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The predatory capability of three nematophagous fungi in the control of *Haemonchus contortus* infective larvae in ovine faeces

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Abstract

The effect of oral administration of three different nematode-trapping fungi, in aqueous suspension containing either *Dactylaria* sp. or *Arthrobotrys oligospora* conidia or *Duddingtonia flagrans* chlamydospores, on the number of *Haemonchus contortus* infective larvae in sheep faeces, was evaluated. The three selected species of fungi produce three-dimensional adhesive nets in the presence of nematodes. Sixteen Creole sheep were divided into four groups of four animals each. Groups 1 and 2 were orally drenched with a suspension containing 2×10^7 conidia of either *A. oligospora* or *Dactylaria* sp. Group 3, received a similar treatment, with *D. flagrans* chlamydospores, instead of conidia, being administered, at the same dose. Group 4 acted as control, without any fungi. Faecal samples were collected directly from the rectum of each sheep and faecal cultures were prepared and incubated at 15 and 21 days. Larvae were recovered from faecal cultures and counted. The highest reduction of the nematode population occurred in the *D. flagrans* group, reaching reductions of 96.3% and 91.4% in individual samplings in plates incubated for 15 and 21 days, respectively. *Arthrobotrys oligospora* showed moderate reductions in the faecal larval population, ranging between 25–64% at 15 days incubation. In general, *Dactylaria* sp., was less efficient in its trapping ability. Despite the inconsistent results with *Dactylaria* sp., reduction percentages of 73.4% and 80.7% were recorded in individual samplings during the first and second days, in plates incubated for 15 days. *Duddingtonia flagrans*, was shown to be a potential biological control agent of *H. contortus* infective larvae.

Introduction

Gastro-intestinal nematodiasis is considered to be one of the main problems which considerably increase production costs in the sheep industry worldwide (Soulsby, 1987; Nansen, 1986, 1987). Different nematodes cause different levels of damage, especially in young animals in which malnutrition, loss of both appetite and weight gain and even the death of animals in the case of massive attack, can occur (Cheville, 1988; Githigia *et al.*, 1995). *Haemonchus contortus* is considered to be one of the most pathogenic parasites mainly because of its world-wide distribution, especially in tropical and subtropical areas. The intensive sucking-blood activity of *H. contortus*

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from the gastric mucosa of the abomasum can produce a severe state of anaemia and even the death of young animals (Charleston, 1994; Sauer, 1996).

Traditionally, the use of chemical anthelmintic drugs against gastro-intestinal nematodes has been considered the most common de-worming practice in the livestock industry (Bisset *et al.*, 1997). However, new control strategies alternative to chemical drugs have been investigated during the past decades, especially as anthelmintic resistance is becoming an increasing problem, to lower the risk of damage to non-target organisms in the ecosystem (Larsen *et al.*, 1991; Waller, 1993, Waller & Faedo, 1993). The use of nematode-trapping fungi as natural enemies has been investigated as an alternative control method for ruminant parasitic nematodes (Barron, 1977; Larsen *et al.*, 1991, 1992, 1994, 1998).

The easiest way to achieve close contact between nematode-trapping fungi and gastro-intestinal free-living nematodes in faeces is through the ingestion of bio-preparations containing such fungi. Once fungi have successfully passed through the gastro-intestinal tract of sheep, the fungi colonize the faecal material and produce traps to capture and destroy nematode larvae recently hatched from eggs (Pryadko & Osipov 1986; Grønvold *et al.*, 1988, 1993; Larsen, 2000).

A large number of genera and species of nematode-trapping fungi have been identified. *Duddingtonia* and *Arthrobotrys* are some of the most widely studied genera and such fungi have showed a strong ability to trap and destroy ruminant gastro-intestinal nematode larvae, i.e. *H. contortus*, *Cooperia* spp., *Ostertagia ostertagi* and others (Faedo *et al.*, 1998; Mendoza de Gives *et al.*, 1998; Grønvold *et al.*, 1989; Flores-Crespo *et al.*, 2001). On the other hand, species of the genus *Dactylaria* are not widely studied, and they could be explored in both *in vitro* and in field trials to evaluate their potential in the control of either animal or plant parasite nematodes. The present investigation was performed to evaluate the effect of the oral administration of *Dactylaria* sp., *Arthrobotrys oligospora* conidia or *Duddingtonia flagrans* chlamydospores to sheep, in the number of *Haemonchus contortus* infective larvae on faeces.

Material and methods

Location

This investigation was carried out at the National Centre of Disciplinary Researches in Veterinary Parasitology (CENID-PAVET) from INIFAP, in Jiutepec, State of Morelos, Mexico. This area has a sub-tropical humid climate, with 800 to 1400 mm of rain per year, with June to September being the rainiest period and December to May the driest period. The yearly average temperature ranges between 18 and 21°C (Síntesis geográfica de Morelos, 1981).

Haemonchus contortus *infective larvae*

A sheep infected with *Haemonchus contortus* acted as the donor for nematode eggs. Larvae were recovered from faecal cultures following the technique described by Liébano-Hernández (1989).

Sheep

Sixteen 2- to 3-year-old male and female Creole sheep, ranging between 30 and 36 kg body weight and free of gastro-intestinal parasitic nematodes, were orally infected with 5×10^3 *H. contortus* infective larvae. Four groups of four sheep were formed, depending upon the average number of eggs deposited per gram of faeces after the pre-patent period.

Nematophagous fungi

The nematode-trapping fungi, *Duddingtonia flagrans* (FTHO-8) and *Arthrobotrys oligospora* (FTHO-6), were originally isolated from sheep faecal samples collected directly from the rectum of sheep on a farm located in Fierro del Toro, Huitzilac, State of Morelos, Mexico (Llerandi-Juárez & Mendoza de Gives, 1998). Fungi were maintained in the laboratory on water agar plates (20 g of agar in 1000 ml of distilled water). Fungal biomass was increased by transferring either conidia or chlamydospores to potato-dextrose agar plates (extract of 20 g of potato + dextrose 20 g + 20 g agar in 1000 ml of distilled water) (Ulloa & Hanlin, 1978). Fungi were incubated for 8 weeks at room temperature (25–30°C). Then, 10 ml of distilled water were added to each plate and fungal material (either conidia or chlamydospores) was removed from the agar surface with a metallic scalpel and collected into crystal flasks. Fungal quantification was estimated using the Neubauer chamber's technique (Medway *et al.*, 1980). *Dactylaria* sp. (DAC), was isolated from a soil garden sample from the Post-graduate College in Chapingo, State of Mexico. This fungus produces three-dimensional adhesive nets, like the other fungi under study. Conidia or chlamydospores were obtained following the method as previously described.

Experimental procedure

Once the four groups were established, every sheep from groups 1 to 3 received an oral suspension of distilled water containing 2×10^7 spores of *A. oligospora* (FTHO-6), *Dactylaria* sp. (DAC) and *D. flagrans* (FTHO-8), respectively. Group 4 as a control, only received distilled water as a placebo.

Before the treatments started and during the seven subsequent days, samples of faeces were taken directly from the rectum of every sheep from all four groups. Faecal samples were collected three times a day (08:00 am, 16:00 pm and 24:00 pm) and macerated in a mortar with a pestle. Macerated faeces were mixed in a blender to obtain a homogeneous mixture. The McMaster technique performed at the time zero revealed a consistent mean number of eggs per gram of faeces in the four groups (1981.2 ± 187.5 epg). Two 2-g faecal cultures per sample were prepared every day using disposable Petri dishes. Sterile distilled water (0.5 ml) was added to each plate and then mixed to maintain adequate moisture in the faecal cultures. The incubation periods were 15 and

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21 days at room temperature (25–30°C) and 60–80% relative humidity.

Infective larvae of *H. contortus* were extracted from the faecal cultures using the Baermann funnel technique, over a period of 24 h. The total number of larvae was estimated by counting the number of larvae in ten 5 μl-aliquots under a microscope (40 ×). Results were compared between groups and a reduction percentage was estimated for each group (Larsen *et al.*, 1992).

In order to verify the viability of fungi after passing through the gastro-intestinal tract of animals, 2 g of faeces from each sheep were deposited in a plastic Petri dish containing water agar. One hundred and fifty *H. contortus* infective larvae were added to each plate and finally incubated at room temperature (25–30°C) for three weeks. The presence of fungi and predatory activity were registered twice a week under a microscope.

Larval reduction percentage on faecal cultures was calculated using the following formula:

$$\text{Larval reduction percentage} = \frac{r - s}{r} \times 100$$

where: r = mean number of recovered larvae in control group and s = mean number of recovered larvae in treated group.

Statistical analysis

Analysis of variance using the Tukey test to compare the means of the groups was used (Hurley *et al.*, 1981).

Results

The three nematophagous fungi were seen on the water-agar plates either 15 or 21 days after adding samples of faecal material. *Duddingtonia flagrans* showed the most abundant growth, followed by *A. oligospora* and *Dactylaria* sp. Seven days after starting the experiment, the fungi showed the development of three-dimensional adhesive nets, mainly on those containing *D. flagrans* and in a smaller number on plates with *A. oligospora*. Again, the fewest traps were observed in *Dactylaria* sp. No fungal growth was observed in the control group plates.

Reduction percentages in larval populations of *H. contortus* in sheep faecal cultures after 15 and 21 days of incubation are shown in tables 1 and 2. The McMaster method showed no significant changes in faecal egg counting in samples from groups, either before or after treatment. Significant reductions in the faecal larval populations were observed with the three fungi; particularly, within the first three days after fungal treatments in cultures incubated for 15 days (table 1). The highest reduction percentages of nematode populations were observed in *D. flagrans* plates on the second day of sampling, reaching 96.3% and 91.4% individual reduction percentages in 15 and 21 incubation days, respectively (tables 1 and 2). *Arthrobotrys oligospora* showed moderate reduction percentages of 64.0% and 51.9% in the faecal larval population in cultures from the second and third sampling days, incubated for 15 days. Similarly, this fungus reached a 40.2% average reduction at the first

Table 1. Individual and daily mean reduction percentages of *Haemonchus contortus* infective larvae in faecal cultures of sheep, treated with either *Duddingtonia flagrans*, *Arthrobotrys oligospora* or *Dactylaria* sp., following an incubation period of 15 days.

		Group 1 *A. oligospora* (strain FTHO-6)		Group 2 *Dactylaria* sp. (strain DAC)		Group 3 *D. flagrans* (strain FTHO-8)	
Sampling days	Time (h)	Individual sampling	Mean	Individual sampling	Mean	Individual sampling	Mean
1	8	13.0		13.0		47.8	
	16	60.4	39.5 c	73.4	52.8 b	74.4	66.5 a
	24	62.3		72.1		88.5	
2	8	38.6		11.3		75.0	
	16	69.0	64.0 b	50.9	47.6 c	96.3	86.5 a
	24	84.6		80.7		88.4	
3	8	58.3		12.5		87.5	
	16	78.9	51.9 a	43.8	18.7 b	38.6	54.3 a
	24	18.5		–		37.0	
4	8	22.2		22.2		44.4	
	16	26.2	25.3 a	–	7.4 b	46.1	30.0 a
	24	27.7		–		–	
5	8	03.7		–		29.6	
	16	–	0.0	–	4.3*	17.3	19.9*
	24	08.7		13.0		13.0	
6	8	26.1		–		–	
	16	–	8.7*	14.2	8.4*	9.5	5.6*
	24	–		11.1		7.4	
7	8	–		–		6.6	
	16	15.0	11.6*	33.0	11.0*	–	7.4*
	24	20.0		–		15.7	

Values followed by a different letter indicate statistical differences ($P < 0.05$).
*Not significant.

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Table 2. Individual and daily mean reduction percentages of *Haemonchus contortus* infective larvae in faecal cultures of sheep treated with either *Duddingtonia flagrans*, *Arthrobotrys oligospora* or *Dactylaria* sp., following an incubation period of 21 days.

		Group 1 *A. oligospora* (strain FTHO-6)		Group 2 *Dactylaria* sp. (strain DAC)		Group 3 *D. flagrans* (strain FTHO-8)	
Sampling days	Time (h)	Individual sampling	Mean	Individual sampling	Mean	Individual sampling	Mean
1	8	5.8		–		23.5	
	16	38.8	40.2 a	–	15.0 b	–	34.0 a
	24	76.1		45.2		78.5	
2	8	6.4		34.5		80.6	
	16	40.0	25.7 b	–	11.8 b	91.4	85.5 a
	24	30.7		–		84.6	
3	8	78.5		57.1		75.0	
	16	–	37.2 a	–	19.0 b	27.7	34.2 a
	24	33.3		–		–	
4	8	–		–		27.2	
	16	27.2	13.0 b*	–	0.0 b	29.5	34.9 a
	24	12.0		–		48.0	
5	8	8.5		29.7		48.9	
	16	21.8	10.1 c	28.5	21.1 b	42.0	37.3 a
	24	–		5.2		21.0	
6	8	11.4		21.3		26.2	
	16	9.0	8.8*	27.2	18.2*	18.1	14.7*
	24	6.2		6.2		–	
7	8	–		–		3.5	
	16	11.9*	3.9*	–	0.0*	26.1	9.8*
	24	–		–		–	

Values followed by a different letter indicate statistical differences ($P < 0.05$).
*Not significant.

sampling day in plates incubated for 21 days (table 2). After the third sampling day, reduction percentages declined in all three groups in both incubation periods.

In general, *Dactylaria* sp. (group 2) was less efficient in the ability to trap *H. contortus* larvae, as shown in the corresponding average sampling reduction percentages (table 1). However, despite the inconsistent results with *Dactylaria* sp., 73.4% and 80.7% maximum reduction percentages were recorded in individual samplings, in cultures from the first and second day, incubated for 15 days.

Discussion

The increasing concern of anthelmintic resistance in ruminant parasitic nematodes around the world demands new alternatives for parasitic control. Nematode-trapping fungi are considered to be a useful biological tool against parasitic nematodes in cattle and sheep (Mendoza de Gives *et al.*, 1998; Larsen, 2000; Flores-Crespo *et al.*, 2001). Results in the present investigation have shown that oral administration to sheep of a suspension containing *D. flagrans* chlamydospores considerably reduced the population of *H. contortus* infective larvae on faeces, especially in samples taken on the second day following treatment, irrespective of the incubation period. Treatment of sheep with *D. flagrans* was more effective than *A. oligospora* and *Dactylaria* sp. in reducing the larval population in faecal cultures. The Tukey test indicated that group 3 (*D. flagrans*) was significantly different from the control group and groups 1 and 2 (*A. oligospora* and *Dactylaria* sp.). The highest reduction percentage of larvae was observed during the first two days after oral treatment, when maximum elimination of fungal material into the faeces occurs. Similar results were reported by Mendoza de Gives *et al.* (1998). On the third and fourth days following after treatment, plates with *D. flagrans* showed lower average control percentages (30 to 54% and 34%) for 15 and 21 days incubation periods, respectively. After the fourth day, reductions were diminishing in all the groups, although in plates with *D. flagrans* corresponding to the fifth day, an average reduction of 37.3% in plates incubated for 21 days, was recorded.

It seems to be clear that the highest larval reduction percentage in faecal cultures is related to the maximum elimination of fungal material into faeces. Future trials should consider a continuous schedule of fungal intake, i.e. through incorporation of *D. flagrans* chlamydospores into multi-nutritional pelleted animal food.

On the other hand, although *A. oligospora* and *Dactylaria* sp. have not been previously reported as fungi resistant to conditions in the sheep gastro-intestinal tract, in the present study, both isolates of such fungi were found in water agar plates supplied with faecal samples from fungi inoculated sheep. This demonstrates that these fungi were able to survive passage through the gastro-intestinal tract of sheep and, although less aggressive against nematodes *in situ* than *D. flagrans*, could also have some

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potential as possible biological control agents of nematodes in future trials. Other genera and species of nematode-trapping fungi, i.e. *Arthrobotrys robusta*, *Harposporium anguillulae* and *Monacrosporium gephyropagum*, which have also displayed remarkable *in vitro* effects in reducing the gastro-intestinal larval population on sheep and cattle faeces (Mendoza de Gives *et al.*, 1992; Araújo *et al.*, 1993; González-Cruz *et al.*, 1998) could be evaluated in similar trials to investigate their possible survival after passing through the gastro-intestinal tract of either cattle or sheep. Establishing an optimum amount of chlamydospores to be administrated in the animals is difficult, because some factors can alter the time of elimination of chlamydospores and also their distribution into faecal material. These factors include the quality and quantity of ingested food and the amount of drinking water consumed by the animals, which can modify the time spent by the chlamydospores in the alimentary tract. Obviously, with many chlamydospores eliminated into faeces, a higher rate of fungal colonization of faecal material takes place, resulting in a more efficient predatory activity and a higher reduction in the number of nematode larvae occurring *in situ* in the faeces. In any case, a standard amount of chlamydospores must be established in any laboratory trial. In the present study, the amount of *D. flagrans* chlamydospores administrated to sheep is lower than other fungal inocula used previously which resulted in 61.3, 72.1 and 61.9% reductions in the *H. contortus* larval population on faeces, after 7, 14 and 21 days post-treatment, respectively (Flores-Crespo *et al.*, 2001). In another investigation, by Mendoza de Gives *et al.* (1998) a unique dose of 11,350,000 chlamydospores, was administrated to sheep, producing 71% and 88% reductions in the *H. contortus* larval population in sheep faecal cultures after 15 and 21 incubation days, respectively.

Reduction percentages in the present investigation are similar, irrespective of the amount of chlamydospores administered; sometimes even a lower amount of chlamydospores resulted in a higher reduction percentage. Faedo *et al.* (1997) reported that large amounts of chlamydospores are not necessary to achieve high reductions in the number of parasites. These workers considered that the capability of *D. flagrans* for resisting the adverse conditions of the ruminant digestive tract colonizing faecal material and being expelled with faeces of animals treated with 1 to 5×10^5, is sufficient to achieve high reduction percentages in the larval population.

On the other hand, both *A. oligospora* and *Dactylaria* sp. showed a lower reduction percentage of larvae, which is similar to results recorded by Flores-Crespo *et al.* (2001) with *A. oligospora*. Such low reduction percentages are likely to be due to the high susceptibility of conidia to the adverse conditions in the digestive tract of sheep. Larsen (2000) mentions that the conidia of nematophagous fungi possess a thin and simple coat wall, which is easily degraded by digestive juices and then assimilated. In contrast, the thick-walled chlamydospores of *D. flagrans* are very resistant to adverse conditions, and pass through the gastro-intestinal tract of sheep without being affected. Gruner *et al.* (1985) found that only a small number of *A. oligospora* conidia is able to survive after passing through the gastro-intestinal tract of sheep and thus, any reduction percentage in the number of larvae in faeces is due to the capability of conidia to colonize faecal material and to trigger a potential predatory activity *in situ*. Pryadko *et al.* (1985) and Pryadko & Osipov (1986) established that most conidia of nematophagous fungi which are in direct contact with the lumen of the digestive tract are easily degraded by digestive juices. However, some conidia are protected within the digestive contents and are not directly exposed to the digestive lumen following oral administration into sheep. Eventually, such conidia are expelled alive with the faeces to the environment, germinating and colonizing faecal material. On the other hand, *Dactylaria* sp. has not been widely studied and only a few records are known of its predatory activity. In the present investigation, *Dactylaria* sp. and *A. oligospora* showed a similar behaviour in reducing the number of *H. contortus* larvae.Variations in the predatory activity of fungi have been found against different nematodes. For instance, Flores-Crespo *et al.* (1999) found differences in the predatory effect of *Dactylaria* sp. and *A. oligospora* against *Panagrellus redivivus*, recording 95.7% and 50.1%, respectively. The predatory mechanism of trapping fungi starts with a glycoprotein receptor recognition system present both in fungal cells and on the external antigenic coat of nematodes. Thus, differences in the ability of fungi to trap nematodes could be due to different protein patterns in the cuticle (Mendoza de Gives *et al.*, 1999). Results in the present paper have shown that the fungus *Duddingtonia flagrans* is a potential biological control agent of gastro-intestinal parasitic nematodes in sheep, and it has implications in the control and prevention of such important parasites in sheep worldwide.

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