The role of anaerobic gut fungi in ruminants

Geoffrey L. R. Gordon and Michael W. Phillips

Commonwealth Scientific and Industrial Research Organisation (C.S.I.R.O.), Division of Animal Production, Locked Bag 1, Delivery Centre, Blacktown, New South Wales 2148, Australia

Abstract

Anaerobic chytridiomycete fungi are found in the gastrointestinal tracts of sheep, cattle and goats, as well as in many other domesticated ruminant and nonruminant herbivores and a wide variety of wild herbivorous mammals. They are principally found associated with the fibrous plant particles of digesta and as free swimming zoospores in the fluid phase. The presence of large fungal populations in animals consuming mature pasture or diets largely composed of hay or straw together with the production of highly active fibre degrading enzymes lead to the belief that anaerobic fungi may have a significant role to play in the assimilation of fibrous feeds by ruminants. While many early studies focused on anaerobic fungi because of their unusual biology and metabolism, the large part of subsequent research has emphasized the biotechnological potential of their cellulases, xylanases and phenolic esterases. In recent years, the extent of the contribution of anaerobic fungi to the nutrition of ruminants has also been established through studies of fungal populations in the rumen and the dietary factors which influence them, as presented in this review. Further, we discuss the evidence supporting an important contribution of anaerobic fungal populations in the rumen to feed intake and digestion of poor quality feed by domesticated ruminants. In conclusion, the review explores some different methods for manipulating fungi in the rumen for increased feed intake and digestion.

Introduction

A feature of the evolution of mammals has been the inability to produce the complex enzyme systems necessary to utilize lignocellulose, the most abundant carbon and energy source in the terrestrial environment. Instead, there has been an evolution of the gastrointestinal tracts of herbivorous mammals to provide microorganisms with environments conducive to microbial fermentation of plant fibre. Such alimentary systems are synergic, with the host providing inputs of heat, moisture and food while the microorganisms contribute protein as microbial biomass and by-products of digestion such as volatile fatty acids for utilization by the animal. The microbial ecosystems in the gut of herbivores are invariably very complex both in terms of types of microorganisms present and their interactions with each other and the host. For most of this century, the normal rumen microbiota were considered to be composed primarily of bacteria with subsidiary populations of ciliate and flagellate protozoa (Hungate, 1966). How-

https://doi.org/10.1079/NRR19980009 Published online by Cambridge University Press

ever, in 1975 Orpin recognized that at least some of the flagellate protozoa were, in fact, the motile zoospore stages of a new class of microorganisms, the anaerobic chytridiomycete fungi. Although fungi are regarded as the primary colonizers and degraders of plant fibre in most terrestrial environments, prior to Orpin's observations of fungi in the rumen members of this otherwise ubiquitous group of microorganisms had never been reported to proliferate in anoxic environments. The unusual nature of anaerobic fungi and the potential importance of fibre degrading fungi to herbivore nutrition have made them the subject of many studies over recent years. In this review, we discuss the evidence which indicates an important role for anaerobic fungi in the rumen of herbage fed ruminants. Even though several general reviews (Fonty & Joblin, 1991; Orpin & Ho, 1991; Li & Heath, 1993; Wubah *et al.* 1993) and more specific review articles (chapters in Mountfort & Orpin, 1994) have been published of late, a description of the morphology and metabolism of anaerobic fungi will assist the understanding of the influence of diet on anaerobic fungi in the rumen and the extent of their contribution to the nutrition of ruminants which are the subjects of this review.

Characteristics of anaerobic fungi

Biology

Life cycle and cell structure

The biology of anaerobic fungi is characterized by a complex life cycle similar to that seen with aerobic chytrid fungi (Heath *et al.* 1983). The most commonly observed growth cycle alternates between a motile zoospore stage and an immobilized thallus bearing one or more sporangia (Orpin, 1975; Bauchop, 1979; Ho & Barr, 1995; see Fig. 1). Depending on the fungal genus, the zoospores have either a single flagellum or a bundle of about fifteen flagella (range: 7–30) beating in synchrony (Orpin, 1994; Ho & Barr, 1995). The zoospores are attracted to pieces of freshly ingested plant material (Orpin, 1977*a*), presumably by a mechanism of chemotaxis (Orpin & Bountiff, 1978; Wubah & Kim, 1996). After attaching to the feed particle, they encyst and germinate, eventually to form a larger vegetative structure (the fungal thallus), which is composed of the sporangium and the rhizoid. A mature sporangium contains from as few as one or two to as many as 88 mononucleate zoospores (Heath *et al.* 1983; Lowe *et al.* 1987*a*) which are formed by repeated division of the single fungal nucleus contained in the original zoospore body (Heath *et al.* 1983). The rhizoid is the structure which attaches to the growth substratum and, when appropriate, penetrates into it (Ho *et al.* 1988). When these fungi are cultured, their life cycle varies between 24 and 32 h (Lowe *et al.* 1987*a*; Wubah *et al.* 1991*a*) though zoosporogenesis has been reported to occur as little as 8 h after germination (Orpin 1977*d*). The time span of a typical fungal growth cycle in the rumen has not been reported but it is likely to be similar to the mean retention time of digesta particles in the rumen (about 18–24 h). The ability to produce multiple zoospores would enable a longer life span, as the multiple zoospores from one sporangium may be able to replace others washed out prior to completion of their cycle.

Variations on this normal life cycle can be observed in culture, with some types of fungi being able to grow vegetatively without producing zoospores (Phillips, 1989; Ho & Bauchop, 1991) although it is not known whether vegetative growth occurs in the rumen. An important but less understood phase of the life cycle is a resting stage that can survive long periods of desiccation and exposure to oxygen (Lowe *et al.* 1987*b*). Davies *et al.* (1993) demonstrated that

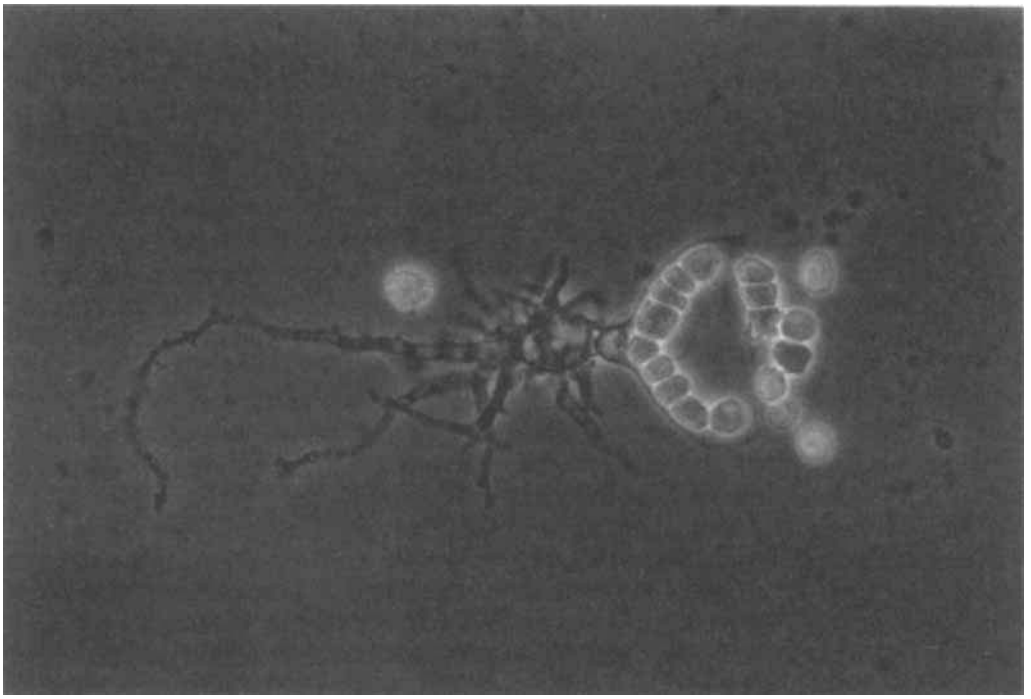

Figure 1. Phase contrast micrograph of uniflagellate zoospores being released from the sporangium of a *Piromyces* sp. isolated from the rumen of cattle.

the resting stage was virtually absent from the rumen but was found in increasing numbers as digesta passed down the gastrointestinal tract and reached a maximum level in faeces. Melanized sporangia, similar to resistant sporangia seen in aerobic chytrids, have been observed in cultures of anaerobic fungi, and Wubah *et al.* (1991*b*) have suggested that these were the specialized resting stage.

The cell wall of anaerobic fungi, like that of aerobic chytrids, has chitin as the main structural component (Orpin 1977*c*) but, unlike aerobic chytrids, mitochondria have not been seen (Munn, 1994) nor has mitochondrial DNA been detected (Brownlee, 1994). Instead of mitochondria there are other ATP-generating organelles, the hydrogenosomes, which produce H_2 and are similar to those seen in anaerobic protozoa (Yarlett *et al.* 1986). Recent biochemical evidence has suggested that these fungal hydrogenosomes evolved from mitochondria (van der Giezen *et al.* 1997). Further details of the growth cycle and ultrastructure of anaerobic fungi can be found in several review papers (Wubah *et al.* 1993; Li & Heath, 1993; Munn, 1994).

Classification

The taxonomy of the anaerobic fungi has until recently been based on the traditional classification methods used for chytrid fungi which rely on studies of morphology and ultrastructure, particularly of the zoospore, and the chemistry of cell walls and the metabolic characteristics of the fungi. Recently much useful information on the taxonomy of these fungi and their relatedness to other fungi has been gained by studies of their nucleic acids.

The anaerobic fungi were assigned by Heath *et al.* (1983) to a new family of chytrids, the Neocallimastigaceae, following a formal description. Comparison of fungal gene sequences (Doré & Stahl, 1991; Bowman *et al.* 1992; Li & Heath, 1992) and isozyme analysis (Ho *et al.* 1994) have confirmed this classification. Until the late 1980s only monocentric types of anaerobic fungi had been isolated from the rumen; these have a zoospore that produces only a single sporangium, normally with all of the dividing nuclei contained inside it. There are three genera of monocentric fungi: *Neocallimastix* includes fungi that have multiflagellate zoospores (7–30 flagella) and develop a relatively large highly branched rhizoid (Heath *et al.* 1983), whereas *Piromyces* spp. have zoospores with generally one or occasionally two flagella and a rhizoid of varying size and degree of branching (Ho & Barr, 1995). *Caecomyces* spp. also have zoospores with one or two flagella but they produce a bulbous rather than filamentous rhizoid (Ho & Barr, 1995). In 1989, there was simultaneous recognition by several groups of the existence of polycentric types of anaerobic fungi in the rumen (Barr *et al.* 1989; Borneman *et al.* 1989; Breton *et al.* 1989; Phillips, 1989). These fungi produced a very extensive branched rhizoid which both contained nuclei and developed multiple sporangia at various intervals along the same rhizoid. Two genera of polycentric fungi have been described: *Orpinomyces* spp. which have multiflagellate zoospores (Barr *et al.* 1989) and *Anaeromyces* spp. which produce zoospores bearing a single flagellum (Breton *et al.* 1990).

Taxonomic studies using fungal nucleic acid have predominantly been of DNA encoding for ribosomal RNA (Brownlee, 1994). These genes have the advantage of being present as multiple copies and also of combining adjacent areas which are highly conserved with others that show greater variation (Brownlee, 1994). Studies of restriction fragment length polymorphisms (Brownlee, 1994) and those using polymerase chain reaction amplification of specific gene sequences (Bowman *et al.* 1992; Doré *et al.* 1993) have indicated that the Neocallimastigaceae is a closely related family even though there are clear genus and species differences. Of the monocentric genera assayed, *Piromyces* had the greatest variation (Doré *et al.* 1993) and this corresponds to the diversity seen morphologically (Ho & Barr, 1995), biochemically (Phillips & Gordon, 1988; Gordon & Phillips, 1989*b*) and using isozyme analysis (Ho *et al.* 1994).

Metabolism

Carbohydrate fermentation. Anaerobic fungi are obligate anaerobes and gain energy from the fermentation of carbohydrates (Orpin, 1994). Of the common plant monosaccharides, fructose, glucose, xylose, cellobiose and gentiobiose were used by all isolates in several different studies, whereas galactose and mannose utilization varied and L-arabinose was not used (Phillips & Gordon, 1988, Gordon & Phillips, 1989*b*; Stewart *et al.* 1995; Dijkerman *et al.* 1997*a*). Utilization of oligosaccharides and polysaccharides was more varied though the plant polysaccharides cellulose and xylan were used by all isolates (Phillips & Gordon, 1988). Pectin fermentation has not been reliably detected despite the ability of both monocentric and polycentric fungi to degrade this compound (Gordon & Phillips, 1992; Kopečný & Hodrová, 1995; Dijkerman *et al.* 1997*a*).

When glucose was used as the substrate the major fermentation end products were acetate, ethanol, formate, D(−)-lactate, succinate, CO_2 and H_2 (Lowe *et al.* 1987*c*) though the relative amounts of each differed between genera (Phillips & Gordon, 1988; 1995*a*). Generally, polycentric anaerobic fungi produced much less lactate than monocentric fungi (Borneman *et al.* 1989; Phillips & Gordon, 1995*a*), although some recent isolates of *Piromyces* from the rumen did not produce any lactate (Ho *et al.* 1996). Anaerobic fungi have been shown to use only the Embden–Meyerhof–Parnas pathway (glycolysis) for the catabolism of glucose to

pyruvate or phospho*enol*pyruvate (O'Fallon *et al.* 1991; Marvin-Sikkema *et al.* 1993*a*; Phillips & Gordon, 1995*a*). The carbon was then converted to lactate, ethanol, formate and succinate in the cytoplasm or directed into specialized organelles, the hydrogenosomes, where the end products were acetate, CO_2 and H_2 (Yarlett *et al.* 1986; O'Fallon *et al.* 1991; Marvin-Sikkema *et al.* 1993*a*).

Anaerobic fungi are not nutritionally fastidious and will grow in a prereduced medium of simple composition with ammonia as the nitrogen source, sulphide as the source of sulphur, as well as thiamin, biotin and haemin (Orpin & Greenwood, 1986; Orpin, 1988). Although better fungal growth has been observed in a more complex medium (Orpin & Greenwood, 1986), ruminal fluid is not essential for growth in culture (Gordon & Ashes, 1984; Phillips & Gordon, 1988; Gordon & Phillips, 1989a).

Polysaccharide degradation. While the anaerobic fungi are likely to encounter small amounts of free sugars, most of the carbohydrate available in the rumen is as complex polysaccharides, principally the major structural carbohydrates cellulose, hemicellulose, and pectin and storage polymers such as starch and inulin. Isolates of each genus can degrade amorphous bacterial cellulose, though some *Caecomyces*, *Piromyces* and *Anaeromyces* isolates had difficulty degrading celluloses with higher degrees of crystallinity (Phillips & Gordon, 1988, 1995*a*). Xylan, a major component of hemicellulose, is degraded by all fungi although some isolates grow on this substrate relatively poorly (Phillips & Gordon, 1988, 1995*a*). Some anaerobic fungi have been shown to possess an endo-acting pectin lyase (Gordon & Phillips, 1991, 1992; Kopečný & Hodrová, 1995) and also a polygalacturonase (Kopečný & Hodrová, 1995) but neither pectin nor its purified core molecule polygalacturonate were fermented.

While most anaerobic fungi ferment starch and glycogen, some isolates of *Caecomyces*, *Piromyces* and *Anaeromyces* do not ferment either of these polymers nor the component disaccharide maltose (Phillips & Gordon, 1988, 1995*a*). A variety of other polysaccharides have been shown to be fermented (Phillips & Gordon, 1988, 1995*a*) or depolymerized (Williams & Orpin, 1987). The ability of cultures of anaerobic fungi to degrade and utilize pure compounds was reflected in the disappearance of the equivalent fractions of plant fibre when incubated with similar cultures. After fungal growth, substantial amounts of acid-detergent fibre and cellulose were removed from plant material (Gordon & Phillips, 1989*a*). Analyses of plant material digested by anaerobic fungi indicated that there was also a similar rate of solubilization of the monomeric sugars constituting cellulose and hemicellulose (glucose, xylose, galactose and arabinose) even though the latter two were not fermented by the fungi (Theodorou *et al.* 1989; Sijtsma & Tan, 1996).

The production of fibre degrading enzymes. The anaerobic fungi produce a wide range of polysaccharide degrading enzymes. Enzymes have been found associated with the rhizomycelium and many were also secreted into the surrounding environment (Williams & Orpin, 1987; Lowe *et al.* 1987*d*; Breton *et al.* 1995; Gerbi *et al.* 1996*b*). The presence and activity of some surface associated enzymes have been shown to fluctuate according to the stage of the life cycle (Breton *et al.* 1995; Gerbi *et al.* 1996*a*). Also growth conditions greatly influence enzyme production, with three times the level of fibrolytic enzymes being produced in a stirred fermenter compared with static batch cultures in bottles (Dijkerman *et al.* 1996*a*) whereas other continuous flow cultures produced up to twenty times the level of enzymes of batch cultures (Zhu *et al.* 1996). Fibrolytic enzymes were generally repressed by the presence of the sugar monomers resulting from degradation of the polysaccharide: glucose for cellulases and xylose and arabinose for xylanases (Mountfort, 1994). Although there is evidence that

some or all of the enzymes are partly constitutive, they can also be induced in the presence of cellulose (Mountfort, 1994; Dijkerman *et al.* 1997*a*) or other cellulose-containing fibre (Gordon & Phillips, 1989*a*; Teunissen *et al.* 1993*a*).

There has been considerable interest, in terms of biotechnology, in the fibre degrading enzymes of anaerobic fungi particularly because of the demonstrated ability of cellulases to attack crystalline cellulose rapidly (Teunissen & Op den Camp, 1993). Wood and coworkers have shown that isolates of both *Neocallimastix* (Wilson & Wood, 1992) and *Piromyces* (Wood & Wilson, 1995) had cellulases that were the most rapid degraders of crystalline cellulose yet found. The cellulases have been characterized as multicomponent complexes (Wood *et al.* 1995; Fanutti *et al.* 1995; Ali *et al.* 1995; Dijkerman *et al.* 1997*b*) that incorporate a wide range of enzyme activities (Wood *et al.* 1995; Ali *et al.* 1995), enzyme to enzyme associations (Wood *et al.* 1995; Ali *et al.* 1995) and enzyme to substrate associations (Ali *et al.* 1995; Dijkerman *et al.* 1996*b*). The principal cellulose degrading components are a mixture of several endoglucanases and cellobiohydrolases which work in combination with β-glucosidase (Wood *et al.* 1995). Similarly the xylanases can also be found associated with a high molecular weight complex containing both endo- and exo-acting enzymes that have been identified and characterized (Teunissen *et al.* 1993*b*; Garcia-Campayo *et al.* 1994).

Cloning of fungal genes has helped to elucidate the characteristics of different components of the enzyme complex (Denman *et al.* 1996; Li *et al.* 1997). Several gene sequences of xylanases (Black *et al.* 1994) and cellulases (Zhou *et al.* 1994) from anaerobic fungi have demonstrated similarities to rumen bacteria, whereas other cellulase sequences had close homology to aerobic fungi such as *Trichoderma* spp. (Denman *et al.* 1996). By analysing subunits of the enzyme complex and examining their activity and characteristics, Wood *et al.* (1995) suggested that the mechanism of action of the cellulase system of *Neocallimastix* was similar to that of aerobic fungi.

Solubilization of lignin. The close association of anaerobic fungi with lignocellulose and the ability of these organisms to weaken plant particles physically (Akin *et al.* 1983) indicated a possible role in the digestion of lignin. Microscopy studies have shown that the fungi had a preference for colonizing highly lignified tissues such as mestome sheath and xylem (Akin & Rigsby, 1987; Grenet *et al.* 1989*a, b*). Furthermore these tissues were degraded to a much higher degree when fungi were present than when plants were exposed to rumen bacteria only (Borneman & Akin, 1990). While it seems clear that the fungi are involved in lignin degradation the precise role has yet to be determined. Fungi have been shown to solubilize part of the lignin component of plant cell walls in culture (Gordon, 1987; Akin & Benner, 1988; Gordon & Phillips, 1989*a*; McSweeney *et al.* 1994; Bernard-Vailhé *et al.* 1995) though there was no evidence of the fermentation of lignin (Gordon & Phillips, 1989*b*; Bernard-Vailhé *et al.* 1995). Studies using model compounds and modified plant components suggested that the fungi were unable to break either the ether bonds between the phenolic moieties and polysaccharides or the interphenolic bonds found in the lignin complex (McSweeney *et al.* 1994). In contrast, there is considerable evidence that the anaerobic fungi are able to break the ester linkages that connect lignin to hemicellulose. The fungi produced feruloyl and *p*-coumaroyl esterases (Borneman *et al.* 1990) that cleaved feruloyl and *p*-coumaroyl arabinoxylans *in vitro* (Borneman *et al.* 1992). When acting on plant cell walls, these enzymes worked synergically with other fungal enzymes such as xylanase (Wubah *et al.* 1993). From analyses of degraded plant material (McSweeney *et al.* 1994) or model lignin compounds (Wubah *et al.* 1993) it seems likely that lignin is solubilized as polysaccharide—lignin complexes that have been cleaved at the phenolic to hemicellulose interface.

Distribution

Anaerobic fungi colonize the alimentary tracts of herbivorous animals that consume a fibrous diet and have a digestive retention time sufficient for a complete fungal life cycle. Thus anaerobic fungi are present in the more important species of domesticated ruminants (sheep, goats, cattle and water buffalo), as well as occurring widely among many different species of herbivorous mammals, both ruminant (such as antelopes and deer), ruminant-like (such as camelids) and other foregut fermenting animals (such as kangaroos) as well as large hindgut fermenters (such as horses and elephants) (Orpin, 1994). Owing to this wide range of host animal species, anaerobic fungi have been found in all the geographical regions where they have been sought. Within ruminants, anaerobic fungi have been isolated from many sites along the digestive tract (Grenet *et al.* 1989*c*; Davies *et al.* 1993; Trinci *et al.* 1994). Davies *et al.* (1993) found the highest concentration of fungi in the rumen and omasum with numbers declining to a minimum in the small intestine; the concentration of fungi increased then as digesta progressed through the large intestine to the faeces. While isolates of the same species have been found throughout the tract (Wong *et al.* 1995), there is evidence that the fungi present in the large intestine can have a different population structure from that occurring in the rumen (Breton *et al.* 1994).

Knowledge of the occurrence of different fungal genera and species in different animal species is incomplete and, apart from the better studied domestic species, is often based on observations from one or two animals. In sheep and cattle, the populations of the different genera can vary considerably over time and be influenced by diet, frequency of feeding, and geographic separation. Species from each fungal genus can generally be found in cattle though not necessarily in the same animal at the same time. Monocentric fungi are usually present in sheep, and polycentric fungi have been found in sheep in France (Breton *et al.* 1989) but not in some geographical areas such as Australia (Phillips & Gordon, 1995*a*).

Transfer of fungi between individual animals

The transfer of fungi between animals can be considered as occurring in two phases: the initial acquisition of a fungal population, which is followed by the addition to or replacement of this population later in life. A number of means of transfer between mature animals have been reported. Anaerobic fungi have been consistently isolated from both fresh and dried faeces (Lowe *et al.* 1987*b*; Wubah *et al.* 1991*b*; Trinci *et al.* 1994) and melanized sporangia, which could be the resistant form, have been observed in smears from cattle faeces that grew isolates of *Neocallimastix*, *Piromyces*, *Caecomyces*, and *Orpinomyces* when cultured (Wubah *et al.* 1991*b*). Saliva has been found to contain viable fungi (Lowe *et al.* 1987*b*) and aerosols have also been indicated as possible means of dissemination between animals (unpublished observations cited by Orpin, 1989).

Fonty *et al.* (1987) found that the rumens of lambs were colonized by substantial numbers of anaerobic fungi within the first two weeks of life even when the lambs were separated from other sheep soon after birth; this would suggest that the most likely initial transfer of fungi would occur between juveniles and their dam. Saliva would be a likely vehicle for transfer through close mouth-to-mouth contact. Subsequent fungal transfer could occur in many ways. Ingestion of faecal matter from pasture could be an important means of transfer within and between flocks and herds. Similarly, despite unsuccessful attempts to culture anaerobic fungi from feed (Bauchop, 1979), resistant forms of the fungi originating from saliva or faeces could

be present at very low levels in cut feed. Aerosols could also be significant in transferring between individuals in a herd or flock (Orpin, 1989) although fungi would be transferred slowly.

Anaerobic fungi in the rumen microbial ecosystem

Interactions with other rumen microorganisms

The gastrointestinal tract and its associated microbiota can be considered to be an open ecosystem with continuous inputs of nutrients and microorganisms from the external environment. When existing knowledge of interactions of fungi with other microorganisms is considered, it is important to relate results obtained *in vitro* to the niches that the fungi are likely to occupy in the rumen. Like many rumen microorganisms, the anaerobic fungi have an affinity for the particulate phase of the rumen digesta even though the zoosporic stage of the life cycle is spent in the fluid phase. Therefore interactions of the zoospores with other microorganisms could be expected to involve organisms that inhabit the fluid phase and also those that colonize the solid—liquid interface that is the external surface of plant particles. In contrast, the opportunities for direct interactions with other microorganisms may be limited for a major part of the fungal biomass when the fungi develop extensive rhizoid systems within plant particles.

Ciliate protozoa

The interaction of fungi with ciliate protozoa would predominantly occur in the fluid phase of digesta and on the surface of plant particles where ciliate protozoa are often observed (Fonty & Joblin, 1991). One of the observed consequences in most experiments where protozoa have been removed from the rumen has been an increase in the numbers of fungal zoospores (Orpin 1977e; Soetanto *et al.* 1985; Romulo *et al.* 1986, 1989; Newbold & Hillman, 1990; Ushida *et al.* 1990: Hsu *et al.* 1991). Newbold & Hillman (1990) found that the presence of protozoa increased the turnover of radiolabelled fungal protein by a substantial, though not significant, amount (40%) and the authors suggested that the observed change in fungal numbers arose by protozoal predation rather than by competition for substrates. Evidence for the ability of protozoa to digest fungal biomass has been obtained by Morgavi *et al.* (1993, 1994*a*) who demonstrated the ability of protozoa to turn over both chitin and protein from dead fungal biomass, and by Williams *et al.* (1994*a*) who reported that a mixed protozoal population from the rumen significantly increased the release of radiolabel from ^{14}C-labelled fungi.

The effects of protozoal—fungal interactions on the functioning of the rumen can be inferred from observations of co-cultures. Generally the presence of protozoa inhibits the level of cellulolytic activity in the cultures (Fonty & Joblin, 1991; Morgavi *et al.* 1994*b*; Widyastuti *et al.* 1995), even though the latter demonstrated that dry matter loss from straw was not affected and Morgavi *et al.* (1994*b*) calculated that the amount of cellulolysis per unit of fungal biomass was actually higher. There is evidence that the presence of protozoa can also increase xylanolysis by anaerobic fungi (Williams *et al.* 1994*b*).

Fibrolytic bacteria

It could be expected that fibrolytic bacteria would compete with fungi when colonizing plant particles, though established hyphal growth may result in the fungi accessing different tissues than the bacteria. Interpretation of many experiments is difficult because fungi tend to grow on the surface of plant particles when grown *in vitro* compared to growing inside the particles in

the rumen. Co-cultures of anaerobic fungi and a variety of fibrolytic bacteria have yielded differing results. With *Ruminococcus albus* and *R. flavefaciens* there was less efficient degradation of a variety of fibrous substrates, such as xylan (Williams *et al.* 1991), filter paper (Bernalier *et al.* 1993*b*) or straw (Roger *et al.* 1993) compared with fungal monocultures. While it is possible that the loss in activity could be caused by bacteria overgrowing the fungal component of cultures or producing levels of volatile fatty acids that are inhibitory for fungi (Joblin & Naylor, 1993), there is evidence of more specific inhibition. Bernalier *et al.* (1993*b*) have described extracellular factors of *R. flavefaciens* that inhibited the growth of anaerobic fungi on cellulosic substrates. Subsequently the factors were isolated and identified as two proteins that did not affect growth of the fungi but instead interfered with the functioning of the fungal cellulases. It has also been demonstrated that supernatants of fungal cultures inhibited xylan degradation by *R. flavefaciens* (Joblin & Naylor, 1996). The inhibiting factor from fungi was not isolated but it did not survive autoclaving and did not inhibit growth on soluble sugars. The factor appeared to be specific for *R. flavefaciens* as it did not affect xylan utilization by *R. albus*. An interpretation of these results is that fungal zoospores and ruminococci compete for the same niches on the particle surface and therefore have evolved mechanisms for each to gain a competitive advantage over the other.

In contrast to interactions with ruminococci, co-cultures of other fibrolytic bacteria with fungi have given increases in fibrolytic activity over what occurs with fungi or bacteria alone. Thus the digestion of straw was greater with a mixture of *Neocallimastix* and *Fibrobacter succinogenes* and likewise the digestion of xylan was greater when fungi were grown with *Butyrivibrio fibrisolvens* or *Prevotella ruminicola* (Williams *et al.* 1991).

Interspecies hydrogen transfer

Methanogens. The anaerobic fungi are fermentative and when in pure culture produce a variety of end products with formate, acetate, D(−)-lactate, ethanol, CO_2 and H_2 being the major components formed in culture. The nature of the products formed is greatly influenced by the closed environment and the negative feedback caused by the build up of these products, particularly H_2, in the growth medium. Hydrogenase is inhibited by the presence of low levels of H_2 and as a consequence the amount of carbon passing through the hydrogenosome is limited to about 30% of the total carbon flux (Marvin-Sikkema *et al.* 1993*b*). This results in a higher production of ethanol and lactate which act as electron acceptors. If H_2 is removed from the environment and hydrogenase is not inhibited, around 70% of carbon will flow through the hydrogenosomes with a resulting increase in the production of H_2, acetate and CO_2 and concomitant decrease in ethanol and lactate. Numerous workers (Stewart & Richardson, 1989; Joblin *et al.* 1990; Marvin-Sikkema *et al.* 1993*b*) have demonstrated that co-culture of methanogenic archaea with anaerobic fungi results in interspecies hydrogen transfer, with the methanogen providing the low H_2 environment that encourages the flow of carbon through hydrogenosomes and results in a shift in the end product profile towards the production of acetate, H_2 and formate (though the latter two are utilized by the methanogens as precursors of methane production). The changes in the production of end products was associated with increased enzyme activity in the hydrogenosomes but not by an increased number of hydrogenosomes and the increased flow of carbon through the hydrogenosomes also generated more substrate level ATP than did the carbon flowing to lactate or ethanol (Marvin-Sikkema *et al.* 1993*b*).

The ability of co-cultures of methanogenic archaea with anaerobic fungi to enhance the fibre digesting capabilities of the fungi is well documented (Bernalier *et al.* 1991; Teunissen *et al.* 1992). This effect was pronounced with pure substrates such as cellulose (Marvin-Sikkema

et al. 1990) or xylan (Joblin *et al.* 1990), although there was little increase in the rate of degradation of plant material such as lucerne stems, and the normal lag phase seen with fungi was absent with co-cultures (Joblin & Williams, 1991). Studies have also indicated that co-cultures with methanogens increased the activity and yield of polysaccharide degrading enzymes (Joblin & Williams, 1991; Teunissen *et al.* 1992). A likely explanation of the increase in fibrolytic activity is that it simply reflects an increase in fungal biomass resulting from more energy productive fermentation and lower levels of the inhibiting end products such as lactate seen in co-cultures (Williams *et al.* 1994*a*). The stability of the co-cultures and the observation that populations of methanogens can be found attached to the external surfaces of protozoa (Vogels *et al.* 1980) has led to speculation that a similar relationship occurs between anaerobic fungi and methanogens. While there is ample evidence that the fungi and methanogens can form stable co-cultures *in vitro*, the spatial distribution of the populations is likely to be considerably different in the rumen. Fungal zoospores and plant germs on the surface of particles are likely to be in close contact with methanogens. However the rhizoids, and the hydrogenosomes within them (Munn, 1994), that are inside the plant particles may be physically separated from the external methanogenic populations. Nevertheless the sporangia with their hydrogenosomes usually project into the fluid phase of digesta where the methanogens are located.

Other hydrogen utilizing bacteria. Co-cultures of anaerobic fungi with H_2 utilizing bacteria have yielded similar results to experiments with methanogens. Thus, the acetogenic species such as *Eubacterium limosum* (Bernalier *et al.* 1993*a*; Hodrová *et al.* 1995), *Acetitomaculum ruminis* (Rees *et al.* 1995) and *Clostridium* spp. (Morvan *et al.* 1996) and the sulphate reducing bacterium *Desulfovibrio* sp. (Morvan *et al.* 1996) shifted the fermentation products away from the production of lactate towards acetate and H_2 (converted either to acetate by acetogens or H_2S by sulphate reducers). However, the overall change to the fermentation pattern was not as pronounced as found with methanogens.

Interactions with other ruminal bacteria

Because fungi inhabit a complex ecosystem, there are many possible interactions that could occur, for example microorganisms may use by-products of fungal metabolism and nutrition. Examples are bacteria that use fermentation end products such as lactate and bacteria that utilize by-products of fungal fibre digestion such as sugars not fermented by fungi. It is likely that such relationships in the rumen are indirect, and *in vitro* studies of representative co-cultures have given varied results. For example, the saccharolytic bacterium *Succinivibrio dextrinosolvens* stimulated hemicellulose degradation by anaerobic fungi possibly by fermenting arabinose with resultant minimization of feedback inhibition by this sugar (Williams *et al.* 1991). The lactate utilizing bacteria such as *Megasphaera elsdenii* and some strains of *Selenomonas ruminantium* stimulated fungal cellulolysis in co-culture (Bernalier *et al.* 1991; Hodrová *et al.* 1995) whereas other *S. ruminantium* strains inhibited this process. Chitinolytic bacteria and their cell free culture medium were also shown to inhibit both the growth of and cellulose degradation by polycentric fungi (Kopečný *et al.* 1996). While the interactions between anaerobic fungi and other microorganisms found to occur in co-cultures probably do occur in the rumen, it is likely that many such interactions occur as part of substrate pools in the rumen rather than as direct one to one processes.

Estimation of fungal populations in the rumen

The combination of a complex life cycle and the preferential colonization of plant particles by vegetative growth has meant that it has been very difficult to estimate either fungal population size or biomass. Although the free swimming zoospores are easily isolated and enumerated from the fluid phase of digesta, the vegetative cells are irreversibly attached and therefore are much more difficult to assess. The variations in the size and the numbers of zoospores released by the sporangia (Orpin, 1994) and the unknown survival and germination rates of zoospores in the rumen make the association between zoospore numbers, thallus numbers and biomass problematic. Consequently the methods used to count anaerobic fungi are better described as estimators of fungal populations than definitive quantitation.

Microscopy methods

Direct counting of fungal zoospores by microscopy is best done with fresh rumen fluid as fungal zoospores can be identified by their distinct twitching form of motility and refractile appearance under phase contrast illumination. Examination of dead zoospores, such as those fixed in formaldehyde, can be complicated by the presence of flagellate protozoa which are similar in size and general appearance to zoospores (Ogimoto & Imai, 1981). Zoospore size and the number of attached flagella have also been used to estimate the proportion of different types of fungi in the rumen (Orpin, 1975,1977*b*).

Sporangia counts. Fungal sporangia can be seen by light microscopy on particles of rumen digesta after staining with a suitable dye such as cotton blue in lactophenol (Akin *et al.* 1983; Akin, 1987*a*). However the variation in such material makes it unsuitable for quantitation of fungi. This variation has been overcome by incubating pieces of a suitable substrate such as grass leaves for a constant time period (normally 18 or 24 h) in the rumen inside small bags made from nylon or polyester cloth. Counts of sporangia over a measured area of leaf surface can then provide an estimate of the fungal population size in the rumen. This is a useful and practical method for comparing the density of anaerobic fungi in the rumen of animals subjected to diet changes (Gulati *et al.* 1985; Weston *et al.* 1988). Alternatively, strips of agar medium have been used to replace grass leaves (Ushida *et al.* 1989) but the relative fragility of the agar means that the strips must be protected from mechanical damage in the rumen by being placed inside a rigid container. Identification of the types of fungi present on the leaves by assessing sporangial morphology is unreliable, although the sporangia of some types of polycentric fungi have been distinguished from one another by light microscopy (Breton *et al.* 1992). Scanning electron microscopy has also been used to assess different morphological groups of anaerobic fungi present on feed particles taken from the rumen (Bauchop, 1979; Akin & Rigsby, 1987; Grenet *et al.* 1989*a, b*). Microscopy methods have the disadvantage that the isolation of those fungal strains that have been observed is not possible, since aerobic handling of the specimens and staining procedures kill the fungi.

Culture methods

Zoospore counts. The number of zoospores in samples of strained ruminal fluid has been used as a relative measure of the overall number of anaerobic fungi present. A widely used method of assessing the number of fungal zoospores in ruminal samples is the agar roll tube technique (Bauchop, 1979; Joblin, 1981; Akin *et al.* 1983; Soetanto *et al.* 1985; Romulo *et al.* 1989). Like other assessments, the method is only an estimate of relative numbers as there are considerable fluctuations in the numbers of zoospores present in the rumen over a 24 h period and the

relation between numbers produced *per* sporangium in the rumen is not known. However, this method has the advantage that the different colony morphology of some genera of anaerobic fungi allows the preliminary identification of the types of anaerobic fungi present in the sample. It has also proved to be useful for the isolation of strains of the different types of anaerobic fungi that are present in a sample of ruminal fluid, even of those fungi which are present only in relatively low numbers (Phillips & Gordon, 1988; Borneman *et al.* 1989; Phillips & Gordon, 1995*a*). A variation on the roll tube method is to inoculate plate media in an anaerobic chamber (Borneman *et al.* 1989).

Most-probable-number (MPN) counts. Another culture method for estimating the population of anaerobic fungi is by adapting the statistical principles of MPN to assess rumen digesta (Theodorou *et al.* 1990*b*; Obispo & Dehority, 1992). Whole rumen digesta is diluted serially and sets of culture tubes are inoculated with each dilution of digesta. By determining the number of tubes showing fungal growth at each dilution and consulting statistical tables, it is possible to determine a probable number of fungi in the original sample. This method has the advantage that, in addition to free zoospores, fungi adherent to plant particles also contribute to the count which therefore is usually higher than that obtained by roll tubes (Obispo & Dehority, 1992). However, the MPN method is also subject to variables that restrict its accuracy as a measure of both population and biomass. It is very difficult to serially dilute digesta from fibrous diets and maintain the same proportion of different particle sizes in successive dilutions. As individual particles can have one or many fungi attached to them, the end points of replicate dilution series will generally be underestimates of the true number of fungi present, although sporangia on plant particles will occasionally release zoospores during the processing of the dilution series, which can lead to artificially high counts. Also, this method is unsuitable for assessing and isolating the different types of fungi in a sample since the contents of each culture tube will be dominated by the most numerous or the quickest growing strains.

Fungal biomass measurement

Chemical markers. The presence of chitin (poly-*N*-acetylglucosamine) in the cell walls of both monocentric (Orpin, 1977*c*; Phillips & Gordon, 1989; Gay, 1991) and polycentric anaerobic fungi (M.W. Phillips & G.L.R. Gordon, unpublished) has suggested that this structural polysaccharide may be useful as a chemical marker for fungi in the rumen, in a similar way to the use of diaminopimelic acid as a marker for bacterial biomass. One chemical method for measuring chitin after deacetylation to chitosan has been found unsuitable (Argyle & Douglas, 1989), but another method for the colorimetric analysis of chitin as *N*-acetylglucosamine after acid hydrolysis of fungal cell walls in pure culture (Phillips & Gordon, 1989) remains untested. Chitin in the diet, either as mould-contaminated feed (Orpin, 1994) or pasture containing endophytic fungi, would decrease the accuracy of the result. Any use of chemical methods to determine chitin (whether as glucosamine or as *N*-acetylglucosamine) in samples of ruminal digesta must account for the probable interference to the result by bacterial cell wall murein which is composed of glucosamine and muramic acid as disaccharide repeats. Alternatively, chitinase has been used to measure chitin in anaerobic fungi grown in culture on fibrous substrates (Akin, 1987*b*) and, in theory, it would provide a specific assay for chitin. However, this method has not been applied to samples of ruminal digesta, possibly because of difficulty in determining the very low amounts of chitin to be found. Any method which was successfully used to measure chitin in rumen digesta would still provide a relatively inaccurate estimate of fungal biomass because of the different chitin composition in each of the several monocentric

genera of anaerobic fungi, ranging from 100–130 mg/g for *Neocallimastix* to 250–300 mg/g for *Piromyces* and *Caecomyces* (Phillips & Gordon, 1989).

Nucleic acid quantitation. The application of molecular biology techniques to the study of microorganisms in complex environments like the rumen is an exciting new area of research with the main focus falling on the bacterial populations through the use of probes derived from the nucleotide sequences of prokaryotic 16S ribosomal (r) RNA genes (for a review, see Mackie, 1996). Quite recently, the development of oligonucleotide probes specific for nucleic acids from anaerobic fungi has provided the opportunity for a more precise determination of both the total fungal biomass and also the proportions of specific fungal subpopulations within the rumen (Faichney *et al.* 1991; Doré *et al.* 1993; Brownlee *et al.* 1996; Millet *et al.* 1996; Faichney *et al.* 1997). The oligonucleotide probes, which were based on the DNA sequence of the eukaryotic 18S rRNA gene (18S rDNA) or on the varying internal transcribed spacer regions between the different rRNA genes (Brownlee, 1994), have been directed at hybridizing to either rRNA or rDNA. One oligonucleotide probe, with a sequence that was common to all fungal isolates, gave an estimate of the total fungal population (Millet *et al.* 1996; Faichney *et al.* 1997) whereas some other rRNA-directed probes had a sequence found only in a subpopulation such as a genus (Millet *et al.* 1996). By using a combination of such probes in a preliminary study, Millet *et al.* (1996) were able to determine the proportions in rumen digesta of some genera compared with total fungi as revealed by a general fungal probe, though there was no estimate of fungal biomass. In a more detailed report, Faichney *et al.* (1997) used a synthetic oligonucleotide probe for total anaerobic fungi to calculate a rumen biomass for them. After hybridization to rRNA from a known amount of rumen digesta, a ^{32}P-labelled probe on membranes was quantified by liquid scintillation counting. These results were then compared with a standard curve of the probe bound to serial dilutions of specific fungal RNA applied to the same membrane and the amount of rRNA in digesta calculated. The results were then allied with estimates of the nitrogen content of anaerobic fungi to give the amount of fungal nitrogen in the rumen. In two sheep fed a hay diet, fungi comprised 1·1% and 3·6% of the microbial nitrogen in the rumen. In an alternative approach, DNA oligonucleotide probes have been developed to be either general for anaerobic fungi (based on rDNA sequences) or specific for individual fungal strains and isolates based on sequences obtained from the internal transcribed spacer regions (Brownlee *et al.* 1996). Gordon *et al.* (1996) have used a strain specific DNA probe to follow the population density of a nonindigenous *Piromyces* isolate after it was introduced into the rumen of sheep. Methods for measuring the biomass of anaerobic fungi, both total and specific strains, in rumen digesta with DNA oligonucleotide probes are presently under development.

Effect of diet or rumen environment on fungal populations

Diet composition

Forage

From the earliest work on anaerobic fungi, diet was determined to have a substantial effect on fungal populations in the rumen (Orpin, 1977*a*; Bauchop, 1979) and subsequent studies corroborated this principle. The fibre or lignocellulose content of the diet was a critical factor in determining the presence of normal ruminal populations of anaerobic fungi. Few anaerobic fungi were seen in the rumen of animals that were fed lush pasture (either legume or grass when

green and leafy) compared with the same pasture when it was mature (Bauchop, 1989; Kostyukovsky *et al.* 1991). Fungal counts in the rumen increased when a greater proportion of hay was included in a diet for cattle (Kostyukovsky *et al.* 1991). One study of the effect of hay type on anaerobic fungi found that these microorganisms were more prevalent and had greater digestive capability in the rumen of cattle fed lucerne when compared with those fed bermuda grass (*Cynodon dactylon*; Akin & Windham, 1989). In contrast, Sekine *et al.* (1995) showed no effect on the normal size of the fungal population in goats when they were fed three different hays, but a trend for slightly higher zoospore counts and sporangia numbers with timothy (*Phleum pratense*), followed by lucerne (*Medicago sativa*) and oaten (*Avena sativa*) hays was found. Diets of silage made from either sorghum (*Sorghum bicolor*) or maize (*Zea mays*) reduced the numbers of anaerobic fungi in the rumen (Akin *et al.* 1988; Grenet *et al.* 1989*a*). Anaerobic fungi colonized refractory palm press fibre in the rumen (Ho *et al.* 1991), whereas they have not been detected in the rumen of sheep eating seaweed (Orpin *et al.* 1985).

Grain and other concentrates

The addition of grain to herbage being eaten by ruminants is a common means of increasing both the energy density of the diet and the intake of available carbohydrates, but the inclusion of rapidly fermented starchy concentrates in the diet had several different effects on anaerobic fungal populations. In one respect, grain additions supported slightly lower populations of anaerobic fungi in the rumen (Orpin, 1977*a*; Gordon, 1985; Grenet *et al.* 1989*a*). On the other hand, the addition of maize to a sorghum silage diet was found to increase the degradative activity of anaerobic fungi when tested *in vitro* (Akin & Windham, 1989). Also, the addition of a largely grain-containing concentrate to a hay diet substantially increased the count of fungal zoospores in the rumen of sheep (over 20-fold increase) but the accompanying increase in fungal biomass was much less at 1·2–2·0-fold (Faichney *et al.* 1997). A possible explanation for these apparent differences may be found in the fact that only some anaerobic fungi produced amylases and had the ability to ferment starch, generally species of the genera *Neocallimastix*, *Piromyces* and *Orpinomyces* (Phillips & Gordon, 1988, 1995*a*; Yanke *et al.* 1993; Mountfort, 1994). The attack on cereal grains by three amylolytic anaerobic fungi has been documented by McAllister *et al.* (1993). Thus, it is apparent from the results presented here that complex interactions occur in the rumen between forages and starchy concentrates and there are no general conclusions relating to effects on ruminal fungal populations to be drawn from the available data.

The feeding of free lipid to ruminants can have a detrimental effect on ruminal fermentation, retarding fibre degradation (see Jenkins, 1993), an important consideration given the increasing use of lipid-containing oilseed meals. Anaerobic fungi, as one component of the rumen microbial population, were adversely affected by the addition of lipid to the diet. The addition of rapeseed oil led to a considerable decrease in the fungal population but the mechanism was not elucidated (Fonty *et al.*, unpublished, as cited by Fonty & Grenet, 1994). Previously, Elliott *et al.* (1987) and Calderon-Cortes *et al.* (1989) found that feeding a supplement of sunflower meal to sheep consuming a barley straw diet resulted in depression of the fungal population in the rumen to below detectable levels. Also, G.J. Faichney, G.L.R. Gordon, M.W. Phillips and A.J. Rintoul (unpublished work) found that fungal zoospore counts in the rumen were reduced below detectable levels and fungal DNA could not be detected when a supplement of cottonseed meal was provided to sheep. In another study, the feeding of calcium salts of medium chain fatty acids (C6–C12) to sheep resulted in reduced numbers of fungal zoospores in the rumen whereas the salts of long chain fatty acids ($C \geq 14$) had no effect on

anaerobic fungi (Ushida *et al.* 1992), indicating that the inhibitory effects of the long chain fatty acids common in oilseed meals can be alleviated, at least partly, by chemical pretreatment.

Sulphur

Early in the study of anaerobic fungi, it was recognized that the sulphur content of hay diets or pasture was a significant factor governing the fungal population in the rumen (Akin *et al.* 1983; Gordon, 1985). When S was present in the diet at levels of ~1·0 g S/kg organic matter or less, anaerobic fungi were apparently absent from the rumen of sheep fed on hay made from the tropical pasture grass *Digitaria pentzii* (Akin *et al.* 1983). The size of the anaerobic fungal populations in the rumen increased dramatically after either an application of a S fertilizer to the pasture used to make the hay (Akin *et al.* 1983) or a S-containing dietary supplement to the low S hay (Gordon *et al.* 1984). Fertilization of the pasture also resulted in an average increase of 38% in *ad lib.* feed intake (Akin *et al.* 1983; Gordon, 1985). Some of the results of these studies are summarized in Table 1. A diet of another tropical grass hay (spear grass, *Heteropogon contortus*), which had a low S content, also resulted in an undetectable fungal population in the rumen (Morrison *et al.* 1990). Alternatively, diets of cereal straw (usually wheat straw) which were low in S supported a low, but detectable, population of anaerobic fungi (Gordon *et al.* 1983; Gulati *et al.* 1985; Weston *et al.* 1988). In another study, the apparent relationship between a reduced S content of pasture and a declining ruminal fungal population did not apply to a ryegrass (*Lolium multiflorum*) pasture in Scotland (Millard *et al.* 1987) where anaerobic fungal populations were higher in sheep consuming an unfertilized hay (containing 0·9 g S/kg dry matter (DM)) than in those fed a hay prepared from fertilized pasture (2·4 g S/kg DM). The additional S of the fertilized pasture was predominantly contained in the sulphate and non-protein fractions which was similar to the S distribution in the fertilized *D. pentzii* used by Akin *et al.* (1983) where the additional S was found in the soluble, nonprotein fraction (Table 2). It is

Table 1. Influence of sulphur in the diet on the size of the anaerobic fungal population in the rumen and the feed intake of poor quality herbages by sheep

Low Sulphur feed	Feed Sulphur[a] (g/kg)	Sulphur added to diet:	Increased fungi after Sulphur added	Intake after Sulphur addition relative to base diet		Reference
				VOMI	DOMI	
Digitaria pentzii	0·8–1·1	F	known	136%	ND	1
		F	presumed	140%	131%	6
		met	known	111%	ND	2
		S^0	unknown	106%	96%	6
Wheat straw	0·7	met	known	106%	123%	3
(alkali-treated)		SO_4	known	107%	116%	7
Heteropogon contortus	0·5	SO_4	known	175%	196%	5
Maize stover	0·2	SO_4	known	271%	336%	4

[a]Sulphur content of diets increases to 1·3–1·7 g S/kg organic matter after either fertilization of the pasture or use of dietary supplement.

DOMI, digestible organic matter intake; F, fertilizer on pasture; met, methionine; ND, no data; S^0, elemental sulphur; SO_4, sulphate; VOMI, voluntary OM intake.

References: 1, Akin & Hogan (1983); 2, Gordon, (1985); 3, Gulati *et al.* (1985); 4, Gutierrez *et al.* (1996); 5, Morrison *et al.* (1990); 6, Rees *et al.* (1982) (cited by Gordon, 1985); 7, Weston *et al.* (1988).

Table 2. Sulphur content of *Digitaria pentzii* hays fed to sheep by Akin *et al.* 1983

	Before fertilizer (g S/kg OM)	After fertilizer (g S/kg OM)
Total Sulphur	0·76	1·29
Protein Sulphur & insoluble Sulphur[a]	0·56	0·57
Soluble non-protein-Sulphur	0·20	0·72

[a]Insoluble fraction after extraction of milled samples with 5% trichloroacetic acid at 60°C for 1 h.
OM, organic matter.

probable that the form and distribution of S in herbage of low total S content was as important as the total S content in determining the size of the fungal population in the rumen during these studies.

In all cases where anaerobic fungi were either apparently absent from the rumen or their numbers were greatly reduced when herbage diets with a low content of S were fed, supplementation of these diets with several different types of S allowed fungi to proliferate in the rumen and resulted in increased voluntary feed intake. At the same time, there was little or no change in the ruminal populations of bacteria and ciliate protozoa due to dietary supplementation (Akin *et al.* 1983; Gulati *et al.* 1985; Morrison *et al.* 1990). Diets of low S *Digitaria* have been successfully supplemented with methionine and elemental S (each about 1 g S/d *per* head; Gordon, 1985; Gordon *et al.* 1984), and cereal straw supplemented with either methionine (Gordon *et al.* 1983; Gulati *et al.* 1985) or sulphate (Weston *et al.* 1988) also supported greatly increased numbers of anaerobic fungi in the rumen. Supplementation of spear grass with sulphate supported a higher fungal population compared with the same hay when unsupplemented (Morrison *et al.* 1990). Gutierrez *et al.* (1996) increased the population of anaerobic fungi in goats fed on maize stover by supplementing the diet with sulphate and urea. Different strains of anaerobic fungi grown *in vitro* required reduced forms of S (Orpin & Greenwood, 1986; Phillips & Gordon, 1991) indicating the need for reduction of supplementary sulphate in the rumen before it would be available for anaerobic fungi. All of the S content of the dietary supplements (elemental S, sulphate or methionine) used so far to stimulate anaerobic fungi in the rumen were potentially available to all of the rumen microbiota. However, a S supplement which is either specific for anaerobic fungi in the rumen or relatively so is still to be discovered.

Feed pretreatment

Alkali (sodium hydroxide, ammonium hydroxide). Pretreatment of low quality fibrous feeds to improve their digestion in the rumen has become an increasingly common practice. Feed is most commonly treated with alkali (sodium or ammonium hydroxides). While there have been many studies of the effect of this pretreatment on the nutrition of ruminants, there are very few instances where the effect on anaerobic fungi in the rumen has been examined. Romulo *et al.* (1986) reported that ammoniation of wheat straw increased the dry matter digestibility of the feed by 8–10 percentage points and normally faunated sheep consuming it had higher fungal counts in the rumen (1·6–3·0-fold). Ammonium hydroxide treatment increased the *in vitro* digestibility of wheat straw by an efficient fibre degrading anaerobic fungus but not that of an inefficient strain (Grenet *et al.* 1993). Because of these two results, it would be interesting to make further observations on the effect on anaerobic fungi in the rumen of alkali pretreatment of poor quality fibrous diets with the likelihood that fungal populations would increase with an

associated enhancement in ruminal fibre degradation. Quite possibly, however, the degree of fungal involvement would depend on the proportion of different types of anaerobic fungi in the rumen during the experiment.

Chlorite. An intriguing result was obtained when barley straw was treated with sodium chlorite and acetic acid to improve its digestibility by reducing its lignin content (Ford *et al.* 1987). Treatment substantially increased DM digestibility in nylon bags suspended in the rumen over 48 h from 58% to 81%. However, reduced voluntary feed intake and slightly lower whole tract digestibility of the treated straw were observed together with an apparent absence of anaerobic fungi from the rumen. The reason for this result is not entirely clear, but it offers further evidence of the potential importance of an active ruminal population of anaerobic fungi in the nutrition of ruminants consuming poor quality fibrous feed. Another experiment with chlorite treated rice straw greatly reduced, but did not eliminate, anaerobic fungi from the rumen (Cann *et al.* 1993*b*), so there is definitely a negative effect of chlorite treated herbage on the development of anaerobic fungi in the rumen. However, there is no direct information on the degree of degradation of chlorite treated straw by anaerobic fungi *in vitro*, even though fungal involvement in this degradation is possible (Cann *et al.* 1994).

Feeding frequency

Offering a diet of hay alone or one composed of up to 80% cereal grain with hay at a frequency of one meal per day resulted in normal zoospore counts in the rumen (10^3–10^4/ml) (Gordon, 1985; Obispo & Dehority, 1992). The same was true for diets offered at multiple times per day (Gordon, 1985; Obispo & Dehority, 1992). However, when larger meals of diets rich in grain were offered at time intervals of 2–3 d, fungal zoospores were apparently absent from the rumen whereas normal zoospore counts were obtained from sheep fed a hay diet on the same time regimen (Gordon, 1985). This was probably due to the depressed pH of the rumen contents (a value of 5·0 or less; Gordon, 1985), caused by rapid bacterial fermentation of the starch, which was likely to prevent zoosporogenesis by fungi and zoospore survival in the rumen (Orpin, 1975, 1976, 1977*b*) and hence led to the apparent disappearance of anaerobic fungi through washout from the rumen.

Defaunation

The treatment of ruminants with defaunating agents (usually ionic detergents) to remove ciliate protozoa from the rumen had a secondary effect; it resulted in increased populations of ruminal fungi (Orpin, 1977*e*; Soetanto *et al.* 1985; Bird, 1989) and bacteria (Orpin, 1977*e*; Orpin & Letcher, 1984). Therefore an inverse relationship between the sizes of the fungal and ciliate populations in the rumen was apparent, at least in herbage fed animals. This relationship has been confirmed in several studies (Romulo *et al.* 1986, 1989; Hsu *et al.* 1991). Defaunation has also been accomplished by other than chemical methods and the same general inverse relationship between anaerobic fungi and ciliates was frequently observed (Newbold & Hillman, 1990; Ushida *et al.* 1990; Mathieu *et al.* 1996), although not under all dietary conditions (Ushida *et al.* 1990; Williams & Withers, 1991; Arakaki *et al.* 1994). The underlying mechanism by which defaunation increases the fungal population is possibly a reduction in the turnover of fungal protein in the rumen (Newbold & Hillman, 1990) and in the absence of

predation on fungal zoospores by ciliate protozoa, a process which has been observed *in vitro* and postulated to occur in the normally faunated rumen (Orpin 1975; Joblin, 1990; Morgavi *et al.* 1994*b*). Therefore, it is likely that the nutritional benefits due to defaunation of animals fed poor quality, low nitrogen herbage (see Bird, 1989) were at least in part because increased fungal numbers were associated with increased degradation of forage fibre. An altered role for fibre degrading bacteria in the defaunated rumen is uncertain since the extent of fibre association by bacteria (presumably the majority of which were fibrolytic) was not altered by defaunation of herbage fed sheep (Orpin & Letcher, 1984) whereas increased numbers of cellulolytic bacteria in the rumen of defaunated sheep fed a concentrate-rich diet was reported recently by Mathieu *et al.* (1996).

Ionophores, antibiotics and feed additives

A range of ionophores have found widespread use as modifiers of ruminal fermentation. Whereas much attention has been paid to the mechanism of inhibition of many different bacteria from the rumen (Russell & Strobel, 1989), there have been relatively few studies on the effects on the growth and metabolism of either ruminal fungi in culture or of zoospore counts *in vivo* (Stewart *et al.* 1987; Bernalier *et al.* 1989; Marounek & Hodrová, 1989; Phillips & Gordon, 1992; Cann *et al.* 1993*a*).

Monensin

This is perhaps the best known of the ionophores used in ruminant feeds. It was reported to eliminate anaerobic fungi from the rumen (Elliott *et al.* 1987) but the result is equivocal. Monensin fed once a day at the rate of 40 mg/kg feed was more inhibitory to anaerobic fungi than the same daily amount fed in multiple meals during the day (Calderon-Cortes *et al.* 1989). Other studies failed to confirm the inhibitory effect of monensin against anaerobic fungi in the rumen (Gordon & Phillips, 1989*c*; Grenet *et al.* 1989*c*; Schlink *et al.* 1989). It has been found that the lowest concentration of monensin required to kill a typical anaerobic fungus, *Neocallimastix* sp. LM1, in culture (16 mg/l) was many times greater than the minimum concentration required to prevent it from growing (fungistatic at 1 mg/l; Phillips & Gordon, 1992) and it is unlikely that sufficient amounts of this ionophore could be fed to ruminants to maintain a fungicidal concentration in the rumen (Gordon & Phillips, 1989*c*). Monensin was even less inhibitory against an anaerobic fungus when methanogenic archaea were also present in the culture (Stewart & Richardson, 1989), suggesting that the inhibitory effect on anaerobic fungi would be diminished in the rumen where many other microorganisms are present. In combination, the results from fungal cultures may explain why monensin has very little effect on zoospore counts of anaerobic fungi in the rumen.

Tetronasin

Another ionophore, tetronasin, was found by Newbold *et al.* (1988) to be inhibitory to the growth of anaerobic fungi. Indeed, this compound was far more inhibitory to an anaerobic fungus growing in culture than monensin since it was fungicidal at very low concentrations (1 mg/l) (Phillips & Gordon, 1992). When administered at the relatively high rates of 64 mg/d to sheep receiving hay diets it reduced zoospore counts in the rumen (Gordon & Phillips, 1993, unpublished work). However, constant administration of lower amounts (16 mg/d) had only an insignificant effect on the zoospore counts (G.L.R. Gordon & M.W. Phillips, unpublished work).

Salinomycin

This ionophore was more toxic than monensin to mixed anaerobic fungi when tested *in vitro* but its inhibition of the growth of one strain each of *Piromyces* and *Neocallimastix* was similar to that found for monensin (Cann *et al.* 1993*b*). When fed to a sheep at the rate of 30 mg/kg feed (lucerne hay and concentrate), the number of fungal sporangia growing on agar strips declined substantially (from $\sim 15\,000$ to $< 100/cm^2$) and remained low for the period of ionophore administration (23 d; Cann *et al.* 1993*a*).

Polyoxins

A different class of antifungal compounds, the polyoxins, have been investigated for their action against anaerobic fungi, both in culture and in the rumen (Cann *et al.* 1993*a*, *b*). These chitin synthesis inhibitors were active against mixed cultures of anaerobic fungi *in vitro* (Cann *et al.* 1993*a*), whereas their antifungal activity *in vivo* was slight and apparently transitory, not lasting for more than a few days even though large amounts were fed daily to the sheep for the duration of the treatment (8·8 g by Cann *et al.* 1993*a*; 17·6 g by Cann *et al.* 1993*b*).

Propionate

Sodium propionate, which can be fungistatic when used to treat stored grain (Elliott *et al.* 1987), was tested as a possible affecter of anaerobic fungi in the rumen but had no effect on the numbers of fungi in sheep at a rumen dose rate of 20–40 g/d (Elliott *et al.* 1987; Ushida *et al.* 1993). In the experiment of Ushida *et al.* (1993), *Neocallimastix* became dominant over *Piromyces* and *Caecomyces* in the rumen after treatment with propionate. A possible reason for the dominance of *Neocallimastix* under these circumstances can be found in the observation that the growth of *Neocallimastix frontalis* was unaffected by 20 mM propionate in the culture medium (Joblin & Naylor, 1993). As no information is available on the effect of propionate on the growth in culture of either *Piromyces* or *Caecomyces*, the mechanism for the differential effect of propionate *in vivo* remains unexplained.

Probiotics or direct-fed microbials

In recent years, several microbial feed additives have been investigated as promoters of ruminant digestion and production, principally fermentation extracts of the fungus *Aspergillus oryzae* and live cultures of the yeast *Saccharomyces cerevisiae* (for reviews, see Martin & Nisbet, 1992; Newbold, 1996). However, production responses to these microbial products have varied as did the associated changes in the populations of microorganisms in the rumen. Many studies have focused on the effects of the microbials on bacteria (Martin & Nisbet, 1992; Newbold, 1996) but the effects on anaerobic fungi have also been tested in a few studies both in the rumen and with pure cultures. *Aspergillus oryzae* fermentation extract has been reported to increase ruminal fibre digestion but it had no effect on fibre digestion *in vitro* by anaerobic fungi selected in rumen fluid with antibiotics (Beharka & Nagaraja, 1993). The counts of anaerobic fungi in the rumen were unaffected by *A. oryzae* extract (Newbold *et al.* 1992; Mathieu *et al.* 1996), and hydrogen production, as an indicator of fermentation and growth, by two strains of *Neocallimastix* and one *Piromyces* was not affected by the presence of the extract in the culture medium (Newbold *et al.* 1992). A stimulatory effect of a heat- and proteinase-labile *A. oryzae* extract on growth and cellulase production by pure cultures of *Neocallimastix*, *Orpinomyces* and *Piromyces* has been reported in other studies (Harper *et al.* 1996; Welch *et al.* 1996). Chaucheyras *et al.* (1995) found that live yeast stimulated the growth in poor medium of a culture of *N. frontalis* in a dose dependent manner, most probably by providing B-group vitamins (mainly thiamin), but the feeding of live *S. cerevisiae* to sheep had no effect on the

numbers of fungal zoospores in the rumen (Mathieu *et al.* 1996). Therefore, both of these microbial agents can stimulate the growth of at least some anaerobic fungi in culture but the failure to demonstrate any effect on fungal populations in the rumen suggests that any positive effect on rumen fermentation is most likely due to a stimulation of the bacterial populations by the direct-fed microbial products.

Dietary phenolics

Early studies showed that anaerobic fungi preferentially colonized the sclerenchyma patches of tropical grass leaves (Akin *et al.* 1989; Akin & Hogan, 1983), suggesting an affinity for lignified tissues. Subsequently, selected strains of anaerobic fungi were found to solubilize about 35% of the label from a ^{14}C[lignin]lignocellulose preparation (Gordon, 1987; Gordon & Phillips, 1989*b*) even though the consistent loss of acid-detergent lignin from wheat straw could not be demonstrated (Gordon & Ashes 1984; Gordon & Phillips, 1989*a*). Nevertheless, $\sim$34% of the lignin component of sorghum stem was removed by the anaerobic fungus *Neocallimastix patriciarum* (McSweeney *et al.* 1994) confirming that 'core# lignin could be attacked by at least some anaerobic fungi. Pure cultures of anaerobic fungi were unable to metabolize lignin derived phenolic compounds to CO_2 (Gordon, 1987; Gordon & Phillips, 1989*b*; Bernard-Vailhé *et al.* 1995).

Phenolic compounds, derived from lignocellulose within the rumen, are a potential barrier to the degradation of structural polysaccharides (Wilson, 1994) although the demonstration by Wubah & Kim (1996) of chemoattraction to ferulic and *p*-coumaric acids by zoospores of both monocentric (*Neocallimastix* and *Piromyces*) and polycentric (*Anaeromyces* and *Orpinomyces*) anaerobic fungi may provide an explanation for the apparent fungal preferential colonization of highly lignified grass tissues. Ferulic and *p*-coumaric acids inhibited fibre degradation by mixed anaerobic fungi *in vitro* (Akin & Rigsby, 1985; Tanaka *et al.* 1991). These phenolic compounds inhibited fibre degradation by pure cultures of both monocentric and polycentric anaerobic fungi, with polycentric strains generally being less sensitive than the monocentric strains (Gordon *et al.* 1995*a*) and having a lower chemotactic response to phenolic acids (Wubah & Kim, 1996). The inhibition by plant phenolics is potentially significant because anaerobic fungi produced powerful hydrolytic enzymes for releasing ferulic and *p*-coumaric acids from plant cell walls (Borneman *et al.* 1990). Even though the infusion of free phenolic acids into the rumen of sheep had no effect on digestion of herbage by sheep (Lowry *et al.* 1993), the localized concentrations of phenolics liberated by fungal enzymes in the rumen may be sufficiently high to hinder fungal growth and activity.

The deleterious effects of another form of dietary phenolics, the plant tannins, on rumen function have been broadly characterized but the ability of the rumen microbial population to interact with and adapt to the presence of tannins has received little attention. Since the minimum inhibitory concentration of tannin appeared to be much higher for fungi than for bacteria (Scalbert, 1991), anaerobic ruminal fungi may be more tolerant of tannin than the bacteria which compete with them. Indeed, the tolerance of anaerobic fungi to tannins was demonstrated by the ability of *Neocallimastix patriciarum* to degrade cellulose effectively in the presence of 100 μg/ml condensed tannin (CT) from birdsfoot trefoil, *Lotus corniculatus* (McAllister *et al.* 1994). Also, several rumen anaerobic fungi have been screened for their tolerance to CT from the shrub legume *Calliandra calothyrsus* and their ability to utilize protein from tannin–protein complexes prepared with bovine serum albumin (unpublished work by C.S. McSweeney, as cited in Gordon *et al.* 1995*b*). Of five anaerobic fungi, *Neocallimastix patriciarum* grew best in the presence of free CT and, with two other *Neocallimastix* spp., it grew when nitrogen was present only as a CT–protein complex whereas *Piromyces* and

Caecomyces did not grow. In another study, anaerobic fungal populations were shown by sporangium counts to increase up to 7–8-fold in the rumen of sheep which were gradually subjected to increasing amounts in the diet of the tannin-containing tropical browse tree *Acacia angustissima* (Odenyo *et al.* 1997). This plant was toxic to sheep unless they were gradually adapted. However, the role of tannin-tolerant anaerobic fungi in the rumen of animals consuming tannin-containing plants remains to be determined.

Contribution of fungi to digestion in ruminants

Feed intake

There is now a body of evidence to support the existence of a definite positive relationship between the presence of anaerobic fungi in the rumen and the voluntary intake of herbage diets of low digestibility (Akin *et al.* 1989; Gordon, 1985; Weston *et al.* 1988; Morrison *et al.* 1990; Gordon & Phillips, 1993). This relationship is quite possibly a result of fungal attack of lignified plant tissues (Akin, 1987*a*; Akin & Borneman, 1990) combined with the resultant weakening of these tough plant components (Akin & Hogan, 1983; Akin *et al.* 1989). Weaker feed fragments in the rumen may be expected to lead to less effort by the animal in eating and ruminating the feed. Indeed, Weston *et al.* (1988) have shown a correlation between the size of the population of rumen anaerobic fungi and the rumination efficiency for each feed bolus in sheep fed a wheat straw diet. Therefore, it is probable that anaerobic fungi exert their major effect in the rumen by facilitating the physical disruption during rumination of the fibrous particles of poor quality feed which leads to a more rapid clearance of these particles from the rumen. In this regard, Sekine *et al.* (1995) have suggested that the medium to large digesta particles (those retained on a sieve of 0·3 mm mesh size), which had a slower outflow rate from the rumen of hay fed sheep, offered greater opportunities for fungal growth and zoospore production.

The removal of anaerobic fungi from the rumen, by various means, has permitted quantitation of the contribution of anaerobic fungi to feed intake. A change in diet from barley straw to one of chlorite treated barley straw reduced fungal populations in the rumen to undetectable levels and resulted in decreased voluntary feed intake by sheep to 49% (790–385 g DM/d; Ford *et al.* 1987). In a later study, the removal of anaerobic fungi from the rumen of sheep reduced the voluntary intake of poor quality feed to about 70%, with little effect on the populations of both total and cellulolytic bacteria and of ciliate protozoa (Gordon & Phillips, 1993), as shown in Table 3. Additional evidence for an important contribution by anaerobic fungi to the intake of fibrous feed has been provided by the experimental addition of fungal cultures to the rumen of sheep and cattle which were previously without fungal populations. The intake of forage by early weaned calves was 35% higher in those which had been dosed with *Neocallimastix* sp. R1 (1·35–1·85 kg DM/d; Theodorou *et al.* 1990*a*) and the dosing of fungus-free sheep with *Neocallimastix* sp. SL1 produced a 40% increase in intake of a straw based diet (Gordon & Phillips, 1993; see Table 3).

Fibre digestion and fermentation in the rumen

Experiments in which anaerobic fungi were absent from the rumen or were greatly reduced in numbers have provided evidence for the contribution of fungi to fibre digestion and the overall

Table 3. The effect of the removal of anaerobic fungi from the rumen of sheep fed on a straw-based diet and the subsequent reinoculation of *Neocallimastix* sp. SL1 into the rumen on voluntary feed intake, feed digestion, and rumen microbial populations (data from Gordon and Phillips, 1993)

	Pretreatment	No fungi	With fungi added
Intake (g/d)			
OM	894 ± 30·8	628 ± 36·4	877 ± 52·6
ADF	390 ± 9·3	264 ± 16·4	373 ± 22·6
Digestibility (%)			
OM	53·2 ± 1·30	50·3 ± 1·38	57·3 ± 1·02
ADF	51·2 ± 1·03	46·5 ± 1·28	55·0 ± 1·64
Anaerobic fungi (zoospores/ml; $\times 10^3$)	7·6 ± 4·0	UD (< 0·001)	19 ± 5·7
Bacteria (cells/ml)			
total viable ($\times 10^9$)	0·8 ± 0·18	1·4 ± 0·37	1·6 ± 0·33
cellulolytic ($\times 10^8$)	0·4 ± 0·19	2·6 ± 1·48	1·1 ± 0·29
Ciliate protozoa (cells/ml; $\times 10^5$)	3·8 ± 0·92	3·0 ± 0·53	4·8 ± 0·49

Data are mean ± standard error for four sheep.
ADF, acid-detergent fibre; OM, organic matter; UD, undetectable (limit of detection).

ruminal fermentation of carbohydrates in the diet. The presence of active fungal populations in the rumen has often been accompanied by increased feed digestibility *in vivo*. In some instances, the increased digestibility was observed when S-containing feed supplements were fed to animals receiving diets of poor quality herbage of low sulphur content, as detailed earlier (Gulati *et al.* 1985; Weston *et al.* 1988; Morrison *et al.* 1990). Decreases in whole tract digestibility of DM or organic matter of ~3–7 percentage points have been demonstrated when anaerobic fungi were removed from the rumen of sheep fed diets of poor quality herbage (Ford *et al.* 1987; Gordon & Phillips, 1993; see Table 3). Also, the dosing of pure cultures of different strains of *Neocallimastix* into the rumen of fungus free sheep increased feed digestibility by 3–7 percentage points (Elliott *et al.* 1987; Gordon & Phillips, 1993). These digestibility increases reflect those observed during *in vitro* incubations with rumen contents which compared feed fermentation in both the absence and presence of anaerobic fungi. Increases in DM digestibility of 7–9 percentage-points (Hillaire *et al.* 1990) and 7–8 percentage points (Kostyukovsky *et al.* 1995) were recorded in the presence of anaerobic fungi.

Acetate is produced by anaerobic fungi as a major product during carbohydrate fermentation without the production of any propionate or butyrate (Lowe *et al.* 1987*c*; Phillips & Gordon, 1988; Borneman *et al.* 1989). Therefore, Elliott *et al.* (1987) suggested that the presence of an active population of anaerobic fungi in the rumen was responsible for depressed concentrations of propionate and elevated concentrations of acetate in the rumen. The suggestion was based on a change in the proportion of propionate to total ruminal volatile fatty acids from 0·29 to 0·15 after the addition of a pure fungal culture (*Neocallimastix* sp. A1) to the rumen of sheep with no fungi (removed by feeding chlorite treated barley straw). Similar changes were found when fungi were removed or greatly reduced by changing the diet from untreated straw to chlorite treated straw (Ford *et al.* 1987; Cann *et al.* 1993*b*). This apparent contribution of anaerobic fungi to depressed ruminal propionate proportions seemed to be supported by Gordon & Phillips (1993) who recorded an increase in propionate proportion from 0·19 to 0·34 when fungi were removed from the rumen and then maintained in the fungus free

state by inclusion of low concentrations of the ionophore tetronasin in the diet (see Table 3). In a subsequent experiment, the proportion of propionate only changed from 0·32 to 0·37 when fungi were removed and tetronasin was present both before and after the treatment to remove anaerobic fungi (G.L.R. Gordon & M.W. Phillips, unpublished work). For comparison, *in vitro* incubations with rumen contents which compared feed fermentation in both the absence and presence of anaerobic fungi found a small average change in propionate proportion from 0·23 to 0·28 (Hillaire *et al.* 1990). It was not possible to assess the change in this parameter in two other studies (Hillaire & Jouany, 1989; Kostyukovsky *et al.* 1995) although the ratios of acetate to propionate (*i.e.* C2/C3) were higher, indicating a depressed propionate proportion, in both studies when anaerobic fungi were present in the fermenters. Therefore, it is not possible to conclude that carbohydrate fermentation by anaerobic fungi in the rumen greatly influences the concentrations of volatile fatty acids there when anaerobic fungi constitute only a small proportion of the rumen microbial biomass in sheep consuming good quality hay (Faichney *et al.* 1997), unless their contribution to fermentation is much greater in the rumen of ruminants consuming poor quality herbage such as cereal straw.

Protein metabolism

Anaerobic fungi have the potential of contributing to the protein supply of the host animal, both through the production of proteolytic enzymes in the rumen and as a proportion of the microbial protein synthesized in the rumen which passes to the abomasum and intestines for digestion and absorption.

Proteolysis

A number of different anaerobic fungi produced extracellular proteinases in culture (Wallace & Joblin 1985; Asao *et al.* 1993; Michel *et al.* 1993; Yanke *et al.* 1993) but the majority of strains tested in one study did not degrade casein while producing several peptidase activities (Michel *et al.* 1993). One isolate of *Neocallimastix frontalis* has been shown to produce an extracellular metalloproteinase active under neutral to slightly acid conditions (Wallace & Joblin 1985) whereas another *Neocallimastix* sp. and a *Piromyces* sp. both secreted into the culture medium a wide range of proteolytic enzymes which were also most active at the pH levels found in the normal rumen (Asao *et al.* 1993). In addition, a strain of the polycentric fungus *Orpinomyces joyonii* was able to degrade casein (Yanke *et al.* 1993) whereas two unnamed polycentric anaerobic fungi were not proteolytic (Michel *et al.* 1993).

In vitro studies with defined populations of both proteolytic and nonproteolytic rumen bacteria and a proteolytic *Neocallimastix frontalis* have shown that the anaerobic fungus was able to contribute to ruminal protein degradation, particularly where the protein was associated with feed particles (Wallace & Munro, 1986). However, the extent of this possible fungal contribution to proteolysis in the rumen remains to be conclusively determined since the only study to examine ruminal proteolysis both in the presence and absence of anaerobic fungi (Bonnemoy *et al.* 1993) used seven strains of anaerobic fungi of which only one was weakly proteolytic in culture (Michel *et al.* 1993). Clearly, anaerobic fungi possess a wide range of proteolytic abilities which may allow some of them to contribute to protein degradation in the rumen. Evidence in support of a fungal contribution was obtained from an experiment, conducted with fungus free sheep and a design similar to that described by Gordon & Phillips (1993), in which anaerobic fungi significantly contributed to the amount of nitrogen apparently digested in the rumen of sheep consuming poor quality herbage while the partition of organic

matter digestion between the rumen and intestines was independent of a rumen population of anaerobic fungi (G.L.R. Gordon, M.W. Phillips & G.J. Faichney, unpublished work).

Protein supply

Kemp *et al.* (1985), Gulati *et al.* (1989*a*) and Onoda *et al.* (1993) showed that fungal cells were composed of proteins with a well balanced combination of amino acids which were highly digestible and available to the ruminant host (Gulati *et al.* 1988, 1989*b*). A high proportion of the protein component of three monocentric anaerobic fungi, *Neocallimastix* sp. LM1, *Piromyces* sp. SM1 and *Caecomyces* sp. NM1, was digested and absorbed in the intestines of sheep, with digestibility factors of 0·91–0·98 (Gulati *et al.* 1988, 1989*b*). These high *in vivo* digestibility values for fungal protein compared favourably with a value of 0·77 for mixed ruminal bacteria measured in a similar manner (Gulati *et al.* 1990).

Previously, the lack of a precise method of measuring the biomass in the rumen delayed an accurate assessment of the potential extent of the fungal contribution to protein supply (Faichney *et al.* 1991). The advent of reliable measurements of fungal biomass in ruminant digesta has shown that in sheep fed either hay or hay/grain diets, anaerobic fungi comprised 1·1–3·6% (average 2·4%) of the microbial nitrogen in rumen digesta (Faichney *et al.* 1997). As a result, the contribution of anaerobic fungi to the supply of microbial protein to the animal was minor as they comprised 0·7–2·7% (average 1·6%) of the microbial nitrogen in digesta flowing to the duodenum. However, this microbial protein was of high quality and readily available to the animal. Should an increase in the biomass of anaerobic fungi in the rumen prove to be feasible, it is likely that the supply of high quality microbial protein to the host ruminant would be enhanced.

Possible manipulation of fungal populations in the rumen

Anaerobic fungi are important to ruminants consuming diets of poor quality, mature herbage by increasing the voluntary intake of feed. Therefore, a considerable potential exists for the manipulation of fungal numbers and activity in the rumen to improve the utilization of poor quality herbage by domesticated ruminants for increased production. Two approaches for increasing fungal influence on forage intake and digestion are possible: the use of a dietary supplement that is specific for the nutrition and growth of the indigenous anaerobic fungi in the rumen, and the inoculation of very efficient strains directly into the rumen. Efficiency in this context refers to the fungal ability to degrade plant fibre relative to degradation by the species or strains of fungi that are indigenous to the rumen of the animal species under investigation.

Selection of fungal strains appropriate for dosing

The significance of anaerobic fungi to ruminant nutrition is probably to weaken the structure of forage plants so that less effort is expended by the animal in rumination, as originally observed by Weston *et al.* (1988). The net effect would be that chewing time is more efficiently used, and that digesta can more rapidly leave the rumen and thus stimulate feed intake. An *in vitro* study showed that substantial variations existed among fungal isolates in their ability to weaken grass stems (Akin *et al.* 1990), supported by Gordon (1990) who determined degradation rates for

^{14}C-labelled plant fibre substrates as a measure of the relative cellulolytic ability of different anaerobic fungi. Subsequently it was shown that the cellulolytic ability and other fibre degrading ability of fungal strains was related to their overall abilities to reduce the strength of forages (G.L.R. Gordon & D.E. Akin, unpublished). Therefore, degradation rates for ^{14}C-labelled plant fibre substrates provided a means of selecting appropriate strains of anaerobic fungi for inoculation into the rumen of mature ruminants at pasture (Gordon, 1990). Generally, the most rapid degradation of fibrous plant tissues by some *Piromyces* strains has been observed, together with fungal isolates from the genera *Neocallimastix* and *Orpinomyces* (Akin *et al.* 1990; Gordon, 1990), so the most likely candidate fungi for dosing into ruminants would come from these genera.

Dosing of fungi between host species

There are additional factors which must also be considered once a small group of efficient fungi has been found. Primary among these is the need to determine that the efficient fungi are able to establish themselves in the rumen of the species of ruminant which is the subject of the modification, in competition with the indigenous anaerobic fungi. The feasibility of cross-species transfer of anaerobic fungi was demonstrated by Orpin (unpublished work; cited by Orpin, 1989 and Orpin & Ho, 1991) who reported the successful separate colonization of the fungus free rumen of lambs (reared in isolation from adult sheep) by a *Piromyces* sp. isolated from the caecum of a horse and by a *Neocallimastix* sp. from reindeer. Also, an oral inoculum of a *Neocallimastix* sp. from sheep presumably established a population in the rumen of early weaned calves because of the stimulation to the amount of hay eaten by the dosed animals (Theodorou *et al.* 1990*a*). In this regard, our own work has shown that some isolates of monocentric fungi from cattle, deer and several species of African antelope established themselves in the fungus free rumen of adult sheep (M.W. Phillips & G.L.R. Gordon, unpublished). Further research is required to determine the ability of the efficient fungi to compete with the ruminal populations of indigenous fungi.

Polycentric strains dosed into sheep

Although some polycentric anaerobic fungi have been observed in sheep ruminal digesta and isolated in pure culture from this source (Breton *et al.* 1989; Michel *et al.* 1993), the only reports are from France and polycentric fungi have never been cultured from sheep digesta samples in Australia. Therefore, the ability of Australian isolates of polycentric fungi to colonize the rumen of sheep was tested. Interspecies transfer of polycentric anaerobic fungi was successful when several *Orpinomyces* freshly isolated from cattle were dosed *via* the cannula into the rumen of fungus free sheep (Phillips & Gordon, 1995*b*). We have been able to establish several polycentric anaerobic fungi from cattle in the rumen of adult sheep previously treated to eliminate their indigenous anaerobic fungi. The polycentric fungi were present in the rumen for at least six weeks after transfer. However, the subsequent dosing experiments with these and other freshly isolated *Orpinomyces* strains from cattle have shown that polycentric anaerobic fungi are unable to establish detectable populations in the rumen of sheep in competition with the indigenous monocentric fungi (G.L.R. Gordon & M.W. Phillips, unpublished work). In these experiments, tracking was performed by both culture methods (see earlier) and strain specific oligonucleotide probes (Brownlee *et al.* 1996).

Kangaroo strains of fungi dosed into sheep

Several strains of *Piromyces* which were isolated from the forestomach digesta of red kangaroos (*Macropus rufus*), eastern grey kangaroos (*M. giganteus*) or western grey kangaroos (*M. fuliginosus*), and which degraded cellulose very rapidly when tested in the laboratory with pure [^{14}C]cellulose, were not able to colonize the rumen of fungus free sheep (G.L.R. Gordon & M.W. Phillips, unpublished work). Verification that any monocentric fungi isolated from the rumen of these sheep were not those dosed into the sheep was accomplished with strain specific oligonucleotide probes directed at DNA sequences from the genome of kangaroo derived fungi (Gordon *et al.* 1996). Thus, it is likely that the conditions in the forestomach of kangaroos are sufficiently different from those found in the rumen to render the fungi, which have evolved there, unable to grow in the rumen of sheep or any other ruminant. In this regard, it is interesting to note that the forestomach of kangaroos contains unique ciliate protozoa which were unable to colonize the defaunated rumen of sheep (Baker *et al.* 1995).

Potential benefits

One experiment with sheep, which retained their indigenous populations of monocentric ruminal fungi (*Neocallimastix* spp., *Piromyces* spp. and *Caecomyces* spp.), showed an increase in the voluntary intake of a straw based diet of ~7–12% when the sheep were dosed by mouth with cultures of monocentric fungi originally isolated from herbivores other than sheep (G.L.R. Gordon & M.W. Phillips, unpublished). Also, an oral inoculum of a *Neocallimastix* sp. from sheep stimulated forage intake by 35% in early weaned calves under conditions where the undosed calves probably remained free of anaerobic fungi (Theodorou *et al.* 1990*a*). Both of these studies used freshly grown cultures of anaerobic fungi so, in the future, it will be important to maintain the viability of an anaerobic fungal inoculum for a sufficiently long time so that it can be used to improve the nutritional status of ruminants at pasture remote from the laboratory. Therefore, much more research effort must be expended in providing answers to these questions before the potential for modifying ruminal populations and activity of anaerobic fungi can be realized.

Another possible method of manipulating the population of anaerobic fungi in the rumen is through increasing the growth and degradative activity of those anaerobic fungi indigenous to the rumen of the particular species of herbivore in question. The early work of Akin & Hogan (1983) and Akin *et al.* (1983) on the beneficial effects of supplementing a hay of low S content on the number of anaerobic fungi in the rumen of sheep and their relative contribution to fibre degrading activity, together with the subsequent work on alkali treated wheat straw (Gordon *et al.* 1983; Gulati *et al.* 1985; Weston *et al.* 1988) and other poor quality feeds (Morrison *et al.* 1990; Gutierrez *et al.* 1996), suggested a means of manipulating anaerobic fungi by provision of an appropriate dietary supplement which contains a S compound. For a supplement of this type to be most effective it should contain a single S compound, or a mixture of several compounds which are most readily utilized by the rumen anaerobic fungi and are initially not readily available or only slowly metabolized by other components of the rumen microbiota (bacteria and protozoa). A search for such S compounds is currently under way.

The removal of ciliate protozoa from the rumen could be of potential use as a means of increasing the size of the fungal population for improved utilization of poor quality, low nitrogen herbage by grazing ruminants, as revealed by many experiments conducted with sheep and cattle defaunated with detergents or maintained protozoa free from birth by quarantine

(Bird, 1989, 1991; Jouany, 1996). The development of practical defaunating agents is a necessary precursor to further advances in this area.

Conclusions

There have been many studies on the biology of anaerobic fungi, some of which have dealt with the contribution, both as measured and as perceived, that is made by these unusual micro-organisms to the availability of nutrients for the host ruminant animal. Some of the features of these studies have been reviewed here. They suggest that anaerobic fungi are an extremely important component of the rumen microbiota in livestock consuming diets of poor quality, mature herbage by increasing the voluntary consumption of feed. This influence is most apparent when additional S is added to tropical pasture grasses that have a low content of S. The high lignocellulose content of tropical pastures (Wilson, 1994; Wilson & Kennedy, 1996) together with the relatively high tolerance of anaerobic fungi to plant phenolics may be determining factors in the effectiveness of these microorganisms in the rumen. Therefore, a considerable potential exists for the manipulation of fungal numbers and activity in the rumen to benefit the utilization of poor quality herbage by domesticated ruminants (cattle, goats, sheep, buffaloes and camels), particularly in the semi-arid regions, for the sustained and improved production of milk, meat, wool, hair and hide in both developed and developing countries.

The original research reported in this review was supported in part by Australian wool-growers through the Australian Wool Research and Promotion Organisation as projects CPB018, CPB040, CPB293 and CPB420, and by Australian beef and lamb producers through the Meat Research Corporation as project CS39.

References

Akin, D. E. (1987*a*). Association of rumen fungi with various forage grasses. *Animal Feed Science and Technology* **16**, 273–285.

Akin, D. E. (1987*b*). Use of chitinase to assess ruminal fungi associated with plant residues in vitro. *Applied and Environmental Microbiology* **53**, 1955–1958.

Akin, D. E. & Benner, R. (1988). Degradation of polysaccharides and lignin by ruminal bacteria and fungi. *Applied and Environmental Microbiology* **54**, 1117–1125.

Akin, D. E. & Borneman, W. S. (1990). Role of rumen fungi in fiber degradation. *Journal of Dairy Science* **73**, 3023–3032.

Akin, D. E., Borneman, W. S. & Lyon, C. E. (1990). Degradation of leaf blades and stems by monocentric and polycentric isolates of ruminal fungi. *Animal Feed Science and Technology* **31**, 205–221.

Akin, D. E., Borneman, W. S. & Windham, W. R. (1988). Rumen fungi: morphological types from Georgia cattle and the attack on forage cell walls. *BioSystems* **21**, 385–391.

Akin, D. E., Gordon, G. L. R. & Hogan, J. P. (1983). Rumen bacterial and fungal degradation of *Digitaria pentzii* grown with or without sulfur. *Applied and Environmental Microbiology* **46**, 738–748.

Akin, D. E. & Hogan, J. P. (1983). Sulfur fertilization and rumen microbial degradation of cell walls in *Digitaria pentzii* (Stent). *Crop Science* **23**, 851–858.

Akin, D. E., Lyon, C. E., Windham, W. R. & Rigsby, L. L. (1989). Physical degradation of lignified stem tissues by ruminal fungi. *Applied and Environmental Microbiology* **55**, 611–616.

Akin, D. E. & Rigsby, L. L. (1985). Influence of phenolic acids on rumen fungi. *Agronomy Journal* **77**, 180–182.

Akin, D. E. & Rigsby, L. L. (1987). Mixed fungal populations and lignocellulosic tissue degradation in the bovine rumen. *Applied and Environmental Microbiology* **53**, 1987–1995.

Akin, D. E. & Windham, W. R. (1989). Influence of diet on rumen fungi. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 75–81. [J. A. Nolan, R. A. Leng and D. L. Demeyer, editors]. Armidale, Australia: Penambul Books.

Ali, B. R. S., Zhou, L., Graves, F. M., Freedman, R. B., Black, G. W., Gilbert, H. J. & Hazlewood, G. P. (1995). Cellulases and hemicellulases of the anaerobic fungus *Piromyces* constitute a multiprotein cellulose-binding complex and are encoded by multigene families. *FEMS Microbiology Letters* **125**, 15–21.

Arakaki, C., Mitsumori, M., Itabashi, H., Shirasaka, S. & Minato, H. (1994). Influence of the presence of protozoa on the rumen microbial population of cattle. *Journal of General and Applied Microbiology* **40**, 215–226.

Argyle, J. L. & Douglas, L. (1989). Chitin as a rumen fungal marker. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 289–290 [J. A. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul Books.

Asao, N., Ushida, K. & Kojima, Y. (1993). Proteolytic activity of rumen fungi belonging to the genera *Neocallimastix* and *Piromyces*. *Letters in Applied Microbiology* **16**, 247–250.

Baker, S. K., Dehority, B. A., Chamberlain, N. L. & Purser, D. B. (1995). Inability of protozoa from the kangaroo forestomach to establish in the rumen of sheep. *Annales de Zootechnie* **44** (Suppl.), 143.

Barr, D. J. S., Kudo, H., Jackober, K. D. & Cheng, K.-J. (1989). Morphology and development of rumen fungi: *Neocallimastix* sp., *Piromyces communis*, and *Orpinomyces bovis* gen. nov., sp. nov. *Canadian Journal of Botany* **67**, 2815–2824.

Bauchop, T. (1979). Rumen anaerobic fungi of sheep and cattle. *Applied and Environmental Microbiology* **38**, 148–158.

Bauchop, T. (1989). Biology of gut anaerobic fungi. *BioSystems* **23**, 53–64.

Beharka, A. A. & Nagaraja, T. G. (1993). Effect of *Aspergillus oryzae* fermentation extract (Amaferm©) on in vitro fiber degradation. *Journal of Dairy Science* **76**, 812–818.

Bernalier, A., Bogaert, C., Fonty, G. & Jouany, J.-P. (1989). Effect of ionophore antibiotics on rumen anaerobic fungi. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 273–275 [J. V. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul Books.

Bernalier, A., Fonty, G., Bonnemoy, F. & Gouet, P. (1993*a*). Effect of *Eubacterium limosum*, a ruminal hydrogenotrophic bacterium, on the degradation and fermentation of cellulose by three species of rumen anaerobic fungi. *Reproduction Nutrition Development* **33**, 577–584.

Bernalier, A., Fonty, G., Bonnemoy, F. & Gouet, P. (1993*b*). Inhibition of the cellulolytic activity of *Neocallimastix frontalis* by *Ruminococcus flavefaciens*. *Journal of General Microbiology* **139**, 873–880.

Bernalier, A., Fonty, G. & Gouet, P. (1991). Cellulose degradation by two rumen anaerobic fungi in monoculture or in coculture with rumen bacteria. *Animal Feed Science and Technology* **32**, 131–136.

Bernard-Vailhé, M. A., Besle, J. M. & Doré, J. (1995). Transformation of C^{14}-lignin-labeled cell walls of wheat by *Syntrophococcus sucromutans*, *Eubacterium oxidoreducens*, and *Neocallimastix frontalis*. *Applied and Environmental Microbiology* **61**, 379–381.

Bird, S. H. (1989). Production from ciliate-free ruminants. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 233–246 [J. V. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul Books.

Bird, S. H. (1991). Role of protozoa in relation to the nutrition of the host animal. In *Recent Advances in the Nutrition of Herbivores*, pp. 171–180 [Y. W. Ho, H. K. Wong, N. Abdullah and Z. A. Tajuddin, editors]. Serdang, Malaysia: Malaysian Society of Animal Production.

Black, G. W., Hazlewood, G. P., Xue, G.-P., Orpin, C. G. & Gilbert, H. J. (1994). Xylanase B from *Neocallimastix patriciarum* contains a non-catalytic 455-residue linker sequence comprised of 57 repeats of an octapeptide. *Biochemical Journal* **299**, 381–387.

Bonnemoy, F., Fonty, G., Michel, V. & Gouet, P. (1993). Effect of anaerobic fungi on the ruminal proteolysis in gnotobiotic lambs. *Reproduction Nutrition Development* **33**, 551–555.

Borneman, W. S. & Akin, D. E. (1990). Lignocellulose degradation by rumen fungi and bacteria: ultrastructure and cell wall degrading enzymes. In *Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Ruminants* (Tri-National Workshop), pp. 325–339 [D. E. Akin, L. G. Ljungdahl, J. R. Wilson and P. J. Harris, editors]. New York: Elsevier.

Borneman, W. S., Akin, D. E. & Ljungdahl, L. G. (1989). Fermentation products and plant cell wall-degrading enzymes produced by monocentric and polycentric anaerobic ruminal fungi. *Applied and Environmental Microbiology* **55**, 1066–1073.

Borneman, W. S., Hartley, R. D., Morrison, W. H., Akin, D. E. & Ljungdahl, L. G. (1990). Feruloyl and *p*-coumaroyl esterase from anaerobic fungi in relation to plant cell wall degradation. *Applied Microbiology and Biotechnology* **33**, 345–351.

Borneman, W. S., Ljungdahl, L. G., Hartley, R. D. & Akin, D. E. (1992). Purification and partial characterization of two feruloyl esterases from the anaerobic fungus *Neocallimastix* strain MC-2. *Applied and Environmental Microbiology* **58**, 3762–3766.

Bowman, B. H., Taylor, J. W., Brownlee, A. G., Lee, J., Lu, S. D. & White, T. J. (1992). Molecular evolution of the fungi. Relationships of the Basidiomycetes, Ascomycetes, and Chytridiomycetes. *Molecular Biology and Evolution* **9**, 285–296.

Breton, A., Bernalier, A., Bonnemoy, F., Fonty, G., Gaillard, B. & Gouet, P. (1989). Morphological and metabolic characterization of a new species of strictly anaerobic rumen fungus: *Neocallimastix joyonii*. *FEMS Microbiology Letters* **58**, 309–314.

Breton, A., Bernalier, A., Dusser, M., Fonty, G., Gaillard-Martinie, B. & Guillot, J. (1990). *Anaeromyces mucronatus* nov. gen., nov. sp. A new strictly anaerobic rumen fungus with polycentric thallus. *FEMS Microbiology Letters* **70**, 177–182.

Breton, A., Confesson, I., Dusser, M. & Gaillard-Martinie, B. (1994). [Comparison of the fungal populations in the rumen, duodenum and faeces of sheep.] *Annales de Zootechnie* **43**, 262.

Breton, A., Dusser, M., Gaillard-Martinie, B., Guillot, J. & Millet, L. (1992). [Characterization of rumen polycentric fungi observed *in vivo*.] *Annales de Zootechnie* **41**, 79–80.

Breton, A., Gaillard-Martinie, B., Gerbi, C., De Segura, B. G., Durand, R. & Kherratia, B. (1995). Location by fluorescence microscopy of glycosidases and a xylanase in the anaerobic gut fungi *Caecomyces communis*, *Neocallimastix frontalis*, and *Piromyces rhizinflata*. *Current Microbiology* **31**, 224–227.

Brownlee, A. G. (1994). The nucleic acids of anaerobic fungi. In *Anaerobic Fungi*, pp. 241–256 [D. O. Mountfort and C. G. Orpin, editors]. New York: Marcel Dekker.

Brownlee, A. G., Rintoul, A. J., Gordon, G. L. R. & Phillips, M. W. (1996). Monitoring rumen fungal populations with PCR and DNA probes. *Microbiology Australia* **17**, A38 (Abstr.).

Calderon-Cortes, J. F., Elliott, R. & Ford, C. W. (1989). Influence of rumen fungi on the nutrition of sheep fed forage diets. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 181–187 [J. V. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul Books.

Cann, I. K. O., Kobayashi, Y., Onoda, A., Wakita, M. & Hoshino, S. (1993*a*). Effects of some ionophore antibiotics and polyoxins on the growth of anaerobic rumen fungi. *Journal of Applied Bacteriology* **74**, 127–133.

Cann, I. K. O., Kobayashi, Y., Wakita, M. & Hoshino, S. (1993*b*). Fungal supression and its effects on some ruminal parameters. *Animal Science and Technology* **64**, 233–238.

Cann, I. K. O., Kobayashi, Y., Wakita, M. & Hoshino, S. (1994). Effects of three chemical treatments on *in vitro* fermentation of rice straw by mixed rumen microbes in the presence or absence of anaerobic rumen fungi. *Reproduction Nutrition Development* **34**, 47–56.

Chaucheyras, F., Fonty, G., Bertin, G. & Gouet, P. (1995). Effects of live *Saccharomyces cerevisiae* cells on zoospore germination, growth, and cellulolytic activity of the rumen anaerobic fungus *Neocallimastix frontalis* MCH3. *Current Microbiology* **31**, 201–205.

Davies, D. R., Theodorou, M. K., Lawrence, M. I. G. & Trinci, A. P. J. (1993). Distribution of anaerobic fungi in the digestive tract of cattle and their survival in faeces. *Journal of General Microbiology* **139**, 1395–1400.

Denman, S., Xue, G.-P. & Patel, B. (1996). Characterization of a *Neocallimastix patriciarum* cellulase cDNA (*celA*) homologous to *Trichoderma reesei* cellobiohydrolase II. *Applied and Environmental Microbiology* **62**, 1889–1896.

Dijkerman, R., Ledeboer, J., Op den Camp, H. J. M., Prins, R. A. & Van der Drift, C. (1997*a*). The anaerobic fungus *Neocallimastix* sp. strain L2 - growth and production of (hemi)cellulolytic enzymes on a range of carbohydrate substrates. *Current Microbiology* **34**, 91–96.

Dijkerman, R., Op den Camp, H. J. M. & Van der Drift, C. (1996*a*). Cultivation of anaerobic fungi in a 10-l fermenter system for the production of (hemi)-cellulolytic enzymes. *Applied Microbiology and Biotechnology* **46**, 85–91.

Dijkerman, R., Op den Camp, H. J. M., Van der Drift, C. & Vogels, G. D. (1997*b*). The role of the cellulolytic high molecular mass (HMM) complex of the anaerobic fungus *Piromyces* sp. strain E2 in the hydrolysis of microcrystalline cellulose. *Archives of Microbiology* **167**, 137–142.

Dijkerman, R., Vervuren, M. B. F., Op den Camp, H. J. M. & Van der Drift, C. (1996*b*). Adsorption characteristics of cellulolytic enzymes from the anaerobic fungus *Piromyces* sp. strain E2 on microcrystalline cellulose. *Applied and Environmental Microbiology* **62**, 20–25.

Doré, J., Brownlee, A. G., Millet, L., Virlogeux, I., Saigne, M., Fonty, G. & Gouet, P. (1993). Ribosomal DNA-targeted hybridization probes for the detection, identification and quantitation of anaerobic rumen fungi. *Proceedings of the Nutrition Society* **52**, 176A.

Doré, J. & Stahl, D. A. (1991). Phylogeny of anaerobic rumen chytridiomycetes inferred from small subunit ribosomal RNA sequence comparisons. *Canadian Journal of Botany* **69**, 1964–1971.

Elliott, R., Ash, A. J., Calderon-Cortes, F., Norton, B. W. & Bauchop, T. (1987). The influence of anaerobic fungi on rumen volatile fatty acid concentrations *in vivo*. *Journal of Agricultural Science* **109**, 13–17.

Faichney, G. J., Brownlee, A. G., Gordon, G. L. R., Phillips, M. W. & Welch, R. J. (1991). Contribution of protozoa and anaerobic fungi to digesta N in sheep given a pelleted hay/grain diet. *Proceedings of the Nutrition Society of Australia* **16**, 209.

Faichney, G. J., Poncet, C., Lassalas, B., Jouany, J. P., Millet, L., Doré, J. & Brownlee, A. G. (1997). Effects of concentrates in a hay diet on the contribution of anaerobic fungi, protozoa and bacteria to nitrogen in rumen and duodenal digesta in sheep. *Animal Feed Science and Technology* **64**, 193–213.

Fanutti, C., Ponyi, T., Black, G. W., Hazlewood, G. P. & Gilbert, H. J. (1995). The conserved noncatalytic 40-residue sequence in cellulases and hemicellulases from anaerobic fungi functions as a protein docking domain. *Journal of Biological Chemistry* **270**, 29314–29322.

Fonty, G., Gouet, P., Jouany, J.-P. & Senaud, J. (1987). Establishment of the microflora and anaerobic fungi in the rumen of lambs. *Journal of General Microbiology* **133**, 1835–1843.

Fonty, G. & Grenet, E. (1994). Effects of diet on the fungal population of the digestive tract of ruminants. In *Anaerobic Fungi*, pp. 229–239 [D. O. Mountfort and C. G. Orpin, editors]. New York: Marcel Dekker.

Fonty, G. & Joblin, K. N. (1991). Rumen anaerobic fungi: their role and interaction with other rumen microorganisms in relation to fibre digestion. In *Physiological Aspects of Digestion and Metabolism in Ruminants*, pp. 655–680 [T. Tsuda, Y. Sasaki and R. Kawashima, editors]. San Diego: Academic Press.

Ford, C. W., Elliott, R. & Maynard, P. J. (1987). The effect of chlorite delignification on digestibility of some grass forages and on intake and rumen microbial activity in sheep fed barley straw. *Journal of Agricultural Science* **108**, 129–136.

Garcia-Campayo, V., McCrae, S. I. & Wood, T. M. (1994). Hydrolysis of oligosaccharides of the β-(1 $\rightarrow$ 4)-linked D-xylose series by an endo(1 $\rightarrow$ 4)-β-D-xylanase from the anaerobic rumen fungus *Neocallimastix frontalis*. *World Journal of Microbiology & Biotechnology* **10**, 64–68.

Gay, L. (1991). Chitin content and chitin synthase activity as indicators of the growth of three different anaerobic rumen fungi. *FEMS Microbiology Letters* **80**, 99–102.

Gerbi, C., Bata, J., Breton, A. & Prensier, G. (1996*a*). Glycoside and polysaccharide hydrolase activity of the rumen anaerobic fungus *Caecomyces communis* (*Sphaeromonas communis* sensu Orpin) at early and final stages of the developmental cycle. *Current Microbiology* **32**, 256–259.

Gerbi, C., Bata, J., Breton, A. & Prensier, G. (1996*b*). Polysaccharide hydrolase production by the rumen fungus *Caecomyces communis*. *Research in Microbiology* **147**, 363–370.

Gordon, G. L. R. (1985). The potential for manipulation of rumen fungi. In *Biotechnology and Recombinant DNA Technology in the Animal Production Industries. Reviews in Rural Science*, volume 6, pp. 124–128 [R. A. Leng, J. S. F. Barker, D. B. Adams and K. J. Hutchinson, editors]. Armidale, Australia: University of New England.

Gordon, G. L. R. (1987). Degradation of ^{14}C-labelled lignocelluloses by rumen anaerobic fungi. In *Herbivore Nutrition Research*, pp. 115–116 [M. Rose, editor]. Brisbane: Australian Society for Animal Production.

Gordon, G. L. R. (1990). Selection of anaerobic fungi for better fiber degradation in the rumen. In *Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Ruminants*, pp. 301–309 [D. E. Akin, L. G. Ljungdahl, J. R. Wilson and P. J. Harris, editors]. New York: Elsevier.

Gordon, G. L. R., Akin, D. E. & Hogan, J. P. (1984). Rumen anaerobic fungi in sheep fed methionine supplemented *Digitaria pentzii* hay. In *Ruminant Physiology—Concepts and Consequences*, p. 174 [S. K. Baker, J. M. Gawthorne, J. B. Mackintosh and D. B. Purser, editors]. Perth: University of Western Australia.

Gordon, G. L. R. & Ashes, J. R. (1984). *In vitro* digestion of wheat straw by different rumen anaerobic fungi. *Canadian Journal of Animal Science* **64** (Suppl.), 156–157.

Gordon, G. L. R., Gulati, S. K. & Ashes, J. R. (1983). Influence of low-sulphur straw on anaerobic fungal numbers in a sheep rumen. *Proceedings of the Nutrition Society of Australia* **8**, 188.

Gordon, G. L. R., McSweeney, C. S. & Phillips, M. W. (1995*b*). An important role for ruminal anaerobic fungi in the voluntary intake of poor quality forages by ruminants. In *Rumen Ecology Research Planning. Proceedings of a Workshop held at ILRI, Addis Ababa, Ethiopia*, pp. 91–101 [R. J. Wallace and A. Lahlou-Kassi, editors]. Nairobi, Kenya: International Livestock Research Institute.

Gordon, G. L. R. & Phillips, M. W. (1989*a*). Degradation and utilization of cellulose and straw by three different anaerobic fungi from the ovine rumen. *Applied and Environmental Microbiology* **55**, 1703–1710.

Gordon, G. L. R. & Phillips, M. W. (1989*b*). Comparative fermentation properties of anaerobic fungi from the rumen. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 127–137 [J. V. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul Books.

Gordon, G. L. R. & Phillips, M. W. (1989*c*). Anaerobic fungi are not always eliminated from the rumen by short-term treatment with monensin. *Conference on Rumen Function, Chicago* **20**, 11.

Gordon, G. L. R. & Phillips, M. W. (1991). Pectin degradation by some ruminal anaerobic fungi. In *Proceedings of the Third International Symposium on the Nutrition of Herbivorves*, p. 27. [M. W. Zahari, Z. A. Tajuddin, N. Abdullah and H. K. Wong, editors]. Serdang, Malaysia: Malaysian Society for Animal Production.

Gordon, G. L. R. & Phillips, M. W. (1992). Extracellular pectin lyase produced by *Neocallimastix* sp. LM1, a rumen anaerobic fungus. *Letters in Applied Microbiology* **15**, 113–115.

Gordon, G. L. R. & Phillips, M. W. (1993). Removal of anaerobic fungi from the rumen of sheep by chemical treatment and the effect on feed consumption and *in vivo* fibre digestion. *Letters in Applied Microbiology* **17**, 220–223.

Gordon, G. L. R., Phillips, M. W., Brownlee, A. G., Rintoul, A. J. & White, S. W. (1996). Dosing of anaerobic fungi from kangaroos into sheep. *Microbiology Australia* **17**, A36 (Abstr.).

Gordon, G. L. R., Wong, H. K. & Phillips, M. W. (1995*a*). *In vitro* degradation of [^{14}C]lignocellulose by polycentric and monocentric ruminal anaerobic fungi is inhibited differently by phenolic monomers. *Annales de Zootechnie* **44** (Suppl.), 152.

Grenet, E., Bernalier, A., Jamot, J. & Fonty, G. (1993). Degradation of untreated and anhydrous ammonia-treated wheat straw by two strains of rumen anaerobic fungi. *Annales de Zootechnie* **42**, 180.

Grenet, E., Breton, A., Barry, P. & Fonty, G. (1989*a*). Rumen anaerobic fungi and plant substrate colonization as affected by diet composition. *Animal Feed Science and Technology* **26**, 55–70.

Grenet, E., Fonty, G. & Barry, P. (1989*b*). SEM study of the degradation of maize and lucerne stems in the rumen of gnotobiotic lambs harbouring only fungi as cellulolytic micro-organisms. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 265–267 [J. V. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul Books.

Grenet, E., Fonty, G., Jamot, J. & Bonnemoy, F. (1989*c*). Influence of diet and monensin on development of anaerobic fungi in the rumen, duodenum, cecum, and feces of cows. *Applied and Environmental Microbiology* **55**, 2360–2364.

Gulati, S. K., Ashes, J. R., Connell, P. J. & Gordon, G. L. R. (1989*a*). Amino acid profiles of anaerobic rumen fungi. *Proceedings of the Nutrition Society of Australia* **14**, 134.

Gulati, S. K., Ashes, J. R. & Gordon, G. L. R. (1988). Digestibility of sulphur amino acids in rumen fungal species. *Proceedings of the Nutrition Society of Australia* **13**, 133.

Gulati, S. K., Ashes, J. R. & Gordon, G. L. R. (1990). Comparative digestibility of sulphur amino acids in rumen bacteria and fungal proteins by sheep. *Proceedings of the Nutrition Society of Australia* **15**, 128–131.

Gulati, S. K., Ashes, J. R., Gordon, G. L. R., Connell, P. J. & Rogers, P. L. (1989*b*). Nutritional availability of amino acids from the rumen anaerobic fungus *Neocallimastix* sp. LM1 in sheep. *Journal of Agricultural Science* **113**, 383–387.

Gulati, S. K., Ashes, J. R., Gordon, G. L. R. & Phillips, M. W. (1985). Possible contribution of rumen fungi to fibre digestion in sheep. *Proceedings of the Nutrition Society of Australia* **10**, 96.

Gutierrez, Ch. L., Contreras-Lara, D., Ramirez, C. J. T., Sanchez, F. & Gonzalez, C. H. (1996). Sulphur supplementation improves rumen activity. *Feed Mix* **4**, 18–19.

Harper, E. G., Welch, R. P., Contreras-Lara, D., Chang, J. S. & Calza, R. E. (1996). The effect of *Aspergillus oryzae* fermentation extract on the anaerobic fungi *Neocallimastix frontalis* EB 188, *Piromyces communis* DC 193 and *Orpinomyces* SP. RW 206: generalized effects and component analysis. *Applied Microbiology and Biotechnology* **45**, 817–821.

Heath, I. B., Bauchop, T. & Skipp, R. A. (1983). Assignment of the rumen anaerobe *Neocallimastix frontalis* to the Spizellomycetales (Chytridiomycetes) on the basis of its polyflagellate zoospore ultrastructure. *Canadian Journal of Botany* **61**, 295–307.

Hillaire, M. C. & Jouany, J.-P. (1989). Effects of rumen anaerobic fungi on the digestion of wheat straw and the end products of microbial metabolism: studies in a semi-continuous in vitro system. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 269–271 [J. V. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul Books.

Hillaire, M. C., Jouany, J.-P. & Fonty, G. (1990). Wheat straw degradation, in Rusitec, in the presence or absence of rumen anaerobic fungi. *Proceedings of the Nutrition Society* **49**, 127A.

Ho, Y. W., Abdullah, N. & Jalaludin, S. (1988). Penetrating structures of anaerobic rumen fungi in cattle and swamp buffalo. *Journal of General Microbiology* **134**, 177–182.

Ho, Y. W., Abdullah, N. & Jalaludin, S. (1991). Fungal colonisation of rice straw and palm press fibre in the rumen of cattle and buffalo. *Animal Feed Science and Technology* **34**, 311–321.

Ho, Y. W. & Barr, D. J. S. (1995). Classification of anaerobic gut fungi from herbivores with emphasis on rumen fungi from Malaysia. *Mycologia* **87**, 655–677.

Ho, Y. W. & Bauchop, T. (1991). Morphology of three polycentric rumen fungi and description of a procedure for the induction of zoosporogenesis and release of zoospores in cultures. *Journal of General Microbiology* **137**, 213–217.

Ho, Y. W., Khoo, I. Y. S., Tan, S. G., Abdullah, N., Jalaludin, S. & Kudo, H. (1994). Isozyme analysis of anaerobic rumen fungi and their relationship to aerobic chytrids. *Microbiology UK* **140**, 1495–1504.

Ho, Y. W., Wong, M. V. L., Abdullah, N., Kudo, H. & Jalaludin, S. (1996). Fermentation activities of some new species of anaerobic rumen fungi from Malaysia. *Journal of General and Applied Microbiology* **42**, 51–59.

Hodrová, B., Kopečný, J. & Petr, O. (1995). Interaction of the rumen fungus *Orpinomyces joyonii* with *Megasphaera elsdenii* and *Eubacterium limosum*. *Letters in Applied Microbiology* **21**, 34–37.

Hsu, J. T., Fahey, G. C., Merchen, N. R. & Mackie, R. I. (1991). Effects of defaunation and various nitrogen supplementation regimens on microbial numbers and activity in the rumen of sheep. *Journal of Animal Science* **69**, 1279–1289.

Hungate, R. E. (1966). *The Rumen and its Microbes*. New York: Academic Press.

Jenkins, T. C. (1993). Lipid metabolism in the rumen. *Journal of Dairy Science* **76**, 3851–3863.

Joblin, K. N. (1981). Isolation, enumeration, and maintenance of rumen anaerobic fungi in roll tubes. *Applied and Environmental Microbiology* **42**, 1119–1122.

Joblin, K. N. (1990). Bacterial and protozoal interactions with ruminal fungi. In *Microbial and Plant Opportunities to Improve Lignocellulose Utilization by Ruminants*, pp. 311–324 [D. E. Akin, L. G. Ljungdahl, J. R. Wilson and P. J. Harris, editors]. New York: Elsevier.

Joblin, K. N. & Naylor, G. E. (1993). Inhibition of the rumen anaerobic fungus *Neocallimastix frontalis* by fermentation products. *Letters in Applied Microbiology* **16**, 254–256.

Joblin, K. N. & Naylor, G. E. (1996). Inhibition of the ruminal bacterium *Ruminococcus flavefaciens* by ruminal fungi. *Annales de Zootechnie* **45** (Suppl.), 289.

Joblin, K. N., Naylor, G. E. & Williams, A. G. (1990). Effect of *Methanobrevibacter smithii* on xylanolytic activity of anaerobic ruminal fungi. *Applied and Environmental Microbiology* **56**, 2287–2295.

Joblin, K. N. & Williams, A. G. (1991). Effect of cocultivation of ruminal chytrid fungi with *Methanobrevibacter smithii* on lucerne stem degradation and extracellular fungal enzyme activities. *Letters in Applied Microbiology* **12**, 121–124.

Jouany, J.-P. (1996). Effect of rumen protozoa on nitrogen utilization by ruminants. *Journal of Nutrition* **126**, S1335–S1346.

Kemp, P., Jordan, D. J. & Orpin, C. G. (1985). The free- and protein-amino acids of the rumen phycomycete fungi *Neocallimastix frontalis* and *Piromonas communis*. *Journal of Agricultural Science* **105**, 523–526.

Kopečný, J. & Hodrová, B. (1995). Pectinolytic enzymes of anaerobic fungi. *Letters in Applied Microbiology* **20**, 312–316.

Kopečný, J., Hodrová, B. & Stewart, C. S. (1996). The effect of rumen chitinolytic bacteria on cellulolytic anaerobic fungi. *Letters in Applied Microbiology* **23**, 199–202.

Kostyukovsky, V., Inamoto, T., Ando, T., Nakai, Y. & Ogimoto, K. (1995). Degradation of hay by rumen fungi in artificial rumen (Rusitec). *Journal of General and Applied Microbiology* **41**, 83–86.

Kostyukovsky, V. A., Okunev, O. N. & Tarakanov, B. V. (1991). Description of two anaerobic fungal strains from the bovine rumen and influence of diet on the fungal population in vivo. *Journal of General Microbiology* **137**, 1759–1764.

Li, J. & Heath, I. B. (1992). The phylogenetic relationships of the anaerobic chytridiomycetous gut fungi (Neocallimasticaceae) and the Chytridiomycota. 1. Cladistic analysis of rRNA sequences. *Canadian Journal of Botany* **70**, 1738–1746.

Li, J. & Heath, I. B. (1993). Chytridiomycetous gut fungi, oft overlooked contributors to herbivore digestion. *Canadian Journal of Microbiology* **39**, 1003–1013.

Li, X.-L., Chen, H. & Ljungdahl, L. G. (1997). Monocentric and polycentric anaerobic fungi produce structurally related cellulases and xylanases. *Applied and Environmental Microbiology* **63**, 628–635.

Lowe, S. E., Griffith, G. G., Milne, A., Theodorou, M. K. & Trinci, A. P. J. (1987*a*). The life cycle and growth kinetics of an anaerobic rumen fungus. *Journal of General Microbiology* **133**, 1815–1827.

Lowe, S. E., Theodorou, M. K. & Trinci, A. P. J. (1987*b*). Isolation of anaerobic fungi from saliva and faeces of sheep. *Journal of General Microbiology* **133**, 1829–1834.

Lowe, S. E., Theodorou, M. K. & Trinci, A. P. J. (1987*c*). Growth and fermentation of an anaerobic rumen fungus on various carbon sources and effect of temperature on development. *Applied and Environmental Microbiology* **53**, 1210–1215.

Lowe, S. E., Theodorou, M. K. & Trinci, A. P. J. (1987*d*). Cellulases and xylanase of an anaerobic rumen fungus grown on wheat straw, wheat straw holocellulose, cellulose, and xylan. *Applied and Environmental Microbiology* **53**, 1216–1223.

Lowry, J. B., Sumpter, E. A., McSweeney, C. S., Schlink, A. C. & Bowden, B. (1993). Phenolic acids in the fibre of some tropical grasses, effect on feed quality, and their metabolism by sheep. *Australian Journal of Agricultural Research* **44**, 1123–1133.

McAllister, T. A., Bae, H. D., Yanke, L. J., Cheng, K.-J. & Muir, A. (1994). Effect of condensed tannins from birdsfoot trefoil on endoglucanase activity and the digestion of cellulose filter paper by ruminal fungi. *Canadian Journal of Microbiology* **40**, 298–305.

McAllister, T. A., Dong, Y., Yanke, L. J., Bae, H. D., Cheng, K.-J. & Costerton, J. W. (1993). Cereal grain digestion by selected strains of ruminal fungi. *Canadian Journal of Microbiology* **39**, 367–376.

Mackie, R. I. (1996). The use of oligonucleotide probes to study the ecology of ruminal microbial populations. *Annales de Zootechnie* **45** (Suppl.), 281–285.

McSweeney, C. S., Dulieu, A., Katayama, Y. & Lowry, J. B. (1994). Solubilization of lignin by the ruminal anaerobic fungus *Neocallimastix patriciarum*. *Applied and Environmental Microbiology* **60**, 2985–2989.

Marounek, M. & Hodrová, B. (1989). Susceptibility and resistance of anaerobic rumen fungi to antimicrobial feed additives. *Letters in Applied Microbiology* **9**, 173–175.

Martin, S. A. & Nisbet, D. J. (1992). Effect of direct-fed microbials on rumen microbial fermentation. *Journal of Dairy Science* **75**, 1736–1744.

Marvin-Sikkema, F. D., Gomes, T. M. P., Grivet, J. P., Gottschal, J. C. & Prins, R. A. (1993*a*). Characterization of hydrogenosomes and their role in glucose metabolism of *Neocallimastix* sp. L2. *Archives of Microbiology* **160**, 388–396.

Marvin-Sikkema, F. D., Rees, E., Kraak, M. N., Gottschal, J. C. & Prins, R. A. (1993*b*). Influence of metronidazole, CO, CO_2, and methanogens on the fermentative metabolism of the anaerobic fungus *Neocallimastix* sp. strain L2. *Applied and Environmental Microbiology* **59**, 2678–2683.

Marvin-Sikkema, F. D., Richardson, A. J., Stewart, C. S., Gottschal, J. C. & Prins, R. A. (1990). Influence of hydrogen-consuming bacteria on cellulose degradation by anaerobic fungi. *Applied and Environmental Microbiology* **56**, 3793–3797.

Mathieu, F., Jouany, J.-P., Senaud, J., Bohatier, J., Bertin, G. & Mercier, M. (1996). The effect of *Saccharomyces cerevisiae* and *Aspergillus oryzae* on fermentations in the rumen of faunated and defaunated sheep: protozoal and probiotic interactions. *Reproduction Nutrition Development* **36**, 271–287.

Michel, V., Fonty, G., Millet, L., Bonnemoy, F. & Gouet, P. (1993). *In vitro* study of the proteolytic activity of rumen anaerobic fungi. *FEMS Microbiology Letters* **110**, 5–9.

Millard, P., Gordon, A. H., Richardson, A. J. & Chesson, A. (1987). Reduced ruminal degradation of ryegrass caused by sulphur limitation. *Journal of the Science of Food and Agriculture* **40**, 305–314.

Millet, L., Fonty, G. & Gouet, P. (1996). Use of 18S-rRNA-targeted oligonucleotide probes for detection and quantification of anaerobic fungi in the rumen of different animals. *Annales de Zootechnie* **45** (Suppl.), 286.

Morgavi, D. P., Onodera, R. & Nagasawa, T. (1993). *In vitro* metabolism of chitin and protein from ruminal fungi by ruminal protozoa. *Animal Science and Technology* **64**, 584–592.

Morgavi, D. P., Sakurada, M., Mizokami, M., Tomita, Y. & Onodera, R. (1994*b*). Effects of ruminal protozoa on cellulose degradation and the growth of an anaerobic ruminal fungus, *Piromyces* sp. strain OTS1, in vitro. *Applied and Environmental Microbiology* **60**, 3718–3723.

Morgavi, D. P., Sakurada, M., Tomita, Y. & Onodera, R. (1994*a*). Presence in rumen bacterial and protozoal populations of enzymes capable of degrading fungal cell walls. *Microbiology UK* **140**, 631–636.

Morrison, M., Murray, R. M. & Boniface, A. N. (1990). Nutrient metabolism and rumen micro-organisms in sheep fed a poor-quality tropical grass hay supplemented with sulphate. *Journal of Agricultural Science* **115**, 269–275.

Morvan, B., Rieu-Lesme, F., Fonty, G. & Gouet, P. (1996). *In vitro* interactions between rumen H_2-producing cellulolytic microorganisms and H_2-utilizing acetogenic and sulfate-reducing bacteria. *Anaerobe* **2**, 175–180.

Mountfort, D. O. (1994). Regulatory constraints in the degradation and fermentation of carbohydrate by anaerobic fungi. In *Anaerobic Fungi*, pp. 147–168 [D. O. Mountfort and C. G. Orpin, editors]. New York: Marcel Dekker.

Mountfort, D. O. & Orpin, C. G. (Eds) (1994). *Anaerobic Fungi: Biology, Ecology and Function*. New York: Marcel Dekker.

Munn, E. A. (1994). The ultrastructure of anaerobic fungi. In *Anaerobic Fungi*, pp. 47–105 [D. O. Mountfort and C. G. Orpin, editors]. New York: Marcel Dekker.

Newbold, C. J. (1996). Probiotics for ruminants. *Annales de Zootechnie* **45** (Suppl.), 329–335.

Newbold, C. J., Brock, R. & Wallace, R. J. (1992). The effect of *Aspergillus oryzae* fermentation extract on the growth of fungi and ciliate protozoa in the rumen. *Letters in Applied Microbiology* **15**, 109–112.

Newbold, C. J. & Hillman, K. (1990). The effect of ciliate protozoa on the turnover of bacterial and fungal protein in the rumen of sheep. *Letters in Applied Microbiology* **11**, 100–102.

Newbold, C. J., Wallace, R. J., Watt, N. D. & Richardson, A. J. (1988). Effect of the novel ionophore tetronasin (ICI 139603) on ruminal microorganisms. *Applied and Environmental Microbiology* **54**, 544–547.

O'Fallon, J. V., Wright, R. W. & Calza, R. E. (1991). Glucose metabolic pathways in the anaerobic rumen fungus *Neocallimastix frontalis* EB188. *Biochemical Journal* **274**, 595–599.

Obispo, N. E. & Dehority, B. A. (1992). A most probable number method for enumeration of rumen fungi with studies on factors affecting their concentration in the rumen. *Journal of Microbiological Methods* **16**, 259–270.

Odenyo, A. A., Osuji, P. O., Karanfil, O. & Adinew, K. (1997). Microbiological evaluation of *Acacia angustissima* as a protein supplement for sheep. *Animal Feed Science and Technology* **65**, 99–112.

Ogimoto, K. & Imai S. (1981). *Atlas of Rumen Microbiology*. Tokyo: Japan Scientific Societies Press.

Onoda, A., Kobayashi, U., Wakita, M. & Hoshino, S. (1993). Isolation of anaerobic phycomycete fungi from some herbivores and their chemical composition. *Animal Science and Technology* **64**, 115–120.

Orpin, C. G. (1975). Studies on the rumen flagellate *Neocallimastix frontalis*. *Journal of General Microbiology* **91**, 249–262.

Orpin, C. G. (1976). Studies on the rumen flagellate *Sphaeromonas communis*. *Journal of General Microbiology* **94**, 270–280.

Orpin, C. G. (1977*a*). Invasion of plant tissue in the rumen by the flagellate *Neocallimastix frontalis*. *Journal of General Microbiology* **98**, 423–430.

Orpin, C. G. (1977*b*). The rumen flagellate *Piromonas communis*: its life-history and invasion of plant material in the rumen. *Journal of General Microbiology* **99**, 107–117.

Orpin, C. G. (1977*c*). The occurrence of chitin in the cell walls of the rumen organisms *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis*. *Journal of General Microbiology* **99**, 215–218.

Orpin, C. G. (1977*d*). On the induction of zoosporogenesis in the rumen phycomycetes *Neocallimastix frontalis*, *Piromonas communis* and *Sphaeromonas communis*. *Journal of General Microbiology* **101**, 181–189.

Orpin, C. G. (1977*e*). Studies on the defaunation of the ovine rumen using dioctyl sodium sulphosuccinate. *Journal of Applied Bacteriology* **43**, 309–318.

Orpin, C. G. (1988). Nutrition and biochemistry of anaerobic chytridiomycetes. *BioSystems* **21**, 365–370.

Orpin, C. G. (1989). Ecology of rumen anaerobic fungi in relation to the nutrition of the host animal. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 29–37 [J. V. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul books.

Orpin, C. G. (1994). Anaerobic fungi - taxonomy, biology, and distribution in nature. In *Anaerobic Fungi*, pp. 1–45 [D. O. Mountfort and C. G. Orpin, editors]. New York: Marcel Dekker.

Orpin, C. G. & Bountiff, L. (1978). Zoospore chemotaxis in the rumen phycomycete *Neocallimastix frontalis*. *Journal of General Microbiology* **104**, 113–122.

Orpin, C. G. & Greenwood, Y. (1986). Nutritional and germination requirements of the rumen chytridiomycete *Neocallimastix patriciarum*. *Transactions of the British Mycological Society* **86**, 103–109.

Orpin, C. G., Greenwood, Y., Hall, F. J. & Paterson, I. W. (1985). The rumen microbiology of seaweed digestion in Orkney sheep. *Journal of Applied Bacteriology* **58**, 585–596.

Orpin, C. G. & Ho, Y. W. (1991). Ecology and function of the anaerobic rumen fungi. In *Recent Advances in the Nutrition of Herbivores*, pp. 163–170 [Y. W. Ho, H. K. Wong, N. Abdullah and Z. A. Tajuddin, editors]. Serdang, Malaysia: Malaysian Society of Animal Production.

Orpin, C. G. & Letcher, A. J. (1984). Effect of absence of ciliate protozoa on rumen fluid volume, flow rate and bacterial populations in sheep. *Animal Feed Science and Technology* **10**, 145–153.

Phillips, M. W. (1989). Unusual rumen fungi isolated from northern Australian cattle and water buffalo. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 247–249 [J. V. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul Books.

Phillips, M. W. & Gordon, G. L. R. (1988). Sugar and polysaccharide fermentation by rumen anaerobic fungi from Australia, Britain and New Zealand. *BioSystems* **21**, 377–383.

Phillips, M. W. & Gordon, G. L. R. (1989). Growth characteristics on cellobiose of three different anaerobic fungi isolated from the ovine rumen. *Applied and Environmental Microbiology* **55**, 1695–1702.

Phillips, M. W. & Gordon, G. L. R. (1991). Growth responses to reduced sulphur compounds of a ruminal fungus, *Neocallimastix* sp. LM1. In *Proceedings of the Third International Symposium on the Nutrition of Herbivores*, p. 26 [M. W. Zahari, Z. A. Tajuddin, N. Abdullah and H. K. Wong, editors]. Serdang, Malaysia: Malaysian Socity for Animal Production.

Phillips, M. W. & Gordon, G. L. R. (1992). Fungistatic and fungicidal effects of the ionophores monensin and tetronasin on the rumen fungus *Neocallimastix* sp. LM1. *Letters in Applied Microbiology* **15**, 116–119.

Phillips, M. W. & Gordon, G. L. R. (1995*a*). Carbohydrate fermentation by three species of polycentric ruminal fungi from cattle and water buffalo in tropical Australia. *Anaerobe* **1**, 41–47.

Phillips, M. W. & Gordon, G. L. R. (1995*b*). Colonisation of the sheep rumen with polycentric anaerobic fungi isolated from cattle. *Annales de Zootechnie* **44** (Suppl.), 141.

Rees, E. M. R., Lloyd, D. & Williams, A. G. (1995). The effects of co-cultivation with the acetogen *Acetitomaculum ruminis* on the fermentative metabolism of the rumen fungi *Neocallimastix patriciarum* and *Neocallimastix* sp. strain L2. *FEMS Microbiology Letters* **133**, 175–180.

Roger, V., Bernalier, A., Grenet, E., Fonty, G., Jamot, J. & Gouet, P. (1993). Degradation of wheat straw and maize stem by a monocentric and a polycentric rumen fungi, alone or in association with rumen cellulolytic bacteria. *Animal Feed Science and Technology* **42**, 69–82.

Romulo, B., Bird, S. H. & Leng, R. A. (1986). The effects of defaunation on digestibility and rumen fungi counts in sheep fed high-fibre diets. *Proceedings of the Australian Society for Animal Production* **16**, 327–330.

Romulo, B., Bird, S. W. & Leng, R. A. (1989). Effects of defaunation and protein supplementation on intake, digestibility, N-retention and fungal numbers in sheep fed straw-based diets. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 285–288 [J. V. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul Books.

Russell, J. B. & Strobel, H. J. (1989). Effect of ionophores on ruminal fermentation. *Applied and Environmental Microbiology* **55**, 1–6.

Scalbert, A. (1991). Antimicrobial properties of tannins. *Phytochemistry* **30**, 3875–3883.

Schlink, A. C., Gordon, G. L. R., Phillips, M. W. & Sutherland, S. (1989). Effects of rumen modifiers and formaldehyde-treated casein on rumen microbial populations of cattle fed sorghum hay. In *The Roles of Protozoa and Fungi in Ruminant Digestion*, pp. 355–357 [J. V. Nolan, R. A. Leng and D. I. Demeyer, editors]. Armidale, Australia: Penambul Books.

Sekine, J., Shinoda, M., Kamel, H. E. M. & Oura, R. (1995). Effect of kinds of hay on population densities of rumen anaerobic fungi. *Indian Journal of Animal Sciences* **65**, 1352–1355.

Sijtsma, L. & Tan, B. (1996). Degradation of perennial ryegrass leaf and stem cell walls by the anaerobic fungus *Neocallimastix* sp. strain CS3b. *Applied and Environmental Microbiology* **62**, 1437–1440.

Soetanto, H., Gordon, G. L. R., Hume, I. D. & Leng, R. A. (1985). The role of protozoa and fungi in fibre digestion in the rumen of sheep. In *Efficient Animal Production for Asian Welfare (Proceedings of the Third Congress of the Asian-Australian Association of Animal Production Societies, Seoul, Korea)*, volume 2, pp. 805–807.

Stewart, C. S., Duncan, S. H., Richardson, A. J., Calder, A. G. & Dewey, P. J. S. (1995). The effect of the presence of glucose on the fermentation of mannose by the anaerobic fungus *Neocallimastix frontalis* strain RE1. *FEMS Microbiology Letters* **127**, 57–63.

Stewart, C. S., McPherson, C. A. & Cansunar, E. (1987). The effect of lasalocid on glucose uptake, hydrogen production and the solubilization of straw by the anaerobic rumen fungus *Neocallimastix frontalis*. *Letters in Applied Microbiology* **5**, 5–7.

Stewart, C. S. & Richardson, A. J. (1989). Enhanced resistance of anaerobic rumen fungi to the ionophores monensin and lasalocid in the presence of methanogenic bacteria. *Journal of Applied Bacteriology* **66**, 85–93.

Tanaka, H., Matsui, H., Ushida, K. & Kojima, Y. (1991). Effect of *p*-coumaric acid on growth and cellulolytic activity of rumen fungi with and without methanogens. In *Proceedings of the Third International Symposium on the Nutrition of Herbivores*, p. 35 [M. W. Zahari, Z. A. Tajuddin and H. K. Wong, editors]. Serdang, Selangor, Malaysia: Malaysian Society of Animal Production.

Teunissen, M. J., De Kort, G. V. M., Op Den Camp, H. J. M. & Vogels, G. D. (1993*a*). Production of cellulolytic and xylanolytic enzymes during growth of anaerobic fungi from ruminant and nonruminant herbivores on different substrates. *Applied Biochemistry and Biotechnology* **39**, 177–189.

Teunissen, M. J., Hermans, J. M. H., Huis in 't Veld, J. H. J. & Vogels, G. D. (1993*b*). Purification and characterization of a complex-bound and a free β-1,4-endoxylanase from the culture fluid of the anaerobic fungus *Piromyces* sp. strain E2. *Archives of Microbiology* **159**, 265–271.

Teunissen, M. J., Kets, E. P. W., Op den Camp, H. J. M., Huis in 't Veld, J. H. J. & Vogels, G. D. (1992). Effect of coculture of anaerobic fungi isolated from ruminants and non-ruminants with methanogenic bacteria on cellulolytic and xylanolytic enzyme activities. *Archives of Microbiology* **157**, 176–182.

Teunissen, M. J. & Op den Camp, H. J. M. (1993). Anaerobic fungi and their cellulolytic and xylanolytic enzymes. *Antonie Van Leeuwenhoek* **63**, 63–76.

Theodorou, M. K., Beever, D. E., Haines, M. J. & Brooks, A. (1990*a*). The effect of a fungal probiotic on intake and performance of early weaned calves. *Animal Production* **50**, 577 (Abstr.).

Theodorou, M. K., Gill, M., King-Spooner, C. & Beever, D. E. (1990b). Enumeration of anaerobic chytridiomycetes as thallus-forming units: novel method for quantification of fibrolytic fungal populations from the digestive tract ecosystem. *Applied and Environmental Microbiology* **56**, 1073–1078.

Theodorou, M. K., Longland, A. C., Dhanoa, M. S., Lowe, S. E. & Trinci, A. P. J. (1989). Growth of *Neocallimastix* sp. strain R1 on Italian ryegrass hay: removal of neutral sugars from plant cell walls. *Applied and Environmental Microbiology* **55**, 1363–1367.

Trinci, A. P. J., Davies, D. R., Gull, K., Lawrence, M. I., Nielsen, B. B., Rickers, A. & Theodorou, M. K. (1994). Anaerobic fungi in herbivorous animals. *Mycological Research* **98**, 129–152.

Ushida, K., Kayouli, C., de Smet, S. & Jouany, J.-P. (1990). Effect of defaunation on protein and fibre digestion in sheep fed on ammonia-treated straw-based diets with or without maize. *British Journal of Nutrition* **64**, 765–775.

Ushida, K., Okutani, A. & Kojima, Y. (1993). Effect of sodium propionate on the population size and generic distribution of phycomycete fungi in sheep rumen. *Animal Science and Technology* **64**, 250–253.

Ushida, K., Tanaka, H. & Kojima, Y. (1989). A simple *in situ* method for estimating fungal population size in the rumen. *Letters in Applied Microbiology* **9**, 109–111.

Ushida, K., Umeda, M., Kishigami, N. & Kojima, Y. (1992). Effect of medium-chain and long-chain fatty acid calcium salts on rumen microorganisms and fiber digestion in sheep. *Animal Science and Technology* **63**, 591–597.

van der Giezen, M., Rechinger, K. B., Svendsen, I., Durand, R., Hirt, R. P., Fèvre, M., Embley, T. M. & Prins, R. A. (1997). A mitochondrial-like targeting signal on the hydrogenosomal malic enzyme from the anaerobic fungus *Neocallimastix frontalis*: support for the hypothesis that hydrogenosomes are modified mitochondria. *Molecular Microbiology* **23**, 11–21.

Vogels, G. D., Hoppe, W. F. & Stumm, C. K. (1980). Association of methanogenic bacteria with rumen ciliates. *Applied and Environmental Microbiology* **40**, 608–612.

Wallace, R. J. & Joblin, K. N. (1985). Proteolytic activity of a rumen anaerobic fungus. *FEMS Microbiology Letters* **29**, 19–26.

Wallace, R. J. & Munro, C. A. (1986). Influence of the rumen anaerobic fungus *Neocallimastix frontalis* on the proteolytic activity of a defined mixture of rumen bacteria growing on a solid substrate. *Letters in Applied Microbiology* **3**, 23–26.

Welch, R. P., Tsai, K.-P., Harper, E. G., Chang, J. S. & Calza, R. E. (1996). The effect of *Aspergillus oryzae* fermentation extract on the anaerobic fungus *Neocallimastix frontalis* EB 188: effects on physiology. *Applied Microbiology and Biotechnology* **45**, 811–816.

Weston, R. H., Lindsay, J. R., Purser, D. B., Gordon, G. L. R. & Davis, P. (1988). Feed intake and digestion responses in sheep to the addition of inorganic sulfur to a herbage diet of low sulfur content. *Australian Journal of Agricultural Research* **39**, 1107–1119.

Widyastuti, Y., Newbold, C. J., Stewart, C. S. & Ørskov, E. R. (1995). Interactions between rumen anaerobic fungi and ciliate protozoa in the degradation of rice straw cell walls. *Letters in Applied Microbiology* **20**, 61–64.

Williams, A. G., Joblin, K. N. & Fonty, G. (1994*a*). Interactions between the rumen chytrid fungi and other microorganisms. In *Anaerobic Fungi*, pp. 191–227 [D. O. Mountfort and C. G. Orpin, editors]. New York: Marcel Dekker.

Williams, A. G. & Orpin, C. G. (1987). Polysaccharide-degrading enzymes formed by three species of anaerobic rumen fungi grown on a range of carbohydrate substrates. *Canadian Journal of Microbiology* **33**, 418–426.

Williams, A. G. & Withers, S. E. (1991). Effect of ciliate protozoa on the activity of polysaccharide-degrading enzymes and fibre breakdown in the rumen ecosystem. *Journal of Applied Bacteriology* **70**, 144–155.

Williams, A. G., Withers, S. E. & Joblin, K. N. (1991). Xylanolysis by cocultures of the rumen fungus *Neocallimastix frontalis* and ruminal bacteria. *Letters in Applied Microbiology* **12**, 232–235.

Williams, A. G., Withers, S. E., Naylor, G. E. & Joblin, K. N. (1994*b*). Effect of heterotrophic ruminal bacteria on xylan metabolism by the anaerobic fungus *Piromyces communis*. *Letters in Applied Microbiology* **19**, 105–109.

Wilson, C. A. & Wood, T. M. (1992). Studies on the cellulase of the rumen anaerobic fungus *Neocallimastix frontalis*, with special reference to the capacity of the enzyme to degrade crystalline cellulose. *Enzyme and Microbial Technology* **14**, 258–264.

Wilson, J. R. (1994). Cell wall characteristics in relation to forage digestion by ruminants. *Journal of Agricultural Science* **122**, 173–182.

Wilson, J. R. & Kennedy, P. M. (1996). Plant and animal constraints to voluntary feed intake associated with fibre characteristics and particle breakdown and passage in ruminants. *Australian Journal of Agricultural Research* **47**, 199–225.

Wong, M. V. L., Ho, Y. W., Tan, S. G., Abdullah, N. & Jalaludin, S. (1995). Isozyme and morphological characteristics of the anaerobic fungus *Piromyces mae* isolated from the duodenum, rumen and faeces of sheep. *FEMS Microbiology Letters* **134**, 9–14.

Wood, T. M. & Wilson, C. A. (1995). Studies on the capacity of the cellulase of the anaerobic rumen fungus *Piromonas communis* P to degrade hydrogen bond-ordered cellulose. *Applied Microbiology and Biotechnology* **43**, 572–578.

Wood, T. M., Wilson, C. A. & McCrae, S. I. (1995). The cellulase system of the anaerobic rumen fungus *Neocallimastix frontalis*. Studies on the properties of fractions rich in endo (1 → 4)-β-D-glucanase activity. *Applied Microbiology and Biotechnology* **44**, 177–184.

Wubah, D. A., Akin, D. E. & Borneman, W. S. (1993). Biology, fiber-degradation, and enzymology of anaerobic zoosporic fungi. *Critical Reviews in Microbiology* **19**, 99–115.

Wubah, D. A., Fuller, M. S. & Akin, D. E. (1991*a*). *Neocallimastix*: a comparative morphological study. *Canadian Journal of Botany* **69**, 835–843.

Wubah, D. A., Fuller, M. S. & Akin, D. E. (1991*b*). Resistant body formation in *Neocallimastix* sp., an anaerobic fungus from the rumen of a cow. *Mycologia* **83**, 40–47.

Wubah, D. A. & Kim, D. S. H. (1996). Chemoattraction of anaerobic ruminal fungi zoospores to selected phenolic acids. *Microbiological Research* **151**, 257–262.

Yanke, L. J., Dong, Y., McAllister, T. A., Bae, H. D. & Cheng, K.-J. (1993). Comparison of amylolytic and proteolytic activities of ruminal fungi grown on cereal grains. *Canadian Journal of Microbiology* **39**, 817–820.

Yarlett, N., Orpin, C. G., Munn, E. A., Yarlett, N. C. & Greenwood, C. A. (1986). Hydrogenosomes in the rumen fungus *Neocallimastix patriciarum*. *Biochemical Journal* **236**, 729–739.

Zhou, L., Xue, G.-P., Orpin, C. G., Black, G. W., Gilbert, H. J. & Hazlewood, G. P. (1994). Intronless *celB* from the anaerobi fungus *Neocallimastix patriciarum* encodes a modular family A endoglucanase. *Biochemical Journal* **297**, 359–364.

Zhu, W. Y., Theodorou, M. K., Longland, A. C., Nielsen, B. B., Dijkstra, J. & Trinci, A. P. J. (1996). Growth and survival of anaerobic fungi in batch and continuous-flow cultures. *Anaerobe* **2**, 29–37.