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ANAEROBIC RUMEN FUNGI: POTENTIAL AND APPLICATIONS

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Anaerobic fungi are the significant constituent of rumen microbiota in livestock that rely on poor-quality fibrous diets. Such fungi colonize plant fragments in the rumen of cattle and other herbivores. Through rhizoidal growth, they penetrate the plant cell wall and increase the area susceptible to enzymatic attack. The fibrolytic enzymes produced perform in concert to efficiently degrade cellulose and hemicellulose to simple sugars with the end-products acetate, lactate, ethanol, formate, CO_2 and H_2. In contrast to the other rumen microbes, fungal enzymes also break ester linkages between lignin and hemicelluloses. Therefore, these fungi are found attached mainly with the lignified tissues that remain in the rumen for extended periods and maintain highest number in animals receiving high-fibre diets but lack in rumen of animals receiving leafy forage, due to the shorter retention period of such feedstuffs. These qualities of anaerobic fungi together with the degree of colonization and growth on fibrous plant fragments collectively suggest that manipulation of such a group of microbes has immense potential for boosting digestive performance in the rumen and ultimately higher animal production response. The present chapter deals with history, nomenclature, life cycle and other characteristics of anaerobic fungi along with their contribution to the livestock.

1. PREAMBLE

The unquestionable role of domesticated ruminants in agriculture has generated significant interest into the process of digestion of plant structural carbohydrates so as to improve the production efficiency of the consumer animals. A majority of the livestock in tropical countries subsists on poor quality fibrous grasses, bulky crop-residues and agro-industrial byproducts poorly digested in the rumen with the ultimate low voluntary intakes. Therefore, attempts are being made to improve the digestibility of poor quality feeds by various feed additives (Nagpal et al. 2007a). A clear positive relationship observed between counts of rumen anaerobic fungi and the voluntary intake of low digestibility herbage suggests the possibility of utilizing anaerobic fungi as probiotic feed additives to enhance the microbial activity.

Rumen anaerobic fungi actively colonize plant cell walls and account for up to 8-12% of the microbial biomass in rumen (Rezaeian et al. 2004). Prior to their discovery, it was assumed that only rumen anaerobic bacteria and protozoa were involved in hydrolysis of plant biomass. But now, it is well established that these ruminal fungi effectively take part in fibre digestion in ruminants (Dey et al. 2004, Lee et al. 2004). The rhizoids of their vegetative thalli penetrate deep into plant tissues better than bacteria and protozoa, and thus achieve access to plant materials otherwise unavailable to other rumen microorganisms. This infiltration leads to a more rapid degradation of forage entering the rumen (Orpin and Joblin, 1988; Nagpal et al. 2007b). These fungi secrete high levels of very active fibre-degrading enzymes (cellulases, hemicellulases, xylanases, avicelases, glycosidases etc.) found to be associated with rhizomycelia (Williams et al. 1994; Lee et al. 2001).

2. HISTORICAL MILIEU

The ruminal anaerobic fungi, reported as early as 1910, were thought to be flagellate protozoa (Liebetanz, 1910; Braune, 1913) and placed in the genera *Callimastix, Sphaeromonas* and *Oikomonas*. These flagellates were recognized as fungi for the first time in the 1970s (Orpin, 1975) with the first named species *Neocallimastix frontalis*. The flagellate zoospores encyst and germinate on ingested forage with radiating rhizoids that produce a single zoosporangium. In terms of lifecycle and morphology, *N. frontalis* is similar to members of Chytridiomycota and its fungal affinities are confirmed by chitin in the cell wall (Orpin, 1975); though uniquely among the fungi it is an obligate anaerobe. About twenty different species of anaerobic rumen fungi have been reported in various ruminant and hindgut-fermenting mammals. It is established that removal of these fungi from the rumen results in a significant diminution in *in-vitro* gas production and degradation of fibrous feeds, signifying a vital role such fungi play in fibre degradation (Lee et al. 2004). The enzyme profiles of various fungi studied indicated secretion of a wide range of ligno-cellulolytic enzymes. Scanning electron microscopic studies ascertained that these fungi preferably attach to most lignified tissues of plant feed (Akin, 1987). Hence, fibre-based diets stimulate their proliferation in the rumen compared to diets rich in easily fermentable carbohydrates (Paul et al. 2003). Pelleted diets generally have a shorter transit time through the gastro-intestinal tract and therefore do not support good anaerobic growth of rumen fungi *in-situ*. High soluble sugar content inhibits germination of fungal zoospores on plant tissues (Roger et al. 1992), and this might be due to lowered pH of rumen liquor (Orpin, 1977).

3. TAXONOMIC STATUS

Anaerobic zoospore-producing fungi are very recently assigned to Chytridiomycota, a basal group within kingdom Fungi and subdivided into five orders i.e., Blastocladiales, Monoblepharidiales, Chytridales, Spizellomycetales and Neocallimastigales (Barr, 1990). Based on the ultrastructural characteristics of zoospores, anaerobic fungi were originally placed in the order Spizellomycetales (Barr, 1980; Barr and Desaulniers, 1988) but later transferred to a separate order (Neocallimastigales) by Li et al (1993). The precise relatedness of the Neocallimastigales to other chytrid fungi is at present unclear since they possess a number of features not common with other chytrid taxa (hydrogenosomes, polyflagellate zoospores, distinctive flagellar attachment; Barr, 1990; James et al., 2000), and are distinctive in other respects too (e.g. very high AT [ca. 70%] DNA base ratio; Brownlee, 1989). The recent international collaborative effort (AFTOL project: All Fungus Tree Of Life; http://aftol.org/) to establish a multiple gene genealogy for kingdom Fungi found Chytridiomycota to be polyphyletic (with loss of flagellate zoospores on several occasions) but confirmed anaerobic fungi basal to the 'core' chytrid clade (James et al., 2006a; b). On the basis

of many distinctive features of anaerobic fungi relative to other chytrids, these may be assigned to a new phylum *Neocallimastigomycota* phylum nov. (MycoBank no.: MB 501279), containing a single class *Neocallimastigomycetes* nov. (MycoBank no.: MB 501280) (Hibbett et al., 2007).

Gold et al (1988) recommended subdivision of anaerobic fungi into three genera *Neocallimastix, Piromyces* (formerly *Piromonas*) and *Caecomyces* (formerly *Sphaeromonas*). However, three other genera with more complex growth morphology have subsequently been discovered i.e. *Orpinomyces* (Barr et al. 1989), *Anaeromyces* (Breton et al. 1990) and *Cyllamyces* (Ozkose et al. 2001). In all such genera, multiple sporangia are formed in *Orpinomyces* and *Anaeromyces* on a more extensive polycentric thallus. *Cyllamyces*, like *Caecomyces*, forms bulbous holdfasts (which can cause rupture of plant tissues; Joblin, 1989) rather than a rhizoidal system with up to 12 sporangia formed on branched sporangiophores. Thus, the six genera can be divided into three groups based on colony morphology (monocentric, polycentric or bulbous). However, it is likely that zoospore flagellation represents a more fundamental division within the anaerobic fungi with *Neocallimastix* and *Orpinomyces* having multiflagellate (5-20 flagella) zoospores while other genera being uniflagellate. Table 1 represents the classification and different morphological characteristics of ruminal anaerobic fungi.

Table 1. Classification and morphological characteristics of anaerobic fungi (after Barr, 1990; James et al., 2000; Hibbett et al., 2007).

Kingdom:		Fungi
Phylum:		*Neocallimastigomycota*
Class:		*Neocallimastigomycetes*
Order:		Neocallimastigales
Genera:	**A.**	Monocentric: ***Neocallimastix***: zoospore with 4 - 20 flagella; thallus with filamentous branching rhizoids; ***Piromyces***: zoospore with 1 - 4 flagella and thallus with filamentous branching rhizoids, and
	B.	Polycentric: ***Orpinomyces***: multiflagellate zoospores; ***Anaeromyces***: zoospore with single flagellum; ***Cyllamyces***: zoospore with 1 - 2 flagella with thalloid branched sporangiophore. Bulbous: ***Caecomyces***: zoospores with 1 - 2 flagella; thallus with globular rhizoid; ***Cyllamyces***: zoospore with 1 - 2 flagella, thalloid branched sporangiophores

Presently, 18 species of anaerobic fungi have been identified and described (Table 2). No anaerobic fungi have hitherto, been reported to have a sexual stage.

4. THE LIFE CYCLE

The life cycle of monocentric fungi is asexual and shifts between a motile, zoosporic and a vegetative, zoosporangial stage (Figure 1). The flagellate zoospores move by chemotaxis to colonize the fibre material (Orpin and Bountiff, 1978; Munn et al. 1988). *N. patriciarum* zoospores show chemotaxis towards several carbohydrates as receptors e.g. glucose, mannose, sorbitol and sucrose (Orpin and Bountiff, 1978), and move across the plant surface, presumably to stumble on the right location for encystment. After release, these get encysted and germinate exogenously to form a germ tube from which the rhizoids emerge (Orpin, 1977). The cell mass develops into a

Table 2. Classification of anaerobic fungi (after Cabe, 1998; Ozkose et al. 2001; Harhangi, 2002).

Genus/ Species	Source(s)	Reference(s)
Caecomyces:		
C. communis; C. equi	Sheep; Horse	Gold et al. (1988)
Piromyces:		
P. communis ; P. mae; P. dumbonica ; P. rhizinflata; P. minutus; P. spiralis; P. citronii	Sheep; cow; Horse; Elephant; Deer; Goat; Donkey	Gold et al. (1988); Julliand et al. (1998); Li et al. (1990); Breton et al. (1991); Ho et al. (1993a & b); Gaillard-Martinie et al. (1995)
Neocallimastix:		
N. frontalis ; N. patriciarum ; N. hrleyensis; N. variabilis	Sheep; Cow	Heath et al. (1983); Orpin and Munn, (1986); Webb and Theodorou, (1991); Ho et al. (1993c)
Anaeromyces:		
A. elegans; A. mucronatus	Cow; Sheep	Ho et al. (1993d); Breton et al. (1990)
Orpinomyces:		
O. joyonii; O. intercalaris	Sheep; Cow	Breton et al. (1989); Ho et al. (1994)
Cyllamyces:		
C. aberensis	Cow	Ozkose et al. (2001)

sporangium and cytokinesis results in uninucleate zoospores to be released to complete the cycle. From studies on *Neocallimastix*, it is established that the life cycle lasts about 23-32 hours (Lowe et al. 1987a), whilst in *Cyllamyces aberensis,* it is slightly shorter to 18-24 hrs (Ozkose et al. 2001). Zoospores development from young sporangia may occur within 8 hours after encystment under appropriate conditions (Orpin, 1977).

Unlike monocentric and bulbous taxa, polycentric fungi have less determinate lifecycles and can differentiate multiple sporangia over periods of several days. Nuclei are visible within rhizoids but it is unclear to what extent these resemble hyphae of higher fungi (Ho and Bauchop, 1991). Zoosporogenesis is asynchronous as in other taxa and in culture, zoospores are often very rare (Phillips, 1989 Fliegerova et al., 2004).

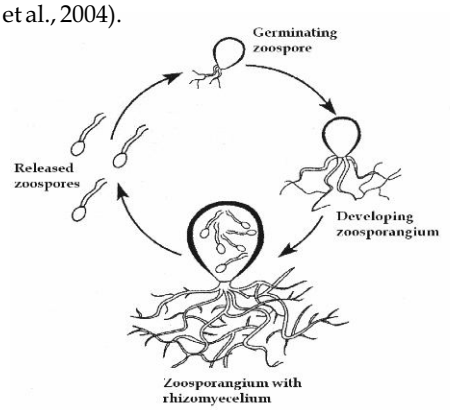

Fig.1. Life cycle of anaerobic fungi (Source: Teunissen and Op den Camp, 1993; Harhangi, 2002).

5. DISTRIBUTION IN NATURE

Anaerobic fungi have been reported in all geographic regions of the world, being ubiquitous among fore-gut fermenters and ruminants such as cattle, buffalo, goat (Ho et al. 1993a; 1993c; Singhal, 2000; Thareja et al. 2006), red deer, impala (Bauchop, 1979) and wild Bluebull (*Boselaphus tragocamelus)* (Paul et al. 2004; Tripathi et al. 2007a) as well as marsupials including kangaroo, wallaroo and swamp wallaby (Breton et al. 1989). These fungi have also been isolated from faecal samples of the horse, zebra, donkey, rhinoceros and Indian elephant (Bauchop, 1983; Breton et al. 1990; Li et al. 1990; Orpin, 1994; Theodorou et al. 1994), all being hindgut fermenters. Therefore, these fungi appear to be a standard constituent of the gut microflora in many herbivores fed on a highly fibrous diet.

6. METABOLISM AND PHYSIOLOGY

Anaerobic fungi derive energy by anaerobic fermentation of carbohydrates (Trinci et al. 1994). A large number of poly-, oligo-, and monosaccharides including glucose, cellobiose, fructose, maltose, sucrose and xylose, support their growth (Orpin, 1975, 1976; Mountfort and Asher, 1983; Phillips and Gordon, 1988). Anaerobic fungi follow a mixed-acid fermentation profile similar to enterobacteria such as *E. coli* with the conversion of hexose to acetate, formate, lactate, succinate, ethanol, CO_2 and H_2 (Borneman et al. 1989, Trinci et al. 1994). These products may fluctuate among different genera i.e. high malate and lactate by *Anaeromyces* spp. compared to *Orpinomyces* (Phillips and Gordon, 1988). Table 3 shows the outline of cellulose fermentation by *N. frontalis*:

Table 3. Fermentation of cellulose by *N. frontalis* (Bauchop and Mountfort, 1981).

Fermentation product	mol/ 100 mol hexose
Acetate	72.7
Lactate	67.0
Ethanol	37.4
Formate	83.1
Carbon dioxide	37.6
Hydrogen	35.3
Methane	0.0

Anaerobic fungi lack mitochondria, cytochromes and other biochemical features of the oxidative phosphorylation pathway. In cytosol, all major enzymes required for glycolysis through the Embden-Meyerhof-Parnas pathway are present while glucose-6-phosphate dehydrogenase and the other enzymes of Entner-Dodouroff pathway are absent (Yarlett et al. 1986; O'Fallon et al. 1991; Marvin-Sikkema et al. 1993). The group possesses organelles (hydrogenosomes) for a major part of anaerobic energy metabolism (Muller, 1993; Trinci et al. 1994; Benchimol et al. 1996). Hydrogenosomes are spherical double-membrane bound redox organelles (0.2 – 1 μm), and have been reported in phylogenetically distant amitochondriate eukaryotes that inhabit anaerobic or microaerophilic environments (Muller, 1993).

7. TRANSFER AND SURVIVAL BETWEEN DIFFERENT HOSTS

Anaerobic fungi can be isolated from alimentary tracts of ruminants (Davies et al. 1990, 1993) and propagules survive in air-dried faeces up to 10 months (Milne et al. 1989; Theodorou et al. 1990; McGranaghan et al. 1999). There have been attempts to identify putative aero-tolerant resting structures (Wubah et al., 1991b; Nielsen et al., 1995; Richardson et al., 1998), though only in a single unnamed *Anaeromyces* species have spores been unequivocally identified (Ozkose, 2001;

Brookman et al., 2000b). In the latter case, fungi could be resuscitated from pure cultures after incubation at 39°C for up to 11 months (anaerobic fungi, however, do not survive beyond 10 days without subculturing) (Ozkose, 2001). Furthermore, thick-walled, elongate and septate spores are readily visible in these cultures. The ability of anaerobic fungi to form spores and possibly, other resting structures in large part explain why they can be transferred so readily between host animals, and far more easily than rumen protozoans which require direct animal contact (Williams, 1986). In fact, during an early study on rumen protozoans, Becker and Hsiung (1929) prevented re-infection by "Callimastix" [sic] of isolated defaunated goats for more than a few weeks (even if the feed was sterilized). As is the case with rumen protozoans, transfer of anaerobic fungi from mother to neonates can also occur by direct contact with saliva during grooming and licking, or by coprophagy (Lowe et al. 1987b; Milne et al. 1989). In sheep, anaerobic fungal populations are established by 8-10 days of birth (Fonty et al. 1987). As ruminal fungi can utilize lactose as a carbon and energy source, their population, along with other microorganisms, gets accumulated and develops in the under developed rumen of young lambs fed on milk; and subsequently stabilizes with the development of rumen as the animal starts consuming fibrous diet (Fonty et al. 1987; Fonty and Grenet, 1994).

Fungal transfer in nature is not only inter-ruminant, but also from non-ruminant to ruminant as these can also be transmitted by aerosols and dried faeces (Orpin, 1989; Dehority and Orpin, 1996). Interspecies transfer has been demonstrated by the establishment in sheep of high ruminal populations of *Piromyces* sp. isolated from horse faeces and also a strain of *Neocallimastix* from the reindeer rumen (Orpin, 1989). *Orpinomyces* sp. and *Piromyces* sp., from cow's rumen and faeces of wild blue bull, respectively, establish in the rumen after administrating to male buffalo calves fed with wheat straw based diets (Tripathi et al. 2007b). As with the rumen protozoans, where evidence of host specificity is equivocal (Williams, 1986), the various anaerobic fungal taxa show little geographic structure with diet having a far greater effect on fungal population than the host. However, Phillips and Gordon (1988) reported that polycentric taxa could not be isolated from sheep in Australia.

8. FIBRE DEGRADATION POTENTIAL

The role of rumen fungi in the degradation of plant fibre has been examined extensively (Theodorou et al. 1989; Samanta et al. 2001; Paul et al. 2004; Dey et al. 2004; Lee et al. 2004; Thareja et al. 2006; Dayanand et al. 2007; Tripathi et al. 2007a, b). The rhizoids or bulbous holdfasts of vegetative thalli are better at penetrating plant tissue than are bacteria and protozoa, so they gain access to the plant material not accessible to other rumen microorganisms (Orpin and Joblin, 1988). Bauchop and Mountfort (1981) suggested that such penetrations lead to a faster and complete degradation of forage entering the rumen. Degradation of lignified plant cell walls is an important characteristic of rumen fungi (Mountfort et al. 1982; Akin and Benner, 1988). Zoospores of many species colonize the lignified tissues preferentially and establish colonies localized on sclerenchyma and xylem cells. Early observations indicated that lignified cell walls were degraded to a greater extent by rumen fungi than by rumen bacteria and protozoa. Experiments with specifically labeled ^{14}C polysaccharides or lignin indicated that the rumen fungi solubilize phenolics and degrade lignocellulose, although cannot consume the lignin moiety (Gordon and Phillips, 1989).

Anaerobic fungi penetrate the cuticle, the rigid structural barrier on the outside of the plant epidermis. These fungi often enter the leaf interior through stomata in the epidermal layer (Akin et al. 1983) giving these fungi an advantage in degrading plant fibre through substantial increase

in the area available for infection. Rumen fungi also show protease activity that may have role in degradation, because the plant structural proteins increase the integrity of plant cell wall (Wallace and Joblin, 1985). Species of *Piromyces, Neocallimastix, Orpinomyces* and *Anaeromyces* degrade fibre to a substantial degree. *Caecomyces* species degrade fibres but lesser than other genera (Gordon, 1990), perhaps because of the lack of an extensive rhizoidal system. In Bermuda grass (*Cynodon dactylon*), stems and leaves harbour *Neocallimastix* and *Orpinomyces* species to degrade the plant material most effectively by weakening the textural strength of the residue (Akin et al. 1990). These findings suggest that the ability to degrade fibre varies among fungal genera, and that plants differ in their support for fungal growth. The greater ability of rumen fungi, compared to rumen bacteria, to weaken forage fibres may be vital to enhancing its utilization by the host animal (Borneman and Akin, 1990).

Ito et al. (1994) studied sheep rumen fungi for degradability and digestibility of rice straw and observed significant decrease in lignin residue that in turn increased digestibility of the feed. Studies by Manikumar et al. (2002) and Dey et al. (2004) indicated increased *in vitro* dry matter digestibility, and decreased cell wall contents of straws by different anaerobic fungi viz, *Orpinomyces, Piromyces* and *Anaeromyces* relative to untreated controls. Also, the molar yield of acetate increased with simultaneous decrease for propionate and butyrate. In a subsequent report, hydrolysis of rice and wheat straw by *Orpinomyces* sp. (C14) was superior to that of *Piromyces* or *Anaeromyces* (Manikumar et al. 2003).

Gordon and Phillips (1998) reported a 7-12% increase in voluntary intake of straw based diet by sheep dosed with monocentric fungi from other herbivores. In contrast, no such effect on feed intake was observed in crossbred calves dosed with polycentric *Orpinomyces* sp. However, the growth rate and nutrient digestion in cow calves improved in the fungus-administered sets. There was also a two and a half fold increase in the fungal count in rumen liquor of fungus-administered animals (Dey et al. 2004).

Lee et al. (2000, 2004) studied the effect of administration of *Orpinomyces* strain KNGF2 from Korean native goats, or their enzymes on the extent of ruminal fermentation, microbial population, enzyme activities and nutrient digestion in sheep rumen. There was a 2-3 fold increase in cellulolytic bacterial count in case of anaerobic fungal treatment of silage based ratios compared to the control.

9. FIBROLYTIC ENZYMES

The ability of ruminants in digesting the plant structural polysaccharides, primarily cellulose and hemicelluloses, depends on the capacity of microorganisms inhabiting rumen, and rumen fungi play a major role in degradation of lignified plant tissues (Akin and Benner, 1988). For plant cell wall degradation, such anaerobic fungi produce a wide range of hydrolytic enzymes, cellulases (Barichievich and Calza, 1990; Yanke et al. 1993), hemicellulases (Mountfort and Asher, 1989), proteases (Michel et al. 1993), amylases, amyloglycosidases (Paul et al. 2004), feruloyl and p-coumaryl esterases (Borneman et al. 1992), various disaccharidases (Chen et al. 1994), pectinases (Gordon and Phillips, 1992) and exonucleases (Cabe, 1998).

Three enzymes viz, endo-1,4--glucanase, exo-1,4--glucanase and -glucosidase act synergistically for efficient cellulose hydrolysis. These enzymes often bind to the substrate prior to hydrolysis, but they may also bind to other plant cell wall polymers such as xylan (Gilkes et al. 1991). Aryl esterases, viz. *p*-coumaroyl esterases and feruloyl esterases are other important enzymes that hydrolyze the ester linkages between hemicellulose and lignin in plant cell walls,

and thus separating hemicelluloses and cellulose from lignin (Borneman and Akin, 1990) and render them accessible to hemicellulases and cellulases. Thus, through esterases, anaerobic fungi play a more important role over bacteria in separating the lignin-polysaccharide linkages in the plant particles by extensive rhizoidal elongation, and are thus ahead of bacteria. *N. patriciarum* solubilized lignin (up to 34%) in sorghum (McSweeney et al. 1994). Unlike rumen cellulolytic bacteria, rumen fungi also have proteases that facilitate penetration of the plant proteinaceous components by fungal rhizoids (Engels and Brice, 1985).

Most fibrolytic enzymes have been found associated with rhizomycelium while some secreted into the surroundings (Gordon and Phillips, 1992; Williams et al. 1994). The activities of these enzymes are common to both the zoosporic and vegetative stages as well as in the cell-free spent culture fluid (Williams and Orpin, 1987); but depend on the stage of the life cycle (Martin and Nisbet, 1992; Lee et al. 2001). A few fibrolytic enzymes are constitutive, and regulated by the presence of soluble sugars (Mountfort and Asher, 1983, 1985, 1989), e.g. production of cellulase repressed by glucose (Mountfort and Asher, 1985). Growth conditions greatly influence enzyme production as the level of fibrolytic enzymes was 3-fold in a stirred fermenter compared to static batch cultures (Morgavi et al. 1994; Paul et al. 2003).

Because of the ability of cellulases to rapidly attack crystalline cellulose, there has been considerable interest in the fibre degrading enzymes of anaerobic fungi (Teunissen et al. 1993). Wilson and Wood (1992) reported that isolates of *Neocallimastix* and *Piromyces* were the most rapid degraders of crystalline cellulose. The weakening of tissues by fungal enzymes may accelerate digestion, and thus making rumination more effective in reducing particle size and increasing protozoal and bacterial digestion in the rumen.

10. MICROECOLOGY IN THE RUMEN

The interactions of anaerobic fungi with other rumen microbes can be positive, negative or neutral, depending on the microbial group involved and the type of substrate used. Since, rumen fungi produce appreciable amounts of H_2; they can interact with H_2 utilizers which in turn alter their metabolite production. Methanogens are the principal H_2 utilizers in rumen; and stable cocultures of fungi and methanogens have been established *in vitro* (Fonty and Joblin, 1991; Orpin and Joblin, 1997). Such cocultures resulted in increased fungal biomass (Bernalier et al. 1989) and also an increase in the rate and extent of cellulose degradation (Wood et al. 1986; Joblin et al. 1989; Bernalier et al. 1991). Interspecies H_2 transfer between the cellulolytic H_2-producing anaerobic fungi and methanogens resulted in increased CO_2 and acetate formation but decreased ethanol and lactate output (Bauchop and Mountfort, 1981; Mountfort et al. 1982). The presence of *Methanobacterium arboriphilus, Methanobacterium bryantii,* or *Methanobrevibacter smithii* also increased (5 to 10%) the level of cellulose fermentation by anaerobic fungi (Marvin-Sikkema et al. 1990). By contrast, cellulose degradation and lactate production by *N. frontalis* decreased in cocultures with non-lactate utilizing *Selenomonas ruminantium*, the sugar fermenting, H_2 consuming rumen bacterium, thus indicating interspecies hydrogen transfer (Richardson and Stewart, 1990).

The fungi are involved in cross-feeding in that they release free sugars, which in addition to several of their normal metabolites (except acetate), serve as energy sources for other bacterial species. The fungi themselves may also depend on the bacteria for supply of B vitamins, heme and amino acids, as the nutritional requirement (Williams et al. 1994). On the other hand, coculture of anaerobic fungi with rumen bacteria inhibits the cellulolytic activity (Bernalier et al. 1992; Roger et

al. 1993) and growth (Dehority and Tirabasso, 2000) of the former. Stewart et al. (1992) and Bernalier et al. (1993) reported an extracellular, thermo-labile protein produced by ruminococci, which inhibits fungal cellulase activity. Dehority and Tirabasso (1993) also reported that mixed rumen bacteria produce a heat stable compound *in vitro*, which markedly inhibits growth of rumen fungi.

Since chitin is the main structural component of fungal cell wall, their growth is likely to be inhibited by rumen chitinolytic microorganisms such as *Clostridium* sp. Co-culturing of the anaerobic fungi with chitinolytic *Clostridium tertium* significantly reduced solubilization of crystalline cellulose, production of short-chain fatty acids and release of endoglucanase (Hodrova et al. 1995), suggesting the role of chitinolytic bacteria in controlling fungal activities *in vivo*. Thus, rumen fungi do not appear to attain their optimal fibre-degrading potential in rumen due to the inhibition by some bacteria. Small sized fungal zoospores are likely to be a prey for protozoa. Co-incubation of protozoa with fungi revealed that protozoa are able to ingest and digest fungi (Morgavi et al. 1994). The fungal growth and cellulolysis is negatively affected by rumen protozoa, possibly because of protozoal predation on zoospores.

11. RESPONSE TO DIVERSE DIETS

The forage rich diets such as hay and silage, with a long ruminal transit time, consistently result in high population density of anaerobic fungi (Fonty and Grenet, 1994). The addition of maize to sorghum silage enhanced degradation by anaerobic fungi (Akin and Windham, 1989). Also, the addition of grain concentrate to the hay diet significantly increased the count of fungal zoospores in sheep rumen (Faichney et al. 1997). In sheep consuming appreciable amounts of fibres, a large number of sporangia are found attached to stem fragments before morning feeding (Bauchop, 1979). Fungal populations were also stimulated to a much greater extent with alfalfa diet than with coastal Bermuda grass (Windham and Akin, 1984).

By contrast, diet rich in soluble carbohydrates (i.e. young pasture, whey and fodder beet) result in a relatively low population density of anaerobic fungi (Grenet et al. 1989). Silage diet from sorghum or maize reduced the numbers of anaerobic fungi in the rumen (Akin et al. 1988). Similarly, rye grass at the leafy stage, was also found unfavorable for fungi (Grenet et al. 1989). Anaerobic fungi are generally adversely affected by the addition of lipid to the diet (Jenkins, 1993). Feeding a supplement of sunflower, cottonseed or rapeseed oil meal to animals also depressed fungal population in the rumen (Elliott et al. 1987; Fonty and Grenet, 1994).

12. CONTRIBUTION OF FUNGI TO HOST NUTRITION

Various reports suggest a positive correlation between anaerobic fungi and voluntary intake of low digestible herbage diet (Akin et al. 1983; Weston et al. 1988; Gordon and Phillips, 1993). This association is an outcome of fungal attack on lignified tissues (Akin, 1987; Akin and Borneman, 1990) combined with the weakening of more recalcitrant plant components (Akin et al. 1983, 1989). Soft feed fragments in the rumen may be anticipated to lead to less effort by the animal in eating and ruminating. A positive correlation between populations of rumen anaerobic fungi and rumination efficiency in sheep fed a wheat straw diet has been reported by Weston et al. (1988). Therefore, anaerobic fungi apparently facilitate the physical disruption during rumination of the fibrous particles of poor-quality feed leading to a lower residence of such particles in the rumen. The intake of forage by early weaned calves was 35% higher in calves dosed with *Neocallimastix* sp. (Theodorou et al. 1990), and the dosing of fungus-free sheep with *Neocallimastix* sp. resulted in a 40% increased intake of a straw-based diet (Gordon and Phillips, 1993).

These fungi also supply protein to the host through the action of proteolytic enzymes and also as a proportion of the microbial protein synthesized in the rumen that passes to abomasum and intestines for digestion and absorption (Gordon and Phillips, 1998). Kemp et al. (1985), Gulati et al. (1989) and Onoda et al. (1993) reported that fungal cells are composed of proteins with a well-balanced combination of amino acids which were highly accessible to and digestible by the ruminant; and a high proportion of fungal protein is digested and absorbed in the intestines of sheep with higher digestibility compared to ruminal bacteria (Gulati et al. 1990). Moreover, the advent of dependable measurements of fungal biomass in ruminant digesta has shown that in sheep fed with either hay or grain diets, anaerobic fungi averaged 2.4% of the microbial nitrogen in ruminant digesta (Faichney et al. 1997). However, the contribution of anaerobic fungi towards supply of microbial proteins to the animal was minor as these averaged 1.6% of the microbial nitrogen in digesta flowing to duodenum. Yet, this microbial protein was of high quality and voluntarily available to animal. Therefore, if the biomass of anaerobic fungi in the rumen is enhanced, it is likely that the supply of high-quality microbial protein to host ruminant would be possible (Gordon and Phillips, 1998).

13. ISOLATION, CHARACTERIZATION AND PERSISTENCE

To culture the rumen anaerobic fungi, one of the methods involves overlaying the partially molten agar with filtered rumen fluid and incubation at 39°C (48 hrs) as by this time, zoospores settle and produce individual thalli to yield pure cultures (Orpin, 1975). Lowe et al. (1985) suggested a plate culture technique to isolate rumen fungi from rumen digesta of sheep and cattle. The roll-bottle method of Joblin (1981) involves inoculating a dilution series of molten agar medium with filtered rumen fluid. After a period of incubation, axenic cultures could be obtained from the individual colonies produced. Antibiotics penicillin, streptomycin, neomycin and chloramphenicol can be added to the isolation media to suppress bacterial growth (Wubah et al. 1991a). It is difficult to maintain these fungi as they require an oxygen-free atmosphere. Hence, the cultures are to be maintained under CO_2 atmosphere during growth.

The paucity of morphological features presents a problem regarding the taxonomy of anaerobic fungi. While examining plant material from the digestive tract, fungi often appear as the complex cluster and this makes the classification even up to genus level difficult. At a time when there is little disagreement as to the status of the six genera, subgeneric classification is problematic since difficulties associated with exchange and long-term maintenance of cultures impeded direct morphological and physiological comparisons among isolates. With the advent of molecular taxonomy, it is hoped that DNA sequence comparisons and phylogenetic reconstruction will elucidate the relatedness of the various taxa. Indeed, a number of molecular phylogenetic papers are on record (Brookman et al. 2000a; Fliegerova et al. 2004; Tuckwell et al. 2005), and over 100 nucleotide sequences deposited with Genebank (http://www.ncbi.nlm.nih.gov).

Majority of the sequences deposited relate to the ribosomal RNA genes widely used in phylogenetic reconstruction. The small ribosomal (18S) subunit is highly conserved in different taxa and thus contains little phylogenetically useful information for subgeneric classification (Li and Heath, 1992). In contrast, the internal transcribed spacer (ITS) regions, widely used for study of closely related fungal taxa, show a high level of variability (Li and Heath, 1992; Brookman et al. 2000a; Fliegerova et al. 2004), and has been used to differentiate the morphologically similar monocentric (*Neocallimastix*, *Piromyces*) and polycentric (*Anaeromyces, Orpinomyces*) genera. Brookman et al. (2000a) also reported that the two multiflagellated taxa (*Neocallimastix*,

Orpinomyces) were closely related based on the ultrastructure of the zoospores. Unfortunately, various problems including the presence of divergent ITS sequences within individual isolates has hampered widespread use of this locus for taxonomic studies (Ozkose, 2001), though PCR amplification of DNA from environmental samples (rumen fluid, digesta etc.) using ITS primers may prove valuable for ecological studies (Tuckwell et al. 2005).

Counts of individual zoospores and zoosporangia have been used to estimate fungal populations *in vitro* (Joblin, 1981) and *in vivo* (Ushida et al. 1989). Breton et al. (1991) used colony-forming units per gram dry weight of faeces as the basis for quantifying species of *Piromyces*. An endpoint dilution practice, based on the technique of most probable numbers, was developed to enumerate rumen fungi as thallus-forming units (Theodorou et al. 1990). The procedure involves a 10-fold dilution series of sample in an antibiotic-containing basal anaerobic medium. Defined medium plus 10% clarified rumen fluid was used for dilution series, and fungal population represented as thallus-forming units per gram of dry weight.

An indirect method based on zoospore concentration and life cycle parameters has been used to quantify fungi in the rumen. Exploiting the life history parameters and growth kinetics of these fungi, France et al. (1990) proposed a mathematical model of the life cycle in a steady state so that the population of the particle-attached fungal thalli could be calculated from the concentration of free-swimming zoospores in rumen fluid. The values obtained were reliable for samples from rumen and faeces. However, the inadequate knowledge of the life cycle of anaerobic fungi makes it complicated to evaluate the consistency of this technique.

For long-term maintenance of these fungi, cultures are usually stored in liquid nitrogen using anaerobic glycerol as the cryoprotectant. Pure cultures of anaerobic fungi can also be maintained in a defined medium consisting of cell-free rumen fluid, tryptone, yeast extract, a carbon source, buffer, L-cysteine as the reducing agent, and vitamins (Wubah et al. 1991a). *Neocallimastix frontalis* has been maintained in a similar medium but without the yeast extract and rumen fluid (Lowe et al. 1985). For prolonged maintenance in the laboratory, pure cultures of anaerobic fungi are transferred into fresh basal anaerobic medium every 3-4 days. Joblin (1981), however, reported that cultures could be maintained for several months on plant tissues stored at 39°C without sub-culturing. Yarlett et al. (1986) reported cryopreservation of the anaerobic fungus *Neocallimastix patriciarum* at -80°C with dimethyl sulphoxide as the cryoprotectant, but the survival rate was only 40% after one year. However, in a similar study by Sakurada et al. (1995), the survival reached 80% after one year of storage at -84°C with ethylene glycol and cell-free rumen fluid.

14. FUTURE PROSPECTS

Rumen microbiologists have constantly shown curiosity in manipulation of the rumen microbial ecosystem to boost feedstuff utilization and improved milk production. It is now a well-established fact that anaerobic fungi participate in hydrolysis of plant biomass in ruminants, based on superior penetration of plant tissues over bacteria or protozoa, and thus leading to an enhanced degradation of forage in the rumen. These fungi are well equipped with enzymes important for rumen fermentation, and represent group of dynamic cellulolytic organisms that explicitly colonize fibrous plant fragments. The properties taken together with perceptible extent of rumen populations in animals on high-fibre diets indicates a significant role of such heterotrophs in fibre digestion. In addition, fungi may bring special changes to plant materials in the rumen with the resultant improved feed intake, body weight gain, enhanced milk output, and improved animal productivity. As efforts are still in its early stages regarding stimulation of rumen fermentation by anaerobic fungi, more studies are imperative to assess the extent of their

contribution to the ruminal digestive event. However, the development of direct-fed microbials for improved rumen performance is a pre-requisite for sustainable animal production. Therefore, a substantial potential exists for the manipulation of fungal population and activity in the rumen to benefit even from poor quality herbages.

CONCLUSION

In the lack of efficient feed materials, utilization of high fibrous crop-residues and agricultural by-products along with the tested animal probiotic could be a better alternative over the existing feeding practices. With the onset of technology for production and administration of direct-fed microbials, it seems feasible to effectively utilize the poor quality fibrous feeds for higher productivity of animals. Since, there is considerable disparity among the fungal isolates from domestic as well as wild animals in their fibre degrading potential; there is immense scope to isolate efficient fibrolytic fungal strains with elevated levels of fibrolytic enzymes so that they can be posted in the rumen for optimum feed utilization. Therefore, more work is needed to study the diversity of these fungi among domestic and wild ruminants, and to isolate/ select the elite strains with high fibrolytic activity which can get established in the rumen to facilitate digestion of low grade roughages for enhanced meat/ milk production, as the case may be.

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