The role of anaerobic gut fungi in ruminants

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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 synergetic, 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-
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, 1977a), 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. 1987a) 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. 1987a; Wubah et al. 1991a) though zoosporogenesis has been reported to occur as little as 8 h after germination (Orpin 1977d). 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. 1987b). Davies et al. (1993) demonstrated that
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. (1991b) 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 1977c) 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 H₂ 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, 1989b) 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 1-arabinose was not used (Phillips & Gordon, 1988, Gordon & Phillips, 1989b; Stewart et al. 1995; Dijkerman et al. 1997a). 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. 1997a).

When glucose was used as the substrate the major fermentation end products were acetate, ethanol, formate, D(−)-lactate, succinate, CO₂ and H₂ (Lowe et al. 1987c) though the relative amounts of each differed between genera (Phillips & Gordon, 1988; 1995a). Generally, polycentric anaerobic fungi produced much less lactate than monocentric fungi (Borneman et al. 1989; Phillips & Gordon, 1995a), 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...
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, 1995a). Xylan, a major component of hemicellulose, is degraded by all fungi although some isolates grow on this substrate relatively poorly (Phillips & Gordon, 1988, 1995a). 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, 1995a). A variety of other polysaccharides have been shown to be fermented (Phillips & Gordon, 1988, 1995a) 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, 1989a). 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. 1987d; Breton et al. 1995; Gerbi et al. 1996b). 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. 1996a). 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. 1996a) 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. 1997a) or other cellulose-containing fibre (Gordon & Phillips, 1989a; Teunissen et al. 1993a).

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. 1997b) 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. 1996b). The principal cellulose degrading components are a mixture of several endo-glucanases and celllobiohydrolases 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. 1993b; 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. 1989a, 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, 1989a; McSweeney et al. 1994; Bernard-Vailhé et al. 1995) though there was no evidence of the fermentation of lignin (Gordon & Phillips, 1989b; 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.
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. 1989c; 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, 1995a).

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. 1987b; Wubah et al. 1991b; 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. 1991b). Saliva has been found to contain viable fungi (Lowe et al. 1987b) 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 1977; 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, 1994a) who demonstrated the ability of protozoa to turn over both chitin and protein from dead fungal biomass, and by Williams *et al.* (1994a) who reported that a mixed protozoal population from the rumen significantly increased the release of radiolabel from 14C-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.* 1994b; Widyastuti *et al.* 1995), even though the latter demonstrated that dry matter loss from straw was not affected and Morgavi *et al.* (1994b) 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.* 1994b).

#### 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
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The role of anaerobic gut fungi in ruminants. 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. 1993b) 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. (1993b) 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, CO2 and H2 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 H2, in the growth medium. Hydrogenase is inhibited by the presence of low levels of H2 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. 1993b). This results in a higher production of ethanol and lactate which act as electron acceptors. If H2 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 H2, acetate and CO2 and concomitant decrease in ethanol and lactate. Numerous workers (Stewart & Richardson, 1989; Joblin et al. 1990; Marvin-Sikkema et al. 1993b) have demonstrated that co-culture of methanogenic archaea with anaerobic fungi results in interspecies hydrogen transfer, with the methanogen providing the low H2 environment that encourages the flow of carbon through hydrogenosomes and results in a shift in the end product profile towards the production of acetate, H2 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. 1993b).

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 cocultures 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. 1994a). 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 H₂ utilizing bacteria have yielded similar results to experiments with methanogens. Thus, the acetogenic species such as Eubacterium limosum (Bernalier et al. 1993a; 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 H₂ (converted either to acetate by acetogens or H₂S 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. 1992). 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.
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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).

**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, 1987a). 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. 1989a, 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, 1995a). 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. 1990b; 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, 1977c; 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, 1987b) 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...
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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, 1977a; 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 oat (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. 1989a). 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, 1977a; Gordon, 1985; Grenet et al. 1989a). 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, 1995a; 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 ≥ 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 \( \sim 1.0 \) 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>
<thead>
<tr>
<th>Feed Sulphur added to diet:</th>
<th>Increased fungi after Sulphur added</th>
<th>Intake after Sulphur addition relative to base diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low Sulphur feed</td>
<td>Sulphur (g/kg)</td>
<td>VOMI</td>
</tr>
<tr>
<td><em>Digitaria pentzii</em></td>
<td>0.8–1.1</td>
<td>F known</td>
</tr>
<tr>
<td></td>
<td></td>
<td>F presumed</td>
</tr>
<tr>
<td></td>
<td></td>
<td>met known</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S(^0) unknown</td>
</tr>
<tr>
<td>Wheat straw (alkali-treated)</td>
<td>0.7</td>
<td>met known</td>
</tr>
<tr>
<td><em>Heteropogon contortus</em></td>
<td>0.5</td>
<td>SO(_4) known</td>
</tr>
<tr>
<td>Maize stover</td>
<td>0.2</td>
<td>SO(_4) known</td>
</tr>
</tbody>
</table>

\(^a\)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

<table>
<thead>
<tr>
<th></th>
<th>Before fertilizer (g S/kg OM)</th>
<th>After fertilizer (g S/kg OM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Sulphur</td>
<td>0.76</td>
<td>1.29</td>
</tr>
<tr>
<td>Protein Sulphur &amp; insoluble Sulphur(^a)</td>
<td>0.56</td>
<td>0.57</td>
</tr>
<tr>
<td>Soluble non-protein-Sulphur</td>
<td>0.20</td>
<td>0.72</td>
</tr>
</tbody>
</table>

\(^a\) 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.* 1993b), 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, 1977b) 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, 1977a; Soetanto *et al.* 1985; Bird, 1989) and bacteria (Orpin, 1977a; 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 \textit{in vitro} and postulated to occur in the normally faunated rumen (Orpin 1975; Joblin, 1990; Morgavi \textit{et al.} 1994b). 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 \textit{et al.} (1996).

\textbf{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 \textit{in vivo} (Stewart \textit{et al.} 1987; Bernalier \textit{et al.} 1989; Marounek & Hodrová, 1989; Phillips & Gordon, 1992; Cann \textit{et al.} 1993a).

\textbf{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 \textit{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 \textit{et al.} 1989). Other studies failed to confirm the inhibitory effect of monensin against anaerobic fungi in the rumen (Gordon & Phillips, 1989c; Grenet \textit{et al.} 1989c; Schlink \textit{et al.} 1989). It has been found that the lowest concentration of monensin required to kill a typical anaerobic fungus, \textit{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, 1989c). 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.

\textbf{Tetronasin}

Another ionophore, tetronasin, was found by Newbold \textit{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).
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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. 1993b). 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 ~15,000 to <100/cm²) and remained low for the period of ionophore administration (23 d; Cann et al. 1993a).

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. 1993a, b). These chitin synthesis inhibitors were active against mixed cultures of anaerobic fungi in vitro (Cann et al. 1993a), 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. 1993a; 17.6 g by Cann et al. 1993b).

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, 1989b) even though the consistent loss of acid-detergent lignin from wheat straw could not be demonstrated (Gordon & Ashes 1984; Gordon & Phillips, 1989a). Nevertheless, ~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, 1989b; 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 chemotaxis attraction 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. 1995a) 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. 1995b). 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
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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, 1987a; 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 quantification 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. 1990a) 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)

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>No fungi</th>
<th>With fungi added</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intake (g/d)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>894 ± 30·8</td>
<td>628 ± 36·4</td>
</tr>
<tr>
<td>ADF</td>
<td>390 ± 9·3</td>
<td>264 ± 16·4</td>
</tr>
<tr>
<td>Digestibility (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>53·2 ± 1·30</td>
<td>50·3 ± 1·38</td>
</tr>
<tr>
<td>ADF</td>
<td>51·2 ± 1·03</td>
<td>46·5 ± 1·28</td>
</tr>
<tr>
<td>Anaerobic fungi (zoospores/ml; × 10^3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>7·6 ± 4·0</td>
<td>UD (&lt; 0·001)</td>
</tr>
<tr>
<td>Bacteria (cells/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>total viable (× 10^9)</td>
<td>0·8 ± 0·18</td>
<td>1·4 ± 0·37</td>
</tr>
<tr>
<td>cellulolytic (× 10^9)</td>
<td>0·4 ± 0·19</td>
<td>2·6 ± 1·48</td>
</tr>
<tr>
<td>Ciliate protozoa (cells/ml; × 10^5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3·8 ± 0·92</td>
<td>3·0 ± 0·53</td>
</tr>
</tbody>
</table>

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. 1987c; 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. 1993b). 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
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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 juyonii* 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. (1989a) 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, 1989b). 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, 1989b). 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
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$^{14}$C-labelled plant fibre substrates as a measure of the relative cellulytic ability of different anaerobic fungi. Subsequently it was shown that the cellulytic 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. 1990a). 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, 1995b). 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 $\sim$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. 1990a). 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 microorganisms 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.

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References


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