New Approach for the Manipulation of Anaerobic Fungi in the Rumen

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Summary

Ruminal anaerobic fungi are important contributors to the utilization of poor-quality, high fibre pastures and crop residues by ruminants. The sensitivity of these unusual microorganisms to a low sulfur content in the diet suggested a means for manipulating them in the rumen by the use of dietary sulfur supplements. However, this approach has remained undeveloped to date. An alternative method for manipulating the fungal contribution to fibre utilization, which involves the oral inoculation of "superior" strains of anaerobic fungi into the sheep rumen, is currently under investigation.

Introduction

For many years the normal rumen microbiota was considered to be composed primarily of bacteria, ciliate protozoa and flagellate protozoa (Hungate, 1966) until it was recognized that at least some of the flagellate protozoa were, in fact, the motile zoospore stages of a new group of microorganisms, the anaerobic chytridiomycete fungi (Orpin, 1975). The potential importance of these unusual microorganisms to herbivore nutrition, as well as the biotechnological potential of their fibre-degrading enzymes, has made them the subject of many studies over recent years. The belief that anaerobic fungi are of considerable importance to the nutrition of ruminants is based on their demonstrated ability to colonize lignified cell walls and to weaken fibrous plant tissues in the rumen (Akin and Borneman, 1990; Akin et *al.,* 1983; 1990), as well as their degradation of the structural components of plant cell walls (Gordon and Phillips, 1989a; Teunissen and Op den Camp, 1993; Wubah et *al.,* 1993) and fermentation of the resulting monosaccharides (fructose, glucose and xylose; Phillips and Gordon, 1988). Therefore, an important contribution is likely when the diet is high in plant fibre. Recently, efforts have been made to measure the contribution of anaerobic fungi to ruminant nutrition. An understanding of the relationship between the diet of the host animal and the population of anaerobic fungi in the rumen is fundamental to appreciating the potential importance of anaerobic fungi and the possibilities for manipulating them in the rumen.

Biology and Ecology of Anaerobic Fungi

Morphology and growth cycle

These fungi exist in the rumen as both zoospores and sporangia (Bauchop, 1979; Orpin, 1975). Depending on the genus to which the individual fungal isolate belongs, the motile zoospores have either a single flagellum or a bundle of about fifteen flagella beating in synchrony (Ho and Bauchop, 1991; Or-pin, 1975, 1976, 1977b). After attaching to a piece of freshly ingested plant material, the zoospore encysts before it germinates to grow and eventually form a larger vegetative structure (the fungal thallus), composed of the sporangium and the rhizoid. A mature sporangium contains from a few to many mononucleate zoospores which are formed by repeated division of the single fungal nucleus contained in the original zoospore body (Li and Heath, 1993). The rhizoid is the structure which attaches to the growth substratum and then penetrates into it (Ho et *al.,* 1988). The time span of a typical fungal growth cycle *in vivo* has not been reported. However, it is probably less than the 26-32 hours reported for one fungus growing *in vitro* (Lowe *et al.,* 1987).

Types of anaerobic fungi

Initially, all of the isolates of anaerobic fungi from the rumen were of the monocentric type. Monocentric anaerobic fungi are those in which a zoospore produces only a single sporangium, normally with all of the dividing nuclei contained inside it. There are three genera of monocentric anaerobic fungi which are

distinguished from one another by the production of either uniflagellate or multiflagellate zoospores, together with the possession of either a filamentous and highly branched rhizoid or thick and bulbous rhizoid. The names given to these genera, with their previous name in brackets, are: Caecomyces (Sphaeromonas), Neocallimastix, and Piromyces (Piromonas) (Li and Heath, 1993; Orpin, 1975, 1976, 1977b), with each of these genera containing several species. Currently, around twelve species are recognised and the number is steadily increasing as new forms are described, particularly for the two genera Piromyces and Neocallimastix.

In 1989, there was simultaneous recognition in Australia, Canada, France and the United States of America 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). Polycentric anaerobic fungi produce a very extensive branched rhizoid which characteristically contains nuclei inside it and multiple sporangia develop at various intervals along the same rhizoid. The new genera Orpinomyces and Anaeromyces were created to accommodate four species of these fungi (Barr et al., 1989; Breton et al., 1990; Ho et al., 1993). There is relatively little known about polycentric anaerobic fungi compared to monocentric fungi because of the relatively short time that polycentric ruminal fungi have been known.

Distribution. of fungi and their transfer between individual animals

Anaerobic fungi have been isolated from many sites along the digestive tract of ruminants (Trinci et al., 1994). As well as being present in the more important species of domesticated ruminants (sheep, goats, cattle and water buffalo), anaerobic fungi occur widely among many different species of herbivorous mammal, including ruminant (such as various species of antelope and deer), ruminant-like (such as camels and llamas) and other foregut fermenting non-ruminant animals (such as kangaroos) as well as hindgut fermenting animals (such as horses and elephants) (Fonty and Joblin, 199 1; Orpin and Joblin, 1988). In addition, anaerobic fungi have been found on all continents (except for Antarctica) and in all of the geographical regions where they have been sought.

Anaerobic fungi are present in the saliva of adult ruminants, and they have been consistently isolated from both fresh and dried faeces (Trinci et al., 1994). The presence of viable anaerobic fungi in dried faeces suggested the existence of a resistant stage which was confirmed when resistant sporangia were observed both in cultures and in cattle faeces (Wubah et al., 1993). The most likely transfer of anaerobic fungi between individual ruminants would occur between juveniles and their dam. Saliva is the likely vehicle for transfer through close mouth-to-mouth contact. Although there is no known evidence for intentional coprophagy in ruminants, curiosity ingestion of fresh faeces by juveniles is possible and inadvertent ingestion of faeces containing resistant sporangia by animals at pasture is also a possible route for transfer between adult animals in the same herd or flock.

Effect of defaunation of the rumen on populations of anaerobic fungi

The treatment of ruminants with defaunating agents (usually ionic detergents) to remove ciliate protozoa from the rumen (Bird, 1989) has a secondary effect; it results in increased populations of ruminal fungi (Hsu et al., 1991; Newbold and Hillman, 1990; Romulo et al., 1989; Soetanto et al., 1985; Ushida et al., 1990). Therefore an inverse relationship between the sizes of the fungal and the ciliate populations in the rumen is apparent. However, this general inverse relationship is not observed with all diets (Ushida et al., 1990; Williams and Withers, 199 1). The underlying mechanism by which defaunation increases the fungal population is possibly a reduction in the turnover of fungal protein by protozoa in the rumen (Newbold and Hillman, 1990). Therefore, it is likely that the nutritional benefits due to defaunation of animals being fed poor-quality, low-nitrogen herbage is at least in part a result of increased fungal numbers with their associated degradative activity against plant fibre.

Influence of diet components on the ruminal populations of anaerobic fungi

Lignocellulose content

The fibre or lignocellulose content of the diet is a critical factor which determines the presence of normal populations of anaerobic fungi in the rumen (Bauchop, 1979; Orpin, 1977a). There were few anaerobic fungi in the rumen of animals that are fed lush pasture (either legume or grass when green and leafy) compared with the same pasture when it is mature (Bauchop, 1979). Silage diets reduced the numbers of anaerobic fungi in the rumen (Grenet et al., 1989). Also, diets rich in rapidly fermented carbohydrates, such as the starch in grain, supported lower populations of anaerobic fungi in the rumen (Gordon, 1985; Grenet et al., 1989; Orpin, 1977a).

Sulfur content

Early in the study of anaerobic fungi, it was recognized that the sulfur content of hay diets or pasture was a significant factor governing the fungal population in the rumen (Akin et *al.,* 1983; Gordon, 1985). When sulfur was present in the diet at levels of around 1 .0 g S per 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 sulfur fertilizer to the pasture used to make the hay (Akin *et al.*, 1983) or a sulfur supplement to the low-S hay (Gordon, 1985; Table 1). Fertilization of the pasture with sulfur resulted in an average increase of 38% in *ad libitum* feed intake (Akin *et al.*, 1983; Gordon, 1985). A diet of another tropical grass hay (spear grass, *Heteropogon contortus),* which had a low sulfur content, also resulted in an undetectable fungal population in the rumen (Morrison *et al.*, 1990). On the other hand, diets of wheat straw which were low in sulfur supported a low, but detectable, population of anaerobic fungi (Gordon, 1985; Gulati *et al.*, 1985; Weston *et al.*, 1988). In all cases, supplementation of these forages with several different types of sulfur allowed fungi to proliferate in the rumen and resulted in an increased voluntary intake of digestible feed (Table 1). 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-sulfur *Digitaria* have been successfully supplemented with methionine (about 1 g S/d per sheep; Gordon, 1985), whereas elemental sulfur had an unknown influence on ruminal fungi and did not improve feed intake (see Table 1). Wheat straw supplemented with either methionine (Gulati *et al.*, 1985) or sulfate (Weston *et al.* 1988) supported greatly increased numbers of anaerobic fungi in the rumen. Spear grass supplemented with sulfate supported a higher fungal population in the rumen compared with the same hay when unsupplemented (Morrison *et al.*, 1990). Anaerobic fungi grown *in vitro* require reduced forms of sulfur (Orpin, 1988; Phillips and Gordon, 1991) indicating the need for reduction of supplementary sulfate in the rumen before it can be available for anaerobic fungi. Despite the potential impact of feed supplemented with sulfur, this method for manipulating ruminal anaerobic fungi has been little studied in recent years. Therefore, a sulfur supplement which is either specific for anaerobic fungi in the rumen, or relatively so, is still to be discovered.

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

Low S feed	Feed S[a] (g/kg)	S added to diet:	Increased fungi after S added	Change due to S addition		Ref
				VOMI	DOMI	
Digitaria pentzi	0.8-1.1	F	known	+36%	ND	1
			presumed	+40%	+31%	5
		met	known	+11%	ND	2
		S^o	unknown	+6%	-4%	5
Wheat straw (alkali-treated)	0.7	met	known	+6%	+23%	3
		SO_4	known	+7%	+16%	6
Heteropogon contortus	0.5	SO_4	known	+75%	+96%	4

[a]S content of diets increases to 1.3-1.7 gS/kg OM after either fertilization of the pasture or use of dietary

Abbreviations: DOMI, digestible organic matter intake; F, fertilizer on pasture; met, methionine; ND, no data; S^o, elemental sulfur; SO_4, sulfate; VOMI, voluntary OM intake.

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

Contribution of Anaerobic Fungi to Ruminant Nutrition

A definite positive relationship exists between the presence of anaerobic fungi in the rumen and the voluntary intake of herbage diets of low digestibility (Akin et al., 1983; Gordon, 1985; Gordon and Phillips, 1993; Morrison et al., 1990; Weston et al., 1988). This is quite possibly a result of fungal attack of lignified plant tissues (Akin and Borneman, 1990) with the resultant weakening of these tough plant components (Akin et al. 1983, 1990). Table 2 shows that the removal of anaerobic fungi from the rumen of sheep reduced the voluntary intake of poor quality feed by about 30%, with little effect on the populations of bacteria and ciliate protozoa. Ruminal fungal activity is often accompanied by increased feed digestibility in vivo, in the order of 2-3 percentage points (Gordon and Phillips, 1993; Weston et al., 1988). The positive effect of anaerobic fungi in the rumen on fibre utilization has been exploited when an oral fungal inoculum stimulated hay intake by early weaned calves (Theodorou et al., 1990). Also, anaerobic fungi have the potential of contributing to the protein supply of the host animal. Fungal cells are composed of proteins with a well balanced combination of amino acids (Kemp et al., 1985) which are highly digestible and available to the ruminant host (Gulati et al., 1989). Until an accurate method of measuring the biomass in the rumen is developed (see Faichney *et al.*, 199 1), the possible extent of the fungal contribution to protein supply is largely conjecture. However, should an increase in the biomass of ruminal fungi prove to be feasible, it is unlikely that the supply of high-quality microbial protein to the host ruminant would be diminished. Therefore, a plan for selecting appropriate strains of anaerobic fungi for inoculation into the rumen of mature ruminants at pasture was proposed (Gordon, 1990) and the remainder of this paper is devoted to a brief discussion of the progress towards this goal.

Screening Cultures of Anaerobic Fungi for Fibre Degradation Ability

The screening of many isolates of anaerobic fungi from a wide variety of mammalian herbivores has been conducted over recent years. The aim of this process has been to obtain cultures of "superior" anaerobic fungi which may be suitable for oral inoculation into domestic livestock (particularly sheep). Selection has been on the basis of the ability to degrade plant fibre.

Weakening of plant structures by fungal growth

Weakening of plant structures by the action of anaerobic fungi is likely to be an important factor in fibre digestion in the rumen. Some strains of anaerobic fungi exert a greater weakening effect on lucerne stems (Medicago sativa) than other strains (Akin et al., 1990). The greatest weakening effect was observed for anaerobic fungi producing filamentous rhizoids (i.e. Neocallimastix, Orpinomyces and Piromyces) rather than those fungi which produce a bulbous rhizoid (i.e. Caecomyces). Also it was found that polycentric fungi were more efficient at weakening stems than were monocentric strains. Unfortunately,

Table 2. The effect of the removal of anaerobic fungi from the rumen of sheep fed on a straw-based diet and the subsequent reinoculation of a *Neocallimastix* sp. into the rumen on voluntary feed intake, feed digestion, and rumen microbial populations (from Gordon and Phillips, 1993).

Parameter	A. Pretreatment	B. No fungi	C. With fungi added
Intake (g/d)			
OM	894±30.8	628±36.4	877±52.6
ADF	390±9.3	264±16.4	373±22.6
Digestibility (%)			
OM	53.2±1.30	50.3±1.38	57.3±1.02
ADF	51.2±1.03	46.5±1.28	55.0±1.64
Anaerobic fungi			
(zoospores/ml; $x10^3$)	7.6±4.0	UD (<0.001)	19±5.7
Bacteria (cells/ml)			
total viable ($x10^9$)	0.8±0.18	1.4±0.37	1.6±0.33
cellulolytic ($x10^8$)	0.4±0.19	2.6±1.48	1.1±0.29
Ciliate protozoa			
(cells/ml; $x10^5$)	3.8±0.92	3.0±0.53	4.8±0.49

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

the specialized nature of the equipment needed to make the appropriate measurements precludes the widespread screening of many different strains of anaerobic fungi in any more than a few laboratories.

Rates of fibre degradation

The rate of degradation of plant fibre is considered to be a more important property of an anaerobic fungus than the extent to which the same strain eventually degrades the substrate. When applied to pure cultures of anaerobic fungi grown on straw, conventional techniques of gravimetric fiber analysis have shown that Neocallimastix spp. degrade more plant fiber than Piromyces spp. which, in turn, degrade more than Caecomyces spp. (Gordon, 1985; Gordon and Phillips, 1989a, 1989b). These methods also measure the degradation rate of the dry matter and the polysaccharide components of plant cell walls by anaerobic fungi (Gordon and Phillips, 1989b; Table 3). However considerable amounts of laboratory time and materials are needed to conduct these types of experiment for the provision of accurate rate data by different strains of anaerobic fungi. Therefore, an obvious need exists for a more rapid method to screen many different isolates of anaerobic fungi which can lead to the selection of those which most efficiently attack plant fibre from a rank in order of degradation rate.

Radioactive fibre substrates provide a useful means of rapidly screening a large number of fungi for their relative abilities to degrade plant fibre (Gordon, 1987, 1990). Pure [U-^{14}C]cellulose prepared from a bacterial culture (Du Preez and Kistner, 1986) and a preparation of plant fibre specifically labelled with [U-^{14}C]glucose in the polysaccharide component (Crawford and Crawford, 1988) have been used as growth substrates for anaerobic fungi to determination of the degradation rate of plant fibre by these microorganisms (Gordon, 1990). Some typical solubilization curves for different anaerobic fungi growing on [^{14}C-glucan]lignocellulose are given in Gordon (1990). A ranking scheme for several anaerobic fungi based on solubilization of these substrates is shown in Table 4. The ranking order obtained in this experiment is the same as that obtained, much more laboriously, by the gravimetric procedures (see Table 3). When subjected to this same ranking procedure, certain strains of polycentric anaerobic fungi *(Orpinomyces* spp.) are also rapid degraders of plant fibre.

Table 4. Comparison of maximum solubilization rates by three monocentric anaerobic fungi obtained for two different ^{14}C-labeled fibre substrates

Fungus	Maximum solubilization rate (dpm thousands, per 24 h)	
	[^{14}C]Cellulose	[^{14}C-glucan] Lignocellulose
Neocallimastix sp. LM1	42.75	40.64
Piromyces sp. SM1	29.96	38.45
Caecomyces sp. NM1	19.92	29.84

The specific activities of both [^{14}C]cellulose and [^{14}C-glucan]lignocellulose were 9,000 disintegrations per minute (dpm) per mg.

Table 3. In vitro degradation of dry matter and cellulose from milled wheat straw by several pure cultures of monocentric anaerobic fungi (Gordon and Phillips, 1989a, 1989b)

Straw component[a]	Fungus	Strain	Percent loss of component after:			Maximum degradation rate
			3 d	5 d	7 d	(mg/d)[b]
Dry matter	*Neocallimastix*	LM1	36	44	44	56
	Piromyces	SM1	14	35	41	28
	Caecomyces	NM1	8	22	31	18
Cellulose	*Neocallimastix*	LM1	49	54	54	48
	Piromyces	SM1	14	46	50	22
	Caecomyces	NM1	3	29	38	11

[a] Individual cultures contained 1% (w/v) milled wheat straw. Residual dry matter was determined at 100 °C and cellulose was determined separately in cultures after treatment with acid detergent solution and permanganate solution.

[b] Degradation rate was determined as the maximum slope of the line drawn between any two data points which were at least 12 h apart.

Potential Benefits from the Inoculation of Superior Anaerobic Fungi into the Rumen

It is apparent that anaerobic fungi are important to ruminants that are 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 benefit the utilization of poor-quality herbage by domesticated ruminants for the increased production responses. One potential means of increasing fungal influence on forage intake and digestion is 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.

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 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 (1989) who reported the successful separate colonization of the rumen of lambs by a *Piromyces* sp. isolated from the caecum of a horse and by a *Neocallimastix* sp. from reindeer. In this regard, 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. Some isolates of monocentric fungi from cattle, deer and several species of African antelope also established in the "fungus-free" sheep rumen. Further research is required to determine the ability of the efficient fungi to compete with the ruminal populations of indigenous fungi. However, a preliminary experiment with sheep which had not been treated to eliminate indigenous ruminal fungi found an increase in the voluntary intake of a straw-based diet of around 7% when the sheep were dosed by mouth with cultures of either monocentric or polycentric fungi originally isolated from herbivores other than sheep. 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. 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.

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