CHAPTER V

COMPARATIVE STUDIES ON THE ROLE OF RUMEN PROTOZOA AND FUNGI IN FIBRE DIGESTION IN SHEEP GIVEN HIGH-FIBRE DIETS

5.1 Introduction

The role of rumen fungi in fibre digestion in ruminants fed high-fibre diets appears to be important; however, few studies have quantitatively assessed the role of fungi. Early observations suggested that there is a marked interaction between fungi, protozoa and bacteria. In these experiments the interaction between fungi and protozoa was studied. It was not possible to study the effect on the bacterial population of defaunation, however, it is known that numbers of bacteria free in the medium increase when protozoa are removed from the rumen

A series of digestion and metabolism studies was conducted in which the digestion of feed and rumen fermentation parameters including concentrations of ammonia, and VFA, and rumen pH of defaunated and refaunated sheep were compared. The number of fungal sporangia which appeared on feed particles (suspended in the rumen in nylon bags) and the number of zoospores in rumen fluid were taken to be an indication of the relative size of the fungal biomass in the rumen of the two groups of animals.

Since fibrous materials are generally low in fermentable N (Preston and Leng, 1984) a small amount of urea was included in the diet.

5.2 Experiment 1

DIGESTION OF OATEN CHAFF BY DEFAUNATED AND REFAUNATED SHEEP

5.3 Experimental

5.3.1 Experimental animals

Six merino wethers (approximately 3 years old) weighing between 35 and 40 kg were each fitted with a ruminal cannula. They were housed individually on slatted floors under continuous lighting. The sheep were divided randomly into two groups of three sheep and were fed diet once a day at 0915 h. They had continuous access to water.

5.3.2 Diet

All animals were fed a fixed amount of feed during both periods when they were unfaunated and also following refaunation. The basal diet consistent of 700 g oaten-chaff (OC) (no grain was present) and 3.5% mineral mixture (see Table 5.1.1 for composition). One group was supplemented with 1% urea. The OC was obtained by separating oaten chaff from the grain by a mechanical separator.

5.3.3 Experimental procedure

Before the experiments were commenced, the animals were subjected to a routine drenching programme to control internal parasites as described in Section 3.2.

Studies were initially done with sheep that had been defaunated and then repeated after these animals had been refaunated. The defaunating agent used and the defaunation procedure were as described in Section 3.4.1. The choice of this defaunating agent was based on the results of previous studies in this laboratory (Burggraaf, 1980; Bird, 1982). Feed was removed one day prior to treatment, but water was freely available. The treatment proved to be successful in maintaining the defaunated state of the rumen during the data collection period. Animals were allowed a four-week period (two weeks both before and after treatment) to adjust to the experimental diet before the measurements were begun. In each period the following measurements were made:

(1) dry-matter disappearance *in sacco* of oaten-chaff fibre,
(2) numbers of fungal sporangia appearing on feed particles,
(3) rumen pH, ammonia and VFA concentrations in rumen fluid.

The procedure of measuring feed dry-matter disappearance *in sacco* was as described in Section 3.4.4. Approximately 3.0 g air-dry ground OC (to pass a 1 mm screen) was placed in the nylong bag (7X15 cm, pore size 25 μm), tied with nylon line after being weighted with a glass marble. The bags were suspended in the rumen at feeding time and one bag was withdrawn after 6, 12, 24, 48 and 72 h. The measurements of rumen pH, the concentrations of rumen ammonia and VFA were done at -3, -2, -1, 0, 3, 6, 9, 12 and 15 h relative to feeding. Fungal sporangia were counted on pieces of OC (1 cm lengths and 20-25 pieces per bag) that had been suspended in the rumen in nylon bags for 6, 12, 24 and 48 h, and enumerated under a light microscope after those feed particles were stained with lactophenol cotton blue. The counting procedure has been outlined in Section 3.4.5.a.

In the period following refaunation the numbers of protozoa in he rumen were counted at timed intervals as already described in Section 3.4.3.a.

At the end of the defaunation period, an effort was made to eliminate the rumen fungi using actidione (cyclohexamide, SIGMA), an anti-fungal agent, as used by Orpin (1975), at 50 μg/ml of rumen fluid. Unlike the procedure of Orpin (1975), which dissolved the actidione in rumen fluid and then returned the solution to the rumen in this study actidione was dissolved in distilled water and injected directly into the rumen. Unfortunately, the sheep lost appetite following this administration, and fungal zoospores were still observed 24 h post-injection. Moreover, one sheep died when the second dose of actidione was given on the following day, so this procedure was abandoned. The surviving sheep were therefore refaunated immediately. Consequently, data collection in the refaunated state was made using five animals instead of six.

5.3.4 Statistical analysis

Differences between treatment effects were analysed statistically as a non-orthogonal experiment with repeated measures and computed using a package programme of BMDP2V (BMDP Statistical Software,

1981) and subjected to analysis of variance followed by least significant difference (Snedecor and Cochran, 1967).

Since there was no significant effect of urea supplementation on dry-matter disappearance *in sacco* of OC, the data were pooled and compared between defaunated and refaunated periods using a paired t-test with unequal numbers (Snedecor and Cochran, 1967).

Table 5.1.1 Composition of the diets used in Experiment 1

Item	Quantity fed (g)	Chemical analysis*)				
		DM	OM	NDF	ADF	CP (NX6.25)
		----------	----------	%	----------	----------
Group A						
OC	700	92	92	70	46	6,5
Urea	7	-	-	-	-	-
Mineral mix.+)	22.5	-	-	-	-	-
Group B						
OC	700	92	92	70	46	6.5
Mineral mix.+)	22.5	-	-	-	-	-

*) apart from DM the figures are on a DM basis

+) mineral mix composed of: 57% CaH_2PO_4; 14.3% Na_2SO_4; 14.3% NaCl; 14.3% ruminant premix (Pfizer quote 422). Each kg premix contained (based on factory analysis):

Ca	max 16% min 15%	Cu	4.4 g
F	max 13%	Co	0.09 g
Mn	7.04 g	I	0.35 g
Zn	17.6 g	Vit.A	4,400,000 IU
Mg	105.6 g	Vit.D	1,760,000 IU
Fe	17.6 g		

5.4 Results

As reported in previous studies in this laboratory (Burggraaf, 1980; Bird, 1982) the animals lost appetite following the administration

of alkanate, but all animals returned to full appetite one day after completion of the treatment. The treatment proved to be very effective as no protozoa were detected during the data collection period.

5.4.1 Dry-matter disappearance (DMD) *in sacco* of OC

There was a significant ($P < 0.01$) increase in DMD in sheep that had been defaunated with and without urea in the diet. Supplementation with 1% urea resulted in no differences in DMD within treatment ($P < 0.05$). The differences between treatments were, however, significant at $P < 0.01$ for any incubation time (Table 5.1.2).

5.4.2 Sporangial counts

The numbers of sporangia on feed particles in the defaunated animals were higher ($P < 0.05$) than those of refaunated sheep, at least until 24 h incubation. The differences became less as the incubation period was prolonged up to 48 h. Supplementation with 1% urea apparently stimulated the growth of rumen sporangia on feed particles in the absence of protozoa (Table 5.1.3).

5.4.3 Composition of rumen contents

i. Rumen pH

The mean values for rumen pH throughout the measurement period were 6.70 and 6.65 respectively in the defaunated and refaunated animals (see Table 5.1.4 and Figure 5.1.1).

ii. Rumen ammonia concentrations

The absence of protozoa from the rumen of sheep tended to decrease the rumen ammonia concentrations even though the differences were not statistically significant between treatments. Addition of 1% urea to the basal diet increased ($P < 0.01$) rumen ammonia concentrations within treatments (Table 5.1.4 and Figure 5.1.2).

iii. VFA concentrations

There were no significant differences in the concentrations of either total VFA (TVFA) or individual acids due to defaunation. In the defaunated sheep, however, there was a trend for the proportion of acetate to increase, while the proportions of propionate and butyrate decreased (Table 5.1.4 and Figure 5.1.3).

iv. Protozoal numbers in refaunated sheep

The predominant species of rumen ciliates found in the refaunated state of both supplemented and unsupplemented groups of sheep were small Entodiniomorphs. Greatest numbers were generally obtained at feeding time. The numbers that could be sampled decreased rapidly a few hours after feeding, but increased again at about 10-12 h post feeding (Figure 5.1.4).

Table 5.1.2 Effect of defaunation on dry-matter disappearance of OC (g/100g) in Experiment 1. Values are means of pooled dry matter disappearance within treatment (with and without 1% urea supplements)

Incubation (h)	Treatment		SED
	Defaunated +)	Refaunated #)	
6	38**	29	1.2
12	44**	37	1.8
24	58**	52	1.6
48	67**	59	1.1
72	69**	62	1.4

+) mean values of six sheep; #) mean values of five sheep

** significantly different from refaunated sheep at $P < 0.01$

SED=Standard Error of Mean difference from t-test

Table 5.1.3 Effect of defaunation on the numbers of fungal sporangia appearing on feed particles in Experiment 1. A=with urea B=without urea. Refaunated A are mean values of two sheep, others are three sheep,

Treatment	Incubation time (h)				SEM
	6	12	24	48	
	------ No.of colonies/mm^2 ----------				
Defaunated					
A	203^{b}	185^{c}	114^{bc}	52^{a}	
B	117^{a}	124^{b}	91^{b}	47^{a}	
Refaunated					
A	83^{a}	73^{a}	55^{ab}	36^{a}	
B	131^{a}	30^{a}	34^{a}	23^{a}	
					12.4

Values in the same coloumn with unlike superscripts are significantly different(at least $P < 0.05$) SEM= Standard Error of the mean

Table 5.1.4 Effect of defaunation on rumen contents of sheep in Experiment 1. A:with urea; B:without urea supplements. Refaunated A are mean values of two sheep,others are three sheep in each case

Treatments	TVFA (mmol/1)	Ac.	Pr.	But.	NH_3-N (mgN/1)	pH
		molar %				
Defaunated						
A	62^a	76^a	19^a	4^a	88^b	6.78^a
B	61^a	74^a	18^a	5^a	46^a	6.81^a
Refaunated						
A	67^a	76^a	20^a	6^a	112^b	6.67^a
B	60^a	71^a	21^a	6^a	53^a	6.70^a
SEM	1.3	0.8	0.4	0.1	2.6	0.02

Values in the same coloumn with unlike superscripts are significantly different ($P < 0.05$). SEM=Standard Error of the mean.
Iso-VFA's were never more than 1% of TVFA,and therefore were not included in the Table.

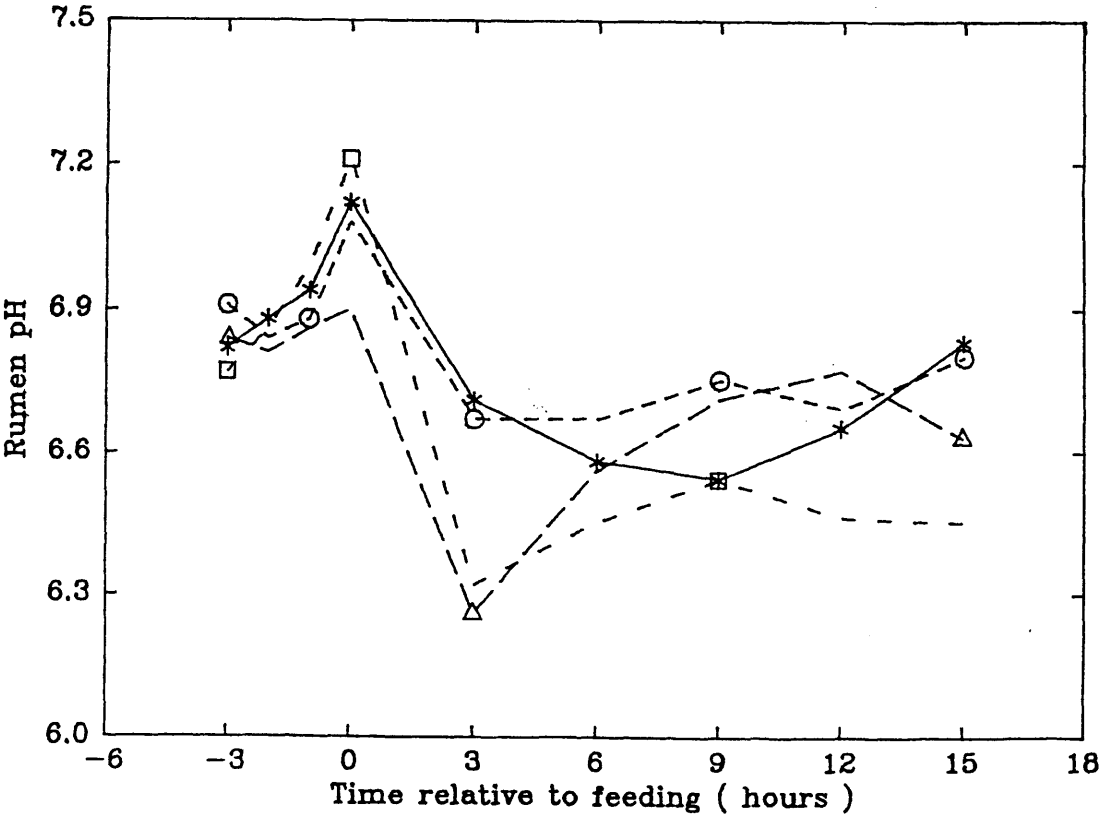


Figure 5.1.1 Variation in rumen pH in Experiment 1 .
* defaunated + urea; 0 defaunated without urea;
□ refaunated + urea ;Δ refaunated without urea

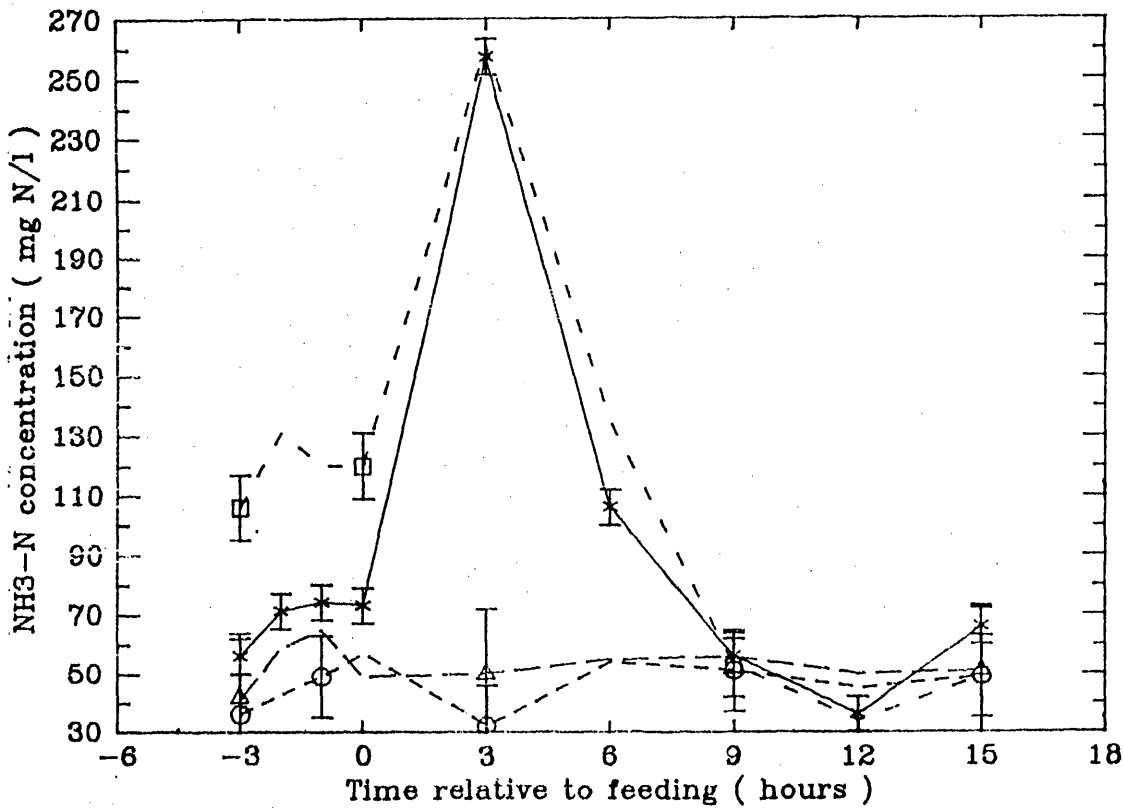
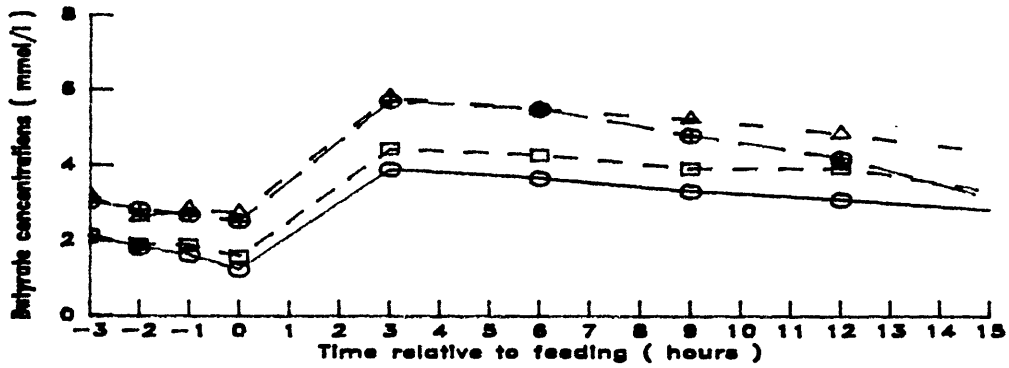
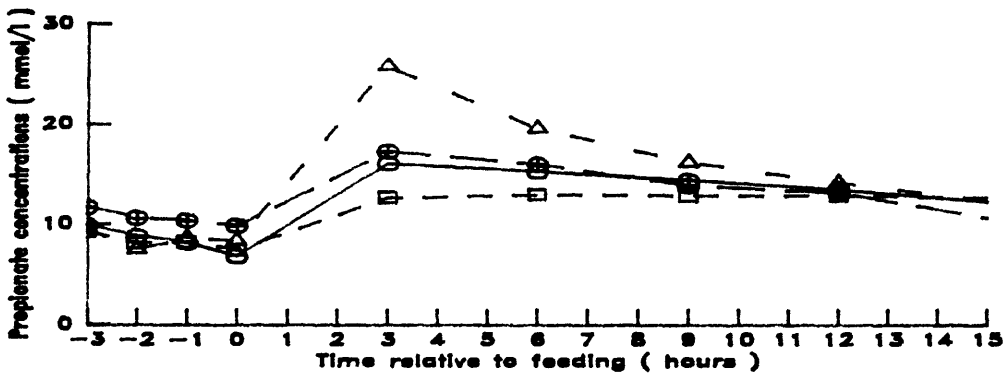
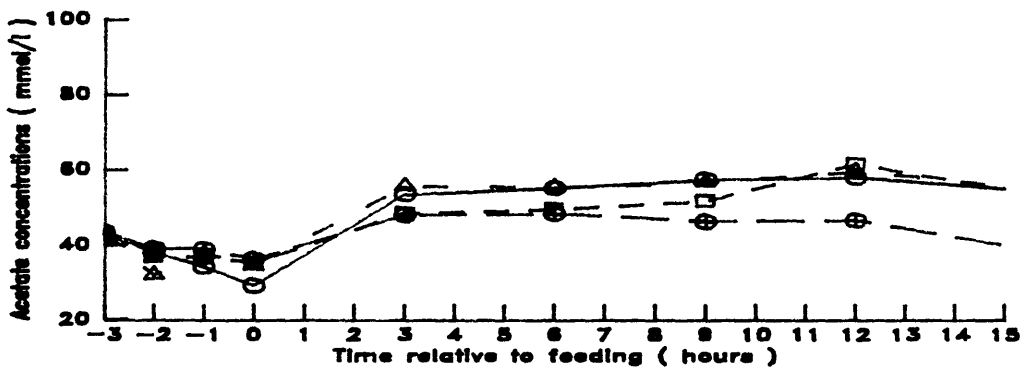


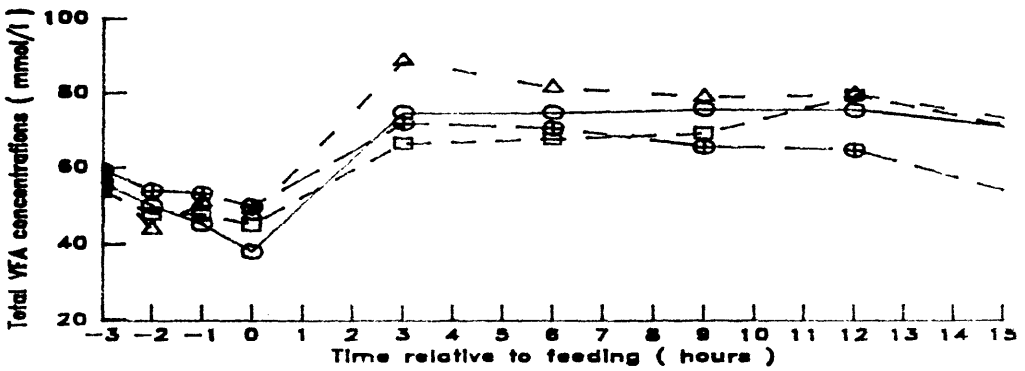
Figure 5.1.2 Variation in rumen NH_3-N concentrations in Experiment 1.
* defaunated + urea; 0 defaunated without urea ;
□ refaunated + urea; Δ refaunated without urea

Figure 5.1.3 Variations in TVFA and the individual acid concentrations in defaunated and refaunated sheep fed a diet of oaten chaff with or without urea supplements (Experiment 1).
O defaunated + urea ;□ defaunated without urea ;△ refaunated + urea ;● refaunated without urea


Butyrate concentrations (mmol/l)
0
2
4
6
8
−3 −2 −1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time relative to feeding (hours)


Propionate concentrations (mmol/l)
0
10
20
30
−3 −2 −1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time relative to feeding (hours)


Acetate concentrations (mmol/l)
20
40
60
80
100
−3 −2 −1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time relative to feeding (hours)


Total VFA concentrations (mmol/l)
20
40
60
80
100
−3 −2 −1 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time relative to feeding (hours)

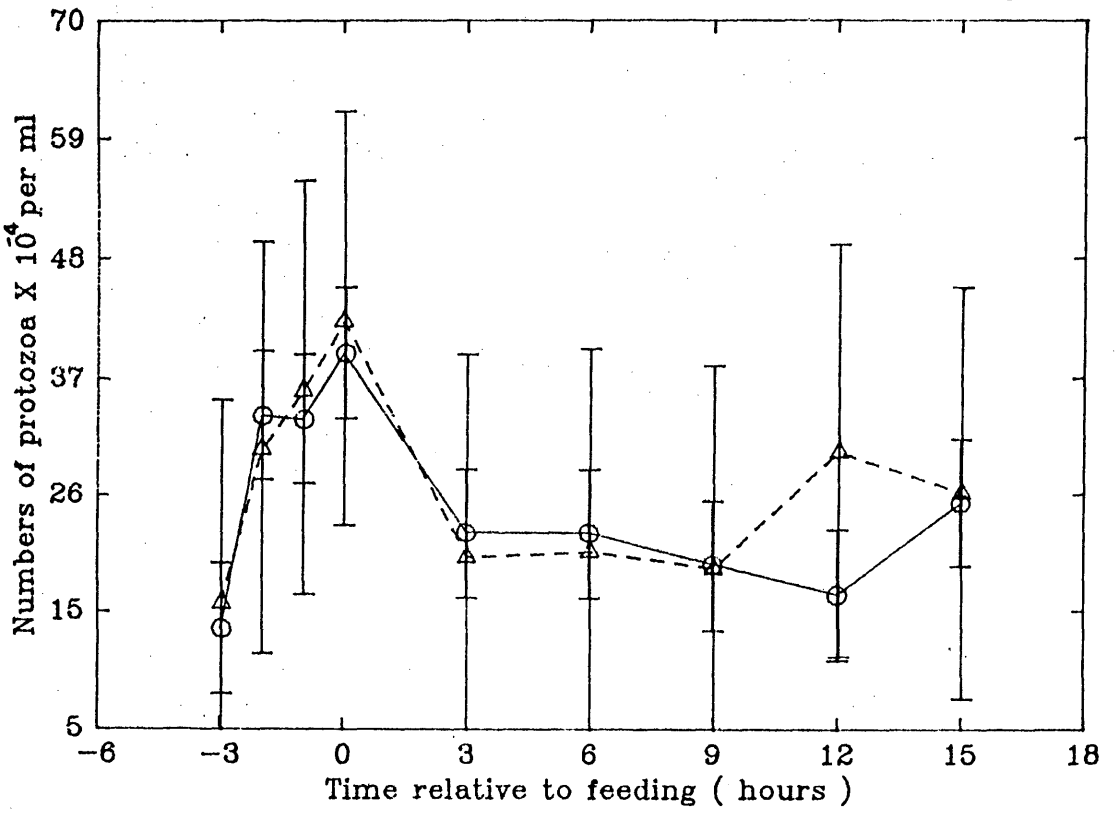


Figure 5.1.4 Variation in the number of rumen protozoa in Experiment 1. Vertical bars are standard deviations. Solid line (———) is refaunated sheep with urea supplement; dashed line (- - - - - - -) is refaunated sheep without urea

5.5 Discussion

The use of alkanate 3SL3 (ICI, Pty. Ltd., Australia) as an anti-protozoal agent proved to be effective as no protozoa were detected in the rumen fluid of defaunated sheep during the data collection period. The 24 h fasting period used in this study prior to alkanate treatment may improve the efficacy of anti-protozoal agents, as also reported by Burggraaf (1980).

As discussed in Chapter II, the removal of protozoa from the rumen has often been reported to decrease fibre digestibility in the rumen. The rate of dry-matter disappearance *in sacco* in the present experiment is not in agreement with results reported in a number of studies reviewed by Demeyer (1981), or the more recent results of Kayouli *et al.* (1984). This difference may be due to the diet used in this study which was fibre-based, in contrast to other experiments reviewed by Demeyer (1981) in which animals were often fed substantial amounts of concentrates.

The high-fibre diets used in the present study supported high numbers of fungal sporangia on feed particles. In addition, fungal sporangia numbers were increased in the absence of protozoa, suggesting that competition occurs between these microorganisms. This may have a profound effect on the loss of dry matter of oaten chaff determined *in sacco*; Akin *et al.*, (1983) have shown that an increase in the number of sporangia from 0.6 to 3.3 per 0.75 mm^2 on the leaf blades of grass caused an increase of at least two-fold in the fragility of plant particles when incubated *in vitro*. In addition, although the numbers of rumen bacteria were not determined in this study, there is ample evidence in the literature that defaunation causes an increase in the numbers of rumen bacteria free in the medium, and on feed particles (see for example Bryant and Small, 1960; Eadie and Hobson, 1962; Hungate, 1966; Orpin and Letcher, 1984). Thus the higher rate of disappearance of fibre upon defaunation in the present study appears to be due to increases in numbers of both cellulolytic bacteria and anaerobic fungi in the absence of competition from protozoa.

There is no strong evidence that rumen protozoa digest significant amounts of fibre; only a few large protozoa (e.g. *Epidinium spp., Polyplastron spp.)* appear to be actively cellulolytic (Jouany and Senaud, 1979).

Although the proportion of active cellulolytic bacteria has been reported by some workers to increase in the presence of protozoa (Yoder *et al.*, 1966; Kurihara *et al.*, 1978; Jouany and Senaud, 1979), it is clear that neither cellulolytic bacteria nor rumen ciliates are able to penetrate the epidermal layer of plant tissues which provide an initial barrier to digestion (Brazle and Harbers, 1977). In contrast, Bauchop (1980) has demonstrated that anerobic fungi do penetrate plant epidermal layers resulting in mechanical weakness and increased susceptibility to colonization by cellulolytic bacteria.

In general, rumen ammonia concentrations are directly proportional to protozoal concentrations (Christiansen *et al.*, 1963; Luther *et al.*, 1966). Thus, rumen ammonia concentrations are higher in faunated animals than in protozoa-free animals (Christiansen *et al.*, 1965; Klopfenstein *et al.*, 1966; Males and Purser, 1970; Lindsay and Hogan, 1972). The general appearance of lower ammonia concentrations in defaunated animals is often related to an increase in ammonia utilization by bacteria (Males and Purser, 1970).

The concentrations of rumen ammonia found in this experiment are in agreement with those reported in previous studies, even though the effect of defaunation in this regard was not statistically significant ($P > 0.05$). The mean values of ammonia concentrations (Table 5.1.4) both in the defaunated (except for sheep which received no urea supplements) and refaunated animals, were however still in the range of levels for maximum rates of fermentation (i.e.: 50-60 mgN/l) as suggested by Allison (1970) and Satter and Slyter (1974), even though other workers found that the optimum concentrations for maximal rate of fermentation were in the order of 88-133 mgN/l (Hume *et al.*, 1970) and up to 235 mgN/l (Ørskov *et al.*, 1977). Nevertheless, the mean ammonia levels in the defaunated sheep which

received no urea supplements may not impair microbial growth; Hespell and Bryant (1979) have stated that the ammonia level in the rumen would seldom limit growth of ammonia-requiring bacteria significantly because the K_s values of rumen ammonia were in the order of 0.1 to 0.2 mM. Satter and Roffler (1977) stated that the critical level of NH_3-N for maintaining maximum rumen microbial growth was about 20 mgN per litre. Thus, whether or not the ammonia concentrations found in this study were sufficient for maximal rates of fermentation in the rumen cannot be determined.

Although Whitelaw *et al.*, (1972) have reported that, relative to defaunated cattle, the presence of ciliates generally resulted in decreases in TVFA concentrations and in the ratio of propionic to butyric concentrations in rumen fluid, other workers were unable to demonstrate the effect of defaunation on the VFA pattern in either lambs (Luther *et al.*, 1966; Eadie and Gill, 1971) or calves (see for example Williams and Dinusson, 1973). In the present study (Table 5.1.3) there were no significant effects of defaunation on either TVFA or individual acid concentrations; this tends to support the recent conclusion drawn by Bird (1982) that the concentrations of TVFA in the rumen are generally not affected by defaunation.

5.6 Experiment 2

DIGESTION OF WHEAT STRAW BY DEFAUNATED AND REFAUNATED SHEEP

5.6.1 Introduction

Dry-matter disappearance of oaten chaff in the rumen of sheep in Experiment 1 was significantly higher in the defaunated state compared with the refaunated state. The increase in dry-matter disappearance may be related indirectly to increased numbers of sporangial colonies which appeared on feed particles (see Table 5.1.3), although an increased bacterial population may also be involved. This finding tends to support the results of previous workers who suggested that rumen fungi may play an important role in fibre digestion (Bauchop, 1979a; Akin *et al.*, 1983).

A second experiment was designed to examine whether similar results could be achieved with a different source of high-fibre feed such as wheat straw which is more resistant to ruminal fermentation than is oaten chaff. The influence of supplements (urea and cotton seed meal) that potentially provide essential nutrients for micro-organisms was also studied. The hypothesis was that the decrease in fungal growth in the presence of protozoa was due to a competition for essential nutrients between fungi and protozoa. For this reason, the same procedures as used in the first experiment were applied to this current study, unless otherwise stated in a relevant section.

5.6.2 Experimental

i. Animals and feeding

Four mature cross-bred wethers (approximately 3 years old), weighing between 33-36 kg, and fitted with permanent ruminal cannulae, were housed individually on slatted floors under continuous lighting. They were fed a fixed amountof a diet of wheat straw, cotton seed meal (CSM), urea and mineral mixture as shown in Table 5.2.1. The ration was given at 1000 h and 1200 h.

Table 5.2.1 Composition of the diet used in Experiment 2 (apart of dry-matter, values are on a dry-matter basis)

Ingredients	Quantity (g)	Chemical analysis				
		DM	OM	NDF	ADF	CP(NX6.25)
		%				
Wheat straw	700	92	92	85	56	3.9
CSM	50	92	93	nd	nd	42.5
Urea	14	-	-	-	-	-
Mineral mix.+)	24	-	-	-	-	-

nd = not determined

+) mineral mix. composed of: 57% CaH_2SO_4, 14.3% Na_2SO_4, 14.3% NaCl, 14.3% Ruminant premix (Pfizer quote 422). Each kg premix contained macro- and trace elements and vitamins as indicated in the foot note to Table 5.1.1.

ii. Experimental procedures

Dry-matter disappearance of feed in the rumen was determined by incubating in nylon bags approximately 5.0 g air-dried wheat straw (ground to pass a 1 mm screen) in the rumen for 6, 12, 24, 48 or 72 h. In both the defaunated and refaunated periods the residue in the nylon bags after incubation were analysed for acid-detergent fibre (ADF) using the method of Goering and Van Soest (1970) as already described in Section 3.5.3. The results were calculated as ADF disappearance (ADFD) against time.

The numbers of fungal sporangia were counted on the basis of visible sporangia which appeared on wheat straw suspended in nylon bag in the rumen for 6, 12, 24 or 48 h.

iii. Statistical analysis

Differences between treatments were analysed statistically using a paired t-test (Snedecor and Cochran, 1967). Differences in dry-matter disappearance and acid-detergent fibre disappearance were compared between treatments using regression analysis. For ruminal ammonia concentrations, pH, VFA and individual acid concentrations, the two treatments were compared at each sampling time.

5.6.3 Results

No protozoa were detected during data collection in the defaunated animals.

a) Rates of dry-matter and acid-detergent fibre disappearance

The DMD of wheat straw used here was not as high as the DMD of the oaten chaff used in Experiment 1. However, defaunation resulted in a marked increase in the disappearance rate of DM from the nylon bags, suggesting that the rate of fibre digestion of straw was improved in the absence of rumen ciliates (Figures 5.2.1 and 5.2.2). The differences were more prominent at 6 and 12 h for both DMD and ADFD than at other times.

Regression analysis of the mean difference between treatments showed a significant difference for both DMD and ADFD ($P < 0.01$). The regression equation for the mean difference in DMD was $Y = 6.06 - 0.09\ X$ ($r^2 = 0.92$) and in ADFD was $Y = 7.38 - 0.115X$ ($r^2 = 0.85$) where Y = mean difference between treatment and X = incubation time.

b) Fungal sporangia counts

Removal of protozoa from the rumen increased the numbers of fungal sporangia attached to feed particles (at least $P < 0.05$) at all incubation times except 24 h (Table 5.2.2). These differences may be due to a reduction in the length of the life cycle of these microorganisms in the absence of competition for essential nutrients from rumen ciliates.

Table 5.2.2 Effect of defaunation on the numbers of fungal sporangia appearing on feed particles in Experiment 2. Values are means of four sheep per treatment

Treatment	Incubation time (h)			
	6	12	24	48
	No. of colonies/mm^2			
Defaunated	136	68	61	35
Refaunated	73	57	57	13
SED	19.0	3.2	1.2	3.4
Significance	*	*	ns	**

* significant at $P < 0.05$; ** significant at $P < 0.01$; ns = non-significant
SED = Standard Error of Mean Difference.

C) Composition of rumen contents

i. pH

There were no significant differences (P >0.05) due to defaunation in this experiment. The pH in defaunated animals varied between 6.4 and 7.0, with a mean value of 6.64, while in the refaunated animals the corresponding figures were 6.4 - 7.0 with a mean value of 6.74 (Figure 5.2.3).

ii. Ammonia concentrations

In contrast to Experiment 1, the mean ammonia concentrations in the defaunated animals in this experiment were higher than those in the refaunated sheep, even though the differences were only statistically significant at 2 h before and at feeding time (P <0.01) (Figure 5.2.4).

iii. VFA concentrations

There were no significant differences in TVFA or individual acid concentrations due to defaunation. Neither diet nor defaunation had any significant effect on the proportions of the individual acids (Figures 5.2.5 and 5.2.6). The proportions of valerate and iso-VFA's (i.e.: iso-butyrate and iso-valerate) were negligible (never more than 1.5%) in both the defaunated and refaunated states.

iv. Protozoal numbers in refaunated sheep

As found in the first experiment, the predominant species of rumen ciliates of this study were small Entodiniomorphs. The minimum number was found at 12 h after feeding (26 x 10^3/ml rumen fluid), while the highest number was obtained at feeding time (59 x 10^3/ml rumen fluid) (Figure 5.2.7).

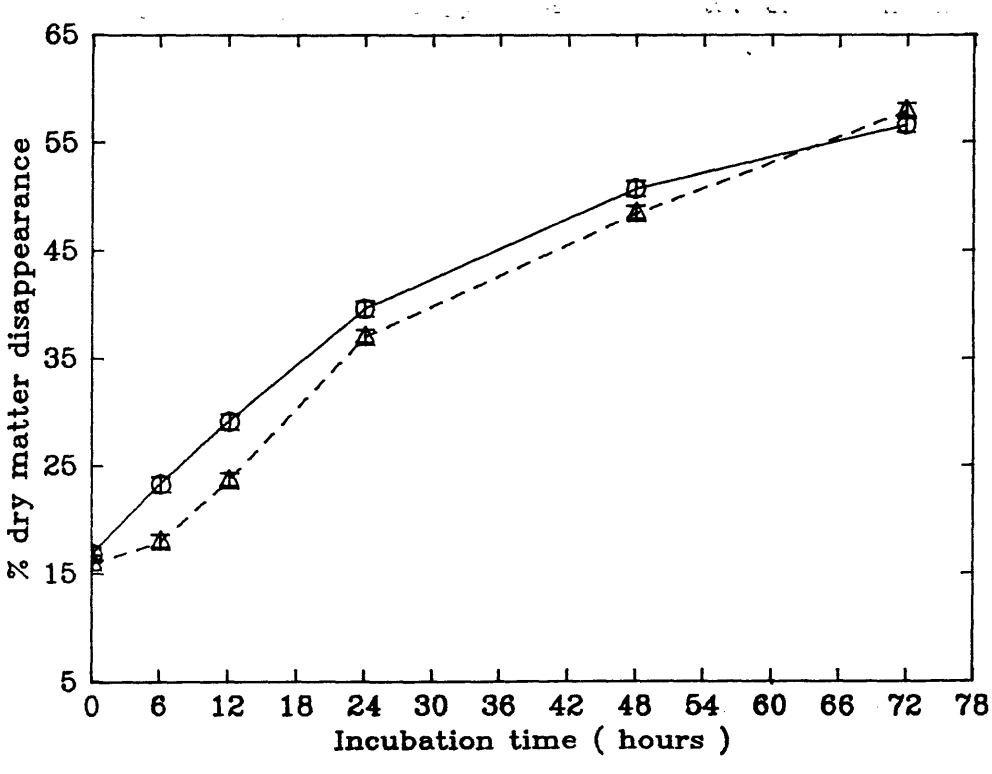


Figure 5.2.1 Dry matter disappearance in sacco of wheat straw in Experiment 2. Solid line (——) and dashed line (-----) denote defaunated and refaunated animals respectively. Vertical bars are standard deviations.

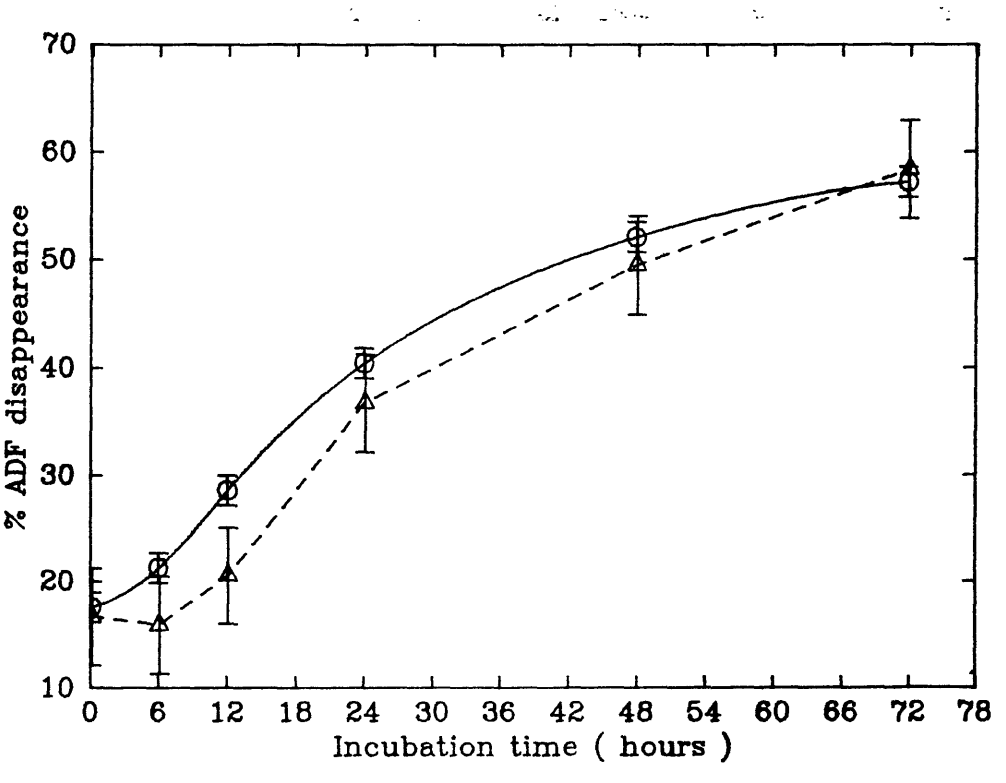


Figure 5.2.2 ADF disappearance in sacco of wheat straw in Experiment 2. Solid line (———) and dashed line (------) denote defaunated and refaunated animals respectively. Vertical bars are standard deviations.

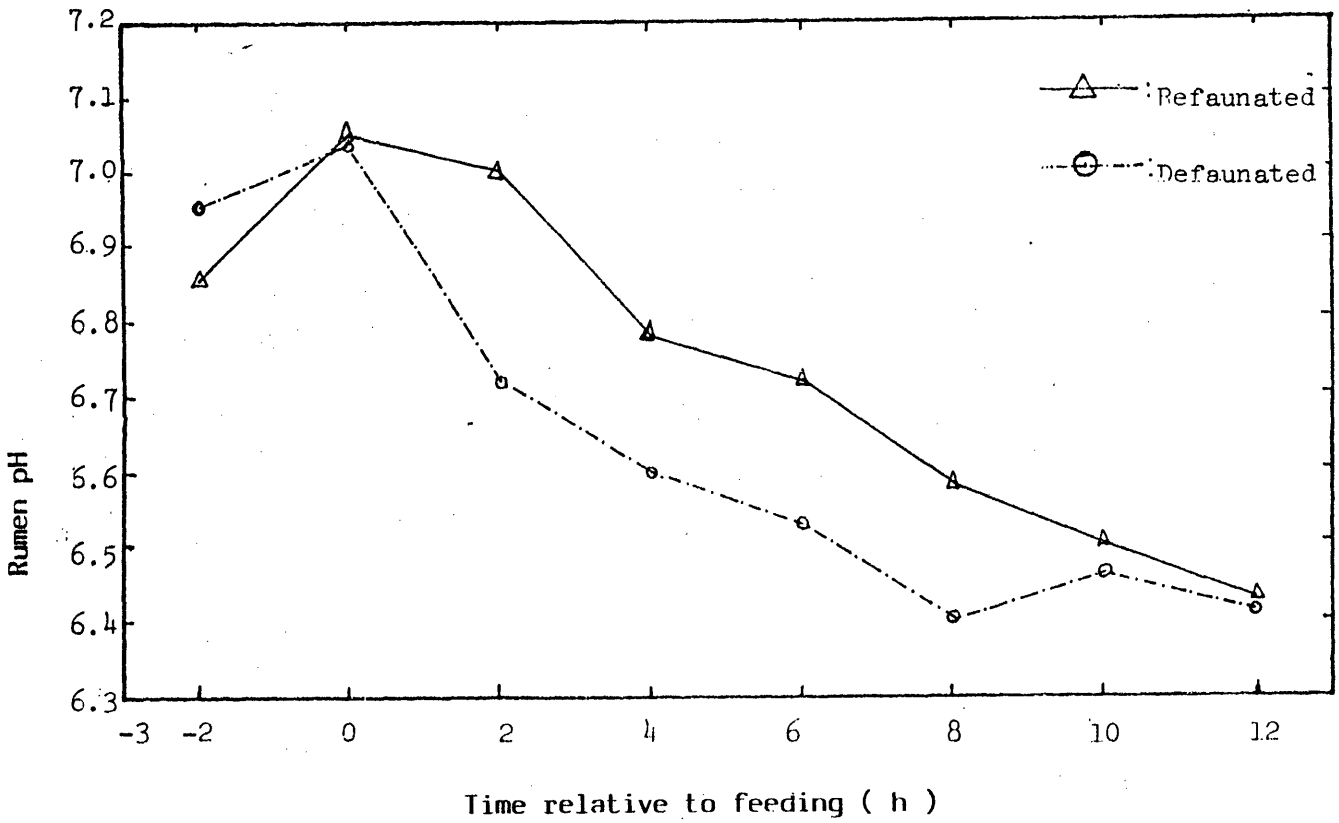


Figure 5.2.3. Variation in rumen pH in Experiment 2

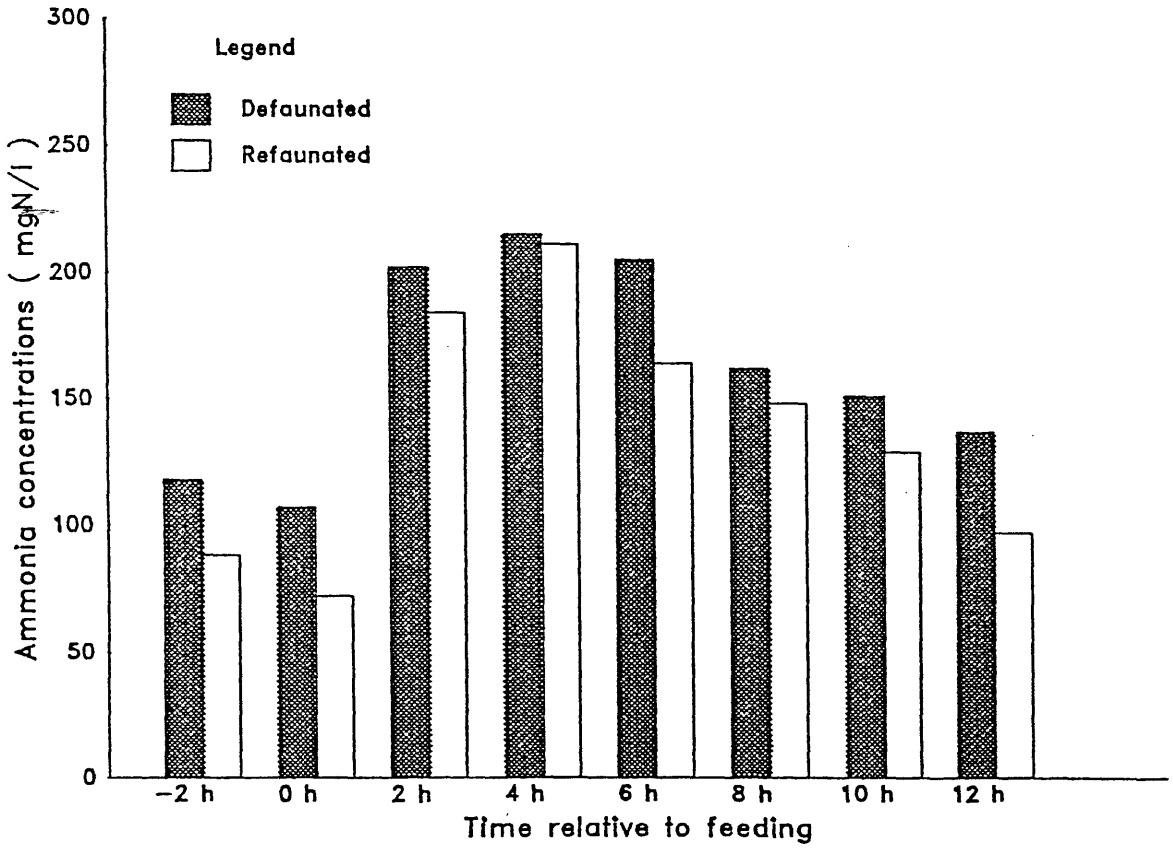


Figure 5.2.4 Variation in rumen NH_3-N concentration in Experiment 2

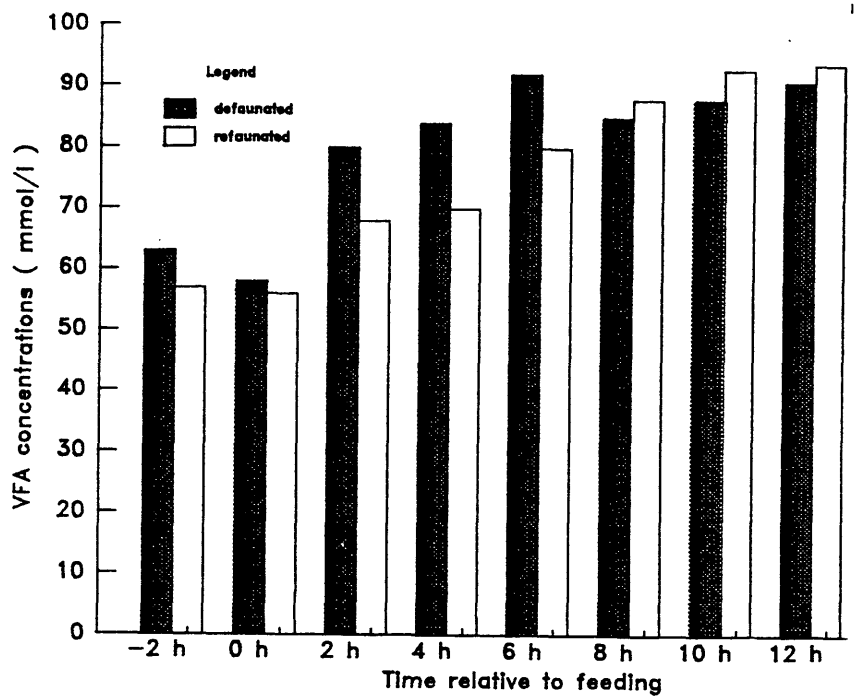


Figure 5.2.5 Variation in total VFA concentration in Experiment 2

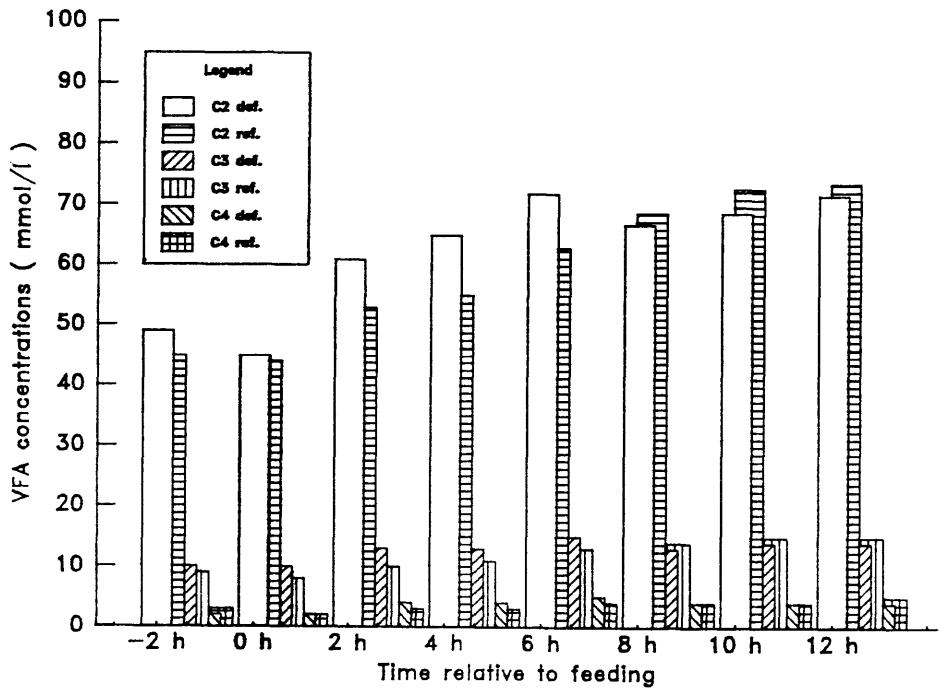


Figure 5.2.6 Variation in individual VFA concentration in Experiment 2. C_2 , C_3 and C_4 denote acetate, propionate and butyrate , respectively. Def.= defaunated; ref.= refaunated

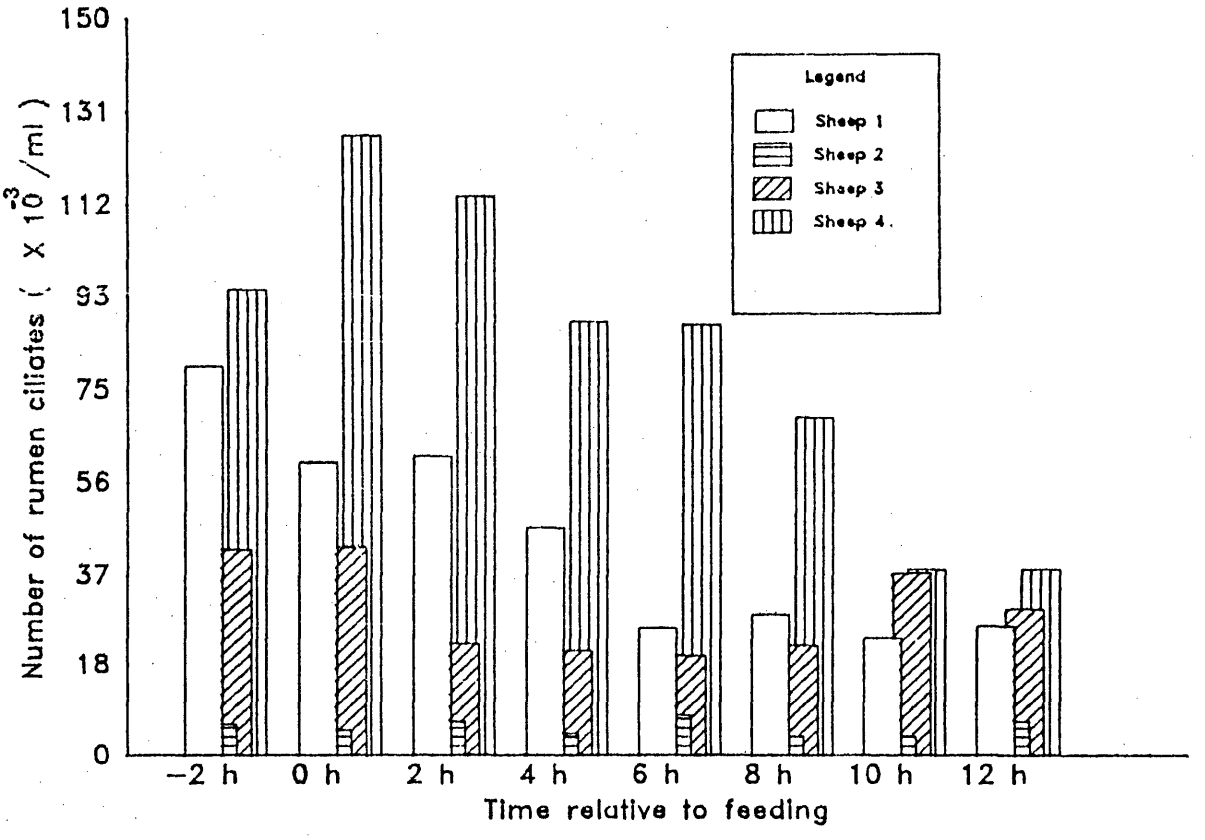


Figure 5.2.7 Fluctuation in the numbers of rumen ciliates in refaunated sheep used in Experiment 2

5.6.4 Discussion

As can be seen in Figures 5.2.1 and 5.2.2 the defaunated state resulted in an increase in the rate of disappearance of DM and ADF even though the difference narrowed as incubation time in the rumen increased. This effect may be important since the rate of digestion is known to influence voluntary feed intake which is a dominant factor in maximizing animal productivity.

The difference between results reported here and those of previous work from other laboratories (see Demeyer, 1981) and reported by Kayouli *et al.* (1984) may be attributed to different proportions of roughage included in the diet. From the present study, it is clear that on high-fibre diets the numbers of rumen protozoa do not reach population densities found in the rumen of animals fed substantial amounts of concentrates which is often as high as 10^6/ml (see for example Kayouli *et al.*, 1984). Kurihara *et al.* (1978) found that on purified diets the numbers of rumen ciliates dropped to as low as 10^3/ml of rumen contents, indicating a need for essential nutrients for protozoa which were perhaps absent in the purified diet.

Kurihara et al. (1978) also reported that cellulose digestion in sacco in the rumen of faunated sheep was about twice greater than that of in defaunated sheep, which is contrary to the results reported herein. According to the authors this was related to both a marked increase in the concentrations of cellulolytic bacteria and a decrease in pH from 6.5 to 6.3 in the rumen of the faunated sheep. This is surprising, since reports by Stewart (1977) and Hiltner and Dehority (1983) indicate that a decrease in rumen pH from 6.5 to 6.3 or below has a profound negative effect on the activity of cellulolytic microorganisms.

Alternatively, independent of pH, the decrease in cellulolytic bacterial numbers in defaunated sheep reported by Kurihara et al.(1978) may be related to the inclusion of starch in the diet. The resultant rapid growth of amylolytic bacteria (Kurihara et al.,1978) would be

expected to depress cellulolysis as has generally been found in other similar studies (see Orpin and Letcher, 1979; Stewart *et al.*, 1979; Mertens and Loften, 1980). On the other hand, although the numbers of rumen protozoa in the faunated sheep were very low (Kurihara *et al.*, 1978), they may have helped to reduce the concentration of starch in the rumen by ingestion of these materials, thereby supressing the amylolytic bacteria and allowing faster growth of the cellulolytic bacteria.

In the present experiment, although priority was given to enumerating the rumen fungi and protozoa rather than the bacteria, the higher DM and ADF disappearance in the defaunated animals might be indirectly attributable to the increase in numbers of rumen fungi as indicated by the numbers of sporangial colonies on feed particles. Thus, the apparent conflicting results of this study and that of Kurihara *et al.* (1978) can be tentatively explained on the basis of the different diets used, and the resultant different microbial populations in the rumen.

Gordon and Ashes (1984) reported that some species of rumen fungi could digest the ADF fractions of wheat straw *in vitro* to as much as 28%. However, Widham and Akin (1984) recently demonstrated that rumen protozoa wereunable to digest ADF or Cellulose of any substrate used, suggesting that rumen protozoa play a minimal role in degrading fibre.

The higher ammonia concentrations at 2 h before and at feeding time in the defaunated animals in this experiment are in contrast to the lower ammonia concentrations following defaunation in Experiment 1. It is therefore concluded that defaunation is not always associated with a decrease in rumen ammonia concentrations. This is supported by reports in the literature, some of which indicate that defaunation decreases ammonia concentrations (e.g. Abou Akkada and El-Shazly, 1964; Christiansen *et al.*, 1965; Klopfenstein *et al.*, 1966; Luther *et al.*, 1968; Males and Purser, 1970; Eadie and Gill, 1971; Williams and Dinnuson, 1973; Demeyer and Van Nevel, 1979; Veira *et al.*, 1983; 1984)

while some do not (e.g. Kurihara *et al.*, 1978; Demeyer *et al.*, 1982). This lack of agreement is probably associated with inclusion of protein and urea in the diets to ensure that ammonia concentrations were sufficiently high to sustain maximal microbial growth (Satter and Slyter, 1974).

As in the first experiment, the effect of defaunation on TVFA and individual acid concentrations was not significant. Other workers have reported that defaunation did alter the concentrations of either TVFA or the three main acids (Klopfenstein *et al.*, 1966; Males and Purser, 1970; Kurihara *et al.*, 1978; Demeyer and Van Nevel, 1979; Veira *et al.*,1983; Kayouli *et al.*, 1984). However, Hobson and Wallace, (1982) suggested that, as with ammonia concentrations, the effects of defaunation on TVFA and the individual acids are not always predictable. Variations are due not only to the different diets used in the experiments, but also to variations in animals and the composition of the microflora, both of which are greatly influenced by both internal and external environments.

5.7 Experiment 3

EFFECTS OF DEFAUNATION ON THE GROWTH OF RUMEN FUNGI AND ON FIBRE DIGESTION IN SHEEP

5.7.1 Introduction

The results of Experiments 1 and 2 indicated that when rumen protozoa were removed, fibre digestion was improved with no apparent systematic changes in rumen fermentative products. It was also found that the numbers of sporangial colonies appearing on feed particles suspended in the rumen were higher in defaunated sheep. These findings strengthen the suggestion that these fungi play a significant role in fibre digestion, especially through their ability to penetrate deeply into plant tissue by expansion of their rhizoids. It is apparent that this initial penetration of plant tissue by fungi allows bacteria to colonize underlying substrates which, in the absence of fungi, may escape fermentation in the rumen (Akin, 1981).

Enumeration of fungal sporangia attached to feed particles in order to measure the relative numbers of fungi in the rumen is somewhat unsatisfactory, since rumen fungi show selective attachment to certain plant tissues (Chapter IV). Similarly, to quantify the fungal population by enumerating the viable zoospores in fresh rumen fluid, as done by Orpin (1975; 1976a; 1977b) may be imprecise, particularly when rumen protozoa are present, due to the difficulty of distinguishing between viable zoospores and some protozoa. The use of roll tubes to grow rumen anerobic fungi has been reported by Joblin (1981); this author has stated that the technique gives results comparable to those from the direct counting of zoospores.

The main objective of this experiment was to examine the reproductability of the results obtained in Experiment 2. The roll tube technique, together with enumeration of sporangial colonies attached to feed particles, were used to compare numbers of rumen fungi both in the presence and the absence of protozoa. The studies were also

expanded to test whether an apparent increase in digestibility in the rumen as measured using nylon bags resulted in an overall increase in digestibility in the animal. Rumen fluid kinetics were also studied by reference to the decline in concentration of a single dose of Cr-EDTA over time.

5.7.2 Experimental

i. Animals and Feeding

Three mature crossbred wethers from Experiment 2 were used in this study, and were fed the same diet as in that experiment. The composition and chemical analysis of the diet used in this experiment were similar to those used in Experiment 2 (see Table 5.2.1).

ii. Experimental procedures

The experimental procedures employed in this experiment were similar to those used in Experiment 2 except for the following additional parameters: counting the numbers of zoospores in culture media in roll tubes; determination of apparent digestibility *in vivo*; and rumen fluid kinetics. These procedures are outlined in Sections 3.4.5.a, 3.4.4.b and 3.6. Apparent digestibility *in vivo* was determined over six days at the end of each period. Rumen fluid which was used as an inoculum for anaerobic culturing of rumen fungi was taken 2 h before and 2 h after feeding.

iii. Statistical analysis

All statistical procedures which were used in Experiment 2 (Section 5.6.2) were applied to the data obtained from this study.

5.7.3 Results

a. Feed digestibility

The results of the *in sacco* digestion study indicate that DM and ADF disappearances in the defaunated sheep were higher (see Figures 5.3.1 and 5.3.2) but the differences were not statistically

significant. This may be associated with the small numbers of sheep used in this study, even though the differences in DMD and ADF disappearances were up to 4 and 5 percentage units, respectively. In this study, regression analysis of the mean difference between treatments both for DM and ADF disappearances were not statistically significant and were best described by quadratic equations: $Y=3.03 - 0.0386 X 0.0003 X^2$ ($r^2=0.11$) for DM disappearance; and $Y= 5.37 - 0.172 X +0.018 X^2$ ($r^2=0.57$) for ADF disappearance, where Y = mean difference between treatment and X = incubation time.

The effect of defaunation on apparent dry matter and organic matter digestibilities *in vivo*, however, were statistically significant at $P > 0.05$ and $P > 0.01$, respectively. There was no statistical difference in feed intake due to defaunation (Table 5.3.1).

b. Fungal sporangia and zoospore counts

Numbers of fungal sporangia were higher in the absence of rumen protozoa than in the refaunated sheep (Table 5.3.2). This result is in accord with results obtained from the first two experiments. The differences between treatments in the numbers of fungal sporangia were significant ($P < 0.05$) except at 12 and 24 h. Fungal zoospores developed on agar medium containing antibiotics in roll tubes indicated that defaunation caused an increase in the numbers of zoospores in the rumen by 12-fold (Table 5.3.2 and Plate 5.3.1). Surprisingly, however, the differences were not statistically significant ($P > 0.05$).

c. Composition of rumen contents

i) pH

In general, rumen pH was not different between defaunated and refaunated animals, except at 4 h after feeding when defaunation cased an increase in rumen pH ($P > 0.05$). The mean pH ranged from 6.5 to 7.0 (Figure 5.3.3).

Table 5.3.1 Intake and apparent digestibility in vivo of dry matter and organic matter by defaunated and refaunated sheep (Experiment 3)

Items	Treatment		SED
	Defaunated	Refaunated	
Mean body weight (kg)	34	32	
Dry matter intake(g)	661	556	52.5
Organic matter intake(g)	607	510	48.6
App.Dry Matter Dig.(%)	54*	45	1.2
App.Organic Matter Dig.(%)	59**	49	1.0

* significant from refaunated sheep at $P < 0.05$

** significant from refaunated sheep at $P < 0.01$

Table 5.3.2 Effect of defaunation on the numbers of fungal sporangia appearing on feed particles and culturable fungal zoospores developing on agar medium containing antibiotics

Treatment	No.of sporangia appearing on straw particles(colonies/mm^2)				No.of culturable zoospores ($X10^{-3}$/ml)	
	6h	12h	24h	48h	2h before feeding	2h after feeding
Defaunated	235	206	59	39	24	17
Refaunated	160	75	49	12	2	3
SED	13.4	33.3	9.9	5.5	13.2	6.1
Significance	*	ns	ns	*	ns	ns

*significant difference between defaunated and refaunated states ($P < 0.05$)

ns = not significant

ii) Ammonia concentration

As found in Experiment 2, ammonia concentration in this study was generally higher in the defaunated animals at almost all sampling times compared with those in the refaunated animals, although not significantly so. Such differences may be related to variation in feeding behaviour and thus in rates of ingestion of different nitrogen sources (Figure 5.3.4).

iii) VFA

Defaunation significantly increased the concentrations of TVFA (Figure 5.3.5) at 2, 4, 8 and 10 h after feeding (at least $P < 0.05$) and, concomitantly, the concentrations of acetate and propionate at all sampling times after feeding. There were no significant differences in butyrate concentrations due to defaunation (Figure 4.3.6). As found in Experiment 2 the concentrations of iso VFA's and valerate were negligible (less than 1%) in both the defaunated and refaunated states.

iv) Protozoal population density

The protozoal population densities measured in the rumen fluid samples collected from the refaunated sheep are presented in Figure 5.3.7. As found in previous experiments, the predominant species (99%) was small *Entodinium spp.*, with the remaining 1% being made up of *Polyplastron spp.* and *Isotricha spp.*

d. Rumen fluid kinetics

Rumen fluid kinetics, estimated by the rate of decline in concentrations of Cr-EDTA in rumen fluid, showed that defaunation reduced the half life ($t_{\frac{1}{2}}$) of rumen fluid ($P < 0.01$), but had no significant effect on rumen volume or outflow rates (Table 5.3.3).

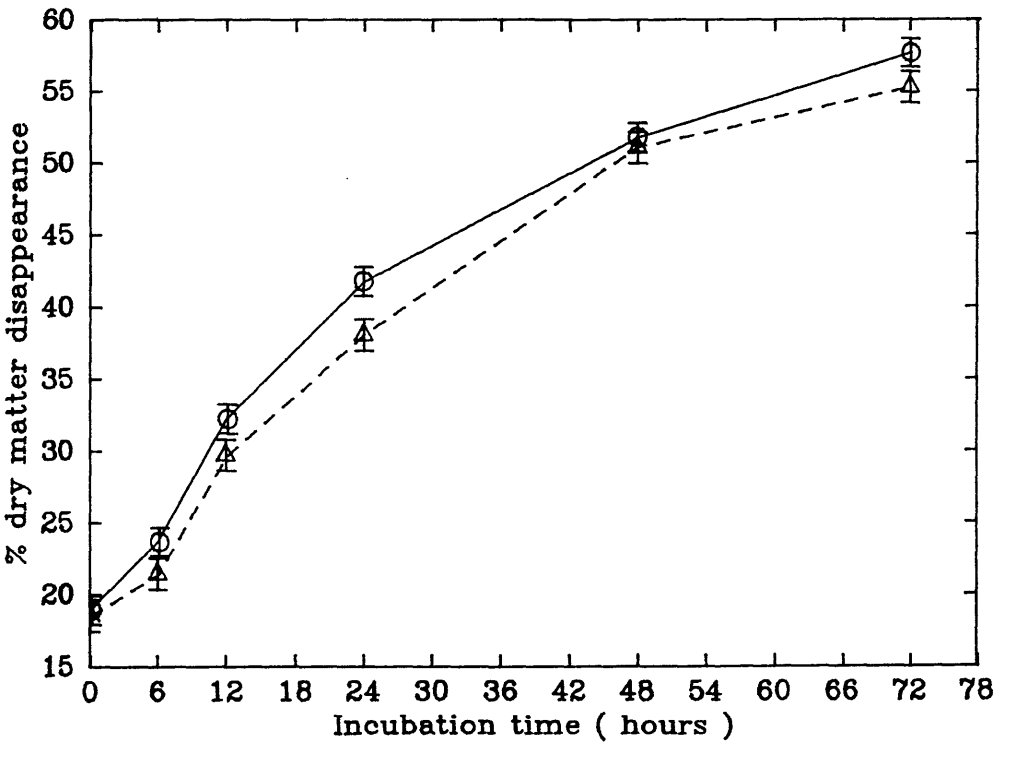


Figure 5.3.1 Dry matter disappearance in sacco of wheat straw in Experiment 3. Solid line (———) and dashed line (-----) denote defaunated and refaunated animals respectively. Vertical bars are standard deviations.

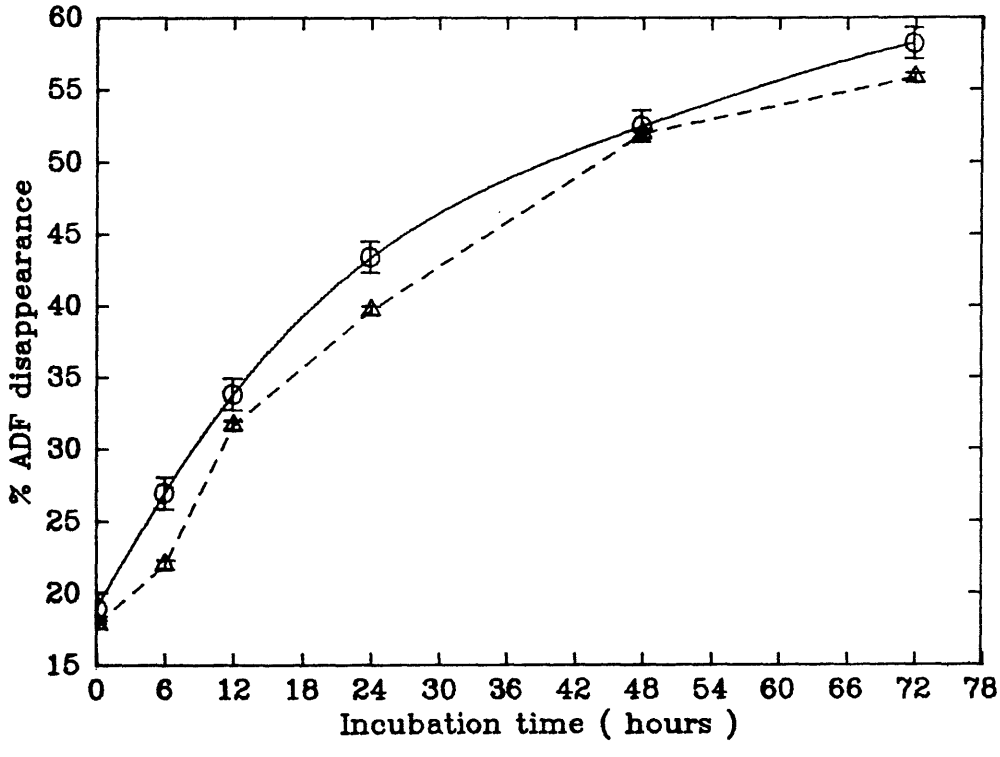


Figure 5.3.2 ADF disappearance <u>in sacco</u> of wheat straw in Experiment 3. Solid line (———) and dashed line (-----) denote defaunated and refaunated animals respectively. Vertical bars are standard deviations.

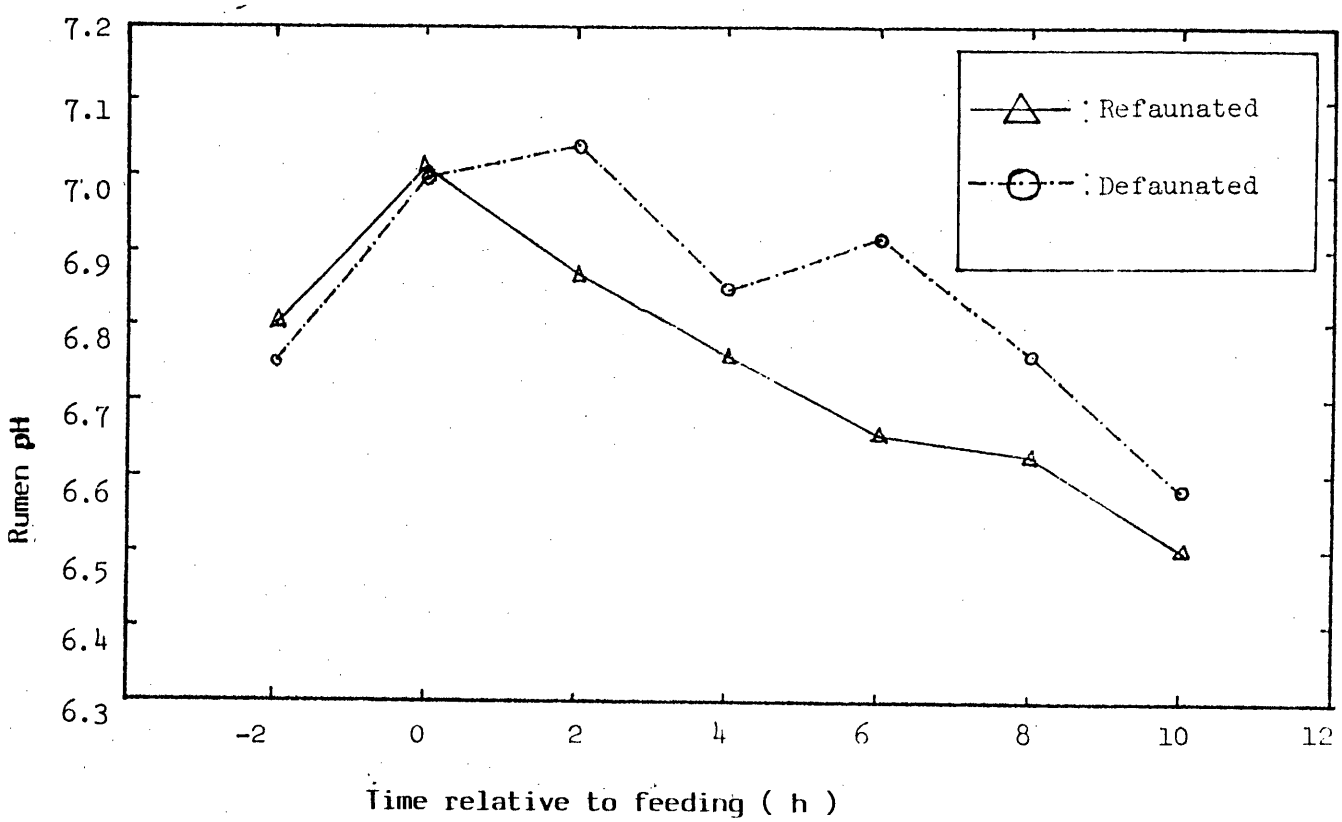


Figure 5.3.3 Variation in rumen pH in Experiment 3

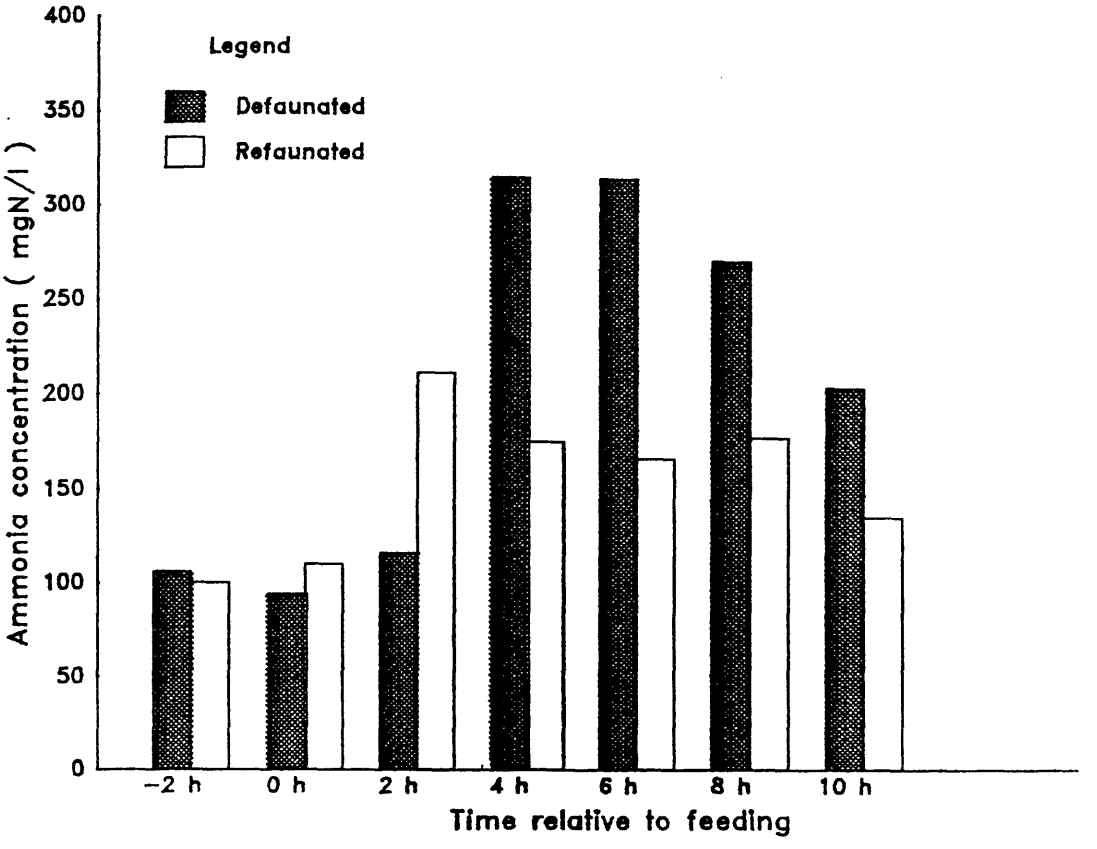


Figure 5.3.4 Variation in rumen NH_3-N concentration in Experiment 3

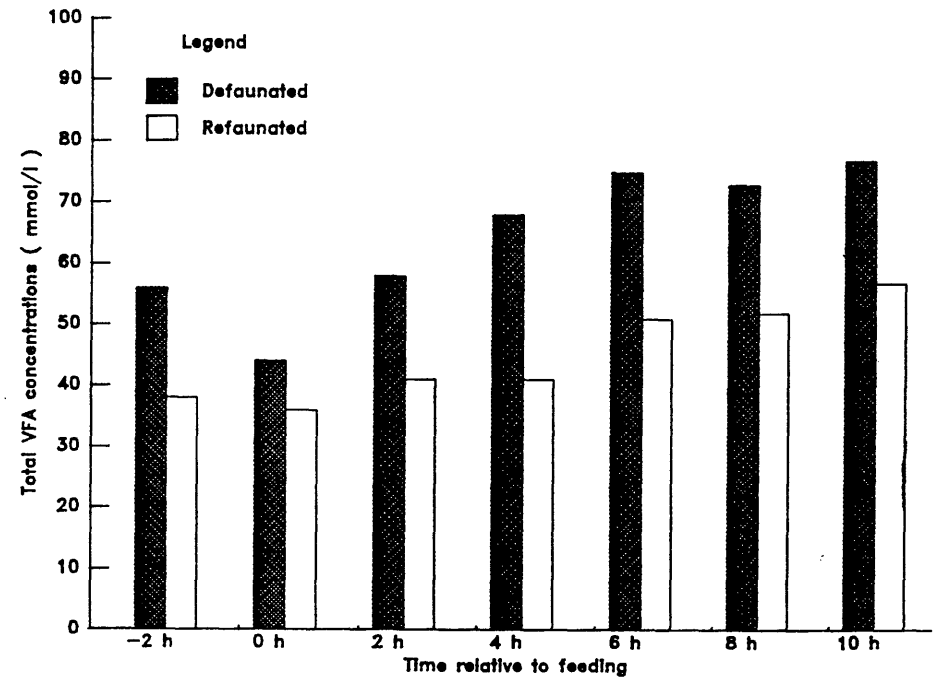


Figure 5.3.5 Variation in total VFA concentration in Experiment 3

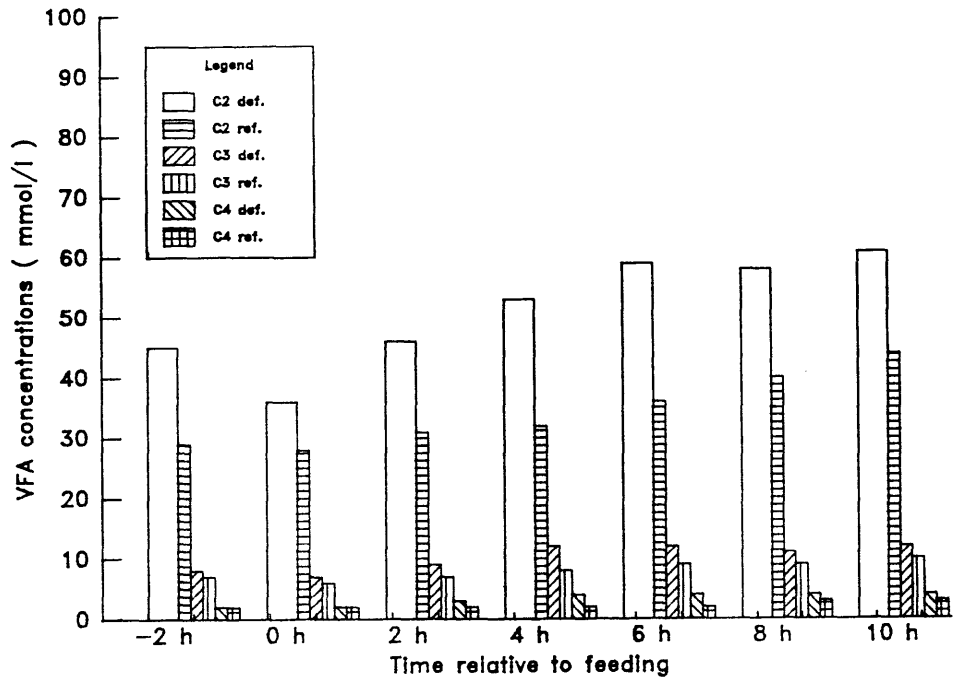


Figure 5.3.6 Variation in individual VFA concentration in Experiment 3. C_2 , C_3 and C_4 denote acetate, propionate and butyrate, respectively. Def.= defaunated ; ref.= refaunated

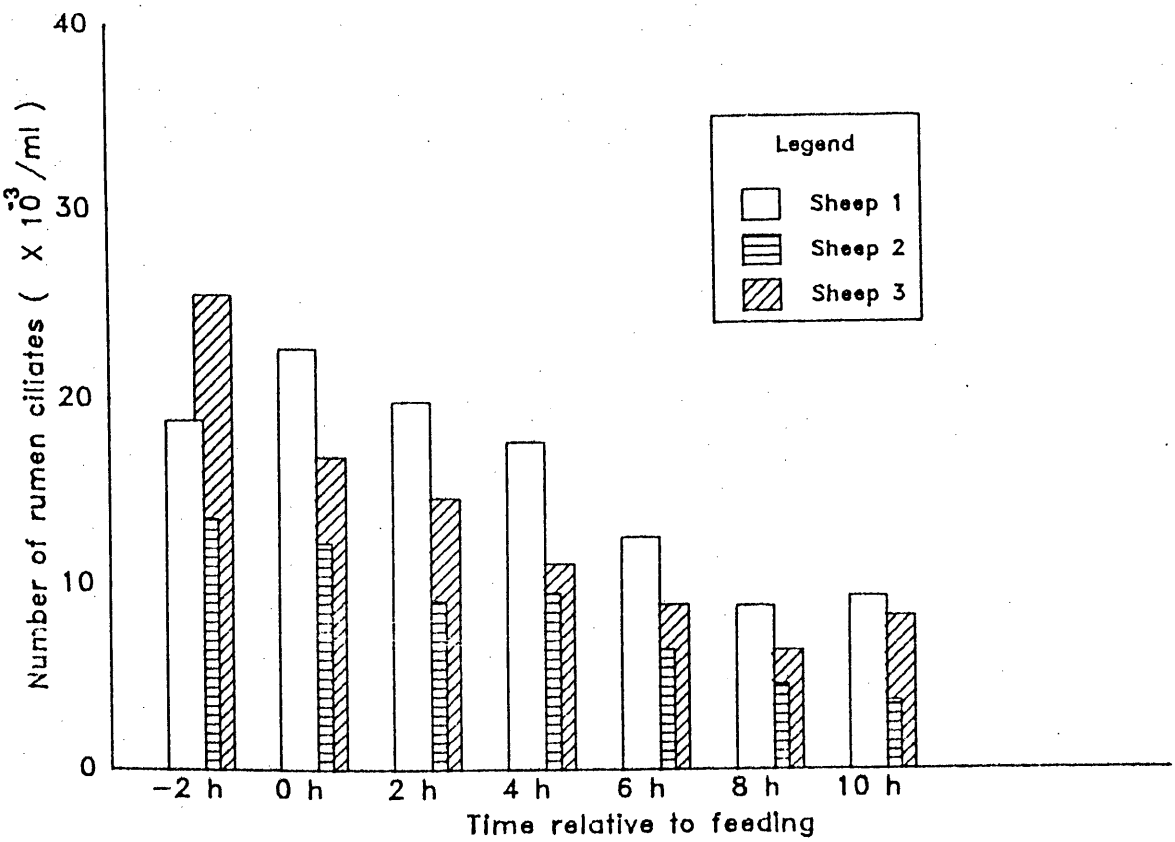


Figure 5.3.7 Fluctuation in the numbers of rumen ciliates in refaunated sheep used in Experiment 3

Table 5.3.3 Rumen fluid kinetics in defaunated and refaunated sheep (Experiment 3)

I t e m s	Defaunated	Refaunated	SED
Rumen volume (l)	6.81	6.90	1.08
Outflow (l/d)	9.95	8.91	1.43
$T\frac{1}{2}$ (min.)	683.21	772.43 **	6.74
Dilution rate (l/h)	0.06	0.05	-

** Significantly different from defaunated sheep (P 0.01)

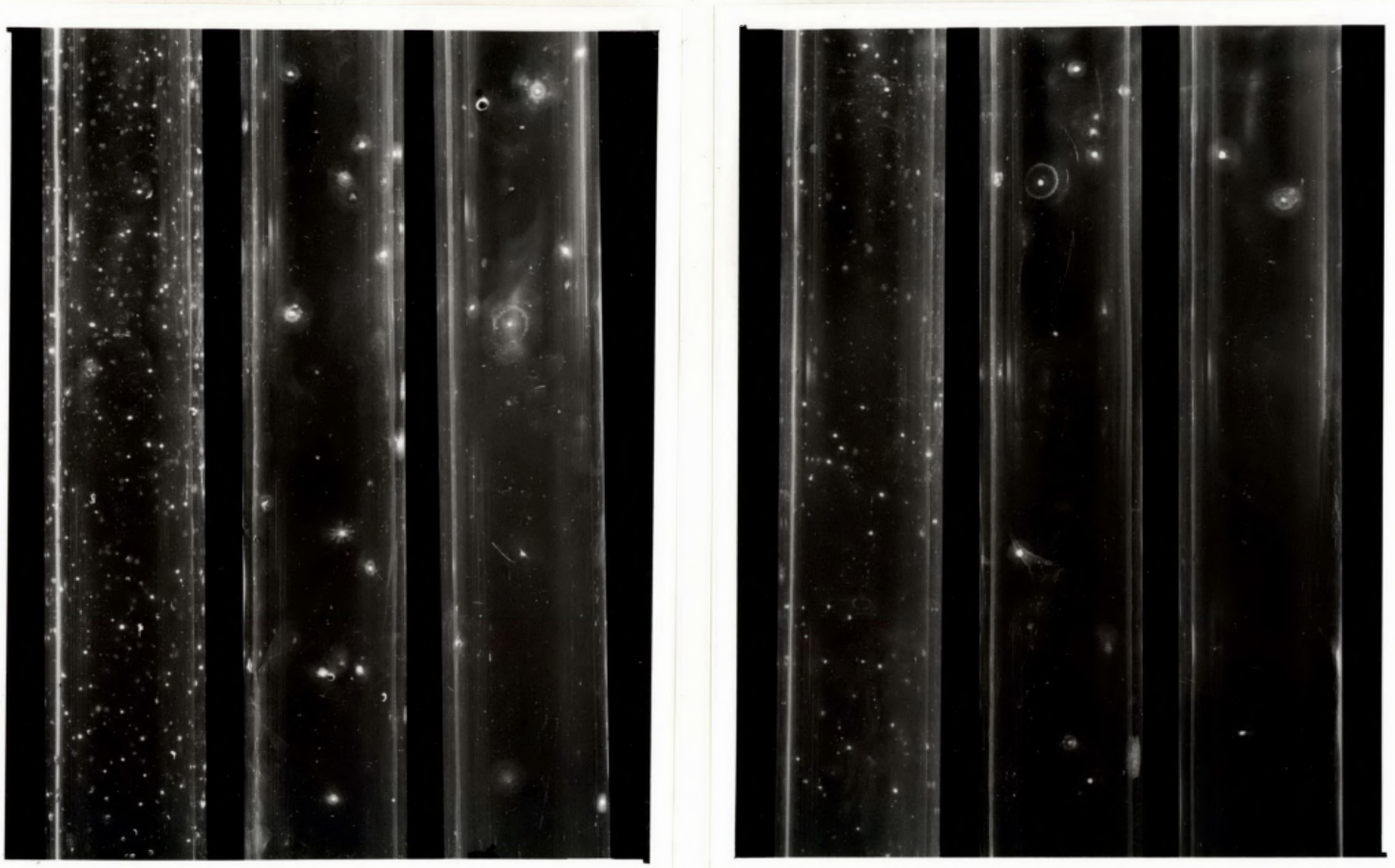


Plate 5.3.1 Rumen-anaerobic fungi developing on agar medium containing antibiotics in roll tubes. From left to right: defaunated inoculum at 10^{0},10^{-1},10^{-2} dilution rates;refaunated inoculum at 10^{0},10^{-1},10^{-2} dilution rates. Rumen fluid for inoculum was taken 2 h before feeding and incubated at $39^{\circ}C$ for 4 days (Magnification 1.5 X)

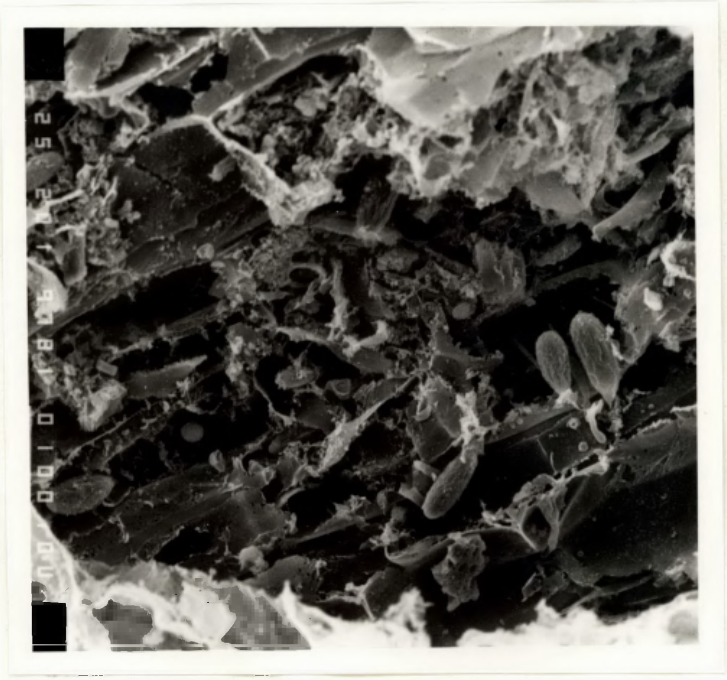


Plate 5.3.2 SEM of rumen fungi growing on inner surface of stem of wheat straw incubated in the rumen of defaunated sheep in nylon bags for 24 h. Bar = 100 μm.

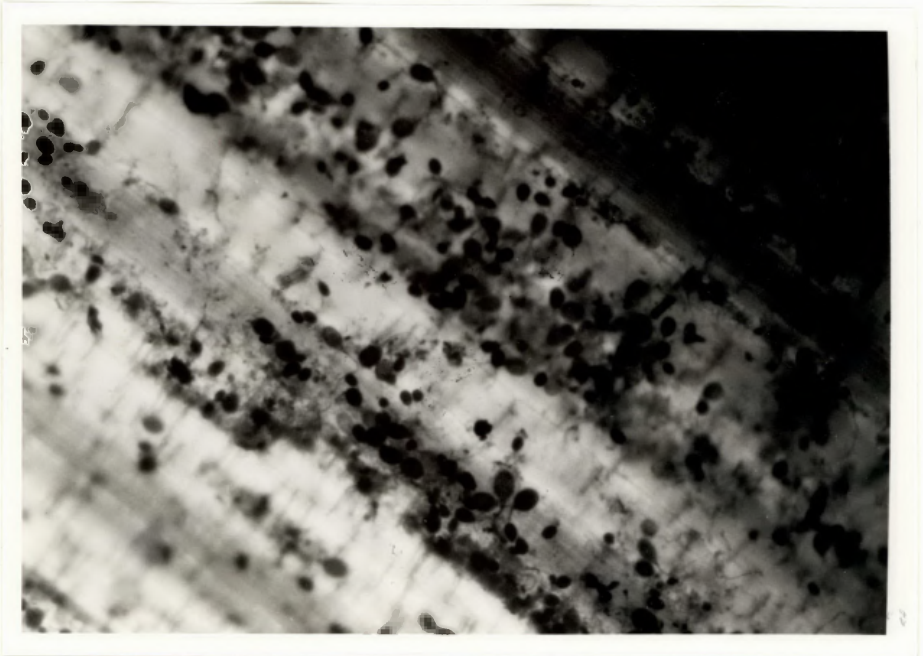


Plate 5.3.3 An extensive colonization by rumen-anaerobic fungi on stem materials of wheat straw incubated in the rumen of defaunated sheep in nylon bags for 22 h (Maginification 40 X).

5.7.4 Discussion

Although differences in DM and ADF disappearance in this study, unlike those found in Experiment 2, were not statistically significant there was an indication that removal of protozoa enhanced fibre degradation in the rumen. The lack of statistical significance is probably a result of insufficient numbers of animals. However, such improvement in the disappearance of DM and ADF in defaunated sheep suggests that the increased fibre digestion rate within the rumen resulted in significant increases in overall digestibility *in vivo*. The present results confirm those from the previous two experiments in which defaunation resulted in significant increases in the rate of disappearance of DM and ADF. Together they support the argument that the discrepancy between the present studies and those reported by others (see Demeyer, 1981; Kayouli *et al.*, 1984; Veira et al., 1983; 1984) is probably due to the use of different diets. Thus, it is clear that when the major constituent of the diet is fibre, removal of protozoa from the rumen is often beneficial to the host animal by improving fibre digestion, due to increases in the numbers of rumen fungi and, possibly also cellulolytic bacteria in the absence of competition from protozoa. As shown in Plate 5.3.2 the growth of rumen fungi on wheat straw suspended in the rumen of sheep in nylon bags was associated with extensive digestion suggesting that rumen fungi can result in significant degradation of fibre, as reported by Bauchop (1979a), Akin *et al.* (1983) and Gordon and Ashes (1984).

The finding that defaunation caused a substantial increase in feed digestibility may have significant implications for animal production, especially where agricultural crop residues are used as the basal diet. Jackson (1981) stated that feed utilization by ruminants could be increased by 100% if the digestibility and protein content of straw-based diet could be increased by 5% and 3%, respectively.

Despite the report of Bauchop (1979a) that fungal colonization on wheat straw stem was low and developed slowly (2-4d), this study has shown (Plate 5.3.3) that wheat straw stems were heavily colonized by rumen fungi by 22 h incubation. This is not only an

indication that animal variation exists, but also that geographical factors may also be important in determining the ease with which fibrous feed materials can be degraded.

The results of the rumen fluid kinetic study (Table 5.3.3) support the argument that an increase in rate of digestion in the rumen of defaunated animals reduced the retention time of fibre in the rumen. This is supported by shortened half-times of Cr-EDTA in the defaunated sheep, although Cr-EDTA marks only the fluid phase of digesta. In addition, since rumen fluid volume in the defaunated animals was not significantly different from that in the refaunated sheep, but outflow rates were slightly increased (although not statistically so), this suggests that more small particles escaped through the reticulo-omasal orifice in defaunated animals, presumably, due to the increased rate and extent of fibre breakdown (Grovum, 1984).

As shown in Figure 5.3.5, the greater TVFA concentration in the defaunated animals may be related to increased ruminal cellulolysis, helping the animal by reducing rumen distension through fermenting fibre to VFA (Grovum, 1984). Although acetate and propionate concentrations were increased in the defaunated sheep, butyrate concentration was not altered, a result often reported with defaunated animals (Bird, 1982). The fact that removal of protozoa caused an increase in acetate concentrations has been reported in anumber of other studies (Luther *et al.*, 1966; Eadie and Gill, 1971; Demeyer and Van Nevel, 1979). An increase in acetate concentration in defaunated animals, particularly when it is accompanied by an increase in numbers of rumen fungi, is not surprising, since Bauchop and Mountfort (1981) have shown that the major product of cellulose fermentation by rumen fungi is acetate.

The effect of defaunation on propionate is not constant, even though several authors observed that high propionate levels were associated with negligible numbers of protozoa in the rumen (Eadie *et al.*, 1970; Whitelaw *et al.*, 1972; Demeyer and Van Nevel, 1979; Kayouli *et al.*, 1984). In view of the complex interaction of factors controlling the pattern of fermentation in the rumen, and the

concomitant changes in microbial population due to defaunation, it is impossible to attribute the change in VFA pattern to the removal of protozoa *per se*.

As found in Experiment 2, the higher ammonia concentrations in the defaunated animals from this study are contrary to those reported in a number of other studies (see Section 5.6.4). It is generally agreed that the ammonia pool in the rumen arises from several sources: (1) degradation of dietary protein and hydrolysis of dietary NPN; (2) hydrolysis of urea recycled to the rumen; and (3) degradation of microbial protoplasm. In addition, rumen ammonia can be utilized in a number of different ways: (1) by incorporation into microbial cells; (2) by absorption through the rumen wall; and (3) by flow to the omasum (Owens and Bergen, 1983). Leng and Nolan (1984) have recently stated that ammonia derived from protozoa is other addition to the ammonia pool. Thus, rumen ammonia concentrations can be altered by changes in any of these seven factors mentioned.

Both in defaunated and refaunated animals, mean pH values were in the range favourable for the growth of cellulolytic microorganisms in the rumen (Kaufmann *et al.*, 1980). The differences in rumen pH between treatments would not be expected to have any significant effect on the induction of fungal zoospores; Orpin (1975; 1977a) demonstrated that an increase in rumen pH from 6.5 to 7.5 had little effect on the production of fungal zoospores. Thus the differences found in the numbers of fungal sporangia and zoospores between treatments are attributable to other factors, such as competition from rumen ciliates.

CHAPTER VI

THE CONTRIBUTION OF RUMEN-ANAEROBIC FUNGI TO CELL WALL DIGESTION IN VITRO

6.1 Introduction

In the experiments reported in Chapter V an increase in digestibility of fibrous residues was consistently achieved in defaunated compared with refaunated sheep. The increases in dry matter digestibility and rates of ADF disappearance in the rumen may be attributed to an increase in the rumen fungal population. These findings indicate that rumen fungi can be significant degraders of fibre, presumably through their unique role in attacking lignicellulosic tissues which are resistant to rumen bacteria (Akin *et al.*, 1983).

Present information on the role of rumen fungi as fibre degraders has been mostly obtained from studies conducted using pure cultures of fungi (e.g. Bauchop and Mountfort, 1981; Mountfort *et al.*, 1982; Akin *et al.* 1983; Gordon and Ashes, 1984). There is now a need to establish the role of rumen fungi in cell wall degradation both with and without bacteria and protozoa.

The objectives of the present work were therefore: (i) to determine the relative contribution of individual microbial groups to fibre degradation, using antibiotics to suppress growth of specific microbial populations; (ii) to quantify fibre degradation by anerobic fungi; and (iii) to investigate the relationship between colonization and fibre-degrading activity of rumen fungi, using inoculum sources from defaunated and faunated sheep.

6.2 Experimental

6.2.1 Animals and feeding

Three defaunated and two faunated mature crossbred wethers, each fitted with a permanent ruminal cannula, were housed individually on slatted floors under continuous lighting. The defaunated sheep

were kept in a separate room. They were offered once a day at 1000h a ration containing 600 g oat straw, 100 g cotton seed meal, 2% urea and 3.5% mineral mixture as used in previous experiments (Chapter V). All sheep had been fed on the experimental diet for three weeks prior to the study.

6.2.2 Preparation of inoculum

Unless stated otherwise, inoculum in this study means a mixture of one part of strained rumen fluid which was taken from either defaunated or faunated sheep, and three parts of warmed (39°C) McDougall's buffer (McDougall, 1948) which had been saturated with CO_2 to bring the pH at 6.9. Rumen fluid was collected through the cannula of sheep using a sampling probe positioned in the dorsal sac of the rumen and taken at 3-4 h after feeding, unless otherwise specified. The rumen fluid was collected into a 1 litre conical flask which had been flushed with CO_2 and placed in a bucket containing water at 40°C. The flask was stoppered by a rubber bung fitted with a bicycle valve and a metal tube which was joined with a plastic tube to deliver the rumen fluid into the flask using a disposable syringe. Immediately after collection, the flask containing rumen fluid was brought to the laboratory and mixed with McDougall's buffer. The flask containing inoculum was placed in a water bath at 39°C under continuous flushing of CO_2 while the inoculum was dispensed by a liquid dispenser to the samples. Inoculum was shaken regularly by hand.

6.2.3 Experimental procedures

a. Preliminary observations

To establish the minimum effective dose of actidione (cyclohexamide, Calbiochem) required to inhibt the growth of rumen fungi a roll tube containing agar and antibiotics (Section 3.2.5.a) was inoculated with a solution containing actidione at various concentrations: 0, 10, 15, 25, 50, 75 and 100 µg/ml of the conents of the roll tubes. The study was conducted at three dilutions: 10^{o}, 10^{-1}, 10^{-2}, with four replicates for each treatment. The tubes were incubated in a shaking water bath at 39°C for 4 days. Observations of fungal colonies

developed on the agar medium were conducted under a dissecting microscope. No fungal colony was detected at any concentration of actidione tested except in the control treatment (without actidione). The results suggested that actidione inhibits growth of rumen fungi at concentrations as low as 10 µg/ml.

In order to check the validity of this result, 15-20 pieces of grass leaf (fresh-frozen *Phalaris aquatica*) were placed in 100 ml plastic centrifuge tubes containing inoculum in the ratio of 1 part of strained rumen fluid to 4 parts of McDougall's buffer. Rumen fluid was taken at 3 h after feeding. Actidione was then added to make a final concentration of :0, 5, 10 and 15 µg/ml of inoculum, then the tubes were saturated with CO_2 and stoppered with Bunsen valves for gas release (rubber policemen with 6mm longitudinal slits). The tubes were incubated in a shaking water bath at 39°C. The incubation was terminated at 12, 24 or 48 h. At the end of incubation the leaves were transferred to a scintillation vial and rinsed twice with normal saline (0.9% NaCl), followed by staining with lactophenol cotton blue (see Section 3. .5.a). The appearance of sporangia on the leaves was observed under a light microscope. Results indicated that fungal sporangia were present on the leaf blades regardless of the level of actidione added to the inoculum. However, the size of sporangia on leaf blades treated with actidione at 5, 10 and 15 µg/ml was much smaller than that of the control treatment (no actidione added) by 24 and 48 h. These results tended to support the first observation that actidione inhibits the growth of rumen sporangia.

The effect of actidione on fungal zoospore production was then studied by incubating 50 ml of inoculum in a shaking water bath with or without 10 µg actidione/ml of inoculum. Rumen fluid was taken at feeding time. Approximately 0.3 g cellobiose was used as substrate in a 125 ml conical flask containing the inoculum with or without actidione (10 µg/ml). The inoculum was sampled after 0, 6 and 24 h incubation and used to inoculate roll tubes containing agar medium plus antibiotics (Section 3.4.5.b) in quadruplicate. The tubes were then incubated for 3 days. No zoospores were detected at any

incubation time when 10 µg actidione/ml of inoculum had been added, suggesting that although sporangia were still detected, no zoospores were liberated.

On the basis of these findings, the concentration of actidione used in the subsequent study was 10 µg/ml of inoculum. Before the experiment was commenced the zoospore population density in the defaunated and faunated sheep was determined at 3 h after the morning feeding in quadruplicate using the method described in Section 3.4.5.b.

b. Forage fibre digestion *in vitro*

The oat straw used in this study was ground and sieved with a test sieve (Endecotts Ltd., London England Mesh no. 30, apperture 0234 ins, 595 mics serial number 131851). The straw contained: 93% OM and 81% cell wall (NDF) on a DM basis. Samples were digested by a one-step technique (Grahman and Amon, 1984) for 6, 18, 24, 48 and 72 h incubation in 125ml conical flasks containing 50ml inoculum from two batches, with duplicate samples from each treatment at each incubation time. The treatments used in this study were:

ID : inoculum from defaunated sheep + 0.5 g air-dried oat straw
IDC : ID + 10 µg actidione (cyclohexamide)/ml of inoculum
IDS : ID + 1.6 mg streptomycin/ml of inoculum
IF : inoculum from faunated sheep + 0.5 g air-dried oat straw
IFC : IF + 10 µg actidione/ml of inoculum
IFS : IF + 1.6 mg streptomycin/ml of inoculum.

The concentration of streptomycin used was based on the study of Amos and Akin (1978) for inhibiting the activity of cellulolytic bacteria. The incubation was terminated by immersing the flask in iced water. Following incubation, the residues were collected in a scintered porcelain crucible (Vitreosil, England) porosity no. 1, in which they were washed with distilled water, then dried at 80°C to constant weight. In each batch duplicate blanks (containing inoculum only or inoculum plus either 10 µg actidione or 1.6 mg streptomycin/ml of inoculum) of each treatment and duplicate flasks containing standard samples of known digestibility were included. The dried residues were

then stored in a scintillation vial until subsequent analysis for cell-wall components (Goering and Van Soest, 1970).
Dry-matter digestibility *in vitro* was calculated as follows:

$$\text{DM digestibility (\%)} = \frac{\text{Sample DM (g)} - (\text{undigested DM residue (g)} - \text{DM blank (g)}) \times 100}{\text{Sample DM (g)}}$$

The model used for kinetics of cell wall disappearance was a first-order kinetic equation (Smith *et al.*,1972). Rates of cell wall digestion were calculated from the regression of the natural logarithmic (ln) transformation of percentages of potentially digestible material remaining against hours of fermentation (Smith *et al.*, 1971).

c. Pattern of fermentation of oat straw *in vitro*

All treatments used in Section 6.2.3.b were used. Approximately 0.3 g air-dried oaten straw was digested with 30 ml of inoculum in a plastic centrifuge tube and stoppered with a rubber bung fitted with a bicycle valve. This study was conducted in a single run only. The duplicate tubes for each treatment were incubated in a shaking water bath for 6, 18 and 24 h. Fermentation was terminated by immersing the tubes in iced water and the contents were acidified with 3 drops of concentrated H_2SO_4. The tubes were then centrifuged at 3000 rpm for 15 min. The supernatant was transferred to a McCartney bottle and stored at -20°C until analysed for ammonia-nitrogen, total VFA and individual acid concentrations as described in Sections 3.5.4 and 3.5.5.

d. Sporangia counts

To evaluate rumen fungi, 20-30 pieces of *Phalaris* leaf (1-2 cm long) were incubated in 250ml conical flasks containing 100 ml inoculum. Sporangial counts were conducted for all treatments except for IDC and IFC. They were incubated in a shaking water bath at 39°C and the leaf blades were sampled after 6, 18 and 24 h of incubation. Sampling was performed under continuous flushing of CO_2 to ensure that anaerobic conditions were maintained. The sampled leaf blades were then transferred to a scintillation vial and treated as described in Section 3.4.5.a.

e. Statistical analysis

Data were subjected to analysis of variance for a factorial design (Snedecor and Cochran, 1967), with inoculum, treatment and replication as sources of variance, and computed with a package program (Neva; Burr, 1980). Differences between treatments were compared by Least Significant Difference (Snedecor and Cochran, 1967).

6.3 Results

6.3.1 Anaerobic Culturing of Fungal Zoospores

As shown in Table 6.1 the number of zoospores which developed on agar medium containing antibiotics was approximately double when rumen fluid was taken from defaunated compared with faunated sheep. In the defaunated sheep, the mycelial-type colonies appeared to develop faster than those of non-mycelial type fungi (see Appendix 2).

6.3.2 Rumen Protozoal Density in Faunated Sheep

The protozoal population density in the rumen of faunated sheep ranged from 2 to 6 x 10^4/ml of rumen fluid 3 h after feeding.

6.3.3 Straw Digestion *in vitro*

The source of inoculum (defaunated Vs faunated) and the treatments used in this study caused significant differences ($P < 0.01$) in dry-matter digestibility *in vitro* (IVDMD) at 48 h incubation, but an extension of incubation time up to 72 h produced no further significant effect due to source of inoculum ($P > 0.05$) (Table 6.2). There was no indication of bacterial movement in the inoculum at any incubation time when 1.6 mg streptomycin was added/ml inoculum.

6.3.4 Fermentation Patterns of Straw Digestion *in vitro*

The concentrations of total VFA (TVFA) and individual acids were not affected significantly ($P > 0.05$) by incubation time, but the effect

of treatments was significant ($P < 0.01$). As shown in Table 6.3 the proportion of acetate in inocula from defaunated sheep was higher than in those from faunated sheep. Addition of actidione to the inoculum was associated with a decrease in acetate concentration. Propionate and butyrate concentrations were higher in IF ($P < 0.01$ and $P < 0.05$, respectively) compared with those in other treatments.

Ammonia concentrations were significantly affected by the source of inoculum (lower in the defaunated animals) and by treatments ($P < 0.01$). In the absence of rumen bacteria the ammonia concentrations were substantially increased ($P < 0.05$).

6.3.5 Numbers of Sporangia Appearing on Grass Leaves Incubated *in vitro*

Table 6.4 shows the numer of funal sporangia appearing on the leaf blades of *Phalaris* grass incubated *in vitro*. It is surprising that addition of 1.6 mg streptomycin/ml of inoculum did not result in an increased number of sporangia. Protozoa were often seen attached to the leaf blades which had been incubated in inoculum from faunated sheep after 6 h incubation. Sporangia of large size were detected after 18 h incubation attached to leaf blades incubated with the faunated inoculum. Leaf blades observed after 24 h incubation with the defaunated inoculum showed considerable damage due to digestion (see Plate 6.1).

Table 6.1 Fungal colonies developing on agar medium plus antibiotics from rumen fluid of defaunated and faunated sheep. MT and NMT denote mycelial type and non-mycelial type colonies, respectively. Rumen fluid was taken at 3 h after feeding.

Source of inoculum	No. of sheep	No. of fungal colonies ($X10^{-3}$/ml) ± SD		
		MT	NMT	Total
Defaunated	3	11 ± 3	3 ± 2	14 ± 2
Faunated	2	3 ± 1	3 ± 2	6 ± 2

Table 6.2 Dry matter (IVDMD) and cell wall (IVNDF) digestibilities, and rates of cell wall digestion (k) of oat straw when incubated with two sources of inoculum and three treatments (ID=no antibiotics added to defaunated inoculum; IDS=plus streptomycin; IDC=plus actidione; IF=no antibiotics added to faunated inoculum; IFS=plus streptomycin; IFC=plus actidione)

	Treatment						SED
	ID	IDS	IDC	IF	IFS	IFC	
IVDMD (%)							
48h	41^{c}	12^{a}	25^{b}	45^{d}	13^{a}	42^{c}	6.3
72h	48^{c}	13^{a}	41^{b}	48^{c}	13^{a}	44^{b}	0.2
IVNDF (%)							
48h	44^{c}	12^{a}	22^{b}	47^{c}	10^{a}	45^{c}	0.1
72h	52^{d}	11^{a}	41^{b}	52^{d}	11^{a}	47^{c}	0.3
k (%/h)	0.0094^{d}	0.0004^{a}	0.0061^{b}	0.0095^{d}	0.0006^{a}	0.0076^{c}	0.0001
r^2	0.98	0.54	0.89	0.94	0.50	0.87	

Values in the same row with unlike superscripts are different ($P < 0.05$)

r^2 is the coefficient of determination

Table 6.3 Fermentation products of oat straw incubated in vitro with two sources of inoculum and three treatments (ID=no antibiotics added to defaunated inoculum;IDS=plus streptomycin; IDC=plus actidione;IF=no antibiotics added to faunated inoculum;IFS=plus streptomycin;IFC=plus actidione)

	Treatment						SED
	ID	IDS	IDC	IF	IFS	IFC	
TVFA (mmol/1)	33^{b}	31^{ab}	28^{a}	34^{b}	31^{ab}	27^{a}	0.5
Acetate (%)	76^{c}	76^{bc}	74^{ab}	70^{bc}	74^{abc}	73^{a}	0.3
Propionate (%)	17^{b}	16^{a}	18^{a}	21^{c}	18^{b}	20^{a}	0.01
Butyrate (%)	6^{a}	6^{ab}	6^{ab}	7^{c}	6^{ab}	6^{a}	0.00
Ammonia (mgN/1)	154^{a}	192^{b}	178^{ab}	272^{c}	303^{c}	147^{a}	19.3

Values in the same row with unlike superscripts are different ($P < 0.05$)

Table 6.4 Numbers of sporangia on grass leaves incubated in vitro with two sources of inoculum and three treatments

Source of inoculum	Treatment	No.of colonies/mm^2	SED
Defaunated	nil	15^{a}	
	streptomycin	16^{a}	
	actidione	0^{a}	
Faunated	nil	5^{a}	
	streptomycin	6^{a}	
	actidione	0^{a}	5.4

Values with unlike superscripts are significantly different ($P < 0.05$)

Plate 6.1 Sporangia growing on Phalaris leaves after 24 h incubation with inoculum from defaunated animals (Magnification 200 X)

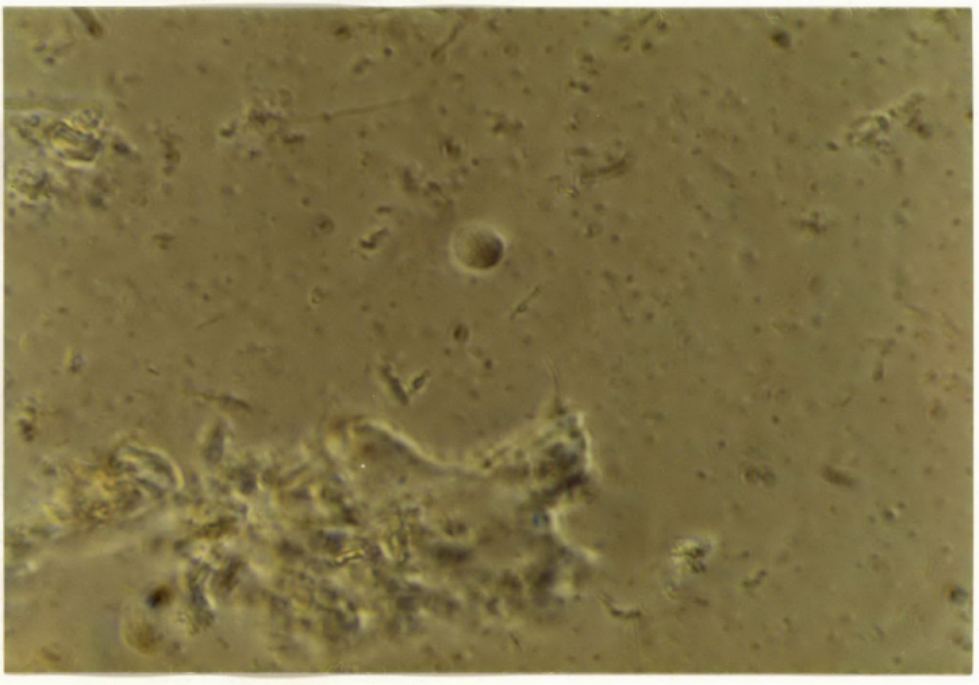


Plate 6.2 Spherical-bodied microorganisms bearing polyflagella. Photographed from inoculum of faunated animals after 48 H incubation in vitro (Magnification 400 X)

6.4 Discussion

Defaunation apparently resulted in an increase in the fungal zoospore population and more mycelial type fungi were found in the rumen (Table 6.1); the number of sporangia appearing on the grass leaves also increased under *in vitro* conditions (Table 6.4). These findings support previous results from Chapter V that the number of fungal sporangia appearing on plant particles, and of fungal zoospores, were substantially increased when protozoa were removed from the rumen of sheep. Nevertheless, when the results of straw digestion *in vitro* using the inoculum prepared from the rumen fluid of defaunated (ID) and faunated (IF) sheep were compared, they were not in agreement in terms of an increased digestibility of fibre in defaunated sheep found in *in vivo* studies in which the rate of digestion of fibrous materials was measured by nylon bag technique and apparent digestibility was measured by total collection as reported in Chapter V. The IVDMD at 48 h of oat straw obtained from ID was less than that from IF (41 Vs 45 percentage units), although this difference was not significant at 72 h incubation (Table 6.2). The rate of cell wall digestion was, however, not significantly different between ID and IF.

Addition of streptomycin (IDS and IFS) virtually inhibited the activity of cellulolytic bacteria; no bacterial movement could be detected in these inocula when observed under a light microscope. Amos and Akin (1978) also reported that similar additions of streptomycin stopped the activity of cellulolytic bacteria *in vitro*. Thus, it can be assumed that in the absence of cellulolytic bacteria (when performed in IDS) the straw disappearance was largely caused by the activities of rumen fungi or rumen fungi together with protozoa (when performed in IFS). The results indicate that under these conditions IVDMD and rates of cell wall digestion were decreased substantially (by 28 and 32 percentage units in IDS and IFS, respectively at 48 h incubation) (Table 6.2).

In contrast, when 10 Ug cyclohexamide per ml of inoculum was needed to inhibit the growth of rumen fungi (IDC and IFC), the IVDMD from treatment IFC was decreased only slightly (approximately

3 percentage units at 48 h incubation) compared with that of IF, but the corresponding figure obtained from treatment IDC was much greater (approximately 15 percentage units at 48 h incubation) when compared with that of ID. Unfortunately the data on straw digestion obtained after 48 h from both IDC and IFC in this experiment may have been confounded by the presence of a large population of actively moving spherical bodies bearing polyflagella. These microorganisms were more prominent in IFC (Plate 6.2), and they were, presumbaly, zoospores of rumen fungi (Bauchop, 1984, personal communication). If this is true, the differences obtained between IDC and IFC cannot be attribued to rumen bacteria (in IDC) and rumen bacteria and protozoa (in IFC) *per se*. Although Dekker (1969) stated that cyclohexamide is a powerful antifungal agent even at low concentrations (1.0 ug/ml of medium), and Orpin (1977a) found cyclohexamide to inhibit the growth of rumen fungi at even lower levels (10 µg/ml of rumen fluid), other workers found that higher concentrations of this antifungal agent (1.5 mg/ml of inoculum) were required (Windham and Akin, 1984). In addition, as reported in Chapter V, injection of 50 µg cyclohexamide per ml of rumen fluid into the rumen of sheep on two consecutive days killed the sheep instead of the rumen fungi. Thus, the efficacy of cyclohexamide in inhibiting the growth of rumen fungi may depend on the species present. Although our knowledge of rumen fungi is rudimentary, the fungal species present are, as with other rumen microbes, known to vary greatly between animals, between diets, and possibly between geographical locations (Kistner, 1965; Leng , 1984).

When the data on straw digestion were related to the number of sporangia on leaf blades, there was no link between an increased sporangial population, IVDMD, and rates of cell wall digestion (Tables 6.2 and 6.4). Addition of streptomycin to the inoculum had no signficiant effect on increasing the number of fungal sporangia. These findings are in accord with the recent results of Windham and Akin (1984) who demonstrated that the sporangial population appearing on leaf blades of Coastal Bermuda grass was not increased when the leaves were incubated *in vitro* in an antibiotic-containing inoculum (streptomycin and penicillin) compared with control inocula (no antibiotics added), and IVDMD at 48 h of incubation did not relate to the number of rumen sporangia. At this stage, the differences

in growth and the number of sporangia in the absence of cellulolytic bacteria suggest a biological interaction between the bacteria and fungi and the protozoa and fungi.

The fact that fibre digestion was substantially lower in the absence of rumen cellulolytic bacteria indicates that these micro-organisms form the most active fibre-digesting population in the rumen. It seems likely that rumen fungi require certain essential nutrients, since the fungal colonies are substantially greater when incubations are carried out in roll tubes containing complex media (Joblin, 1981; Bauchop and Mountfort, 1981; Gordon and Ashes, 1984) than when carried out under these present experimental conditions (see also Wiłdham and Akin,1984). Moreover, it is clear that rumen fungi are dependent on some rumen bacteria such as methanogens for utilizing some of the fermentation products (Bauchop and Mountfort, 1981). Thus, the lack of potential to digest a substrate by rumen fungi observed herein *in vitro* may be related to the accumulation of fermentation products such as ammonia whose concentration was relatively high in the absence of rumen bacteria (Table 6.3). On the other hand there is no evidence at present to suggest that the levels of ammonia found in treatments IDS and IFS inhibited the growth of rumen fugi, despite the substantial decrease in IVDMD and rates of cell wall digestion on these treatments.

Although TVFA concentrations were not affected by treatments used in this study, IF was associated with increases in propionate and butyrate concentrations. In the absence of rumen fungi, acetate concentrations were reduced by 18% compared with those on treatments ID and IF; Bauchop and Mountfort (1981) reported that the major fermentation product of cellulose digestion by a pure culture of rumen fungi was acetate.

In summary, it is likely that rumen fungi are very sensitive to changes in the rumen environment. If so, this will make it difficult to elucidate the contribution of these microorganisms to fibre digestion. Substrate digestion by rumen fungi in the present study was less than that by inoculum without antibiotics (ID and IF) or inoculum plus cyclohexamide (IDC and IFC). These results suggest that

rumen bacteria are the most active fibre degraders. Rumen protozoa, however, show no obvious evidence of contributing to fibre digestion in the absence of rumen bacteria (IFS). Nevertheless, protozoa may have degraded the soluble carbohydrate components in the DM and NDF fractions of oat straw, accounting for the small loss of those components in the presence of cyclohexamide (Table 6.2).

CHAPTER VII

GENERAL DISCUSSION

7.1 Research Findings

To study the contribution of anaerobic fungi to feed digestion in the rumen, it is necessary to determine the pool size of these organisms relative to other rumen inhabitants. The methods available at present to measurethe biomass of fungi in the rumen are unsatisfactory because they are imprecise and demanding in terms of equipment and labour (see Chapters II and IV for review).

Two methods used in the present studies, viz. counting the numbers of sporangia on feed particles (Akin *et al.*, 1983) and culturing the zoospores in roll tubes (Joblin, 1981) resulted in a large variation in results (mean value ± 70%) even allowing for variations between animals and diets, suggesting that the methods cannot be used to determine the fungal biomass in the rumen precisely. It is not possible to estimate the pool size of rumen fungi from these studies. However, the use of those methods to enumerate the numbers of sporangia and zoospores throughout the studies presented here gave an indication of relative fungal population density within the rumen between treatments under the same experimental conditions.

The use of defaunated animals to study rumen-anaerobic fungi indicated that the growth of rumen fungi was enhanced in the absence of protozoa in the rumen of sheep. This was demonstrated by both enumerating the sporangia appearing on feed particles suspended in the rumen in nylon bags, and by culturing the zoospores in rumen fluid in roll tubes *in vitro*. At this stage, it can be safely assumed that there is a marked competition between anaerobic fungi and rumen protozoa for essential nutrients. Thus, the presence of protozoa in the rumen not only reduces the numbers of rumen bacteria both free in the medium and associated with feed particles (Orpin and Letcher, 1984), but also appears to reduce substantially the pool size of rumen

fungi, as much as 12 fold, (see Chapter V). The present of protozoa therefore have a profound negative effect on fibre degradation in the rumen. The results of these studies also confirmed a report of Orpin (1975) that protozoa prey on fungi in the rumen.

It has been demonstrated in a number of studies from other laboratories that rumen fungi are actively cellulolytic (Orpin and Letcher, 1979; Orpin and Hart, 1980; Bauchop and Mountfort, 1981). An increase int he number of sporangia colonizing grass leaves from 0.6 to 3.3 per 0.75 mm^2 resulted in a 100% increase in the fragility of grass leaves (Akin *et al.*, 1983). In contrast, the role of rumen protozoa in fibre digestion is not clear (see Delfosse-Debusccher *et al.*, 1979; Bauchop, 1982; Widham and Akin, 1984). Hungate (1975) showed that the actively-cellulolytic protozoa *Polyplastron multivesienlatum* (Jouany and Senaud, 1979) constituted only a small part of the total protozoal population in the rumen. This was also found in the present studies. Thus the presence of protozoa in the rumen of animals fed on high-fibre diets may be disadvantageous.

The results from the three experiments reported in Chapter V have shown that on fibre-based diets, removal of protozoa from the rumen increased fibre digestion, both in the rumen as measured in nylon bags and in the whole animal *in vivo*. Such increases in digestibility may be associated with increases in the fungal population in the rumen (as reflected by numbers of sporangia and zoospores) and, presumably, rumen cellulolytic bacteria as well. Orpin and Letcher (1984) demonstrated that defaunation resulted in increased numbers of rumen bacteria, both free in the medium (by 480%) and associated with particles (by 40%).

In some previous reports in which defaunation decreased fibre digestibility in both sheep and cattle, and was associated with a lower concentration of rumen ammonia, the diets used contained substantial amounts of concentrates (Demeyer, 1981; Veira *et al.*, 1983; 1984; Kayouli *et al.*, 1984). Under these conditions it can be safely assumed that the numbers of cellulolytic bacteria (Leedle *et al.*, 1982), and

of anaerobic fungi (Bauchop, 1979a) were relatively low because of high populations of amylolytic bacteria in the rumen. Surprisingly, in several reports the mean rumen pH of animals used for studying the effects of defaunation on digestibility was below 6.3 (see Males and Purser, 1969; Demeyer and Van Nevel, 1979; Veira *et al.*, 1983; 1984; Kayouli *et al.*, 1984). Cellulolytic activity in the rumen was almost completely destroyed and the numbers of culturable cellylolytic bacteria were reduced when Stewart (1977) and Hitner and Dehority (1983) reduced the pH of rumen contents below 6.5. A reduction in zoosporegenesis at pH values below 6.0 has also been reported (Orpin, 1975; 1976a; 1977b). Thus, the lower fibre degradation *in sacco* in the defaunated rumen by Demeyer (1981) and others (e.g. Kayouli *et al.*, 1984) was probably associated with a decrease in activity of cellulolytic bacteria and anaerobic fungi in the rumen rather than to the removal of protozoa.

Although an increase in feed digestibility in defaunated sheep was associated with a higher density of the fungal populations in the rumen compared with those in the faunated sheep (Chapter V) a significant role of rumen fungi in fibre digestion could not be demonstrated under *in vitro* conditions (Chapter VI of this thesis and Windham and Akin (1984). The reason for this is not clear, but it is presumably associated with accumulation of fermentation end-products such as ammonia and H_2 which depressed the growth of rumen fungi in the absence of rumen bacteria. Bauchop and Mountfort (1981) showed that the rate and extent of cellulose degradation by monocultures of rumen fungus were less than by co-cultures with rumen Methonogens (53 versus 82%), suggesting that some fungal fermentation end-products may have been inhibitory to growth of the fungus. There is no evidence at present to suggest that high ammonia concentrations (more than 250 mg N/l; see Chapter VI) depress the growth of rumen fungi *in vitro*, but from preliminary observations (Chapter IV, Experiment 2) the ammoniated rice-straw was poorly colonized by rumen fungi, indicating that this material may not be attractive to fungi.

There was no systematic change in rumen ammonia concentrations due to defaunation. The lower ammonia levels found in the protozoa-free animals from Experiment 1 (Chapter V) and in *in vitro* studies (Chapter VI)

have often been reported (e.g. Klopfenstein *et al.*, 1966; Moles and Purser, 1970; Demeyer and Van Nevel, 1979; Bird, 1982). However, the opposite result, as found in Experiments 2 and 3 (Chapter V), has also been reported by others (Kurihara *et al.*, 1978; Demeyer *et al.*, 1982). The higher ammonia levels in the defaunated state than in the refaunated state in Experiments 2 and 3 apparently were not due to any alteration in NH_3 utilization by the bacterial population, but instead may have been associated with the inclusion of cotton seed meal and urea in the diets to ensure that ammonia concentrations were sufficiently high to sustain normal microbial growth (Satter and Slyter, 1974; Preston and Leng, 1984). Bryant (1973) reported that the principal cellulolytic bacterial species in the rumen utilize ammonia as their main source of nitrogen. Thus the growth of cellylolytic bacteria was probably more rapid in Experiments 2 and 3 than in Experiment 1. A requirement for high rumen ammonia levels for rapid growth of cellulolytic bacteria may be more apparent in the defaunated sheep as the bacterial pool size in the rumen generally increases when protozoa are removed (Orpin and Letcher, 1984).

The effect of defaunation on the concentrations of total VFA (TVFA) and of the individual acids was also not consistent between experiments reported in Chapters V and VI. Changes in TVFA concentrations due to defaunation in Experiment 1 were irregular but in Experiments 2 and 3 TVFA and individual acid concentrations were higher in protozoa-free animals (see Chapter V). This lack of consistency in the effect of defaunations on TVFA and individual acid concentrations has also been reported in the literature (see Demeyer, 1981). In addition, defaunation had apparently no effect on TVFA concentrations *in vitro* (Chapter VI). The most consistent effect of defaunation on the fermentation products in the present studies was an increase in acetate concentrations in the rumen of defaunated sheep compared with that in refaunated sheep. This may have been associated with an increase in the fungal population in the defaunated rumen, since Bauchop and Mountfort (1981) have shown that acetate is the major fermentation product of cellulose degradation by rumen fungi.

From the present studies, it is apparent that defaunation of ruminants on low quality fibrous feeds can lead to large increases in productivity. Demeyer *et al.* (1982) reported a 53% increase in

growth rate of defaunated lambs compared with faunated lambs given alkali-treated straw diets. The main reason for these increases in animal growth rates is an improvement in microbial protein flow to small intestine and presumably in digestibility of fibre in the defaunated rumen. Part of the improvement in fibre digestibility can be attributed to an increase in the rumen fungal population, although it is known that the bacterial population also increases in the defaunated state.

7.2 Future Research

The effect of defaunation in increasing feed digestibility in sheep fed on high-fibre diets was the most striking result arising from the present studies. Of equal importance was the finding that removal of protozoa from the rumen increased both the numbers of sporangia appearing on feed particles and of zoospores in rumen fluid. However, further research is necessary to quantify the contribution of rumen fungi to fibre digestion *in vivo*.

The development of methods for quantifying the fungal biomass in the rumen is now a major research priority. As suggested by Bauchop (1984), the size of fungal populations cannot be assessed accurately by enumeration of sporangia or zoospores.

Future research on the nutrient requirements of rumen fungi for rapid growth is also important. This knowledge should enable us to manipulate the rumen fermentation in order to substantially improve the utilization of fibrous diets by ruminants.

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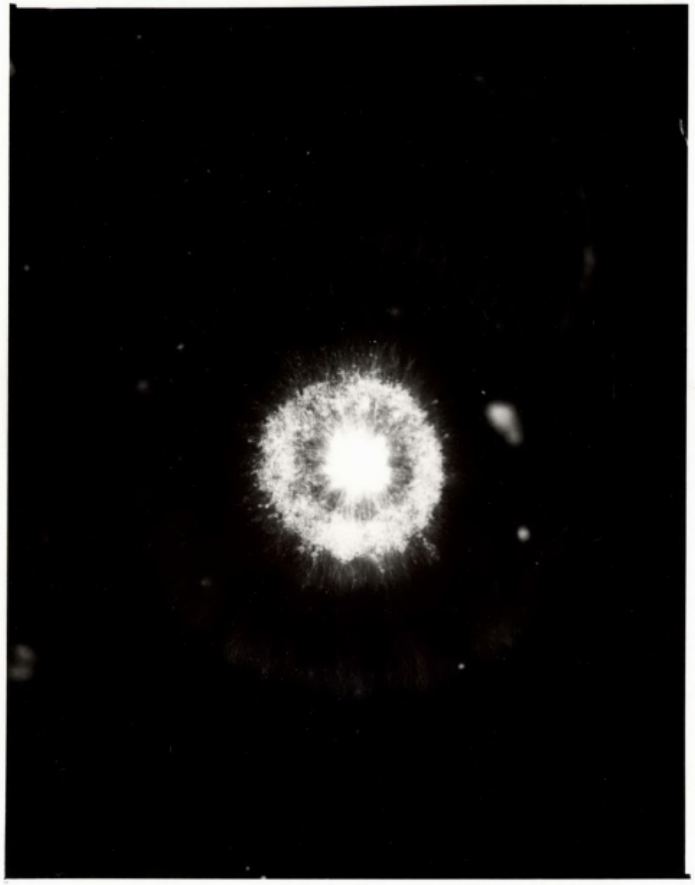
A



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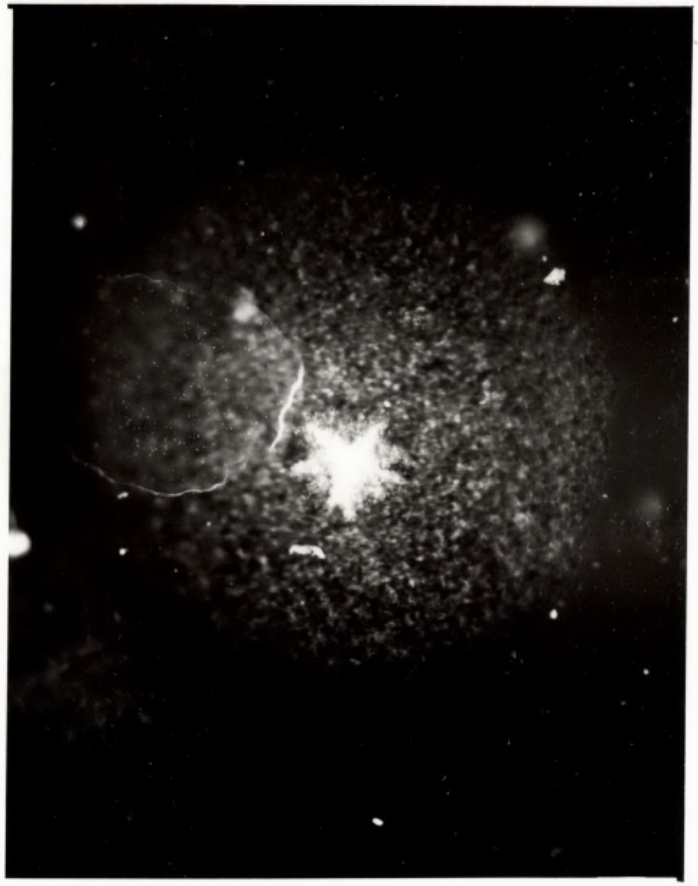
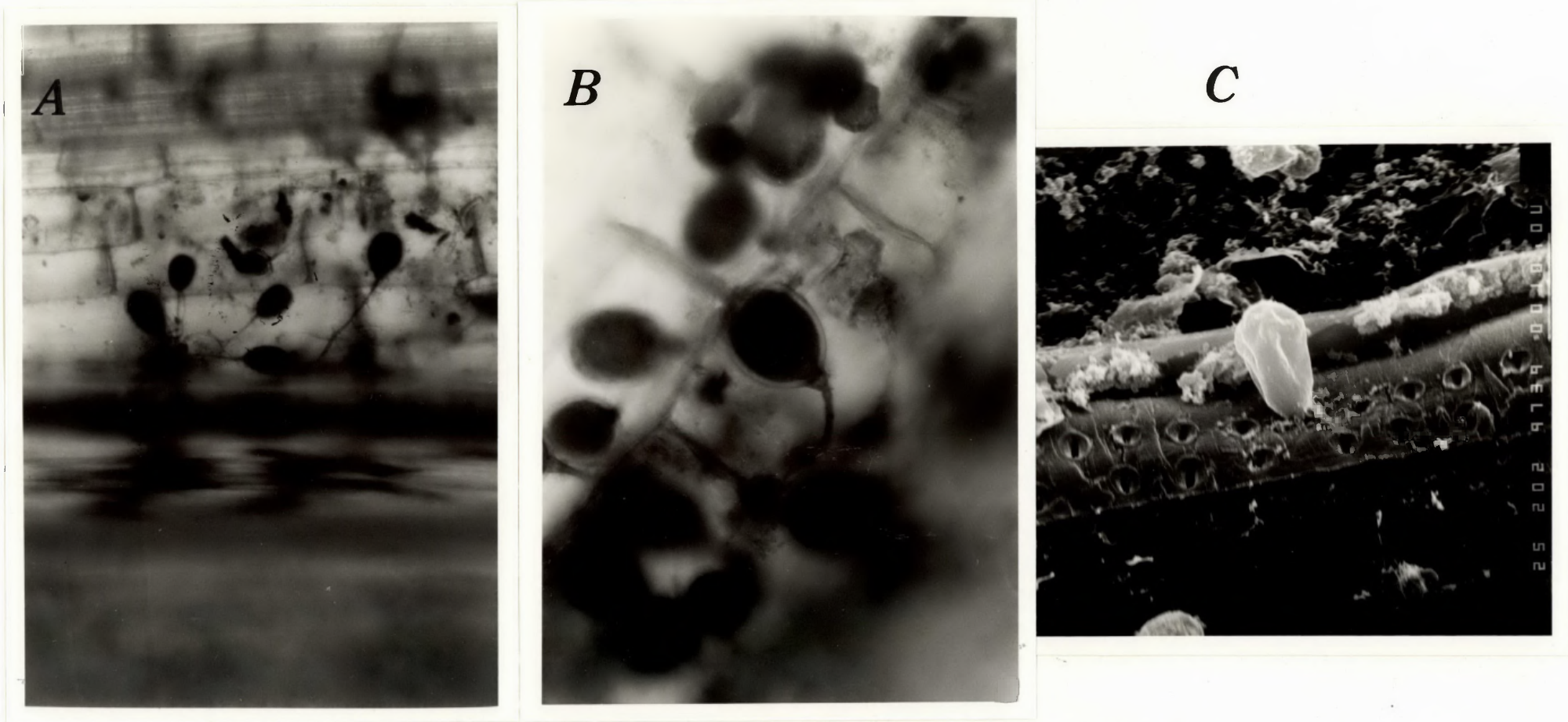


Mycelial (A) and non-mycelial (B) colonies of rumen fungi developing on agar medium containing antibiotics



Mature rumen fungal sporangia (A & B) attaching on wheat straw suspended in the rumen of sheep after 22 h (magnification 200 X and 400 X respectively). SEM of rumen protozoa attached to wheat straw particles suspended in the rumen of sheep after 6 h (C)