Improvements in nematophagous fungi to control gastro-intestinal parasites

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Abstract

Gastro-intestinal parasites are a major cause of production loss in New Zealand livestock, and the continuing development of anthelmintic-resistant strains represents a significant threat to the future New Zealand agricultural economy. This has led to an increased interest in alternative (non-chemotherapeutic) controls, including potential application of the nematode-trapping fungi *Duddingtonia flagrans* and *Arthrobotrys oligospora*. These species are capable of reducing the number of free-living stages of trichostrongylid nematodes developing in faeces, following oral administration or the addition of fungal material to faeces. However, high spore mortality through the gastro-intestinal tract currently limits the development of a commercial product, even for the robust chlamydospores of *D. flagrans*. The potential to reduce spore mortality by applying a protective coating to the spores was investigated, and an *in vitro* rumen simulation bioassay was used to quantitatively evaluate and compare the survival of *D. flagrans* and *A. oligospora* spores in a series of experiments. These experiments revealed that unprotected *D. flagrans* chlamydospores were superior to *A. oligospora* conidia in their ability to withstand the debilitating effects of rumen fluid. However, the survival of *A. oligospora* was improved by integration into a biopolymer formulation. Dried *D. flagrans* chlamydospores were more resistant to a simulated rumen environment than freshly harvested chlamydospores, and exposure to water as an incubation medium was less detrimental than rumen fluid to the survival of both fresh and dried *D. flagrans* chlamydospores. The application of a stearic acid coating to dried *D. flagrans* chlamydospores failed to improve spore survival in either a simulated rumen environment, or efficacy during subsequent *in vivo* testing. However, as the application of a biopolymer formulation successfully improved the survival of *A. oligospora* conidia, it is likely that similar formulations may be successfully applied to other fungal species. These results highlight the potential for development of formulations containing multiple species of nematophagous fungi, including the application of fungal species that were previously unsuitable due to very high spore mortality.

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"Horses know nothing of money, status, beauty or accomplishment……
Horses see only our hearts, and they accept us or reject us on what they find within……
In short, horses do naturally what humans can pass a lifetime without ever mastering."

Author Mary Midkiff quoted in *The Denver Post*

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1. Introduction and Literature Review

1.1 Introduction

Gastro-intestinal parasites were introduced to New Zealand by early settlers along with sheep and cattle, in the absence of any reliable anthelmintic treatment. They are a major cause of production loss in grazing livestock both in New Zealand (Vlassoff *et al.*, 2001) and around the world (Waller, 1999). This is particularly important in New Zealand, where a large portion of the economy is based on the agricultural industry, particularly that involving small ruminants. There are estimates that up to one third of sheep production in New Zealand is dependent upon effective parasite control (Brunsdon, 1988).

New Zealand farmers currently spend about $93M on chemical anthelmintics each year (Charleston and McKenna, 2002). While increasing levels of drench resistance force the implementation of non-chemotherapeutic helminth controls in sheep production, this is not the case with cattle (Waller, 1997). However, there is a growing concern over the sustainability of current practices, in light of problems faced by the sheep industry (Vercruysse and Dorny, 1999). There is also a strong public demand for animal products that have minimal chemical residues (Knox *et al.*, 2002; Yeates *et al.*, 1997).

Over the last 10 years, the interest in biological control of parasites has increased considerably, in response to the increasing problem of resistance to chemical anthelmintics, especially in sheep and goat parasites (Leathwick *et al.*, 2001; Waller and Faedo, 1993). A range of non-chemical control options are being investigated as adjuncts to the use of anthelmintics for parasite control. These include plants with anthelmintic properties (Alawa *et al.*, 2003; Niezen *et al.*, 1998); the breeding of sheep that are less reliant on anthelmintics (Bisset *et al.*, 2001; Woolaston and Baker, 1996); the production of anthelmintic vaccines (Knox *et al.*, 2001; Knox, 2000) and the commercial application of nematophagous fungi (Larsen, 2000).

Many coprophilic fauna, including dung beetles, earthworms and flies are capable of influencing the number of pre-infective larvae developing in the faecal mass. However, these organisms are difficult to apply and control. For example, dung beetles tend to be

unevenly distributed in areas where they are present (Dymock, 1993), with large numbers occupying discrete areas (pers. obs.). Also, the effectiveness of dung microfauna at reducing larval development is highly subject to environmental conditions (Christie, 1963).

The nematophagous fungi are a diverse group that are able to utilise nematodes as part of their food source. In doing so, they are capable of reducing parasite burdens in livestock by reducing the level of exposure to infective larvae, thereby altering the epidemiology of infection. As the application of nematophagous fungi involves treating the environment rather than the animal, the effectiveness of treatment will be influenced by the presence, or absence of other dung microfauna. One group, the nematode-trapping fungi, produce varying forms of specialised hyphal structures enabling them to trap and kill nematode larvae. Two species that have received particular attention are *Duddingtonia flagrans* and *Arthrobotrys oligospora*. These two species trap nematodes by forming 3-dimensional adhesive hyphal nets.

In order for a fungal biological control to be useful as a parasite control tool, it must be able to reduce significantly the number of parasite larvae developing in the faeces and migrating onto herbage. This is most likely to be achieved if the fungus is delivered to the faeces as the parasite eggs hatch and the larvae develop, which is most easily accomplished by passing them through the host animal. Unfortunately, this approach is limited by the ability of the fungal spores to survive passage through the ruminant gut, as spores of some fungal species can suffer high to complete mortality during gut passage.

Further, commercialisation of this approach would be greatly enhanced by the development of a spore-releasing slow-release bolus. Early research on this technology has found that a major constraint to the commercial success of such boluses is the retention of viability of spores while they are resident within the rumen (R.A. Skipp, pers. comm.).

It is evident, that delivery of fungal spores through the ruminant gut would be greatly enhanced if it were possible to provide the spores with an enteric, protective coating which would increase their survival rates, thus reducing the minimum effective dose rate, and in turn extending the bolus life.

1.2 Objectives and Limitations

1.2.1 Objectives

This thesis aims to evaluate a range of protective coatings for their ability to enhance spore survival through the gastro-intestinal tract of ruminants. To achieve this, the rate of decline in survival of untreated and treated spores exposed to simulated rumen conditions must be defined. The resulting information can then be used to evaluate the effectiveness of the coatings, and to determine which warrant further investigation.

The objectives of the research described in this thesis can be summarised (by Chapter) as follows:

1. To modify an existing *in vitro* bioassay, that simulates the conditions within the rumen and use the assay to evaluate spore survival through the rumen (Chapter 2).

2. To establish decay curves for the nematophagous fungi *Duddingtonia flagrans* and *Arthrobotrys oligospora* in order to determine the length of incubation (in rumen fluid) necessary to reduce spore germination by at least 80%, (Chapter 2).

3. To evaluate survival of spores of *Arthrobotrys oligospora* (Chapter 3) and *Duddingtonia flagrans* (Chapter 4) following treatment with a protective biopolymer formulation. The previously determined *in vitro* rumen fluid assay will be used to determine which formulations warrant further (*in vivo*) investigation.

4. To evaluate the efficacy of the formulations at protecting spores during *in vivo* passage through the gastro-intestinal tract of sheep (Chapter 5).

5. To consider the potential of these (or similar) formulations for commercial application as a biological control (Chapter 6).

1.2.2 Limitations

The experimental work carried out for this thesis does not aim to cover all the aspects necessary for development of a product with coated spores, but merely to establish which coatings warrant further investigation and development. It does not aim to review all background material on the epidemiology of nematodosis, but there will be a review of primary factors influencing the percentage and rate of development of common trichostrongylids of sheep and cattle, and how these factors relate to the ecology of the fungi.

1.3 Literature Review

1.3.1 The parasites & their lifecycles.

Most parasitic nematodes of sheep and cattle follow a similar direct lifecycle, illustrated in Figure 1-1, below. The following account is largely taken from Vlassoff *et al.* (2001). Eggs are passed in faeces and these hatch and develop through two feeding stages, each of which is concluded by a moulting of the cuticle. The second moult is incomplete; leaving the non-feeding, infective, third stage larvae with the retained second stage cuticle acting as a protective sheath. These larvae then migrate onto the herbage and following ingestion by a suitable host, ex-sheath. This occurs in response to pH, temperature and oxygen concentrations in the appropriate organ. Two more moults usually occur before development is complete.

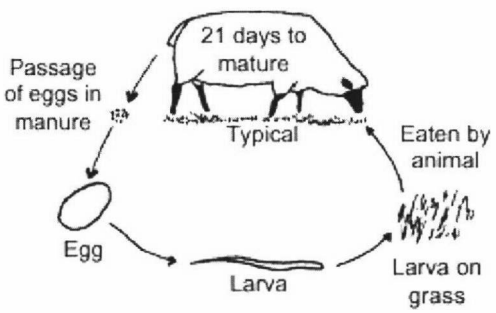


Figure 1-1.General lifecycle of trichostrongylid parasites of sheep and cattle (adapted from Department of Animal Science, Oklahoma State University website, http://www.ansi.okstate.edu/exten/sheep/f3858/f-3858.html).

The percentage of eggs which develop successfully to infective larvae, and the rate at which this occurs, will vary according to climatic conditions, particularly moisture and temperature. Moisture is considered to be a more significant limiting factor for sheep

parasites, due to the lower moisture levels of ovine faeces compared to that of cattle.

1.3.2 Parasites of sheep

There are several species of nematodes that parasitise sheep in New Zealand. These are summarised in Table 1-1 (McKenna, 1997). The presence of some species (e.g. *Ostertagia ostertagi* and *Trichostrongylus capricola*) is due to the cross-grazing of sheep and goats with cattle, and these incidental infections generally occur in low numbers. Another species, *Oesophagostomum columbianum*, has only been found in individuals that were imported from Australia. Production losses are generally associated with infections of *Haemonchus contortus, Ostertagia (Teladorsagia) circumcincta* and *Trichostrongylus axei* in the abomasum, and *Trichostrongylus* spp., *Nematodirus* spp. and to a lesser degree *Cooperia* spp. in the small intestine. Clinical lungworm infections are relatively rare in New Zealand (Vlassoff *et al.*, 2001). Recent classifications of the genera in the sub-family Ostertagiinae, have indicated that the name *Teladorsagia circumcincta* is more appropriate than *Ostertagia circumcincta*, due to certain anatomical features of the adult worms. However, as most of the literature reviewed in this thesis refers to the parasite as *Ostertagia circumcincta*, this name has been used to avoid confusion.

Table 1-1 List of nematode parasites recorded from sheep in New Zealand

Lung	
Dictyocaulus filaria	*Protostrongylus rufescens*
Muellerius capillaries	
Abomasum	
Haemonchus contortus	*Ostertagia trifurcata*
Ostertagia (Teladorsagia) circumcincta	*Ostertagia pinnata*
Trichostrongylus axei	*Ostertagia ostertagi**
Small Intestine	
Brunostomum trigonocephalum	*Capillaria bovis**
Cooperia curticei	*Cooperia surnabada (mcmasteri)**
*Cooperia oncophora**	*Cooperia punctata**
Nematodirus abnormalis	*Nematodirus filicollis*
*Nematodirus helvetianus**	*Nematodirus spathiger*
Trichostrongylus columbriformis	*Strongyloides papillosus*
Trichostrongylus capricola	*Trichostrongylus vitrinus*
Large Intestine	
Chabertia ovina	*Trichuris ovis*
Oesophagostomum columbianum	*Oesophagostomum venulosum*

* Indicates incidental infections picked up from other host species. These are generally considered of minor importance and rarely result in clinical disease.

1.3.3 Parasites of Cattle

Twenty seven species of parasitic nematodes have been identified in cattle in New Zealand, these are listed in Table 1-2 (Bisset, 1994). Similar to the situation with sheep, several species represent incidental infections as a result of cross-grazing with sheep (e.g. *C. curticei*) or deer (e.g. *Ostertagia leptospicularis*). The species that are most widespread and of most economic importance in cattle are *O. ostertagi*, *C. oncophora* and *T. axei*. *O. ostertagi* is considered to be the most pathogenic nematode species infecting cattle, both in New Zealand and around the world (Bisset, 1994). Clinical infection of cattle with lungworm (*D. viviparus*) is relatively more common than in sheep, however, a strong host resistance is usually developed by young calves within a month of initial infection (Bisset, 1994).

Table 1-2. List of nematode parasites recorded from cattle in New Zealand

Lung	
Dictyocaulus viviparus	
Abomasum	
*Haemonchus contortus**	*Ostertagia leptospicularis*
*Ostertagia kolchida**	*Ostertagia ostertagi*
Ostertagia lyrata	*Teladorsagia circumcincta**
*Teladorsagia trifurcata**	*Trichostrongylus axei*
Small Intestine	
Bunostomum phlebotomum	*Capillaria bovis*
*Cooperia curticei**	*Cooperia surnabada (mcmasteri)*
Cooperia oncophora	*Cooperia punctata*
*Nematodirus filicollis**	*Nematodirus helvetianus*
*Nematodirus spathiger**	*Strongyloides papillosus*
Trichostrongylus longispicularis	*Trichostrongylus vitrinus**
*Trichostrongylus colubriformis**	
Large Intestine	
Chabertia ovina	*Oesophagostomum radiatum*
*Oesophagostomum venulosum**	*Trichuris ovis*
Trichuris discolor	

* Indicates incidental infections picked up from other host species. These are generally considered of minor importance and rarely result in clinical disease.

1.3.4 Environmental factors influencing pre-parasitic development and transmission of larvae onto herbage.

1.3.4.1 Micro-environmental factors

The rate of larval development outside the host is influenced by three primary environmental factors; oxygen, temperature and moisture. Correlations have also been found

with other factors, such as faecal dry matter (D.M. Leathwick, unpublished data), illumination and windspeed (Krecek *et al.*, 1992) and dietary forage (Marley *et al.*, 2003). In addition, meteorological conditions such as barometric pressure and cloud cover, and the effect of birds, insects and wild mammals may influence larval development and transmission, and subsequently the likelihood of ingestion by the definitive host (Stromberg, 1997). In order for the infective larvae to migrate on to the herbage, a moisture film is necessary. Larval development, and migration of infective third-stage larvae stops in unsuitable conditions (Romero *et al.*, 1997).

Oxygen is considered the limiting factor that prevents egg development past the morula stage in the gastro-intestinal tract of the animal (Silverman and Campbell, 1959). This is supported by Rose (1961), who reported that *O. ostertagi* eggs suspended in oxygen rich medium develop faster than eggs suspended in medium with low oxygen content. Further, Rose (1961) found that *O. ostertagi* larvae near the edge of the cow pat developed faster than those near the centre, and greater numbers of third stage larvae occurred near the top of the pat, despite an even initial distribution of the eggs. This indicates vertical migration of the larvae. By comparison, oxygen is not considered a major limiting factor for larvae developing in sheep faeces, due to the small size of the faecal pellet and the relatively rapid degradation of sheep faeces.

For parasites of sheep, moisture levels are more likely to affect larval development. As reported by Rossanigo and Gruner (1995) sheep faeces have lower moisture content than cattle faeces and so their parasites are more susceptible to desiccation. Embryonated eggs are more resistant than un-embryonated eggs and pre-infective larvae (Rose, 1961). Studies have shown that third-stage larvae are most resistant to desiccation (Rose, 1961) and this is thought to be due to the protection offered by the retained L2 sheath (Stromberg, 1997). Furthermore, developing larvae of sheep parasites are more tolerant to lower relative humidity than parasites of cattle and deer (Rossanigo and Gruner, 1995). Research in Australia (Beveridge *et al.*, 1989) reports differences in the tolerance of the two small intestinal sheep species, *T. rugatus* and *T. vitrinus* to desiccation and the influence this has on the relative predominance and epidemiology of each species.

Temperature is considered the main limiting factor for larval development in cattle faeces, particularly in areas with high rainfall. Larval development increases with temperature between 10°C - 35°C. However, survival decreases again if temperatures become too high with the optimum temperature for development 25°-27°C. The exact minimum temperature for development varies between species and strains (Stromberg, 1997). An Australian study (Besier and Dunsmore, 1993) found the minimum temperature for development of *H. contortus* to be lower than that reported in a similar study carried out in Kenya (Dinnik and Dinnik, 1958). It is likely that the Western Australia strain of *H. contortus* has become more tolerant to the cooler Australian temperatures, through natural selection. Temperature also influences the rate of migration of infective larvae onto herbage, which increases as temperature decreases (Skinner and Todd, 1980).

1.3.4.2 Dung Microfauna

1.3.4.2.1 Flies (Diptera)

There are surprisingly few published studies on the presence of developing diptera larvae in faeces, and their direct or indirect effect on the development of trichostrongylid larvae. A large portion of the literature that is available was not published in English. Consequently, only the abstracts of these studies are available, at best, for the majority of studies (Sudhaus *et al.*, 1988; Koslov and Chel'tsov, 1986; 1987). Despite this, it is highly likely that the presence of fly larvae in the dung pat will influence the development of trichostrongylid larvae by influencing the degree of aeration and moisture content.

Larvae of several Dipteran species are known to inhabit the faeces of sheep or cattle during their development (Laurence, 1954). Sudhaus *et al.* (1988) suggested it is likely that some of fly larvae facilitate the development of parasitic nematodes by causing aeration of the dung, when oxygen is a limiting factor. The authors stated that this was dependent on the site of pupation. It is assumed that larvae of species which pupate in the faeces will cause more aeration of the faeces than larvae which pupate in the soil. The effect of aeration of the faeces on development of parasite larvae is discussed in more detail for earthworms.

In contrast, Koslov and Chel'tsov (1987) reported that larvae of the Muscid fly *Pyrellia cadeverina* may significantly reduce the number of strongylid eggs and larvae successfully developing and migrating onto herbage. In another study, the same authors (Koslov and

Chel'tsov, 1986) reported that strongylid eggs in water were eaten by 10 to 12 day old larvae of another Muscid species *P. anea.* The authors suggested that the fly larvae were able to control the nematode population by eating nematode eggs and inhibiting hatching. Unfortunately as only a brief abstract was available for these references, they could not be critiqued further.

1.3.4.2.2 Earthworms (Annelida)

Earthworms feed on faeces of many grazing animals (Kraglund *et al.*, 1998; Gronvold, 1979). Several species of earthworms are known to inhabit the soil surrounding and directly under dung pats in the field (Martin *et al.*, 1979). Consequently, they can play an important role in the disintegration and removal of dung pats from the soil surface (Waghorn *et al.*, 2002; Gronvold *et al.*, 1996b; Holter, 1979). Earthworms are very sensitive to factors such as moisture content in the dung (Barth *et al.*, 1995) and so rainfall may have a considerable effect on the number of earthworms invading dung. The seasonal activity of earthworms peaks in autumn and spring, about the same time as parasite larvae numbers. This seasonal variation in earthworm activity has been attributed to fluctuations in soil moisture and temperature (Sharpley and Syers, 1977).

Earthworms influence the rate at which faeces degrades both directly, through ingestion and removal of dung, and indirectly through stimulation of microbial activity and facilitation of weathering due to destruction of the crust (Holter, 1979). By removing dung containing trichostrongylid eggs and/or developing larvae, from the soil surface, earthworms may in turn reduce the number of infective larvae contaminating the herbage (Gronvold, 1987). This was confirmed in a study by Waghorn *et al.* (2002) who reported that earthworms reduced the number of larvae developing from dung deposited on the soil surface. Further, they proposed that parasite eggs were damaged by ingestion (and passage through the digestive tract) of an earthworm. Several studies have indicated that the earthworm *Aporrectodea longa* may be responsible (D.M. Leathwick, unpublished data). In contrast, Gronvold (1979) reported that the eggs and/or larvae of *O. ostertagi* are capable of passing through an earthworm, with no detrimental effects. That experiment involved a mixture of *Lumbricidae* species, including *A. longa*, however the species from which viable *O. ostertagi* were recovered was not stated. Further research would enable positive identification of the species responsible for these differences.

When environmental conditions favour earthworm activity, dung may be disintegrated before nematode larvae reach the infective stage. This, combined with the aeration of the dung through earthworm activity, may result in desiccation of susceptible stages (Gronvold, 1987; Christie, 1963). Conversely, dung aeration may enhance the development of larvae in deeper parts of the dung, which would otherwise be limited by oxygen supply. However, this situation would only occur when rainfall is sufficient to prevent desiccation of the larvae (Gronvold, 1996b). In addition, any stages of larvae that are carried into the soil by earthworms may complete development to the infective stage in a relatively protected environment. This supports the view that soil may act as a reservoir for nematodes, providing protection from desiccation and the environment (Roepstorff *et al.*, 2001; Stromberg, 1997), thereby favouring survival and development of larvae.

There are more publications regarding the interactions between earthworms and ascarids of pigs and dogs, than earthworms and trichostrongylids of sheep and cattle. It should be noted that trichostrongylid eggs are relatively fragile, compared to the eggs of ascarids, the latter being particularly resistant to adverse environmental conditions due to their proteinaceous outer shell. A study by Kraglund *et al.* (1998), reported that *Ascaris suum* eggs transported into the soil by *A. longa* were subject to conditions favourable for survival, but also moved the eggs away from potential hosts. Another study by Mizgajska (2001), found that over 80% of worm casts sampled from suburban areas of Poznan, Poland, contained viable *Toxocara* eggs. This indicates that it is possible for ascarid eggs to remain viable after passing through an earthworm, effectively using the earthworm as a transport host. A study by Oakley (1981) reported that earthworms carried only small numbers of viable *Dictyocaulus viviparus* larvae from the soil to the surface.

1.3.4.2.3 Dung Beetles (Coleoptera)

Several species of dung-burying beetles are capable of reducing the number of infective parasite larvae developing in faeces (Houston *et al.*, 1984; Fincher, 1975; 1973; Bryan, 1972). Consequently, they have been investigated as a possible biological control in their own right (Fincher, 1973). It has also been proposed that additional, exotic species of dung beetles should be introduced to New Zealand in order to help control nematode parasitism of farm livestock (Dymock, 1993).

Throughout the world, there are approximately 7000 species of dung-burying beetles (Coleoptera: Scarabaeidae). Of these, the sub-family Scarabaeinae are the most coprophagous. The tunnelling and feeding activities of adult dung beetles causes aeration of the faeces, which can result in desiccation of the pre-infective stages of parasite larvae under dry conditions (Bryan, 1972). In contrast, a separate study reported that the majority of larvae contained in faeces that were buried while it was fresh, did develop to ensheathed 3rd stage larvae. However, these larvae were then unavailable to infect a potential host (Dymock, 1993). This would be subject to environmental conditions and the species of dung beetle responsible. Some species of dung beetle bury the dung in the top 5 centimetres of soil which would not be sufficient to prevent migration back into herbage. In fact, this shallow burial may assist larval development by offering protection from adverse environmental conditions (Chirico *et al.*, 2003; Bryan, 1976; Houston *et al.*, 1984).

A recent study involving the burial of faeces (Waghorn *et al.*, 2002) to simulate the action of dung beetles reported that the number of larvae recovered increased as a result of burial. This supports the hypothesis that the soil is a more suitable environment for larval survival than on the herbage, and that burial alone does not reduce the development of larvae. It was therefore proposed by Waghorn *et al.* (2002) that the reduction in larval development described above may be due to a direct effect of the beetles, whereby the eggs and/or larvae are detrimentally affected by ingestion of faeces by adult beetles. This possibility has been confirmed by Miller (1961), who reported that helminth eggs may be destroyed by the mandibles of feeding dung beetles.

1.3.5 Nematophagous microfungi

Most fungi (nematophagous fungi included) produce a multi-cellular feeding structure known as mycelium. Mycelium consists of many connected, branching, tubular threads called hyphae, the individual cells of which are separated by cellular cross-walls called septa. Nematophagous fungi may be saprophytic (feed primarily on dead organic matter) and facultative nematophagous predators, or obligate predators of nematodes. The taxonomic classification of fungi is based on the type of sexual spore that is produced. Most nematophagous fungi belong to the Deuteromycota or Fungi Imperfecti, as they have no

known perfect (sexually reproducing) stage. The reproductive structures of the Deuteromycota are asexual spores, known as conidia. Conidia are produced on specialised spore-forming structures called conidiophores.

Nematophagous fungi can be classified into three basic groups; endo-parasitic fungi, fungi capable of infecting parasite eggs, and predaceous (nematode-trapping) fungi. Screening studies in New Zealand (Hay *et al.*, 2002) identified local isolates of several species that were capable of significantly reducing the number of *Trichostrongylus colubriformis* larvae developing in laboratory faecal cultures. These included isolates of *Verticillium* sp., *Harposporium* spp., *Arthrobotrys* spp. *Monacrosporium* spp. and *Nematoctonus* spp.

Endo-parasitic fungi are obligate parasites, and have very limited ability to develop in the absence of, or outside the prey (Larsen, 1999). For example, *Drechmeria coniospora* is an endoparasitic fungus which produces very small conidia that adhere to the cuticle of a passing nematode, which it subsequently penetrates and digests. Another endo-parasite, *Harposporium anguillulae* produces very small conidia, that when eaten by a nematode, settle in the digestive tract. Upon germination, they grow and digest the nematode from the inside out. On the other hand, the nematode-egg infecting fungi, *Verticillium* spp. are able to enzymatically degrade and subsequently infect the egg of *Ascaris suum* (Larsen, 1999).

Predaceous fungi trap and kill nematodes by producing specialised hyphae. The structures that predaceous fungi produce may vary considerably in form depending on the species involved, for example: *Monacrosporium gephyropagum* forms adhesive knobs, *Duddingtonia flagrans* and *Arthrobotrys oligospora* form adhesive nets, *Arthrobotrys dactyloides* forms constricting rings (Kaplan *et al.*, 1991), and *Dactylaria candida* forms non-constricting rings (Saikawa and Takahashi, 2002). As only two species were investigated in the following experimental chapters, only *Arthrobotrys oligospora* and *Duddingtonia flagrans* will be reviewed in detail here.

1.3.5.1 *Arthrobotrys oligospora*

1.3.5.1.1 Taxonomy, morphology and factors influencing growth

Arthrobotrys oligospora (Fresenius 1850) dominated many of the preliminary studies in search of a prospective biological control for parasitic nematodes. This saprophytic,

facultative predaceous nematode-trapping fungus is commonly found in New Zealand soils and naturally invades animal dung under field conditions (Hay *et al.*, 1997a; 1997b; 2000). *A. oligospora* readily forms 3-dimensional adhesive nets from anastomosing vegetative hyphae in the presence of nematodes, this process is described in more detail for *D. flagrans* (section 1.3.5.2.2).

The primary means of reproduction in *A. oligospora* is through the production of conidia. The conidia of *A. oligospora* are thin-walled and uniseptate (possess a single cross wall), and are abundantly produced in clusters along the conidiophore. The conidiophores are tall structures that grow perpendicular to the parent mycelium. The morphology of *A. oligospora* is illustrated below in Figure 1-2. Chlamydospores, or resting spores, are produced in low numbers on the mycelium (Gronvold *et al.*, 1993b).

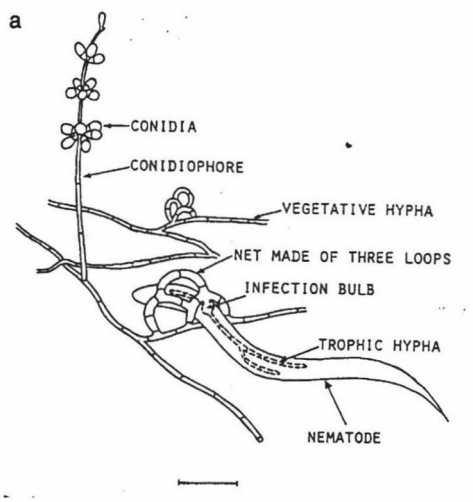


Figure 1-2 – Illustration of *Arthrobotrys oligospora*, scale bar = 100 µm (Gronvold *et al.*, 1993a)

A. oligospora only produces nematode traps when induced to do so due to competition from other saprophytic fungi (Quinn, 1987), or stimulated by the presence of nematodes (Nansen *et al.*, 1988). The nematode traps provide an alternative source of nutrition when nutrient availability is limited (Morgan *et al.*, 1997). Consequently, the nutrient level of the substrate has an important influence on the saprophytic growth and nematode-trapping behaviour of *A. oligospora* (Gronvold, 1989).

Trap stimulation is thought to be by direct physical contact of the nematodes, exposure of fungi to metabolic products excreted by the nematodes, or a combination of both (Nordbring-Hertz, 1977; Nordbring-Hertz and Jansson, 1984). Nematodes are trapped when they stick to the adhesive surface of the mycelium nets as they try to swim through. *A. oligospora* then dissolves the cuticle and penetrates the nematode body, and with specialised hyphae, form an 'infection bulb'. Hyphae then grow from this infection bulb, to fill the entire length of the nematode (Nansen *et al.*, 1986). As death of the nematode often occurs faster than would be expected from hyphal growth, early researchers (Shepherd, 1955; Olthof and Estey, 1963) suggested the fungus secretes a toxin which paralyses the nematode. Further research by Anke *et al.* (1995) reported that linoleic acid is produced by the infection bulb. This substance is nematicidal and may play a role in the killing process.

A correlation between locomotive behaviour and the ability to induce trap formation was reported by Nansen *et al.* (1988). Furthermore, the ability of *Cooperia oncophora* larvae to induce trap formation by *A. oligospora* was similar to two species of soil nematode (Nansen *et al.*, 1986). In the former study, rapidly moving intestinal trichostrongylids of cattle and sheep were reported to be potent trap inducers, while the slow-moving *Dictyocaulus viviparus* was not. Further observation found that free-living and pre-infective *C. oncophora* larvae stopped wriggling a few hours after being trapped in *A. oligospora* nets. By comparison, infective larvae of *C. oncophora* struggled more vigorously for much longer, some for over 20 h. Thus, it is possible that the retained sheath of these infective third-stage larvae offers them limited protection against *A. oligospora.*

The optimum temperature for trap formation of *A. oligospora* is 20°C (Gronvold, 1989). The rate of trap formation is halved at 15°C, and trap formation stops at 5°C. The upper limit for trap formation is 30 °C, which is severely compromised at 25 °C. When temperature is maintained below 25°C, a proportion of traps will retain their trapping ability for at least 7 weeks. Traps kept at 30° and 35°C lose their trapping ability after 7 and 4 weeks respectively. However, this may be partially due to drying out of the agar plates (Gronvold, 1989). Nematode larvae readily develop within the temperature ranges that *A. oligospora* produces traps, as larval development increases between 10°C - 35° C , with an optimum of 25-27°C (discussed in Section 1.3.4.1).

The centre of a bovine faecal pat is anaerobic, with oxygen levels increasing from the periphery over time (Holter, 1991), making the environment within fresh dung unfavourable for *A. oligospora*, which requires oxygen for growth and trap formation (Gronvold *et al.*, 1985; Gronvold, 1989). However, parasite larvae also develop more slowly near the centre of the faeces, where oxygen is a limiting factor. This has been discussed in Section 1.3.4.1.

1.3.5.1.2 The application of *Arthrobotrys oligospora*

Results of previous studies regarding both the *in vitro* and *in vivo* application of *A. oligospora* are summarised in Table 1-3, which is largely taken from Hindhede and Jensen (2002). Briefly, *A. oligospora* showed a lot of promise in early, *in vitro* studies where experimental evidence has shown that *A. oligospora* is capable of trapping larvae from a large range of parasitic nematodes (Nansen *et al.*, 1988). However, its ability to pass through the gastro-intestinal tract of livestock and remain viable is at best, highly variable. Consequently, very little literature has been published on the *in vivo* application of *A. oligospora*, and in recent years, interest has shifted to *Duddingtonia flagrans*. Recent developments include an initial study that has proven it is possible to improve the virulence of nematophagous fungi, with particular reference to *A. oligospora*, through genetic engineering (Ahman *et al.*, 2002).

Studies involving the *in vitro* addition of *A. oligospora* conidia or fungal material to faeces are summarised in Tables 1-3 to 1-6. These studies have shown that *A. oligospora* may be highly effective at reducing the number of larvae developing in faecal cultures, with substantial reductions being obtained at doses as low as 20 conidia per gram of faeces (Hashimi and Connan, 1989).

Summary of literature on *in vitro* addition of *Arthrobotrys oligospora* conidia to faeces

Authors	Details	Dose	Efficacy	Parasite
Gronvold *et al.* (1985)	FEC was 600epg, faeces was cultured at room temperature for 13 days.	High: 2500 conidia/g faeces Low: 250 conidia/g faeces.	99% and 25% reduction for high and low dose rates respectively	*Cooperia oncophora*
Gronvold *et al.* (1988)	Study performed under field conditions	2000 conidia/g faeces	89% reduction in larvae on surrounding herbage.	*Ostertagia ostertagi*
Hamishi and Connan (1989)	Study performed under field conditions. FEC not reported.	20 conidia/g faeces	63% reduction in larvae	*Haemonchus contortus*
Waller and Faedo	FEC not reported	100 conidia/g faeces	79% reduction in larvae in faecal cultures	*H. contortus*

(1993)				
Mendoza de Gives and Vazquez-Prats (1994)	Faecal cultures were incubated for 15 days at room temperature. FEC was 11, 680.	20,000 conidia/g faeces	98.3% reduction in faecal cultures	*H. contortus*
Bird and Herd (1995)	Faecal cultures incubated for 8 days at 26°C	Low: 1 spore/egg Medium: 10 spores/egg High: 100 spores/egg	Low: 40.5% reduction in faecal cultures. Medium: 87.4% reduction. High: 95.8% reduction	Cyathostominae
Charles *et al.* (1995)	Trapping efficacy dependant on concentration of conidia	1000 conidia/g faeces	Greater than 90% reduction	Cyathostominae
Chandrawathani *et al.* (1998)	Faeces had a FEC of 500 epg and was cultured for 14 days at 25°C.	High: 2000 conidia /g faeces. Low: 200 conidia/g faeces.	High: 99.6% reduction in faecal cultures. Low: 18.5% reduction	*Strongyloides papillosus*
Sanyal, (2000a)	Faeces cultured at 28°C for 2 weeks in saturated humidity.	1000+ conidia/g	>98% efficacy in faecal cultures	*S. papillosus*

Table 1-3 Summary of literature on *in vitro* addition of *Arthrobotrys oligospora* conidia to faeces

Summary of literature on *in vitro* addition of *Arthrobotrys oligospora* fungal culture to faeces				
Authors	Details	Dose	Efficacy	Parasite
Gronvold *et al.* (1987)	Experiments were run for 29 days, under natural field conditions, during the summertime, in Denmark.	10g mycelium/1kg faeces (with or without fungal growth medium)	86% and 96% reduction on surrounding herbage, with and without growth medium respectively.	*Cooperia oncophora*
Gronvold *et al.* (1989)	This study was carried out under field conditions in Denmark.	0.250g mycelial fragments per kg faeces	42% reduction of larvae in cow pat and 50-71% reduction in surrounding herbage.	*Ostertagia ostertagi*
Larsen *et al.* (1991)	Faecal cultures were incubated for 3 weeks at 22°C	40 barley grains containing fungal material/125g faeces	86% reduction of larvae developing in faecal cultures	*O. ostertagi*
Hay *et al.* (2000)	Faecal cultures were incubated for 44 days at 15°C	Suspension of fungal macerate (2x 5mm plugs of agar)	90.2% reduction of larvae developing in faecal cultures	*Trichostrongylus colubriformis*
Yakchali *et al.* (2001)	Faecal cultures carried out under laboratory conditions.	2 colonies per 100cc, 200cc or 250cc.	Reduction in faecal cultures 49%, 34% and 37% respectively	Cyathostominae
Santos *et al.* (2001)	The effect of various constant temperatures on the predatory activity of *A. oligospora* was investigated. Significantly reduced L3 when mixed in faeces and cultured at temperatures allowing the development of pre-parasitic cyathostomes.	Not stated in abstract (Published in Portugese).	>90% when incubated at 25°C or 30°C. efficacy was >90% when incubated at all temp (10°C, 15°C, 20°C, 25°C and 30°C) if the cultures were incubated for an additional 14 days at 27°C.	Cyathostominae

Table 1-4 Summary of literature on *In vitro* addition of *Arthrobotrys oligospora* fungal culture to faeces

Summary of literature on *in vitro* Addition of *Arthrobotrys oligospora* fungal culture to Petri dish				
Authors	Details	Dose	Efficacy	Parasite
Mendoza *et al.* (1994)	50 infective larvae were incubated at 18°C for 7 days.	Fungus cultured on Corn Meal Agar	25.71% reduction	*Haemonchus contortus*

Table 1-5 Summary of literature on *in vitro* addition of *Arthrobotrys oligospora* fungal culture to Petri dish

Summary of literature on *in vitro* rumen simulation stress selection of *Arthrobotrys oligospora*		
Authors	Details	Parasite
Larsen *et al.* (1991)	Soil and compost containing fungal spores was exposed to a rumen simulation bioassay. An isolate of *A. oligospora* remained viable and exhibited predaceous activity following bioassay treatment.	*Ostertagia ostertagi*

Table 1-6 Summary of literature on *In vitro* rumen simulation stress selection of *Arthrobotrys oligospora*

The publications summarised in Table 1-7 indicate that the ability of *A. oligospora* to survive passage through the gastrointestinal tract is highly variable, at best. The larval reductions tend to be lower than desired (50-60%), and only obtained when unpractical quantities of fungus are administered (Hashimi and Connan, 1989). The single study in which high efficacies were obtained (Larsen *et al.,* 1991) has not been repeated, despite attempts to do so by the authors. However, as *A. oligospora* is capable of effectively reducing larvae *in vitro*, it is an excellent candidate for application with a protective coating that would improve the rate of survival through the gastrointestinal tract.

Summary of literature on *in vivo* passage of spores of *Arthrobotrys oligospora* conidia				
Authors	Details	Dose	Efficacy	Parasite
Hashmi and Connan (1989)	The larval counts were still very high and the authors suggest that the amount of passed fungus was probably small.	$8x10^6$ conidia fed twice weekly for 3 months	51% and 62% reduction of *C. oncophora* and *O. ostertagi* L3 respectively.	*Cooperia oncophora, Ostertagia ostertagi*
Larsen *et al.* (1992)	Calves with FECs of 200-300 epg were fed fungal culture on barley grains. Faeces was collected on days 4 and 5 after feeding, and incubated for 4 weeks at 22°C. The *A. oligospora* isolate used was identified in Larsen *et al.* (1991)	Fungal culture on barley grains (dose unknown)	91% reduction for dung pat bioassay. 99% reduction for faecal cultures	*O. ostertagi*
Gronvold *et al.* (1993a)	Basically, this was a copy of Larsen et al (1991), but there were no significant reductionin larval development, which could not be explained by the authors.	100g barley	No significant effect.	*O. ostertagi*
Waller *et al.* (1994)	A proportion of unprotected conidia fed to calves in a water	$1.2x10^6$ conidia	No significant reduction.	*Haemonchus contortus*

	solution was able to pass through the gastrointestinal canal of sheep in 24h and subsequently give rise to fungal growth. The fungus was selected by the method described in Larsen *et al.* (1991)			
Yakchali *et al.* (2001)	*A. oligospora* survived passage through the horse and reduced the number of larvae developing in faeces from horses that received 250 colonies.	120, 200 or 250 colonies. Details of fungal inoculum not stated.	Not stated in abstract (published in Arabic)	Cyathostominae

Table 1-7 Summary of literature on *in vivo* passage of *Arthrobotrys oligospora* conidia

A. oligospora produces conidia abundantly when grown on Corn Meal Agar (personal observation), a relatively low-nutrient medium. In contrast to *D. flagrans*, chlamydospores, or resting spores are produced only in low numbers (R.A. Skipp, personal communication). Consequently, the experimental inoculum of *A. oligospora* consisted of either conidia or mycelium, which are usually produced on cereal grain cultures.

Studies investigating *in vivo* passage of conidia or fungal material have not been encouraging. Since Larsen *et al.* (1992) there has been only one report (Yakchali *et al.*, 2001) of successful larval reduction following passage through the gastrointestinal tract. That particular study (Yakchali *et al.*, 2001) could not be critiqued fully as it was not published in English, only the abstract was available.

1.3.5.2 *Duddingtonia flagrans*

1.3.5.2.1 Taxonomy, morphology and factors influencing growth

Duddingtonia flagrans was initially classified by C.L. Duddington (1949) as *Trichothecium flagrans*. Later, it was renamed by Cooke (1969) as *Duddingtonia flagrans* the single species in the genus *Duddingtonia*. More recent studies (Barron, 1981; Bird and Herd, 1995; Scholler *et al.*, 1999) have referred to the species as *Arthrobotrys flagrans*. However, as most of the literature reviewed in this thesis refers to this fungus as *Duddingtonia flagrans*, this name has been used to avoid confusion.

D. flagrans has received more attention than other species in recent years, and is considered the most likely prospect for commercial application as a biological control

(Larsen, 2000). This is due to its superior ability to pass through the gastro-intestinal tract of an animal and remain viable. It has been reported as occurring naturally in several countries including New Zealand (Skipp *et al.*, 2002), Australia (Faedo *et al.*, 1997), Denmark (Larsen *et al.*, 1991) and South Africa (Faedo and Krecek, 2002), usually in association with organic matter such as compost or faeces.

The mycelium of *D. flagrans* is formed from branched, septate, smooth-walled hyphae. As with many nematode-trapping fungi, *D. flagrans* only produces traps when induced to do so, by the presence of migrating nematodes (Gronvold *et al.*, 1996a). The traps are formed from specialised hyphae, a process described in Gronvold *et al.* (1996a) and illustrated in Figure 1-3. Initially, a lateral branch will grow out from the parent hypha (figure 1-3a), this branch will then curl back towards the parent hypha and anastomose, forming a loop (figure 1-3b). From this loop, more loops may form (figure 1.3c), eventually forming a 3-dimensional adhesive trap network.

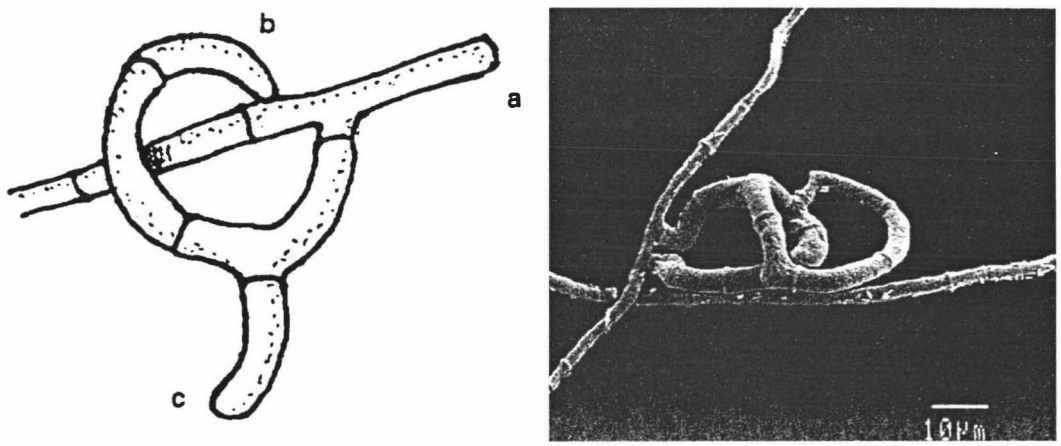


Figure 1-3a thru c –Formation of *D. flagrans* traps; a- lateral branch growing from parent hyphae, b- branch curls back and anastomoses with parent branch, c- secondary loops form off primary loop (from Gronvold *et al.*, 1996a). Figure 3d – Scanning Electron Microscope image of *D. flagrans* net (Gronvold *et al.*, 1993).

D. flagrans produces two types of spores. The type produced is largely dependant on the age of the fungi, although it may also be influenced by nutrient availability. Young cultures produce obconical to ellipsoidal thin-walled conidia, with a single septum (illustrated in Figure 1-4). These are 25-50 by 10-15 μm in size and are formed, one at a time, on the tips of single, unbranched conidiophores. By comparison, mature cultures produce large numbers of intercalary (= between cells), thick-walled chlamydospores, or resting spores (illustrated in

Figure 1-5). These chlamydospores are spherical, about 20-30 µm and are red-brown in colour. They commonly have a rough, knobbed appearance and are resistant to unfavourable environmental conditions (Larsen *et al.*, 1991).

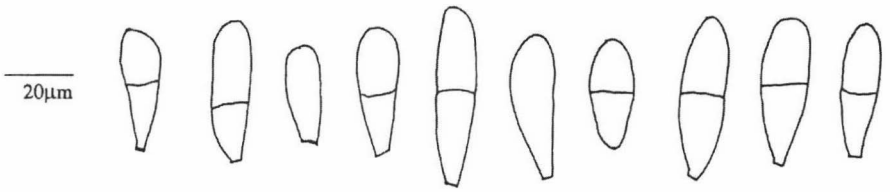


Figure 1-4 Variations of conidia of *Duddingtonia flagrans* (Skipp *et al.*, 2002)

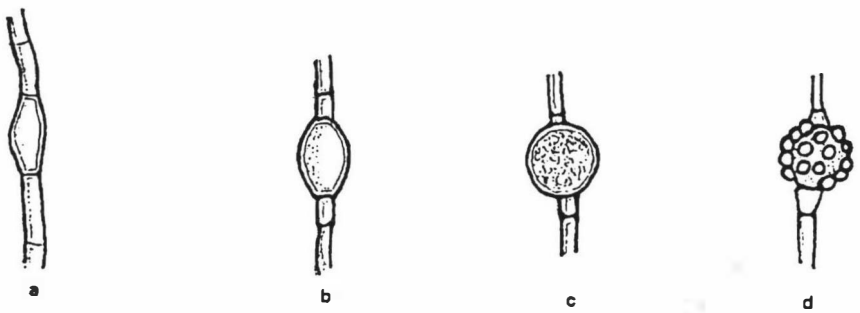


Figure 1-5 Formation of intracalary chlamydospores of *Duddingtonia flagrans* (Gronvold *et al*, 1996a)

D. flagrans grows well and produces large numbers of conidia when grown on Corn Meal Agar, a relatively low-nutrient medium, for 2 weeks at 20°C (R.A. Skipp, pers. comm.). Alternatively, *D. flagrans* produces abundant chlamydospores when grown on sterile barley grain for at least 3 weeks, at 20°C (Clarke, 2003). Morgan *et al.* (1997) reported peak growth between 25-33°C. The optimum temperature for mycelial growth of *D. flagrans* occurs at temperatures of approximately constant 20°C (maximum used) or fluctuating about 15°C (10-25°C) (Fernandez *et al.*, 1999e). *D. flagrans* is capable of producing nets at temperatures between 10-30°C when induced by *C. oncophora* larvae. These high optimum temperatures increase survival in compost and dung.

Mycelial growth is largely unaffected by pH, and remains high between pH 6.3-9.3 (Gronvold *et al.*, 1999). Measurements were not taken below pH 6.3 as the agar was liquid at pH 5.1. In contrast, production of nets was affected by pH, with an optimum at pH 7 and less vigorous net production at 6.3-9.3 (Gronvold *et al.*, 1999). The pH of a cow pat (pH 6-9) is thus well tolerated by *D. flagrans*.

A recent study by Gronvold *et al.* (2004) assessed the effect of several species of faecal bacteria and fungi on the behaviour of *D. flagrans*. They demonstrated that the presence of selected bacteria or fungal species previously isolated from cattle faeces reduced the growth of *D.* flagrans on agar plates. However, when high numbers of the same fungal and bacterial species were added to cattle faeces containing *D. flagrans* chlamydospores and infective *C. oncophora* larvae, they failed to reduce the ability of *D. flagrans* to trap larvae in faecal cultures. Furthermore, increasing the concentration of *D. flagrans* chlamydospores increased larval reduction with no indication of inter-specific competition.

D. flagrans continues to grow at a normal rate in low-oxygen conditions, with a lower limit of 6% O_2, by volume (Gronvold *et al.*, 1999). Thus, *D. flagrans* only grows in the outer 5 mm of the cattle faecal pat, due to reduced oxygen tension within the faecal mass. As the dung pat ages, and oxygen tension increases, *D. flagrans* may become more active in the central part of the pat. Although *D. flagrans* does not grow under anaerobic conditions, spores remain viable and it remains capable of producing traps for at least 3 weeks.

1.3.5.2.2 The application of *Duddingtonia flagrans*

Despite a large number of *in vivo* studies and considerable interest in the use of this fungus, there is limited literature on the behaviour of spores under some form of *in vitro* simulated rumen environment. Literature regarding both the *in vitro* and *in vivo* application of *D. flagrans* will be discussed in more detail in the relevant chapters (Chapters 2, 4 and 5). For review see Hindhede and Jensen (2002). Briefly, there have been many publications reporting successful larval reduction following *in vivo* passage of spores through the gastrointestinal tract of various animals. *D. flagrans* has been proven effective for a large range of host and parasite species, including *Haemonchus contortus* in sheep (Pena *et al.*, 2002), strongyles in horses (Fernandez *et al.*, 1997), *Oesophagostomum dentatum* and *Hyostrongylus rubidus* in pigs (Nansen *et al.*, 1996) and trichostrongylid infections in calves (Sarkunas *et al.*, 2000).

Studies involving the *in vitro* evaluation of *D. flagrans* are summarised in Tables 1-8 and 1-9. These highlight the variation around dose rate and resultant efficacy. For example, Waruiru (2001) reported a 75% reduction of *H. contortus, T. axei* and *C. oncophora*

following the addition of 1000 or 5000 chlamydospores/g. By comparison, Gronvold *et al.* (2004) demonstrated >93% efficacy when 250 chlamydospores/g were added to the faeces. Although substantial reductions have been reported from the addition of mycelium to faeces (Larsen *et al.*, 1991), it is difficult to quantify the amount of fungal material in such inoculations. Only one publication (Pountney, 1999) reports quantitative analysis of chlamydospore survival like the experiments reported in this thesis. Pountney (1999) carried out a series of experiments demonstrating the negative effect of *in vitro* rumen simulation and *in vivo* passage on chlamydospore survival.

Summary of literature on *in vitro* addition of conidia/chlamydospores to faeces				
Authors	Details	Dose	Efficacy	Parasite
Henriksen *et al.* (1997)	Artificial cow pats of 200g .	$5x10^4$ chlamydospores /g	86%	*Dictyocaulus viviparus*
Petkevicius *et al.* (1998)	High and similar reduction capacity of 3 isolates of *D. flagrans*, irrespective of undigested fibre and dry matter (DM) content of faeces. Greater L3 development in faeces with high fibre/DM content.	$5x10^3$ chlamydospores /g	60 to 89%	*Oesophagostomum dentatum*
Fernandez *et al.* (1999e)	The growth rate and efficacy for 5 isolates of *D. flagrans* was compared at 3 temperatures, under fluctuating and constant conditions.	$6.250x10^3$ chlamydospores /g	Efficacies; 18-25% and 48-80% at 10°C, 70-96% and 93-95% at 15°C, and 63-98 and 0-25% at 20°C, constant and fluctuating respectively.	*Cooperia oncophera*
Fendandez *et al.* (1999c)	Fungal treatment was more effective for gastrointestinal parasites than on *D. viviparus*.	$6x10^3$ and $1.3x10^4$ chlamydospores /g	Up to 81% on L3	*D. viviparus, C. oncophora, Ostertagia ostertagi*
Gronvold *et al.* (1999)	Light, temperature and biochemical properties in dung affect the fungus. Trap formation is induced by parasites, and growth occurs under micro-aerophilic conditions.	Not stated.	Not discussed.	*C. oncophora*
Santos *et al.* (2001)	The effect of various constant temperatures on the predatory activity of *D. flagrans* was investigated. Significantly reduced L3 when mixed in faeces and cultured at temperatures allowing the development of pre-parasitic cyathostomes.	Not stated in abstract (published in Portuguese).	47.5% and 41.8% when incubated at 10°C and 20°C respectively.	*Cyathostominae*

Waruiru (2001)	The efficacy of *D. flagrans* in 4 FEC levels and 4 fungal concentrations, for 4 species of parasitic nematode was investigated. Percentage reductions in faecal cultures increased with corresponding increase in fungal concentration and epg levels for all 4 species of nematode.	1000, 5000 and 25,000 chlamydospores /g	>75% for *H. placei, T. axei* and *C. oncophora* at 200-280 epg. 53% for *O. radiatum* at 1000 and 5000 spores/g	*Haemonchus placei, Trichostrongylus axei, Cooperia oncophora, Oesophagostomum radiatum*
Waghorn *et al.* (2002)	Powdered alginate inoculum of *D. flagrans* added to faeces and homogenised and 40g of faeces added to plots. More of same inoculum added to surface of faeces.	Total of 0.01g of alginate formulation/g faeces.	79%	*Ostertagia circumcincta*
Clarke (2003)	Fresh spores were mixed into laboratory faecal cultures. Cultures were incubated for 14 days at 20°C at saturated humidity.	100, 1000, 3000 and 10,000 chlamydospores /g	55%, 91%, 98%, and 99% respectively.	*Cooperia, Ostertagia, Trichostrongylus, Haemonchus*
Gronvold *et al.* (2004)	Increasing chlamydospore concentration increased nematode mortality to 99%, indicating no severe intraspecific competition between the *D. flagrans* chlamydospores.	250, 2500, 25,000, 50,000, 200,000 chlamydospores /g	Over 93% when at least 250 spores/g are added.	*C. oncophora*

Table 1-8 Summary of literature on *in vitro* addition of *Duddingtonia flagrans* conidia/chlamydospores to faeces

Summary of literature on *in vitro* stress selection/ *In vitro* trap formation with/without larval reduction				
Authors	Details	Dose	Efficacy	Parasite
Larsen *et al.* (1991)	Isolation of *D. flagrans* from compost and compost soil. *In vitro* stress selection of fungi with verification of predacious efficacy of surviving isolates in a dung pat bioassay.	40 barley grains with fungal material/125 g faeces.	Approx 90% reduction of L3	*Ostertagia ostertagi*
Gronvold *et al.* (1996a)	Rate of trap formation has an optimum temperature of 30°C, where 200 L3/cm2 induces 700-800 traps/cm2/2days	N.A. Culture grown on agar.	Not discussed	*O. ostertagi*
Carvalho and Bernardo (1998)	Cyathostome larvae were added to plate cultures of *D. flagrans.* Plates were examined 48h, 7, 15 and 30 days later or baermannised 24, 48 and 72h later	N.A. Culture grown on agar.	Larvae in plates reduced by 84.8, 94.9, 97.3% respectively. Baermann recoveries were 98.7, 98.7 and 96.9% reduced respectively.	Cyathostominae
Flores Crespo *et al.* (1999a)	Comparison of 2 isolates of *D. flagrans,* added to Petri dishes containing free-living nematodes on flour-maize agar. The nematophagous capacity of *D. flagrans* was not affected in spite of being kept for one year at laboratory temperature.	Not stated in abstract (Published in Spanish)	Efficacy not stated. Nematophagous capacity of both cultures were similar but significantly different from controls.	*Panagrellus redivivus*

Flores Crespo *et al.* (1999b)	Isolates of *D. flagrans, Monacrosporium eudermatum, Arthrobotrys* spp and *Dactylaria* spp showed excellent (<90%) predatory activity.	Not stated in abstract (Published in Spanish)	>90% reduction of nematodes on agar plates.	*P. redivivus*
Flores Crespo *et al.* (1999c)	150 nematodes were added to petri dishes containing either a French or a Mexican strain of *D. flagrans* growing on corn flour agar. Efficacy determined by counting nematodes 5 days after addition to agar plates.	N.A. Fungal culture grown on agar.	98.9% efficacy for Mexican strain. 97.7% efficacy for French strain.	*P. redivivus*
Mendoza de Gives *et al.* (1999)	*D. flagrans* showed superior trapping efficiency compared to isolates of *Monacrosporium* and *Arthrobotrys* spp.	N.A. Fungal culture grown on agar.	Efficacy >90% for *C. elegans*, 41-7-% for *H. contortus*, 5-22% for *T. axei*, and 30-57% for *O. circumcincta*.	*Caenorhabditis elegans, Haemonchus contortus, O. circumcincta, Trichostrongylus axei*
Pountney (1999)	Quantitative evaluation of chlamydospore survival following *in vitro* rumen simulation and *in vivo* passage through bovine and equine gastro-intestinal tract	Not relevant	Not discussed	N.A.

Table 1-9 Summary of literature on *in vitro* stress selection/*in vitro* trap formation of *Duddingtonia flagrans* with/without larval reduction

The interest in biological control of parasites, with particular reference to *D. flagrans,* has increased recently, with the number of publications of *in vivo* studies more than doubling over the last 3-4 years. The standard dose rate for cattle is $5x10^5$ chlamydospores/kg, twice that usually administered to sheep in order to obtain an efficacy of at least 90% (R.A. Skipp, personal communication). When a very high efficacy (>98%) is desired, it is generally necessary to use a dose several magnitudes higher than expected to obtain a moderate (>75%) efficacy. However, there is a considerable degree of variation in efficacy for any given dose rate. The studies included in Table 1-10 demonstrate that *D. flagrans* is capable of reducing the number of larvae developing in faecal cultures by 80-90% following *in vivo* application, provided a dose of at least $1x10^5$ chlamydospores/kg live weight is administered. Efficacies in excess of 90% were obtained by increasing the dose rate. This dose is less than half the recommended dose rate for sheep and goats.

Summary of literature on *in vivo* passage of spores with/without larval reduction				
Authors	Details	Dose	Efficacy	Parasite
Larsen *et al.* (1992)	Calves fed barley fungal culture. Fungi isolated from faeces which was also cultured to demonstrated larval reduction	The dose rate was not stated, but it was assumed (by association) to be chlamydospores grown on barley	L3 reduced by 76-99%	*O. ostertagi*

		grain, fed at 200g/calf/day.		
Gronvold *et al.* (1993a)	Calves were orally dosed with *D. flagrans* and 1 kg artificial dung pats made from their faeces.	10^9 spores/calf/day	74-85% reduction in infectivity of herbage	*O. ostertagi*
Wolstrup *et al.* (1994)	Calves orally dosed with *D. flagrans*. Results included reduced herbage infectivity and abomasal larval count, and better weight gain.	100g fungal barley culture, twice daily.	Up to 87% reduction in abomasal worm count	*O. ostertagi*
Larsen *et al.* (1995a)	Oral administration of *D. flagrans* to horses. Positive correlation between dose rate and larval reduction	10^3 10^6 10^7 fungal unit/kg. Dose was <5% conidia and >95% chlamydospores.	Larval development (based on FEC) was 0.1-10.8% for 2 higher doses.	Cyathostominae
Larsen *et al.* (1995b)	Oral dosing of calves every day for 2 months resulted in absence of clinical parasitosis, increased weight gain, low pepsiongen levels, decreased herbage contamination, decreased larval counts in faecal culture	The dose rate was not stated, but was assumed (by association) to be 200g barley. Barley culture yielded approximately 10^6 spores/g	Not stated.	*O. ostertagi, C. oncophora, Nematodirus*
Nansen *et al.* (1995)	Feeding *D. flagrans* to first season grazing cattle, during the initial 3 months of the grazing season prevented clinical trichostrongylidosis. 200g fungal barley fed to each animal per day. Concentration of spores approximately 10^6 chlamydospores/g of fungal barley.	Approximately 10^6 chlamydospores/kg live weight/day	>95% efficacy	*O. ostertagi, C. oncophora*
Larsen *et al.* (1996)	FECs were similar in treated and control groups. The percentage of larvae developing to L3 in faecal cultures was 1-14% for treated animals, and 19-52% in controls. Treatment also resulted in low herbage contamination, and significantly lower worm counts, as documented by tracer foals. Clinical signs in controls included ill thrift and lower weight gain.	$5x10^6$ chlamydospores/kg	Several species approached total reduction in fungus fed treatments, with significant reductions in FEC, worm counts and pasture contamination. Efficacy not discussed.	Strongylidae spp., Cyathostominae spp.
Nansen *et al.* (1996)	Addition of *D. flagrans* to daily feed ration resulted in decreased larval counts.	$5x10^6$ chlamydospores/kg/day	86% and 72% (*O. dentatum* and *H. rubidus* respectively) reduction in worm counts of tracer pigs.	*O. dentatum and Hyostrongylus rubidus*
Faedo *et al.* (1997)	Sheep were orally dosed with *D. flagrans*. High dose rates obtained similar efficacy to lower dose rates	10^5-$5x10^6$ chlamydospores per sheep. Dose was ½ conidia	Efficacy approaching 100%, L3 reduced to	*Trichostrongylus colubriformis*

		and ½ chlamydospores.	negligible numbers at 1-$5x10^5$	
Fernandez *et al.* (1997)	Horses were orally dosed with *D. flagrans*. Reduction in larval recoveries from faecal cultures varied with dose level and time of year	10^6 and $5x10^6$ chlamydospores/kg live weight	65-99.7% efficacy in faecal cultures, 65-95% reduction of pasture contamination	Strongylidae spp., Cyathostominae spp.
Githigia *et al.* (1997)	Lambs were offered *D. flagrans* in feed. L3 development in treated groups 1-28%, compared to 60-80% in untreated groups.	10^6 chlamydospores/kg	Tracer worm counts; treated paddocks had 86% fewer parasites.	*Ostertagia, Trichostrongylus*, and *Nematodirus* spp.
Faedo *et al.* (1998)	The fairly low effect may be explained by dry faeces (sheep pellets) and dry weather (plots had to be irrigated).	$5x10^6$ chlamydospores/sheep/day	43% reduction in faecal field plots	*T. colubriformis*
Larsen *et al.* (1998)	Oral dosing of normal sheep and inter-cannulae dosing of surgically modified sheep. A compartmentalised study of the gastrointestinal system showed no specific viability limiting gut compartment.	$5x10^5$ - 10^6 propagules (chlamydospores 70/ conidia 30) per sheep per day	>80% larval reduction	*T. colubriformis, H. contortus.*
Llerandi-Juarez & Mendoza de Gives (1998)	Sheep dosed orally, spores of *D. flagrans* isolated from faeces 22—32h post administration. Re-isolated spores were capable of larval reduction on agar plates.	$5x10^5$ spores/sheep/day	Efficacy not stated.	*H. contortus*
Mendoza de Gives *et al.* (1998)	A single dose of *D. flagrans* spores resulted in a reduction of L3 that persisted for 4-5 days	10^7 chlamydospores/animal	88% efficacy in laboratory faecal cultures	*H. contortus*
Fernandez *et al.* (1999a)	The activity of *D. flagrans*is higher in horse faeces indicating a more favourable micro-environment for fungal growth (*c.f.* cattle faeces). Survival through the gastrointestinal tract may also be different.	10^6 chlamydospores/kg live weight	L3 reduction of 98.4%, herbage infectivity reduction of 85.8-99.4%	Strongylidae spp., Cyathostominae spp.
Fernandez *et al.* (1999b)	Calves dosed orally with *D. flagrans*, at different dose rates and different stocking densities. Effect was less obvious at lower stocking density.	2 dose levels: 10^6 and $2.5\text{-}5x10^5$ chlamydospores/kg live weight	81.9% larval reduction at higher dose rates	*O. ostertagi*
Fernandez *et al.* (1999d)	Calves dosed orally and faeces mixed 1:1 with faeces containing parasites. Artificial dung pats placed on pasture plots and subsequent L3 herbage contamination was quantified. Large variation in efficacy between years. No significant difference between efficacy of isolates.	10^6 chlamydospores/kg live weight/day	30.3 - 95.7% for C13 isolate, 18.1 - 98.1% for Troll A isolate.	*O. ostertagi*
Baudena *et al.* (2000)	Horses dosed orally. Faeces mixed with faeces from parasite infected horses	$2x10^6$ spores/kg live weight ($1x10^6$ twice daily)	66-99% reduction of pasture contamination	Strongylidae spp., Cyathostominae spp.
Faedo *et al.* (2000)	Significant reductions were seen if spores and parasite eggs were deposited simultaneously (sim plots)	10^6chlamydospores/g for sim plot and 10^7 for post plot in 1998. In	Not discussed, but L3 recovery was significantly	*Nematodirus* spp. *Trichostrongylus* spp., *Ostertagia*

		1999 all treatments were 10^6 spores/g.	lower in sim plots	spp.
Lukyanchenko (2000)	*D. flagrans* fed to horses reduced the number of strongylid larvae developing in faecal cultures following passage through the digestive tract.	Not stated in abstract (published in Russian)	98.6% at 7 days incubation and 99.9% at 14 days	Strongylid
Sarkunas *et al.* (2000)	Oral dosing of calves with *D. flagrans* prevented clinical parasitosis, increased weight gain, decreased pasture contamination. Reduction larval recoveries from faecal culture varied with time of year.	10^6 chlamydospores/kg live weight/day	61-97% reduction in faecal cultures	*Ostertagia* spp., *Cooperia* spp., *Nematodirus* spp.
Sanyal, (2000b)	Isolates from sheep and buffalo proved superior in growth, predaceous activity, germination potential, and survival through ruminant gastrointestinal tract, compared to other fungi.	$2x10^7$ chlamydospores/animal (equivalent to $4x10^5$ and $1.5x10^6$ chlamydospores/kg for cattle and sheep respectively)	Spores were detected, but efficacy was not measured following *in vivo* passage.	*H. contortus*
Flores Crespo *et al.* (2001)	Sheep dosed orally with chlamydospores.	$120x10^6$ chlamydospores per sheep	95.4% reduction in faecal cultures	*H. contortus*
Knox & Faedo (2001)	Lambs offered barley grains containing *D. flagrans* culture. Large variation between treated groups. Good "consumers" benefited from fungal treatment, while poor "consumers" did not.	$5x10^7$ – $1x10^6$ chlamydospores per 10 sheep	No significant effect of fungal treatment in worm counts.	*O. circumcincta T. colubriformis, T. axei, Oesophogostomum spp., H. contortus,*
Sanyal (2001)	Sheep orally dosed with spore suspension twice daily. Differences in larval recoveries from faecal cultures were significant from 1 week into the trial.	$5x10^5$spores/kg twice daily	L3 in faecal cultures was approx. 750 LPG in controls and approx. 150 LPG in fungus fed group.	*H. contortus*
Waller *et al.* (2001b)	Sheep offered barley grains containing *D. flagrans* culture as feeding block or supplementary diet. As little as 5g barley culture/sheep/day may virtually eliminate larvae from faecal culture..	$3x10^6$ chlamydospores/day	Almost total reduction obtained by feeding 10g barley/sheep/day (3x106 spores/day)	*T. colubriformis*
Chandrawathani *et al.* (2002)	*D. flagrans* administered either as a grain supplement or incorporated into a feed block.	$1x10^6$ spores/animal/day	>90% efficacy in faecal cultures.	*H. contortus*
Kahn *et al.* (2002)	Faeces was collected 2-3 times/day and cultures performed for each collection. Faecal cultures were incubated for 14 days at 25°C. % reduction based on FEC, however % development of controls was not stated (control = same sheep prior to fungal treatment).	$1.5x10^6$ once or $3x10^6$ twice (2h between doses)	Larval development was 7.1% and 16.6% for 2 dose rates respectively for 25h post dosing cultures	*H. contortus*
Pena *et al.*	Sheep orally dosed with *D. flagrans.*	$2.5x10^5$ - $2.5x10^4$	97-100%	*H. contortus*

(2002)	All doses obtained a high level of larval reduction from 2 days after commencement of feeding.	spores/kg	reduction in faecal cultures	
Sanyal & Mukhopadhyaya (2002)	Faeces from sheep and calves was cultured on agar plates baited with *H. contortus*. Faeces from goats was cultured and spread over herbage. Larval recoveries were not stated in terms of efficacy.	Sheep and calves $1x10^7$ each. Goats $1x10^6$ chlamydospores/kg	Total reduction of L3 on agar at 72h by DFS2550 & 80% reduction by DFS2507 at 96h. Goats; treated groups approached total reduction in faecal cultures and herbage contamination.	*Haemonchus* spp., *Cooperia* spp., *Oesophogostomum* spp.
Dimander *et al.* (2003)	Evaluation of 4 management strategies to control parasitism in first season grazing cattle, over 3 consecutive years. Significant benefits of treatment with *D. flagrans* chlamydospores was reported at ½ the previously reported minimum effective dose. Fungal treatment did not perform well when high rainfall coincided with high FEC.	$1x10^6$ chlamydospores/kg in 1998. $0.5x10^6$ chlamydospores/kg in 1999 and 2000.	Significant reduction in pasture contamination compared to untreated controls, over 3 years.	*O. ostertagi, C. oncophera.*
Chartier & Pors (2003)	Pasture plot study investigating herbage contamination. Goats were dosed orally with *D. flagrans* chlamydospores and the faeces was used to contaminate plots.	$2.5x10^5$ chlamydospores/kg	50-60% in May, July and November, 80-90% in September	*O. circumcincta, T. colubriformis*
Chandrawathani *et al.* (2003)	Two methods of delivering the fungal dose to sheep and goats were investigated (i.e. daily feed supplement and feed blocks).	$1.25x10^5$ (low) and $2.5x10^5$ (high) chlamydospores/kg per day.	Low: 80-90% High: >99%	*H. contortus*
Flores-Crespo *et al.* (2003)	Sheep were orally dosed with spores and faeces collected. Laboratory faecal samples were incubated for 15 or 21 days. *D. flagrans* performed better than *Arthrobotrys oligospora* and *Dactylaria* spp.	$2x10^7$ chlamydospores	96.3% and 91.4% for 15 and 21 day faecal cultures respectively.	*H. contortus*
Fontenot *et al.* (2003)	Fewer L3 developing in faecal cultures and lower pasture contamination.	$5x10^5$ chlamydospores/kg	96.8% fewer worms in tracers	*H. contortus*
Paraud & Chartier (2003)	Goats offered *D. flagrans* in feed. Efficacy tested against 2 strains of *O. circumcincta* (resistant and susceptible to benzimidazoles) and the lungworm *M. capillaries.*	$5x10^5$ chlamydospores/kg	84% and 90% against BZ susceptible and resistant *O. circumcincta*, respectively. No significant reduction of *M. capillaries*	*O. circumcincta, Muellerius capillaries*

Sanyal & Mukhopadhyaya (2003a)	Pasture plot study investigating herbage contamination. Sheep were fed *D. flagrans* chlamydospores and the faeces was used to contaminate pasture plots.	10^6 chlamydospores/ kg	Significantly lower herbage contamination on plots when *D. flagrans* and eggs delivered simultaneously	*H. contortus*
Sanyal & Mukhopadhyaya (2003b)	Pooled faeces from groups of sheep fed *D. flagrans* spores were spread on to pasture plots. Larval recoveries were monitored from herbage and in vitro faecal cultures.	$1x10^5$, $5x10^5$, $1x10^6$ or $2x10^6$ chlamydospores/ kg	Larval recoveries were greatly reduced at $1x10^5$ spores/kg and was almost prevented at $1x10^6$ spores/kg.	*H. contortus*
Waghorn *et al.* (2003)	Sheep and goats were orally dosed with *D. flagrans*. Efficacy was dose dependant for *O. circumcincta* but not *H. contortus* or *T. colubriformis*.	$2.5x10^5$ or $5x10^5$ chlamydospores/ kg	78% (40-93%)	*O. circumcincta, T. colubriformis*
Wright *et al.* (2003)	Goats orally dosed with *D. flagrans* chlamydospores. Reduced pasture contamination reflected in worm counts of tracers.	$5x10^7$ chlamydospores/ animal daily	Tracer worm counts; 54.8% for *O. circumcincta*, 85.0% for *H. contortus*.	*O. circumcincta, H. contortus*
Chandrawathani *et al.* (2004)	Sheep were offered *D. flagrans* in feed. FEC of controls increased thoughout the trial, while FEC of treated groups remained at the initial level	$5x10^5$ chlamydospores/ kg	Efficacy not stated, but worm burdens were significantly lower than controls.	*H. contortus*
Paraud *et al.* (2004)	Goats were orally dosed with *D. flagrans* spores. Treatment with a benzimidazole drench resulted in suppression of fungal growth for 24-36h, however full predatory activity was restored by 96h post drenching.	$5x10^5$ chlamydospores/kg	86-96% reduction in faecal cultures (similar to sheep at this dose rate)	*T. colubriformis,*
Terrill *et al.* (2004)	Goats offered *D. flagrans* in feed. All doses were effective at controlling L3 development in a dose dependant manner. L3 development was reduced at same rate for *H. contortus, T. colubriformis* and *Cooperia* spp. Larval reduction was consistently higher when goats were fed daily (not every 2 or3 days).	$5x10^5$, $2.5x10^5$, 10^5 & $5x10^4$ chlamydospores/ kg	93.6, 80.2, 84.1 & 60.8% for highest-lowest.	*H. contortus, T. colubriformis, Cooperia* spp.

Table 1-10 Summary of literature on *in vivo* passage of *Duddingtonia flagrans* spores with/without larval reduction

The application of *D. flagrans* spores refers almost exclusively to the use of chlamydospores rather than conidia. This is because chlamydospores are known to be more resistant to adverse environmental conditions, therefore they are considered to be the

inoculum of choice (R.A. Skipp, personal communication). Although the viability of chlamydospores decreases with age (R.A. Skipp, personal communication), the maximum survival of air-dried chlamydospores is more than 20 months (Gronvold *et al.*, 1996a). The warty proturberances on the surface of chlamydospores may be wiped off if the spores are young, but not from older chlamydospores (Gronvold *et al.*, 1996a), supporting the view that as the chlamydospores dry out the outer wall becomes harder and more resistant (Pountney, 1999).

The results described in Table 1-10 above highlight the level of inconsistency that occurs when similar doses of chlamydospores are experimentally administered by different researchers. The cause/s of this is inconsistency have not yet been identified, but remain under investigation. Unfortunately, studies which result in no significant larval reduction are rarely published. Nevertheless, these studies demonstrate the ability of *D. flagrans* to pass through the gastro-intestinal tract, and remain viable in numbers sufficient to significantly reduce the number of larvae developing in faeces and moving onto herbage (Pena *et al.*, 2002; Sarkunas *et al.*, 2000; Fernandez *et al.*, 1997; Nansen et al, 1996). However, the harmful effects of rumen fluid may cause the dose rate necessary to obtain efficacy to be unreasonably high. If the number of spores killed en-route through the digestive tract can be reduced through protective coatings, the minimum oral dose necessary to obtain efficacy will also be reduced (this prospect is considered in Chapters 3, 5 and 6). Reducing the loss of viable spores in the rumen could allow reduction in the spore loading of slow release devices, and/or increase the time they remain effective in the animal. Both would improve the commercial potential of such devices (Waller *et al.* 2001; Skipp, unpublished data)

1.3.5.2.3 Potential environmental implications of un-naturally high levels of *D. flagrans* on non-target organisms.

Until recently, studies have focused on the effectiveness of nematode-trapping fungi against reducing parasite larvae in faeces. There has been little concern for the possible detrimental effects to non-target organisms such as beneficial soil nematodes. Although dung fauna such as earthworms and dung beetles play an obvious role in the breakdown of dung pats and integration of organic matter soil nematodes also play an important role in the

recycling of nutrients (Yeates *et al.*, 1997). Soil nematode populations are also a valuable indication of soil condition and processes (Yeates and King, 1997).

Initial studies have focused on *D. flagrans* as this species is the most likely candidate for commercial application. Faeces contaminated with *D. flagrans* by feeding chlamydospores to animals failed to show any significant effect on soil nematode numbers (Yeates *et al.*, 2003; 2002; 1997; Knox *et al.*, 2002) or composition (Faedo *et al.*, 2002) when compared to no-fungal-treatment controls and/or faeces from animals treated with anthelmintics.

Further investigation by Faedo *et al.* (2002) found that *D. flagrans* had little growth beyond the faecal deposit and did not establish in significant numbers in the soil. As *D. flagrans* is often found in association with rich organic matter such as compost (Skipp *et al.*, 2002) or faeces (Larsen *et al.*, 1994) it has been proposed by Faedo *et al.* (2002) that *D. flagrans* is less effectively able to grow as a saprophyte compared to other nematode trapping fungi. This is based on investigations by Gray (1985) who reported that net-trapping fungi are usually associated with soils of lower pH, moisture and organic matter content. This observation, that *D. flagrans* appears to be a poor competitor in the soil-pasture ecosystem, has been reported by other researchers (G. W. Yeates, pers. comm.).

A. oligospora has been documented naturally invading animal dung as early as three days following deposition (Hay *et al.*, 1997a). Although *A. oligospora* is capable of moving to and growing into dung unaided (Hay *et al.*, 2000) the process may be hastened by soil nematodes or insects carrying spores with them as they migrate into the dung. The rate at which nematophagous fungi invade freshly deposited dung varies according to the time of year (Hay *et al.*, 1997b); for example in New Zealand fungal infestation 2 weeks after dung deposition in March was significantly lower than 2 weeks after dung deposition in January, and significantly higher than the 2 week sampling for dung deposited in May.

In addition, Knox *et al.* (2002) reported that environmental contamination of faeces from sheep fed *D. flagrans* at a rate of 1×10^7 chlamydospores/sheep/day (double the dose rate required for effective control of parasitic nematodes in sheep) had no significant effect on the number of Collembola (springtails) or Acarina (mites) inhabiting the soil/pasture interface. In that study, *D. flagrans* did not spread horizontally into the soil. However it was observed

to penetrate down the soil profile, possibly by chlamydospores being washed down the burrows of earthworms and arthropods, during periods of heavy rain. Moreover, earthworms also exhibit no detrimental effects after feeding on faeces contaminated with high levels of *D. flagrans* chlamydospores (Gronvold *et al.*, 2000).

1.3.5.3 Limitations on the application of nematode trapping fungi as a biological control

All pest control strategies have their limitations and *D. flagrans* and *A. oligospora* are no exception. In order for a nematophagous fungus to reduce the number of larvae developing in faeces, it must be present in the faecal/soil/pasture environment at the same time as the developing larvae (Faedo *et al.*, 1998, 2000). For this reason, any predaceous nematode trapping fungus will have a limited and unreliable efficacy against *Nematodirus* species, where most of the larval development occurs within the egg (Faedo *et al.*, 2000).

Trap formation of *D. flagrans* appears to cease under dry conditions (Faedo *et al.*, 1998) or when dry matter content of faeces is above 50% (D.M. Leathwick, pers. comm.). Also, trap formation stops when dry matter is low as a result of the high faecal moisture content causing the faeces to be anaerobic. The fresh dung pat of cattle in particular, is a relatively moist, anaerobic environment. As time passes, it dries out, partially due to the meteorological effects and partially due to the effects of invasive coprophilic fauna such as earthworms, flies and dung beetles. Consequently, the environment within the dung pat becomes more favourable for colonisation by *D. flagrans* with time (Gronvold *et al.*, 1999).

The trapping of nematode larvae by *A. oligospora* is probably restricted to surface areas of the cow pat where there are high O_2 tensions and low CO_2 tensions (Gronvold *et al.*, 1989). This is where one would also expect high concentrations of infective *O. ostertagi* because these conditions are most suitable for larval development (Rose, 1961). Also, as light has a negative effect on the number of nets produced by *A. oligospora* (Gronvold, 1989), this may influence trap production by this species on the surface of cow pats. Further, a 3 year study involving *in vivo* application of *D. flagrans* reported the fungal treatment to control parasitism in a range of climatic conditions throughout the year. However, fungal treatment failed when high rainfall coincided with high FECs (Dimander *et al.*, 2003).

Despite the many reports of successful *in vivo* application, some have demonstrated that *D. flagrans* may be less effective at trapping certain species of nematode. Studies have shown *D. flagrans* to be less efficacious against *D. viviparus* and *Ostertagia* sp. compared to other trichostrongylid genera (Waghorn *et al.*, 2003; Clarke, 2003; Fernandez *et al.*, 1999c). This is believed to be because *Ostertagia* are much less active than other trichostrongylids, often exhibiting long periods of lethargy (Crofton, 1963). This is similar to the observations reported with *A. oligospora* and the cattle lungworm *D. viviparus*, which is also less motile than other trichostrongylids (Nansen *et al.*, 1988). Also, the efficacy of *D. flagrans* on *Nematodirus* species is highly variable (Faedo *et al.*, 2000), because the *Nematodirus* larvae complete development to infective stage within the egg. As a result, they are less available to be trapped by *D. flagrans* nets. It is noted from Table 1-10 that many studies with high efficacies at low dose rates involved *H. contortus*, the larval stages of which are known to be particularly motile.

It is unlikely that the application of *D. flagrans* will consistently obtain the >99% efficacy usually associated with anthelmintic products. However, it has been proposed that production losses may be substantially reduced at efficacies considerably below this level (Larsen, 2000; Barnes *et al.*, 1995), through the employment of an integrated pest management strategy (Larsen 1999; 2000). Further, the "trickle" exposure to infective larvae allowed by such a system would stimulate an immune response while minimising production losses.

It is well documented that many coprophilic insects are detrimentally affected by the residues of ivermectin (an anthelmintic drug) in the faeces following anthelmintic treatment, and that these residues have been reported to slow the rate of degradation of the faeces (Floate, 1998; Gronvold *et al.*, 1996b; Strong, 1992). As coprophilic fauna such as earthworms, dungbeetles and fly larvae play an important role in the aeration of faeces, their presence (or absence) will inevitably influence the ability of nematophagous fungi to colonise the faeces and reduce subsequent larval development. Research has indicated that some biological and chemotherapeutic controls are not compatible in integrated control strategies (Gronvold *et al.*, 1996b). However, the extent to which anthelmintic treatment may affect faecal colonisation by nematophagous fungi is largely unknown.

2. *In vitro* rumen simulation bioassay for evaluation of *Arthrobotrys oligospora* and *Duddingtonia flagrans* spores.

2.1 Introduction

In ruminants, ingested food is known to spend longer in the rumen, the major organ of digestion, than in other portions of the gastro-intestinal tract. Exposure of *Arthrobotrys* and *Duddingtonia* spores to the adverse conditions found in the rumen seems to be a major factor limiting their survival and subsequent germination in faeces, or even in culture media (Gronvold *et al.*, 1993b; Pountney 1999). It is difficult to study effects on survival within animals themselves and so an *in vitro* rumen simulation bioassay provides a valuable alternative technique to rapidly determine the effects of stresses associated with rumen residence on the viability of spores. This general approach, with some variations, has been successfully utilised by other researchers investigating spore survival and in the selection of superior species or strains for consideration as biological control agents (Larsen *et al.*, 1991; Waller *et al.*, 1994).

The aims of the present experiment were: (a) to establish the rate at which spores of *D. flagrans* and *A. oligospora* are killed following exposure to rumen fluid and (b) to establish the duration of exposure necessary to cause a minimum of 80% reduction in germination. Also of interest, was to determine if there was any difference in the germination of spores exposed to bovine or ovine rumen fluid. Results of previous studies have suggested that survival of *D. flagrans* spores through the gastro-intestinal tract of calves may be greater than that through sheep, although no direct comparisons had been made between the two species (D.M. Leathwick, unpublished data). Answers to these questions would determine suitable sampling times in the second bioassay series (Chapter 3), in which the germination of untreated spores (similar to those used in this assay) is compared to the germination of spores coated in one of several biopolymer formulations.

2.2 Materials and Methods

2.2.1 Experimental Design

The experiment was of factorial design, with two fungal species (*D. flagrans* and *A. oligospora*), two sources of rumen fluid (ovine and bovine), and five sampling times (1 h, 12 h, 24 h, 36 h and 48 h). This gave 20 treatment combinations and with 3 replicates a total of 60 experimental units.

Treatment	Fungus	Rumen fluid source
1	*D. flagrans*	Ovine
2	*A. oligospora*	Ovine
3	*D. flagrans*	Bovine
4	*A. oligospora*	Bovine

Table 2-1 Treatment groups for *in vitro* evaluation of *Arthrobotrys oligospora* and *Duddingtonia flagrans* spores exposed to rumen simulation assay. 1 h, 12 h, 24 h, 36 h and 48 h samples were taken for each treatment.

2.2.2 Preparation of spore inoculum

The chlamydospore inoculum of *D. flagrans* was grown on sterilised hulled barley for three weeks at 20°C [details in Appendix 2-1]. This barley was then agitated with water, and the contents strained through fine curtain mesh, leaving a suspension of spores. As *A. oligospora* produces very low numbers of chlamydospores, the spore inoculum for this species consisted of conidia. *A. oligospora* was cultured on Corn Meal Agar (Difco) plates at 20°C for ten days [details in Appendix 2-2]. These plates were then flooded with distilled water, and the spore suspension strained off in a similar manner. In each case, the concentration of spores was determined by counting with a haemocytometer [details in Appendix 2-3]. The suspensions were then concentrated by centrifugation at 805 g for 7 minutes and resuspended in water to give a concentration of 5×10^5 spores per ml.

2.2.3 Rumen fluid

Rumen fistulated sheep and cattle grazing ryegrass/clover pastures at AgResearch Grasslands were used to obtain rumen fluid for all incubations. The rumen contents were strained through a double layer of cheesecloth and centrifuged for 2 min at 805 g to reduce fungal and bacterial contamination. The supernatant was used in the assay, and care was

taken to keep the rumen fluid warm and minimise aeration through movement/shaking. The assay was set up within 2 h of rumen fluid collection.

2.2.4 Assay procedure

The *in vitro* rumen simulation bioassay used in this experiment was adapted from a technique developed by G.C. Waghorn (as described in Barrell *et al.*, 2000) in order to compare nutritional aspects of ruminant digestion. Spores were incubated in a simulated rumen environment [a detailed protocol is included in Appendix 2-3 and 2-4]. In brief, sixty 50 ml screw-capped bottles with vented lids were filled with 12 ml of McDougall's Buffer (saturated with carbon dioxide) and warmed to 39°C. The following were then added to each bottle: 3 ml centrifuged rumen liquor, 0.5 ml cysteine sulphide reducing agent, and 1 ml of spore suspension (Figure 2-1). This was carried out under a flow of CO_2, which displaced the oxygen in the bottles. The bottles were maintained under anaerobic conditions and mixed continuously at 90 oscillations per minute (Figure 2-2). Bottles were removed from the incubator and sampled for viable spores at 1 h, 12 h, 24 h, 36 h and 48 h, with the 1 h sample representing the initial germination, as the sample was taken immediately after experimental set up was complete. Each bottle was sampled only once, due to the 60-90 minute time lag in bacterial activity incurred when the bottle is aerated during sampling. Sampling required approximately 300 spores (4 drops) of the contents of each bottle, which was spread onto each of two plates of antibiotic Potato Dextrose Agar (PDA), and incubated for 24 h at 25°C.

Figure 2-1 Rumen simulation bioassay (1 bottle)

Figure 2-2 Rumen simulation bioassay – bottles in incubator

Following incubation, agar plates were examined to determine percentage germination. A total of 100 spores were examined from each plate, 24 h after inoculation. Samples taken at 12 h and 36 h were stored at 4-5°C overnight, and examined the following day. The criterion used to distinguish germinated spores was the presence of a germ tube with a minimum length of the width of the spore. A *D. flagrans* chlamydospore that demonstrates the minimum criteria is shown in Figure 2-3, and a germinated spore with considerable hyphal growth is shown in Figure 2-4.

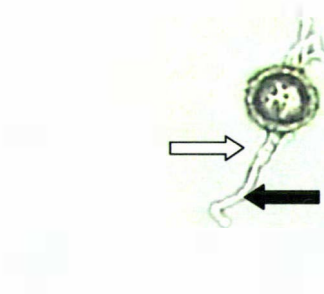


Figure 2-3 Germinated chlamydospore of *D. flagrans* (200x). Germ tube is the thicker portion of initial fungal growth, indicated by white arrow. Hypha is indicated by black arrow.

Figure 2-4 Germinated chlamydospore of *D. flagrans* (100x), with considerable hyphal growth.

2.2.5 Statistical analysis

A general linear model procedure was used to analyse the variance between the samples. As the 12 h samples for *A. oligospora* had no variance (they were all zeros), analysis of this data was limited. However, this was not the case for *D. flagrans*, and several models were considered. The first model considered that the percentage germination is determined by the mean plus the effects of host, time, a host*time interaction, plus the error. When the standard residuals of this model were plotted against the fits, the result indicated a quadratic model would be appropriate. As the effects of host and host*time interaction were not significant, they were dropped from any further model. The second model stated that the percentage germination is determined by the sample mean, time, time^2, plus the error. This model fitted the data much better than the first, confirming the quadratic model and that the data follows a curve. A third model was considered, adding time^3 to the second model, however, the inclusion of time^3 was not significant, and caused time^2 effect to be non-significant. An ANOVA table for the most appropriate (second) model is included in Table 2-2, from which decay curves were calculated [a full printout is included in Appendix 2-5].

2.3 Results

2.3.1 *Arthrobotrys oligospora*

All *A. oligospora* spores failed to germinate after just 12 h exposure to rumen fluid. Viability of the spore inocula was confirmed by high germination of the initial counts, at 1 h incubation in rumen fluid. The arithmetic mean obtained for the initial germination was 96.1%, while the germination was 0% from 12 h onwards, for all treatments. This is illustrated in Figure 2-5, an ANOVA table for the final model is presented in Table 2-2 and the raw data is included in Appendix 2-6. The effect of time was highly significant ($P \leq 0.001$), however as there was no significant difference between ovine and bovine rumen fluid, these results are not presented.

Source	df	MS	P
Time	1	2566.2	0.000
Time^2	1	1570.1	0.000
Error	27	26.7	
Total	29		

Table 2-2 ANOVA table for final model for *Arthrobotrys oligospora*, germination determined by the effects of time and time^2, plus the error

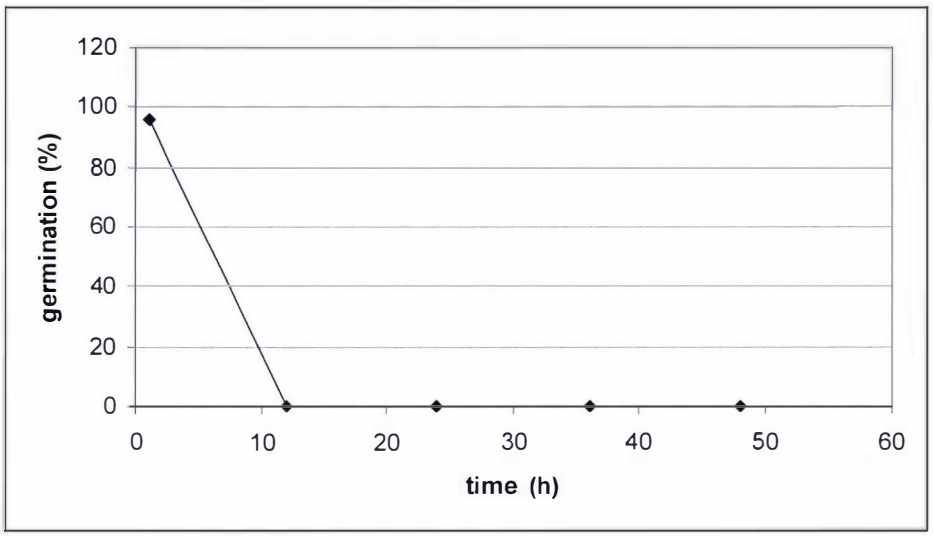


Figure 2-5 Germination of *Arthrobotrys oligospora* conidia following *in vitro* exposure to rumen fluid.

2.3.2 *Duddingtonia flagrans*

The percentage germination for *D. flagrans* decreased as the length of exposure to rumen fluid increased, to form a decay curve. This is illustrated in Figure 2-6, an ANOVA table for the final model is included in Table 2-3 below, and raw data is included in Appendix 2-6.

Source	df	MS	P
Time	1	1948.5	0.000
Time^2	1	545.2	0.007
Error	27	64.5	
Total	29		

Table 2-3 ANOVA table for final model for *Duddingtonia flagrans*, germination determined by the effects of time and time^2, plus the error

As for *A. oligospora*, viability of the spore inocula was confirmed by relatively high germination of spores in the initial samples, with the model finding the mean germination to be 66.9%, which was reduced to 27.3% following 48 h exposure to rumen fluid. There was no significant difference (P> 0.05) between the effect of bovine and ovine rumen fluid on the germination of *D. flagrans* spores, therefore these results are not presented.

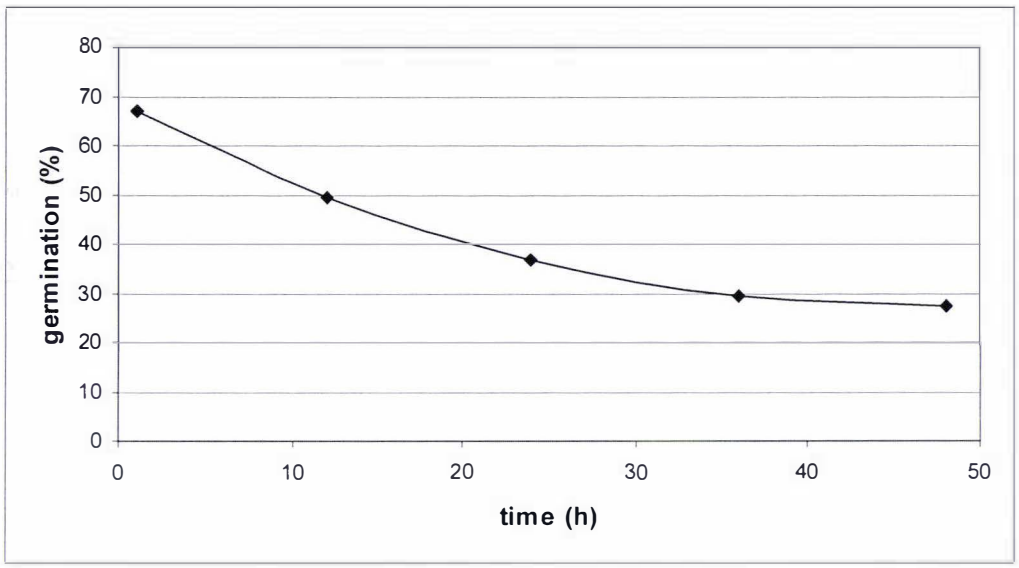


Figure 2-6 Least squares means for germination of *Duddingtonia flagrans* spores following *in vitro* exposure to ovine or bovine rumen fluid

2.4 Discussion

The rumen simulation bioassay used in the present experiment was developed in order to evaluate the nutritional value of ruminant feedstuffs, as described in Barrell *et al.* (2000). However, for the present study, some modifications were made; this involved the exclusion of plant material, and the removal of large particulate matter by coarse filtration, further removal of particulate matter and the majority of bacterial and fungal rumen microflora (which made identification of the spores difficult in the germinations tests) was achieved via centrifugation.

The percentage germination for *D. flagrans* decreased as the length of exposure to rumen fluid increased, to form a decay curve as predicted by previous studies carried out by AgResearch (unpublished data) and overseas (Pountney, 1999). In the present study, the 1 h germination for *D. flagrans* chlamydospores exposed to rumen fluid was 66.9%. This result was slightly lower than the initial germination reported by Pountney (1999), of 84% and 88% for fresh chlamydospores exposed to rumen fluid or water respectively.

Total reduction of *A. oligospora* spore germination occurred after 12 h exposure to either bovine or ovine rumen fluid. This result was in contrast to a study by Waller *et al.* (1994), where an Australian isolate of *A. oligospora* survived both a 24 h *in vitro* assay using dilute

(1:4) rumen fluid, and a subsequent ovine *in vivo* stress selection test. Another study by Larsen *et al.* (1991), refers to unpublished work, in which *A. oligospora* conidia survived for 48h when incubated in water, but for only 4h when exposed to rumen fluid. Although the rumen fluid used in that study is not discussed in detail, it is assumed by association that a 1:4 dilution was used, similar to the current study.

In the present experiment, the *D. flagrans* inoculum included both conidia and chlamydospores flushed from the barley culture but only the chlamydospores were counted. Surprisingly, considerable numbers of conidia were also observed to be germinating following 48 h exposure to rumen fluid. This finding was difficult to explain as the conidia of *D. flagrans* are, superficially at least, very similar in morphology and appearance to those of *A. oligospora.* However, it is possible that the molecular structure of *D. flagrans* conidia differs from that of *A. oligospora* resulting in greater resistance to the debilitating effects of the rumen fluid. The results contrast with those of Pountney (1999), who found that spore germination rates of 92% for centrifuged and 96% for untreated rumen fluid both declined to 0% following 36h incubation in bovine rumen fluid. However, in that study, 1 ml of spore suspension ($1x10^5$ spores /ml) was added to 9 mls of centrifuged rumen fluid. The higher level of germination in the present experiment may be partially due to dilution of the rumen fluid by 1:4 with buffer, which was required to prevent the rumen fluid from denaturing and invalidating the assay procedure. Other studies using *D. flagrans* discuss the effect of a chlamydospore inoculum, despite it being highly likely that conidia are also present.

The variation described above highlights the differences between strains, studies and researchers. Improved performance appears to be obtained from previously stress-selected strains (Waller *et al.*, 1994; Larsen *et al.*, 1992). However, several studies report or refer to unpublished work, in which various strains (including previously stress-selected strains) of *A. oligospora* failed to remain viable following *in vivo* passage through sheep, cattle, goats or pigs (Gronvold *et al.*, 1993a; Gronvold *et al.*, 1993b; Larsen *et al.*, 1992). These results, from *in vivo* studies, support the validity of the stress selection assay used in the present study. Further, the inferior performance of *A. oligospora* spores in this assay, was expected due the thin-walled conidia it produces. This is consistent with studies by other researchers, as summarised by Larsen (2000). By comparison, *D. flagrans* produces thick-walled

chlamydospores that are relatively resistant to adverse environmental conditions (Gronvold *et al.*, 1996a).

Previous studies using similar *in vitro* techniques (Pountney, 1999) have also excluded plant material and attempted to partially clarify the rumen liquor by centrifugation. In that study centrifugation was longer (10 min) and apparently faster than the present study. As the survival of *D. flagrans* spores exposed to centrifuged or untreated rumen fluid was similar, this indicates that centrifugation did not significantly reduce the debilitating effects of rumen fluid. However, centrifugation would be expected to remove more fungal and bacterial contamination which was a problem in the present experiment. In the present experiment the prescribed 100 spores/plate were not counted for the *A. oligospora* treatments due to visibility problems associated with contamination, and the failure of any spores that were visible, to germinate. As a result, only 20-30 spores/plate were observed. Stress selection techniques used in similar *in vitro* studies vary considerably, particularly in methods used for the preparation of the rumen fluid. In some studies, (Pountney, 1999) the rumen fluid was not diluted with buffer at all, while in others (Waller *et al.*, 1994; Larsen *et al.*, 1991) the rumen fluid was diluted 1:4 with McDougall's buffer solution.

Inputs and outputs of the rumen are both complex and intermittent (Czerkawski, 1986). In brief, ingested pasture separates into two main phases in the rumen. The liquid phase is comprised of saliva, rumen fluid, juices from ingested plant material, and suspended small particles. The liquid is digested relatively quickly, before passing out of the rumen, either by being absorbed across the rumen epithelium, or passing through the reticulo-omasal orifice. The particulate phase is comprised of the solid portion of ingested material. This consists of dry matter and cell wall (cellulose), and requires considerable fermentation for digestion. In general, large particles don't leave the rumen (Czerkawski, 1986). Not surprisingly, the rate of digestion of such matter is highly dependant on the type of cellulose (Van Soest, 1982). The rate of outflow from the rumen, and in turn the retention time of material in the rumen, may be influenced by many factors. These include type of diet and rumen conditions as influenced by diet, such as particle size, extent of digestion and particle shape (de Vega and Poppi, 1997). The time a particle spends in the rumen may therefore vary considerably. This appears to be dependent on whether the particle follows the path of fluid state material,

which will usually pass through the rumen in less than 12 h, or that of solid state material, passage of which may take up to 48 h.

A study by Waller *et al.* (1994) using *A. oligospora* found the majority of spores pass through the rumen of sheep 4-12 h following administration. This suggests that the 24+ h of rumen fluid exposure used during *in vitro* assays may not be appropriate. It is probable that the spores pass through the rumen in the fluid phase, rather than with the particulate digesta, allowing a more rapid path through the gastro-intestinal tract. As the rate at which material passes through the rumen is largely dependent on size, it would be reasonable to assume that chlamydospores of *D. flagrans* pass through the rumen at a similar rate to the spores of *A. oligospora*.

3. *In vitro* evaluation of *Arthrobotrys oligospora* formulations using a rumen simulation bioassay.

3.1 Introduction

In New Zealand, *Arthrobotrys oligospora* is often found naturally occurring in the faeces of grazing cattle and sheep. This may occur either through ingestion of spores (Rex Munday, pers. comm.), or as a result of natural invasion from pasture soil (Hay *et al.*, 1998). Fungal material (mycelium and/or conidia) of some isolates of *A. oligospora* have been experimentally shown capable of retaining viability following passage through the gastro-intestinal tract of domestic animals, including sheep (Waller *et al.*, 1994), cattle (Hashmi and Connan, 1989), and a donkey (Soprunov, 1958). However, spore survival is usually not sufficient for there to be any appreciable reduction in larvae developing in faeces and migrating onto herbage (Gronvold *et al.*, 1993a), except in cases where extremely high and impractical dose rates were used (Hashmi and Connan, 1989). In order to improve the survival of *A. oligospora* spores through the gastro-intestinal tract it has been proposed that the spores be protected through an enteric coating and/or incorporated into a controlled release system (Waller *et al.*, 2001a; 2001b).

In plant protection research, alginate preparations have often been used to formulate fungal bio-control agents, including some with activity against plant pathogenic nematodes (Stirling and Mani, 1995). Adoption of this technology was relatively simple as fungal material is easily encapsulated in alginate from a liquid culture or spore suspension, creating a product with good handling properties and increased fungal survival (Lewis and Papavizas, 1987). However, a recent study that used alginate beads as a fungal inoculum for a pot trial compared the trapping ability of the three nematophagous fungi *D. flagrans*, *Monacrosporium gephyropagum*, and *Harposporium helicoides* (Waghorn *et al.*, 2002). The experiment was carried out in autumn, and repeated the following spring. In the autumn experiment, *D. flagrans* significantly reduced the number of *Ostertagia circumcincta* larvae developing in the dung ($P \leq 0.05$). The reduction in larval development dropped considerably for the spring experiment, which the authors proposed was most likely due to lower viability of the fungal inoculum in the spring trial.

Further development in this area has resulted in controlled release biopolymer formulations (NZ Patent Applications No. 506484 to No. 506488), which have proven highly successful in other areas of biological control. For example, Invade® is a granulate biopolymer formulation that contains high concentrations of live cells of the bacterium *Serratia entomophila*, a highly effective biological control agent for the native grass grub *Costelytra zealandica* (White). Although Invade® has been available for some time in liquid form, limitations with storage and application have restricted its commercial success. Stabilising the bacterium in a biopolymer matrix has overcome these limitations and offered several benefits including extended shelf life, and ease of application with conventional farm machinery (Johnson *et al.*, 2001). Extending this technology to biological control of nematode parasites has been initiated by incorporating viable spores of *A. oligospora* into several different biopolymer formulations.

An *in vitro* rumen simulation bioassay (as described in Chapter 2) enables each coating to be evaluated and compared in terms of protective ability and spore survival specifically under conditions similar to those encountered in the rumen. In the previous experiment (Chapter 2), unprotected spores of *A. oligospora* lost viability completely following 12 h exposure to rumen fluid. Thus, any survival of coated spores at 12 h would indicate a protective ability of the coating. Another factor to consider is the initial germination of biopolymer-coated spores; a measure of how many spores remain viable following the coating process and/or influence of the coating on spore germination.

3.2 Materials and Methods

3.2.1 Experimental Design

Four formulations of *A. oligospora* (three biopolymer, one alginate beads) were assessed in an artificial rumen environment, and compared with unprotected control spores exposed to either rumen fluid or water (Table 3-1). Each bottle was sampled only once and samples were taken at set up (1h) and following 12h of exposure to bovine rumen fluid. This gave 12 treatment combinations which with 3 replicates gave 36 experimental units.

Treatment	Spore coating	Medium
1	Uncoated (UW)	Water
2	Uncoated (UR)	Rumen fluid
3	A	Rumen fluid
4	B	Rumen fluid
5	C	Rumen fluid
6	D	Rumen fluid

Table 3-1 Treatment groups for *in vitro* evaluation of *Arthrobotrys oligospora* biopolymer formulations exposed to rumen simulation assay. 1 h, and 12 h samples were taken for each treatment.

3.2.2 Preparation of uncoated spore inoculum

The spore inoculum of *A. oligospora* was grown on Corn Meal Agar (Difco) plates at 20°C for ten days. These plates were then flooded with water, and the spore suspension strained through fine curtain mesh leaving a suspension of spores. The concentration of spores was then determined by counting with a haemocytometer and adjusted by centrifugation and reducing the supernatant to give a concentration of $5x10^5$ spores per ml. Uncoated spores were used in two treatments; the rumen fluid control (UR) and the water control (UW).

3.2.3 Preparation of coated spore inoculum

The spore inoculum was grown in a similar manner to that described above and transported to AgResearch Lincoln where the spore formulations were prepared. One plate of PDA containing approximately 400 spores was incubated for 24 h at 30°C to confirm viability of the spores prior to the formulation process. The remaining spore suspension was then divided into four equal parts, each of which was then coated by one of four protective biopolymer matrices. Coatings consisted of (A) a dry-gel, B Alginate beads, C a soft prill formulation, and D a harder prill formulation.

A series of 10-fold dilutions were performed on Coatings A, C and D, to establish the number of viable spores/g. Due to the nature of alginate beads and the limited quantity available, serial dilutions were not carried out for Coating B. Serial dilutions involved thoroughly mixing 1g of spore formulation with 9mls distilled water to create a suspension of $1x10^{-1}$ dilution. One millilitre of this suspension was then added to 9 ml of distilled water to create a $1x10^{-2}$ suspension. This was continued to obtain serial dilutions to a concentration of $1x10^{-7}$ g/ml. One hundred microlitres of each suspension was spread onto each of 2 PDA plates, except the $1x10^{-1}$ and $1x10^{-2}$ suspensions, which were too viscous. The plates were

incubated for 66 h at 30°C, and the number of germinated spores was observed under a stereomicroscope.

The quantity of fungal inoculum added to each bottle was adjusted for the concentration of viable spores in the formulations. For example; for Coating A, the inocula weighed 0.25 g, for Coating B, the inocula weighed 0.1 g, and for Coatings C and D, the inocula weighed 1 g each.

3.2.4 Rumen fluid

A rumen-fistulated cow on ryegrass/clover based pasture at AgResearch Grasslands was used to obtain rumen fluid for all incubations. The rumen contents were strained through a double layer of cheesecloth and the collected rumen fluid was centrifuged for 2 min at 805 g to reduce fungal and bacterial contamination. The supernatant was used in the assay, and care was taken to keep the rumen fluid warm and minimise aeration through movement/shaking. The assay was set up within 2 h of rumen fluid collection.

3.2.5 Assay procedure

The assay procedure for this experiment was similar to that described in Chapter 2 (Section 2.2.4) and Appendix 2-3, with the following modifications;

- Thirty-six 50 ml bottles were prepared
- Samples were taken at 1h and 12h only
- Samples were spread onto Acid PDA rather than antibiotic PDA in order to reduce bacterial growth which was caused visibility problems in the work described in Chapter 2.

All 1 h samples were incubated for the prescribed 24 h at 25°C, however, as the germination was lower than expected one replicate was incubated for a further 24 h. These results were then used to estimate 48 h counts for the two remaining replicates. All 12 h plates were incubated for 48 h at 25°C.

3.2.6 Statistical analysis

The effect of the coating process on spore germination was determined by comparing the initial germination percentages. Again, a general linear model was initially used to compare

the variation. The model for this analysis states that final germination is determined by replicate and coating plus the error. Again, replicate was not significant, therefore the model was replaced by one-way ANOVA stating that spore germination is determined by coating plus the error. The results for Coating B were removed as the variance within this treatment group was not equal to other treatments. Similar analysis was also carried out on the final germination percentages. However, as the final germination percentages for Coatings B and C were 0% the results from these treatment groups were excluded. The ANOVA tables for these comparisons are shown in Tables 3-3 and 3-4

The protective ability of the coatings, was defined as final germination as a percentage of the initial germination for each treatment. A General Linear Model was used to analyse the variance between the treatment groups. The model for this analysis states that the change in germination is determined by replicate and treatment number plus the error. As replicate was not significant, this variable was removed and the model was replaced with a one-way ANOVA which states that the change in spore germination is determined by the effect of the treatment number, plus the error. The results for Coatings B and C were removed from the analysis as their 12 h germinations were 0%, which left four treatment groups, as presented in the ANOVA, Table 3-6. A full printout of the statistical analysis described here is included in Appendix 3-1.

3.3 Results

3.3.1 Spore germination

The germination test performed prior to formulation processing resulted in 97% germination verifying viability of the fungal inoculum. Table 3-2 shows the arithmetic means for the 1h samples, following 24 h and 48 h incubation. The germination percentages of treatments 3 and 6 increased by greater than 10% following the additional 24 h incubation. The germination of treatment 2 (UR) treated spores increased by slightly less than 10%.

Treatment	Coating	24 hours	48 hours
1	Uncoated (UW)	90.6	93.1
2	Uncoated (UR)	86.5	96.0
3	A	53	64.3
4	B	4.2	4.7
5	C	68.8	64.9
6	D	86.5	97.0

Table 3-2 Arithmetic means of germination percentage following incubation at 25°C

The 1 h germination for treatments 3 and 5 was 64% while treatments 1, 2 and 6 had higher 1 h germinations of 90-98%. The ANOVA describing the model used for this analysis is included in Table 3-3, the least squares means are presented in Table 3-4, and raw data is included in Appendix 3-2. Treatment 4 had only 4.7% 1 h germination and consequently results with this coating were excluded from the statistical analysis. However, as the germination for this coating was well below the 95% confidence intervals for all other coatings, it was considered significantly different. The 1 h germination percentages for treatments 3-6 show the effect of each biopolymer formulation process on spore viability. Differences between germination of treatment 2 and treatments 3-6 demonstrate the effect of the biopolymer coating process on germination. This effect was significant for treatments 3, 4 and 5 ($P \leq 0.001$). There was no significant effect of rumen fluid on the survival of uncoated spores, indicated by similar germinations for treatments 1 and 2.

Source	df	MS	P
Trt	4	856.0	0.000
Error	10	10.1	
Total	14		

Table 3-3 ANOVA table for one-way ANOVA whereby the 1 h germination is determined by the treatment group, for treatment numbers 1, 2, 3, 5 and 6.

Treatment	Coating	1 h Germination (%)	12 h Germination (%)
1	Uncoated (UW)	93.6 a	7.2 a
2	Uncoated (UR)	96.0 a	1.5 b
3	A	64.3 b	4.2 b
4	B	4.7 c	0 b
5	C	64.9 b	0 b
6	D	95.8 a	15.0 c

Table 3-4 Least-squares means for germination percentage of *Arthrobotrys oligospora* conidia following 1 h and 12 h exposure to rumen fluid and 48h incubation. Means with the same letter were not significant different, as determined by LSD ($P \leq 0.05$).

As demonstrated by the least-squares means for this model shown in Table 3-4, the 12 h germination of treatment 6 was significantly higher than treatment 1, which was significantly higher than treatment 2 ($P \leq 0.05$). The 12 h germination for treatments 2, and 3, was very low (1.5% and 4.2% respectively), and germination of treatments 4 and 5 was reduced to 0%. The final germination of treatments 2, 3, 4, and 5 were not significantly different as determined by LSD ($P > 0.05$). The ANOVA describing the model used for this analysis is included in Table 3-5.

Source	df	MS	P
Trt	3	102.96	0.001
Error	8	6.16	
Total	11		

Table 3-5 ANOVA table for one-way ANOVA where the model states that 12 h germination is determined by the treatment group, for treatment numbers 1, 2, 3, and 6

3.3.2 Protective ability

The protective ability of each coating was determined by the 12 h germination as a percentage of the 1 h germination, for each coating. An ANOVA for this analysis is shown in Table 3-6, the results are presented in Figure 3-1, and raw data is included in Appendix 3-2. The mean relative germination for treatments 1, 2, 3, 4, 5 and 6 were 7.8%, 1.6%, 6.6%, 0%, 0%, and 15.3% respectively. Relative germination of spores treated with treatment 6 was significantly different ($P \leq 0.005$) from all other treatments. Treatments 1 and 2 were significantly different from each other and treatment 6, but treatments 1 or 2 were not significantly different from treatment 3.

Source	df	MS	P
Treatment	3	95.55	0.005
Error	8	9.89	
Total	11		

Table 3-6 ANOVA table for protective ability of coatings, whereby model states the relative germination (12 h germination as a percentage of 1h germination) is determined by treatment plus the error, for treatment numbers 1, 2, 3, and 6.

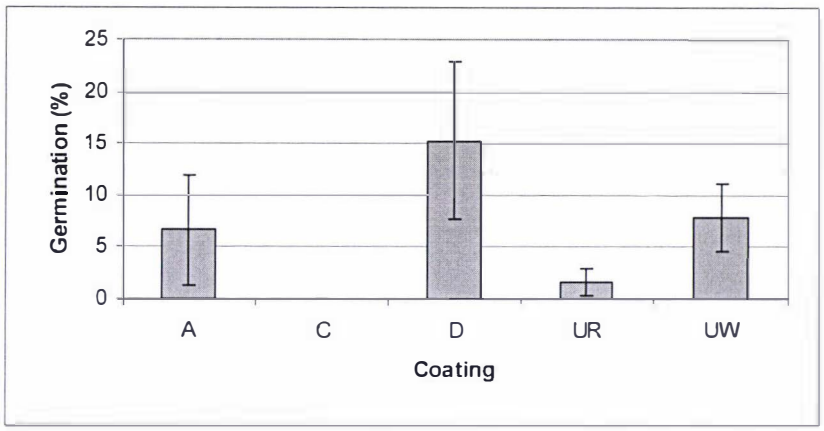


Figure 3-1 12 h germination of *Arthrobotrys oligospora* spores as a percentage of the 1 h germination. Error bars show 95% confidence intervals of the mean as determined by SD. LSD = 5.92

3.3.3 Serial dilutions

The serial dilutions carried out at AgResearch Lincoln are shown in Table 3-7 below. Dry gel formulations typically had much higher spore concentrations than prill formulations (Von Johnson, pers. comm.), as reported here.

Formulation	Number of spores/g
A (flake)	1.41×10^7
C (prill)	1.72×10^5
D (prill)	2×10^5

Table 3-7 Number of germinated spores/g of product in formulations A, C and D, following 66 h incubation at 30°C.

3.4 Discussion

This is the first published report of a biopolymer formulation significantly improving the survival of spores in an *in vitro* simulated rumen environment. This was determined by the 12 h germination as a percentage of the 1 h germination, as the 1 h germination varied significantly between treatment groups. Treatment 6, with 15.3% relative germination was the only coating treatment that was significantly different to the uncoated spores exposed to rumen fluid (treatment 2) at 1.6% relative germination. This means that 15.3% of the initial number of viable spores remained viable following exposure to formulation processing followed by 12 h in a simulated rumen environment. This difference, of almost 10-fold, can be attributed to the protective ability of Coating D. Should this improvement carry through

for *in vivo* trials, it would have a considerable effect on the minimum effective dose rate necessary to achieve the desired efficacy. Although the relative germination of Coating A (treatment 3) was not significantly different to treatment 2, this coating warrants further investigation as results were very similar to treatment 1 (UW) which was significantly greater than treatment 2 (UR). This result, whereby exposure to water was less damaging than exposure to rumen fluid, is consistent with previous studies on the more robust chlamydospores of *D. flagrans*, (Pountney, 1999).

Also of interest was the 1 h germination percentage as this indicates any effect of the coating process on spore viability. The 1 h germination for uncoated spores exposed to water or rumen fluid and Coating D exposed to rumen fluid (treatments 1, 2 and 6) were similar at 93%, 96% and 95% respectively. At 96% the 1 h germination for uncoated *A. oligospora* conidia exposed to rumen fluid in the present experiment was similar to the 98% predicted by the previous experiment (Chapter 2). The 1 h germination of coated spore treatments 3 and 5 were 64%, significantly below treatments 1, 2 and 6 ($P \leq 0.001$) indicating that the coating process had a detrimental effect on spore viablility. However, while all spores receiving Coating C (treatment 5) failed to germinate following 12 h exposure to the rumen simulation assay, a proportion of those getting Coating A (treatment 3) survived. The coating process was severely detrimental for Coating B (alginate beads), and as the 1 h germination was very low (4.7%) the 0% final germination was not surprising. Coating D had high 1 h germination (98%), and the highest 12 h germination (15.0%).

This experiment demonstrates that controlled release biopolymer formulation technology (NZ Patent Applications No. 506484 to No. 506488; Johnson *et al.*, 2001) may be adapted and applied to another area of biological control. It is encouraging when on a first attempt such as this, one formulation significantly increases spore survival and another warrants further investigation. Also, the conidia of *A. oligospora* are considered to be relatively fragile, compared with the thick-walled chlamydospores of *D. flagrans* (Faedo *et al.*, 1997). Thus, it is anticipated that if these fragile conidia remain viable following the formulation process then it is likely that conidia of other nematode trapping fungi would also survive a formulation process.

The formulations used in the present experiment consist of *A. oligospora* conidia protected within a biodegradable bio-matrix. The spores were incorporated into a biopolymer gel which was then mixed with clays and extruded to form clay pellets and air-dried to a desired moisture level. Subsequently, the formulations offer two coatings; the primary physical barrier of the carrier (clay) formulation, and a secondary protective (dry gel) component surrounding individual spores (Von Johnson, pers. comm.). The rate at which the carrier formulation breaks down is easily manipulated as it is dependent upon the binding properties of the bio-matrix ingredients (NZ Patent Applications No. 506484 to No. 506488).

Interestingly, biopolymer coating seemed to slow the germination of *A. oligospora* conidia (pers. obs.). The germination of serial dilutions was low following 24 h incubation, therefore they were subsequently incubated for longer than initially prescribed. This was also done for the 12 h germination samples in the present experiment, which were all subsequently incubated for 48 h.

When the uncoated spores of *A. oligospora* were exposed to rumen fluid for 12 h total reduction of germination did not occur (see Table 3-6) as predicted by the previous experiment (Chapter 2). The high level of contamination of plates in Chapter 2 (despite the addition of chloramphenicol and streptomycin antibiotic to the PDA) severely compromised the visibility of spores. Consequently, only 20-30 spores per plate were observed (not the planned 100 spores per plate) and apparently caused false-zeros to be read. The substitution of antibiotic PDA with acid PDA in the present experiment successfully resolved this problem, slowing bacterial growth considerably and allowing accurate readings to be made.

Although *Arthrobotrys* sp. have been shown to pass through the gastro-intestinal tract of domestic animals and reduce the number of larvae developing in faeces, the dose rates required to do so are not practical. For example, Hashmi and Connan (1989) obtained a 50% reduction in herbage contamination following the weekly dosing of calves with 16 million *A. oligospora* conidia each, for 3 months. Comparable results were obtained by Gruner *et al.* (1985) as described in Larsen (2000) whereby significant reduction of larvae developing in faecal cultures was reported. In that study, housed lambs were mono-specifically infected with *Haemonchus contortus* or *Ostertagia circumcincta* and fed between 470 g and 680 g of

A. tortor, *A. musiformis* or *Dactylaria candida* fungal material on millet. This resulted in a significant reduction of *H. contortus* larvae by *A. tortor*. However, it is probable that this dose rate may be dropped to a more realistic level following the development of a protective coating such as that described in this study. In the absence of such a product, the focus of experimental investigation has shifted to *D. flagrans* over the last 10 years due to its superior ability to pass through the digestive tract (Gronvold *et al.*, 1993b; Faedo *et al.*, 1997; Larsen 2000).

4 *In vitro* evaluation of fresh and dried *Duddingtonia flagrans* chlamydospores using a rumen simulation bioassay.

4.1 Introduction

The germination of freshly harvested *Duddingtonia flagrans* chlamydospores is negatively correlated with time incubated in the rumen simulation bioassay (Chapter 2). However, preliminary studies by AgResearch (unpublished data) indicated that the germination of dried *D. flagrans* chlamydospores was positively correlated. As dried spores were to be coated with stearic acid for the experiments described in Chapters 5 and 6, it was necessary to compare the behaviour of fresh and dried spores independent of coating treatment. Therefore, the aim of the present experiment was to compare the germination of fresh and dried chlamydospores following exposure to a rumen simulation or water.

The germination of dried spores was approximately 40% when incubated for 24 h at 25°C on PDA. By comparison, fresh spores usually exhibited a germination of >75% when incubated under the same conditions (R.A. Skipp, pers. comm.; Pountney, 1999). However, by 72 h the germination percentages from both spore sources were >90% suggesting that while the viability of fresh and dried spores may be similar, dried spores could be slower to germinate. Germination and resistance to adverse conditions is also influenced by the age of the culture, and the conditions under which it has been maintained. In particular, it is thought that drying may cause chlamydospores to become more resistant to adverse environmental conditions (R.A. Skipp, pers. comm.).

4.2 Materials and Methods

4.2.1 Experimental Design

This experiment was a factorial design (Table 4.1). There were two spore sources (dried and fresh), two different media (water and rumen fluid), and four time periods (1 h, 24 h,

48 h and 72 h) that spores were exposed to either medium. With three replicates for each treatment and time period this gave 48 experimental units.

Treatment	Spore source	Medium
1	Fresh	Water
2	Fresh	Rumen fluid
3	Dried	Water
4	Dried	Rumen fluid

Table 4-1 Treatment groups for *in vitro* evaluation of fresh and dried *Duddingtonia flagrans* chlamydospores exposed to water or rumen fluid. 1 h, 24 h, 48 h and 72 h samples were taken for each treatment.

4.2.2 Preparation of spore inoculum

Dried *D. flagrans* chlamydospores were provided by Christian Hansen Biosystems A/S (Hørsholm, Denmark). These were unpurified chlamydospores from batch 2002/05, however further details of these spores were not available. As the number of spores per gram of powder was known, spore suspensions were made up by adding the appropriate weight of dried spore powder to 20 ml of distilled water. The fresh chlamydospore inoculum of *D. flagrans* (Danish isolate) was grown on sterilised hulled barley for three weeks at 20°C [see Appendix 2-1 for full details]. This barley was then agitated with water, and the contents strained off, leaving a suspension of spores, which was centrifuged and adjusted to the appropriate concentration [full details are included in Appendix 2-3].

4.2.3 Assay procedure

The rumen fluid was prepared as described in Chapter 3 (Section 3.2.4). The assay procedure for this experiment was similar to that described in Chapter 2 (Section 2.2.4) and Appendices 2-3 and 2-4, with the following modifications;

Forty-eight 50 ml bottles were prepared

Samples were taken at 1 h, 24 h, 48 h and 72 h

All samples were incubated for 48 h before the germination percentage was observed.

4.2.4 Statistical analysis

An initial plot of the data suggested that 1 h germination of dried and fresh spores might differ. This was tested using a two-way ANOVA; the model stated that 1 h germination was determined by spore source and medium. An ANOVA describing this analysis is included in Table 4-2.

A regression analysis was chosen to model the behaviour of the spores in each of the treatment groups over time. This required that the dried and fresh spores be analysed separately, as the spore sources behaved differently in response to treatment so a single model was not appropriate (F. Potter, pers. comm.). In essence, this then compared incubation medium for each of the spore sources separately over time.

As an alternative analysis, the overall effect (after 72 h) of incubation was considered by calculating the change in germination for each treatment group. This was established by subtracting the 1 h arithmetic mean from the 72 h value for each bottle, and a one-way ANOVA was used to compare the differences between treatments. This then compared the change in germination over the incubation period, thereby adjusting for differences in 1 h germination. An ANOVA table describing this analysis is included in Table 4-3 and a full printout of the statistical analysis is included in Appendix 4-1.

4.3 Results

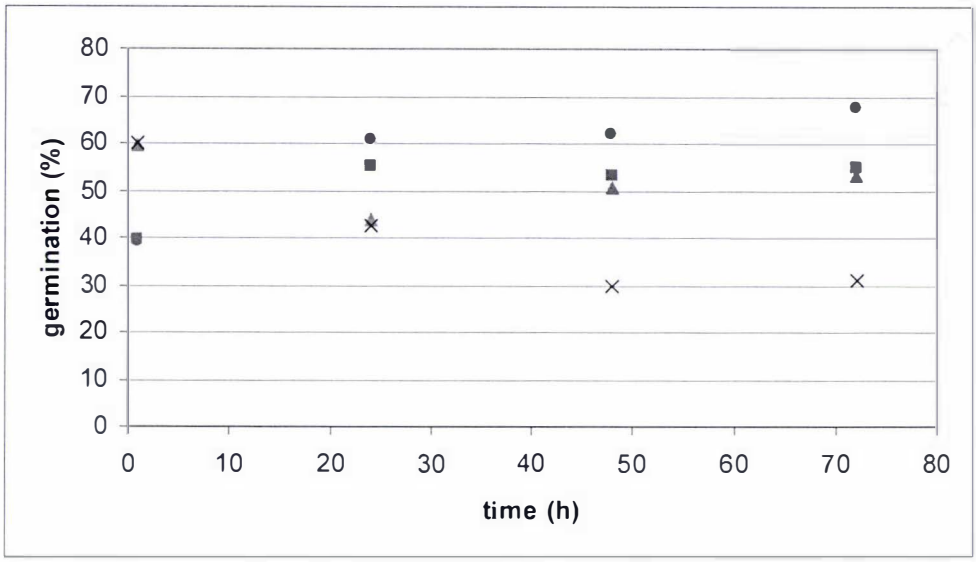
The mean 1h germination was 39.3% and 59.9% for dried and fresh spores respectively. As shown in Table 4-2 below, the two-way ANOVA showed that spore source significantly affected spore germinations ($P \leq 0.001$) while medium and the medium*spores interaction did not.

Source	df	MS	P
Spores	1	1277.63	0.000
Medium	1	0.55	0.781
Interaction	1	0.03	0.951
Error	8	6.68	
Total	11		

Table 4-2 ANOVA table for two-way ANOVA, where 1 h germination is determined by spores, medium, and medium*spores interaction, plus the error.

Curves fitted to the data from different spore sources were sufficiently different in shape to preclude a single regression analysis of the combined data. This indicates that the two spore sources responded differently to the assay i.e. dried spore germination increased over time while fresh spore germination declined. Regression analysis of fresh and dried spores

both showed significant divergence with time as a response to incubation medium. This is illustrated in Figure 4.1 below and the raw data are included in Appendix 4-2.



x Treatment 1 (fresh spores in rumen fluid) ▲ Treatment 2 (fresh spores in water)
● Treatment 3 (dried spores in water) ■ Treatment 4 (dried spores in rumen fluid)

Figure 4-1 Arithmetic means of germination percentage for *D. flagrans* chlamydospores following the rumen simulation bioassay and a 48 h incubation at 20°C.

As shown in Table 4-3, spore treatment (spore source/medium) was highly significant ($P \leq 0.001$) as a predictor of the change in variation over 72 h ($P \leq 0.001$). The change in germination of Treatment 3 at 28.3% was significantly higher than Treatment 4 at 15.3%, which was significantly higher than treatment 1 at -6.7% which was significantly higher than treatment 2 at -29.0% ($P \leq 0.05$).

Source	df	MS	P
Treatment	3	1907.3	0.000
Error	8	13.0	
Total	11		

Table 4-3 ANOVA table for one-way ANOVA, change in germination is determined by treatment (spore source/medium), plus the error.

4.4 Discussion

A study similar to the present experiment investigated the germination of chlamydospores of *D. flagrans* (Danish 'Troll A' isolate) when exposed to a rumen simulation assay containing either rumen fluid or water (Pountney, 1999). In that study,

spores were harvested from *D. flagrans* cultures grown on Sabouraud Dextrose Agar (SDA), and were similar to fresh spores harvested from barley culture in the present experiment. These spores were then exposed to a rumen simulation assay containing either water or rumen fluid at 37°C. Interestingly, Pountney (1999) reported both treatments to have a negative effect on spore germination. Germination in the rumen fluid treatment declined from 84% at 0 h to 9% after 36 h, and for spores in the water treatment germination declined from 88% at 0 h to 21% after 36 h. In both cases the reduction in germination was significant indicating that both temperature and rumen fluid negatively influence spore survival. By comparison, in the present experiment the initial germination was 60% for both treatments (water and rumen fluid) containing fresh spores. The germination of these spores had dropped by half, to approximately 30%, following 72 h exposure to rumen fluid. However, germination of the spores exposed to water at 39°C dropped only slightly to 53% at 72h. Differences between the results in the present experiment and Pountney (1999) can be largely explained by different experimental protocols with particular reference to the concentration of rumen fluid in the rumen simulation assay. The protocol described by Pountney (1999) employed more concentrated rumen fluid, the consequences of which have been discussed in Chapter 2 (Section 2.4). It is also possible that the different culture conditions of spores grown in the present experiment and by Pountney (1999) may have influenced chlamydospore survival.

Furthermore, the reduction in germination of fresh spores following water exposure reported by Pountney (1999) was significantly less than the reduction in germination following rumen fluid exposure indicating that rumen fluid is significantly more damaging to chlamydospore germination than water. Similar results were demonstrated by the present experiment. Although this is not surprising considering the harsh conditions maintained within the rumen environment, and the subsequent nature of rumen fluid, it carries important implications for the *in vivo* application of *D. flagrans*.

In a separate experiment, Pountney (1999) investigated the germination of *D. flagrans* chlamydospores from "granulate" when exposed to her rumen fluid assay. The initial germination was 32%, which rose to 37% at 12 h before falling to 0 by 48h. There is limited detail on the composition of granulate but it is stated that they were recovered from millet seed and therefore could be compared to the dried spores used in the present

experiment. These results are not unlike those in the present experiment where dried spores in rumen fluid had an initial germination of 39%, rising to 55% at 24h. However, in the present experiment the germination remained high, at approximately 54%, until 72 h. Again, this difference in the final germination may be explained by the different concentration of rumen fluid in the assay. Unfortunately, Pountney (1999) did not include a water control in that experiment. However, she did propose that the increase in germination was not a coincidence, but due to hydration of the spores (which must occur before germination), after which point the debilitating properties of rumen fluid were likely to have taken effect. The results from the water control in the present experiment support this hypothesis, as the initial germination was 39% rising to 67% at 72 h. It should be noted that Pountney's experiment and the present experiment both used antibiotic (streptomycin sulphate and chloramphenicol) PDA as a substrate of assaying germination; however, Pountney (1999) incubated the plates for 24 h at 26°C, compared to 48 h at 25°C in the present experiment. This, in addition to the dilute rumen fluid may largely explain the higher peak and slower decline of germination in the present experiment.

Dried chlamydospores used in the present experiment (supplied by Christian Hansen) were harvested from culture a that was grown on millet seed for 4 weeks at 26°C, before being dried and shipped to New Zealand. In addition, these chlamydospores had been stored at AgResearch for 18 months prior to the commencement of this experiment. By comparison, fresh chlamydospores were grown on barley culture at 20°C for 3 weeks immediately prior to the commencement of the experiment. Although the same isolate was obtained from both sources, it is possible that behaviour differences of fresh and dried spores observed in the present experiment may be partially explained by the different culture conditions, and age of the spores. The drying process is believed to be the primary cause of these differences (R. A. Skipp, pers. comm.). However, the effects of culture conditions and age on chlamydospore production and survival are discussed further in Section 1.3.5.2.2 (Chapter 1). Similar studies by Pountney (1999) described above also used fresh and dried chlamydospores from different sources, as reported here.

Only one other published study (Larsen *et al.*, 1991) specifically investigates the behaviour of *D. flagrans* in an *in vitro* rumen simulation bioassay. In that study, chlamydospores were harvested from *D. flagrans* culture grown on tetracycline chloride

water agar. Although the authors reported isolates of *D. flagrans* to be viable following passage through the *in vitro* rumen bioassay the subsequent germination was not quantitatively measured at any time. Also, they did not state the age of the culture from which their chlamydospores were harvested. As the age of the chlamydospores is thought to be a factor in the resistance of the spores to adverse environmental conditions, this may influence the behaviour of the spores in the assay. Spores used in studies reported by Pountney (1999) and Larsen *et al.* (1991) are likely to be more comparable with the fresh spores (harvested from barley culture), than the dried spores (supplied by Christian Hansen Biosystems) used in the present experiment. This is because the moisture content of spores harvested from agar is generally higher than that of spores that have been harvested and dried for storage. It is therefore reasonable to assume that the debilitating effects of rumen fluid will act more quickly on fresh spores than on dried spores. This would enable a superior performance of dried spores in the rumen simulation bioassay.

Further, the bioassay technique used by Larsen *et al.* (1991) was very similar to the method used in the present experiment, in contrast to Pountney (1999). However, their protocol was relatively simple, with a general 24 h rumen simulation that was followed by a 4 h pepsin-HCl stress selection in some treatments. Although this work was qualitative, the addition of a 4 h pepsin-HCl treatment to the 24 h rumen simulation did not reduce the survival of *D. flagrans*.

In conclusion, the germination of dried spores was significantly higher than fresh spores when exposed to a simulated rumen environment for 72 h. In support of Pountney (1999), the results from the present experiment suggest that spore hydration may be an important factor influencing the success of gastro-intestinal tract passage. Further, Gronvold *et al.* (1996a) reported that the warty proturberances on the surface of chlamydospores may be wiped off young spores, but not from older spores, indicating the spore surface becomes harder as it dries. Pountney (1999) also noted that *D. flagrans* chlamydospores harvested from granulate appeared to have a thicker cell wall. She proposed that this might give additional protection against the outside environment. New Zealand researchers (R.A. Skipp, pers. comm.) have noted that dried *D. flagrans* spores on millet seed substrate seem to provide more consistent efficacy data *in vivo* than when fresh spores are harvested and fed to livestock in suspension.

5 *In vitro* evaluation of stearic acid coated *Duddingtonia flagrans* chlamydospores using a rumen simulation bioassay.

5.1 Introduction

One limitation on the application of *D. flagrans* as a biological control agent is that fungal material must be fed on a regular, (preferably daily) basis in order to consistently reduce the development of parasite larvae in faeces (Faedo *et al,* 2000; Terrill *et al.*, 2004). Unfortunately, this is not practical in many farming situations. Thus, the development of controlled release devices containing *D. flagrans* chlamydospores has been proposed to overcome this problem (Waller *et al.*, 2001a; R.A. Skipp, pers. comm.).

While there is very little published information on the performance of experimental controlled release devices it is clear that the manufacturing process could limit the effective life of spores within a bolus to less than that which would be practically and commercially desirable (Waller *et al.*, 2001a; R.A. Skipp, pers. comm). Several properties of *D. flagrans* chlamydospores lend them favourably towards incorporation into controlled release devices. For example, Waller *et al.*, (2001a) found that chlamydospores withstood a long period of anaerobiosis, conditions similar to that occurring in the rumen, and they also withstood the considerable pressure necessary to form tablets for a plastic-bodied 'extender-type' capsule. Also, a measurable proportion remained viable for at least 3 weeks in a prototype bolus resident within sheep (Waller *et al.*, 2001a).

One method of extending spore survival and therefore the effective lifespan of a bolus is through protective coatings. Assuming a 10% survival of unprotected spores through the gut passage, a coating that improved spore survival by a further 10% would essentially half the bolus spore loading and in turn increase the length of action of the bolus. Obviously, a greater than 10% improvement would incur even more benefits. In this Chapter, the protective ability of 5 stearic acid spore coatings were investigated using the rumen simulation bioassay. They were evaluated by comparing germination percentages of each of the coatings to uncoated control spores, following 48 h exposure to rumen fluid. While, there are no published reports of similar attempts to coat *D. flagrans* spores in order to improve

survival, two studies have recognised the need for a sustained delivery system for *D. flagrans*. These studies (Waller *et al.*, 2001a; 2001b) have investigated the potential application of *D. flagrans* in either a controlled release intra-ruminal device or feeding block.

5.2 Materials and Methods

5.2.1 Experimental Design

This experiment was a factorial design (Table 5-1). There were five coatings and an untreated spore control, two exposure media (water and rumen fluid) and two time periods (1 h and 48 h). Each treatment combination was replicated 3 times yielding a total of 72 experimental units.

Treatment	Spore coating	Medium
1	None	Water
2	None	Rumen fluid
3	104	Water
4	104	Rumen fluid
5	106	Water
6	106	Rumen fluid
7	107	Water
8	107	Rumen fluid
9	108	Water
10	108	Rumen fluid
11	109	Water
12	109	Rumen fluid

Table 5-1 Treatment groups for *in vitro* evaluation of stearic acid coated *Duddingtonia flagrans* chlamydospores exposed to water or rumen fluid. Samples were taken at 1 h and 48 h for each treatment.

5.2.2 Preparation of spore inoculum

Dried, unpurified, uncoated *D. flagrans* control chlamydospores were from batch 2002/05. Coated spores had been spray-coated with stearic acid using fluidised bed technology. They were allocated the reference numbers 104, 106, 107, 108, and 109. Coatings 104, 106 and 107 consisted of 50% stearic acid, while coatings 108 and 109 were 30% stearic acid. All spores were provided by Christian Hansen Biosystems (Horsholm, Denmark). It should be noted that the uncoated spores used as positive controls were not from the same batch as the coated spores, as uncoated spores from the same batch were not available. Since the number of spores per gram of powder was known, spore suspensions were made up by adding either

0.2 g or 0.3 g of dried spore powder (as appropriate) to 20 ml of distilled water, in order to make a spore suspension of approximately $4x10^5$ spores/ml.

5.2.3 Assay procedure

The rumen fluid was collected in the same manner as described in Chapter 3 (Section 3.2.4). The assay procedure for this experiment was similar to that described in Chapter 2 (Section 2.2.4) and Appendix 2-3, with the following modifications;

Seventy-two 50 ml bottles were prepared

Samples were taken at 1 h, and 48 h

All samples were incubated for 48 h

5.2.4 Statistical analysis

A general linear model was used to analyse the variance between treatments. The first model considered stated that the germination is determined by the effects of replicate, coating, medium, time, coating*time, medium*time, medium*coating and the medium*coating*time interaction plus the error. As coating*medium and coating*medium*time were not significant, they were dropped from the model. The final model stated that germination was determined by the effects of replicate, coating, medium, time, coating*time, and medium*time plus the error. An ANOVA for this analysis is presented in Table 5-2, and a full printout of the statistical analysis is included in Appendix 5-1.

5.3 Results

Replicate, medium, coating, coating*time and medium*time had a significant effect on the 48 h germination percentage ($P \leq 0.01$), but time and the interaction of coating*medium did not ($P > 0.05$). The ANOVA table for this analysis is shown in Table 5-2 below.

Source	df	MS	P
Replicate	2	580.01	0.000
Time	1	36.91	0.125
Coating	5	123.71	0.000
Medium	1	334.81	0.000
Time*Medium	1	137.98	0.004
Time*Coating	5	115.14	0.000
Error	56	15.24	
Total	71		

Table 5-2 ANOVA table for final model, whereby spore germination is determined by the effects of replicate, time, coating, medium, time*medium and time*coating, plus the error.

The 1 h germination percentages for all spore treatments (including non-coated control) were similar, having least-squares means of between 19% and 21%. This is illustrated in Figure 5-1 below and raw data is included in Appendix 5-2. The 48 h germination of Coatings 106, 107 108 and 109 were very similar to 1 h germination. The 48 h germination of Coating 104 decreased from the 1 h germination, while germination of uncoated spores increased and this difference was significant (P≤ 0.001).

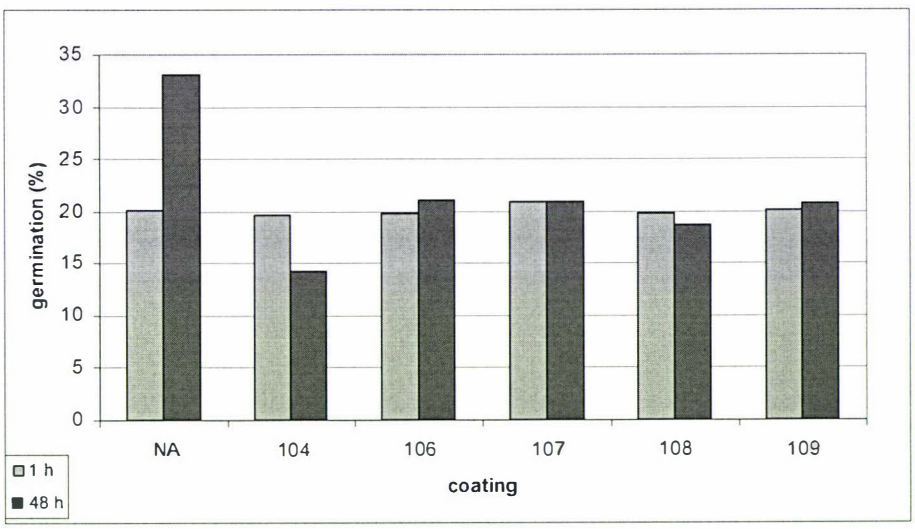


Figure 5-1 Comparison of 1 h and 48 h germination of stearic acid coated spores (referenced 104, 106, 107, 108 & 109) with uncoated controls (NA), illustrating least-squares means for germination percentage of *D. flagrans* chlamydospores following 48 h in rumen simulation bioassay and 48 h incubation at 20°C.

The overall germination of spores exposed to water increased while the germination of spores exposed to rumen fluid decreased between the 1 h and 48 h germinations. The 1 h germination of spores incubated in rumen fluid was significantly higher than that in water (P≤ 0.001) (Figure 5-2).

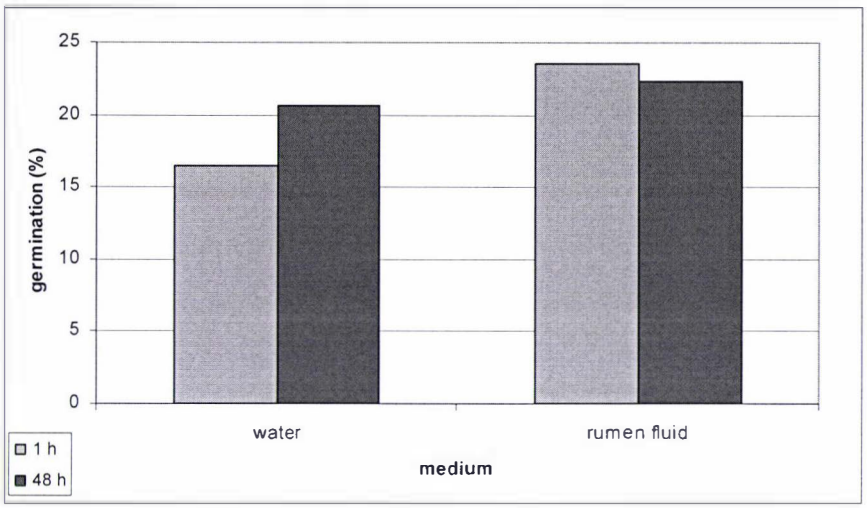


Figure 5-2 Least squares means for comparison of the overall spore germination following 1 h and 48 h, incubation in either media.

5.4 Discussion

In order for *D. flagrans* to consistently reduce the number of larvae developing in faeces, the animals must be supplied with a regular source of spores. This generally requires oral dosing or feeding of formulations added to cereal grain supplements (Faedo *et al.*, 2000; Terrill *et al.*, 2004). However, such practices are not feasible for application on commercial farms in New Zealand and Australia where livestock are farmed extensively on pasture, often in large numbers, and are not handled on a daily basis. However, both countries have significant problems associated with anthelmintic resistance (Leathwick and Vlassoff, 1996; Waller, 1997; Leathwick *et al.*, 2001) making them an ideal marketplace for a biological control product in controlled release formulation. It is commercially desirable for such a product to have as long an effective lifetime as possible.

Limitations on the effective life of a bolus are determined by several constraints including; the maximum dimensions that can be orally administered to the target animal and the maximum spore loading. The rate at which spores are released by the bolus is determined by the bolus formulation, which may contain up to 50% v/v chlamydospores without the integrity of the bolus being compromised. The growing lamb increases in weight throughout the effective period of the bolus and spores are released by the bolus at a constant rate. This pushes the limitations further, as the bolus must be formulated to release spores appropriate for the lamb weight at the end of the effective period, rather than weight at bolus application.

Obviously, one area for possible improvement is spore survival through the gastrointestinal tract following release from the bolus. Many publications report that doses between $1.5x10^5$ and $1x10^6$ *D. flagrans* chlamydospores per kilogram liveweight are required to obtain >90% reduction in larval development in sheep, cattle, horses and pigs. However, despite *D. flagrans* spores being considered the most robust of any studied to date, researchers believe that up to 90% of spores are killed by passage through the gastro-intestinal tract of ruminants (Gronvold *et al.*, 2004; D.M. Leathwick, pers. comm).

Unfortunately, biopolymer formulations similar to those used in the coated *Arthrobotrys oligospora* experiment (Chapter 3) were not available for the present *D. flagrans* experiment. Instead, chlamydospores coated with variations of an experimental stearic acid coating provided by Christian Hansen Biosystems (Horsholm, Denmark), were made available. These spores had arrived from Denmark in March 2002 and as this experiment was carried out in March 2004, the spores were at least 2 years old before they were subject to the rumen simulation bioassay. Although the maximum survival of air-dried chlamydospores is more than 20 months (Gronvold *et al.*, 1996a), the viability for these spores would be expected to decline over this period (R.A. Skipp, pers. comm.). The germination of these spores in 2002 was between 30-40%, following 24 h incubation at 25°C on PDA. However, in the present experiment the 1h germination of uncoated spores exposed to water, and incubated for 48h was only 20%.

It should be noted that the uncoated control spores used in this experiment were from the same batch as those used in Chapter 4 (batch 2002/5). However, in the present experiment the 48 h germination of 33% was substantially below that of 54%, for the same treatment reported in Chapter 4. This difference may be explained by the 7 months that passed between the running of these two experiments. Spore viability is known to decline over time, and a marked reduction in viability of these spores had been noted prior to the commencement of the present experiment (R.A Skipp, pers. comm.).

Surprisingly, the 48 h germination percentages for the coated spores were all below that of the uncoated spores, and Coating 104 was significantly so ($P \leq 0.001$). These results suggest that although these coatings may be providing some form of protection in the rumen

simulation assay, either the coating, or some part of the coating process is reducing the ability of the spores to germinate. Stearic acid is a saturated fatty acid that has been used to protect bacteria (R.A. Skipp, pers. comm.). The coating process could have been detrimental to spores at the same time that it applied a coating that might protect against adverse effects in the rumen environment. Dried *D. flagrans* chlamydospores as received from Christian Hansen are particularly resistant to adverse environmental conditions. However, additional heating and rapid drying during the fluidised bed, spray coating process could have been injurious.

In Chapter 4, the germination percentage for uncoated, dried *D. flagrans* spores following 48 h in the rumen simulation bioassay was higher than the 1 h germination. This was also true for the present experiment, and 48 h germination of uncoated spores was significantly higher than that of Coating 104 ($P \leq 0.001$). This indicates that the coating process for Coating 104 detrimentally affected spore survival compared to uncoated spores. Interestingly, this was not consistent with the germinations for Coatings 106 and 107 in which spores were coated with a similar 50% stearic acid coating. In contrast to Chapter 4, the 1 h germination of spores was significantly influenced by medium (23.6% and 16.5% germination for rumen fluid and water respectively). This difference may be largely explained by observer error, due to difficulty associated with observing spores on heavily contaminated plates such as those incubated in rumen fluid. When contamination is heavy, ungerminated spores are more likely to be missed which may result in an artificially increased germination percentage.

To conclude, coatings similar to those used in the present experiment potentially offer solutions to problems associated with previous fungal bolus formulations, by offering added protection from the rumen fluid in the form of a secondary coating (R.A. Skipp, pers. comm.). This could potentially double (or better) spore viability, enabling the spore loading to be reduced and the boluses to last longer. To my knowledge this was the first attempt to investigate protective coating of *D. flagrans* chlamydospores using an *in vitro* rumen simulation assay, and absence of any clear response with the material provided does not mean that this approach is unlikely to be rewarding in future. However, it should be considered that the rumen simulation bioassay may not influence spore germination in the same manner as *in vivo* gut passage. As discussed previously in Chapter 2 (section 2.4), the

rumen fluid used in this assay was diluted 1:4. This is considered to explain some of the differences between the results presented in this thesis, and those of Pountney (1999). Also, although every effort was made to follow the correct procedure, this assay is particularly sensitive to even minor technical variations of temperature, maintenance of anaerobiosis etc. Therefore, *in vivo* evaluation was necessary, in order to validate the *in vitro* bioassay results.

6. *In vivo* evaluation of coated *Duddingtonia flagrans* chlamydospores.

6.1 Introduction

Preliminary in vitro evaluation of 5 stearic acid coatings (discussed in Chapter 5) suggested that these coatings would not increase spore survival through the gut passage of ruminants. This is because coated chlamydospores did not have a significantly higher germination percentage than uncoated spores, following exposure to the rumen simulation bioassay. Further, the germination percentage of spores coated with Coating 104 was significantly lower than that of uncoated spores. However, as this is the first time this assay has been applied in this manner, and *in vitro* evaluation does not always accurately predict *in vivo* results, it was considered necessary to validate the assay *in vivo*.

There are few published *in vitro* dose titration studies investigating the behaviour of *D. flagrans* following the addition of chlamydospores to faecal cultures. Such studies are necessary to establish a minimum effective dose rate, without the detrimental effects of in vivo passage on the chlamydospores. One *in vitro* study demonstrated >75% efficacy (reduction in larval development to L3 in the faeces) may be obtained through the addition of 1000 spores/g, and that efficacy was dependant upon spore concentration and FEC (Waruiru, 2001). Pilot studies for this thesis demonstrated a 91% efficacy with a fungal inoculum of 1000 spores/g. Further, a 55% efficacy was obtained from 100 spores/g. By comparison, *in vivo* dose titrations studies demonstrate that a minimum dose rate of $1x10^5$ spores/kg live weight is required to obtain a 90% reduction in larval development for sheep and goats (Chandrawathani *et al.*, 2003; Pena *et al.*, 2002).

The germination percentages of *D. flagrans* chlamydospores cannot be directly observed following *in vivo* gut passage, as they can with *in vitro* evaluation. Therefore, *in vivo* application of *D. flagrans* must be evaluated indirectly, through efficacy data and re-isolation of the fungi through presence/absence testing. Numerous studies (summarised in section 1.3.5.2.3), carried out by various researchers around the world have demonstrated that a dose of $2.5x10^5$ *D. flagrans* chlamydospores/kg live weight is adequate to reduce the number of larvae developing in faecal cultures by >90%.

The aim of the present experiment was to demonstrate any improvement in spore survival through protective stearic acid coatings. As it is difficult to detect improvement in spore viability statistically when the efficacy of uncoated spores is high (>90%), the fungal dose was reduced to $1.25x10^5$ chlamydospores/kg. Local and international data predict an efficacy of approximately 50-60% using this dose rate.

6.2 Materials and Methods

6.2.1 Experimental Design

The purpose of this experiment was to evaluate the protective ability of 5 experimental coatings on their ability to improve survival of *D. flagrans* chlamydospores through the gastro-intestinal tract of sheep. Each of the coatings was evaluated by comparing its efficacy against an uncoated spore control group and a no-fungal-treatment control group. This gave 7 treatment groups, each with 5 sheep/group, forming 35 experimental units. The treatment groups (of 5 sheep each), are shown in Table 6-1 below.

Treatment number	Treatment	Coating
1	Negative control – no fungal treatment	n/a
2	Positive control - uncoated *D. flagrans* chlamydospores	none
3	Coated *D. flagrans* chlamydospores	104
4	Coated *D. flagrans* chlamydospores	106
5	Coated *D. flagrans* chlamydospores	107
6	Coated *D. flagrans* chlamydospores	108
7	Coated *D. flagrans* chlamydospores	109

Table 6 -1 Table of treatment groups and description of treatments

6.2.2 Fungal Treatments

Spores used were the same batches as used previously in Chapter 4. The positive control sheep were dosed with uncoated chlamydospores of *D. flagrans*, while the sheep in the treated groups were dosed with *D. flagrans* chlamydospores treated with one of five variations of a stearic acid coating. Coatings 104, 106 and 107 consisted of 50% stearic acid, while coatings 108 and 109 were 30% stearic acid. No further details of the coatings were available. All spores were obtained from Christian Hansen Biosystems A/S (Hørsholm, Denmark). It

should be noted that the uncoated spores used as positive controls were not from the same batch as the coated spores, as uncoated spores from the same batch were not available.

6.2.3 Experimental Procedure

Thirty five weaned lambs with a mean live weight of 29.4 kg (range 22-35 kg) were each treated with abamectin (Genesis – Ancare, Batch 1050, Expiry 12/2006) and levamisole/albendazole (Arrest Hi Mineral – Ancare, Batch 0685, Expiry 02/2006) sufficient for a 40kg animal on Days -10 and -11, prior to the start of the experiment. A Faecal Egg Count (FEC) was performed on all of these animals on Day -6 to ensure that no animals were passing trichostrongylid eggs. The animals were individually ear-tagged with a unique identifying number, and weighed on Day -3. They were then sorted by weight, allocated to groups of 7 and the animals from each group were randomly allocated to a treatment. Thus, the 7 heaviest animals were randomly allocated, one each, to the 7 treatment groups and so on.

The experiment commenced on Day 0 when all animals except the no-fungal treatment control were dosed to their individual live weight with a suspension of 1.25×10^5 spores/kg of *D. flagrans* chlamydospores. All doses were suspended in tap water immediately prior to administration, and were administered orally with a syringe. Animals were maintained as a single mob on the same pasture and dosed at the same time each day for three days, with their allocated formulation. A single 50g faecal sample was collected from each animal on Day 3, 96 h after the initial dose. From each sample, three 10 g faecal cultures and five 1 g presence/absence tests were carried out.

6.2.4 Faecal Cultures

Three 10 g faecal cultures were made for each animal [details in Appendix 6-1]. Briefly, faeces were placed in an uncovered 5.5 cm diameter Petri dish, which was then placed inside an 8.5 cm tissue culture dish. As the faeces had previously been tested free of trichostrongylid eggs, approximately 2000 *Trichostrongylus colubriformis* eggs were added to each culture. These eggs were obtained from one experimentally-infected sheep carrying a monospecific infection of *T. colubriformis*. The base of the culture dish was then covered with approximately 10 ml of distilled water, to maintain humidity and prevent the larvae

escaping (Figure 6-1). The lid of the culture dish, with its unique identifying label, was then replaced and the cultures were incubated for 14 days at 20° (Figure 6-2).

Figure 6-1 Faecal of culture of *Trichostrongylus colubriformis* with lid off

Figure 6-2 Faecal cultures of *Trichostrongylus colubriformis*

Larvae were extracted by a Baermannisation technique and recovered after 24 h (Figures 6-3 and 6-4). The collected water containing the recovered larvae was examined under a stereo microscope and if less than 100 larvae were present, the entire sample was counted. If more than 100 larvae were present, larvae were counted in a 20% aliquot to estimate the total count. Efficacy of the fungal treatments was determined as a reduction in the number of larvae developing in faecal cultures from treated groups compared to untreated controls.

Figures 6-3 and 6-4, Faecal cultures of *Trichostrongylus colubriformis* on Baermann funnels

6.2.5 Presence/Absence tests

Presence/absence tests were carried out in order to detect viable *D. flagrans* chlamydospores following passage through the gastro-intestinal tract of lambs. Briefly, the presence/absence tests involved spreading 1 g of faeces on water agar and adding ¼ plate of water agar containing *Rhabditis* sp. nematodes that had been cultured for 3 weeks. The plates were incubated at 20°C for several weeks, and observed at regular intervals for the presence of *D. flagrans* nets and/or spores [details in Appendix 6-2]. Five replicates of 1 g presence/absence tests were made for each animal. The presence of *D. flagrans* chlamydospores in the faeces (indicated by the white arrows) is demonstrated in Figure 6-2 and live *Rhabditis* sp. nematode is trapped in the *D. flagrans* net indicated by the black arrow.

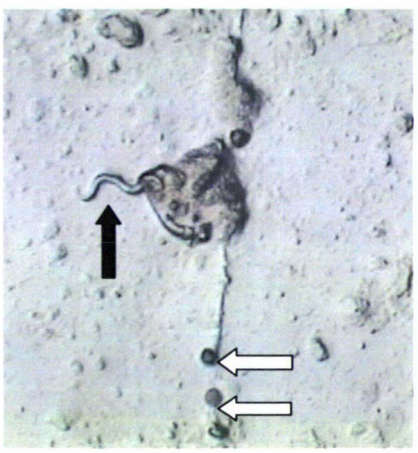


Figure 6-5 Testing for presence of *Duddingtonia flagrans* in faeces (100x magnification). White arrow indicates *D. flagrans* chlamydospore, black arrow indicates *Rhabditis* sp. nematode trapped in net of *D. flagrans*.

6.2.6 Statistical Analysis

Three cultures were made for each animal, and the mean number of larvae recovered was used in the analysis. A one-way ANOVA was used to compare the treatment groups. The model states that the number of larvae recovered is determined by the sample mean for each treatment, plus the error. A full printout of the statistical analysis is included in Appendix 6-3.

6.3 Results

6.3.1 Faecal Cultures

A summary of the numbers of larvae recovered is shown figure 6-6 below, and the raw data in Appendix 6-4. The coatings were evaluated by comparing the number of larvae developing in each treatment group. In this experiment, the uncoated spores did not significantly reduce the number of larvae developing in the faecal cultures, and no spore coating caused a significant change in the number of larvae recovered between treatments. In addition, the number of larvae developing in the no-spore control faecal cultures was lower than expected, and not significantly greater than the treated groups. Consequently, efficacy comparisons were not appropriate.

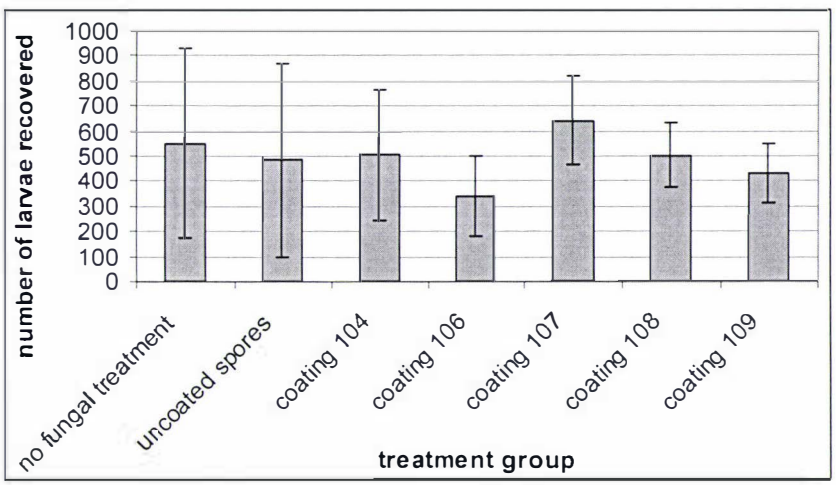


Figure 6-6 Mean number of L3 recovered from 10 g laboratory faecal cultures, from sheep orally dosed with *Duddingtonia flagrans* chlamydospores. Coated spores were spray coated with stearic acid and allocated the reference numbers 104-109. 2000 *Trichostrongylus colubriformis* eggs were added to each culture. Error bars show 95% confidence intervals of the means.

6.3.2 Presence/absence tests

Presence/absence testing revealed that at least three animals in each spore-treated group tested positive on at least one of the five replicate plates (Table 6-2 and raw data in Appendix 6-5). Presence/absence tests were carried out on all sheep except for one in the Coating 107 spore-treated group, as there were not enough faeces collected from this animal. One animal in the negative controls tested positive for *D. flagrans* on 2 out of 5 plates.

Treatment	No. of animals positive / No. of animals tested
No fungal treatment	1/5
Uncoated *D. flagrans* spores	4/5
D. flagrans coating 104	5/5
D. flagrans coating 106	3/4
D. flagrans coating 107	4/5
D. flagrans coating 108	3/5
D. flagrans coating 109	4/5

Table 6-2 showing the number of animals testing positive in presence/absence test for each treatment group, over the number of animals tested.

6.4 Discussion

The present experiment failed to demonstrate any significant reduction in larval development, either by the uncoated control spores, or by any of the 5 variations of coated spores. Interestingly, these results are similar to those predicted when these spore coatings were evaluated *in vitro* (Chapter 5). However, it is in contrast to a previous study by AgResearch using the same spores, whereby an efficacy of approximately 70% was obtained for coatings 104 and 107, compared to an uncoated spore efficacy of 38% (unpublished data). In that study, the spores were orally administered to calves at a dose rate of $5x10^{4}$ spores/kg.

Sheep are usually dosed at $2.5x10^{5}$ spores/kg, half the dose rate for cattle, in order to obtain an efficacy of 90% or better (R.A. Skipp, pers. comm.). The aim of the present experiment was to investigate any improvement in spore survival resulting from the stearic acid coatings applied to the spores. As it is difficult to demonstrate a statistically significant improvement in spore survival when the efficacy of uncoated spores is high (above 90%), it was decided to reduce the dose to a level that was expected to have obtained 50-60% efficacy. An approximate dose-response relationship has been generated by various studies carried out by AgResearch (unpublished data), and international data. Until recently, the vast majority of studies involved high dose rates ($1x10^{6}$ - $1x10^{7}$spores/kg), in order to obtain the desired level of larval reduction.

Numerous publications report high efficacies when large doses (approximately $1x10^{6}$ chlamydospores/kg) of spores are administered. These publications are summarised in Section 1.3.5.2.3 and Table 1.10 (Chapter 1). However, recent dose titration studies have revealed that a much lower dose may reduce larval development to an acceptable level. In

support of this, Pena *et al.* (2002) reported that a dose of $5x10^4$ chlamydospores/kg consistently resulted in 97% efficacy against *H. contortus* larvae developing in faecal cultures of sheep. Another study by Terrill *et al.* (2004) reported this dose ($5x10^4$ chlamydospores/kg) obtained 70-93% efficacy for *Haemonchus contortus*, *T. colubriformis* and *Cooperia* sp. in goats. Furthermore, the authors reported similar efficacies for doses of $5x10^4$ and $5x10^5$ chlamydospores/kg, despite the 10-fold difference between doses. This is consistent with a general understanding by researchers, whereby the dose required to obtain very high efficacies (i.e. >99%) is several-fold higher than the dose required to obtain a modest efficacy of 50-70% (R.A. Skipp, pers. comm.).

A recent study carried out in Malaysia by Chandrawathani *et al.* (2003) reported that $1.25x10^5$ spores/kg consistently reduced development of *H. contortus* by greater than 90% following *in vivo* passage through sheep and goats. A similar study carried out in India, involved supplemental feeding sheep with pellets top-dressed with decreasing quantities of dried *D. flagrans* chlamydospores (Sanyal and Mukhopadhyaya, 2003b). The dose titration resulted in the lowest dose ($1x10^5$ chlamydospores/kg) obtained approximately 70% efficacy and the second-lowest dose ($5x10^5$ chlamydospores/kg) obtained approximately 75% efficacy. By comparison, the two highest doses ($1x10^6$ and $2x10^6$ chlamydospores/kg) approached total reduction of larvae developing.

One group of veterinary anthelmintics, the benzimidazoles, contain known fungicides. The animals in the present experiment were administered a full therapeutic dose of 3 active anthelmintics (levamisole, abamectin, albendazole), 10 days prior to the commencement of fungal dosing. However, the level of benzimidazole residues in faeces 10 days after dosing (as described in Chapter 6) would not be high enough to negatively affect germination, or subsequent larval reduction. In support of this hypothesis, Paraud *et al.* (2004) reported that *D. flagrans* displayed full predatory activity 96 h following oral administration of thiabendazole or albendazole.

One animal in the untreated controls tested positive for *D. flagrans* chlamydospores in its faeces. It is suspected that another animal from the same group (which also had a low larval recovery) may also have also tested positive, but a degree of observer error compromised this result and it remains uncertain. Contamination is a common problem in field studies despite

every care being taken, including the changing of gloves between animals, to prevent this occurrence. It is also possible that there was some degree of operator error. In any case, this occurrence potentially contributed to the low larval development in some cultures, and emphasises the importance of controls.

The methodology used to culture larvae during the present experiment is commonly used by the Parasitology group at AgResearch Grasslands. Larval recoveries from individual cultures using this methodology can be quite variable, but generally vary between 25% - 50% of the number of eggs added to each untreated control culture (unpublished data). In the present experiment, the mean larval development for untreated controls in the present experiment was 27% of eggs added (range of 10-56% between replicates), which is within the range expected for this methodology.

The high level of variation in larval recoveries between experiments using this methodology may be partially explained by the source of the eggs. Obviously, the viability of the eggs will have a considerable impact on larval development, and subsequent larval recovery. Egg viability, or developmental success from egg to L3 may be influenced by several factors that are not controlled by the experimental protocol. For example, the source of the eggs (donor animals) is not consistent, and is often selected on convenience at the time. Donor animals may be infected either naturally or experimentally, with monospecific or mixed infections, they may be lambs or adults and thus differ in immunological response to infection, and they may or may not be from a line of sheep that has been selected for low FEC. Each of these factors may significantly affect developmental success of nematode eggs (Jorgensen *et al.*, 1998).

Nevertheless, the effect of animal on larval development in faecal cultures is often highly significant (Waghorn *et al.*, 2003; Jorgensen *et al.*, 1998; D.M. Leathwick, pers. comm.). Despite this, it is also often overlooked by researchers. In the present experiment, all eggs were sourced from a single donor animal harbouring a monospecific infection of *T. colubriformis*. This would be expected to reduce the level of variation within treatment groups. However, many factors of the immune status of the donor animal, and the effect they had on development success are not known.

T. colubriformis eggs used in the present experiment were collected over 1 day between 0800 h and 1600 h, and transported from AgResearch Wallaceville to Grasslands overnight. They were packaged on an ice-pack to minimise the number of eggs hatching during transport. However, chilling may detrimentally affected the developmental success of the eggs. This is supported by McKenna (1998) who demonstrated that trichostrongylid eggs are negatively affected by refrigeration at 4°C, and the magnitude of this effect varied between genera. Although that study reported a 12 day refrigeration period necessary to cause a significant reduction in *Trichostrongylus* development, it is possible that the overnight exposure to low temperatures had some effect in the present experiment.

In conclusion, the absence of a significant difference in larval recoveries between treatment groups in the present experiment may have been caused by several factors. Although this result was not surprising for stearic coated chlamydospores considering the preliminary nature of this work, it was not expected for the uncoated-spore control group. Potential explanations include; the fungal dose may have been too low; low viability of the chlamydospores (due to age); contamination of no-fungal-treatment controls; lower than expected larval recoveries from no-fungal-treatment controls; large variation in larval recoveries within treatment groups; or a combination of these factors. As the studies reviewed above suggest, a moderate but significant larval reduction should have occurred in the uncoated-spore controls at the dose applied in the present experiment, the low dose alone is an unlikely explanation. Unfortunately, a more recent batch of spores was not available, and the source of contamination in no-fungal-treatment controls remains unknown. Three cultures were carried out for each animal, in order to reduce the variation between animals within treated groups, however this variation was still large. Also, vermiculite was not utilised in the present experiment, the addition of which may have prevented the low larval development reported here and which is common when cultures have a high moisture content. It is therefore proposed that the failure of uncoated *D. flagrans* chlamydospores to reduce larval development significantly was due to a combined effect of low spore viability, contamination of the no-fungal-treatment controls (the larval recoveries of which were lower than expected), large variation in larval recoveries, and minimal dose of the fungal inoculum.

7. General Discussion

The number of researchers working with nematophagous fungi has increased markedly in recent years, with many groups around the world now publishing studies on the possible application of fungal biocontrol to veterinary parasitology. This interest in alternative parasite controls may be attributed to increasing awareness of, and pressure to control, drench-resistant parasites. The nematode-trapping fungus *Arthrobotrys oligospora* dominated early research, with numerous *in vitro* studies demonstrating its ability to reduce larval development following the addition of conidia to faeces. However, interest shifted to *Duddingtonia flagrans* when it was realised that its ability to produce large numbers of chlamydospores could provide fungal inoculum with superior ability to survive passage through the gastro-intestinal tract of livestock. While no biological control is likely to give almost complete kill of parasites in dung (as well as in the animal) that is currently the norm expected of any new chemical anthelmintic, it is also unlikely that such extremely high efficacy will be necessary. Modelling suggests that production losses can still be substantially reduced when only 75% of larvae are prevented from developing in faeces (Larsen, 1999; 2000).

Given the above, a minimum effective dose rate of a biocontrol fungus administered to animals can be defined as that which consistently reduces the number of developing larvae by 75%. Estimates in the literature of the dose rate required to achieve this level of efficacy vary considerably. For example, Gronvold *et al.* (2004) reported that a minimum dose of $2.5x10^5$ *D. flagrans* chlamydospores/kg liveweight was necessary to consistently obtain at least 75% efficacy in cattle. By comparison, Chandrawathani *et al.* (2002) reported that in sheep a 5 fold lower dose of $5x10^4$ chlamydospores/kg would achieve similar efficacy. These differences may be partially explained by the properties of different isolates and effects of different preparation methods used by various researchers, as the preparation method is known to influence spore viability (R.A. Skipp, pers. comm.). However, there is a tendency for researchers to quote doses as total spore numbers without considering the percentage of viable spores in each dose. As discussed in Chapter 1 (Section 1.2.1) investigation of the potential of applying coatings to spores of nematophagous fungi was attempted to improve their survival through the gastrointestinal tract of livestock. This in turn could have provided a way to reduce the dose of spores administered.

D. flagrans did not significantly reduce larval development of *T. colubriformis* when orally administered at $1.25x10^5$ spores/kg live weight (Chapter 6), although numerous publications have demonstrated high efficacies at similar dose rates (see Chapter 1, Section 1.3.5.2.3). Interestingly, high efficacies obtained at low dose rates tend to be against predominantly *Haemonchus* infections. The larval stages of *Haemonchus* are known to be highly motile and thus likely to stimulate trap production by nematophagous fungi. Dimander *et al.* (2003) reported successful application of $5x10^5$ *D. flagrans* chlamydospores/kg against *Ostertagia ostertagi* and *Cooperia oncophora* under field conditions but efficacy was lost during periods of heavy rainfall. It is likely that factors which might affect efficacy such as consistency of spore intake, nematode species, and rainfall will be most influential as the threshold for minimum effective dose is approached. However, although most studies have been carried out with the Danish strain, and there is not much variation among isolates world wide, recent results suggest that there may be some scope for increasing efficacy through selection of *D. flagrans* isolates (Sanyal & Mukhopadhyaya, 2002).

The amount of faeces produced by sheep over a 24 h period is determined by dry matter intake and food digestibility. A 30 kg lamb maintained under pastoral conditions, as described in Chapter 6 will produce approximately 1.5 kg faeces /day. Thus, the faeces of such a lamb dosed with $1.25x10^5$ spores/kg each day could theoretically contain approximately 2500 *D. flagrans* spores/g. *In vitro* tests using this dose has achieved 75-90% reductions in numbers of larvae developing in faeces (Waruiru, 2001; Clarke, 2003). Further, as few as 250 chlamydospores/g gave 93% reduction when these were placed directly in cattle faeces (Gronvold *et al.*, 2004). The authors proposed that the need to dose spores at a higher rate than would be needed in the faeces reflects the more than 90% of mortality of spores in the alimentary tract of cattle. The experimental work described in Chapter 6 suggests similar spore mortality occurs in sheep.

As mentioned previously, the rumen simulation bioassay employed in Chapters 2-5 of this thesis was developed to evaluate and compare aspects of ruminant nutrition. For the purposes of this thesis, the assay protocol was modified to facilitate the observation of spores that had been sampled from medium containing rumen fluid. The assay protocol described in

Chapter 2 (and Appendix 2-3) is similar to that used for ruminant nutrition studies except it omits the addition 1g of finely chopped forage (e.g. ryegrass/white clover, lucerne, or cereal grain) to each bottle (often the variable under investigation for digestion studies which generally rely on chemical analysis), Such material was considered unnecessary in the present experiments and would have obscured spores making counts far more difficult. Other researchers investigating *in vitro* spore survival have also excluded forage and/or centrifuged rumen fluid to facilitate spore recovery/observation (Larsen *et al.*, 1991; Pountney, 1999). Although the exclusion of forage in the rumen simulation protocol is not considered to have significantly affected results, the potential for further manipulation of this assay may be investigated in the future.

Preliminary studies have demonstrated successful incorporation of *D. flagrans* chlamydospores into prototype intra-ruminal controlled release devices (CRDs)(Waller *et al.*, 2001a) and feed blocks (Waller *et al.*, 2001b) removing the need for daily feeding of spores. However, studies by Agresearch (unpublished data) suggest that the effective period of CRDs is constrained by high spore mortality in the gastrointestinal tract of livestock (following release from the CRD). This was the reason for investigating the potential of applying protective coatings to extend the lifespan of chlamydospores.

Some of the factors that may negatively affect the ability of *D. flagrans* to reduce larval development in faeces remain undefined. Thus even though researchers try to keep to consistent experimental methodology (discussed in Section 1.3.5.2.3), results from field trials and, indoor and laboratory experiments can be highly variable and difficult to interpret and form generalisations. Recent studies (Gronvold *et al.*, 2004) have investigated potential sources of this variation, but have failed to identify causative organisms and/or circumstances. As there is a tendency to publish only studies in which *D. flagrans* successfully reduced larval development, this may give the impression that *D. flagrans* reduces larval development more consistently than it does.

Although in this study stearic acid coating of *D. flagrans* chlamydospores was unsuccessful in increasing survival, the results for *A. oligospora* biopolymer formulations were encouraging. It was unfortunate that biopolymer formulations were not available for *D. flagrans*. Nevertheless, formulations like those used in Chapter 3 potentially offer solutions

to problems associated with previous fungal bolus formulations by offering added protection from the rumen fluid in the form of a secondary coating (RA Skipp, personal communication). Such a coating could potentially improve spore viability, enabling the spore loading to be reduced and the effective period of the boluses to be increased as desired.

The successful formulation of *A. oligospora* is promising, as the conidia of *A. oligospora* are considered fragile compared to the chlamydospores of *D. flagrans*. It is anticipated that this, or similar technology could be applied to a range of different species of nematophagous fungi. This would present the possibility of formulations containing a 'cocktail' of nematophagous fungi. The major advantage this would bring would be the possibility of efficacy over a wide range of climatic conditions because of the range of optimum temperature and other environmental factors of the component species. This concept was successfully applied in a plot trial under New Zealand field conditions (Waghorn *et al.*, 2002), in which the greatest larval reduction occurred on plots treated with a combination of fungal species.

9. List of Appendices

List of abbreviations used in Statistical Analysis and Raw Data

Trt	=	Treatment number
Rep	=	Replicate number
% germ	=	Percentage germination of spores
Plate	=	Plate number 1 or 2 (of 2) sampled from each bottle
mean % germ	=	mean of 2 plates (used for analysis)
Time	=	Time (in hours) exposed to water or rumen fluid in assay prior to sampling
Time 2	=	$Time^2$
Fungus	=	Species of fungal inoculum
Df	=	*Duddingtonia flagrans*
Ao	=	*Arthrobotrys oligospora*
Host	=	Source of rumen fluid
S	=	Sheep
C	=	Cattle
Medium	=	Rumen fluid or water
R	=	Rumen fluid
W	=	Water
Spores	=	Source of spores
D	=	Dried (provided by Christian Hansen)
F	=	Fresh (grown on fresh barley culture)
Coating	=	Reference number or letter for each coating
U	=	Uncoated
A	=	Coating A (dry gel) used in Chapter 3
B	=	Coating B (alginate beads) used in Chapter 3
C	=	Coating C (prill) used in Chapter 3
D	=	Coating D (prill) used in Chapter 3
NA	=	Not applicable (no fungal inoculum)
None	=	No protective coating applied
104	=	% stearic acid coating used in Chapters 5 and 6
106	=	% stearic acid coating used in Chapters 5 and 6
107	=	% stearic acid coating used in Chapters 5 and 6
108	=	% stearic acid coating used in Chapters 5 and 6
109	=	% stearic acid coating used in Chapters 5 and 6

Culture	=	Larval culture number 1, 2 or 3 (of 3) carried out for each sheep
1	=	total number in 1st 20% aliquot
2	=	total number in 2nd 20% aliquot
3	=	total number in 3rd 20% aliquot
4	=	total number in 4th 20% aliquot
5	=	total number in 5th 20% aliquot
total	=	total number of larvae in entire sample (estimated if >100/culture)
mean total	=	mean of 3 culture totals for each animal
+	=	animal passed viable *D. flagrans* chlamydospores in faeces
-	=	animal did not pass detectable numbers of viable *D. flagrans* chlamydospores in faeces

Appendix 2-1 Procedure to culture *Duddingtonia flagrans* on barley grain

Laboratory Procedure

Materials

- Less than 2-week-old fungal culture maintained on corn meal agar (CMA) or potato dextrose agar (PDA) [as described in Appendix 2-2]
- 100g hulled barley grain
- 100ml distilled water
- 500 ml conical flask
- Cotton wool and aluminium foil
- Autoclave
- Metal spatula

Method

- 1st day: soak barley in water in a 500ml flask for 24 h
- 2nd day: autoclave
- 3rd day: autoclave again
- Inoculate flask with five blocks of agar (3.5mm x 3.5mm)
- incubate at 20°C for 3 weeks
- 1 week after inoculation, mix culture in the flask with a metal spatula

References:

Lysek, G., and Nordbring-Hertz, B. 1981. An endogenous rhythm of trap formation in the nematophagous fungus *Arthrobotrys oligospora*. *Nematologica* 23: 443-451.

Gronvold, J., Wolstrup, J., Larsen, M., Hendriksen, S.A., and Nansen, P. 1993. Biological control of *Ostertagia ostertagi* by feeding selected nematode-trapping fungi to calves. *Journal of Helminthology*. 67: 31-3

Appendix 2-2 Procedure to Prepare Agar Plates and culture fungi on agar

Procedure to prepare agar plates

Corn Meal Agar

Materials

- 500 ml conical flask
- water bath
- approximately 20 sterile Petri dishes
- 400ml of distilled water
- Corn Meal Agar (CMA) powder (Difco)
- Laminar flow cabinet
- Cotton wool and aluminium foil
- Autoclave

Method

- Warm water bath to 50°C.
- Measure distilled water into a 500ml conical flask.
- Add 17g/l of CMA (Difco).
- Mix thoroughly, cap with cotton wool and aluminium foil and autoclave at 121°C for 15 minutes.
- Remove from the autoclave and immediately place the flask into water bath
- Under laminar flow, pour agar into petri dish until base is covered, approximately 20mls.
- Label and store in refrigerator 4°C until required

Antibiotic PDA

Materials

- 500 ml conical flask
- water bath
- approximately 20 sterile Petri dishes
- 400ml of distilled water
- Potato Dextrose Agar (PDA) powder (Difco)
- Laminar flow cabinet
- Chloramphenicol (Sigma)
- Streptomycin sulphate (Sigma) stock solution (0.5g of streptomycin sulphate dissolved into 50ml of sterile, distilled water)
- Cotton wool an aluminium foil
- Autoclave

Method

- Measure 400ml of distilled water into a 500ml conical flask.
- Add 39g/l of PDA (Difco) and 0.05g/l of chloramphenicol.
- Mix thoroughly, cap with cotton wool and aluminium foil and autoclave at 121°C for 15 minutes.
- Remove from the autoclave and immediately place the flask in water bath.
- Once cooled to 50°C aseptically add 10m/l streptomycin sulphate stock solution.
- Pour agar into petri dish until base is covered, approximately 20mls.

Acid PDA

Materials

- 500 ml conical flask
- water bath
- approximately 20 sterile Petri dishes
- 400ml of distilled water
- Potato Dextrose Agar (PDA) powder (Difco)
- Laminar flow cabinet
- Sterile disposable pipette
- Lactic acid (BDH laboratory reagents)
- Cotton wool and aluminium foil
- Autoclave

Method

- Measure 400ml of distilled water into a 500ml conical flask
- Add 19.5g of PDA (Difco)
- Mix thoroughly, cap with cotton wool and aluminium foil and autoclave at 121°C for 15 minutes
- Remove from the autoclave and immediately place the flask in a pre-warned 50°C water bath
- Add 3 drops of Lactic acid to each sterile Petri dish, via sterile disposable pipette
- Pour agar into Petri dish until base is covered, approximately 20mls

Procedure to culture fungi on agar plates

Materials

- Actively growing fungal culture on agar medium.
- Alcohol burner/flame
- Mounted needle or scalpel and blade
- Alcohol (98% ethanol)
- Remove from incubator after 2 weeks and store in refrigerator (4-5°C).

Method

- Flame mounted needle or scalpel blade
- Dip mounted needle/scalpel blade in alcohol
- Flame again (briefly, to burn off alcohol)
- Cut 1 block (approximately 3.5mm X 3.5mm) from the edge of the culture.
- Place block upside-down (to ensure good contact of fungus with medium) in the centre of each agar plate.
- Replace lid on Petri dish, label and incubate at 20-25°C for up to 2 weeks.
- Remove from incubator after 2 weeks and store in refrigerator (4-5°C).

Appendix 2-3 Procedure to determine the concentration of spores in a solution

Materials

- Fuchs Rosenthal haemocytometer and cover slip
- Disposable pipette
- Compound microscope
- Counter

Method

- Apply cover slip until the interference rings are seen
- Ensuring the suspension is thoroughly mixed, load the chambers with spore suspension using a disposable pipette.
- Determine the number of chlamydospores present in eight large squares (each comprising of 16 smaller squares) by direct counts at a magnification of 200 using phase contrast microscopy.
- Load the chamber 3 more times and if the counts differ by more than 10%, further counts are made.
- The mean number of spores per large square is determined by dividing the total number of spores by the number of squares counted.
- Spore concentration = 10,000 x mean no. spores/large square

Example:

1. Add about 5 ml of sterile water to each plate, wash off spores with sterile glass rod and tip into sterile 250 ml beaker. The suspension was filtered though 1 layer of sterile muslin, measure spore concentration (so we know the need to concentrate or dilute).

No. spores in 8 large squares (each comprising 16 smaller squares) of a haemocytometer:

Rep 1:

2		9
0		5

5		1
10		12

Rep 2:

7		4
6		5

6		5
4		1

Rep 3:

2		0
2		2

3		1
6		5

So spore concentration (1) 10^4 x (2+9+0+5+5+1+10+12)/8= $5.5x10^4$/ml
So spore concentration (2) 10^4 x (7+4+6+5+6+5+4+1)/8= $4.75x10^4$/ml
So spore concentration (3) 10^4 x (2+0+2+2+3+1+6+5)/8= $2.6x10^4$/ml

Mean spore concentration = [(1) + (2) + (3)]/3 = $4.28x10^4$/ml

Appendix 2-4 Protocol for *in vitro* rumen simulation bioassay

Materials

- 39°C incubator with vibrating stage (90 oscillations per minute)
- 50 ml Schott bottles with vented lids
- McDougall's Buffer
- Reducing agent
- Carbon Dioxide in gas cylinder
- Acid or antibiotic potato dextrose agar plates
- 25°C incubator
- Rumen fluid (supernatant from liquor following centrifugation at 805g for 2 minutes)
- Alchohol burner/flame
- Glass spreader (glass rod with flattened end)
- Disposable pipettes

McDougall's Buffer (Artificial Saliva):

To make 1 litre:
9.8g $NaHCO_3$
3.67g Na_2HPO_4 anhydrous
0.47g NaCl
0.57g KCl
0.128g $MgCl_2.6H_20$
0.04g $CaCl_2$ (add last)
1 litre distilled water

Combine altogether and mix well. Prior to use, bubble with CO_2 until pH is approx 6.8. Can be made day before and stored in fridge over night but needs to be made fresh each time (i.e. do not use >24 hours after being made).

Reducing Agent:

To make 50ml:
315mg cysteine HCl
48ml distilled water
2ml 1M NaOH
315mg sodium sulphide
Add together and mix well.

Method

To prepare assay

- Saturate McDougall's buffer with carbon dioxide by bubbling for at least 30 minutes
- Place 12 ml of McDougall's buffer in each bottle and warm to 39°C in the incubator.
- Collect rumen fluid, strain through a double layer of cheese cloth and centrifuge, taking care to minimise aeration through shaking and keep fluid warm.
- Add 0.5 ml reducing agent, 3 ml centrifuged rumen liquor, and 1 ml of spore suspension under a flow of CO_2 (to displace O_2 in the bottles).
- Replace lid and place bottles in incubator for designated period
- At appropriate sample time, remove bottles from incubator
- Ensuring bottle is thoroughly mixed, apply four drops of the contents onto each of 2 agar plates using a disposable pipette
- Use a separate pipette for each bottle
- Spread sample over agar plate with a glass spreader, flaming the spreader and dipping in alcohol between samples.
- Place in 25°C incubator for 24h
- Following incubation, observe germination of 100 spores from each plate using a compound microscope (germination determined by the presence of a germ tube)

Appendix 2-5 Statistical Analysis – *In vitro* evaluation of *Duddingtonia flagrans* and *Arthrobotrys oligospora* spores

Unequal variances between *A. oligospora* and *D. flagrans* over time, thus both spore sources cannot be included in the same model.

Test for Equal Variances

```
Response    % germ
Factors     Fungus  Time
ConfLvl     95.0000

Bonferroni confidence intervals for standard deviations

  Lower     Sigma     Upper     N  Factor Levels

 0.53224    1.0206    4.1157    6  Ao      0
 0.00000    0.0000    0.0000    6  Ao     12
 0.00000    0.0000    0.0000    6  Ao     24
 0.00000    0.0000    0.0000    6  Ao     36
 0.00000    0.0000    0.0000    6  Ao     48
 4.64024    8.8980   35.8814    6  Df      0
 6.40694   12.2858   49.5427    6  Df     12
 3.74149    7.1746   28.9317    6  Df     24
 2.91949    5.5984   22.5754    6  Df     36
 2.19656    4.2121   16.9853    6  Df     48


Bartlett's Test (normal distribution)


Test Statistic: 80.165
P-Value       :  0.000


Levene's Test (any continuous distribution)


Test Statistic: 10.020
P-Value       :  0.000
```

Final Model for *A. oligospora* germination

General Linear Model: % germ versus

```
Factor     Type Levels Values

Analysis of Variance for % germ, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS     Adj MS       F      P
Time        1     2140.6      2566.2     2566.2   96.07  0.000
time 2      1     1570.1      1570.1     1570.1   58.78  0.000
Error      27      721.2       721.2       26.7
Total      29     4431.9

Term          Coef   SE Coef        T      P
Constant    89.963     6.713    13.40  0.000
Time       -6.4812    0.6613    -9.80  0.000
time 2     0.09963   0.01300     7.67  0.000
```

Final Model for *D. flagrans* germination

General Linear Model: % germ versus

```
Factor     Type Levels Values

Analysis of Variance for % germ, using Adjusted SS for Tests

Source     DF     Seq SS     Adj SS     Adj MS       F      P
Time        1     5880.6     1948.5     1948.5   30.21  0.000
time 2      1      545.2      545.2      545.2    8.45  0.007
Error      27     1741.5     1741.5       64.5
Total      29     8167.2

Term          Coef   SE Coef        T      P
Constant    66.912     3.086    21.68  0.000
Time       -1.6742    0.3046    -5.50  0.000
time 2    0.017692  0.006085     2.91  0.007
```

Appendix 2-6 Raw Data (Spore Germinations) - *In vitro* evaluation of *Duddingtonia flagrans* and *Arthrobotrys oligospora* spores

Germination (%) following 24 h incubation at 25°C

Trt #	Rep	Host	Fungus	Time	Plate	% Germ	Mean % germ
1	1	S	Df	1	1	74	
1	1	S	Df	1	2	70	72
2	1	S	Ao	1	1	98	
2	1	S	Ao	1	2	97	97.5
3	1	S	Df	1	1	78	
3	1	S	Df	1	2	77	77.5
4	1	S	Ao	1	1	97	
4	1	S	Ao	1	2	93	95
1	2	S	Df	1	1	56	
1	2	S	Df	1	2	53	54.5
2	2	S	Ao	1	1	93	
2	2	S	Ao	1	2	97	95
3	2	C	Df	1	1	69	
3	2	C	Df	1	2	76	72.5
4	2	C	Ao	1	1	95	
4	2	C	Ao	1	2	97	96
1	3	C	Df	1	1	65	
1	3	C	Df	1	2	66	65.5
2	3	C	Ao	1	1	94	
2	3	C	Ao	1	2	98	96
3	3	C	Df	1	1	53	
3	3	C	Df	1	2	64	58.5
4	3	C	Ao	1	1	97	
4	3	C	Ao	1	2	97	97
1	1	S	Df	12	1	34	
1	1	S	Df	12	2	34	34
2	1	S	Ao	12	1	0	
2	1	S	Ao	12	2	0	0
3	1	S	Df	12	1	42	
3	1	S	Df	12	2	36	39
4	1	S	Ao	12	1	0	
4	1	S	Ao	12	2	0	0
1	2	S	Df	12	1	40	
1	2	S	Df	12	2	55	47.5
2	2	S	Ao	12	1	0	
2	2	S	Ao	12	2	0	0
3	2	C	Df	12	1	62	
3	2	C	Df	12	2	56	59
4	2	C	Ao	12	1	0	
4	2	C	Ao	12	2	0	0
1	3	C	Df	12	1	65	
1	3	C	Df	12	2	62	63.5
2	3	C	Ao	12	1	0	
2	3	C	Ao	12	2	0	0

3	3	C	Df	12	1	61	
3	3	C	Df	12	2	60	60.5
4	3	C	Ao	12	1	0	
4	3	C	Ao	12	2	0	0
1	1	S	Df	24	1	39	
1	1	S	Df	24	2	40	39.5
2	1	S	Ao	24	1	0	
2	1	S	Ao	24	2	0	0
3	1	S	Df	24	1	38	
3	1	S	Df	24	2	43	40.5
4	1	S	Ao	24	1	0	
4	1	S	Ao	24	2	0	0
1	2	S	Df	24	1	26	
1	2	S	Df	24	2	19	22.5
2	2	S	Ao	24	1	0	
2	2	S	Ao	24	2	0	0
3	2	C	Df	24	1	41	
3	2	C	Df	24	2	39	40
4	2	C	Ao	24	1	0	
4	2	C	Ao	24	2	0	0
1	3	C	Df	24	1	29	
1	3	C	Df	24	2	37	33
2	3	C	Ao	24	1	0	
2	3	C	Ao	24	2	0	0
3	3	C	Df	24	1	26	
3	3	C	Df	24	2	34	30
4	3	C	Ao	24	1	0	
4	3	C	Ao	24	2	0	0
1	1	S	Df	36	1	25	
1	1	S	Df	36	2	27	26
2	1	S	Ao	36	1	0	
2	1	S	Ao	36	2	0	0
3	1	S	Df	36	1	28	
3	1	S	Df	36	2	27	27.5
4	1	S	Ao	36	1	0	
4	1	S	Ao	36	2	0	0
1	2	S	Df	36	1	45	
1	2	S	Df	36	2	32	38.5
2	2	S	Ao	36	1	0	
2	2	S	Ao	36	2	0	0
3	2	C	Df	36	1	34	
3	2	C	Df	36	2	24	29
4	2	C	Ao	36	1	0	
4	2	C	Ao	36	2	0	0
1	3	C	Df	36	1	36	
1	3	C	Df	36	2	42	39
2	3	C	Ao	36	1	0	
2	3	C	Ao	36	2	0	0
3	3	C	Df	36	1	35	
3	3	C	Df	36	2	28	31.5
4	3	C	Ao	36	1	0	
4	3	C	Ao	36	2	0	0
1	1	S	Df	48	1	20	

1	1	S	Df	48	2	24	22
2	1	S	Ao	48	1	0	
2	1	S	Ao	48	2	0	0
3	1	S	Df	48	1	29	
3	1	S	Df	48	2	35	32
4	1	S	Ao	48	1	0	
4	1	S	Ao	48	2	0	0
1	2	S	Df	48	1	21	
1	2	S	Df	48	2	23	22
2	2	S	Ao	48	1	0	
2	2	S	Ao	48	2	0	0
3	2	C	Df	48	1	30	
3	2	C	Df	48	2	21	25.5
4	2	C	Ao	48	1	0	
4	2	C	Ao	48	2	0	0
1	3	C	Df	48	1	29	
1	3	C	Df	48	2	32	30.5
2	3	C	Ao	48	1	0	
2	3	C	Ao	48	2	0	0
3	3	C	Df	48	1	26	
3	3	C	Df	48	2	29	27.5
4	3	C	Ao	48	1	0	
4	3	C	Ao	48	2	0	0

Appendix 3-1 Statistical Analysis – *In vitro* evaluation of *Arthrobotrys oligospora* formulations

48 h germination as a percentage of initial germination

One-way ANOVA: 12h germ as % of initial germ versus trt

```
Analysis of Variance for C5
Source     DF        SS        MS        F        P
trt         3    286.65     95.55     9.66    0.005
Error       8     79.14      9.89
Total      11    365.78

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  -----+---------+---------+---------+-
1           3     7.778     2.723            (------*------)
2           3     1.598     0.869   (------*------)
3           3     6.628     5.230          (------*------)
6           3    15.264     2.011                        (------*------)
                                    -----+---------+---------+---------+-
Pooled StDev =    3.145               0.0       6.0      12.0      18.0
```

LSD = 5.92

Comparison of initial germinations across treatment groups

One-way ANOVA: germ % versus trt

```
Analysis of Variance for germ %
Source     DF        SS        MS        F        P
trt         4    3424.1     856.0    84.87    0.000
Error      10     100.9      10.1
Total      14    3524.9

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ----------+---------+---------+------
1           3     93.06      3.50                          (---*--)
2           3     96.02      0.53                             (--*--)
3           3     64.33      1.85  (---*--)
5           3     64.94      2.73   (--*---)
6           3     97.00      5.20                             (---*--)
                                    ----------+---------+---------+------
Pooled StDev =    3.18               72        84        96
```

LSD = 5.78

Comparison of final germinations across treatment groups

Test for Equal Variances

```
Response    germ %
Factors     trt
ConfLvl     95.0000

Bonferroni confidence intervals for standard deviations

  Lower     Sigma     Upper     N  Factor Levels

 1.04887   2.36291   29.8419    3    1
 0.38442   0.86603   10.9373    3    2
 1.47778   3.32916   42.0450    3    3
 1.19302   2.68765   33.9431    3    6


Bartlett's Test (normal distribution)


Test Statistic: 2.412
P-Value       : 0.491


Levene's Test (any continuous distribution)


Test Statistic: 0.288
P-Value       : 0.833
```

One-way ANOVA: germ % versus trt

```
Analysis of Variance for germ %
Source     DF        SS        MS        F        P
trt         3    308.87    102.96    16.71    0.001
Error       8     49.28      6.16
Total      11    358.15
                                  Individual 95% CIs For Mean
                                  Based on Pooled StDev
Level       N      Mean     StDev  ----+---------+---------+---------+--
1           3     7.167     2.363            (-----*----)
2           3     1.500     0.866   (----*-----)
3           3     4.167     3.329       (-----*----)
6           3    15.041     2.688                          (----*-----)
                                  ----+---------+---------+---------+--
Pooled StDev =    2.482              0.0       6.0      12.0      18.0
```

LSD = 4.67

Appendix 3-2 Raw Data (Spore Germinations) - *In vitro* evaluation of *Arthrobotrys oligospora* formulations

1h germinations following 24h incubation

Trt	Rep	Coating	Medium	Time	Plate	% Germ
1	1	U	W	1	1	95
1	1	U	W	1	2	93
1	2	U	W	1	1	89
1	2	U	W	1	2	92
1	3	U	W	1	1	89
1	3	U	W	1	2	85
2	1	U	R	1	1	85
2	1	U	R	1	2	87
2	2	U	R	1	1	85
2	2	U	R	1	2	89
2	3	U	R	1	1	80
2	3	U	R	1	2	93
3	1	A	R	1	1	44
3	1	A	R	1	2	58
3	2	A	R	1	1	54
3	2	A	R	1	2	55
3	3	A	R	1	1	61
3	3	A	R	1	2	44
4	1	B	R	1	1	4
4	1	B	R	1	2	2
4	2	B	R	1	1	7
4	2	B	R	1	2	3
4	3	B	R	1	1	7
4	3	B	R	1	2	2
5	1	C	R	1	1	67
5	1	C	R	1	2	65
5	2	C	R	1	1	72
5	2	C	R	1	2	66
5	3	C	R	1	1	71
5	3	C	R	1	2	72
6	1	D	R	1	1	75
6	1	D	R	1	2	84
6	2	D	R	1	1	90
6	2	D	R	1	2	89
6	3	D	R	1	1	89
6	3	D	R	1	2	92

1h germination following 48h incubation (reps 2 and 3 estimated from rep 1 observatons)

Trt	Rep	Coating	Medium	Time	Plate	% Germ	mean % germ
1	1	U	W	1	1	97.0	
1	1	U	W	1	2	96.0	96.5
1	2	U	W	1	1	91.4	
1	2	U	W	1	2	95.0	93.2
1	3	U	W	1	1	91.5	
1	3	U	W	1	2	87.5	89.5
2	1	U	R	1	1	93.0	
2	1	U	R	1	2	98.0	95.5
2	2	U	R	1	1	94.5	
2	2	U	R	1	2	98.6	96.6
2	3	U	R	1	1	89.5	
2	3	U	R	1	2	102.5	96.0
3	1	A	R	1	1	67.0	
3	1	A	R	1	2	58.0	62.5
3	2	A	R	1	1	65.7	
3	2	A	R	1	2	66.7	66.2
3	3	A	R	1	1	72.7	
3	3	A	R	1	2	55.8	64.3
4	1	B	R	1	1	2.0	
4	1	B	R	1	2	5.0	3.5
4	2	B	R	1	1	7.5	
4	2	B	R	1	2	3.5	5.5
4	3	B	R	1	1	7.6	
4	3	B	R	1	2	2.5	5.1
5	1	C	R	1	1	63.5	
5	1	C	R	1	2	60.8	62.1
5	2	C	R	1	1	68.1	
5	2	C	R	1	2	62.1	65.1
5	3	C	R	1	1	67.0	
5	3	C	R	1	2	68.1	67.6
6	1	D	R	1	1	89.0	
6	1	D	R	1	2	93.0	91.0
6	2	D	R	1	1	100.0	
6	2	D	R	1	2	100.0	100.0
6	3	D	R	1	1	100.0	
6	3	D	R	1	2	100.0	100.0

12 h germination following 48h incubation

Trt	Rep	Coating	Medium	Time	Plate	% Germ	mean % germ	12h germ rel to 1h	mean rel germ
1	1	U	W	12	1	4		4.1	
1	1	U	W	12	2	5	4.50	5.2	4.6
1	2	U	W	12	1	12		13.1	
1	2	U	W	12	2	6	9.00	6.3	9.7
1	3	U	W	12	1	8		8.7	
1	3	U	W	12	2	8	8.00	9.1	8.9
2	1	U	R	12	1	2		2.2	
2	1	U	R	12	2	0	1.00	0.0	1.0
2	2	U	R	12	1	3		3.2	
2	2	U	R	12	2	2	2.50	2.0	2.6
2	3	U	R	12	1	2		2.2	
2	3	U	R	12	2	0	1.00	0.0	1.1
3	1	A	R	12	1	0		0.0	
3	1	A	R	12	2	4	2.00	6.9	3.4
3	2	A	R	12	1	2		3.0	
3	2	A	R	12	2	3	2.50	4.5	3.7
3	3	A	R	12	1	8		11.0	
3	3	A	R	12	2	8	8.00	14.3	12.6
4	1	B	R	12	1	0		0.0	
4	1	B	R	12	2	0	0.00	0.0	0
4	2	B	R	12	1	0		0.0	
4	2	B	R	12	2	0	0.00	0.0	0
4	3	B	R	12	1	0		0.0	
4	3	B	R	12	2	0	0.00	0.0	0
5	1	C	R	12	1	0		0.0	
5	1	C	R	12	2	0	0.00	0.0	0
5	2	C	R	12	1	0		0.0	
5	2	C	R	12	2	0	0.00	0.0	0
5	3	C	R	12	1	0		0.0	
5	3	C	R	12	2	0	0.00	0.0	0
6	1	D	R	12	1	6		6.7	
6	1	D	R	12	2	18	12.00	19.4	13.0
6	2	D	R	12	1	8		8.2	
6	2	D	R	12	2	26	17.10	26.0	16.9
6	3	D	R	12	1	20		20.0	
6	3	D	R	12	2	12	16.0	12.0	15.7

Serial Dilutions

Formulations				**spores/g dry product**
dilution	-5	-6		
A (dry gel)	**10**	**2.5**		1.41×10^7
Dilution	-3	-4		
C (prill)	**10.5**	**2.5**		1.72×10^5
Dilution	-3	-4		
D (prill)	**20.5**	**2**		2×10^5

The count for each formula and dilution is the mean of the two plates

Appendix 4-1 Statistical Analysis – *In vitro* evaluation of fresh and dried *Duddingtonia flagrans* chlamydospores

Statistical output for dried spores

*** Estimates of parameters ***

	estimate	s.e.
R	0.9025	0.0357
B	-20.40	
A Trt 1	63.03	
A Trt 2	56.23	

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ Time	2	3538.72	1769.36	59.14	<.001
+ Trt	1	556.14	556.14	18.59	<.001
Residual	44	1316.42	29.92		
Total	47	5411.28	115.13		

Statistical output for fresh Spores

*** Estimates of parameters ***

	estimate	s.e.
R Trt 1	0.	1780813.
B Trt 1	10.48	2.70
A Trt 1	49.17	1.56
R Trt 2	0.9637	0.0117
B Trt 2	33.17	4.20
A Trt 2	27.26	3.89

*** Accumulated analysis of variance ***

Change	d.f.	s.s.	m.s.	v.r.	F pr.
+ Time	2	2960.38	1480.19	50.61	<.001
+ Trt	1	1406.85	1406.85	48.11	<.001
+ Time.Trt	1	747.93	747.93	25.58	<.001
+ Separate nonlinear	1	228.93	228.93	7.83	0.008
Residual	42	1228.26	29.24		
Total	47	6572.35	139.84		

Alternative analysis

One-way ANOVA: change germ versus trt

```
Analysis of Variance for change g
Source     DF        SS        MS        F        P
trt         3    5722.0    1907.3   146.72    0.000
Error       8     104.0      13.0
Total      11    5826.0

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  -------+---------+---------+---------
1           3    28.333     4.619                                  (-*--)
2           3    15.333     4.163                        (--*-)
3           3    -6.667     2.517              (--*-)
4           3   -29.000     2.646  (--*-)
                                    -------+---------+---------+---------
Pooled StDev =    3.606                -20         0        20
```

Test for Equal Variances

```
Response    change germ
Factors     trt
ConfLvl     95.0000

Bonferroni confidence intervals for standard deviations

  Lower      Sigma      Upper     N  Factor Levels

 2.05024   4.61880   58.3323     3    1
 1.84806   4.16333   52.5800     3    2
 1.11710   2.51661   31.7831     3    3
 1.17442   2.64575   33.4140     3    4


Bartlett's Test (normal distribution)


Test Statistic: 0.919
P-Value       : 0.821


Levene's Test (any continuous distribution)


Test Statistic: 0.107
P-Value       : 0.954
```

Appendix 4-2 Raw Data (Spore Germination) - *In vitro* evaluation of fresh and dried *Duddingtonia flagrans* chlamydospores

1 h and 24 h germinations following 48h incubation

Trt	Rep	Spores	Medium	Time	Plate	% Germ	mean % germ
1	1	D	W	1	1	41.0	
1	1	D	W	1	2	35.0	38.0
1	2	D	W	1	1	44.2	
1	2	D	W	1	2	36.0	40.1
1	3	D	W	1	1	35.9	
1	3	D	W	1	2	42.5	39.2
2	1	D	R	1	1	40.2	
2	1	D	R	1	2	47.5	43.8
2	2	D	R	1	1	41.0	
2	2	D	R	1	2	32.0	36.5
2	3	D	R	1	1	41.0	
2	3	D	R	1	2	35.0	38.0
3	1	F	W	1	1	56.9	
3	1	F	W	1	2	61.0	58.9
3	2	F	W	1	1	57.0	
3	2	F	W	1	2	68.0	62.5
3	3	F	W	1	1	60.0	
3	3	F	W	1	2	55.0	57.5
4	1	F	R	1	1	61.0	
4	1	F	R	1	2	57.0	59.0
4	2	F	R	1	1	64.0	
4	2	F	R	1	2	61.0	62.5
4	3	F	R	1	1	51.0	
4	3	F	R	1	2	67.0	59.0
1	1	D	W	24	1	63.0	
1	1	D	W	24	2	58.0	60.5
1	2	D	W	24	1	64.1	
1	2	D	W	24	2	68.0	66.1
1	3	D	W	24	1	58.0	
1	3	D	W	24	2	55.0	56.5
2	1	D	R	24	1	51.0	
2	1	D	R	24	2	59.0	55.0
2	2	D	R	24	1	60.0	
2	2	D	R	24	2	56.0	58.0
2	3	D	R	24	1	58.0	
2	3	D	R	24	2	48.1	53.0
3	1	F	W	24	1	49.0	
3	1	F	W	24	2	49.0	49.0
3	2	F	W	24	1	42.0	
3	2	F	W	24	2	42.0	42.0
3	3	F	W	24	1	39.0	
3	3	F	W	24	2	43.0	41.0
4	1	F	R	24	1	56.0	
4	1	F	R	24	2	40.0	48.0
4	2	F	R	24	1	47.0	
4	2	F	R	24	2	33.0	40.0
4	3	F	R	24	1	41.0	
4	3	F	R	24	2	38.0	39.5

48 h and 72 h germinations following 48 h incubation

Trt	Rep	Spores	W/R	Time	Plate	% Germ	mean % germ
1	1	D	W	48	1	62.0	
1	1	D	W	48	2	61.0	61.5
1	2	D	W	48	1	64.0	
1	2	D	W	48	2	61.0	62.5
1	3	D	W	48	1	57.0	
1	3	D	W	48	2	69.0	63.0
2	1	D	R	48	1	59.0	
2	1	D	R	48	2	51.0	55.0
2	2	D	R	48	1	55.0	
2	2	D	R	48	2	55.8	55.4
2	3	D	R	48	1	42.0	
2	3	D	R	48	2	55.3	48.7
3	1	F	W	48	1	46.0	
3	1	F	W	48	2	53.0	49.5
3	2	F	W	48	1	50.0	
3	2	F	W	48	2	55.0	52.5
3	3	F	W	48	1	46.0	
3	3	F	W	48	2	53.0	49.5
4	1	F	R	48	1	32.0	
4	1	F	R	48	2	31.0	31.5
4	2	F	R	48	1	29.0	
4	2	F	R	48	2	27.0	28.0
4	3	F	R	48	1	33.0	
4	3	F	R	48	2	28.0	30.5
1	1	D	W	72	1	64.0	
1	1	D	W	72	2	60.0	62.0
1	2	D	W	72	1	68.0	
1	2	D	W	72	2	73.0	70.5
1	3	D	W	72	1	68.0	
1	3	D	W	72	2	72.0	70.0
2	1	D	R	72	1	50.0	
2	1	D	R	72	2	53.0	51.5
2	2	D	R	72	1	58.0	
2	2	D	R	72	2	61.0	59.5
2	3	D	R	72	1	59.0	
2	3	D	R	72	2	48.0	53.5
3	1	F	W	72	1	51.0	
3	1	F	W	72	2	50.0	50.5
3	2	F	W	72	1	50.0	
3	2	F	W	72	2	55.0	52.5
3	3	F	W	72	1	59.0	
3	3	F	W	72	2	53.0	56.0
4	1	F	R	72	1	33.0	
4	1	F	R	72	2	36.0	34.5
4	2	F	R	72	1	31.0	
4	2	F	R	72	2	27.0	29.0
4	3	F	R	72	1	28.0	
4	3	F	R	72	2	32.0	30.0

Appendix 5-1 Statistical Analysis - *In vitro* evaluation of coated *Duddingtonia flagrans* chlamydospores

General Linear Model: % germ versus replicate, coating, medium

```
Factor     Type Levels Values
rep       fixed      3 1 2 3
coating   fixed      6 104 106 107 108 109 na
medium    fixed      2 r w

Analysis of Variance for % germ, using Adjusted SS for Tests

Source          DF     Seq SS     Adj SS     Adj MS       F      P
rep              2     498.49     498.49     249.25   13.86  0.000
coating          5    1187.89    1187.89     237.58   13.21  0.000
medium           1      22.09      22.09      22.09    1.23  0.280
coating*medium   5     182.94     182.94      36.59    2.03  0.113
Error           22     395.69     395.69      17.99
Total           35    2287.10


Unusual Observations for % germ

Obs    % germ       Fit      SE Fit  Residual   St Resid
  5   45.5000   36.2000      2.6447    9.3000      2.81R
 21   27.0000   19.7875      2.6447    7.2125      2.18R

R denotes an observation with a large standardized residual.

Least Squares Means for % germ

coating    Mean   SE Mean
104       14.25     1.731
106       20.75     1.731
107       21.29     1.731
108       18.67     1.731
109       20.68     1.731
none      33.17     1.731
```

Appendix 5-2 Raw Data (Spore Germination) - *In vitro* evaluation of coated *Duddingtonia flagrans* chlamydospores

1 h counts following 48h incubation at 25°C

Trt	Rep	Medium	Coating	Time	Plate	% germ
1	1	W	none	1	1	21
1	1	W	none	1	2	15
1	2	W	none	1	1	17
1	2	W	none	1	2	21
1	3	W	none	1	1	12
1	3	W	none	1	2	8
2	1	R	none	1	1	41
2	1	R	none	1	2	27
2	2	R	none	1	1	23
2	2	R	none	1	2	18
2	3	R	none	1	1	24
2	3	R	none	1	2	17
3	1	W	104	1	1	22
3	1	W	104	1	2	21
3	2	W	104	1	1	12
3	2	W	104	1	2	17
3	3	W	104	1	1	16
3	3	W	104	1	2	16
4	1	R	104	1	1	25
4	1	R	104	1	2	30
4	2	R	104	1	1	17
4	2	R	104	1	2	19
4	3	R	104	1	1	19
4	3	R	104	1	2	13
5	1	W	106	1	1	20
5	1	W	106	1	2	24
5	2	W	106	1	1	13
5	2	W	106	1	2	17
5	3	W	106	1	1	21
5	3	W	106	1	2	15
6	1	R	106	1	1	28
6	1	R	106	1	2	32
6	2	R	106	1	1	19
6	2	R	106	1	2	13
6	3	R	106	1	1	17
6	3	R	107	1	2	18
7	1	W	107	1	1	22
7	1	W	107	1	2	25
7	2	W	107	1	1	12
7	2	W	107	1	2	9.5
7	3	W	107	1	1	17
7	3	W	107	1	2	12
8	1	R	107	1	1	35
8	1	R	107	1	2	39

8	2	R	107	1	1	20
8	2	R	107	1	2	24
8	3	R	107	1	1	10
8	3	R	107	1	2	24
9	1	W	108	1	1	19
9	1	W	108	1	2	31
9	2	W	108	1	1	13
9	2	W	108	1	2	18
9	3	W	108	1	1	17
9	3	W	108	1	2	13
10	1	R	108	1	1	26
10	1	R	108	1	2	35
10	2	R	108	1	1	19
10	2	R	108	1	2	16
10	3	R	108	1	1	22
10	3	R	108	1	2	17
11	1	W	109	1	1	18
11	1	W	109	1	2	18
11	2	W	109	1	1	14
11	2	W	109	1	2	14
11	3	W	109	1	1	11
11	3	W	109	1	2	9
12	1	R	109	1	1	35
12	1	R	109	1	2	30
12	2	R	109	1	1	32
12	2	R	109	1	2	21
12	3	R	109	1	1	19
12	3	R	109	1	2	19

48h counts following 48h incubation at 25°C

Trt	Rep	Medium	Coating	Time	Plate	% germ
1	1	W	NA	48	1	28
1	1	W	NA	48	2	30
1	2	W	NA	48	1	22
1	2	W	NA	48	2	27
1	3	W	NA	48	1	31
1	3	W	NA	48	2	27
2	1	R	NA	48	1	42
2	1	R	NA	48	2	37
2	2	R	NA	48	1	44
2	2	R	NA	48	2	47
2	3	R	NA	48	1	32
2	3	R	NA	48	2	31
3	1	W	104	48	1	16
3	1	W	104	48	2	22
3	2	W	104	48	1	8
3	2	W	104	48	2	9
3	3	W	104	48	1	14
3	3	W	104	48	2	14
4	1	R	104	48	1	20
4	1	R	104	48	2	28
4	2	R	104	48	1	11
4	2	R	104	48	2	16
4	3	R	104	48	1	6
4	3	R	104	48	2	7
5	1	W	106	48	1	28
5	1	W	106	48	2	29
5	2	W	106	48	1	19
5	2	W	106	48	2	11
5	3	W	106	48	1	24
5	3	W	106	48	2	17
6	1	R	106	48	1	29
6	1	R	106	48	2	23
6	2	R	106	48	1	15
6	2	R	106	48	2	20
6	3	R	106	48	1	21
6	3	R	107	48	2	13
7	1	W	107	48	1	22
7	1	W	107	48	2	28
7	2	W	107	48	1	9
7	2	W	107	48	2	16
7	3	W	107	48	1	26
7	3	W	107	48	2	28
8	1	R	107	48	1	29
8	1	R	107	48	2	26
8	2	R	107	48	1	18
8	2	R	107	48	2	17
8	3	R	107	48	1	17
8	3	R	107	48	2	14
9	1	W	108	48	1	23
9	1	W	108	48	2	18
9	2	W	108	48	1	18

9	2	W	108	48	2	20
9	3	W	108	48	1	19
9	3	W	108	48	2	16
10	1	R	108	48	1	29
10	1	R	108	48	2	21
10	2	R	108	48	1	15
10	2	R	108	48	2	18
10	3	R	108	48	1	14
10	3	R	108	48	2	18
11	1	W	109	48	1	24
11	1	W	109	48	2	27
11	2	W	109	48	1	14
11	2	W	109	48	2	16
11	3	W	109	48	1	17
11	3	W	109	48	2	18
12	1	R	109	48	1	19
12	1	R	109	48	2	34
12	2	R	109	48	1	23
12	2	R	109	48	2	18
12	3	R	109	48	1	13
12	3	R	109	48	2	16

Appendix 6-1 - Procedure to determine the larval development of Parasitic Nematodes in faeces of sheep

Materials

- 85 mm x 25 mm cell culture dishes and lids (Corning), 1 per culture
- 55 mm x 10 mm Petri dishes (Corning), 1 per culture
- Metal spatula
- Alcohol burner
- ethanol
- Distilled water (10 ml/culture)
- Baermann funnels (tissues and sieves)
- At least 2000 parasite eggs per culture
- Balance
- 50 ml centrifuge tube (falcon)

Methods

- Weigh 10 g (± 0.1g) of faeces into a 55 mm diameter Petri dish for with no lid using a metal spatula
- Place Petri dish inside a 85 mm cell culture dish and add distilled water to the cell culture dish, until the base is covered (approximately 10 mls/dish)
- Wash spatula with hot water, dip in ethanol and flame between samples from individual animals
- Add 2000-3000 parasite eggs to each culture
- Replace lid of outer, cell culture dish and label
- Incubate for 14 days at 20°C,
- Check water level in cell culture dish at least once during 14 day incubation, and replenish if necessary.
- Following incubation, recover infective third stage larvae by placing cultures on Baermann funnels, with a single layer of tissue and a steel mesh.
- After 24 h, tap off 50 ml into a centrifuge tube, which may then be concentrated to 10ml by standing for at least 8 h and then siphoning off the supernatant.
- 20% of the 10ml sample is placed in a glass chamber slide with a drop of Lugols solution and examined under 40X magnification
- If the number of larvae in this initial proportion is less than 50, then a further 20% will be counted until fifty or more larvae have been counted, or the complete sample has been counted.

Calculations

The total number of third stage larvae in each sample is determined by the following equation;

(Number of larvae recovered ÷ number of proportions counted) x 5= Total number of larvae recovered per sample

This figure can be used to determine the proportion of eggs that developed into third stage larvae, if required, using the following calculations (where a known number of eggs was added to the faeces);

(total # of larvae recovered ÷ # of eggs added) x 100/1 = percent development per sample

The arithmetic mean of the three cultures is then used, as the value recovered per animal or treatment, in further analyses.

Appendix 6-2 - Procedure for detecting nematophagous fungi in faeces

Materials

- Five tap water agar (2%) plates per animal to be tested [agar made from Standard agar (Germantown) (20g/l) and tap water, and ½ strength nutrient agar made from Nutrient broth (Difco) (4g/l), Standard agar (Germantown) (15g/l) and tap water, autoclaved at 15lb/in^2 for 15 minutes and poured into Petri dish plates]
- Three to six-week old cultures of *Rhabditis* sp. nematodes [cultured on ½ strength nutrient agar, incubated at 15°C]

Method to set up presence/absence tests

- Check each *Rhabditis* sp. plate under stereomicroscope to ensure that the nematodes are active
- Gloves are to be worn when handling samples.
- 1g of faeces is spread onto each of at least 5 replicate Petri plates (9cm diameter) of 2% tap water agar
- Add to each sample plate, one-quarter plate of 3 to 6-week-old *Rhabditis* sp. culture (>500 nematodes), while under a laminar flow cabinet.
- Sterilise spatula between samples by first cleaning with tap water, then dipping in ethanol and flaming over an alcohol burner.
- Sealed plates with plastic cling film and incubate at 20°C.
- Examine plates at 2, 3, and 4 week intervals (extended to 6 week if necessary to confirm identification) under a stereomicroscope (16X-60X) for the presence of nematophagous fungi.

Detection Criteria

Nematophagous fungi present on plates are identified on the basis of characteristic structures involved in nematode parasitism (e.g. traps and or hyphae colonising nematode cadavers), or fungal dispersal (e.g. conidiophores and conidia). Characteristics seen under the stereomicroscope, and/or under a compound microscope (125X-500X) on blocks of agar cut from culture plates are compared with those in the identification keys of Cook and Godfrey (1964) and Van Oorschot (1985). Plates are recorded as being positive or negative for the fungus in question and if diagnostic characteristics are not apparent after 6 weeks, identification is regarded as uncertain and results are excluded.

Negative reports are based on failure to detect a specific fungus on all plates of at least 5 (or 2 in the case of detecting nematophagous fungi in feed grains) replicate detection plates.

Appendix 6-3 Statistical Analysis - *In vivo* evaluation of coated *Duddingtonia flagrans* chlamydospores

Test for Equal Variances

```
Response    culture
Factors     trt
ConfLvl     95.0000

Bonferroni confidence intervals for standard deviations

   Lower     Sigma     Upper     N  Factor Levels

 191.417   378.279   1813.78     5    1
 194.721   384.809   1845.09     5    2
 132.839   262.517   1258.73     5    3
  81.073   160.216    768.21     5    4
  89.374   176.622    846.87     5    5
  66.761   131.933    632.60     5    6
  61.043   120.633    578.41     5    7


Bartlett's Test (normal distribution)


Test Statistic:  9.905
P-Value       :  0.129


Levene's Test (any continuous distribution)


Test Statistic: 0.682
P-Value       : 0.666
```

One-way ANOVA: culture versus trt

```
Analysis of Variance for culture
Source     DF        SS        MS        F        P
trt         6    262390     43732     0.68    0.666
Error      28   1795644     64130
Total      34   2058034

                                    Individual 95% CIs For Mean
                                    Based on Pooled StDev
Level       N      Mean     StDev  ------+---------+---------+---------+
1           5     550.2     378.3           (--------*--------)
2           5     484.6     384.8        (--------*---------)
3           5     504.4     262.5         (--------*--------)
4           5     341.6     160.2  (---------*--------)
5           5     641.6     176.6              (---------*--------)
6           5     503.6     131.9         (--------*--------)
7           5     430.6     120.6      (--------*---------)
                                    ------+---------+---------+---------+
Pooled StDev =    253.2              250       500       750      1000
```

Appendix 6-4 Raw Data (Larval Cultures) - *In vivo* evaluation of coated *Duddingtonia flagrans* chlamydospores

Individual larval recoveries

					L3's recovered						
Tag	trt	rep	Culture	Coating	1	2	3	4	5	total	mean total
349	1	1	1	na	38	30	26	23	12	129	
349	1	1	2	na	42	33	32			178	
349	1	1	3	na	56	63				298	202
1723	1	2	1	na	22	32	22	19	7	102	
1723	1	2	2	na	65	71				340	
1723	1	2	3	na	58	66				310	251
3069	1	3	1	na	140					700	
3069	1	3	2	na	177					885	
3069	1	3	3	na	39					195	593
51	1	4	1	na	112					560	
51	1	4	2	na	109					545	
51	1	4	3	na	112					560	555
3412	1	5	1	na	262					1310	
3412	1	5	2	na	226					1130	
3412	1	5	3	na	202					1010	1150
3068	2	1	1	none	262					1310	
3068	2	1	2	none	181					905	
3068	2	1	3	none	252					1260	1158
3378	2	2	1	none	41	25	25	20	12	123	
3378	2	2	2	none	123					615	
3378	2	2	3	none	71	46				293	344
1	2	3	1	none	83	79				405	
1	2	3	2	none	29	15	34	26	10	114	
1	2	3	3	none	61	52				283	267
1317	2	4	1	none	10	16	16	10	6	58	
1317	2	4	2	none	64	74				345	
1317	2	4	3	none	64	41				263	222
5275	2	5	1	none	70	97				418	
5275	2	5	2	none	90	80				425	
5275	2	5	3	none	82	99				453	432
11	3	1	1	104	75	59				335	
11	3	1	2	104	64	66				325	
11	3	1	3	104	54	59				283	314
74	3	2	1	104	180					900	
74	3	2	2	104	189					945	
74	3	2	3	104	84	81				413	753
3008	3	3	1	104	22	34	39	25	0	120	
3008	3	3	2	104	89	129				545	
3008	3	3	3	104	137					685	450
176	3	4	1	104	191					955	
176	3	4	2	104	185					925	
176	3	4	3	104	103					515	798
1319	3	5	1	104	67	51				295	
1319	3	5	2	104	35	36	31			170	
1319	3	5	3	104	35	33	24	45	18	155	207
2092	4	1	1	106	19	39	29	33	5	125	
2092	4	1	2	106	34	40	58	20	20	172	
2092	4	1	3	106	92	111				508	268
177	4	2	1	106	88	93				453	

177	4	2	2	106	90	64				385	
177	4	2	3	106	113					565	468
3325	4	3	1	106	50	46	53			248	
3325	4	3	2	106	27	45	37	15	16	140	
3325	4	3	3	106	48	62				275	221
75	4	4	1	106	4	0	3	2	0	9	
75	4	4	2	106	53	59				280	
75	4	4	3	106	65	55				300	196
3413	4	5	1	106	193					965	
3413	4	5	2	106	27	28	42	41	11	149	
3413	4	5	3	106	110					550	555
1322	5	1	1	107	34	42	39			192	
1322	5	1	2	107	89	70				398	
1322	5	1	3	107	149					745	445
3336	5	2	1	107	101					505	
3336	5	2	2	107	171					855	
3336	5	2	3	107	160					800	720
3390	5	3	1	107	138					690	
3390	5	3	2	107	123					615	
3390	5	3	3	107	144					720	675
3042	5	4	1	107	176					880	
3042	5	4	2	107	207					1035	
3042	5	4	3	107	144					720	878
73	5	5	1	107	104					520	
73	5	5	2	107	101					505	
73	5	5	3	107	93	85				445	490
3067	6	1	1	108	40	63				258	
3067	6	1	2	108	77	77				385	
3067	6	1	3	108	94	78				430	358
5274	6	2	1	108	174					870	
5274	6	2	2	108	155					775	
5274	6	2	3	108	153					293	646
1318	6	3	1	108	54	63				390	
1318	6	3	2	108	88	68				560	
1318	6	3	3	108	112					910	620
1323	6	4	1	108	182					165	
1323	6	4	2	108	28	39	26	59	13	950	
1323	6	4	3	108	190					418	511
1724	6	5	1	108	91	76				370	
1724	6	5	2	108	74					403	
1724	6	5	3	108	77	84				375	383
1325	7	1	1	109	76	74				120	
1325	7	1	2	109	31	25	20	34	10	313	
1325	7	1	3	109	82	43				313	248
447	7	2	1	109	68	79				368	
447	7	2	2	109	83	62				363	
447	7	2	3	109	154					770	500
1320	7	3	1	109	175					875	
1320	7	3	2	109	19	19	17	17	21	93	
1320	7	3	3	109	66	69				338	435
2941	7	4	1	109	90	82				430	
2941	7	4	2	109	22	40	28	39	5	134	
2941	7	4	3	109	128					640	401
1324	7	5	1	109	177					885	
1324	7	5	2	109	85	80				413	
1324	7	5	3	109	83	81				410	569

Appendix 6-5 – Raw Data (Presence/absence test) - *In vivo* evaluation of coated *Duddingtonia flagrans* chlamydospores

Individual test results for the presence of viable *Duddingtonia flagrans* chlamydospores in faeces.

Tag	treatment	replicate	coating	+/-
349	1	1	na	+
1723	1	2	na	-
3069	1	3	na	-
51	1	4	na	-
3412	1	5	na	-
3068	2	1	None	+
3378	2	2	None	+
1	2	3	None	+
1317	2	4	None	+
5275	2	5	None	-
11	3	1	104	+
74	3	2	104	+
3008	3	3	104	+
176	3	4	104	+
1319	3	5	104	+
2092	4	1	106	-
177	4	2	106	+
3325	4	3	106	+
75	4	4	106	-
3413	4	5	106	+
1322	5	1	107	+
3336	5	2	107	+
3390	5	3	107	+
3042	5	4	107	+
73	5	5	107	unknown
3067	6	1	108	+
5274	6	2	108	-
1318	6	3	108	-
1323	6	4	108	+
1724	6	5	108	+
1325	7	1	109	-
447	7	2	109	+
1320	7	3	109	+
2941	7	4	109	+
1324	7	5	109	+