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Efficiency of feeding *Duddingtonia flagrans* chlamydospores to grazing ewes on reducing availability of parasitic nematode larvae on pasture

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Abstract

Gastrointestinal nematodes are of concern in sheep production because of production and economic losses. Control of these nematodes is primarily based on the use of anthelmintic treatment and pasture management. The almost exclusive use of anthelmintic treatment has resulted in development of anthelmintic resistance which has led to the need for other parasite control options to be explored. The blood sucking abomasal parasitic nematode *Haemonchus contortus* causes severe losses in small ruminant production in the warm, humid sub-tropic and tropics. This study evaluated the effectiveness of a nematode trapping fungus, *Duddingtonia flagrans*, in reducing availability of parasitic nematode larvae, specifically *H. contortus*, on pasture. Chlamydospores of *D. flagrans* were mixed with a supplement feed which was fed daily to a group of crossbred ewes for the duration of the summer grazing season. A control group was fed the same supplement feed without chlamydospores. A reduction in infective larval numbers was observed in fecal cultures of the fungus-fed group. Herbage samples from the pasture grazed by the fungus-fed group also showed a reduction in infective larvae. There were no significant ($P > 0.05$) differences in overall fecal egg count, packed cell volume or animal weight between fungus-fed and control groups. Tracer

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animals were placed on the study pastures at the end of the study to assess pasture infectivity. Although tracer animals were only two per group, those that grazed with the fungus-fed group had substantially reduced (96.8%) nematode burdens as compared to those from the control group pasture. Results demonstrated that the fungus did have activity against nematode larvae in the feces which reduced pasture infectivity and subsequently nematode burdens in tracer animals. This study showed that *D. flagrans*, fed daily to grazing ewes, was an effective biological control agent in reducing a predominantly *H. contortus* larval population on pasture.

Keywords: Nematode; Sheep; Fungus; *Duddingtonia flagrans*; Biological control

1. Introduction

Several gastrointestinal nematodes of the family Trichostrongylidea cause parasitism in sheep. Of these, *Haemonchus contortus* is the predominant one in tropical and subtropical regions of the world. It is also the most pathogenic because it is a blood feeder and with heavy infections animals become severely anemic and can die. Otherwise, as a group these nematodes cause production losses (decreased weight gain, weight loss, reproductive inefficiency, etc.) and thus, economic losses. In efforts to reduce or prevent such losses, control of gastrointestinal nematodes has been primarily achieved through the use of anthelmintic treatment programs with some emphasis on pasture management. The almost exclusive use of anthelmintic treatment has resulted in nematode populations that have developed resistance to multiple anthelmintic chemical classes and compromised pasture management due to reduced availability of parasite safe pastures (Sangster, 1999).

A need exists for alternative control measures that do not rely as heavily on these traditional methods. In addition to anthelmintic resistance, there is an increasing awareness of issues involving environmental contamination with chemicals and consumer pressure to reduce drug residues in meat and meat products that may influence the use of anthelmintics (Larsen, 2000). Alternative control options currently available include breeding for genetically resistant animals (Gasbarre and Miller, 1999) and strategic use of anthelmintics in conjunction with an eye color chart that reflects the level of anemia caused by *H. contortus* infection (FAMACHA, Vatta et al., 2001). Alternative control options under development include nematode vaccines (Knox and Smith, 2001), tannin containing plants (Athanasiadou et al., 2001) and the use of various biological control agents. One such biological control measure is using a nematode trapping fungi, which destroys larval stage nematodes in feces by using a sticky hyphal network (Grønvold et al., 1993a; Waller and Faedo, 1996). In order to trap larvae in the feces, the fungus must be able to survive passage through the gastrointestinal tract. The thick walled chlamydospores of *Duddingtonia flagrans* are able to survive environmental rigors and in comparison with other species of nematode trapping fungi, have been shown to be superior in survival through the ruminant gut (Larsen et al., 1992, 1994; Faedo et al., 1997; Llerandi-Juarez and Mendoza de Gives, 1998).

Studies conducted in vitro have demonstrated the trapping ability of *D. flagrans* in feces (culture per pats) (Larsen et al., 1991; Grønvold et al., 1999; Peña et al., 2002). Studies have also shown that feeding *D. flagrans* chlamydospores was successful in reducing levels of

parasitic nematode larvae (predominately *Ostertagia*) on pasture grazed by calves (Larsen et al., 1995; Nansen et al., 1995; Fernandez et al., 1999a) and *Ostertagia* and *Trichostrongylus* infections in lambs (Githigia et al., 1997). There is no information available on using nematode-trapping fungi in ewes and/or for controlling *H. contorus*. The objective of this study was to evaluate the efficiency of daily feeding of *D. flagrans* chlamydospores in reducing the level of parasitic nematode larvae (predominantly *H. contortus*) on pasture grazed by mature dry ewes during the peak period of infection in Louisiana.

2. Materials and methods

2.1. Animals

2.1.1. Ewes

Thirty F_1 (Gulf Coast Native × Suffolk) ewes (1–3 years of age) with a fecal egg count (FEC) of 0 eggs per gram (EPG) of feces were randomly allocated into either a treatment (fungus-fed) or control group consisting of 15 animals each. Ewes were maintained on pasture for a period of 18 weeks from mid-June through October, 2000 at the Ben Hur Research Farm, Louisiana Agricultural Experiment Station, Baton Rouge, Louisiana USA.

2.1.2. Tracer lambs

Four Gulf Coast Native lambs (6 months of age) were used as tracer lambs (two in each pasture) during the last 3 weeks of the grazing period. Three weeks prior to grazing, lambs were placed on concrete (to prevent any further infection) and dewormed (see Section 2.5).

2.2. Pasture

A 2.44 ha perennial bermuda grass pasture was divided equally into two 1.22 ha pastures. Pastures had been grazed previously by nematode parasite infected sheep.

2.3. Fungus treatment protocol

D. flagrans spores were provided by Christian Hansen (Hørsholm, Denmark) as a granular product containing 1.0×10^7 spores per gram. Spore dosage for the fungus-fed group was 5×10^5 spores per kg of body weight (BW). Both groups were fed 6.8 kg of a pelleted maintenance ration each day. The appropriate amount of spore material was mixed with the feed of the fungus-fed group and a small amount of water was added to enhance spore material adherence. Feed was dispensed in bunks under cover that provided adequate space for all animals to have equal access. Ewes were weighed at the beginning and at 2-week intervals to adjust the dose.

2.4. Fecal and blood sampling

Fecal and blood samples were collected weekly from each ewe and tracer lamb. Fecal samples were collected directly from the rectum and processed using a modified

McMaster technique to determine the FEC (Whitlock, 1948). Blood was drawn by jugular venipuncture in 7 ml EDTA vacutainer tubes and the packed cell volume (PCV) value was determined using a microhematocrit centrifuge (Autocrit, Bectin Dickinson, Parsippany, NJ).

2.5. Deworming protocol

Individual ewes were dewormed with albendazole (Valbazen®, 10 mg/kg by oral drench) and levamisole (Levasole®, 8.8 mg/kg by subcutaneous injection) if the PCV value was 15% or lower or if the value dropped five percent or more within the weekly sampling period. Tracer lambs were dewormed with both of these anthelmintics on two consecutive days.

2.6. Animal weights

All ewes were weighed every 2 weeks by passing through a portable sheep scale subsequent to sample collection. Weights were used to determine weight changes between the two groups and the weights of the fungus-fed group were used to adjust the amount of fungal spore material to maintain the dosage.

2.7. Fecal cultures

Feces were cultured from individual animals that had an FEC greater than 0 EPG. Since no larvae could be expected to be recovered from feces with a 0 EPG, the number of samples processed varied from week to week. In a 100 ml plastic cup, 10 g of feces was mixed with approximately 7 g of vermiculite, and water was added until a moist crumbly consistency was achieved. The cup was covered with cheesecloth and inverted into a 200 ml plastic cup which contained 4–5 ml of water. The water and cheesecloth interface was not allowed to touch. Cultures were placed in sealed plastic bags to ensure that an adequate moisture level was maintained. Cultures were kept for 10 days at room temperature (24 °C) at which time they were removed from the plastic bag and warm water (approximately 50 °C) was added to the top of the larger cup completely immersing the culture material. After 24 h, the cup containing the culture material was carefully removed so as not to disturb the sediment containing the larvae in the second cup. After 1–2 h, the supernatant was then carefully siphoned off until approximately 14 ml remained. The solution was then thoroughly mixed and transferred to a 15 ml centrifuge tube. One milliliter of formalin was added to preserve the contents and the tubes were stored at room temperature. Sedimented samples were further processed by carefully siphoning off the supernatant to achieve a volume of 1 ml. The sediment and remaining supernatant were thoroughly mixed and a 100 μl representative sample was removed, placed on a microscope slide, covered with a cover slip and the number of L_3 were counted at 100× power. This number was multiplied by 10 to estimate the total number of L_3 recovered. The first 100L_3 encountered in each sample were identified to genus (Ministry of Agriculture, Fisheries and Food, 1977). The total number of L_3 was divided by 10 to get the number of L_3 per gram (LPG) of feces.

2.8. Percent reduction

The number of L_3 recovered from cultures was used to calculate the percent reduction as:

$$\text{percent reduction} = \left[\frac{\text{control mean LPG} - \text{fungus-fed mean LPG}}{\text{control mean LPG}}\right] \times 100$$

2.9. Herbage sampling

Herbage samples were collected every 2 weeks to determine the infective potential of each pasture. While walking a W-shaped route across each pasture, 25 samples per pasture were collected at approximately equal intervals along the route. All samples as they were collected from each pasture were placed in a plastic bag (20 cm × 55 cm). At each collection point, a wire square (approximate area of 400 cm^2) was tossed in the air and where it landed all the herbage within the square was collected by clipping with a grass cutter to ground level. If the wire square landed on a fecal mass, it was tossed again. A small amount (25–30 g) of herbage from each pasture was removed, weighed (g, wet weight) and oven dried (52 °C) for 7 days. The sample was weighted again (g, dry weight) and the percent dry matter was calculated as wet weight minus dry weight divided by wet weight times 100. The remaining herbage sample for each pasture was weighed (kg) and placed in a 30 l foot tub with enough water to completely cover the herbage. Five milliliters of liquid detergent was added to break surface tension. The samples were allowed to settle overnight. The following day the herbage was removed, rinsed, and discarded. The rinse water was added to the tub. The contents of the tub was then subjected to a series of sedimentations at 3–4 h increments that included carefully pouring off and discarding approximately 75–80% of the supernatant. At each step, the contents were thoroughly mixed and transferred to smaller containers, 30 to 15 to 5 to 1 L and finally to a 500 ml volume from which two 25 ml aliquots were transferred to 50 ml screw top centrifuge tubes. Five milliliters of formalin was added to preserve the contents and the tubes were stored at room temperature. Samples were further processed by carefully vacuuming off the supernatant until 10 ml remained. After thorough mixing, three 100 μl subsamples were taken to count the number of L_3 present. The mean of the three subsamples was multiplied by 500 to estimate the total number of L_3 in the herbage sample. The number of L_3 per kg of dry matter was estimated by dividing the number of L_3 in the herbage sample by the herbage sample dry weight.

2.10. Necropsy of tracer lambs

The gastrointestinal tract was removed for nematode (immature and mature) recovery, enumeration, and identification in accordance with established procedures (Miller et al., 1987). The recovery procedure was modified slightly from that reported in that organ contents were brought to a volume of 5 l (instead of 10 l) and a 500 ml (instead of 1 l) aliquot was taken. After a 2 h sedimenting period, 50 ml (instead of 100 ml) of supernatant was poured off and replaced with 50 ml (instead of 100 ml) of formalin.

2.11. Statistical analysis

FEC data was log transformed to stabilize variance and normalize the data (Winer, 1971). Weight, PCV and herbage sample data were analyzed using raw data. The means of the groups were compared using PROC GLM for repeated measures in SAS for FEC, PCV, and animal weights. Tukey–Kramer was used as the adjustment for multiple comparisons. Differences were considered statistically significant when $P < 0.05$.

3. Results

Mean FEC remained relatively similar between groups through week 12 (Table 1). From weeks 13–15, FEC in the control group increased, after which, it returned to the fungus-fed group level. There was no significance difference in FEC at anytime or in overall mean FEC between the two groups. Two ewes required anthelmintic treatment. One fungus-fed ewe required treatment at 7 and 13 weeks, and one control ewe required treatment at 15 weeks.

There was no significant difference between the two groups in mean blood PCV at anytime or overall, however the control group was consistently lower (2–4%) than the fungus group from week 14 to 17 (data not shown).

The ability of *D. flagrans* to trap nematode larvae in fecal matter was measured by percent reduction of L_3 in fecal culture (Table 1). The percent reduction ranged from 0% (week 6) to 99.3% (week 13). One animal accounted for the 0% at week 6, and if that animal is excluded, the percent reduction was 79.4%. The LPG for the fungus-fed group was consistently lower than the control group, and the percent reduction remained high ($\geq$78.9%). The L_3 populations of the fecal cultures were identified as greater than 90% *H. contortus*.

The L_3 count from the fungus-fed group pasture herbage was slightly higher on week 2, but the control group pasture became higher on week 4 and this difference between the two pastures remained relatively consistent with the fungus-fed group pasture herbage having lower L_3 counts for the duration of the study (Table 1).

The FEC of all tracer animals was 0 EPG when turned out on pasture (Table 2). At necropsy, the FECs of tracers that grazed control and fungus-fed group pastures were 350 and 1150 and 700 and 1600 EPG, respectively. The number of immature and mature nematodes recovered was 14 100 and 28 600 and 400 and 800, respectively. The largest number of nematodes was recovered from the abomasum (*H. contortus*, mostly late L_4 and immature adults with a few mature adults) and a few were recovered from the small intestine (all mature adult *Trichostrongylus* spp.). No nematodes were found in the large intestine. Weights of all the ewes remained relatively similar throughout the study period and were not significantly different between groups (data not shown).

4. Discussion

In this study, daily feeding of 5×10^5 chlamydospores per kg of BW of the nematode trapping fungus *D. flagrans* caused a reduction of the nematode larval population in feces which subsequently resulted in lower pasture infectivity and reduced nematode burdens

Table 1
Mean (±SEM) fecal egg count (FEC), mean (±SEM) L_3 (per gram of feces) and percent reduction of L_3 from fecal cultures of control and fungus-fed ewes, and L_3 (per kg dry matter) recovered from pasture herbage grazed by control and fungus-fed ewes over an 18-week summer/fall grazing period

Week	FEC (eggs per gram)[a]		Fecal culture L_3					Herbage L_3[b]	
	Control	Fungus-fed	Control	n[c]	Fungus-fed	n[c]	Reduction (%)[d]	Control	Fungus-fed
0	0	0	ND		ND			ND	ND
1	13 ± 8	23 ± 12						0	1100
2	373 ± 109	130 ± 43	95 ± 21	10	20 ± 4	8	78.9		
3	290 ± 109	93 ± 53						3200	2100
4	630 ± 247	83 ± 42	153 ± 39	15	18 ± 4	8	88.2		
5	383 ± 134	347 ± 245						4520	1040
6a[e]	180 ± 69	440 ± 306	102 ± 38	10	298 ± 277	9	0.0		
6b[f]					21 ± 6	8	79.4		
7	187 ± 76	1053 ± 958[g]						4380	1450
8	467 ± 327	167 ± 52	122 ± 21	7	13 ± 3	11	89.3		
9	590 ± 298	340 ± 195						6030	2000
10	330 ± 194	263 ± 155	196 ± 88	8	9 ± 3	9	95.4		
11	457 ± 170	820 ± 510						6670	1410
12	963 ± 535	693 ± 491	1254 ± 637	7	22 ± 7	13	98.2		
13	1503 ± 958	950 ± 597[g]						5150	2450
14	2267 ± 1212	660 ± 289	1468 ± 752	11	10 ± 2	13	99.3		
15	2860 ± 1187[h]	653 ± 297						6540	1010
16	440 ± 166	280 ± 117	466 ± 197	8	4 ± 1	9	99.1		
17	120 ± 44	70 ± 34						4520	2760
18	483 ± 127	211 ± 70	295 ± 80	9	11 ± 4	9	96.3		
Mean	686 ± 199	400 ± 77	461 ± 208		45 ± 34		90.2	4600 ± 680 a	1700 ± 210 b
				15 ± 4[f]	96.7[f]				

[a] $n = 15$ per group.
[b] Means with unlike letters are significantly different ($P < 0.05$).
[c] Number of animals with a positive FEC.
[d] [(control L_3 − fungus L_3)/control L_3] × 100.
[e] One fungus-fed ewe had a very high L_3 count which indicated that an adequate amount of fungal spores was not consumed during the day or two before culture which resulted in a 0.0% reduction.
[f] Week 6 data excluding the fungus-fed ewe with the high L_3 count.
[g] One fungus-fed ewe required anthelmintic treatment twice.
[h] One control ewe required anthelmintic treatment.

Table 2
Fecal egg count (FEC) and nematode burden of the tracer animals that grazed pastures of control and fungus-fed ewes

Pasture	Animal	FEC[a]		Nematodes		
		Turnout	Necropsy	Abomasum[b]	Small intestine[c]	Total
Control	1	0	1150	27100	1500	28600
	2	0	350	12700	1400	14100
Mean		0	750	19900	1450	18650
Fungus-fed	1	0	1600	800	0	800
	2	0	700	200	200	400
Mean		0	1150	500	100	600

[a] Eggs per gram of feces.
[b] All were *H. contortus* (mostly late L_4 and immature adults).
[c] All were *Trichostrongylus* spp.

in tracer animals. Larsen et al. (1998) and Peña et al. (2002) reported that this dosage was adequate to produce substantial nematode trapping. When the fungus is deposited in the feces at the same time that nematode eggs are present, such as in feeding or oral administration of the fungal spores, transmission of larvae from fecal material onto pasture herbage is reduced (Faedo et al., 1998, 2000).

The trapping ability of the fungus is evaluated by L_3 recovery from cultured feces and from pasture herbage. The percent reduction gives an indication of how effective the fungus is at trapping the larvae in the fecal material. L_3 reduction in fecal cultures has been reported to range from 30 to greater than 90% with an average percent reduction of 85% (Larsen et al., 1991; Grønvold et al., 1993a; Mendoza de Gives et al., 1998; Fernandez et al., 1999b; Peña et al., 2002). In cattle studies, a summer grazing period demonstrated that the greatest difference in L_3 populations existed from mid- to late August (Grønvold et al., 1993b; Larsen et al., 1995; Nansen et al., 1995; Larsen, 2000). Variation in nematode trapping in both fecal culture and herbage samples can be associated with temperature and larval density. The optimum temperature range for *D. flagrans* is 25–30 °C indicating that the effectiveness of the fungus may be compromised under higher or lower temperature ranges (Fernandez et al., 1999c). An increased L_3 percentage in fecal material is associated with an enhanced trapping ability indicating that a higher FEC may lead to greater larvae reduction (Waruiru, 1998; Furmonavicius, 1998). During the period of this study, the temperature range was 24–35 °C and epidemiological data has shown that at this time of year, development of larvae and infection rates peak with very high FECs (Miller et al., 1998). So, temperature conditions and larval availability were conducive to optimal fungus activity which supports the results observed.

The FEC and PCV results showed no significant difference between the two groups at any time which indicated similar nematode burdens. However, FEC did increase in the control group during weeks 13–15, but then returned to the fungus-fed group level. Concurrently, a slight and consistent decrease was observed in the control group PCV from week 14, which corresponded to the increased FEC of the control group. This observed FEC increase might be expected as potential infectivity increased, as indicated by herbage L_3 counts, on

the control group pasture but it was not sustained. One explanation for this could be that only four ewes accounted for the increase and the ewe with the highest FEC was the one that required an anthelmintic treatment at week 15. Results from cattle (Larsen et al., 1995; Nansen et al., 1995) and sheep (Githigia et al., 1997) experiments in which *D. flagrans* was fed have also shown no difference in FEC and/or infection level between fungus-fed and control groups. However, in both cattle trials the number of L_3 on fungus-fed pasture was lowered, control (not fungus-fed) animals showed clinical signs of nematode parasitism which required anthelmintic treatment, and the weight gain of fungus-fed animals was better than control animals. In the sheep trial, there was a significant reduction in L_3 on pasture but essentially no reduction in nematode burden of fungus-fed resident animals that were slaughtered. The nematode reducing capacity was, however, demonstrated in the nematode burden of the introduced tracer lambs. So it appears that although differences in FEC and/or nematode burden may not be initially detected, the beneficial effect of feeding the grazing animals fungal spores can be evaluated by other parameters such as pasture L_3 counts, reduction capacity in fecal cultures, clinical signs requiring anthelmintic treatment, nematode burden of tracer animals and weight gain. It also appears that it might take an extended period of time to gain the optimum effect of this strategy depending on the initial infection level of the pasture in question.

It might be expected that some control and no fungus-fed ewes would develop signs of clinical haemonchosis that would require deworming, but one ewe in each group met the deworming criteria. Since the fungus-fed ewe required two deworming, it suggests that the ewe was either compromised in some manner that resulted in clinical haemonchosis even when ingesting fewer L_3 or the effect of the fungus had not yet reached a level to prevent clinical disease in relatively susceptible animals. It is important to understand that this method does not result in immediate elimination of infection but requires an extended period of time for reduction of pasture infectivity.

The tracer animals were parasite-free at the time they were turned out to their respective pastures, and any nematode infection was acquired from grazing. In other experiments where tracer animals were used, a significant reduction in nematode burden also occurred where the type of infection and seasonal time frame corresponded to the fungus administration of this study (Wolstrup et al., 1994; Githigia et al., 1997). The presence of mostly larval and immature adult *H. contortus* in tracers at necropsy could be explained by the 2-week maturation period which was not long enough to allow most of the developing stages to become mature adults. This would also explain the lack of difference in FEC between control and fungus-fed group pasture tracers. Although there were only two tracers per group, the substantially reduced infection in both fungus-fed group compared to control group lambs suggested that the fungus did reduce pasture infectivity, and therefore, it should result in decreased ingestion of nematode larvae from grazing areas.

Additional studies are needed to address different age groups and/or breeds of sheep or different climatic conditions in controlling gastrointestinal nematodes by using *D. flagrans* as a biological control. The implementation of the fungal spores into different feed formulations and supplements such as mineral blocks, pellets or boluses should also be addressed to assist ease of administration.

The results of this study have shown that feeding of *D. flagrans* chlamydospores as a biological control reduced larval populations in fecal material and subsequently on pasture

under field grazing conditions. This in turn should theoretically reduce the intake of L_3 from pasture and thereby reduce the nematode burdens. The overall goal of using nematode-trapping fungi as a control measure is to effectively reduce parasitic nematode populations without the extensive use of anthelmintic treatment. However, it should be expected that such larval reduction would take a period of time before infection is reduced to levels below which anthelmintic treatments would not be necessary. The implementation of this biological control is a promising adjunct in gastrointestinal nematode parasite control.

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