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Methods for the isolation, culture and assessment of the status of anaerobic rumen chytrids in both *in vitro* and *in vivo* systems

Mohammad REZAEIAN[1], Gordon W. BEAKES[2] and David S. PARKER[3]

[1] *Department of Animal health and Nutrition, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran.*
[2] *Department of Biological and Nutritional Sciences, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, UK.*
[3] *Novus Europe, Rue Gulledellestraat 94, B-1100 Brussels, Belgium.*
E-mail : *mrezaee@ut.ac.ir*

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Anaerobic fungi were isolated from both the rumen and faeces of nine sheep and a cow. A reliable and simple method for the isolation of anaerobic fungi using 24 h rumen incubated milled straw as the inoculum source was developed. We also evaluate the use of chitin measurements as an assay of rumen fungal biomass. Chitin levels were determined from various sample sources (milled barley straw used as the fungal culture substrate *in vitro*; plant particulate digests from the rumen (PLP) and centrifuged strained rumen fluid (CSRF) using both HPLC and colorimetric methods. Both methods were highly correlated and consequently the simpler colorimetric method was adopted for subsequent studies. There was also a high degree of correlation between anaerobic fungal cellulase activities with the assayed chitin content of milled barley straw cultures over 12 d of an *in vitro* experiment. The colorimetric chitin assay protocol was then used to assess the diurnal variation and abundance of rumen fungi in *in vivo* assays. We assessed the distribution of chitin (mg g^{-1} dry matter) in various fractions of the strained rumen fluid (SRF) and PLP samples from the rumen of sheep. Chitin was detected in all fractions of strained rumen fluid but the main source of chitin in the samples may be attributed to the fungal biomass. We did not detect any significant differences in chitin levels over a 24 h sampling period. Finally, an SEM study on subsamples of milled straw and plant particulate matter used in the chitin assays, revealed that the pattern of the fungal development on substrate material differs from the culture medium to the rumen.

INTRODUCTION

The anaerobic fungi are now known to be one of the most significant groups of rumen microorganisms (Trinci *et al*. 1994, Orpin & Joblin 1997). Rumen fungi were first isolated from a pellet of strained rumen fluid from sheep (Orpin 1975). Since then, various techniques have been developed to isolate these fungi (Theodorou & Trinci 1989). Most methods involve inoculating strained rumen fluid (SRF) into a suitable culture medium under anaerobic conditions. Animal faeces have also been used as a source of inoculum for the isolation of these fungi (Milne *et al*. 1989, McGranaghan *et al*. 1999).

Rumen fungi are able to degrade and utilise plant storage and structural polysaccharides by producing a wide range of polysaccharide depolymerase and glycoside hydrolase enzymes (Hebraud & Fevre 1988, Op den Camp *et al*. 1994, Srinivasan *et al*. 2001). Their contribution to fibre degradation in the host animal is probably the most important role of these microorganisms (Akin & Borneman 1990, Trinci *et al*. 1994, Lee, Ha & Cheng 2000). However, the extent of their contribution in the complex rumen ecosystem has yet to be fully evaluated (Trinci *et al*. 1994, Orpin & Joblin 1997). Measurement of *in situ* fungal populations is therefore needed in order to determine the extent of their contribution to the ruminal digestive process. The life-cycle of these fungi consists of two principle stages in which the motile zoospore alternates with a vegetative stage (sporangium) attached by a rhizoidal system to plant particles (Orpin 1975). Whilst the available methods for the estimation of fungal populations are mostly based on the measurement of the concentration of free zoospores in the rumen fluid (Joblin 1981, Obispo & Dehority 1992), it has been estimated that approximately three-quarters of ruminal microorganisms are attached to or closely associated with the digested solids (Forsberg & Lam 1977, Craig, Broderick & Ricker 1983). Since chitin is present in the cell wall (sporangia and rhizoids) of rumen fungi (Orpin 1977, Gay 1991), measurement of this polymer has been used to estimate fungal biomass and their growth *in vitro* (Gordon & Phillips 1989, Gay 1991), and only in a

single case *in vivo* (Argyle & Douglas 1989). However, no specific information exists on the amount and distribution of chitin in the different fractions of ruminal digests. Scanning electron microscopy (SEM) has also been used in the evaluation of forage digestion in the rumen (Bauchop 1980, Akin, Gordon & Hogan 1983).

This paper describes the development of isolation and assay protocols developed to assess the status and activity of rumen fungi in both *in vitro* culture and *in vivo* systems.

MATERIALS AND METHODS

Animals and diets

Nine sheep and a cow (live weight 35–40 and 420 kg respectively), each fitted with a rumen fistula, were used in this study. To develop the isolation protocols a sheep and a cow were used. They were fed on a daily diet of hay and pelleted lucerne (200 and 400 g) and (2 and 6 kg) respectively. Fractionation was carried out using four sheep as a source of rumen fluid. Their diet consisted of 700 g of pelleted lucerne supplemented with 5% molasses offered once a day at 09.00 h. Diurnal fluctuation of the chitin in the rumen and the SEM studies was performed using four sheep which were fed 150 g hay and 500 g lucerne pellets daily. Diet were offered twice a day in two equal portions at 07.30 and 15.30 h. All sheep were Suffolk Wethers, and were individually housed in metal-framed metabolism crates with a steel mesh floor panel. The cow was also kept in an individual stall. The environmental temperature was maintained at 12–15 °C, and artificial light provided for a 14 h d^{-1}. Clean water and licks of rock salt were always available.

Isolation and culturing of anaerobic rumen fungi

Rumen liquor samples were collected from the sheep and cow into a vacuum flask and returned to the laboratory within 2 h of collection. The presence of motile zoospores in the samples was determined microscopically. Different dilutions of this rumen liquor (10^{-2}–10^{-5}) were then used to inoculate (10% v/v) serum bottles containing 45 ml of culture medium prepared as described by Davies *et al.* (1993). Fresh faeces were also obtained from the same animals and about 5 g of sample was placed in a plastic bag that was flushed with CO_2 gas. 45 ml of culture medium was added to the faeces sample and pummelled with a stomacher (Seaword Medical, London) for 2 min. A serial dilution (10^{-2}–10^{-5}) of this homogenate was then prepared and 5 ml of each dilution series (3 replicates) was inoculated into 45 ml of medium and then incubated at 39 °. Fungal growth was monitored each day for 6 d. In a third procedure, milled straw was incubated in the rumen using the Dacron bag technique (Mehrez & Orskove 1977) and the residual materials were then used as the source of inoculum. Dacron bags (19 × 10 cm) with a 40–50 μm pore size containing 5–7 g of milled (1 mm screen size) barley straw were inserted into the rumen of fistulated sheep prior to feeding. The bags were removed after 24 h incubation, and then washed under warm tap water at about 40 °. A small amount (*ca* 100 mg) of this incubated straw was then inoculated into three replicate serum bottles containing 45 ml of culture medium and 1 ml of antibiotic solution (5 mg ml^{-1} chloramphenicol in 50% aqueous ethanol). The bottles were gassed with oxygen-free CO_2 for about 2 min and then incubated at 39 ° after sealing.

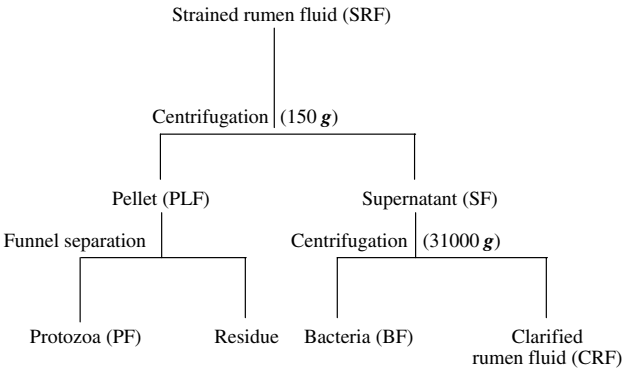


Fig. 1. Illustration of the procedure for the fractionation of the strained rumen fluid and determination of the dry matter content in each fraction.

Fractionation of strained rumen fluid

Rumen contents were collected from the four sheep 2 h before feeding and returned to the laboratory immediately after being strained through two layers of muslin. About 250 ml of the strained rumen fluid (SRF) was used to perform the fractionation procedure. Of this, 10 ml was taken for dry matter determination and 20 ml for chitin analysis. The remaining part was centrifuged at 150 ***g*** for 10 min and samples of the resulting supernatant (fraction SF, 10 ml) and pellet (PLF fraction, about 2 g) were removed for chitin assay. To separate the bacterial fraction the remaining supernatant was transferred into a 250 ml centrifuge tube and centrifuged at 31 000 ***g*** for 30 min and the resulting bacterial fraction (BF) was freeze dried. Separation of the protozoal fraction (PF) was carried out based on the method described by John & Ulyatt (1984). The residue mainly composed of protozoa was then collected, freeze-dried and used for the chitin analysis. A summary of these separation procedures is given in Fig. 1. The whole procedure was also carried out on 40 ml of the SRF separately to determine the dry matter content of the fractions PF and BF. The dry matter content of the fractions SF and clarified rumen fluid (CRF) was also determined by the weight difference of the fractions (SRF with PLF) and (SF with BF) respectively, in the appropriate volume. Then the dry matter content of each fraction was calculated as percentage dry matter of the SRF.

Chitin assay

Analysis of chitin was carried out on freeze dried samples of the colonised milled straw from in vitro cultures, centrifuged strained rumen fluid (CSRF) and the associated plant particulate material (PLP), and other fractions from fractionation procedure. Rumen contents were strained through two layers of muslin. 20 ml of the strained fluid was transferred into the universal tubes and then centrifuged immediately at 3200 rpm for 15 min at 5 °. The pellet was then used for chitin analysis. The chitin content of samples was determined from the glucosamine hydrochloride equivalent resulting from hydrolysis by both HPLC and colorimetric methods and a correlation between them was determined. Dried samples were hydrolysed using the methods of Chen & Johnson (1983) and Lin & Cousin (1985). Between 100–300 mg of dried milled sample was placed into an acid-washed Pyrex tube and hydrolysed with 6 ml of 6 N HCl for 4 h at 105 °. After cooling, the hydrolysate was centrifuged at 3200 rpm for 30 min at 4 °C. About 2 ml of supernatant was filtered using a 0.45 μm syringe filter (Gelman Sciences, Ann Arbor, MI) and 1 ml of this sample was then freeze dried. Triplicate samples of pure crab shell chitin (*ca* 15 mg) were also hydrolysed under the same conditions and examined at hourly intervals for up to 8 h to determine the amount of resulting glucosamine hydrochloride recovery. The linearity of the concentration of glucosamine hydrochloride in the standard solution (5, 10, 15, 25, 30, 35 and 40 $\mu g\ ml^{-1}$ of solution) was determined colorimetrically and a regression analysis between the concentration of glucosamine and the related absorbance (at 530 nm) was performed and the correlation coefficient (r) was calculated. The absorbance of the samples was also measured for varying lengths of time (20, 35, 50, 65 and 80 min) after colour development to determine the optimum stability period.

Detection of glucosamine from the dried hydrolysates was also carried out by HPLC based on the method of Quigley & Englyst (1992). The HPLC system consisted of a Dionex advanced gradient pump, Dionex automated sampler, a pulsed amperometric detector, and a Dionex 4400 integrator for integration. Dried hydrolysate samples were first purified using Dowex 50w-x4 resin suspension (1 : 1 w/v in distilled water) and Pasteur pipettes with Pyrex glass wool as described by Lin & Cousin (1985). The effluents were then freeze dried, redissolved in distilled water and the pH was adjusted between 7–8 using 5 M NaOH solutions. Aliquots (0.8 ml) were transferred to the polythene sample vials which included a 20 μl filter and run onto the Carbopac PA-1 column with a flow rate of 1 $ml\ min^{-1}$ and 15 mM NaOH eluent.

Measurement of cellulase activity and chitin content of the fungal biomass in vitro

The colonised milled straw from culture bottles (three replicates) were collected daily over a 12 d experimental period by centrifugation for 30 min. The pellet was washed on a sintered glass filter crucible (grade 1) before freeze-drying. These samples were used for chitin assays after dry matter measurement of the substrates. The chitin content of the cell walls was determined and expressed as $mg\ g^{-1}$ DM of initial substrate using the colorimetric method as described before.

Cellulase activity in the samples of the culture supernatant was quantified by measuring the generation of reducing sugar from carboxymethyl cellulose (CMC) as the substrate using the procedure described by Miller *et al*. (1960). Enzyme and substrate controls were included in all assays and each sample was analysed in duplicate. Cellulase activity was expressed in terms of international units (IU) where one unit of enzyme activities was defined as the amount of enzyme required to produce one micromole of reducing sugar (glucose) every minute under standard conditions. The results of the CMCase activity in the culture medium and related chitin content during the period of the experiments were then compared and the correlation between them was calculated.

Diurnal fluctuation of chitin in the rumen

Diurnal fluctuation of the chitin in the rumen were measured from the ruminal digesta samples taken at 4 h intervals over a period of 48 h, commencing just before the morning feed (zero time) on the first day and on the second day commencing 2 h after feeding (09.30 h), to make a staggered 2 h sampling series. The pH of the liquor was determined after straining and samples of centrifuged strained rumen fluid (CSRF) and associated plant particulate material (PLP) were collected and prepared for chitin analysis as described above.

SEM study of fungal growth and development in vivo *and* in vitro

The development of anaerobic fungi on *in vitro* and *in sacco* incubated milled straw and the plant particles of rumen digesta samples was studied by scanning electron microscopy. The incubation time for the *in sacco* samples was 24, 48 and 72 h. The *in vitro* samples were taken from the serum bottles of anaerobic fungi cultures incubated for a period of 2, 6, and 12 d at 39 °. Digesta samples were taken directly from the rumen at 08.00 h (before morning feeding) and fixed into 2.5% glutaraldehyde in phosphate buffer (pH 7) for at least 4 h at 4 °. The fixed tissues were dehydrated through a graded series of ethanol concentrations before critical point dried via liquid CO_2 at 36 °, mounted on aluminium stubs, and coated with a thin layer of gold in a Polaron sputter coater (Polaron, Watford). They were then examined under a Cambridge 240 Stereoscan SEM operated at 10–15 kV.

Table 1. Growth of anaerobic fungi in the culture medium inoculated with rumen incubated milled straw and dilutions of strained rumen fluid or faeces samples[a].

	Faeces dilutions[b]		SRF dilutions[b]		
Animal	10^{-2}	10^{-3}	10^{-2}	10^{-3}	Incubated milled straw
sheep	− − −	− + +	− − −	− − −	+ + +
cow	+ + +	+ + +	+ − −	+ − +	ND

[a] Results are from three tests for each dilution. + indicates that fungi were isolated and − indicates that no fungi were isolated in each test. SRF, Strained rumen fluid; and ND, Not determined.

[b] Dilutions of 10^{-4} and 10^{-5} showed no growth.

Statistical analysis

Regression analysis was used to determine the linearity between the absorbance and glucosamine hydrocloride concentration using Microsoft Excel™. The correlation between both cellulase activity and chitin content and the results of the HPLC analysis and colorimetric assay of chitin was also calculated using the same program. The results of the pH and chitin content of strained rumen fluid and plant particles at each sampling time were analysed using the general linear model (GLM) procedure using the Minitab® statistical package.

RESULTS

Fungal isolation

The results of serial dilutions for the isolation of rumen fungi from strained rumen fluid (SRF), faeces, and *in sacco* incubated milled straw after 6 d incubation are summarised in Table 1. Most of the samples inoculated with SRF did not show any growth, particularly when samples from sheep were used as inoculum. However, anaerobic fungi were successfully isolated from faeces samples from both species. We only successfully isolated rumen fungi from the 10^{-2} and 10^{-3} dilutions. In contrast, anaerobic fungi were observed in all of the bottles inoculated with colonised milled straw.

Motile zoospores of anaerobic fungi were found in all of the rumen fluid samples examined microscopically. Their shape was variable but basically ovoid. In rumen fluid samples, zoospore motility was retained for at least 1 h in most suspensions. However, when zoospores ceased swimming their individual flagella separated (Fig. 6) and it was possible to distinguish between genuinely monoflagellate and those with multiflagellate zoospores.

Examination of the isolated cultures under the light microscope showed the range of morphological diversity of the zoospores, sporangia and rhizoidal systems (Figs 4–11). Some of the zoospores observed possessed 1–6 flagella (Fig. 4), whilst others showed more than six flagella (Figs 5–6). All of the isolates were monocentric, but the shape of the sporangia and the extent of rhizoidal development was variable. The initial shape of the sporangia was usually spherical (Figs 8–10) to ovoid (Fig. 11). Mature sporangia contained many zoospores (Fig. 8) and most sporangia showed an extensively branched rhizoid system (Figs 8 and 10). A small number of sporangia with less branched rhizoids were also present (Fig. 11). The morphological characteristics of all the zoospores, sporangia and rhizoidal systems of rumen and faecal isolates were similar.

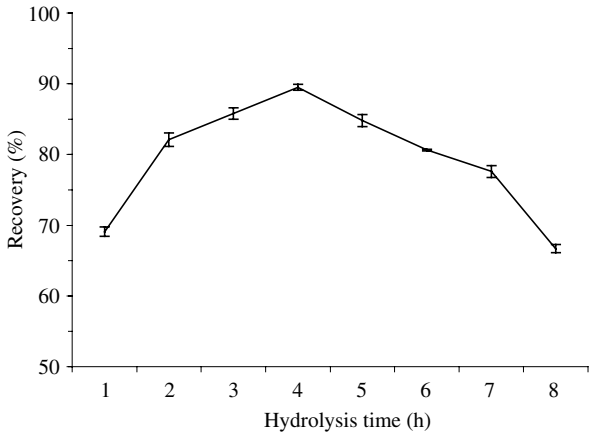


Fig. 2. Recovery of chitin hydrolysis after different times of incubation in 6 M HCl at 105 °C.

Chitin and carboxymethyl cellulase assays

The amount of glucosamine hydrochloride released from the hydrolysis of pure chitin in 6 M HCl for different lengths of times increased steadily up to 4 h incubation and then showed a sharp decrease (Fig. 2). The release was 82.1% of the initial weight of chitin after 2 h, reached a maximum of 89.5% at 4 h, and apparently decreased to 66.7% at 8 h (Fig. 2). A linear relationship ($r = 0.99$) was observed over chitin concentrations of 5–40 μg ml^{-1} of standard solution, and the colour of the reaction solution remained stable up to 80 min after colour development. Measurement of glucosamine hydrochloride from the hydrolysate samples of CSRF, PLP, and colonised milled straw by the colorimetric method generally showed a higher concentration when compared with the results of the HPLC analysis from the same samples but there was a high correlation ($r = 0.98$) between the results of both methods (Fig. 3a).

The carboxy methyl cellulase (CMCase) activity and correlated chitin levels from *in vitro* culture bottle experiments are summarised in Table 2. CMCase activity showed a relatively sharp increase until the 9th day of growth and then decreased slowly. The amount of chitin in the culture bottles also increased rapidly from the first to the fourth day of growth, continued more slowly to reach a maximum level nine days after inoculation, and then followed a similar trend as cellulase activity. The relationship between cellulase activity and chitin content demonstrated a high positive correlation ($r = 0.96$) during the period of *in vitro* growth experiment (Fig. 3b).

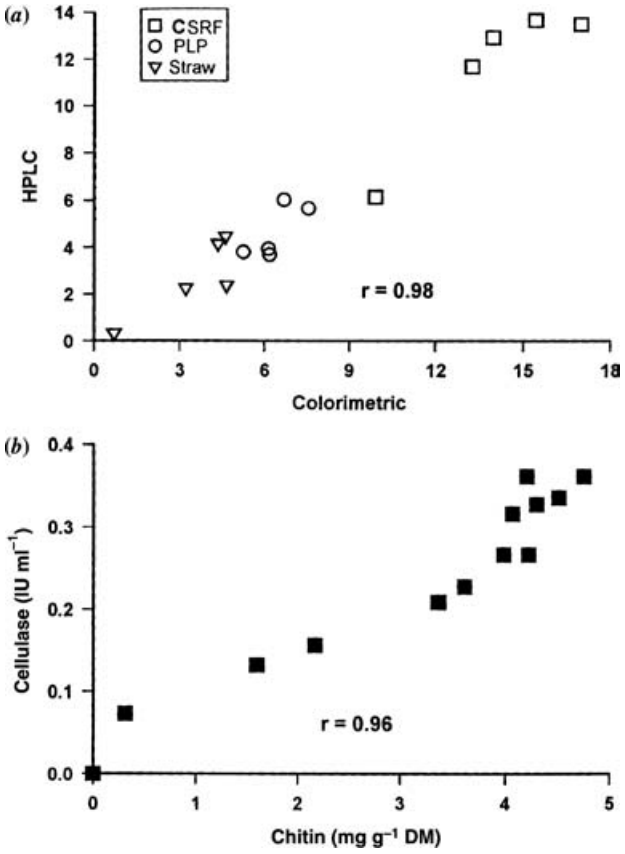


Fig. 3. Correlations between chitin measurement from different samples by HPLC and colorimetric method (*a*) and between cellulase activity and chitin cell wall content of rumen anaerobic fungi in *in vitro* culture (*b*).

Chitin content of the strained rumen fluid fractions

Results of the dry matter and chitin content of each fraction of strained rumen fluid are summarised in Table 3. The percentage dry matter in the fractions PLF, BF and CRF were 37.3, 13.7, and 49 g dry matter of SRF, respectively. The highest amount of chitin was measured from fraction PLF (16.43 mg g^{-1} DM) compared to that of fraction BF and the protozoal fractions, which were 4.18 and 7.66 mg g^{-1} DM, respectively. When the chitin component of the fractions was expressed as mg g^{-1} DM of SRF, fraction PLF contributed 91.5% of the total chitin content of the SRF.

Diurnal variation of pH and chitin content in the rumen

The changes in rumen pH and chitin content of sheep over a 24 h period are summarised in Table 4. There was a decline of pH during the first 4 h after feeding. After this fall, the pH rose and reached the prefeeding level after 8 h. The chitin content of the CSRF and PLP samples was measured per g dry matter of each fraction. In general, the level of chitin in the CSRF was about 4 times higher than that of the PLP samples. The mean value of that for SRF varied and was 12.6–13.8 mg g^{-1} DM compared to that of PLP, which was

Table 2. Carboxymethyl cellulase activity and chitin cell wall content of rumen anaerobic fungi during 12 d incubations at 39 °C[a].

Day of growth	Cellulase activity (IU ml^{-1})	Chitin content (mg g^{-1} DM)
0	0.00	0.00
1	0.07 ± 0.018	0.33 ± 0.091
2	0.13 ± 0.025	1.70 ± 0.432
3	0.16 ± 0.056	2.31 ± 0.517
4	0.21 ± 0.015	3.59 ± 0.170
5	0.23 ± 0.051	3.83 ± 0.603
6	0.27 ± 0.003	4.28 ± 0.639
7	0.32 ± 0.021	4.36 ± 0.851
8	0.34 ± 0.019	4.80 ± 0.329
9	0.36 ± 0.028	5.04 ± 0.490
10	0.37 ± 0.013	4.47 ± 0.784
11	0.33 ± 0.039	4.57 ± 0.287
12	0.27 ± 0.036	4.48 ± 0.293

[a] Values for each day are mean of three replicates samples ± SD.

3.2–4.0 mg g^{-1} DM. There was a slight decrease in chitin component of the CSRF up to 4 h after morning feeding which then increased gradually and reached the prefeeding level 10 h after feeding. After that, it remained fairly constant up to 22 h after feeding. Following the afternoon feeding, no significant changes were observed in this component. The variation in the chitin component of the CSRF and PLP at different sampling times was not statistically significant ($P > 0.05$).

SEM examination of in vitro *and* in vivo *plant particle samples*

Pieces of milled barley straw before incubation showed the very uniform appearance of the intact cuticle and the bumpy ridges of the epidermal cells (Fig. 18). Anaerobic fungi were observed on *in vitro* inoculated straw particles (Figs 12–16) and on both *in sacco* incubated milled straw (Figs 19–22, 24) and plant particle samples taken directly from the rumen (Figs 17, 23). Anaerobic fungi continued to increase in biomass during the 12 d of the *in vitro* experiment in which extensive rhizoidal systems and associated zoosporangia were observed even after 2 d inoculation (Figs 12–13). The density of rhizoids and number of developing zoosporangia increased up to day six of the incubation (Figs 14–15) although there were some discharged sporangia (Fig. 15) observed at this time. Developing sporangia with an abundant rhizoid system were observed over the straw surface in *in vitro* incubated particles, although the sporangia were generally formed on the damaged surfaces of the straw (Fig. 21). Thalli were still observed on the milled straw, especially on the cut ends of the particles after 12 d (Fig. 16). Even after this prolonged incubation period, some parts of the straw fragments remained relatively unaffected by fungal activity (Fig. 16). In the *in sacco* samples sporangia were observed on the cut edges of the straw (Figs 19, 24). In some cases, the penetration of the straw cuticle (Fig. 22) was also seen after 24 h incubation in the

Table 3. Percentage dry matter and chitin content of the strained rumen fluid (SRF) fractions taken from the sheep fed a diet of pelleted Lucerne 2 h before feeding[a].

	DM of the fractions (as % of DM of SRF)			Chitin content (mg g^{-1} DM)				Chitin content (mg g^{-1} DM of SRF)	
	PLF	BF	CRF	SRF	PLF	BF	PF	PLF	BF
	37.3	13.7	49.0	6.70	16.43	4.18	7.66	6.13	0.57
STD	4.17	2.48	9.71	0.53	1.31	0.52	0.93	0.88	0.115

[a] Values are means of four sheep.

DM, dry matter; BF, bacterial fraction; PF, protozoal fraction; PLF, pellet of the centrifuged SRF with 150 ***g***; CRF, clarified rumen fluid; and STD, Standard deviation.

Table 4. Diurnal variation of the rumen pH and chitin component of the centrifuged strained rumen fluid (CSRF) and plant particles (PLP) from the rumen of sheep fed a diet containing hay and pelleted lucerne twice a day[a].

Time after morning feeding (h)	Rumen pH	Chitin component (mg g^{-1} DM) of	
		CSRF	PLP
0.0	7.3 ± 0.15	13.4 ± 0.87	3.3 ± 0.74
2.0	6.3 ± 0.14	13.0 ± 1.10	3.4 ± 1.26
4.0	6.2 ± 0.25	12.6 ± 0.71	3.7 ± 0.36
6.0	6.8 ± 0.14	12.7 ± 1.40	3.8 ± 1.22
8.0	7.1 ± 0.21	13.1 ± 1.20	3.7 ± 0.91
10	6.1 ± 0.17	13.6 ± 1.30	3.2 ± 0.69
12	5.9 ± 0.12	13.3 ± 0.67	3.3 ± 0.76
14	6.4 ± 0.12	13.5 ± 1.83	3.5 ± 0.63
16	6.6 ± 0.09	13.5 ± 1.35	3.7 ± 0.39
18	7.0 ± 0.19	13.8 ± 2.04	4.0 ± 0.57
20	7.0 ± 0.09	13.5 ± 2.17	3.9 ± 0.82
22	7.2 ± 0.04	13.7 ± 1.66	3.6 ± 0.53

[a] Values are means of four sheep ± SD.

rumen. Variations in the shape of fungal sporangia were also observed among the various samples. Rounded forms were dominant in the *in vitro* samples (Fig. 14), whilst elongate forms were mostly seen from the *in sacco* (Figs 19, 21–22, 24) and plant particle samples (Fig. 23). Sporangia were particularly abundant in some of the samples taken directly from the rumen (Fig. 23). Developing sporangia were observed along the line of the cell periclinal walls (Fig. 17). The presence of a complex rhizoid system on the straw incubated in the culture medium (Figs 12–16) was the most obvious difference between these and the *in sacco* incubated straw or *in situ* rumen plant particle samples. No superficial rhizoidal system was observed in *in vivo* specimens (Figs 17, 19–22, 24).

DISCUSSION

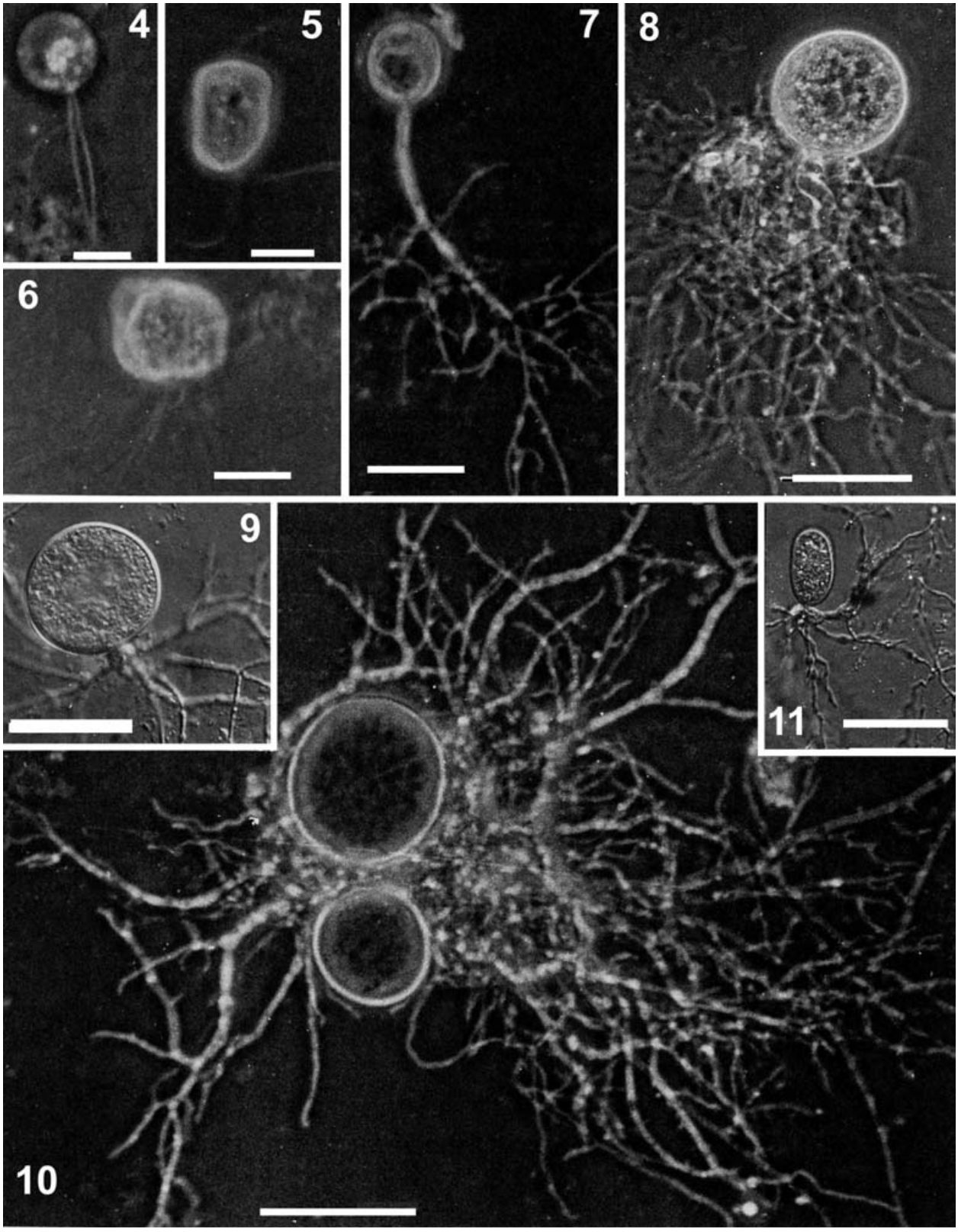
Fungal isolation and identification

The observation of motile zoospores in the strained rumen fluid (SRF) samples confirms the presence of these organisms in the rumen of the animals used for sample collections in this investigation. The successful isolation of anaerobic fungi from some samples indicates that the isolation technique *per se* was satisfactory. The failure to successfully isolate fungi in other samples may have been due to several factors. Fungal zoospores are only reported to be viable 1–4 h after release from the sporangium (Lowe *et al*. 1987a), and their viability is reduced when exposed to air. The lack of establishment of cultures from rumen liquor may be due to the low number of viable zoospores because of the exposure to air of samples at the time of sampling, and (or) the lack of probable resistant form of the fungus in the rumen liquor. These observations suggest it may be the sporangia associated with milled straw that are the most likely source of inoculum. Therefore, baiting with milled straw in dacron bags was used as a reliable inoculum source for the isolation of anaerobic fungi from the rumen. This method has the advantage in that it does not require stringent anaerobic conditions at the time of sample collection from the animal.

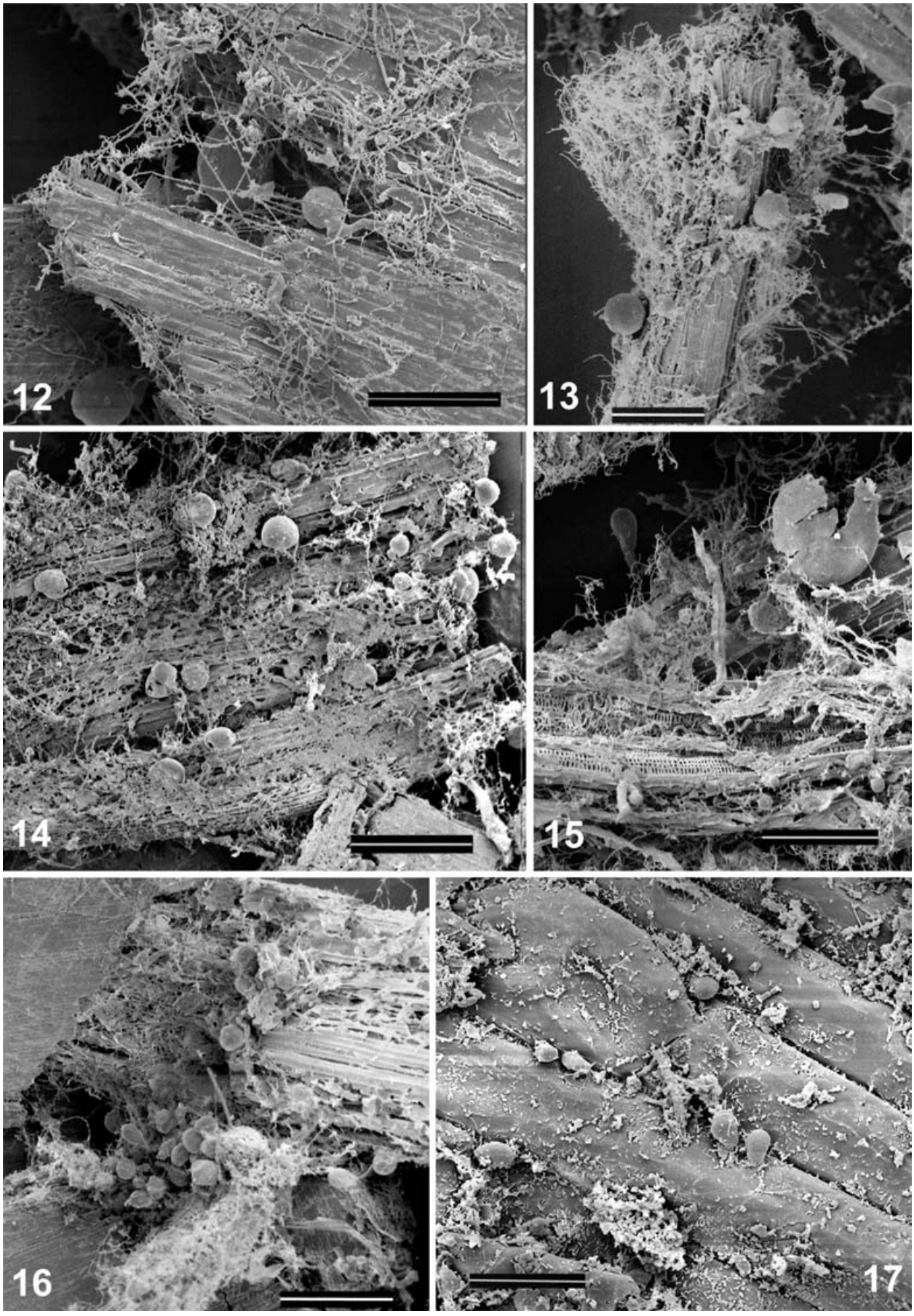
The efficacy of chitin and cellulase assays

Conditions for the hydrolysis procedure is one of the factors which may affect the accuracy of the chitin assay (Toppan, Roby & Esquerre-Tugage 1986). The percentage of hydrolysate recovery depends upon the acid concentration, duration and temperature of the hydrolysis treatment (Chen & Johnson 1983). Our results found are in agreement with the finding of Wu & Stahmann (1975) and Gurusiddaiah, Blanchette & Shaw (1978) who reported that the maximum hydrolysis of chitin occurred after 4 h of incubation. Continued hydrolysis results in destruction of glucosamine (Lin & Cousin 1985) and so the sharp decrease in the recovery of chitin hydrolysis after 4 h may be explained by this. Therefore, the hydrolysis was performed for 4 h and the recovery correction (89.5 %) was applied to all of the samples used for the chitin assay.

The linearity between the absorbance and glucosamine hydrochloride concentrations in the standard solution was comparable to the results of Muzzarelli & Rocchetti (1986). After acid hydrolysis of chitin, the yield of the glucosamine hydrochloride can be measured as total hexosamine colorimetrically (Chen & Johnson 1983) or by HPLC (Lin & Cousin 1985, Quigley & Englyst 1992). The high correlation between the results obtained from the colorimetric and an HPLC method were good. Therefore, the use of the colorimetric method was adapted for the subsequent experiments, since it was the simplest to perform and quicker.



Figs 4–11. Morphology of rumen anaerobic fungi isolated from a sheep with the use of the culture medium C and photographed under phase contrast optics (Figs 4–8, 10) or differential interference contrast optics (Figs 9, 11). **Fig. 4.** An oval zoospore containing less than six flagella. **Fig. 5.** An elongate zoospore with more than six flagella. **Fig. 6.** A multiflagellate zoospore with more than 15 flagella. **Fig. 7.** Zoosporangium at the early stage of development showing the main rhizoid, which is beginning to develop. **Fig. 8.** A spherical mature sporangium with extensively branched rhizoid. **Fig. 9.** A spherical mature sporangium showing the main rhizoid. **Fig. 10.** Two spherical sporangia with their rhizoidal system showing an extensively branched tapering form. **Fig. 11.** An elliptical sporangium and related rhizoids which does not show a highly branched rhizoidal system. Bars Figs 4–6 = 10 μm; Fig. 7 = 20 μm; Figs 8–10 = 50 μm; and Fig. 11 = 40 μm.



Figs 12–17. For legend see opposite page.

In this study, the sharp increase in the amount of chitin during the first four days of growth (Table 2) is in line with other growth studies (Lowe *et al.* 1987a). Increased production of the CMCase enzyme during the same period (Table 2) mirrors the recovered chitin levels. These enzymes are produced by the vegetative rhizoidal systems (Williams & Orpin 1987, Hebraud & Fevre 1988). The amount of enzyme activity after 7 d growth was approximately the same as that reported in *Neocallimastix* sp. strain LM-1 by Gordon & Phillips (1989). The close correlation between enzyme activity and chitin levels confirms that the chitin assay is a valid indicator of fungal biomass for a mixed population of anaerobic fungi in *in vitro* cultures. It can also give an estimation of the fungal activity in relation to cellulose degradation potential.

Chitin contents of the different SRF fractions

One of the major problems in assessing the importance of anaerobic fungi in the rumen has been the difficulty of reliably estimating their biomass. Although different methods have been used, so far, there is no reliable method for estimating these fungal populations in the rumen. The chitin assay has so far only been used by Argyle & Douglas (1989) for the assessment of fungal biomass in the rumen. In their work, chitin was measured from the rumen samples taken from defaunated sheep either with or without anaerobic fungal populations. They found little difference between the chitin values obtained from the two groups of animal, which they attributed factors other than fungal biomass in the rumen (Argyle & Douglas 1989). Gay (1991) pointed out that cell wall of bacteria may release glucosamine during the hydrolysis procedure which may affect the assay. Chitin was present in both bacterial and protozoal fractions (Table 3). We only found about 8.5% was associated with the bacterial fraction. Since the percentage of chitin which may be attributed to bacterial cell walls was very low, the amount of chitin measured from the rumen samples is likely to be from either protozoa or fungi. However, the low amount of chitin associated with the protozoal fraction suggests that the main origin of chitin in the PLF fraction is the fungi. More than 90% of the chitin content of the whole rumen fluid was in this fraction (Table 3). Further qualitative and quantitative measurements of chitin from pure rumen bacteria and protozoa are still needed to confirm this deduction. To do this, a better method of separating and purifying the various microbial fractions from the whole rumen fluid needs to be developed. In addition, the measurement of chitin from axenic and/or mixed bacterial populations may also clarify the efficacy of the chitin assay in *in vivo* experiments.

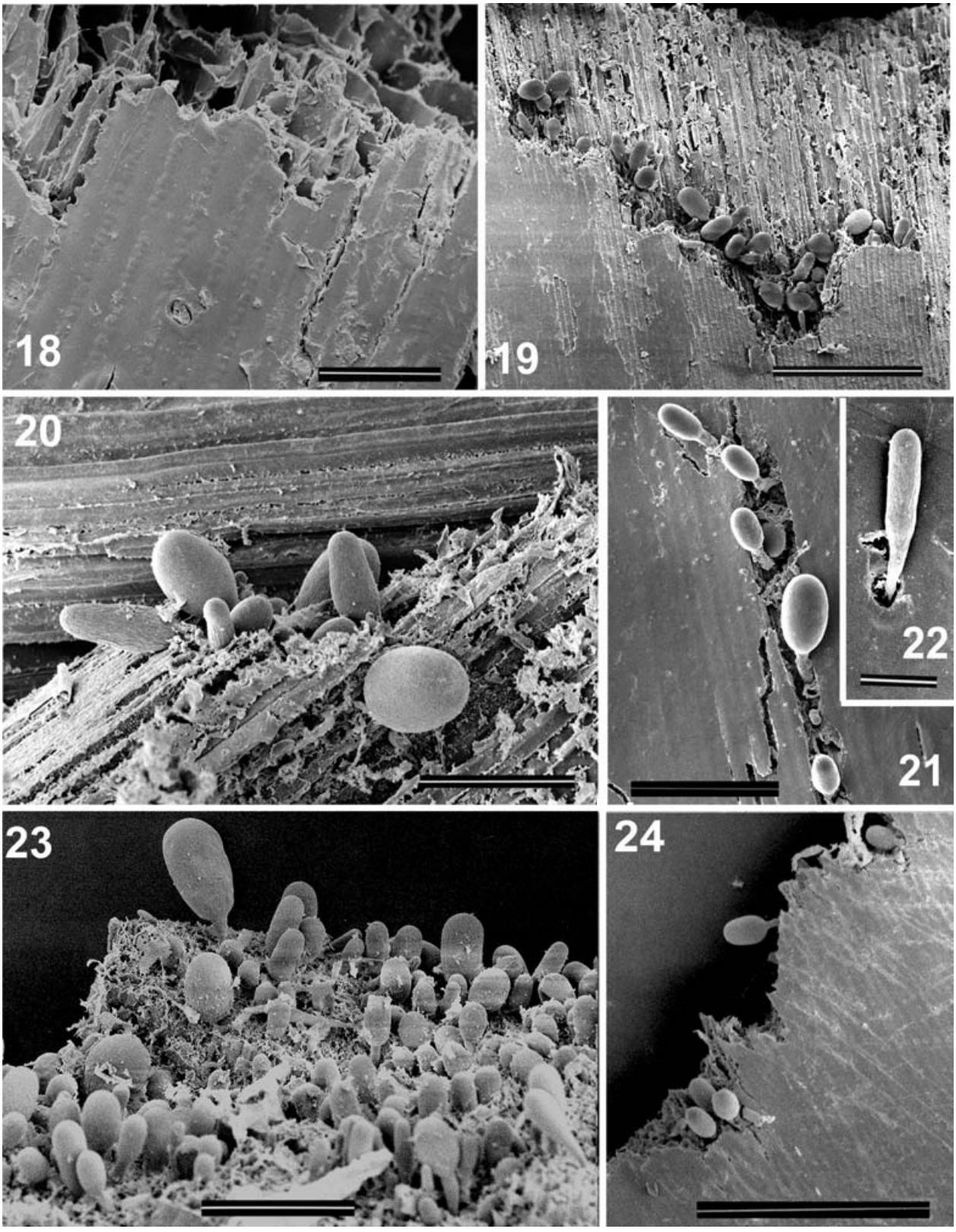
Diurnal variation of pH and chitin content in the rumen

Changes in ruminal pH have been considered as one of the fermentation characteristics of the rumen (Hobson & Wallace 1982). The pH changes we found support this concept. Anaerobic fungi grow optimally at pH 6–7 (Orpin 1975, Grenet *et al.* 1988), with maximal production at pH 6.5 (Dehority & Orpin 1988). The rumen pH values measured in this study was always within the range at which these fungi can thrive.

In this study, the chitin content of the rumen samples in both the centrifuged strained rumen fluid (CSRF) and associated plant particulate material (PLP) fractions did not show any significant change over a 24 h period (Table 4). This is in contrast to the results obtained by the zoospore counting method, in which there was a marked increase in the number of the fungal zoospores during the 2 h period following feeding (Orpin 1975). Clearly, the residual sporangial wall and rhizoids will still be rich in chitin even after zoospore release, and might not therefore be expected to show the fluctuation observed when measuring zoospore populations. This agrees with the findings of Obispo & Dehority (1992), based on the most probable number (MPN) method for enumeration of rumen fungi, which quantifies both sporangia and free zoospores present in rumen. Obispo & Dehority (1992) concluded that regardless of the feeding frequency, fungal numbers remained fairly constant over a 24 h period, as we found.

Since anaerobic fungi colonise plant debris by producing an extensive rhizoidal system (Bauchop 1979, Chaudhry 2000), we expected that the level of chitin in the plant particles would be higher than that in the strained rumen fluid. The apparently greater level of chitin in the CSRF may be due to the fractionation of the sporangia into the rumen fluid. Since in *in situ* studies we observed little evidence of extensive rhizoid development, any rhizoid contribution must be from fungal material within smaller fragments of partially digested plant materials. The greater amounts of undigested residues in the plant particulate samples

Figs 12–17. Series of scanning electron micrographs summarizing and illustrating the growth and development of rumen fungi on straw particles incubated in culture medium (Figs 12–16) and on plant particulates from the rumen (Fig. 17.) **Figs 12–13.** Portion of broken straw fragment 2 d after inoculation showing extensive rhizoid system and developing zoosporangia. **Figs 14–15.** Infestation of the straw particles with fungal rhizoids 6 d after inoculation. Both developing and discharged sporangia are abundant at this time. **Fig. 16.** Infested straw particles 12 d after inoculation showing an abundance of sporangia forming on the cut ends of the straw particles. **Fig. 17.** Developing sporangia along the line of the cell periclinal walls of plant particles from the rumen. Bars Figs 12, 13 and 15 = 100 μm; Figs 14 and 16 = 250 μm; Fig. 17 = 25 μm.



Figs 18–24. Series of scanning electron micrographs summarizing and illustrating intact barley straw (Fig. 18) and the growth and development of rumen fungi on *in sacco* incubated straw particles in the rumen (Figs 19–22, 24) or on plant particulates from the rumen (Fig. 23). **Fig. 18.** Detail of 'control' milled barley straw before being incubated *in sacco* in the rumen. **Fig. 19.** The ellipsoidal sporangia appearing from the cut ends of the straw particles with the complete absence of a superficial or surface rhizoid system after 24 h incubation within the rumen. **Fig. 20.** Rounded ovoid sporangia on straw particles after 24 h incubation in the rumen. Again there is a complete absence of a branched superficial rhizoid system. **Fig. 21.** Details of sporangia associated with the cuticle surface from milled straw particles, after 24 h incubation in the rumen. They have infested a cracked or broken region of the cuticle surface. **Fig. 22.** Elongate finger-like sporangia on the smooth

compared to that of CSRF dry matter content might also account for this difference. The amount of chitin in the present samples was however higher than that reported by Argyle & Douglas (1989).

SEM study of fungal growth and development on in vitro *and* in vivo *samples*

Bauchop (1979) was the first to use the SEM to demonstrate a large population of anaerobic fungi attached to plant fragments from the rumen of cattle and sheep. Further studies *in vitro* (Lowe *et al*. 1987b, Akin *et al*. 1989) and *in vivo* (Windham & Akin 1984, Grenet & Barry 1988) on incubated plant fragments demonstrated that the major route of invasion by fungi appeared to be via areas of epidermal damage. The observations of the *in vitro* and *in sacco* samples in the present study are generally similar to those of previous studies (Bauchop 1980, Grenet & Barry 1988, Roger *et al*. 1993). Sporangia were mostly present on the damaged surfaces (Fig. 21) or on the cut edges of the straw (Figs 19, 24).

The lack of an extensive rhizoidal system associated with sporangia in *in vivo* specimens, which we observed here has also been noted by Roger *et al*. (1993). They concluded that the growth of anaerobic fungi *in vitro* does not follow the same pattern as in the rumen. Whether this difference is due to the rumen conditions (nutrient availability etc.) or due to the interaction with other microorganisms is not known. In an SEM study, Grenet, Fonty & Barry (1989) examined maize and lucerne stems from the rumens of gnotobiotic lambs containing only fungi. Their micrographs do not show any rhizoids, which suggests it may be due to the lack of sufficient nutrients in the rumen environment that inhibits superficial growth of rhizoids. Further studies are needed to identify the factors that are responsible for these differences.

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cuticular surface of straw particles after 24 h incubation. The localised erosion of the cuticle in the region of fungal rhizoid penetration is clearly seen. **Fig. 23.** The abundant ellipsoidal sporangia projecting on the surface of a plant particle from the rumen content of sheep and complete suppression of rhizoidal growth. **Fig. 24.** The ellipsoidal sporangia appearing from the cut ends of the straw particles after 72 h of incubation in the rumen and the complete absence of a superficial or surface rhizoid system. Bars: Figs 18 and 20 = 50 μm; Figs 19 and 24 = 200 μm; Figs 21 and 23 = 100 μm; and Fig. 22 = 20 μm.

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Corresponding Editor: N. P. Money