 ^{ad}	22.3 ^{acd}	22.4 ^{acd}	23.1 ^{ad}	21.0 ^b	20.7 ^b	21.1 ^{bcd}	21.7 ^{acd}
3	22.1 ^{abc}	21.7 ^{ac}	22.0 ^{ac}	22.1 ^{ac}	21.1 ^{bc}	20.2 ^b	21.0 ^{bc}	21.8 ^{ac}	22.0 ^{ac}	22.6 ^{ac}
4	22.3 ^{abc}	22.0 ^{abc}	22.7 ^{ac}	23.0 ^{ac}	21.3 ^{bc}	20.9 ^{bc}	20.9 ^{bc}	20.9 ^{bc}	21.6 ^{abc}	22.5 ^{ac}

S.E.D. = 0.43

Means in the same row not sharing a common superscript are significantly different ($P < 0.001$).

Table 9.3.3 shows that pattern of mean incubation series replicate $ISDMD_{FISHMEAL}$ was different in each period and that these patterns were also different from those observed for $ISDMD_{HAY}$. Thus indicates that errors in the processing of the bags after incubation in the rumen cannot explain the observed variation between replicate incubation series. The SED for the fish meal results were found to be lower than those for the comparable hay $ISDMD$ results indicating that there was less variation between the individual dacron bags incubated in the rumen at the same time, replicate incubation times and between sheep for the fishmeal analysis. This may indicate that the factors affecting the stability of $ISDMD_{HAY}$ in the rumen were having less effect on the $ISDMD$ of fish meal.

Figure 9.2 shows the effect of length of incubation in the rumen and treatment on the $ISDMD_{FISHMEAL}$ and demonstrates that extent of degradation of fishmeal in the rumen increased in a curvilinear manner. The mean incubation series for each period in each sheep was fitted to the model described by Ørskov and McDonald (1979), according to the equation:

$$Y = A + B * (1 - \exp^{-C * T})$$

Where:

- Y = the potential degradability
- T = incubation time
- A = initial losses due to solubilisation and washout of small particles
- B = the amount degraded after an infinite time (Upper asymptote)
- C = the rate of degradation

The mean R^2 (% variance accounted for by regression) to this model was found to be 92.9 %

Figure 9.2 also shows that the YC treatment resulted in a degradability curve that appeared different to those for the other treatments. The $ISDMD_{FISHMEAL}$ degradability curve for this treatment showed that maximum degradation was reached by the 16 hour incubation measurement whereas the other treatments showed increased degradation after 16 hours incubation in the rumen. It was this difference in the 24 hour degradability measurements caused a significant ($P=0.031$) hour x treatment interaction (Table 9.3.4). The depressed 24h $ISDMD_{FISHMEAL}$ may have related to the low (absolute) rumen pH and duration of the pH minima observed during the rumen diurnal fluctuations when YC was included in the diet.

Rumen pH has been shown to be an important factor influencing rumen proteolysis (Erfle *et al.*, 1982; Lindberg 1985) (Section 9.3.7). Low rumen pH may account for the effect of supplementary YC on rumen proteolysis, rather than a direct effect of additional YC on the rumen proteolytic microflora.

Table 9.3.4: The effect of supplemental YC, IYC and PAS on mean ISDMD_{Fishmeal}

Incubation time.	Treatment			
	Control	YC	IYC	PAS
0	_a 15.2 ^a	_a 15.2 ^a	_a 15.1 ^a	_a 15.2 ^a
8	_a 22.4 ^b	_a 22.4 ^b	_a 22.4 ^b	_a 22.5 ^b
16	_a 24.5 ^c	_a 24.5 ^c	_a 24.1 ^c	_a 24.9 ^c
24	_b 25.5 ^d	_a 24.7 ^c	_{ab} 25.2 ^d	_a 26.3 ^d

Incubation time means not sharing a common superscript in the same column are significantly different ($P < 0.05$; S.E.D. = 0.31).

Treatment means not sharing a common subscript in the same row are significantly different ($P < 0.05$; S.E.D. = 0.21)

Analysis of the constants fitted to the Ørskov and McDonald (1979) model (Table 9.3.5) showed that the final asymptote (b term) fishmeal degradation for the PAS treatment was significantly higher than those for the YC and IYC treatments ($P < 0.1$) but were not found to be significantly different from those for the control diet ($P > 0.1$). The rate of fishmeal degradation (c term) noted when the diet was supplemented with YC was found to be higher than that observed when the diet was supplemented with PAS ($P < 0.05$). The effect of IYC on degradation rate was found to be similar to that of additional YC but not as great. However, the control c term mean was not found to differ significantly ($P > 0.05$) from any other treatment.

Table 9.3.5: The effect of treatment on the degradability curve of fishmeal

Curve constant	Treatment				S.E.D.	F.Pr
	Control	YC	IYC	PAS		
Initial losses (a)	15.1	15.2	15.2	15.2	0.04	$P = 0.14$
Asymptote (b)	10.8 ^{ab}	9.9 ^a	10.2 ^a	11.6 ^b	0.49	$P < 0.1$
Rate constant (c)	0.144 ^{ab}	0.166 ^b	0.154 ^{ab}	0.128 ^a	0.01	$P < 0.05$

Means in the same row not sharing a common superscript are significantly different at the stated level of significance.

The rumen effective degradability of fishmeal was calculated for 4 rumen outflow rates according to the equation :

$$P = A + \frac{B * C}{C + k}$$

Where: P = Effective degradability (%)
 A = initial losses
 B = Asymptote
 C = Rate of degradation
 k = Ruminant LDR

The results of this calculation showed that treatment had no effect on the effective degradability of fishmeal (P=0.306) (Table 9.3.6).

Table 9.3.6 shows that as rumen outflow increases, the percentage of fishmeal effectively degraded lessens. This is in accordance with the findings described in Chapter 6, where it was observed that when OMD decreased resulting in a greater outflow of digesta from the rumen the excretion of urinary allantoin increased (Dewhurst and Webster 1992a,b). These results also show the opposite effect on fishmeal degradability noted between the PAS treatment compared to that induced by either the YC or IYC treatment was still present in the calculation of effective degradability. however, the differences between the treatments would only be noted at long rumen retention times (P < 0.05).

Table 9.3.6: The effect of treatment and rumen LDR on the effective degradability (%) of fishmeal.

Rumen LDR (rate constant <i>k</i>)	Treatment			
	Control	YC	IYC	PAS
0.02	24.6 ^{ab}	24.0 ^a	24.1 ^a	25.1 ^b
0.06	22.6 ^a	22.4 ^a	22.1 ^a	22.9 ^a
0.10	21.4 ^a	21.2 ^a	21.3 ^a	21.5 ^a
0.14	20.5 ^a	20.5 ^a	20.5 ^a	20.6 ^a

S.E.D. = 0.26

Means not sharing a common superscript, in the same row are significantly different (P < 0.05).

9.3.3: Measurement of urinary allantoin

Analysis of variance showed that the total daily excretion of allantoin in urine was not significantly effected by treatment ($P= 0.626$) or period ($P= 0.842$). Table 9.3.7 shows the treatment means and Table 9.3.8 the period means.

Table 9.3.7: The effect of additional YC, IYC or PAS on the excretion of urinary allantoin (g/d)

Control	YC	IYC	PAS	S.E.D.	F.Pr.
11.4	11.2	11.2	10.7	0.53	0.626

Table 9.3.8: Mean urinary allantoin excretion for each experimental period (g/d)

1	2	3	4	S.E.D.	F.Pr.
11.1	10.8	11.2	11.3	0.53	0.842

The means for all the sheep on each day were found to be significantly different ($P= 0.052$) and the period x replicate days interaction was also found to be significant ($P < 0.001$). This indicated that the total excretion of urinary allantoin per day varied on a day-to-day basis and that this variation was different in different periods, but that the overall mean total excretion of allantoin in each period was the same. It was assumed that the variation observed between replicate days resulted from the inherent variability in the day-to-day excretion of individual sheep, and was related to daily variations in individual microbial population densities (Wilson and Briggs, 1955; Leedle *et al*, 1982) and microbial growth efficiency as observed and commented on in Chapters 3 and 7.

The treatment means for the total excretion of urinary allantoin per day for the current experiment was found to be lower than that observed in Chapters 3 and 6. This was assumed to relate to the lower ME content (10.5 MJ/kgDM) of the diet in the present experiment as compared to that of the semi-purified diet (ME = 12.5 MJ/kgDM) used in Chapters 3 and 6.

Microbial growth efficiency expressed as MN/ME (g/MJ) was found to be 1.2, 1.5 and 1.1 for the experiments described in Chapters 3, 6 and 9 respectively. This is in accord with the findings of Dewhurst *et al* (1987).

9.3.4: Measurement of rumen and hindgut liquid dilution rates

Table 9.3.9 shows the treatment means for LDR in the rumen and hindgut. Rumen LDR was not found to be significantly affected by treatment ($P = 0.756$). However, a significant hindgut LDR response was found for IYC ($P = 0.096$).

Table 9.3.9: The effect of treatment on the LDR of the rumen and hindgut

Site	Treatment				S.E.D.	F.Pr.
	Control	YC	IYC	PAS		
Rumen	0.051	0.053	0.048	0.049	0.0043	0.756
Hindgut	0.108 ^a	0.12 ^{ab}	0.13 ^b	0.117 ^a	0.0064	0.096

Means in the same row with different superscripts are significantly different at the stated level of significance.

These results suggest that the supplemental YC and IYC treatments significantly increased the transit time of digesta through the hindgut. This is in contrast to the measurements observed in Chapter 7 during which the hindgut outflow rate was observed to be reduced by the YC supplement for both diets ($P > 0.05$). The reasons for this possible mode of action of the YC and IYC treatments is not clear, it may be theorised that the increased outflow of undegraded dietary protein as a result of reduced rumen proteolytic activity may have stimulated the propulsion of digesta through the hindgut. However, if the YC treatment truly affects the hindgut in such away as to increase the rate of transit through the hindgut, the possibility of either of these treatments having a beneficial effect on hindgut digestion appears more remote.

9.3.5: Yeast viability in the rumen

Table 9.3.10 shows the treatment means at each sampling time. Analysis of variance showed that the viable yeast counts ($\text{Log}_{10}\text{CFU/ml}$) in those sheep receiving the viable yeast supplement was significantly ($P < 0.001$) higher than those sheep on the other treatments and also that the viable yeast count was higher 2 and 6 hours post supplementation than 16 hours after administration of the supplement.

Table 9.3.10: Diurnal mean yeast culture recovery from the rumen ($\text{Log}_{10}\text{CFU/ml}$).

Hours post supplementation	Treatment			
	Control	YC	IYC	PAS
2	2.2 ^a	2.5 ^a	2.3 ^a	2.2 ^a
6	1.9 ^a	3.5 ^b	2.5 ^a	2.2 ^a
16	2.2 ^a	3.2 ^b	2.1 ^a	1.9 ^a

S.E.D. = 0.12

Means in the same column not sharing a common superscript are significantly different at the $P < 0.001$ level of significance.

The different sampling days and the sampling day x treatment interaction were not found to be significantly different ($P > 0.05$) (Table 9.3.11).

Table 9.3.11: Variation in viable rumen yeast count ($\text{Log}_{10}\text{CFU/ml}$) within each experimental period.

Period day	Treatment			
	Control	YC	IYC	PAS
1	2.21 ^b	2.92 ^a	2.33 ^b	2.14 ^b
7	1.94 ^b	3.28 ^a	2.14 ^b	2.06 ^b
21	2.16 ^b	2.94 ^a	2.40 ^b	2.05 ^b

S.E.D. = 0.22

Means in the same row not sharing a common superscripts are significantly different ($P < 0.05$).

The relatively high viable yeast counts observed when the diet was not supplemented with viable YC were assumed to relate to microbial contamination of the feedstuff (Lund 1974) and carry over from previous experimental periods where additional YC was fed. However, rumen viable yeast counts were found to be significantly higher ($P < 0.05$) when the diet was supplemented with YC (Table 9.3.11).

The rate of loss of viable yeast cells from the rumen was determined by a linear regression of the viable yeast count against time and was found to be 0.069 CFU/ml/h and fit the linear model (R^2 of 99.3 %). This equates to the rumen LDR which was found to have a rate constant of 0.051. These results are in accord with the findings of Chapter 7 during which the rate of loss of viable yeast cells from the rumen was calculated to be 0.15 CFU/ml/h. These results further support the theory that the yeast supplement is capable of surviving in the rumen but not of forming a stable population at a density equivalent to that at inoculation, in accord with the findings of Newbold *et al.* (1990).

9.3.6: Faecal yeast count

Analysis of variance found that faecal yeast counts measured in sheep ($P = 0.045$) and on different sampling days ($P = 0.79$) were significantly different. The treatment x day interaction was also found to be statistically significant at the $P = 0.023$ level of significance. Table 9.3.12 shows the mean Log_{10} viable yeast count for each treatment on each sampling day.

Table 9.3.12: Mean viable faecal yeast count (Log_{10} CFU/ml) for each treatment on a given day of each experimental period.

Period day	Treatment			
	Control	YC	IYC	PAS
2	2.3 ^{ab}	3.2 ^a	1.9 ^a	1.9 ^a
8	1.9 ^b	2.2 ^b	1.9 ^a	2.1 ^a
21	2.6 ^a	2.4 ^b	2.1 ^a	2.0 ^a

S.E.D. = 0.26

Means in the same column not sharing a common superscript are significantly different ($P < 0.05$).

Table 9.3.12 shows that the viable yeast supplement was capable of surviving transit through the abomasum and hindgut. These results also indicate that the YC supplement may be able to maintain a small population in either the rumen and/or the hindgut for prolonged periods as relatively high viable yeast counts were still being measured in the faeces of animals that had not been fed the YC supplement for at least 21 days. Mean rumen viable yeast counts ($\text{Log}_{10}\text{CFU/ml}$) for the control treatment were found to be higher than the equivalent faecal yeast count ($\text{Log}_{10}\text{CFU/ml}$) on day 21 of each experimental period. This may suggest that the carry over effects observed in faecal yeasts may relate to the establishment of a yeast population in the sheep hindgut. These results are in contrast to those discussed in Chapter 7, where the viable yeast counts of animals not receiving the YC supplement were much lower than the animal receiving the YC treatment. However, in accordance with results of Chapter 7 the viable yeast count of the faeces was found to be significantly higher on the second day of supplementation when compared to the yeast counts obtained on days 8 and 21 of each period. The results of the current experiment support the theory that yeast autolysis in the rumen may promote further autolysis until an equilibrium has been established between the addition of new cells and rate of autolysis, or the fresh addition of YC to the rumen promotes the autolysis of the yeast already present in the rumen.

9.3.7: Measurement of rumen pH

Analysis of variance showed that treatment had no effect on rumen pH. However, the mean rumen pH for the YC treatment was found to be lower than the control treatment mean at $P < 0.1$. Table 9.3.13 also indicates that the IYC treatment induced a similar but lesser effect compared to YC. The treatment means are shown in Table 9.3.13.

Table 9.3.13: The effect of supplemental YC, IYC or PAS on rumen pH

Control	YC	IYC	PAS	S.E.D.	F.Pr.
6.23	6.11	6.15	6.24	0.06	0.224

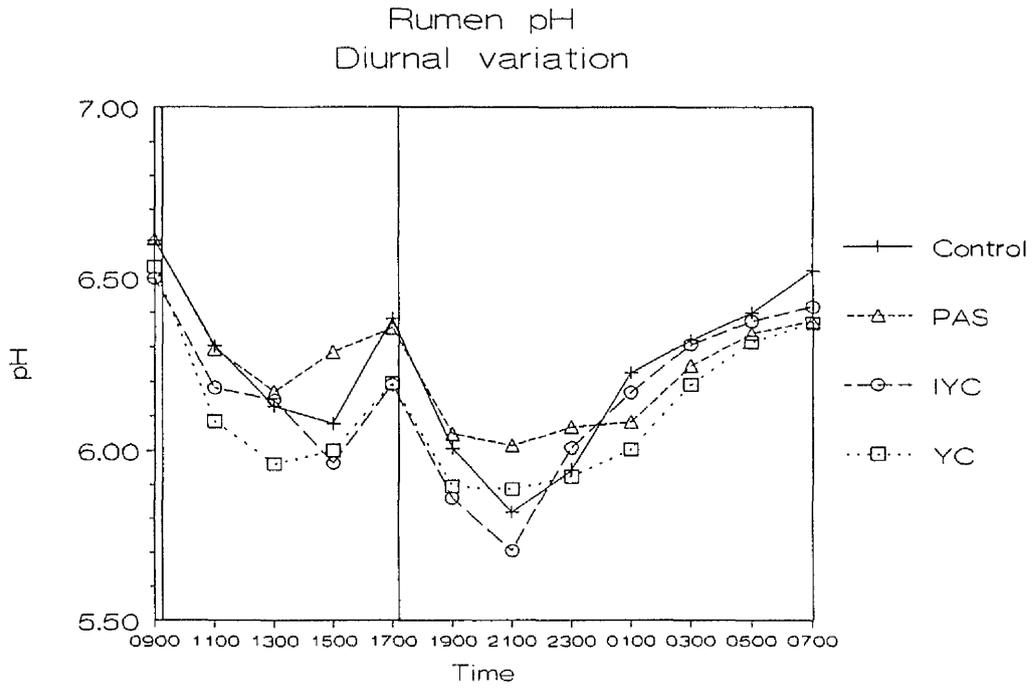
Values for individual sheep and time of sampling were found to be significantly different ($P = 0.063$ and $P < 0.001$ respectively).

Figure 9.3 shows the relationship between rumen pH and time of sampling for each treatment. The vertical lines on this figure show when the animals were fed. This figure shows that all the treatments induced similar diurnal variations in rumen pH. The interaction between treatment and sampling time was not found to be significant ($P=0.520$). Rumen pH prior to feeding PM was observed to be lower in comparison to the AM pre-feeding pH levels. Rumen pH was found to drop to its lowest minima after the PM feeding. This confirms the previously theorised effects of unequal feeding periods postulated in Chapters 3, 7 and 8.

Stewart (1977) and Mould and Ørskov (1983) showed that cellulolysis was inhibited by both a drop in absolute rumen pH below 6.0 and also by the duration of the pH depression. Similarly pH has been found to affect protein degradation. Henderickx and Martin (1963), showed that the extent of protein degradation in the rumen was closely related to the solubility of the protein, and Song and Kennelly (1991) found that protein solubility increased with solvent pH. Lindberg (1985), cites that the optimum rumen pH for proteolytic enzymes is in the range of 6.0-8.0 and Erfle *et al* (1982) observed a decrease in proteolytic activity and the number of proteolytic organisms when they lowered rumen pH (*in vitro*) from 7.0-5.0. The findings of Erfle *et al* (1982) were in accord with those of Brock *et al* (1982) and Kopečný and Wallace (1982) who showed that most rumen proteolytic activity is cell wall-associated. The rumen pH minima at approx. 13:00 and 21:00 observed in the current experiment were in the range at which cellulolysis and proteolysis may be inhibited. The pH after the evening feed was observed to fall further below pH 6.0 and for longer than after the morning feed. This diurnal variation in rumen pH was assumed to be responsible for the anomalous 16h *in sacco* degradation of both washed hay and fishmeal. At these observations it was noted that the ISDMD losses after 8h of incubation were found to be greater than after 16h of rumen incubation and may account for some of the observed variation in the measured degradation parameters for hay and fishmeal.

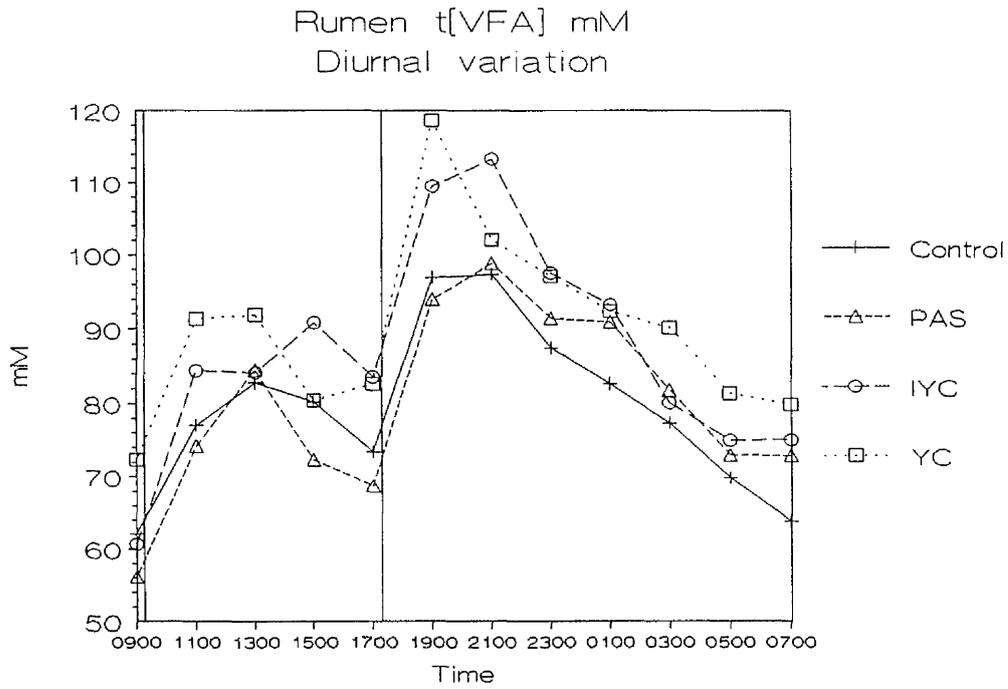
Figure 9.3 also shows that the YC and IYC treatments induced rumen pH levels lower than those found for either the PAS or control treatments. Whereas, the PAS treatment compared to the control was found to induce higher minimum pH levels, although pH values were not found to be consistently higher throughout the 24h period. A statistical analysis which compared the means of YC + IYC against PAS + Control found that supplementation of the diet with yeast (either viable or non viable) significantly ($P= 0.035$) reduced rumen pH. However, this analysis is confounded by the effects of the PAS treatment, which may be opposite to the effects of supplemental YC and IYC as found for the degradation of fishmeal *in sacco*.

Figure 9.3



S.E.D.= 0.116

Figure 9.4



S.E.D.= 7.86

These results indicate that supplementation of a diet with YC and IYC may result in reduced rumen pH levels ($P > 0.05$). Consequently, initial analysis of the effects of treatment on rumen ammonia and the individual VFA concentrations must make careful consideration of the effects of rumen pH on the absorption of these degradation end-products.

9.3.8: Measurement of the rumen volatile fatty acids

9.3.8.1: Rumen t[VFA]

Analysis of variance showed that supplemental YC significantly ($P = 0.096$) increased total VFA concentration in the rumen. The IYC t[VFA] treatment mean was also found to be significantly higher than for the control diet ($P = 0.096$), but failed to reach significance when compared to the PAS treatment mean. The treatment means are shown in Table 9.3.14 and the diurnal variation in t[VFA] for each treatment in Figure 9.3.

Table 9.3.14: The effect of treatment on the rumen fermentation end-products

Rumen Parameter	Treatment				S.E.D.	F.Pr.
	Control	YC	IYC	PAS		
t[VFA]	79.3 ^c	90.0 ^a	87.3 ^{ab}	79.9 ^{bc}	4.09	0.096
Acetate (mM)	53.6	59.9	57.8	53.7	2.55	0.115
Propionate (mM)	14.9	17.6	16.9	16.0	1.19	0.224
Butyrate (mM)	9.6	11.1	11.4	8.9	0.97	0.105
isoButyrate (mM)	0.43	0.48	0.43	0.46	0.024	0.234
Valerate (mM)	0.61	0.65	0.62	0.65	0.056	0.836
isoValerate (mM)	0.24	0.3	0.26	0.27	0.02	0.106
Acetate (molar %)	67.9	66.6	66.3	67.4	0.68	0.162
Propionate (molar %)	18.5	19.6	19.0	19.6	1.14	0.702
Butyrate (molar %)	12.0	12.2	13.2	11.2	0.74	0.162
isoButyrate (molar %)	0.56	0.54	0.51	0.60	0.045	0.357
Valerate (molar %)	0.77	0.72	0.70	0.81	0.063	0.396
A:P ratio	3.78	3.53	3.59	3.54	0.22	0.649

Means in the same row not sharing a common superscript are significantly different at the level of statistical significance shown in the table.

Table 9.3.14 shows that the increased t[VFA] noted on the YC and IYC treatments was due mainly to increases in the absolute concentrations of the three major VFA. Table 9.3.15 shows the correlation coefficients for the comparison of t[VFA], pH and the individual VFA for all treatments throughout the experiment.

Table 9.3.15: Correlation coefficients for the relationship between rumen t[VFA], pH and the individual VFA (mM).

	t[VFA]	Acetate	Propionate	isoButyrate	Butyrate	isoValerate	Valerate
Acetate	0.979 ^{***}						
Propionate	0.864 ^{***}	0.758 ^{***}					
isoButyrate	0.185	0.168	0.139				
Butyrate	0.851 ^{***}	0.830 ^{***}	0.603 ^{***}	0.200*			
isoValerate	-0.037	-0.049	-0.119	0.868 ^{***}	0.141		
Valerate	0.808 ^{***}	0.774 ^{***}	0.767 ^{***}	0.129	0.868 ^{***}	-0.108	
pH	-0.849 ^{***}	-0.790 ^{***}	-0.804 ^{***}	0.078	0.129	0.217*	-0.739 ^{***}

Asterisks show the significance of correlation, (***) = $P < 0.001$, (**) = $P < 0.01$, (*) = $P < 0.05$).

Table 9.3.15 shows that t[VFA] was significantly ($P < 0.001$) increased due to changes in the absolute concentrations of acetate, propionate, butyrate and the minor VFA valerate. Table 9.3.15 also shows that changes in the absolute concentrations of these acids was negatively associated with the observed changes in rumen pH ($P < 0.001$).

Table 9.3.16 shows that as t[VFA] increased, the proportion of acetate and butyrate decreased, whereas, the molar proportion of propionate increased. This was assumed to relate to diurnal changes in rumen fermentation pattern. During the rapid fermentation of the RFC fraction of the diet, post-feeding t[VFA] was found to increase to its maxima and rumen pH decline to its minima. It was during these conditions of rapid fermentation that peak propionate production was observed, whereas the more gradual fermentation of the structural carbohydrate portion of the diet resulted in a slower production of t[VFA] and increased rumen pH levels. It was during these conditions of more gradual degradation of the structural carbohydrates that the relative production of acetate and butyrate was found to increase. The effects of diurnal changes

in the fermentation pattern were assumed to be greater than the effects of rumen pH on the relative absorption rates of the individual VFA, as an increase in rumen pH may have been expected to result in a decrease in the molar proportion of acetate (Ørskov and Ryle; 1990).

Table 9.3.16: Correlation coefficients for the relationship between rumen t[VFA], pH and the individual VFA (molar %).

	t[VFA]	pH	Acetate %	Propionate %	isoButyrate %	Butyrate %	isoValerate %
pH	-0.849***						
Acetate %	-0.407***	0.524***					
Propionate %	0.374***	-0.488***	-0.868***				
isoButyrate %	-0.671***	0.720***	0.233*	-0.274**			
Butyrate %	0.085	-0.069	-0.076	-0.421***	-0.032		
isoValerate %	-0.584***	0.629***	0.188	-0.328***	0.935***	0.169	
Valerate %	0.040	-0.142	-0.122	0.262**	-0.074	-0.364***	-0.180

Asterisks show the significance of correlation, (***) = $P < 0.001$, (**) = $P < 0.01$, (*) = $P < 0.05$).

The treatment x time of sampling interaction for rumen tVFA, acetate, propionate and butyrate concentration was not found to be significant ($P = 0.766, 0.815, 0.900$ and 0.330 respectively) showing that treatment had no overall statistically significant effect on the diurnal pattern of VFA production. Nevertheless, there were some trends in diurnal pattern in response to treatment which are worth consideration. Figure 9.4 shows that the significant ($P < 0.1$) increase in ruminal t[VFA] was noted on the YC and IYC treatments was observed throughout the 24 hour period and that the peak t[VFA] for the IYC treatment occurred later than that for the YC treatment, suggesting that the mode of action for the two treatments may be slightly different. The peak t[VFA] concentration for the YC treatment was found to correspond to peak ruminal acetate, butyrate and propionate concentration whereas, the t[VFA] peak for the IYC diet was found to correspond with an increase in the ruminal acetate and butyrate concentration at this time. It may therefore be theorised that the YC and IYC supplements induced an increased t[VFA], possibly by providing a factor that stimulated either microbial growth and/or microbial activity, and that for the IYC supplement this nutrient was less readily available causing a slower, smaller response than that for YC treatment. As the IYC treatment exerted

its effect more slowly than the YC treatment, it might be expected to favour the production of acetate and butyrate. The PAS treatment was not found to alter the tVFA concentration or the VFA mix in comparison to the control treatment.

9.3.8.2: Major VFA

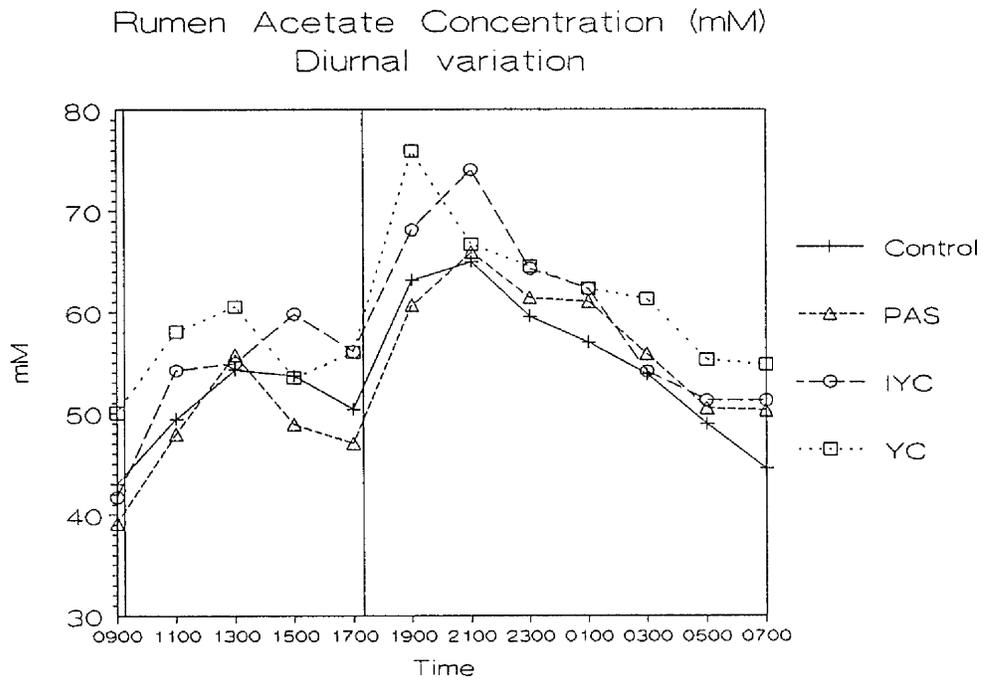
Analysis of variance showed that the absolute concentrations and molar proportions of the major VFA were unaffected ($P > 0.05$). The treatment means for the individual VFA absolute concentrations and molar proportions are given in Table 9.3.14. Figures 9.6 to 9.10 show the diurnal variations for the absolute and molar proportions of the individual VFA.

Figures 9.6, 9.8 and 9.10 indicate that the effect of treatment on the molar proportions of acetate, propionate and butyrate respectively was greater following the PM feed than after the AM feed. The reason for this is not clear and was assumed to be coincidental as the absolute concentrations of the individual acids showed a less clear differentiation between the treatments after either feeding time.

In contrast to the findings of Chapters 3 and 7, supplementation of the diet with YC did not significantly alter the diurnal pattern of propionate concentration or cause a drop in the a molar proportion of propionate. There was a trend towards increased concentration of propionate at the expense of acetate, in accordance with the findings (bent stick trend analysis, section 6.3.5) described in Chapter 6. However, the effect of supplemental YC in the current experiment on acetate production was not great enough to affect the A:P ratio which was not found to be significantly influenced by treatment ($P = 0.649$).

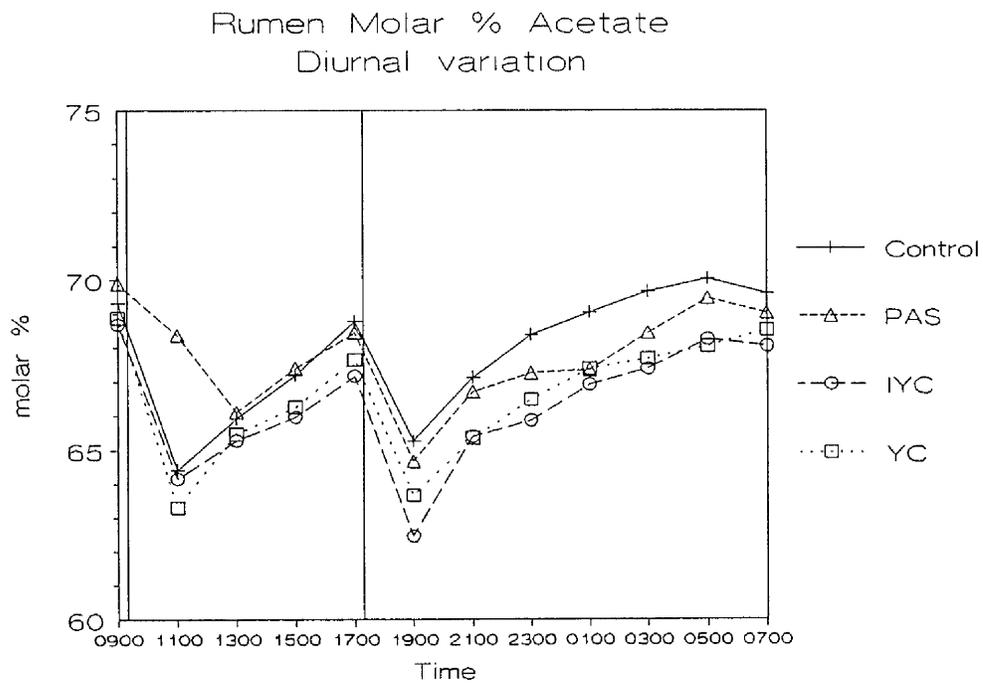
In accordance with the findings of Chapter 3, the IYC treatment tended to elevate ($P > 0.05$) the molar proportion of butyrate to higher levels than were observed for the YC treatment providing further tentative evidence to support the theory that the IYC treatment may act in a slight modified way to that of the YC treatment. Both these treatments were found to have higher absolute ruminal butyrate concentrations in comparison to the other treatments and this nearly reached statistical significance ($P = 0.105$). However, when the results are expressed as a molar proportion of t[VFA] the difference between the treatments was reduced.

Figure 9.5



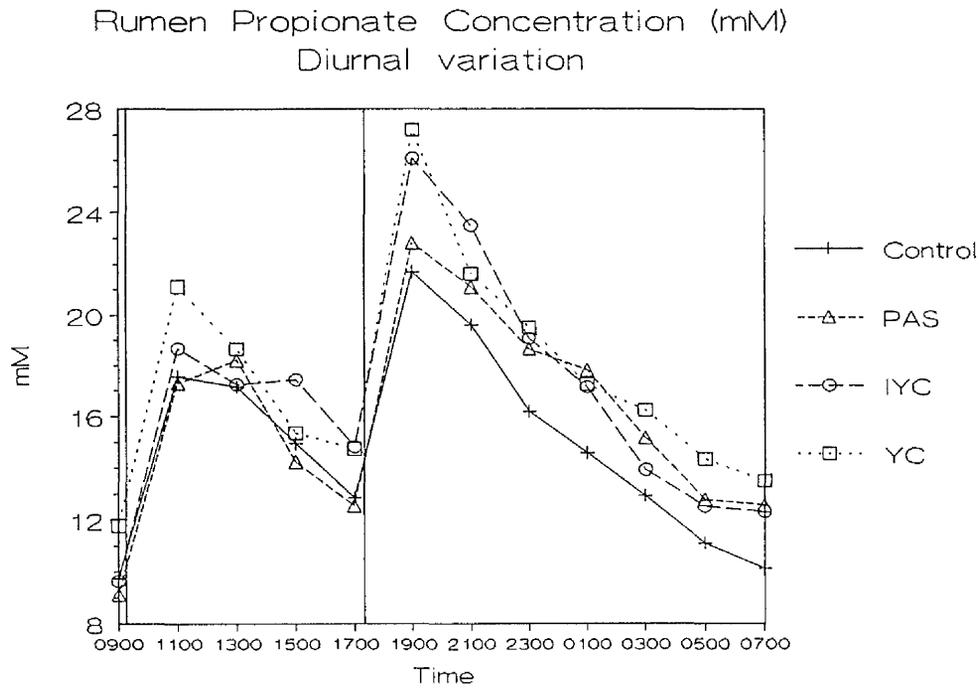
S.E.D.= 5.05

Figure 9.6



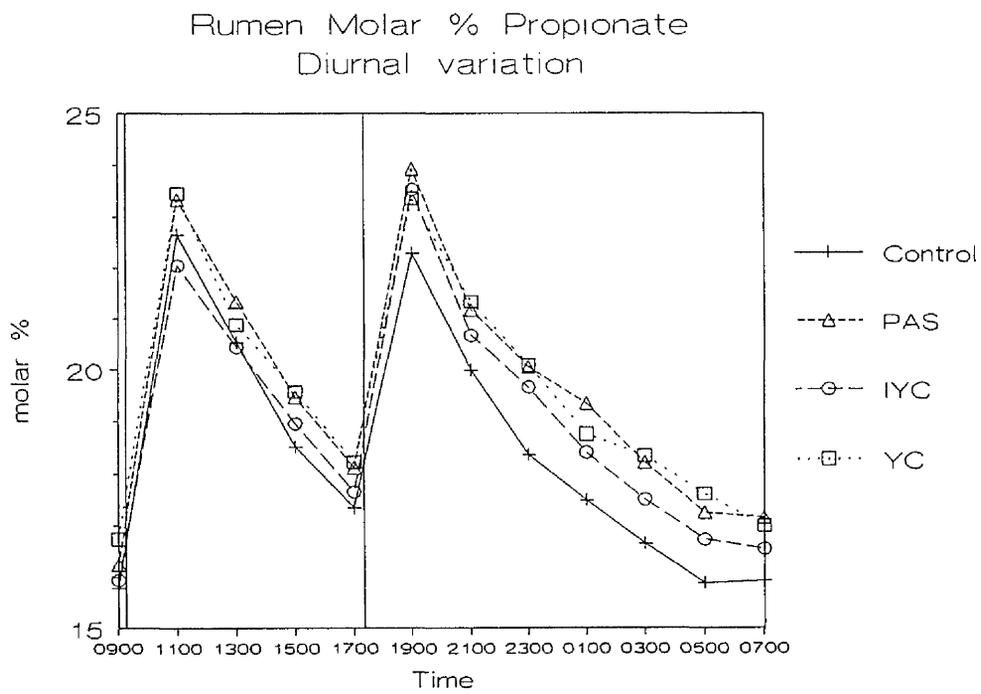
S.E.D.= 0.93

Figure 9.7



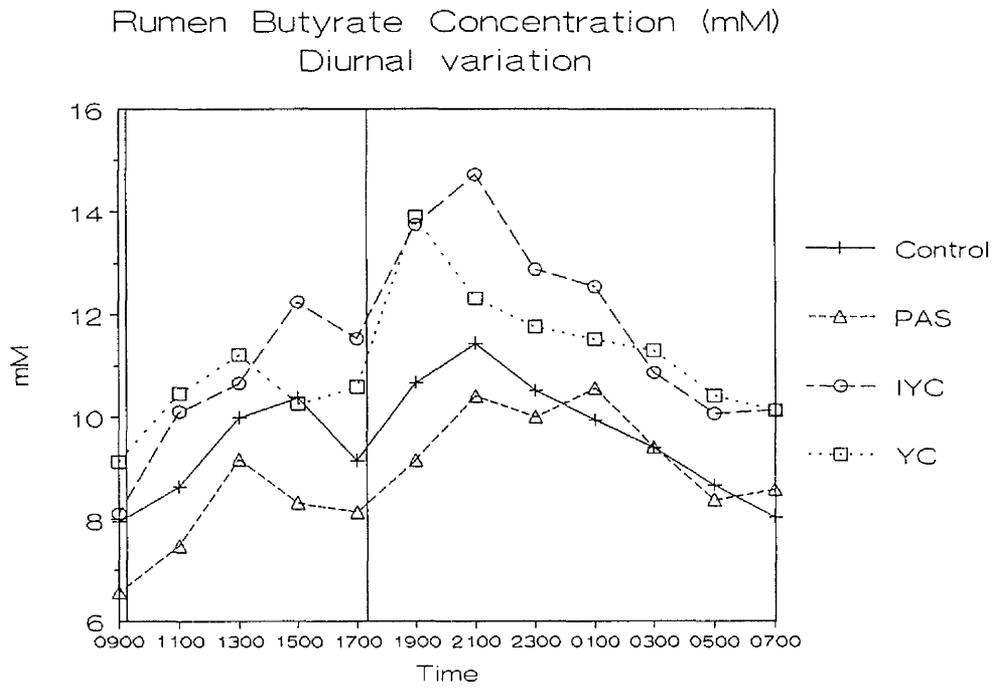
S.E.D.= 2.05

Figure 9.8



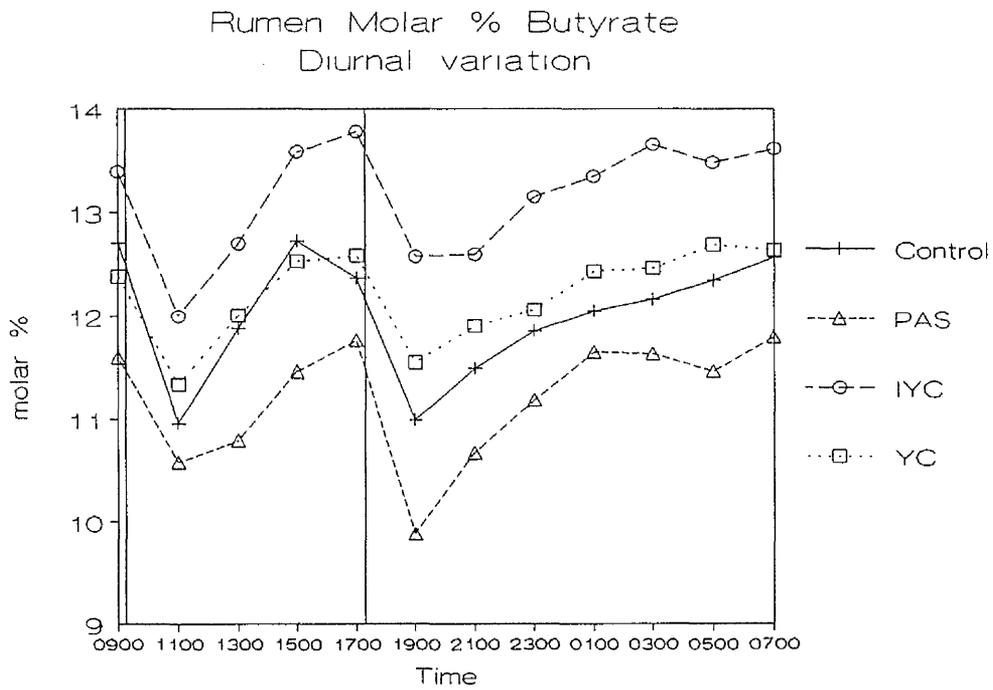
S.E.D.= 1.34

Figure 9.9



S.E.D.= 1.29

Figure 9.10



S.E.D.= 0.84

9.2.8.3: Minor VFA

Analysis of variance showed that the absolute concentrations and the molar proportions of the minor VFA were not significantly affected by treatment ($P > 0.05$). Table 9.3.14 shows the treatment means for these acids. The absolute concentration and the molar proportions of the minor VFA showed a significant ($P < 0.001$) diurnal variation. No other factors or interactions were found to be significant ($P > 0.05$).

9.3.9: The effects of treatment on ruminal ammonia concentration

Analysis of variance showed that treatment had a significant effect ($P = 0.1$) on the ruminal ammonia concentration. Treatment means are shown in Table 9.3.17. The effect of sheep and time of sampling were also found to be significant ($P = 0.029$ and $P < 0.001$ respectively).

Table 9.3.17: The effect of treatment on ruminal ammonia concentration

Control	YC	IYC	PAS	S.E.D.	F.Pr.
77.4 ^b	97.5 ^a	85.3 ^{ab}	77.9 ^b	7.31	0.1

Means in the same row not sharing a common superscript are significantly different at the stated level of statistical significance.

Table 9.3.17 shows that supplementation of the diet with YC significantly increased ($P = 0.1$) ruminal ammonia concentration. The IYC treatment led to a smaller non-significant ($P > 0.05$) increase in rumen ammonia concentration.

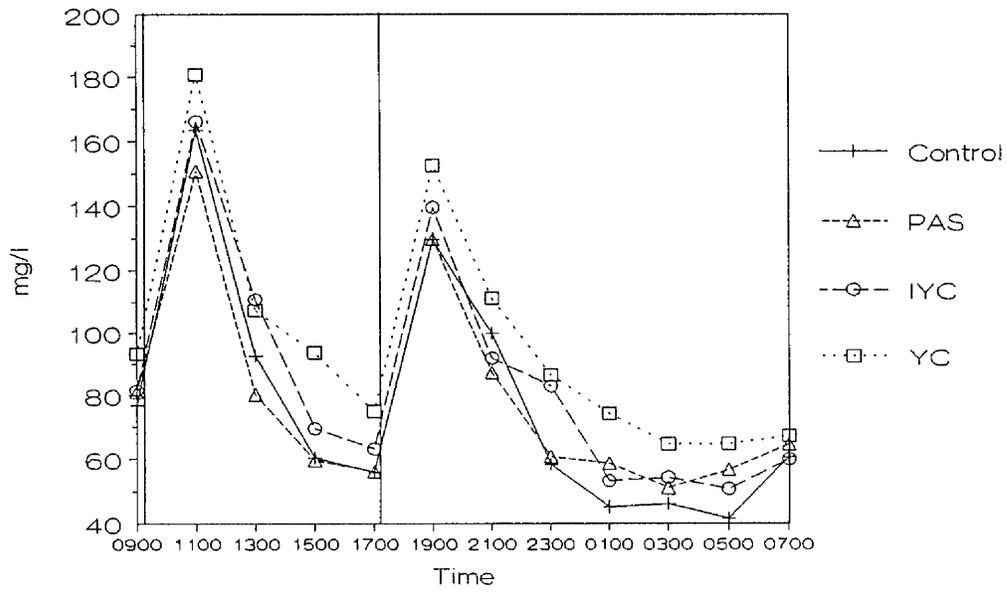
Chalmers *et al* (1976), surmised in their review concerning the passage of nitrogen through the wall of the ruminal digestive tract, that the amount of ammonia absorbed from the rumen increased with either its concentration or rumen pH. The treatment means for ruminal pH and ammonia were found to be significantly negatively correlated ($r = -0.951$; $P < 0.05$). Urinary

allantoin measurements suggested that treatment had no effect on the outflow of ruminal biomass and proteolysis. Therefore, it was assumed that the observed decrease in ruminal pH caused by an increase in t[VFA] was responsible for a reduction in the absorption of ammonia from the rumen and hence elevated the ruminal ammonia concentration.

In contrast to results reported in Chapter 7 peak rumen ammonia concentration was not found to coincide with the peak for t[VFA]. Peak ruminal ammonia concentration was observed at 2 hours post-feeding whereas, peak t[VFA] was observed between 2 and 4 hours post-feeding. However, the peak concentration of ammonia was noted to coincide with the peak concentration of propionate which was assumed to relate to the rapid fermentation of the RFC content of the diet. The t[VFA] peak was assumed to be more prolonged than the ammonia peak due to the further fermentation of the structural carbohydrates 4 hours post feeding. The ruminal ammonia concentration noted in Chapter 7 was observed to be higher when the diet was supplemented with YC and this corresponded with a non-significant reduction in ruminal pH when the diet was supplemented with YC. These findings in Chapter 7 are in accordance with the results of the current experiment and suggest that the elevation of ruminal ammonia concentration may be related to changes in rumen pH and not a direct effect of the treatment.

Figure 9.11

Rumen ammonia concentration (mg/l)
Diurnal variation



S.E.D.= 13.95

9.4: Conclusions

The results of the current experiment indicated that supplementation of the control diet with either PAS, YC or IYC did not significantly affect the rate of hay degradation *in sacco* ($P > 0.05$). Overall rumen protein degradability ($\text{ISDMD}_{\text{FISHMEAL}}$) was not affected by supplementation, however, the effects of the individual treatments were found to be opposite ($P < 0.05$). Additional PAS was noted to increase the final asymptote (b term) and decrease the rate of degradation (c term), whereas, the addition of either YC or IYC was noted to decrease the final asymptote and increase the rate of degradation. It is not clear why this supplement should affect the rumen in this way as it was not found to affect the rumen fermentation (tVFA or VFA mix) in comparison to the control diet. It may be theorised that the PAS supplement provided a stimulus to the rumen proteolytic microflora which increased the duration of their activity, but not their population density as the excretion of urinary allantoin was not found to be significantly affected by treatment ($P > 0.05$).

Urinary allantoin was unaffected by supplementation of the control diet with YC which suggests that microbial efficiency was not increased as a result of YC treatment.

Consecutive degradability curves within a period were found to vary significantly ($P < 0.05$). The S.E.D. noted for the $\text{ISDMD}_{\text{FISHMEAL}}$ was noted to be lower than that for $\text{ISDMD}_{\text{HAY}}$. This may indicate that the factors affecting the stability of rumen $\text{ISDMD}_{\text{HAY}}$ were exerting less effect on the degradation of fishmeal *in sacco*. A possible cause of the variation noted between consecutive degradability curves was assumed to result from the unequal feeding periods which resulted in a minimal rumen pH below 6.0 the threshold level at which rumen cellulolysis may be inhibited (Stewart 1977, Mould and Ørskov 1983, Mould *et al* 1983; Erfle *et al*, 1982; Lindberg, 1985).

Lindberg (1985) noted that the variation between ISDMD measurements of equivalent rumen incubation time was greatest during the period of most rapid degradation as compared to the variation observed between measurements taken at the start or finish of the degradation process. This observation may be expected as the period of most rapid degradation may be assumed to relate to the period of greatest sensitivity of the *in sacco* technique. Hopson *et al* (1963), observed greatest variation between five replicates of duplicate bags in short term incubations in the rumen, with the variation between bags lessened as incubation time increased and maximal degradation was approached (Table 9.4.1).

Table 9.4.1: Coefficients of variation between bags within time periods¹

Time periods (h)	6	12	18	24	30	36	42
C.V. %	42.4	21.4	14.53	10.08	6.78	5.6	4.63

¹ Taken from Hopson *et al* (1963)

The results above indicate that a considerable variation between replicate degradation curves may have existed and support the findings of the present experiment.

The ISDMD results of the current experiment indicate that the use of the *in sacco* technique to predict the *in vivo* cellulose and protein degradability should be carried out with a large degree of replication within an experimental period and the use of a robust experimental design, which involves a high degree of replication in order to prevent confounding effects of inherent variation in different sheep and periods.

Future use of the *in sacco* technique may improve the accuracy of the measurements by limiting the basal diets to those which do not compromise cellulolytic activity. This may be achieved by feeding frequent small meals or a by feeding diets containing low concentrations of RFC. Also, incubations shorter than 24 hours should always start at the same time relative to the time of feeding of the host animal. In addition when a twice-a-day feeding regimen is used the spacing of the meals should be 12h in order to minimise the diurnal variation of rumen pH.

The measurement of yeast viability in the rumen and hindgut indicated that the YC supplement may be present in both these sites at low concentrations for prolonged periods after supplementation has ceased. However, the lack of a significant ($P > 0.05$) treatment x period interaction indicates that affects of carry-over may not have been great as period 1 and would be expected to be different to the other periods.

Peak production of the individual VFA from the current experiment indicated that the YC and IYC supplements may have slightly different modes of action or that the microbial response time to treatment was slower for the IYC supplement. The results of Chapter 4 suggested that the microbial stimulant may be made available to the rumen when the viable yeast supplement autolyses and when the IYC supplement degrades. This difference in the availability of the possible microbial stimulant may have resulted in the observed difference between the YC and IYC treatments.

Summary of results

- 1) Rumen *in sacco* degradability of washed hay was not affected by treatment ($P > 0.05$), but significant differences ($P < 0.05$) between consecutive slopes were found to be significant within a period.
- 2) Rumen *in sacco* degradability of washed fishmeal indicated that the PAS treatment increased the final asymptote and decreased the rate of degradation, whereas, the effects of either the YC or IYC treatments were opposite. However, none of the treatments was found to be significantly affect the effective degradability of fishmeal. Consecutive degradability curves were also found to be significantly ($P < 0.05$) different within a period.
- 3) The total excretion of urinary allantoin per day was not significantly ($P > 0.05$) affected by treatment.
- 4) Rumen LDR and hindgut outflow were not significantly ($P > 0.05$) affected by treatment.
- 5) The sheep receiving the YC supplement was found to have a significantly higher rumen viable yeast count than the other sheep ($P < 0.001$). The rate of loss of viable yeast from the rumen was found to be greater than the rumen LDR.
- 6) The faecal yeast count was found to be significantly higher in the sheep receiving the YC supplement ($P < 0.05$) and was higher on the second day of supplementation in accordance with the results of Chapter 7.
- 7) Rumen pH was not significantly affected by treatment ($P > 0.05$) but the rumen pH tended to be lower when the diet was supplemented with either YC or IYC. The 24 hour diurnal variation of rumen pH confirmed the theory that ruminal pH dropped to its lowest minima post PM feeding due to the unequal feeding periods.
- 8) Ruminal tVFA concentration was found to be significantly ($P = 0.096$) increased by the YC treatment and to a lesser extent by the IYC treatment.
- 9) Concentrations or molar proportions of the individual VFA were not found to be significantly affected by treatment nor was there a significant treatment x time of sampling interaction ($P > 0.1$).
- 10) Ruminal ammonia concentration was found to be significantly ($P = 0.1$) higher when the control diet was supplemented with either YC or IYC. This was assumed to be related to the lower rumen pH levels observed on these treatments as a result of increased rumen t[VFA].

Although small responses to YC supplementation of the diet have been observed in the current experiment and previously in Chapters 3, 6, 7 and 8 the effect of additional YC on rumen function has not been consistent and has been difficult to detect due to the inherent natural variation observed in the rumen. Subsequent work aimed to establish the effect of the YC supplement in a simple *in vitro* system, where the effect of supplemental YC could easily be compared against other supplements in standard conditions.

10.1: Introduction

The development of a robust *in vivo* model for the investigation of potential rumen probiotics (Chapters 3, 6, 7 and 9) has been confounded by the inherent day-to-day variation in rumen function as assessed by changes in rumen pH, VFA and *in sacco* degradability. The results of the *in vivo* experiments indicated that the viability of the YC supplement was not necessary for it to induce small changes in the rumen fermentation pattern and protein degradation. The results of Chapter 4 indicated that the IYC supplement was not metabolically active, yet from the results reported in Chapters 3 and 9 it appeared to have an effect on rumen function. The effect of the IYC supplement on the rumen may arise from a factor within the yeast cell that stimulated either microbial growth and/or activity. An alternative experimental approach may be an *in vitro* test to determine the ability of a potential rumen probiotic to induce measurable effects on rumen microbes.

The aim of this experiment was to develop a simple *in vitro* test that would establish whether or not a potential rumen supplement could affect the growth characteristics of pure cultures of rumen bacteria. To achieve this an pure cultures of rumen bacteria were co-cultured in a clear medium with a range of potential probiotics. Bacterial growth was then determined by recording the change in optical density of the medium. n-Acetyl glucosamine was included as an additional treatment since it is one of the major constituents of yeast autolysate (Belousova *et al.* 1990).

10.2: Materials and Methods

10.2.1: Reagents

Preparation of the anaerobic medium used in the current experiment is detailed in Appendices 2 and 3.

Malt extract agar (Unipath Ltd., Basingstoke) slopes were prepared and inoculated with a 10^{-1} dilution of a viable yeast culture (*Saccharomyces cerevisiae* 1026 - Yea Sacc, Alltech Plc.) received in either 1988 (YC88; batch No. unknown), 1989 (YC89; batch No. 42911) or 1991 (YC91; batch No. 67181).

10.2.2: Procedure

Triplicate media tubes were labelled for control (bacterium only and yeast only) and bacterium plus treatment. The media tubes and treatments were then transferred to an anaerobic cabinet in which each media tube was opened and inoculated with either 0.006g N-Acetyl glucosamine (n-AG), 0.006g irradiated yeast culture (IYC; batch No. 42911) or a sterile disposable loop of yeast culture (YC88, YC89 or YC91).

The media tubes were then resealed and transferred back to the bench where they were inoculated with 0.2 ml of either the rumen bacterium *Butyrivibrio fibrisolvens* strain JW 11 or SH1, or *Ruminococcus albus* strain SY3. Bacterium and yeast only media tubes were also prepared as controls.

The optical absorbance at 560 nm for zero time was recorded using a "Spectronic 20" (Bauch and Lomb, C/O Milton Roy Co., Analytical Products Division, Rochester, NY., USA.) spectrometer adapted to accept 10 ml test tubes. The media tubes were then incubated at 39°C. Thereafter the absorbance of the media tubes was recorded at hourly intervals.

10.2.3: Interpretation, presentation and analysis of the results

The initial optical density values at zero time for each treatment was recorded and subtracted from all subsequent measurements for an individual treatment. The cumulative increase in OD values for each treatment in triplicate were then statistically analyzed using the analysis of variance option in Genstat 5.13. Subsequently, the corrected recordings for the individual co-

cultures were fitted to the Gompertz model (See Chapter 4) using the FITCURVE option available in Genstat 5.13. The curve constants derived from the curve fitting exercise were then statistically analysed by analysis of variance to determine the growth response of the bacterium to treatment. The BLOCK TREATMENT commands used to determine the degrees of freedom are shown in table 10.2.1.

Table 10.2.1: Block - treatment structure used for the analysis of the individual rumen bacteria

```
BLOCK      REPLICATETUBES
TREATMENT TREATMENT * TIME
```

Analysis of Variance

Source of variation	d.f.
replicatetubes stratum	2
replicatetubes . *Units* stratum	
treatment	5
time	8
time.treatment	40
Residual	106
Total	161

Table 10.2.2: Block - treatment structure used for the analysis of the curve constants derived from the Gompertz model

```
BLOCK      BACTERIA / REPLICATETUBES
TREATMENT BACTERIA * TREATMENT
```

Analysis of Variance

Source of variation	d.f.
bacteria. replicatetubes stratum	
bacteria	2
Residual	6
bacteria . replicatetubes . *Units* stratum	
treatment	2
bacteria . treatment	5
Residual	30
Total	53

10.3: Results and discussion

Analysis of variance showed that supplementation of the individual rumen bacterium plus growth medium with either additional viable YC (YC88, YC89 or YC91), irradiated YC (IYC89) or n-acetyl-glucosamine (n-AG) affected the mean optical density of the individual rumen bacteria significantly at the $P < 0.001$ level of statistical significance (Table 10.3.1).

Table 10.3.1: The effect of supplemental YC ('88, '89, '91), IYC or n-AG on the mean optical density (at 560nm) for the growth curves of *Butyvirbio fibrisolvens* JW11 and SH1 and *Ruminococcus albus* SY3

Rumen bacterium	Treatment						SED	FPr
	Bacterium Control	YC88	YC89	YC91	IYC89	n-AG		
JW11	0.43 ^b	0.38 ^c	0.39 ^c	0.40 ^{bc}	0.47 ^a	0.45 ^a	0.008	$P < 0.001$
SH1	0.10 ^b	0.14 ^c	0.15 ^c	0.14 ^c	0.25 ^d	0.09 ^a	0.003	$P < 0.001$
SY3	0.056 ^a	0.077 ^b	0.077 ^b	0.074 ^b	0.055 ^a	0.053 ^a	0.004	$P < 0.001$

Means not sharing a common superscript in the same row are significantly different at the stated level of statistical significance.

Table 10.3.1 suggests that the effects of the individual viable YC supplements on a single rumen bacterium were similar. The effects of the IYC and n-AG treatments were found to have a similar effects on the mean OD of the rumen bacteria *B. fibrisolvens* JW11 (JW11) and *R. albus* SY3 (SY3), but were found to have opposite effects on the mean OD for the rumen bacterium *B. fibrisolvens* SH1 (SH1). IYC supplementation of the rumen bacterium SH1 was noted to increase the mean OD of this bacterium, whilst n-AG decreased the mean OD at 560nm ($P < 0.001$) as compared to the mean OD for the bacterium only control.

Table 10.3.1 also indicates that the mean OD for the individual rumen bacteria were not affected in the same way by the individual supplements. The viable YC supplements YC88 and YC89 reduced the mean OD of JW11 significantly ($P < 0.001$). The YC91 supplement also

reduce the OD mean but this mean was not found to be significantly different from either of the other YC supplements or the control culture. Conversely, additional IYC and n-AG increased the mean OD for the growth curve of JW11 ($P < 0.001$). The mean OD for the growth curve for the rumen bacterium SH1 was increased significantly by the viable YC and IYC supplements ($P < 0.001$). However, the affect of the IYC89 supplement was much greater than that of the viable YC supplements ($P < 0.001$). Supplemental n-AG was not found to significantly affect ($P > 0.05$) the mean OD of the bacterium SH1 in comparison to the control culture. An increased mean OD was observed when the rumen bacterium SY3 was supplemented with viable YC ($P < 0.001$) but not for IYC ($P > 0.05$).

Figure 10.1 shows the change in OD values for the YC88, YC89 and YC91 only cultures over an 8 hour period. These measurements were taken in order to confirm that the differences observed in the bacterial growth curves of the individual treatments was not confounded by the growth of the viable YC in the culture medium. Figure 10.1 indicates that there was a small increase in the OD of the YC only cultures, which, when linearly regressed against time was found to be statistically significant ($P < 0.05$). However, the observed rate of increase in the mean OD of the YC only cultures was assumed not to be sufficiently large to impair comparisons between these supplements and the non viable supplements. Therefore, the increased OD for the cultures containing an isolated rumen bacterium was assumed to relate solely to an increase in bacterial growth.

The treatment x time interaction was also found to be statistically significantly for each rumen bacterium ($P < 0.01$). This suggests that the individual supplements affected the growth of each rumen bacterium differently. Figures 10.1 - 10.3 illustrate the different effects of treatment on the growth curve of the individual rumen bacteria. Further statistical analysis was carried out whereby the cumulative OD values for each replicate was fitted to the mathematical model described by Gompertz (see Chapter 5). The curve constants determine by the curve fitting were recorded and statistically analysed in order to evaluate the effect of the individual treatments on the growth curves of each rumen bacterium. Semi-log plots of log optical density against time showed a "bent stick" relationship. The first component showed growth was initially exponential, but ceased a times which varied between 4-8h for the different treatments.

A statistical analysis comparing the differences between the individual bacteria (Table 10.3.2) and the effect of supplementation of the growth curve constants derived from the Gompertz model was carried out.

Statistical analysis of the Gompertz curve constants

The final asymptote values and slopes of the mean bacterial growth curves were found to be significantly different. The time to the point of inflexion (mid-point time) and the initial OD value for the rumen bacterium JW11 were found to be significantly different ($P < 0.001$) to those of the other two rumen bacteria which had similar values for these parameters ($P > 0.05$) (Table 10.3.2). These results indicate that the rumen bacterium JW11 started from a higher initial intercept, took less time to reach the point of inflexion and had a higher final asymptote than either of the other bacteria. Whilst the rumen bacteria SH1 and SY3 were found to have similar initial intercepts and mid-time values. The rumen bacterium SH1 was observed to grow at a faster rate and have a higher final asymptote value than the rumen bacterium SY3.

Table 10.3.2: Mean growth curve parameters for the individual rumen bacteria

Curve Constant	Rumen Bacterium			S.E.D.	F.Pr.
	JW11	SH1	SY3		
Initial Value (OD)	0.048 ^a	0.007 ^b	0.009 ^b	0.002	< 0.001
Asymptote (OD)	0.66 ^a	0.26 ^b	0.15 ^c	0.014	< 0.001
Slope (OD/h)	0.90 ^a	0.15 ^b	0.09 ^c	0.008	< 0.001
Mid-Time (h)	3.2 ^a	4.9 ^b	5.0 ^b	0.17	< 0.001

Means not sharing a common superscript are significantly different at the stated level of significance.

Individual treatments were found to affect the curve constants for each rumen bacterium differently (Table 10.3.3). This was in accordance with the Time x Treatment interaction noted in the initial statistical analysis. The effect of treatment on the growth curves of the individual rumen bacteria will be discussed separately. However, these values are confounded as the co-cultures were not substrate limited.

Table 10.3.3: The effect of treatment on the growth of individual rumen bacteria.

Rumen bacterium		Treatment						S.E.D.	F.Pr.
		Control	YC88	YC89	YC91	IYC89	n-AG		
JW11	Init. (OD)	0.04	0.06	0.04	0.05	0.06	0.04	0.011	P=0.174
	Asym.(OD)	0.67 ^c	0.57 ^a	0.60 ^a	0.62 ^b	0.71 ^c	0.81 ^d	0.018	P<0.05
Slope (OD/h)		1.03 ^c	0.84 ^{ab}	0.75 ^a	0.85 ^{ab}	1.02 ^c	0.94 ^{bc}	0.074	P=0.023
Mid-point (h)		3.2 ^a	3.1 ^a	3.1 ^a	3.2 ^a	3.1 ^a	3.8 ^b	0.14	P=0.004
SH1	Init. (OD)	0.008 ^{ab}	0.05 ^{ab}	0.02 ^b	0.01 ^{ab}	0.001 ^a	-0.004 ^a	0.005	P=0.002
	Asym.(OD)	0.19 ^{ab}	0.25 ^c	0.24 ^{bc}	0.26 ^c	0.44 ^d	0.17 ^a	0.011	P<0.001
Slope (OD/h)		0.08 ^a	0.16 ^{bc}	0.18 ^c	0.11 ^{ab}	0.32 ^d	0.07 ^a	0.014	P<0.001
Mid point (h)		5.0 ^{ab}	5.0 ^{ab}	4.7 ^a	5.2 ^b	4.7 ^a	4.7 ^a	0.18	P=0.077
SY3	Init. (OD)	0.008	0.017	0.009	0.012	0.004	0.006	0.005	P=0.255
	Asym.(OD)	0.12	0.17	0.17	0.14	0.15	0.15	0.027	P=0.550
Slope (OD/h)		0.08 ^a	0.08 ^a	0.10 ^{ab}	0.13 ^b	0.07 ^a	0.10 ^a	0.015	P=0.028
Mid point (h)		5.0	5.0	4.8	4.4	5.3	5.4	0.48	P=0.372

Means in the same row not sharing a common superscript are significantly different at the stated level of significance.

10.3.1: *B. fibrisolvens* JW11

Results shown in Table 10.3.3 and Figure 10.1 show that initial OD was not significantly different for the individual treatments (P=0.174).

The final asymptote of the rumen bacterium JW11 was noted to be significantly increased (P<0.001) by the addition of n-AG to the growth medium. All three viable YC supplements were observed to reduce the final OD (P<0.05). The effect of supplementary YC91 was found to less substantial than the other two YC supplements but still reached statistical significance at the P<0.05 level. Supplemental IYC89 led to small increase in final asymptote (P<0.1)

The rate of increase in OD (Slope) was observed to be reduced significantly by all three YC supplements ($P < 0.05$) in comparison to the control mean and additional YC89 was found to have the lowest rate OD increase ($P < 0.05$). Additional n-AG was also found to reduce the mean slope of the rumen bacterium JW11, however, it was not found to be significantly different from any other treatment except the YC89 supplement ($P < 0.05$). The IYC89 supplement was not found to have a significant effect on the rate of growth of the rumen bacterium JW11 in comparison to the control ($P > 0.05$).

The time taken to reach the point of inflexion in the bacterial growth curve of JW11 was increased significantly by additional n-AG ($P = 0.004$). No other treatment was found to have an effect ($P > 0.05$).

Summary

A summary of the effects of the individual treatments in comparison are illustrated in Table 10.3.4 and are listed in full below.

YC88, YC89 and YC91

All three viable YC supplements reduced the rate of growth and lowered the final OD of JW11 ($P < 0.05$). However, additional YC was not found to alter the initial intercept or the mid-point time ($P > 0.05$).

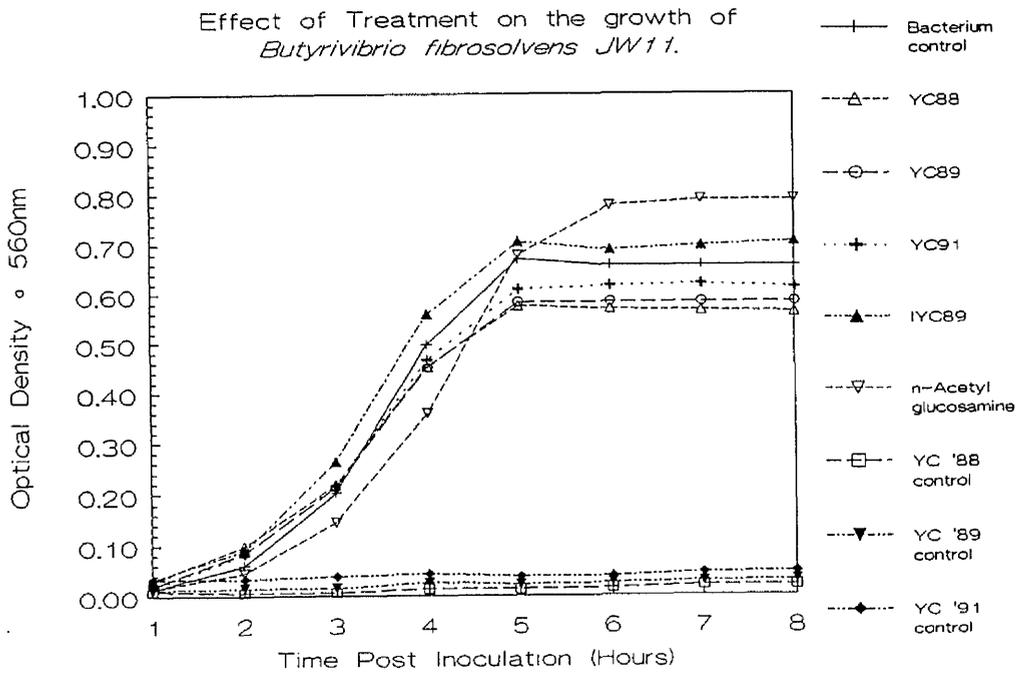
IYC89

Supplemental IYC89 was not found to significantly affect the growth pattern of JW11 ($P > 0.05$).

n-AG

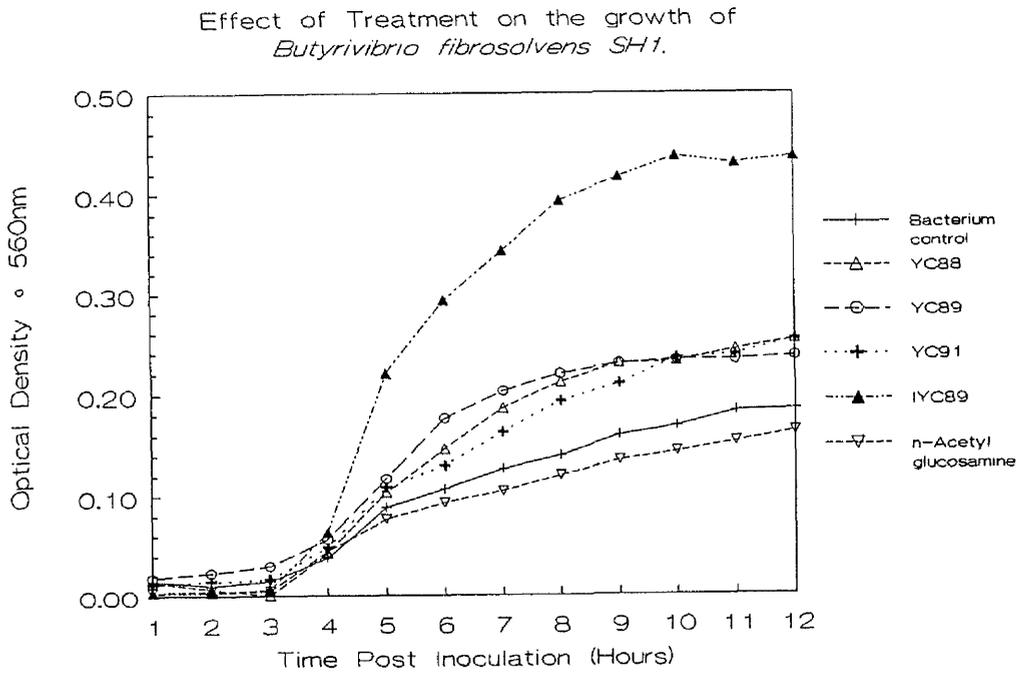
The addition of n-AG to the growth medium of JW11 was found to increase the final asymptote ($P < 0.001$) and mid-point time ($P = 0.004$), but had no affect on the initial intercept or growth rate of this organism.

Figure 10.1



SED= 0.023

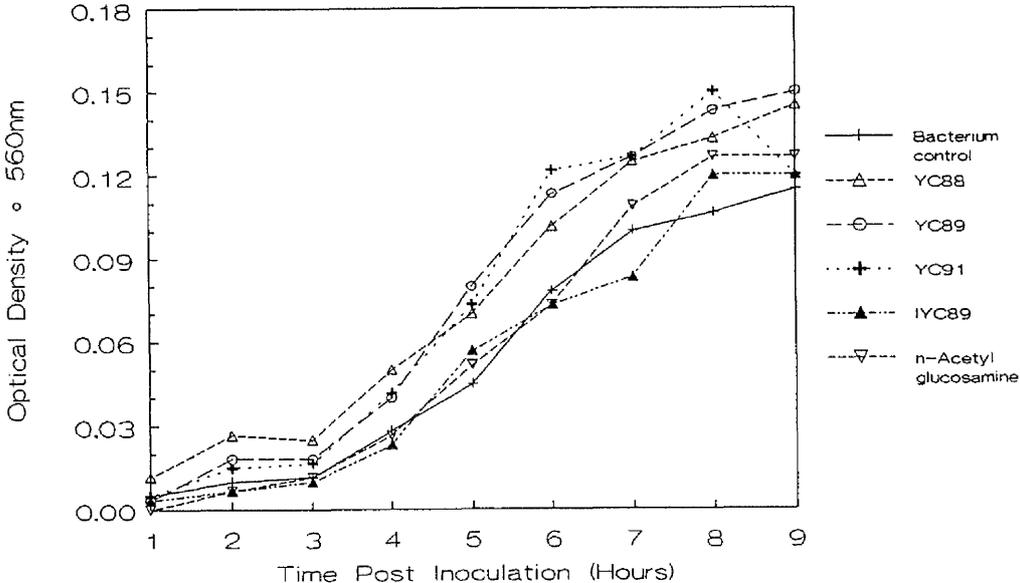
Figure 10.2



SED= 0.010

Figure 10.3

Effect of Treatment on the growth of *Ruminococcus albus* SY3.



SED= 0.011

10.3.2: *B. fibrisolvens* SH1

Results shown in Table 10.3.3 and Figure 10.2 show that in comparison to the bacterium only control culture, none of the supplements affected the initial OD value (calculated by the "FITCURVE" option in Genstat 5.13) significantly ($P > 0.05$). However, the initial mean OD of the YC89 supplement was found to be higher than both the IYC89 and n-AG initial values ($P = 0.002$). The reason for this apparent difference was not clear but was assumed to relate to the different bacterial growth pattern induced by these treatments affecting the mathematically calculated initial OD values.

Additional YC88, YC89, YC91 and IYC89 were all noted to increase the final asymptote in comparison to the bacterial control culture ($P < 0.002$). The increase in final OD induced by the IYC89 supplement was found to be significantly higher than any other treatment. Additional n-AG was not found to alter the final OD of this bacterium in comparison to the control culture.

The bacterial culture containing the IYC89 supplement was noted to have the fastest rate of increase in OD in comparison to all other treatments ($P < 0.001$). The YC88 and YC89 supplements were also noted to increase the rate of growth of the rumen bacterium SH1. The YC91 supplement was not found to be significantly different from the control ($P > 0.05$) or from the YC88 supplement at the $P < 0.001$ level of statistical significance. However, the growth rate of the bacterial culture containing the YC91 supplement was found to be significantly lower than that of the culture containing additional YC88 at the $P < 0.05$ level of statistical significance, suggesting that these two supplements (YC88 and YC91) were in fact mediating different bacterial responses. Additional n-AG was not found to affect the rate of growth of the rumen bacterium SH1 ($P > 0.05$).

The time taken to reach the point of inflexion of the control bacterial culture was not found to differ significantly from any other culture ($P > 0.05$). However, the culture containing the YC91 supplement was found to have a longer mid-point time than the cultures containing either YC89, IYC89 or n-AG ($P < 0.05$).

Summary

A summary of the effects of the individual treatments in comparison are illustrated in Table 10.3.4 and are listed in full below.

YC88

Additional YC88 was noted to increase the final OD ($P < 0.001$) and the rate of growth of SH1 but, did not alter the initial intercept or the time taken to reach the point of inflexion ($P > 0.05$).

YC89

Supplementation of SH1 with YC89 increased the rate of bacterial growth ($P < 0.001$) and final OD ($P < 0.05$), but did not affect the initial OD or the mid-point time ($P > 0.05$).

YC91

YC91 supplementation of the rumen bacterium SH1 was found to increase the final OD ($P < 0.001$) but, in contrast to the other YC supplements additional YC91 did not affect the growth rate of SH1 ($P > 0.05$), or the initial OD intercept and the time taken to reach the point of inflexion in the growth curve ($P > 0.05$).

IYC89

The addition of IYC to the growth medium was found to induce the fastest bacterial growth rate and highest final OD in comparison to all other treatments and the control bacterium culture ($P < 0.001$). The effects were concurrent with the shortest observed mid-point time ($P > 0.05$).

n-AG

The addition of the sugar n-AG to the growth medium was not found to affect the bacterial growth pattern ($P > 0.05$).

10.3.3: *Ruminococcus albus* SY3

Results shown in Table 10.3.3 and Figure 10.3 show that supplementation of the culture medium with any treatment did not affect the initial OD intercept, the final OD or the time taken to reach the point of inflexion ($P > 0.1$).

The growth rate of SY3 was found to be increased by both the YC89 and YC91 supplements but only for YC91 was the rate significantly different from the control culture ($P = 0.028$).

10.3.4: Summary

The only supplement to affect the growth of SY3 was the YC91 supplement, which was found to significantly increase the growth rate of this bacterium (Table 10.3.4).

Table 10.3.4: Summary of the effects of supplementation on the growth patterns of the individual rumen bacteria *in vitro*.

Bacterium / Parameter		Treatment				
		YC88	YC89	YC91	IYC89	n-AG
JW11	Init. (OD)	↔	↔	↔	↔	↔
	Asym.(OD)	↓	↓	↓	↑*	↑
	Slope (OD/h)	↓	↓	↓	↔	↔
	Mid-point (h)	↔	↔	↔	↔	↑
SH1	Init. (OD)	↔	↔	↔	↔	↔
	Asym.(OD)	↑	↑	↑	↑	↔
	Slope (OD/h)	↑	↑	↔	↑	↔
	Mid-point (h)	↔	↔	↔	↔	↔
SY3	Init. (OD)	↔	↔	↔	↔	↔
	Asym.(OD)	↔	↔	↔	↔	↔
	Slope (OD/h)	↔	↔	↑	↔	↔
	Mid-point (h)	↔	↔	↔	↔	↔

* Arrow indicates a statistical response at the $P < 0.1$ level of significance.

Arrows indicate the nature of the difference in response of a treatment from the bacterium only control culture ($P < 0.05$).

10.4: Conclusions

The technique proved to be simple to carry out and sensitive enough to pick up small changes in the growth of individual rumen bacteria. Statistical analysis of changes in the bacterial growth patterns using the curve fitting option in Genstat 5.13 proved a useful technique to quantify the statistical significance of the treatment x time interaction of the treatment means ($P < 0.001$; Table 10.3.1) observed for both JW11 and SH1. The treatment x time interaction for SY3 reached a statistical significance of $P = 0.151$. This was subsequently assumed to relate to the increased growth rate of SY3 induced by the additional YC91 ($P < 0.05$) [no other treatment was observed to have an effect]. The primary statistical analysis carried out on the treatment means (Table 10.3.1) indicated that all 3 YC supplements increased the mean optical density of SY3. This effect was illustrated in Figure 10.3 where it may be seen that overall the viable YC supplements had increased OD values in comparison to all other treatments. However, subsequent analyses failed to find an effect of YC88 and YC89 on the growth pattern of SY3, and it may be argued that the curve fitting procedure was a more reliable test of the effects of supplementation on bacterial growth.

Table 10.3.4 illustrates that supplemental viable YC depressed the growth of JW11 but enhanced the growth of SH1. The reasons for this apparent contradiction were not clear, but indicates bacteria-specific effects. The effect of additional YC in the JW11 cultures may have depressed the growth of this organism due to competitive antagonism between the bacterium and the additional YC for the available substrate. The converse was true for the addition of IYC and n-AG to the JW11 culture medium as competition was absent because the supplements contained no live cells but, a growth factor could have been supplied. For the rumen bacterium SH1, the additional YC may have provided a growth factor which stimulated the growth of this organism, but it is not clear why the bacterial response to the IYC89 treatment should be so great and why the addition of n-AG had no effect. However, The IYC89 supplement and YC supplements differed in two respects:

- 1) The yeast cells present in the IYC89 supplement were not viable (Chapters 1 and 9) and were not metabolically active (Chapter 4).
- (2) The IYC89 supplement also contained the basal medium on which the yeast was originally grown.

This was in contrast to the YC supplements used in this experiment as they consisted solely of viable, metabolising yeast cells. Future experiments may investigate the confounding effect of irradiation and the basal medium by supplementing an individual rumen bacterium with:

- (a) irradiated yeast cells without medium and
- (b) the original basal medium without yeast cells.

Further work may be carried out in order to elucidate the possible mechanisms of the observed responses. Bergey (1986), in his manual of systematic bacteriology, recommends that for the *in vitro* culture of most strains of *B. fibrisolvens* and *R. albus* a chemically defined medium should contain; glucose or cellobiose as the major source of energy plus an amino acid mixture and an ammonium salt as the nitrogen source. The medium should also contain minerals, B-vitamins, cysteine and a solution of VFA containing acetate, propionate, or branched chain volatile acids. The medium used in the current experiment contained a wide range of essential amino acids (Table 10.4.1), vitamins and soluble carbohydrates plus a solution VFA and a solution of an ammonium salt, (Appendices 1 and 2 provide a full list of the medium constituents). The current medium may therefore be considered to contain the requisite mixture constituents required for the growth of the test ruminal organisms used in the present experiment. Subsequent experiments may utilise an altered medium which is deficient in one nutrient. Supplementation of the deficient medium with a desired additive may indicate if the additive contains the missing nutrient and therefore establish a possible mode of action of the additive. Alternatively a complete medium supplemented with purified extracts of the sample additive may be used and ultimately the combination of a deficient medium and purified extracts would confirm the mode of action of a rumen additive.

Growth curves of the bacterial culture containing the viable YC supplements were confounded by the growth of the yeast in the medium and a possible interaction between the bacterium and the yeast. Future work may investigate the effect of additional yeast cells on the growth of the bacterium and the effect of the bacterium on the growth of the yeast. This may be achieved by culturing a single bacterium with a given viable supplement with sufficient replication so that at regular intervals during the growth of the rumen bacterium a number of replicates may be opened (anaerobically) to allow bacterium and yeast counts to be carried out.

The YC88 and YC89 supplements were found to have similar effects on the growth rates of the individual rumen bacteria. However, The bacterial cultures containing additional YC91 were found to differ in their to YC supplementation as compared to the responses observed for

the other YC supplements. It may be theorised that the YC88 and YC89 treatments failed to induce a response when added to the SY3 culture (in contrast to the YC91 supplement), possibly due to their greater age and as the YC88 and YC89 supplements were in fact beyond their shelf life dates. However, it was assumed unlikely that the YC88 and YC89 additives were beyond their active life span due to:

- a) All 3 yeast cultures had shown a limited ability to grow in the culture medium
- b) YC88 and YC89 supplements elicited an effect on the growth pattern of SH1, whereas the YC91 treatment failed in this respect.
- c) All 3 YC supplements induced similar responses on the growth pattern of JW11.

It may therefore be theorised that the differences that exist in the bacterial responses are due to changes in the manufacturing procedure of the YC product (Yea Sacc 1026 - Alltech Plc).

Table 10.4.1: Amino acid composition of rumen bacteria and "Tryptone"

Amino Acid	Rumen Bacteria ^a	Tryptone ^b
Arginine	5.2	3.1
Histidine	2.1	2.1
Isoleucine	5.7	3.8
Leucine	7.6	6.9
Lysine	8.5	7.9
Methionine	2.4	1.8
Cystine	1.2	0.3
Phenylalanine	4.9	3.6
Tyrosine	4.4	1.4
Threonine	5.4	2.3
Valine	6.0	5.1
Tryptophan	1.3	1.0
Alanine	7.1	2.6
Aspartic acid	11.2	6.8
Glutamine	12.6	17.3
Glycine	5.5	1.8
Proline	3.5	8.5
Serine	4.1	1.7

^a Adapted from Ørskov, 1982

^b Adapted from Oxoid manual

Each supplement was found to have different effect on an individual rumen bacteria. An increase in the rate of growth and final OD of a cellulosic bacterium may be reflected in the rumen by an increase in fibre digestion. Whereas, the converse may be true if a depression in the rate of growth and final OD were observed *in vitro*. The variation in response of the individual rumen bacteria to a single treatment may help explain the variable rumen responses noted when a few grams of YC (viable or non-viable) are added to the diet of the animals. Subsequent experiments may include the use of a wider range bacterial species and include a range of protozoal and fungal species in order to evaluate their response to treatment and therefore achieve a greater understanding of the YC mode of action, making the prediction of a response to YC supplementation more possible.

Interactions between rumen bacteria *in vivo* may also affect the way in which a rumen bacterium may respond to a given supplement therefore the use of simple *in vitro* experiments such as the one used in the current experiment have a limited use in the determination of a ruminal response to supplementation with potential rumen probiotics.

CHAPTER 11: General discussion

11.1: The effect of supplementary YC on rumen metabolism

In order for an experimental treatment to be considered to be having a statistically significant effect, the treatment must not only show a consistent effect within an experiment but also between replicate experiments. A robust experiment will highlight small changes induced by a treatment, which within an experiment may be found to be statistically significant, but if the effect of the treatment is found to vary between different experiments the statistical significance of the effect is weakened (Cochran and Cox, 1957).

The effects of supplemental YC on rumen parameters described in Chapters 3,6,7 and 9 are summarised in Table 11.1.1.

The diurnal pattern of molar % and concentration of propionate in Chapter 3 were altered significantly when the control diet was supplemented with YC and IYC resulting in a reduction of propionate post-feeding in comparison to the control treatment. However, the mean concentration and molar proportion of propionate were not significantly affected by either the YC or IYC supplements. The effect of supplemental YC in Chapter 7 was found to induce a similar effect to those observed in Chapter 3 on propionate concentration but in this experiment (Chapter 7) mean concentration and the molar proportion were reduced and in contrast to Chapter 3 there was no significant decrease in the mean concentration of propionate, however a decrease in peak levels of propionate was observed and corresponded to a significant treatment-samptime interaction. The acetate : propionate (A:P) ratio showed a small non-significant increase when the basal diet was supplemented with YC in Chapters 3 and 7. A similar shift in the A:P ratio was observed by Gray and Ryan (1989) who whilst working *in vitro* found a non significant increase in the A:P ratio when they fed hay plus 0.5 gYC/l rumen fluid. However, the increased A:P ratio noted in Chapters 3 and 7 was found to result from a decrease in propionate concentration whereas, the concentration of propionate in the culture experiment of Gray and Ryan (1989) was not found to be affected by additional YC. Molony (1989) compared the effect of YC on 2 contrasting diets and noted a non-significant increase in the production of acetate and no effect on the production of propionate in accordance with Gray and Ryan (1989). Reduced propionate levels were observed in both chapters 3 and 7. These results and are in contrast to the findings of Gray and Ryan (1989) and Molony (1989), although the overall effect on the A:P ratio was similar. Williams (1989) in his review

Table 11.1.1: Summary table showing the effects of supplemental YC on rumen parameters from the experiment Described in Chapters 3, 6, 7 and 9.

Chapter	Parameter											
	OMD	IVOMD	ISDMD	LDR	Allantoin	Ammonia	pH	tVFA	pVFA	Ethanol	Cellulolytic activity	Other
3	↔	↔	↔		↔ YC ↓ IYC	↔	↔	↔	↔	↔	↔	YC and IYC affected Pr & ethanol treatment x samptime significantly (↓ Pr & ↑ ethanol post feeding cf. control).
6	↔		↔		↔	↔	↔	↔	↔			Detection of treatment effects was made difficult by confounding effects of period. Trend analysis suggested additional YC ↑ Pr %.
7			↔	↔		↔	↔	↔	↔	↔		No diet x YC interaction was observed.
9			↔ Hay ↓ Fishmeal (YC 24 hr.)	↔ Rumen	↔	↑ YC	↔	↑ YC ↑ IYC ↔ Pas	↔			YC ↑ hindgut LDR

↑ Significant increase (P<0.1); ↓ significant decrease (P<0.1); ↔ no effect

discussing the biochemical mode of action of YC, presented experimental evidence that showed an increase in the A:P ratio due to small increases in acetate production and decreases in the production of propionate. The evidence presented by Williams (1989) would tend to support the findings of Chapters 3 and 7 but responses to supplementary YC observed by other authors (Table 1.5.1) highlights the variability in the rumen fermentation response to additional YC. Table 1.5.1 shows that supplemental YC may induce a decrease in the A:P ratio (Harrison, 1987a,b, 1988, Martin *et al*, 1989, and Williams *et al* 1991). These authors found a decrease in the A:P ratio due to an increased propionate production and their findings are supported by other similar non-significant responses (Table 1.5.1).

In contrast to the results of Chapters 3 and 7, the effect of supplemental YC on VFA concentration in Chapter 9 was to induce a small increase in the concentrations of acetate, butyrate and propionate resulting in a significant increase in the tVFA concentration. This is in accord with the findings of Arambel *et al* (1987), Edwards *et al* (1991a,b) and Gray and Ryan (1990a,b). In contrast, Dawson *et al* (1990) and Harrison *et al* (1988) found that the addition of YC to a basal diet significantly decreased ($P < 0.05$) the ruminal t[VFA].

Overall, supplementary YC was found to have no consistent effect on the absolute or relative concentrations of the fermentation end products (Table 11.1.1). Therefore, it is not possible to be able to predict the effect of supplementary YC on the rumen fermentation processes.

Rumen *in sacco* degradation of soya hulls (Chapters 3 and 6), ACW (Chapter 7), or hay (Chapters 7 and 9) was not found to be significantly affected by supplemental YC. This is in contrast to the findings of Williams *et al* (1991) and Chademana and Offer (1990), who observed a significant increase in the short term degradation of hay (<24h). However, the results described in this thesis are in accordance with the findings of Huhtanen (1991) who observed no effect due to additional YC on ISDMD of hay. The statistical significance for the results presented by Williams *et al* (1991) who noted a significant ($P < 0.05$) increase in 12h hay ISDMD, is weak due to the lack of a final control period in the switchback experimental design and therefore confounded by time.

Microbial efficiency (gMN/kgDOM) was not significantly affected by the addition of YC to the basal diet (Chapters 3 and 6). The excretion of urinary allantoin in Chapter 9 was also not affected by the supplemental YC. These results are in contrast to the findings of Edwards *et al* (1991a) who observed an increase ($P > 0.05$) in urinary allantoin excretion when YC was added to the silage based diet of steers. In accordance with the findings of Edwards *et al*

(1991a) an increase in rumen microbial protein was observed by Gomez-Alarcon *et al* (1987) and an increase in the total anaerobic microbial count was observed by Harrison *et al* (1987a, 1988; $P > 0.05$) and Wiedmier *et al* (1987; $P < 0.05$). An increased microbial protein concentration resulting from elevated bacterial growth observed when additional YC was fed would cause increased urinary allantoin excretion only if the outflow of digesta from the rumen was not affected by the additional YC treatment. The present experiments suggest that this may be the case since supplemental YC has not been found to have a significant effect on rumen LDR (Table 1.5.1 and Table 11.1.1). Increases in the individual populations of the rumen microbes (Arambel and Wiedmier (1986), Dawson *et al* (1990) and Harrison *et al* (1987a, 1988) would not necessarily be expected to increase the flow of microbial N from the rumen since the rise of one population may be counteracted by a fall in the density of another population.

Ruminal ammonia concentration was not affected significantly ($P > 0.1$) by supplemental YC (Chapters 3 and 7) but increased ammonia concentrations ($P = 0.1$) were observed in Chapter 9 when the control diet was supplemented with YC. This is in contrast to the findings of Chademana and Offer (1990), who observed a NS decrease in ruminal ammonia concentration when the basal diet was supplemented with YC. The results discussed in Chapters 3 and 7 support the findings of Huhtanen (1991), since he noted that supplemental YC had no effect on ruminal ammonia concentration or total N flow through the digestive tract. The slight significant ($P = 0.1$) increase in ruminal ammonia concentration observed in Chapter 9 was assumed to relate to reduced ammonia absorption observed when rumen pH is depressed (Chalmers *et al* 1976) and not due to the direct effects of the additional YC. This theory is supported by the findings of Arambel *et al* (1987) who observed a corresponding increase in ruminal ammonia concentration when t[VFA] increased and Dawson *et al* (1990), who observed a significant ($P < 0.05$) decrease in ruminal ammonia concentration associated with a drop in ruminal t[VFA].

The IYC treatment was found to have a similar but lesser effect to that of the YC treatment where ruminal effects were observed (Chapters 3 and 9) which are in accord with the findings of Newbold *et al* (1991) who found similar results when they compared the effects of irradiation and autoclaving of *A. oryzae* on its ability to exert an effect *in vitro*. They concluded that irradiating the fungal supplement impaired but did not inhibit completely its ability to induce an effect whereas no response to the autoclaved supplement was observed, suggesting that the causal factor was heat labile. The PAS treatment (Chapter 9) did not induce similar effects to YC and IYC supplements (Chapter 9). The reasons for this are not clear but indicate

that the yeast cells need to be administered intact, the causal factor is heat labile (yeast is heat treated during PAS manufacture) and/or that the yeast species used in the distillery industry is not capable of inducing the same response as that used for the manufacture of the YC product. Another possible reason why the PAS treatment failed to show similar responses to that of either the YC or IYC treatments may be because of the copper contained in the PAS supplement (approx. = 60 mg/kg) which may induce anti-microbial properties and therefore exert a different mode of action to that of the YC and IYC supplements. The similarity of the effects exerted by the YC and IYC supplements may also have been because of the inclusion in both supplements of the basal medium on which the yeast was originally grown. It may be theorised that the basal medium has the major effect and the effect of the YC cells is less strong. This is supported by the results of Chapter 10 where the effects of the viable yeast culture supplements without the basal medium were found to be different from that exerted by the IYC supplement where the non-viable yeast was still mixed with the basal medium. Future work must establish the effect of the isolated basal medium on the growth of isolated rumen micro-organisms and if an effect is observed further experiments carried out to note the effect of the basal medium on rumen fermentation. The results described in Chapter 10 also indicate that the yeast autolysate product n-acetyl glucosamine induced similar effects on the growth pattern of the rumen bacterium JW11 but not SH1 or SY3. It may therefore be theorised that there is more than one causal factor inducing a multitude of bacteria-specific effects.

The viable YC supplement was found to be able to survive in the rumen and pass throughout the whole digestive tract. This is in accordance with the findings of Newbold *et al* (1990). The viable faecal yeast count was also found to be higher on the second day of supplementation ($P < 0.05$) than after a week or 3 weeks of supplementation. The reasons for this are not clear but may be related to the addition of fresh YC to the rumen which stimulated the autolysis of the YC present. Alternatively it may be theorised that the products of autolysis promoting further autolysis until an equilibrium had been established. It was considered to be coincidental that Ryan and Gray (1989) found an increased t[VFA] response after 2 days of supplementation as compared to that after a week. This theory was based on the evidence that supplementation with IYC exerts similar ruminal effects to the YC supplement *in vivo*, and since the IYC supplement is reproductively inactive its ruminal concentration would decline at a rate equivalent to LDR. Furthermore, Ryan and Gray (1989), observed that semi-clarification of the rumen before placing it in the culture vessels did not remove residual sugars and fine particles which the rumen microbes and the viable yeast culture could utilise for the production of VFA. They suggested that no further explanation was required to explain the higher tVFA

concentration observed after 2 days of incubation as compared to that after 3 weeks of experimentation.

Rumen pH was found to have a substantial effect on rumen *in sacco* degradation (Chapters 7,8 and 9) and on the absorption of ammonia from the rumen (Chapter 9) as found by Chalmers *et al* 1976. Williams *et al* (1991) found that the response to supplementary YC was greatest when cellulolysis was compromised by low rumen pH levels. However, the results from Chapters 3 and 6 do not support the findings of Williams *et al* (1991). The results of these Chapters plus those of 7 and 9 are in accordance with the findings of Molony (1987) and Chademana and Offer (1990) who observed no interaction between the level of concentrate and the response to supplementary YC. Great care in subsequent work should be taken to ensure that rumen pH levels below 6.0-6.1 are not induced. This would prevent the effects of supplementation being confounded by the effect of low rumen pH on the absorption of the individual VFA and ammonia or its negative associative effects on cellulolysis.

Overall supplementary YC did not affect ($P > 0.1$) rumen microbial efficiency, the overall OMD, rumen pH and the *in sacco* degradation of hay, ACW or soya hulls. Small but statistically significant results ($P < 0.1$) on the VFA mix (Chapters 3 and 7) and t[VFA] (Chapter 9) were noted but these results were not found to be consistent in every experiment and therefore the mode of action of YC in the rumen was not clearly defined. Supplementary YC was also found to reduce the degradation of fishmeal at 24h (Chapter 9) but effective degradability was only found to be affected significantly at long rumen retention times ($P < 0.05$). Williams *et al* (1990) found a greater flow of NAN to the hindgut ($P > 0.05$) but related this to an increased microbial efficiency. The effect of supplementary YC on the ruminal degradation of protein has not yet been established and the results of subsequent experiments may prove to be as contradictory as those found for other rumen parameters. It may be theorised that the effect of additional YC on 24 hr. fishmeal degradation was the result of specific bacterial stimulation or inhibition induced by a causal factor or factors arising from the YC supplement.

Rumen fermentation and the degradation of a standard substrate was found to vary (Chapter 8) substantially between consecutive days in an apparently random manner. This makes the detection of small ruminal changes in response to the inclusion of a rumen stimulant practically impossible and may help explain the observed variation in response to supplementary YC (Table 1.5.1). The response of isolated rumen bacteria to the viable YC supplements (Chapter 10) indicated that YC affects different bacteria in different ways and therefore the response to

the YC supplement may vary according to the relative population strengths at the commencement of supplementation (Wilson and Briggs 1955). The YC supplement may have more than one mode of action depending on: diet type, rumen bacterial population strengths, host-rumen interactions and host behaviour in accordance with the sequential model proposed in Chapter one.

11.2: Inherent variation between consecutive days

The ability of the rumen to degrade a standard substrate was found to vary substantially between consecutive days (Chapters 3, 6, 7 and 8). These variations were unlikely to be caused by systematic experimental error such as errors in drying since the variations between sheep were not found to be synchronised. Also similar variations were observed in the rumen parameters of pH, VFA, OMD and microbial efficiency (Chapters 6,7 and 8). This is accordance with Wilson and Briggs (1955), who found that the rumen microbial population varied within a defined range and that this range was relatively wide and was considered to reflect the dynamic equilibrium prevailing in the rumen. They went on to discuss the importance of establishing a normal range of variation in a single animal on a fixed diet, since previous authors have regarded small overall changes within the range of the "normal" variation to be indicative of significant major changes in the rumen flora. Czerkawski (1980), also observed periods of unexplainable instability in his *in vitro* experiments and found that this instability occurred in all donor animals. He concluded that the periods of instability were widespread and may explain some of the variability observed in long-term animal experiments. The results of Chapter 8 indicated that not only was the "normal" variation within a sheep fed a fixed diet substantial but also that the inherent variations depended on diet, thus supporting the need to clarify the effect of diet type on the inherent variation within an animal. However, the results described in Chapter 8 showed that the variability observed in rumen function between consecutive days could be categorised into 3 main types: 1) short-term noise; 2) medium-term stability; 3) long-term trends. Analytical error, housing stress, temperature and psychological stress were suggested to be the most likely source of the observed variations.

Table 11.2.1 shows the number of bags that are required in order to achieve confidence limits of less than 5% at the $P < 0.05$ level of statistical significance, using the equation described by Mehrez and Ørskov (1977) († see below). A comparison has been made between the number of bags required to achieve $< 5\%$ confidence limits using the variations between bags incubated in the same rumen on the same day, bags incubated in the same rumen on different days and the variation between bags incubated in different rumen on the same day observed by Mehrez and Ørskov (1977), Lindberg (1985) and those observed in Chapter 8.

$$\dagger \quad \text{Variance of the mean (V}_m\text{)} = \frac{V_B + (b * V_D) + (b * d * V_s)}{b * d * s}$$

Where:

b = number of replicate bags V_B = the % variation of the mean of b
s = number of replicate sheep V_S = the % variation of the mean of s
d = number of replicate days V_D = the % variation of the mean of d

$$\ddagger \quad \text{Confidence limits} = \sqrt{V_m} * 1.96$$

Table 11.2.1: The number of *in sacco* bags required to maintain confidence limits below 5% at the $P < 0.05$ level of statistical significance, for the variation between bags, days and sheep observed by Mehrez and Ørskov (1977), Lindberg (1985) and those noted in Chapter 8.

No. of days	No. of sheep	No. of bags required			
		Mehrez and Ørskov (1977)	Lindberg (1985)	Hay	Mix
3	1	> 100	> 100	8	> 100
1	2	2	2	10	> 100
2	2	1	1	1	6
3	2	1	1	1	2
1	3	1	1	3	> 100
2	3	1	1	1	1
1	4	1	1	1	2
1	5	1	1	1	1

Table 11.2.1 shows that the number of bags required to fit confidence limits of < 5% to mean ISDMD values are substantially affected by the number of replicate days, sheep, bags and diet type.

Calculation of the confidence limits for the variances (V_B , V_D and V_S) noted by Mehrez and Ørskov (1977) and Lindberg (1985) generally required fewer nylon bags to achieve the required accuracy than when the calculation was made using the variances observed in Chapter 8. Mehrez and Ørskov (1977), concluded that the use of one bag, 2 days and 3 sheep could be a reasonable combination which could be used without serious loss of precision. It may be noted from Table 11.2.1 that variations observed in Chapter 8 concur with the conclusion of Mehrez and Ørskov (1977). However, when less sheep are used more bags and days are required to achieve the same accuracy using the variances of Chapter 8, in comparison to those of Mehrez and Ørskov (1977) or Lindberg (1985).

It may also be observed from Table 11.2.1 that considerably more bags are required to maintain confidence limits below 5% when a mixed barley, maize, hay diet is fed to sheep than when only hay was fed. This observation necessitates great care to be taken when designing experiments involving the *in sacco* technique, since it may be theorised that more bags, days and/or sheep will be required to maintain accuracy as the diet concentrate : forage ratio increases.

Considerable variation was also observed between consecutive ISDMD degradability slope (hay) and curves (fish-meal) (Chapter 9). The reason for this variation was not clear, however, as it was found to be impracticable to place more than 6 bags in the sheep rumen at any one time, consequently bags for each time period were incubated in the rumen independently. It was theorised that the variation in consecutive slopes and curves related to day-to-day variation and that the unequal feeding periods also contributed to the variation between consecutive slopes and curves.

Marinucci *et al* (1992) have shown that another possible source of variation in ISDMD may relate to a different micro-environments existing inside each nylon bag as compared to ruminal environment outside the bag. Future research may investigate methods of minimising and/or standardising these differences with the aim of improving the accuracy of the *in sacco* technique.

Cusum analysis of the daily variation observed (Chapters 3, 6, 7 and 8) indicates that the variation between consecutive days was not found to be periodic (Figures 3.1, 6.3, 7.1-7.12 and 8.1-8.8) but occasional periods when large consecutive changes in hay digestibility *in sacco* interspersed by periods of random oscillations were observed. The cusum means relating to periods of apparent stability were found to vary in duration and the variation of values within

a cusum mean was also found to be substantial. It was postulated that the unequal diurnal feeding periods (meals at 09:00 and 17:00) were causing rumen pH fluctuations which inhibited cellulolysis, and that these periods of inhibition varied in length and therefore contributed to the observed variation between consecutive days. This theory was confirmed (Chapter 8) for the daily variation of $ISDMD_{HAY}$ and rumen pH. However, a similar pattern of variation was found to exist for the all-hay diet for which diurnal changes in pH were small.

The CV% mean between consecutive days, with individual sheep for $ISDMD_{HAY}$ on the all hay diet was found to be 9.2 % and t[VFA] 27.9 %.

Table 11.2.2: Linear regression analysis ($y = a + b*x$) of the relationship between t[VFA]mM for consecutive days and where the daily results have been randomised for each sheep.

Sheep	a term [§]	b term [⊗]	R ²	F.Pr.
A	24.0	0.47	21.8	<0.001
A randomised			0.0	0.435
B	18.2	0.62	37.6	<0.001
B randomised			0.0	0.510
C	23.6	0.48	23.2	<0.001
C randomised			0.0	0.568
D	28.3	0.40	15.3	<0.001
D randomised			0.0	0.655
E	25.5	0.49	23.0	<0.001
E randomised			0.7	0.176
F	25.6	0.40	15.1	<0.001
F randomised			0.0	0.887
Hay all sheep	21.2	0.54	28.9	<0.001
Hay all sheep randomised			0.0	0.899
Mixed all sheep	24.3	0.48	22.3	<0.001
Mixed all sheep randomised			0.0	0.503

§ a term: y intercept

⊗ b term: slope gradient

Figures 11.1 and 11.3 show the relationship between rumen t[VFA] levels found on consecutive days (Chapter 8) and Figure 11.2 and 11.4 shows the relationship between consecutive values when the different daily values have been randomised, so that consecutive values have no sequential relationship. The regression analysis for these figures and the other sheep (Table 11.2.2) indicates that a significant ($P < 0.001$) relationship between consecutive days exists. Whereas no such relationship was observed where the days had been randomised. This analysis suggests that the variation between consecutive days is not random. That is when a high value is observed there is an increased chance of a high the following day. Figures 11.1 and 11.3 which show the plot of consecutive daily values (n) against the same values lagged by one day ($n+1$). Using this plot it may be theorised that if there was little variation between consecutive days all the points lie in centre. However, it is evident from Figures 11.1 and 11.3 that a relationship between consecutive days exists, as several points (< 4) may be seen in close proximity and that the line between consecutive points may cross to either extreme of the plot very rapidly. Table 11.2.2 shows the linear regression analysis for n against $n+1$ for the consecutive and randomised values of t[VFA].

The auto-correlation function coefficients which are calculated from the correlations between days at different lag times are shown for Sheep A and D (Table 11.2.3).

Table 11.2.3 below suggests that the consecutive days are significantly correlated to each other up to 4 days apart ($P < 0.05$), thereafter the relationship fails to reach statistical significance. Table 11.2.3 also indicates that the variation between consecutive days is not periodic since the statistical significance of the correlations tends to gradually decrease and no subsequent significant correlations were found at periodic intervals.

The variation between consecutive days has therefore been shown not to be periodic or random. May (1987), in his review of chaos and the dynamics of biological populations suggests that dynamical behaviour could be generated by simple deterministic equations that describe the behaviour of single populations with discrete non-overlapping generations (first-order difference equations with one critical point), single populations with discrete but overlapping generations (higher-order difference equations), single populations with continuous growth where regulatory effects contain time lags (time delayed differential equations) and for two or more interacting populations. The rumen may be considered to contain more than two populations interacting in an undefined number of ways. Therefore, it would not be unreasonable to assume that dynamical (chaotic) rhythms may exist in the rumen and that these rhythms may be observed indirectly via the production of t[VFA] and the rumen parameters measured in Chapters 3, 6,

Figure 11.1

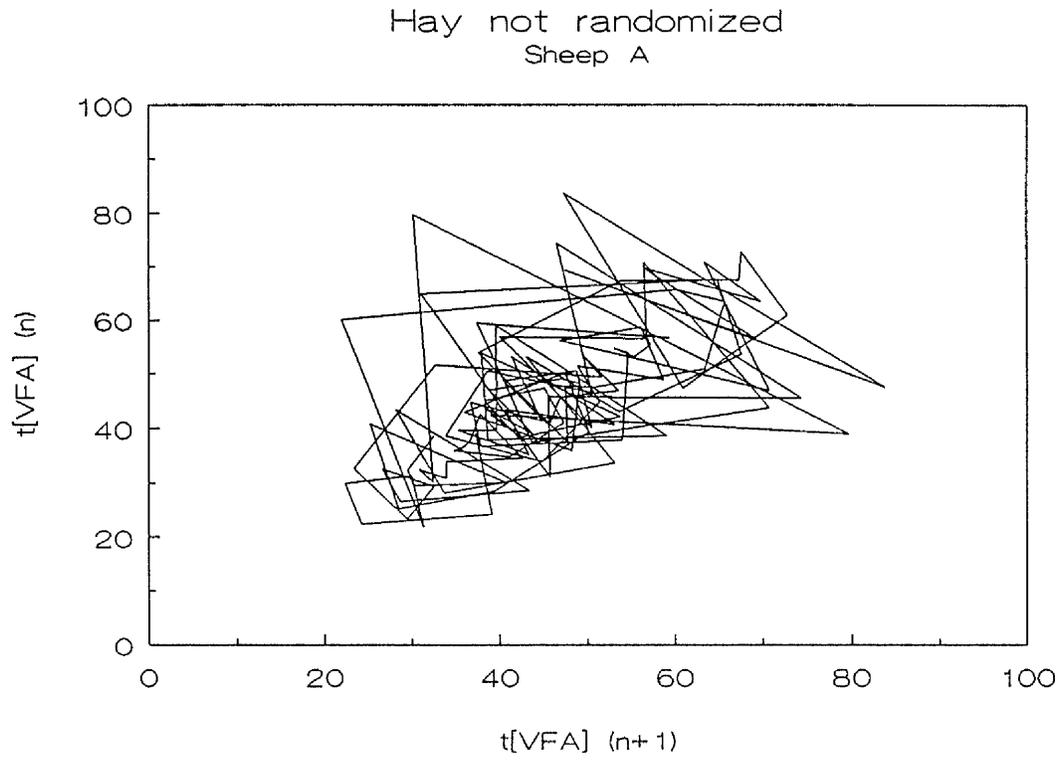


Figure 11.2

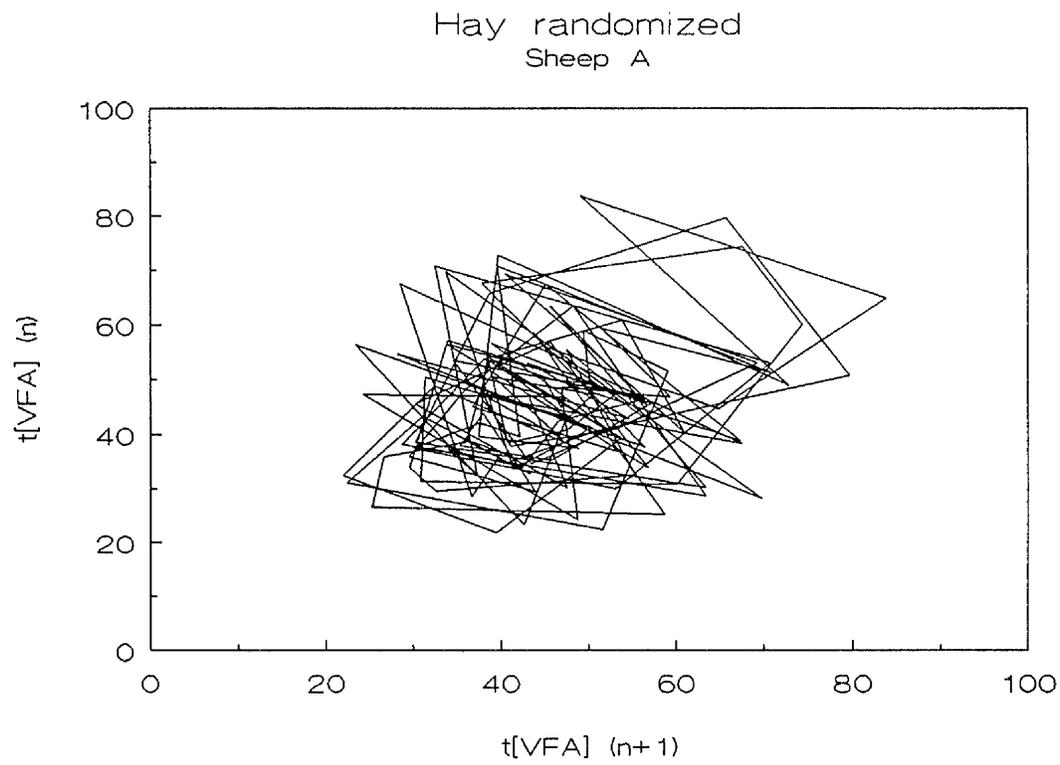


Figure 11.3

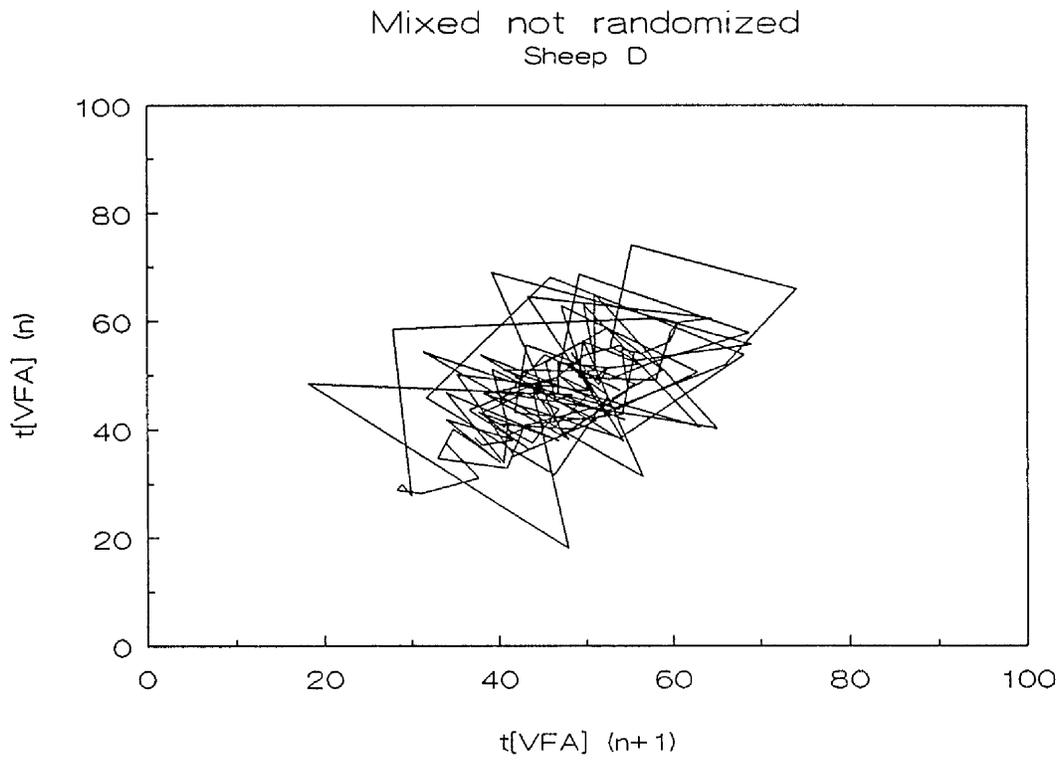
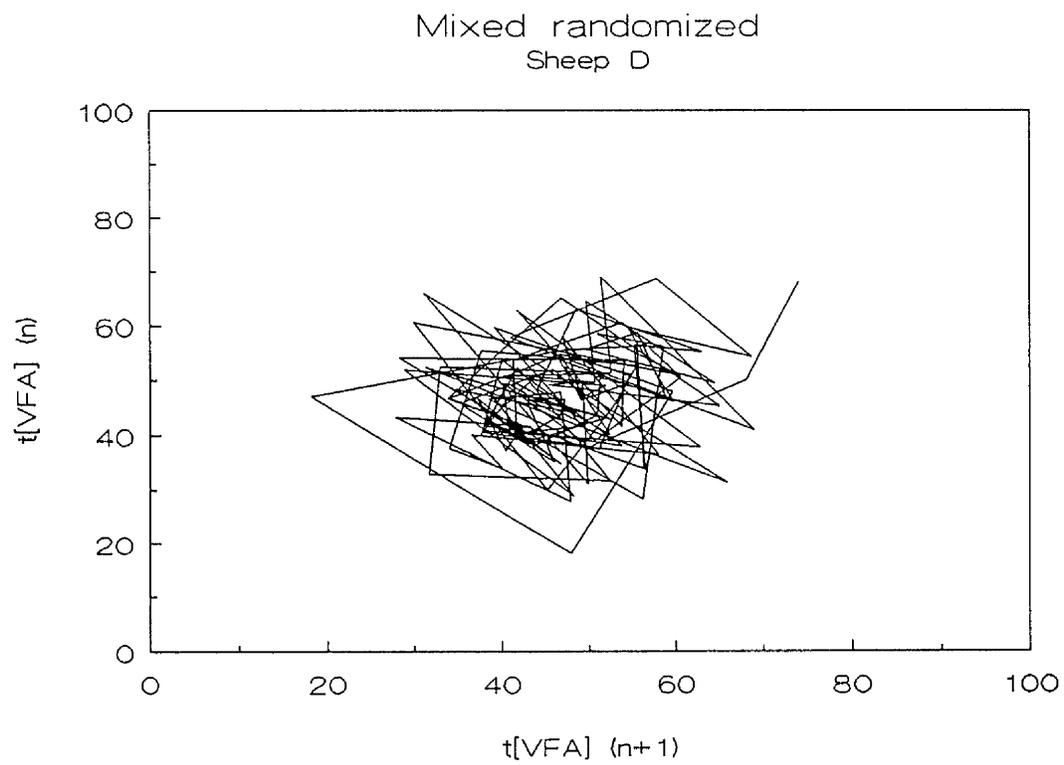


Figure 11.4



7 and 8. This concurs with the theory of May (1989) who suggested that it would be easy to find chaos in complex models containing many interacting species.

The concept of chaos has been reviewed in both the popular press and scientific journals. Gleick (1987), reviewed the principles of chaos in simple systems and then extrapolated these principles to discuss the discovery of chaos in natural systems. The existence of chaos in ecological systems has been reviewed by May 1974, Schaffer and Kot (1986) and Pool (1989a,b,c). The review papers provide an insight into the natural variations that occur in single populations and those containing interactions with other species.

Table 11.2.3: Auto-correlation coefficients for the comparison of t[VFA] on consecutive days at different lag times.

Lag time (Days)	Sheep A	Sheep A Randomised	Sheep D	Sheep D Randomised
1	0.468***	0.069	0.390***	0.040
2	0.381***	-0.022	0.257**	-0.002
3	0.253*	-0.086	0.162	0.058
4	0.269*	-0.116	0.227*	-0.003
5	0.175	-0.112	0.071	0.152
6	0.218*	-0.002	0.029	-0.141
7	0.169	-0.032	-0.020	-0.051
8	0.155	0.098	0.004	-0.058
9	0.088	-0.054	0.070	-0.134
10	0.021	-0.101	0.021	-0.020
11	0.012	0.100	0.114	-0.060
12	-0.039	0.094	-0.046	-0.116
13	0.023	-0.188*	-0.009	-0.136
14	-0.033	-0.146	-0.088	0.004
15	-0.035	-0.072	-0.102	0.085
16	-0.043	-0.144	-0.038	-0.054
17	0.014	0.071	-0.031	-0.112
18	-0.101	0.101	-0.030	-0.024
19	-0.029	0.042	-0.107	0.067
20	0.024	-0.068	-0.086	0.002
21	-0.018	-0.160	-0.033	0.037

Coefficients with a series of asterisks relate to the significance of correlation, (***) = $P < 0.001$, (**) = $P < 0.01$, (*) = $P < 0.05$).

Chaos has been described as: a strange type of mathematical order that appears to be random (Pool, 1989a); the ability of simple models, without inbuilt random features to generate highly irregular behaviour (Stewart, 1989) and as a dynamic phenomena that occurs when the state of a system changes with time (Stewart, 1989). No simple, singular definition has been used to define chaos but the following attributes are seen in chaotic systems (Schaffer *et al.* 1986).

- 1) **Deterministic:**
a system that may be described by a set of mathematical rules.
- 2) **Complex dynamics:**
Chaotic systems exhibit sustained motion. That is they never reach a state of equilibrium or settle into a stable cycle.
- 3) **Sensitive dependence on initial conditions ("The butterfly effect"):**
This may be demonstrated by observing the trajectories of a variate starting at nearly but not quite the same point rapidly diverging from each other and describe a individually unique path.
- 4) **Formation of chaos:**
As a system moves from a steady state to a chaotic one the transition occurs as a series of period doublings in the possible variations. This process is described by the process of bifurcation.

By way of a simple analogy the variations in the rumen parameters may be seen to vary in a similar way to that of the weather which is a dynamic (chaotic) system that conforms to the mathematical model described by Lorenz (1963). Days containing both rain and sun may be seen to predominate, but periods of persistent rain or several consecutive days without rain may also be observed. However, it is not possible knowing what the weather is one day to predict what the weather will be like in a weeks time, but a reasonable guess may be made as to the type of weather that may be experienced on the following day. The same principle is true of the results illustrated in Figures 11.1 and 11.3, short term (< 4 days) but not long term (> 4 days) predictions may be possible. These figures also show that the values are normally distributed since there is a greater concentration of lines in the centre of the figure and less at the extremities.

The reasons for the biological variation observed in the rumen parameters may be seen to result from a combination of various interacting factors not least of which include the eating, drinking

and rumination behaviour of the host animal, but even if these major sources of variation are removed it would merely reveal other underlying sources of variation such as those that exist due to the interactions of the microbial populations that form the rumen microbial ecosystem. May (1987), in his review concluded that for most natural populations, environmental noise and other complications make it difficult to find examples of time series that show period doubling, intermittency, transitions to chaos and other dynamical features that exist in chaotic systems and are clearly exhibited in some physiological and biochemical systems. However, it is conceivable that the rumen t[VFA] (and the other rumen parameters) may be seen to behave in a chaotic manner since their production is dependent on the interaction of many ruminal microbial populations made up of even more individual microbial species.

11.3: Conclusions

The initial aim for this set of experiments was to establish a robust *in vivo* model to determine the mode of action of potential rumen probiotics. In spite of the biological variation observed in the rumen in all experiments, statistically significant responses to supplementary YC and IYC were observed in individual experiments. Ruminal responses were: altered diurnal pattern of propionate molar % ($P=0.055$) (Chapter 3); in Chapter 7 increased mean acetate molar % ($P=0.021$), reduced mean propionate concentration ($P=0.055$) and molar proportion ($P=0.011$); increased tVFA ($P=0.096$) and reduced 24 hr. *in sacco* degradation of fishmeal ($P<0.05$) (Chapter 9). However, the ruminal response to added YC was not consistent between different experiments. In order to overcome the problems of inherent variability *in vivo*, an *in vitro* technique capable of detecting small changes in individual bacterium growth patterns was developed. The results from both the *in vivo* and *in vitro* experiments suggest that the YC and IYC supplements affected rumen function by the provision of one or more bacterial specific factors. These factors may act by stimulating desired groups of bacteria such as, the lactate utilisers or cellulolytic bacteria and/or by inhibiting microbial groups which compete with the desired bacterial populations for example, protozoan species. Hence, the *in vivo* response to additional YC or IYC would depend upon the population strengths of the individual microbial groups and the factors that affect them including: diet; stress of the host; host behaviour.

The observed inherent biological variation in all rumen parameters emphasises the need for well designed experiments such as the "latin square" type design. If "switch back" designs are used it is recommended that the same treatment pattern is not used in all animals.

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APPENDIX 1: Preparation of the anaerobic medium used in Chapter 3.

Anaerobic Media Preparation

0.01g	Cellobiose
2g	Yeast extract
2g	Tryptone
75mls	Mineral solution 1
75mls	Mineral solution 2
1ml	Haemin (1mg/ml.soln)
1ml	Resazurin (1mg/ml.soln)
3.1mls	VFA mix
150mls	Cell free rumen liquor

The above components were mixed together and then gassed with carbon dioxide for 30 minutes. Following gassing, the pH of the solution was checked and corrected to pH 7.0.

Anaerobic Diluent Preparation

45mls	Mineral solution 1
45mls	Mineral solution 2
0.3mls	Resazurin (1mg/ml)
0.15g	Cysteine - HCl
0.9g	Na ₂ CO ₃
300mls	Anaerobic distilled water

The above components were added together and then carbon dioxide gas was passed through to facilitate anaerobiosis and help dissolve the constituents. After 30 minutes of gassing the pH of the solution was corrected to pH 6.6.

Preparation of the Hungate Tubes

Filter paper strips measuring approximately 17.5 x 0.5 mm were used as a source of cellulose for the experiment, these strips were placed in the Hungate tubes. The tubes were then gassed for a few seconds with carbon dioxide and the stoppers subsequently tightly screwed on. The media was prepared as previously described using distilled water that had been boiled and cooled under a stream of carbon dioxide in order to ensure anaerobiosis.

The media was then dispensed into the tubes using a Zipette dispenser with an attached side arm. Anaerobiosis was maintained by attaching a gas line to the side arm of the dispenser so that the gas was blown into each tube during dispensing. 10 mls of media was put into each tube and the cap tightly fastened. Following autoclaving at 121°C for 15 minutes the tubes were ready for a use.

The diluent was dispensed in a similar manner as the media, into pre-gassed Hungate tubes.

APPENDIX 2: Anaerobic media used for the measurement of rumen bacterial growth by optical density (Chapter 10).

Composition of media

	/100 ml
Glucose	0.05 g
Cellobiose	0.05 g
Starch	0.05 g
Tryptone	0.5 g
Mineral solution 3	5.0 ml
Vitamin solution	1.0 ml
Haemin solution	1.0 ml
VFA solution	1.0 ml
Ferrous sulphate solution	1.0 ml
Methionine solution	1.0 ml
Ammonium sulphate solution	1.0 ml
10 % NaOH	1.9 ml
Reasuzurin solution	0.1 ml
Distilled water	80 ml
Sodium carbonate solution	5.0 ml
Cysteine solution	2.0 ml

Preparation

The above components were mixed and brought to the boil. The mixture was then allowed to cool as CO₂ gas was bubbled through it until the mixture was saturated with CO₂. The pH of the mixture was then adjusted to 6.8 using a concentrated NaOH solution. Subsequently the mixture was transferred to an anaerobic cabinet where it was dispensed in 6 ml aliquots into 10 ml screw-capped Hungate tubes. The media tubes were then autoclaved at 121°C for 15 mins. and subsequently stored at room temperature to await use.

APPENDIX 3: Reagents used in the preparation of anaerobic media and diluent used in Chapters 3 and 10.

Mineral Solution 1

2.9g/l K_2HPO_4 stock soln.

This was dissolved in distilled water and added to the media in the proportion of 10 mls soln./100 mls media.

Mineral Solution 2

1.76g/l KH_2PO_4 stock solution

0.44g/l NaCl

4.5g/l $(NH_4)_2SO_4$

0.9g/l $CaCl_2 \cdot 6H_2O$

0.94g/l $MgSO_4 \cdot 7H_2O$

The above components were dissolved in distilled water and added to the media in the proportion of 10 mls soln./ 100 mls media.

Mineral Solution 3a

NaCl 18.0g

KH_2PO_4 18.0g

$CaCl_2$ 0.4g

$MgCl_2 \cdot 6H_2O$ 0.4g

$MnCl_2 \cdot 4H_2O$ 0.2g

$CoCl_2 \cdot 6H_2O$ 0.02g

Volatile Fatty Acid Mix (stock solution)

17mls Acetic acid

6mls Propionic acid

4mls n-Butyric acid

1ml Isobutyric acid

1ml n-Valeric acid

1ml isovaleric acid

1ml DL-Methyl butyric acid

The above solutions were mixed together and added to the media in the proportions of 0.31mls stock soln/ 100mls media.

Vitamin Solution

	/100ml
Pyridoxine—HCl	20mg
Riboflavin	20mg
Thiamine—HCl	20mg
Nicotinamide	20mg
Ca—D—Pantothenate	20mg
p—Aminobenzoic acid	1mg
Folic acid	0.5mg
Biotin	0.5mg
Vit B ₁₂	0.05mg

The above vitamins were added to a 100ml volumetric flask. The flask was then made up to the mark with distilled water and filter sterilised. The filtrate was then frozen and stored at -5°C.

Vitamin K₁ stock solution

Vitamin K₁ (0.15ml) was dissolved in 30ml of 95% ethanol. This solution was then stored at 4°C.

Cell Free Rumen Fluid

A sample of rumen fluid was left at 37°C for 10 days, this ensured that any residual sugars in the rumen fluid were used up by microbes, therefore preventing the carry over of sugars into the media. After incubating the rumen fluid for ten days it was centrifuged at 20,000g for 30 minutes and the supernatant kept.

Cysteine solution (reducing agent)

L—cysteine HCl·H₂O (2.5g) was dissolved in 100ml of distilled H₂O. The solution placed in a round bottomed flask, brought to boil and cooled while bubbling with oxygen free CO₂. The flask were sealed with a butyl rubber stopper, wired in place and autoclave at 120°C for 15 min. Store at room temperature.

Sodium carbonate solution (anaerobic buffer)

Sodium carbonate (anhydrous) (8.0g) was dissolved in 100ml of distilled water, in a round bottomed flask. The solution was brought to boil and cooled under CO₂.

Resazurin solution (oxidation—reduction indicator)

Resazurin (1g) was dissolved in 1 litre of distilled water and stored in the dark at room temperature.

Heamin solution (provides cofactors)

Heamin (0.1g) was dissolved in 1 litre of a mixture containing 1 volume of 95% ethanol and 1 volume of 0.2M KOH. This solution was then stored at 4°C.

The above components were mixed together and dissolved in 1l of distilled water. The resulting solution was stored in the fridge at 4°C.

Ferrous Sulphate Solution

FeSO₄ (1.0g) was dissolved in a 10% solution of concentrated HCl.

Methionine Solution

D—C—Methionine (0.45g) was dissolved in 100ml distilled H₂O.

Ammonium Sulphate Solution

(NH₃)₂SO₄ (9.0g) was dissolved in 100ml of distilled water.

APPENDIX 4: Latin square designs used in Chapter 3.

Square 1:

	Period 1	Period 2	Period 3
Sheep 3	Control	IYC	YC
Sheep 5	IYC	YC	Control
Sheep 6	YC	Control	IYC

Square 2:

	Period 1	Period 2	Period 3
Sheep 1	Control	YC	IYC
Sheep 2	IYC	Control	YC
Sheep 4	YC	IYC	Control

APPENDIX 5: Results of *in sacco* soya hull DMD% analysed by CUSUM (Chapter 3).

	From obs.	To obs.	Stage mean	SD	Combined within stage SD
Sheep 1	1	4	44.71	6.90	2.550
	5	12	35.41	1.88	
	13	25	43.17	2.75	
	26	33	38.92	1.35	
	34	47	36.21	1.89	
	48	53	37.91	1.82	
	54	58	41.92	2.01	
	59	66	35.71	2.66	
	67	75	39.75	2.20	
	76	79	37.75	0.73	
	80	84	34.81	2.93	
Sheep 2	1	8	39.75	2.60	1.912
	9	22	43.49	1.53	
	23	36	39.68	1.77	
	37	50	42.96	1.93	
	51	60	40.20	2.08	
	61	74	42.33	1.53	
	75	84	40.20	2.24	

	From obs.	To obs.	Stage mean	SD	Combined within stage SD
Sheep 3	1	4	37.47	3.30	2.523
	5	19	44.36	2.05	
	20	33	39.67	2.79	
	34	49	48.34	2.14	
	50	58	46.72	2.97	
	59	75	43.97	2.54	
	76	84	40.57	2.64	
Sheep 4	1	4	37.33	0.60	1.727
	5	13	40.26	1.56	
	14	19	36.41	2.61	
	20	24	42.78	0.67	
	25	41	40.79	1.69	
	42	47	34.80	3.05	
	48	53	40.06	1.79	
	54	63	43.72	1.43	
	64	68	42.39	1.00	
	69	75	40.35	1.62	
	76	84	42.68	1.27	

	From obs.	To obs.	Stage mean	SD	Combined within stage SD
Sheep 5	1	8	29.84	2.75	2.668
	9	15	35.04	2.12	
	16	23	33.77	1.55	
	24	29	30.98	2.46	
	30	50	35.32	1.92	
	51	57	39.49	2.12	
	58	65	33.51	5.71	
	66	71	38.01	1.70	
	72	77	33.32	2.02	
	78	82	37.62	2.34	
Sheep 6	1	19	35.01	2.46	3.015
	20	25	39.78	1.58	
	26	36	35.74	2.76	
	37	58	40.90	3.84	
	59	66	37.34	3.22	
	67	73	44.10	2.75	
	74	84	39.64	2.74	

Consecutive means are significantly different at the $P < 0.05$ level of significance.

APPENDIX 6: Individual sheep OMD results (Chapter 6).

	Period	Batchday			
		1	2	3	4
Sheep A	1	796	866	841	820
	2	847	811	769	807
	3	844	822	822	844
	4	806	857	815	847
Sheep B	1	787	841	815	835
	2	862	845	825	810
	3	855	832	814	828
	4	830	872	816	846
Sheep C	1	730	852	831	808
	2	799	803	812	792
	3	820	825	767	809
	4	789	824	787	821

APPENDIX 7: Results of the *in sacco* degradability of soyahulls analysed by CUSUM (Chapter 6).

	From obs.	To obs.	Stage Mean	SD	Combined within stage SD
Sheep A Unwashed	1	5	36.514	2.13	2.701
	6	33	32.709	2.775	
Sheep A Prewashed	1	6	24.766	3.8	2.498
	7	19	20.797	1.829	
	20	26	23.646	2.278	

Sheep B Unwashed	1	6	35.091	1.58	1.95
	7	13	31.632	1.616	
	14	22	33.611	2.302	
	23	28	35.54	1.731	
	29	33	39.271	2.291	
Sheep B Prewashed	1	6	31.671	3.509	3.536
	7	26	24.055	3.543	

Sheep C Unwashed	1	6	34.425	4.249	2.675
	7	10	28.505	2.252	
	11	19	34.508	1.617	
	20	33	30.868	2.498	
Sheep C Prewashed	1	6	23.415	2.337	2.083
	7	20	20.848	1.783	
	21	26	22.894	2.499	

APPENDIX 8: Latin Square Change Over Design used in Chapter 7

Sheep	Period			
	1	2	3	4
A	Diet 1 + YC	Diet 1	Diet 2	Diet 2 + YC
B	Diet 1	Diet 2 + YC	Diet 1 + YC	Diet 2
C	Diet 2 + YC	Diet 2	Diet 1	Diet 1 + YC
D	Diet 2	Diet 1 + YC	Diet 2 + YC	Diet 1

Diet 1: Semi-purified diet used in Trials 1 & 2.

Diet 2: Hay, Barley, Maize plus urea & minerals.

APPENDIX 9: Results of the *in sacco* degradability of absorbent cotton wool analysed by CUSUM for each sheep (Chapter 7).

	From obs.	To obs.	Stage mean	SD	Combined within satge SD
Sheep A	1	4	19.543	10.297	3.931
	5	13	12.561	3.493	
	14	20	6.18	3.072	
	21	44	11.111	3.405	
	45	52	21.906	5.744	
	53	57	18.712	3.672	
	58	62	14.758	3.44	
	63	84	10.271	2.215	
Sheep B	1	19	13.863	3.646	4.202
	20	28	12.083	3.816	
	29	41	19.417	2.957	
	42	51	21.965	8.095	
	52	55	8.648	0.589	
	56	65	11.76	4.206	
	66	84	14.162	3.228	
Sheep C	1	8	18.753	2.241	2.77
	9	15	15.517	2.058	
	16	20	19.036	2.492	
	21	27	24.667	1.157	
	28	44	19.398	3.723	
	45	51	13.653	3.814	
	52	62	7.086	2.872	
	63	84	4.8	2.163	

	From obs.	To obs.	Stage mean	SD	Combined within satge SD
Sheep D	1	17	10.817	2.107	4.182
	18	25	21.035	4.227	
	26	44	8.149	3.052	
	45	67	11.794	4.747	
	68	71	16.135	9.908	

APPENDIX 10: Results of the *in sacco* degradability of washed hay analysed by CUSUM for each sheep (Chapter 7).

	From obs.	To obs.	Stage mean	SD	Combined within stage SD
Sheep A	1	5	26.314	5.41	3.044
	6	15	18.201	2.701	
	16	29	15.196	1.958	
	30	44	24.406	3.138	
	45	59	29.803	3.093	
	60	70	27.338	2.219	
	71	74	31.658	4.633	
	75	84	27.087	3.093	
Sheep B	1	17	17.759	3.028	3.023
	18	24	14.116	3.172	
	25	29	19.67	1.347	
	30	46	30.262	2.124	
	47	55	28.751	3.625	
	56	59	27.798	2.239	
	60	65	25.623	5.806	
	66	72	29.813	3.04	
	72	84	26.884	2.233	
Sheep C	1	5	27.492	2.208	2.561
	6	27	22.939	2.424	
	28	44	30.936	3.05	
	45	64	24.113	2.323	
	65	84	21.742	2.554	

	From obs.	To obs.	Stage mean	SD	Combined within stage SD
Sheep D	1	5	23.362	2.635	3.459
	6	12	15.916	1.894	
	13	30	20.311	3.24	
	31	50	21.474	3.667	
	51	59	28.646	4.123	
	60	71	25.863	3.797	

APPENDIX 11: Daily rumen pH analysed by CUSUM (Chapter 7)

	From obs.	To obs.	Stage mean	SD	Combined within stage SD
Sheep A	1	6	6.68	0.059	0.082
	7	20	6.396	0.097	
	21	45	6.291	0.08	
	46	60	6.729	0.104	
	61	85	6.661	0.062	
Sheep B	1	4	6.55	0.117	0.101
	5	10	6.313	0.162	
	11	17	6.499	0.19	
	18	25	6.316	0.061	
	26	43	6.646	0.087	
	44	64	6.356	0.053	
	65	72	6.558	0.12	
	73	85	6.747	0.085	
Sheep C	1	16	6.724	0.068	0.059
	17	43	6.653	0.042	
	44	55	6.224	0.063	
	56	69	6.149	0.065	
	70	73	6.065	0.024	
	74	85	6.212	0.071	

	From obs.	To obs.	Stage mean	SD	Combined within stage SD
Sheep D	1	6	6.628	0.123	0.163
	7	23	6.475	0.099	
	24	28	6.324	0.147	
	29	47	6.133	0.105	
	48	51	6.343	0.255	
	52	67	6.596	0.156	
	68	71	7.068	0.457	

APPENDIX 12: Individual sheep rumen *in sacco* degradability (ISDMD) cusum means (Chapter 8)

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep A	1	16	33.816	2.414	3.201
	17	39	36.326	1.695	
	40	57	34.388	2.181	
	58	63	30.942	2.375	
	64	81	36.665	2.253	
	82	109	32.836	3.164	
	110	125	35.910	6.286	
Sheep B	1	48	34.994	1.766	2.555
	49	52	30.133	2.293	
	53	64	36.554	1.416	
	65	91	34.627	1.727	
	92	101	31.825	3.134	
	102	109	28.492	2.318	
	110	125	32.108	5.048	
Sheep C	1	8	36.510	2.034	2.850
	9	12	33.738	2.394	
	13	78	33.142	2.304	
	79	94	32.536	2.058	
	95	125	29.853	4.164	

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep D	1	17	29.809	2.803	3.165
	18	26	32.954	2.223	
	27	31	27.168	5.433	
	32	35	29.77	2.057	
	36	70	31.444	2.430	
	71	77	28.490	1.334	
	78	84	31.723	1.687	
	85	90	29.845	2.071	
	91	102	29.013	2.691	
	103	106	24.677	4.098	
	107	125	29.147	5.09	
Sheep E	1	22	28.698	3.146	3.536
	23	31	26.425	3.414	
	32	41	29.535	1.177	
	42	86	27.202	3.105	
	87	102	25.89	3.496	
	103	106	19.742	2.221	
	107	125	26.395	5.475	
Sheep F	1	10	31.824	3.298	3.411
	11	25	27.878	2.252	
	26	39	31.432	2.395	
	40	70	29.100	2.780	
	71	74	22.488	1.158	
	75	125	27.674	4.255	

APPENDIX 13: Individual sheep rumen t[VFA] cusum means (Chapter 8)

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep A	1	8	41.328	7.230	10.234
	9	21	57.946	13.766	
	22	32	43.584	4.310	
	33	51	53.643	7.943	
	52	63	68.233	10.124	
	64	72	59.224	5.879	
	73	81	38.677	7.132	
	82	86	70.527	12.405	
	87	100	60.67	13.384	
	101	112	80.910	13.135	
	113	120	66.664	11.776	
	121	127	36.829	8.743	
Sheep B	1	8	46.812	7.582	9.380
	9	21	65.214	4.762	
	22	49	50.142	7.679	
	50	71	62.659	9.142	
	72	81	43.871	10.610	
	82	89	67.243	8.585	
	90	94	89.452	21.099	
	95	103	66.127	14.702	
	104	107	98.705	12.492	
	108	120	75.443	6.768	
	121	127	37.831	4.621	

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep C	1	9	37.396	13.934	10.513
	10	15	66.905	14.696	
	16	20	41.258	11.181	
	21	56	52.370	10.601	
	57	77	55.261	6.471	
	78	91	60.991	4.472	
	92	97	50.03	14.627	
	98	111	79.824	14.193	
	112	120	67.648	10.146	
	121	127	37.777	6.52	
Sheep D	1	10	47.605	4.272	9.002
	11	35	62.624	9.917	
	36	52	52.071	5.074	
	53	77	57.780	10.909	
	78	100	62.572	9.076	
	101	120	71.261	10.263	
	121	127	38.794	4.432	

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep E	1	8	49.961	11.565	11.247
	9	17	67.137	7.068	
	18	26	49.535	3.965	
	27	45	59.460	8.956	
	46	57	72.622	8.348	
	58	71	63.578	8.542	
	72	80	53.535	10.648	
	81	86	73.211	3.818	
	87	97	54.144	13.683	
	98	120	71.770	17.318	
	121	127	39.736	10.033	
Sheep F	1	12	45.365	7.717	9.693
	13	50	55.423	6.919	
	51	71	51.458	8.526	
	72	80	42.526	7.110	
	81	86	60.177	5.159	
	87	97	46.166	13.762	
	98	105	65.347	10.679	
	106	118	53.757	15.474	
	119	127	34.755	11.748	

APPENDIX 14: Individual sheep rumen pH cusum means for the day-to-day variation (Chapter 8)

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep A	1	10	6.78	0.119	0.086
	11	26	6.91	0.076	
	27	32	6.86	0.021	
	33	37	6.79	0.082	
	38	44	6.65	0.083	
	45	51	6.80	0.076	
	52	67	6.62	0.107	
	68	74	6.65	0.116	
	75	83	6.70	0.087	
	84	93	6.58	0.039	
	94	99	6.45	0.087	
	100	104	6.57	0.033	
	105	127	6.62	0.084	

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep B	1	14	6.98	0.073	0.079
	15	22	6.86	0.080	
	23	32	6.99	0.030	
	33	45	6.87	0.072	
	46	49	6.98	0.070	
	50	53	6.71	0.054	
	54	59	6.96	0.077	
	60	81	6.87	0.075	
	82	97	6.80	0.062	
	98	119	6.63	0.111	
	120	127	6.80	0.088	
Sheep C	1	9	7.12	0.232	0.102
	10	20	6.86	0.108	
	21	37	6.75	0.068	
	38	75	6.78	0.066	
	76	88	6.62	0.08	
	89	97	6.70	0.03	
	98	102	6.43	0.252	
	103	127	6.65	0.073	

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep D	1	9	6.68	0.066	0.051
	10	13	6.51	0.043	
	14	31	6.65	0.036	
	32	36	6.53	0.106	
	37	63	6.64	0.044	
	64	81	6.60	0.045	
	82	93	6.56	0.028	
	94	100	6.45	0.081	
	101	116	6.60	0.046	
	117	120	6.50	0.033	
	121	127	6.63	0.070	
Sheep E	1	9	6.23	0.047	0.069
	10	16	6.48	0.097	
	17	26	6.75	0.056	
	27	34	6.65	0.082	
	35	57	6.51	0.052	
	58	63	6.60	0.024	
	64	67	6.52	0.014	
	68	80	6.43	0.061	
	81	100	6.37	0.049	
	101	105	6.46	0.093	
	106	112	6.30	0.053	
	113	121	6.40	0.096	
122	127	6.50	0.15		

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep F	1	13	6.77	0.053	0.056
	14	17	6.64	0.083	
	18	24	6.71	0.031	
	25	31	6.67	0.081	
	32	36	6.61	0.041	
	37	42	6.71	0.018	
	43	46	6.60	0.030	
	47	62	6.70	0.026	
	63	83	6.66	0.040	
	84	88	6.63	0.022	
	89	93	6.59	0.021	
	94	104	6.51	0.084	
	105	120	6.59	0.048	
	121	127	6.67	0.129	

APPENDIX 15:

Individual sheep rumen ammonia concentration cusum means for the day-to-day variation (Chapter 8)

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep A	1	4	106.025	13.60	16.584
	5	15	85.918	17.27	
	16	27	74.483	8.19	
	28	37	90.58	15.70	
	38	52	68.1	16.28	
	53	59	94.464	35.82	
	60	79	76.815	8.03	
	80	102	62.12	13.55	
	103	107	43.88	9.24	
	108	116	88.167	29.63	
	117	127	51.8	11.73	
Sheep B	1	9	67.90	21.19	23.90
	10	13	153.325	67.02	
	14	20	104.671	27.53	
	21	26	60.183	13.85	
	27	37	87.927	23.23	
	38	44	56.6	7.28	
	45	53	90.611	26.92	
	54	60	120.486	23.47	
	61	84	71.421	14.53	
	85	106	58.078	19.06	
	107	127	73.833	28.61	

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep C	1	13	65.877	22.60	16.615
	14	18	88.98	17.13	
	19	35	62.447	12.68	
	36	43	51.225	10.72	
	44	76	74.897	15.826	
	77	80	67.95	0.947	
	81	106	54.403	19.84	
	107	113	73.5	18.34	
	114	127	45.246	12.47	
Sheep D	1	11	100.082	16.04	21.32
	12	16	130.92	59.39	
	17	45	97.821	14.59	
	46	52	129.457	33.63	
	53	64	106.633	19.71	
	65	75	87.90	12.09	
	76	85	110.52	17.85	
	86	99	90.429	23.54	
	100	104	115.384	17.30	
	105	110	89.083	20.60	
	111	123	111.792	14.23	
	124	127	71.6	20.71	

	From obs.	To obs.	Mean	SD	Combined within stage SD
Sheep E	1	26	86.904	11.99	18.793
	27	33	117.314	14.17	
	34	42	81.067	15.28	
	43	53	113.418	20.93	
	54	59	136.583	26.60	
	60	67	96.75	15.50	
	68	84	135.60	15.0	
	85	97	101.315	19.52	
	98	104	111.647	23.34	
	105	111	153.829	35.86	
	112	122	114.909	20.85	
	123	127	88.28	14.58	
Sheep F	1	9	113.8	32.56	19.183
	10	22	95.931	12.01	
	23	46	107.138	15.41	
	47	60	123.7	21.60	
	61	81	108.343	16.66	
	82	85	142.575	22.19	
	86	106	90.992	19.21	
	107	112	117.367	19.12	
	113	127	92.193	19.73	

APPENDIX 16: Latin square change over design used in Chapter 9.

Sheep	Period			
	1	2	3	4
A	Control	PAS	YC	IYC
B	IYC	YC	PAS	Control
C	PAS	IYC	Control	YC
D	YC	Control	YC	PAS

Control = Basal diet.

YC = Viable yeast culture supplement.

IYC = Irradiated yeast culture supplement.

PAS = Pot ale syrup supplement.

APPENDIX 17: The philosophy of the "butterfly effect"

If a butterfly flaps its wings in Arizona can it cause a typhoon in Japan ?

If the conditions are such that the flap from the butterfly's wings starts a series of events which increase in magnitude as they progress, then it may be possible for a butterfly to start a typhoon in Japan when it flaps its wings in Arizona.

(Adapted from Gleik 1989)

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