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The survival of anaerobic fungi in cattle faeces

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Abstract

The survival of anaerobic fungi in cattle faeces was studied under a range of environmental conditions, in the laboratory, in cowpats, in slurry and in manure. Numbers of anaerobic fungi were enumerated as thallus-forming units with time after defacation and/or storage using the most probable numbers technique. Survival of anaerobic fungi was observed at a wide range of temperatures and moisture regimes but was relatively poor under certain conditions, notably in manure and in cowpats during the summer period. The results are discussed in relation to the survival of anaerobic fungi in their natural environment and the possible interactions with other micro-organisms present in ruminant faeces.

Keywords: Rumen fungus; Survival; Coprophilous; Faeces; Most probable numbers enumeration

1. Introduction

Since their discovery in 1975 [1], obligately anaerobic chytrid fungi (belonging to the order *Neocallimastigales*) have been found to exist in a wide variety of both ruminant and hindgut-fermenting mammalian herbivores [2,3]. As primary colonisers of freshly ingested forage, these fungi (which produce some of the most powerful cellulases known to man) play an important role in the digestion, particularly when animals are fed on a fibrous low-quality diet [2,3]. In most cases, the presence of these fungal symbionts has been established using fresh or air-dried faeces as inoculum [4,5]. Herbivore faeces, particularly when dried, is not anoxic, so the ability of these fungi to survive such conditions has led to the suggestion that they may produce aerotolerant survival structures [6]. The occurrence and longevity of such a stage in the life cycle of anaerobic fungi is of ecological significance, since it is likely to be involved in the transfer of symbionts between host animals and incidentally between mother and offspring. Although short term survival in saliva has been observed for 8 h [4,7] and in aerated in vitro cultures for 18 h [4], a long term survival (up to 10 months) has been observed only in air-dried sheep and cattle faeces [4,8]. Anaerobic fungi were isolated by Milne et al. [4] from

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sheep faeces, allowed to dry at 20°C or 39°C for up to 128 days but similar samples which were kept moist did not yield any anaerobic fungi after 24 h. It has been suggested that rapid desiccation is necessary both to induce the formation of survival structures and also to reduce the competition from bacteria by inhibiting their growth in the faeces [4]. Subsequent work [9] showed a long term survival of anaerobic fungi in air-dried digesta from each compartment of the digestive tract of ruminants with the notable exception of the digesta from the rumen.

The precise nature of these putative aerotolerant structures is not known, although the production of thick-walled pigmented zoosporangia containing increased levels of DNA in vitro under anaerobic conditions has been observed by Wubah et al. [10] and Richardson et al. [11]. However, these structures failed to germinate on subsequent re-inoculation into fresh batch culture medium. Nielsen et al. [12] identified zoosporangia which contained DAPI staining nuclei on plant fragments isolated from cow faeces. Germination of these zoosporangia was not directly observed but anaerobic fungi were cultured from these dried faecal residues. More recently, the production of specialised multicellular spores has been observed from a number of polycentric isolates obtained from cattle faeces and which are associated with a greatly prolonged survival in a laboratory culture (Jayne Brookman, personal communication).

Studies to date have highlighted desiccation as one of the major determinants of the longevity of anaerobic fungi in faeces [4,6,8,9,12]. Here, we report a series of investigations of the dynamics of anaerobic fungal survival in cattle faeces under several temperature and desiccation regimes, including natural conditions (as cowpats) and cattle waste environments.

2. Materials and methods

2.1. Sources and storage of faecal material

Faeces were obtained from Friesian cows kept at research farms at either the Institute of Grassland and Environmental Research (Trawsgoed or Gogerddan Farms) or the University of Wales (Penglais Farm). Freshly produced slurry from a cattle shed and manure from 3–4-days old straw-bedded litter was obtained. In both cases, the faecal waste and slurry was well-mixed, placed into three replicate 250-l plastic barrels (140 cm high × 48 cm diameter) and kept outdoors but under cover.

2.2. Media and culture conditions

Anaerobic fungi were enumerated as thallus forming units (TFU) according to the endpoint dilution procedure of Theodorou et al. [8]. Samples were homogenised in a stomacher, diluted and placed into Hungate tubes and grown in medium C [6] containing chloramphenicol at a concentration of 50 mg l^{-1} with 1% wheat straw (ground through a 1-mm dry mesh screen) as the substrate. Enumerations were performed on 5-g (fresh weight) amounts of faeces, manure or slurry. Dilution tubes were incubated at 39°C for a period of 10 days prior to examination for the presence of anaerobic fungi using an Olympus CK-2 inverted light microscope with bright field illumination. Most probable numbers (MPN) tubes were scored as positive if the thalli of anaerobic fungi were observed. The fungal population density was expressed as TFU g^{-1} dry matter (DM) as determined from the statistical tables of deMann [13]. The DM of all samples was determined by oven drying (80°C) triplicate aliquots until the weight remained constant.

2.3. Experimental design

Samples (5 g) of a well-mixed sample of freshly voided cow faeces from three different cows were weighed onto aluminium trays. Half of these were subsequently placed into sealed (but not airtight) polythene bags and half were left uncovered on the trays. Both the sealed and unsealed samples were stored at laboratory ambient temperatures in the light for 49 days. At intervals, the anaerobic fungi from one exposed and one covered sample from each cow were enumerated. Faeces were also stored uncovered at a range of temperatures, in a laboratory incubator at 39°C, in a freezer at −20°C or outdoors but under cover (during autumn/winter 1996). Samples were taken for enumeration at intervals over 82 days.

Samples of slurry and manure for enumeration of

anaerobic fungi were taken from the top (5 cm deep), middle (30 cm deep for slurry, 60 cm for manure) and bottom (60 cm deep for slurry, 140 cm deep for the manure) of each barrel. A sample corer was used to obtain the manure samples and slurry samples were obtained manually using a stoppered wide-mouth bottle at each depth. The volume of the manure was sufficiently large for thermophilic composting to occur though no measurements were made of composting-induced temperature changes.

To monitor the survival of anaerobic fungi in faeces under field conditions, three freshly voided cowpats (each from a different cow) were covered with protective cages to prevent trampling (autumn 1996). Samples were taken periodically from the edge of the cowpats for the enumeration of anaerobic fungi. Sampling ceased after 22 days by which time the cowpat had been largely washed into the soil by rainfall. In a repeat experiment, three 10-kg mounds of freshly voided faeces (each from a different cow, circa 50 cm diameter × 20 cm high) were placed on an ungrazed grassy embankment. This experiment was performed on three occasions, once in the autumn (30/09/97–14/01/98) and twice in the spring and summer (06/05/98–22/05/98 and 10/6/98–17/06/98). The samples were covered with protective cages and enumeration was conducted at intervals on faecal samples which were taken from the edge of the mounds.

3. Results

3.1. Effect of drying and incubation temperature on survival

Anaerobic fungi could be cultured from freshly voided cow faeces in sealed bags for at least 25 days after collection, while the size of the population in samples which were left uncovered in the laboratory (and which became dry within a few days) declined only marginally within 49 days (Fig. 1). The initial faecal samples (pH 6.8) had a moisture content of 87.6% (12.4% DM), decreasing to less than 5% within 48 h in the uncovered samples but remaining above 80% for the duration of the experiment in the sealed samples. These results differ from those of Milne et al. [4], who were unable to isolate anaerobic

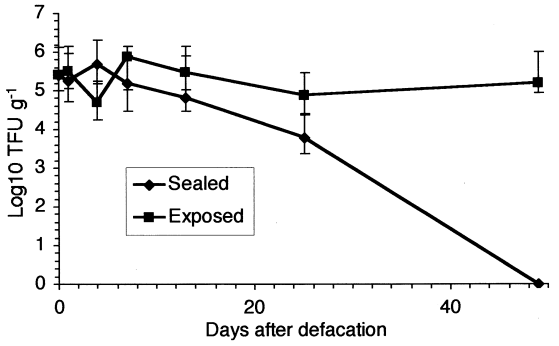

Fig. 1. Survival of anaerobic fungi in 5-g (fresh weight) samples of cattle faeces maintained at 39°C, either exposed or sealed in a polythene bag. Graph of survival of exposed versus sealed. (95% upper and lower confidence limits are indicated as error bars).

fungi from sheep faeces kept in sealed bags for 24 h. A repetition of the experiment of Milne et al. [4] with sheep faeces in the present study showed that survival in sealed bags was less prolonged than in cow faeces with counts decreasing from 60746 TFU g^{-1} after 1 day to 4339 TFU g^{-1}after 4 days and zero at 7 days.

All the samples stored at different temperatures (−20°C, outdoor ambient and 39°C) contained viable anaerobic fungi when the experiment was terminated after 82 days. A 10-fold reduction in the viable count (initially 4×10^5 TFU g^{-1}) was observed for both the samples stored at 39°C and outdoors under cover (in which the temperature ranged from −5°C to +15°C with a mean of 5.5°C over the period of incubation) but the frozen samples showed only a 2-fold decline after 82 days to 1.5×10^5 TFU g^{-1} (Fig. 2). Faeces incubated at 39°C for 7 days (until quite dry) were subsequently incubated at either 50°C or 70°C. MPN counts of 100 TFU g^{-1} were obtained after 3 days only at 50°C.

3.2. Survival in slurry and manure

Anaerobic fungi were found to survive longer in slurry than in manure (Fig. 3). Initial slurry and manure samples gave percentage dry weight figures of 1.2–2.6% (pH 7.1) and 24.6–32.6% (pH 8.5), respectively. The initial viable count in manure (sampled immediately after mixing with straw) was 350 TFU g^{-1}, two orders of magnitude lower than

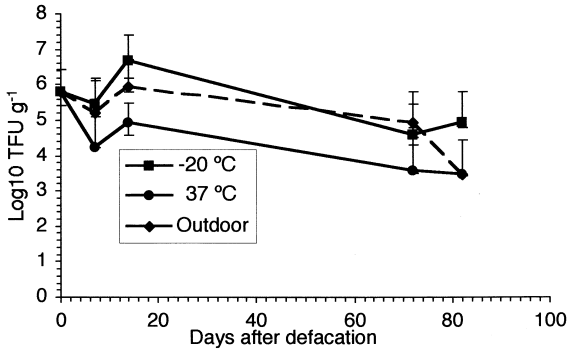

Fig. 2. Survival of anaerobic fungi in 5-g (fresh weight) samples of cattle faeces maintained at either 39°C, outdoor ambient temperature or frozen (−20°C) (95% upper and lower confidence limits are indicated as error bars).

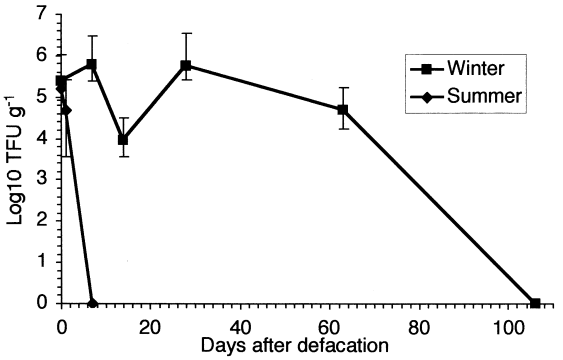

Fig. 4. Survival of anaerobic fungi in cowpats during the winter and summer (95% upper and lower confidence limits are indicated as error bars).

those found in slurry or faeces, and counts subsequently decreased rapidly, with no anaerobic fungi recovered after 14 days. In contrast, counts for slurry samples initially at 6×10^4 TFU g^{-1} increased to 1.2×10^6 TFU g^{-1} after 14 days but decreased to 1200 TFU g^{-1} after 77 days. In both cases, samples taken at depths of 5–140 cm in three replicate storage vessels gave very similar MPN counts.

3.3. Survival in cowpats

An initial experiment using freshly deposited cowpats covered with protective cages (October/November 1996) showed that anaerobic fungi survived for at least 22 days with a small decrease in the recovery from 3×10^5 to 1×10^5 TFU g^{-1}. During this period, the percentage dry weight fluctuated between 9.6 and 12.6%. It was not possible to continue sampling beyond 22 days because heavy rainfall had washed away most of the cowpat. The experiment was therefore repeated in the following autumn with larger (artificial) cowpats. Propagules of anaerobic fungi survived in these samples for between 63 and 106 days (Fig. 4). Over the first 28 days, counts fluctuated around a mean value of 3.5×10^5 TFU g^{-1}. After this time, there was a decline in counts to zero by 106 days. Repetition of this experiment on two further occasions (with two and three replicate cowpats, respectively) in the spring and summer (May and June 1998) revealed a very different pattern of survival. Initials counts of 1.5×10^5 TFU g^{-1} in both cases decreased 3-fold after 24 h and no anaerobic fungi could be isolated after 7 days.

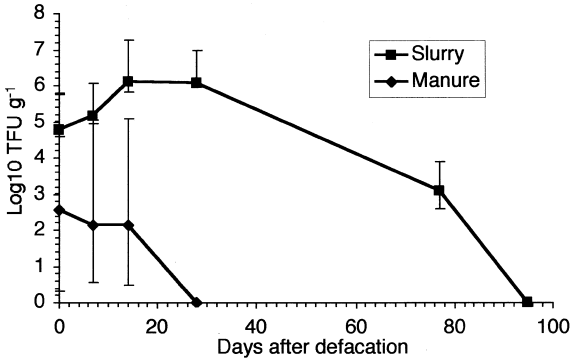

Fig. 3. Survival of anaerobic fungi in slurry and manure (95% upper and lower confidence limits are indicated as error bars).

4. Discussion

The data presented here show that anaerobic fungi can survive in faeces subjected to a wide range of environmental conditions over prolonged periods (>82 days under certain conditions) and that as previously suspected [4,6], the water content of the faeces appears to have a greater effect on the propagule longevity than the temperature of the faeces. Anaerobic fungi were recovered from faeces stored at a range of temperatures (−20°C to 39°C, Fig. 2)

within and beyond those likely to be encountered under natural conditions (including freezing/thawing). In the present study, changes in the water content of faecal samples were recorded. However, in drier samples, measurement of the water availability would be a more relevant determinant of survival.

Milne et al. [4], studying sheep faeces, found that anaerobic fungi could not be recovered beyond 24 h under moist conditions (i.e. sealed in a plastic bag at 39°C). Fig. 1 illustrates how propagules in cattle faeces showed a greater longevity (>25 days) under the same conditions. Repetition of Milne's experiment with sheep faeces confirmed that the survival is reduced compared to cattle faeces, but that anaerobic fungi could be recovered for at least 4 days. A partial explanation for this difference is that Milne et al. [4] used an enrichment procedure without dilution to isolate anaerobic fungi, whereas a serial dilution method was used in the present study. The latter procedure has the advantage that contaminating bacteria are inhibited by the presence of chloramphenicol in the culture medium, thus favouring the growth of anaerobic fungi. It was further observed that a white aerial mycelium (of an unidentified coprophilous species) had covered sheep faeces within a fortnight of incubation in the sealed bags at 39°C, whereas only occasionally was fungal growth observed on cow faeces under similar conditions. It may also be the case that the poorer survival in sheep faeces reflects differences in the anaerobic fungal flora. For example, Gordon and Phillips [14] report that sheep host mainly monocentric taxa and that polycentric species have not been isolated from sheep in Australia. The observation that some polycentric anaerobic fungi produce spores (Jayne Brookman, personal communication) may be significant in this respect.

The prolonged survival of anaerobic fungi in slurry was unexpected, given the relatively rapid loss of viability in sealed plastic bags. The critical difference between these two treatments may be that slurry is highly anoxic, whereas the plastic bags though closed to prevent water loss were not airtight. It therefore appears that the loss of viability of anaerobic fungi associated with storage at high moisture contents, may be related to the aerobic status of the environment.

The rapid decline in anaerobic fungal populations in manure compared to both slurry and freshly voided faeces may be related to the fact that manure heaps are subject initially to an increased pH (up to 8.6 in our samples as a result of ammonia release from urea degradation) and subsequently self-heating (as a result of high levels of aerobic microbial activity during the composting process). The 'heaps' used in the present study did heat up to some degree during the incubation period, though temperature changes were not recorded. However, survival rates at three points (top, middle, bottom) in the manure heap were very similar, suggesting that the increased pH rather than the temperature was a more important factor in the poor survival of anaerobic fungi. Theodorou et al. [8] were able to recover anaerobic fungi from sun-baked Ethiopian cattle faeces and in the present study, survival at 50°C for limited periods was observed in dried faeces.

Anaerobic fungi in cowpats remained viable during the winter for 2 months or more (Fig. 4), despite several frosts and periods of heavy rainfall. Leaching due to rainfall may have been responsible for the reduced counts after a prolonged incubation in the cowpats, as has been observed for the protozoan parasite *Cryptosporidium parvum* [15]. Wubah and Kim [16] have reported the isolation of anaerobic fungi from the sediment of a pond, indicating that leaching may occur from faecal material. However, anaerobic fungi could not be cultured from soil samples taken from around the cowpats in this study.

In contrast to the prolonged survival of anaerobic fungi in cowpats during the winter, survival during the late spring/summer was greatly reduced. Despite high counts in the fresh faeces, fungi were not recovered after 7 days. This poor level of survival was unexpected and not easily explained by the environmental conditions prevailing in the cowpats.

The gut contents and faeces of herbivores contain a diverse array of organisms. In addition to the other anaerobic rumen microbes (bacteria and protozoa) which co-exist with the anaerobic fungi (and which presumably have analogous strategies for survival in faeces), a range of other organisms including coprophilous (dung) fungi [17–19], parasitic nematodes [20,21] and plant seeds [22] also occur in this habitat. The life cycles of many coprophilous fungi are known to involve passage of (usually melanised) spores through the herbivore gut with germination

occurring in freshly voided dung [17]. In the case of zygomycetes, such as *Pilobolus* spp., sporulation may occur as soon as 2 days after defacation. Nutrient-rich faecal material is also rapidly colonised by the larvae of dung flies and other insects. The resulting high levels of metabolic activity and bioturbation by insect larvae and earthworms in the faeces, as well as the complex interactions between these organisms, are likely to affect the survival of anaerobic fungi.

Although the biology of coprophilous organisms in herbivore faeces has received the attention of biologists since the last century (e.g. [23,24]), there have been surprisingly few integrated studies of the decomposition process or the interactions between the various groups of decomposers (e.g. between insect larvae and fungal growth in dung). The succession of coprophilous fungal fruit bodies on herbivore dung (broadly following the sequence: Zygomycota→Ascomycota→Basidiomycota) is well-documented but most studies have been floristic in nature and based on the occurrence of fruit bodies. In one of the few studies of mycelial growth in dung, Dickinson and Underhay [25] found that there was a 3-fold increase in the total hyphal lengths between 5 and 40 days after defacation, with aseptate (zygomycete) hyphae initially predominating. They also observed that fungal growth was inhibited in dung of a reduced water content and that colonisation of the internal regions of the cowpats occurred more gradually (taking 60 days to penetrate 30 mm). Other studies have shown that fruiting of coprophilous fungi is inhibited at very high and low moisture levels, as well as incubation outside the range of 20–30°C [26,27].

A number of positive and negative interactions have been noted between coprophilous fungi (e.g. [28]). For instance, Ikediugwu and Webster [29,30] showed that *Coprinus heptemerus* inhibited the sporulation of several coprophilous fungi in rabbit dung and that this effect was manifest on agar media as hyphal interefence. More recently, Gloer and Truckenbrod [31,32] demonstrated the potent growth inhibitory effect of (+)-isoepoxydon produced by *Poronia punctata* on several coprophilous ascomycetes, an effect which is consistent with negative correlations of fruit body occurrence on cowpats [19]. The complex array of antibiotic and other secondary metabolites resulting from fungal decomposition of the dung could reduce the viability of anaerobic fungal propagules.

The patterns of survival for anaerobic fungi observed in the present study show a negative correlation with the patterns of development for aerobic decomposers. In manure and summer cowpats, there is a rapid proliferation of coprophilous fungi, while in winter, cowpats and laboratory samples insects, low temperatures and/or low moisture contents inhibit the microbial activity. It may also be significant that the bioperturbative effect of insect larvae (especially dung flies) is absent both in winter cowpats and in laboratory samples. The absence of insects from the dung of animals treated with ivermectin has been shown to reduce rates of decomposition [33], while Hay et al. [34] found significant seasonal differences in the proliferation of nematophagous fungi in sheep faeces. Direct correlation between the survival of anaerobic fungi and the proliferation of decomposer organisms in faeces remains to be demonstrated but our results do indicate that abiotic factors alone do not fully explain the observed patterns.

Although the physiology of rumen fungi under anaerobic conditions has been the subject of extensive investigation (reviewed in [3,14,35]), the dynamics of their survival outside their hosts is poorly appreciated. This study has demonstrated that anaerobic fungi are able to remain viable for periods of weeks or months in a wide range of faecal environments. As detailed earlier, it has been established that anaerobic fungi are capable of survival outside their mammalian hosts and various presumably aerotolerant resting structures exist among the *Neocallimastigales*. However, direct evidence that these structures are able to survive in faeces and to germinate under favourable conditions is still lacking. Future work should focus on the differential survival of the various anaerobic fungal taxa in faecal material, the role that resting structures play in this process and the effect of aerobic decomposers on the survival of these propagules.

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