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Distribution and estimation of anaerobic zoosporic fungi along the digestive tracts of sheep

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The status of anaerobic zoosporic (*Chytridiomycota*) fungi along the entire digestive tract of sheep was assessed both analytically and microscopically. Digest samples were taken from different segments of the digestive tracts of three newly killed sheep that previously had been used in experimental dietary studies. These digest samples were tested for the presence of rumen fungi by assessing the recovery of live fungi from the samples, direct observation of digested plant remains under the scanning electron microscope (SEM), and using a chitin assay as an estimation of fungal biomass. Live anaerobic fungi were recovered from the abomasum, small and large intestine, caecum and faeces of sheep, but not from the digest samples of rumen and omasum. However, SEM examination of the samples confirmed the presence of fungal structures from all of these organs. In the large intestine and caecum samples the observed sporangial structures were rounded and showed conspicuous surface pitting. Results of the chitin assay indicated that the anaerobic fungi might account for up to 20% of the total microbial biomass in the rumen of sheep. The results of this study support the view that anaerobic fungi may be present as a resistant stage in the lower reaches of the digestive tract.

INTRODUCTION

Since the discovery of anaerobic rumen fungi by Orpin (1975), considerable research has been directed towards obtaining a better understanding of these organisms and their role within the digestive tract ecosystem of herbivorous animals. They have been isolated from animals from different parts of the world (Trinci *et al.* 1994, Orpin & Joblin 1997) providing evidence to suggest that they may have an important role in the digestion of fibrous materials in the rumen (Akin & Rigsby 1987, Joblin 1989, Lee, Ha & Cheng 2000). However, there is little information on the morphological and physiological state of these fungi whilst passing through the digestive tract of animals and their fate in relation to the digestion process of the host has yet to be determined. Orpin (1989) commented that the determination of the fate of the spent fungal vegetative material within the rumen, and of the fate of any vegetative stages which may pass into the lower alimentary tract would be of great value in determining the possible contribution of these organisms to the nutrition of the animal.

A study of the distribution of these fungi along the digestive tract of cattle have been carried out by Davies *et al.* (1993b). They successfully isolated fungi from fresh digest samples, taken from each segment of the digestive tract. However, attempts to isolate them from dried samples of the rumen digests were unsuccessful. These observations led Theodorou and his colleagues to suggest that anaerobic fungi produce a resistant survival structure during part of their life-cycle which is the likely mechanism for their transfer between animals (Davies *et al.* 1993b, McGranaghan *et al.* 1999, Brookman *et al.* 2000). This was confirmed by Wubah *et al.* (1991) who illustrated resistant structures produced by *Neocallimastix* from the rumen of cattle (Wubah *et al.* 1991). Lowe, Theodorou & Trinci (1987) isolated anaerobic fungi from the fresh faeces and saliva of sheep and further research demonstrated their ability to survive in dry faeces for up to 128 d. The planned slaughter of a number of fistulated experimental animals which had been used in long term nutritional experiments gave us the rare opportunity to investigate the status of anaerobic fungi and their survival within the digestive tract and to assess

measurement of the chitin component of the digest as an indicator of fungal biomass.

MATERIALS AND METHODS

Animals and diets

Three Suffolk wethers sheep (average live weight 62 kg) were used in this investigation. The animals had been kept individually in metal-framed metabolism crates with a steel mesh floor panel. They were habituated to a diet consisting of 1.6 kg chopped and pelleted lucerne (70 and 30 %, on a dry matter basis, respectively) once a day at 09.00 h for 14 d. The pellet included 5 % of molasses that was added to the chopped lucerne. Clean, fresh water and mineral blocks were always available.

Sampling procedure

The sheep were killed by injection of Euthatal (May and Baker, Dagenham) 30 h after feeding. The whole digestive tract was removed from the carcass immediately after death, and the digest sampled as described by Davies *et al*. (1993b). The attachment points between the different organs of the alimentary tract were tied off and then the different parts were separated from each other. Except for the rumen, the tract organs were weighed, emptied and weighed again to calculate the component weight of each part by difference. The rumen contents were weighed directly. Triplicate samples of the digest contents (*ca* 30–50 g) were collected from each part for dry matter determination and chitin assay. The digest contents of each organ were then strained between two layers of intact muslin. The pH of these strained samples (digest fluid) was measured immediately using a digital pH meter. Samples of digest fluid and associated plant particles (digest solid) were also collected for dry matter determination and chitin assay. Faeces samples were collected directly from the end part of large intestine for chitin measurement.

Chitin assay

Analysis of chitin in digest samples was performed on freeze dried samples of digest contents, digest fluid, and digest solid. 20 ml of the digest fluid samples were used and prepared for chitin analysis as described by Rezaeian, Beakes & Parker (2004). Dried samples were hydrolysed using the method described by Lin & Cousin (1985). The chitin content of samples was determined calorimetrically as the glucosamine hydrochloride equivalent resulting from hydrolysis as described by Chen & Johnson (1983). The total amount of chitin in each organ was also calculated from the total digestive dry matter content of each part of the tract and the results of the chitin assay from the samples of whole digest contents taken from the same organ.

Table 1. Results of culture inoculation with three replicate from the samples of different parts of digestive tract and of faeces.

	Animals[a]		
Organ	A	B	C
Rumen	−	−	−
Omasum	−	−	−
Abomasum	+	+	+
Small intestine	−	−	+
Large intestine	+	+	+
Caecum	+	+	+
Faeces	+	+	+

[a] +, fungi isolated from individual sheep; and −, no fungi isolated from the sheep.

Isolation of fungi from the digestive tract

Digest solid from each organ was collected into polythene bags and returned to the laboratory for the isolation procedure. Samples were inoculated, within 4 h of collection, into serum bottles containing 45 ml of medium C as described by Rezaeian (1996). The inoculated serum bottles were monitored for 6 d after inoculation for the appearance of rumen fungi. Microscopical observations were also carried out on the isolated fungi using a Reichert Zetopan phase contrast microscope to compare the morphological characteristics of the isolates in different sections of the gut.

SEM study of digestive tract contents

Samples of plant particles from the digest solid from each segment of the digestive tract were collected and prepared for SEM examination as described by Rezaeian *et al*. (2004).

Statistical analysis

The relevant correlation coefficient (r) between the amounts of chitin (mg g^{-1} DM) in digest contents (DC), digest fluid (DF), and digest solid (DS), fractions in all organs was determined using Microsoft Excel.

RESULTS

Recovery of fungi from the digest samples

The recovery of cultures of rumen fungi from samples taken from each organ of digestive tract are summarised in Table 1. Anaerobic fungi were isolated from the abomasum, large intestine, caecum, and faeces, of all three animals and the small intestine of one. However, no rumen fungi were recovered either from the rumen itself or the omasum. Polyflagellate fungal zoospores, probably of *Neocallimastix*, were however observed when screening the rumen samples under the light microscope before the isolation procedure was undertaken.

Table 2. pH, dry matter and chitin content in each organ of the digestive tract of sheep fed a diet of chopped and pelleted lucerne[a].

Organ	pH	Percentage DM in each organ	Total DM per organ (g)	Chitin content (mg g^{-1} DM) of digest fractions			Total chitin (mg) per organ
				DF	DS	DC	
Rumen	6.9 ± 0.25	11.2 ± 0.32	984 ± 190	17.7 ± 2.09	5.4 ± 0.36	5.7 ± 0.65	5589 ± 1171
Omasum	7.1 ± 0.17	20.0 ± 0.84	50 ± 6.1	19.6 ± 3.36	6.5 ± 0.59	6.6 ± 0.70	338 ± 74
Abomasum	2.7 ± 0.40	10.4 ± 0.24	59 ± 2.0	18.6 ± 1.70	5.5 ± 0.16	6.3 ± 0.57	289 ± 79
Small intestine	7.3 ± 0.10	9.6 ± 1.21	64 ± 16.1	22.2 ± 1.02	7.6 ± 0.94	12.1 ± 0.26	846 ± 204
Large intestine	7.1 ± 0.11	15.3 ± 0.09	88 ± 13.0	20.4 ± 0.88	8.2 ± 0.54	9.5 ± 0.74	911 ± 246
Caecum	7.1 ± 0.09	13.5 ± 0.05	105 ± 17.8	20.3 ± 1.05	6.7 ± 0.61	9.7 ± 1.16	1036 ± 272
Faeces	NA	ND	NA	NA	NA	10.2 ± 0.82	NA

[a] Values are mean of three replicates ± SE.
DM, dry matter; DF, digest fluid; DS, digest solid; DC, digest contents; NA, not applicable; and ND, not determined.

pH and dry matter of digest contents

The pH of the digest fluid in the reticulo-rumen and omasum was 6.9 and 7.1, respectively (Table 2). In the abomasum, the digest was more acidic with a mean pH of 2.7 (Table 2). For the other parts of the digestive tract (i.e. the small intestine, large intestine, and caecum) the pH values were all nearly neutral, ranging from 7.3–7.1 between the small intestine and the caecum respectively. The percentage DM of the digest was different in each organ (Table 2). The lowest value was in the small intestine (9.6%), and the highest was found in the omasum (20%). The total dry matter content also varied between the different parts (Table 2). The highest level was in the rumen, which contained about 72% of the total dry matter content of the alimentary tract.

Fungal status as assessed by chitin measurement

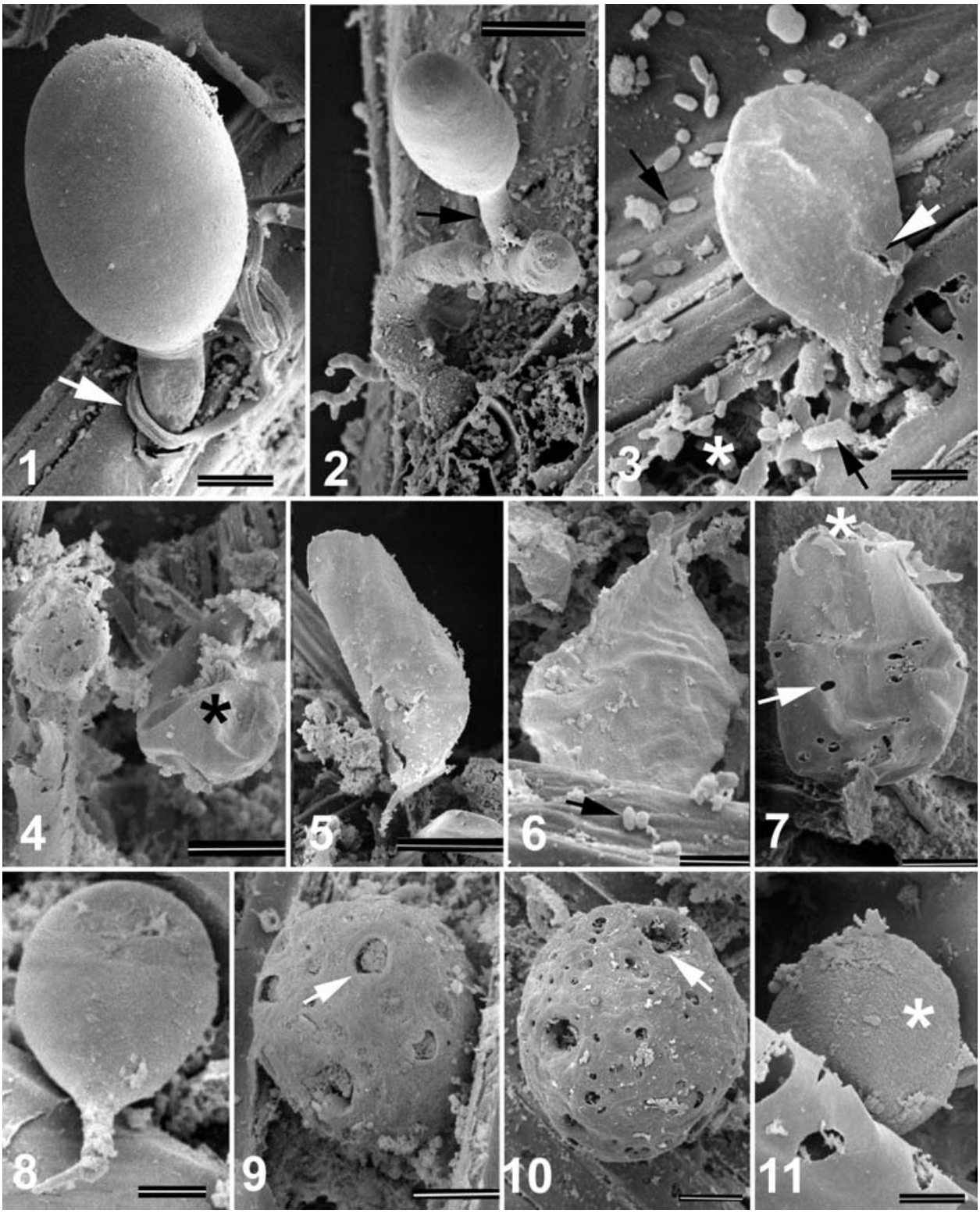
Chitin was present in all of the samples of digest fractions of each organ. The highest amount of chitin was measured in the digest fluid fraction samples, ranging from the 22.2 mg g^{-1} DM in small intestine to 17.7 mg g^{-1} DM in the rumen. This was about 2.5–3 times higher than the level present in the digest contents and the digest solid fractions (Table 2). The level of chitin in digest contents was also generally higher than the solid fraction samples in each organ. In all, three types of sample, the amount of chitin per gram DM in the rumen, omasum and abomasum was nearly the same. However, the levels were always higher in the small and large intestine and caecum, compared with the chitin content of the abomasum and pre-gastric organs. There was a high correlation between the levels of chitin associated with the different fractions within each organ ($r = 0.82$, 0.95, 0.80 and 0.98 for DF and DS, DF and DC, DS and DC, DF + DS and DC respectively). The total amount of chitin in each organ when expressed as mg per organ was highest in the rumen accounting for about 62% of the total chitin content of the digestive tract (Table 2). In the other organs it ranged from 3.2% in the abomasum to 11.5% in the caecum. It was calculated that the entire digestive tract contained 8.5 g chitin. In the faeces samples, chitin content was calculated per gram dry matter and came to 10.2 mg g^{-1} DM (Table 2).

SEM examination of fungal structure in the digestive tract

A different and distinct morphological form of the sporangium was observed on the plant particles taken from the rumen. Most had a typical elongate digit-like morphology (Fig. 2) although some were ellipsoid (Fig. 1) or ovoid. The sporangial stalks often had a curious rather twisted or branched morphology (Fig. 2), but there was no rhizoidal system associated with any of the cell wall fragments. They varied in size from about 12 μm for the elongate digit form, to 20–30 μm for the ellipsoidal and ovoidal form. The site of the fungal rhizoid penetration through the plant walls can be clearly seen (Fig. 1).

On the plant particles isolated from the omasum, sporangia were also observed but their form differed compared with those observed in the rumen. They mostly appeared to be collapsed (Figs 4, 6) and no young developing sporangial stages were observed in this part of the digestive tract. Collapsed sporangia were also observed in the abomasum sample (Fig. 7) in which there are clearly some holes in the collapsed walls. These appeared to be mature structures from which the zoospores had been released from the torn apices (Fig. 7). The size of the collapsed sporangia from the omasum (Fig. 6) and abomasum (Fig. 7) was about 40 and 60 μm, respectively.

No fungal structures were distinguished on plant particles isolated from the small intestine. However, sporangia were again observed on the particles taken from the large intestine (Figs 8–10). These sporangia appeared to be rounded in morphology (Figs 9–10) and showed many circular pits in the wall. The smooth rounded body shown in Fig. 8 was about 18 μm diam and may be a developing sporangium suggesting that colonisation of plant particles is still actively occurring in this part of the digestive tract. A probable fungal sporangium was also observed in the caecum sample (Fig. 11). It has a rounded shape and a rather fibrillar



Figs 1–11. Series of scanning electron micrographs illustrating sporangial morphology of anaerobic fungi from plant particles samples taken from the rumen **(Figs 1–3)**, osmasum **(Figs 4–6)**, abomasums **(Fig. 7)**, large intestine **(Figs 8–10)**, and caecum **(Fig. 11)** of sacrificed sheep fed a mixture of chopped and pelleted lucerne (70: 30 ratio) and killed 30 h after feeding. **Fig. 1.** Ellipsoidal sporangium and the coil of undegraded lignified spiral thickening (arrow), which has wrapped itself around the base of the sporangium. **Fig. 2.** Sporangial stalks (arrows) with a curious rather twisted or branched morphology. **Fig. 3.** The abundance of bacteria (arrows) associated with both the surface of the plant particle and the sporangial wall. **Fig. 4.** Collapsed ovoid sporangium. **Fig. 5.** Elongate sporangium. **Fig. 6.** Collapsed sporangium may has released zoospores. **Fig. 7.** Large collapsed (discharged) sporangium with holes (arrow) on its wall. The zoospores appear to have been released from the torn apex of this sporangium (*). **Fig. 8.** Rounded developing sporangium. **Figs 9–10.** Rounded sporangia showing circular surface pitting (arrow). **Fig. 11.** Rounded fungal sporangium with a rather granular texture (*) to its wall. Bars: Figs 3, 8 and 11 = 5 μm; Figs 1–2, 6 and 9–10 = 10 μm; Fig. 7 = 20 μm; Fig. 4 = 25 μm; and Fig. 5 = 50 μm.

texture to its wall. No rhizoidal structures were identified from any of the samples taken from the digestive tract of sheep.

DISCUSSION

Isolation of fungi from the digestive tract and their survival form

The occurrence of anaerobic fungi in all parts of the digestive tract of sheep was confirmed and supports previous studies (Theodorou *et al*. 1990, Wubah, Fuller & Akin 1991, Davies *et al*. 1993b). The isolation of anaerobic fungi from the abomasum samples indicate that they are also present in this part of the digestive tract although no fungal structures directly were observed in the SEM study. This is in agreement with the results of Davies *et al*. (1993b) who reported the isolation of these fungi for the first time from the abomasum of cattle. However, only a very small volume of digest samples can be directly examined in the electron microscope. It seems these fungi are able to survive the very acidic environmental conditions found in the abomasums (Grenet *et al*. 1989, Davies *et al*. 1993b).

The failure to isolate viable fungi from the rumen and omasum samples, in spite of the apparent presence of their zoosporangia on particles examined in the SEM (Figs 1–3), contrasts with their successful isolation and observed presence in the lower segments of digestive tract and faeces. It is possible that zoospores and normal vegetative thalli may not survive 4 h of exposure to oxygen. Fungal growths possibly arise only from putative survival structures under these isolation conditions. This suggests that physiological changes may have occurred to enable them to be recovered from what appear to be less favourable environments. The enhanced survival of anaerobic fungi in faeces has been reported by a number of authors (Trinci, Lowe & Theodorou 1988, Milne *et al*. 1989, Theodorou *et al*. 1993). The presence of a thick-walled sporangium has been reported in batch cultures of *Neocallimastix* (Wubah *et al*. 1991) although these putative resistant structures were never observed to germinate. The post-ruminal formation of such structures was speculated upon by Davies *et al*. (1993b) and this study provides further evidence in support of it. Nielsen, Zhu & Trinci (1995) has also demonstrated the occurrence of zoosporangia attached to the plant particles in cow faeces and concluded that they may be responsible for the growth of the anaerobic fungi from faeces. The shape and size of the fungal structures in Figs 9–11 suggest that they are possibly thalli in this putative survival form. This is the first direct observation of fungal structures from the large intestine and caecum of animals. These findings support the hypothesis that anaerobic fungi may have a resting stage in their life-cycle and that the resting structures may be formed outside the rumen (Wubah *et al*. 1991, Davies *et al*. 1993a, Theodorou *et al*. 1996, Brookman *et al*. 2000). However, no information is available on the factors, which may lead to their formation.

Chitin measurements as a marker of fungal status

Comparative estimations of fungal populations in the rumen, lower parts of the digestive tract (duodenum and caecum) and the faeces of cows was first carried out by Grenet *et al*. (1989) using the roll tube method. By using the thallus forming unit (TFU) method, Davies *et al*. (1993b) also studied the distribution of these organisms along the digestive tract of cattle. This study reports for the first time changes in chitin levels along the digestive tract of sheep. We have shown that chitin measurements may be used as an indicator of rumen fungal populations (Rezaeian *et al*. 2004). Our data indicate there is an increase in the chitin content of the digest samples after passing into the small intestine from the abomasums (Table 2). This is in contrast to the results of Davies *et al*. (1993b) who found a lower value of TFU for the fungal population in the abomasum and the other lower parts of the digestive tract compared to that of the rumen and omasum. This may be the result of the thickening of the sporangial walls as resting stages develop (Wubah *et al*. 1991). Our SEM observations (Figs 9–11) suggest that the fungal wall structure appears different from typical sporangia found in the rumen (Figs 1–3). It is also possible that active growth of these fungi may still be occurring in the lower regions of the digestive tract (Breton *et al*. 1994). We also have observed what appear to be normal typical sporangia in the large intestine (Fig. 8).

The correlation between the amounts of chitin in digest contents, digest fluid and digest solid fractions in each organ indicate that the measurement of chitin from the strained digest fluid or digest solid may also be used to compare the chitin content of the digestive tract parts. However, the values for the DF were always higher than that of DC in all organs. Nevertheless, these results showed that the chitin content of the DS fractions is nearly the same as the related digest contents. This is probably due to the very low dry matter content of the fluid samples suggesting a better estimate of fungal population from the digest solid samples compared with that of the strained digest fluid.

The presence of the holes in the wall of the discharged sporangium (Fig. 7) suggests the active degradation of these structures. Reports indicate that chitin can be degraded anaerobically (Sturz & Robinson 1986). In an *in sacco* degradability measurement, a partial digestion of pure chitin has also been reported (Patton & Chandler 1975). Detection of enzymes capable of degrading fungal cell walls from rumen bacteria and protozoa has also reported (Morgavi *et al*. 1994). However, the precise site and the extent of chitin digestion in the gut have yet to be identified.

Our analysis indicates that the rumen of the sheep contains between 4–6 g chitin under these experimental conditions. The chitin content of fungal dry weight varies according to the species (Orpin 1977, Phillips & Gordon 1989). Assuming a mean chitin content of 20% (Orpin 1981) for the fungal dry weight suggests that the fungal biomass may have a value of 20–25 g dry weight in the rumen. When comparing this with a value of 120 g for the whole microbial dry matter in sheep's rumen (Czerkawski 1986), it can be concluded that anaerobic fungi may represent around 20% of the microbial biomass in the rumen. This is higher than the estimation of Orpin (1981) who reported the fungal biomass to be up to 8% of the total biomass. Although the assumption for the mean chitin content in both studies is the same (20%), no value has been reported for the total microbial mass of the rumen by Orpin (1981). These findings support the suggestion that anaerobic fungi are a significant group of rumen microorganisms, which contribute particularly to the digestion of fibre material throughout the digestive tract (Gordon & Phillips 1993, Fonty & Gouet 1994).

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