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ANIMAL SCIENCE

Evaluation of the supply of *Duddingtonia flagrans* for the control of gastrointestinal parasites in sheep

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Abstract: Parasitic resistance imposes alternative control methods, like nematophagous fungi. In this study, two experiments were conducted supplying *Duddingtonia flagrans* aiming to evaluate the biological control of parasites in sheep. In the first, 24 sheep naturally infected by gastrointestinal nematodes were allocated, in randomized blocks, following the treatments: control or treated group, 0.5g/animal product containing *D. flagrans*, chlamydospores. Weight, body score, Famacha©, egg count per gram of feces (EPG), and larval percentage were evaluated. In the second experiment, *D. flagrans* (0.25 and 0.5g product) was infested with manure, plus or not protein concentrate, in a completely randomized design. In both experiments the dose was intentionally lower than recommended. Recovery and larval identification were performed. The SAS analyzed the variables by the MIXED procedure, repeated measures in time. Weight, body score, hematocrit, and Famacha© did not show differences between treatments ($p>0.05$); however, EPG ($p<0.001$) and the percentage of larvae identified in coproculture were different. In the second experiment, the inclusion of the fungus did not influence the recovery of larvae ($p>0.05$). In both experiments, colonization and advancement of the fungus were visualized. Under the experimental conditions, the fungus *D. flagrans* was not effective in the biological control of parasitic infection in sheep.

Key words: Animal production, biological control, *Duddingtonia flagrans*, *Ovis aries*, sustainable animal production.

INTRODUCTION

Sheep farming has always had numerous challenges, such as selection of better adapted breeds, improvement of pastures and nutritional increment. However, infestation by worms continues to be one of the biggest problems. Gastrointestinal helminths in sheep include, among the most prominent genera, *Haemonchus*, *Trichonstrogylus*, *Strongyloides*, *Cooperia* and *Oesophagostomum*. Many factors affect the severity of infestations, such as physiological and nutritional status, animal stocking rate, quality of forage offered, quality of facilities, and hygiene (Amarante et al. 2014, Toigo et al. 2018).

Since the intensive use of anthelmintics causes parasitic resistance, biological control is a valuable tool. This can involve the inclusion of microorganisms in feed and concentrates so they are ingested and propagated (Vieira 2003).

The use of nematophagous fungi for the control of worms is not recent. In general, they do not cause undesirable effects in the environment, allowing longer permanence. Nematophages can disperse through structures known as "dry spores", or in some cases chlamydospores, which are highly resistant

and can be commercially produced (Baron 1977). Chlamydospores are thick-walled spores, differentiated from hyphae, and usually appear under extreme stress conditions. They can give rise to hyphae, conidiophores and conidia, so they are present in most predatory fungi, especially those most used for parasite control (Barbosa et al. 2019).

Predatory fungi produce adhesive traps, which propagate randomly through the soil. When coming into contact with larval worms, they adhere and immobilize them, allowing the fungus in question to penetrate the cell wall and feed on it (Carneiro 1992). The main fungal genera that produce sticky traps or rings are *Arthrobotrys* spp, *Duddingtonia* spp, *Dactylaria* spp and *Dactylella* spp, while those that produce adhesive mycelia are *Cystophaga* spp and *Stylophaga* spp. The genera *Duddingtonia* and *Arthrobotrys* are used the most for control of parasites, due to their success and resistance to the weather (Vilela et al. 2012).

In the study, the first experiment had the objective of verifying the action of the nematophagous fungus *Duddingtonia flagrans* for the control of infective larvae in pastures, along with improvement in physical and physiological conditions (weight, body score and Famacha™) of treated animals, at very low doses (0.5g animal-day). In the second experiment, the objective was to verify again the action of the fungus *D. flagrans*, in the reduction of populations of larvae in manure (infectious and free-living), obtained from sheep bedding kept at room temperature, as well as inside a temperature-controlled greenhouse, fixed at 25 °C. This time, using half the dose of the experiment 1 and same one as well (0.25g and 0.5g).

MATERIALS AND METHODS

The experiment was approved by the Ethics Committee for the Use of Animals of the Animal Science Institute/APTA– CEUA/IZ, under technical opinion n. 309-2020.

Two experiments were carried out, both in the sheep sector of the Instituto de Zootecnia, Nova Odessa, São Paulo state, Brazil, at geographic coordinates of 22°42'S and 47°18'W, 528 m altitude. Climate data were obtained from the CIIAGRO online database and are compiled in Table I.

Experiment 1

Eight paddocks of 600 m^2 each were implemented, divided by wire fences, with *ad libitum* water supply. The predominant forage in the paddocks consisted of Aruana grass (*Megathyrsus maximus*, cv. IZ 05). Twenty-four crossbred sheep (20 males and 4 females) were allocated in the paddocks, balanced through initial weight The animal numbers inside the paddocks were 2, 3, 3 and 3 (receiving fungus) and 4, 3, 3 and 3 (control, not receiving fungus). The distribution was completely random, only considering

Table I. Humidity (%) Temperature (Cº) and Precipitation (mm) in the experiment months.

Months (2021)	Humidity (%)	Temperature (Cº)	Precipitation (mm)
January	75.28	26.27	7.17
February	72.52	25.5	2.98
March	72.7	25.6	3.05
April	71.1	22	0.67
May	69.58	19.87	0.69
June	72.78	18.48	0.68
July	64.23	16.17	0.92

male and female separation. Once a day, always at 2:00 p.m., protein feed was provided, consisting of 300g/protein concentrate/animal, together with 0.5 g of a commercial product, containing chlamydospores of the predatory fungus *Duddingtonia flagrans*. Dosed used was low, mainly with the intention to observe the development of the fungus is such amounts offered, compared to previously work, in which the amount of product used was higher (Healey et al. 2018a). The product was introduced with an inert component (chicken bran) along with 10^5 chlamydospores per gram of product. The manufacturer's recommended dose is 1 g of product per kg of live weight. The animals were divided in the group treated with the fungus and a control group, only receiving feed with concentrate.

The data were collected monthly for seven months, including the rainy and dry seasons (January-July). At this time, sheep were also weighed and evaluated for body condition score and Famacha ©.

Collection of feces and blood

Parasitological analyses were performed at the Laboratory for Fecal Analysis of the Animal Science Institute of Nova Odessa/APTA, while the hematological analyses were carried out in the Laboratory for Clinical Analysis at the University of Santo Amaro, São Paulo.

Every month, all the animals were moved from their paddocks to a containment corral, with separation between males and females. Stool samples were collected directly from the rectal ampoule, identified, and stored in a cooler for later fecal egg counts (EPG), conducted according to the modified technique of Gordon & Whitlock (1939). Infective larvae were identified to the genus level by fecal cultures (Roberts & O'Sullivan 1950), with separation by paddocks and treatments.

Blood samples were collected by venipuncture of the jugular vein of all animals in two tubes: Vacutainer tubes (5 ml) containing ethylenediaminetetraacetic acid (EDTA) for hematologic analysis (packed cell volume carried out every month; blood count, performed at the beginning of the experiment and then every two months); and serum separator Vacutainer tubes for biochemical analysis. The blood count was performed using an Abbott Cell Dyn 3500 Automated Hematology Analyzer (Abbott Laboratories, Chicago, USA). Differential leukocyte counts were confirmed by analyzing blood smears with the aid of an optical microscope at 100x magnification. Hematologic analyses were completed within 24 hours.

Samples for biochemical analysis were centrifuged at 3000 rpm for 15 minutes. The serum was separated and stored in 2 ml Eppendorf tubes and frozen for later processing in an automatic device (Labmax 100, Labtest, Minas Gerais, Brazil). The serum for biochemical analysis was collected and analyzed each month. Eight values were analyzed, consisting of Urea, Albumin, Total protein, Glucose, Gama GT, Phosphatase, Cholesterol, and Creatinine.

Collection of forage

To perform the larval counting in each paddock, as well as forage mass and bromatological evaluations, samples were collected monthly, always between 7:00 and 9:30 a.m. (Amarante et al. 2014). Three successive movements were made with an iron square measuring 50 cm x 50 cm, completely at random. At each movement, the entire portion of the forage inside the square was cut using pruning shears with undulated for precise cutting. Each forage sample was weighed individually, placed in a clean bag and taken to the laboratory for processing.

In the laboratory, a portion was removed for bromatological evaluation, which was carried out

in the Bromatology Laboratory of the Institute of Animal Science. The rest of the material was again weighed, followed by collection of the infective larvae present. The analyses consisted of measuring dry matter (DM) – dried in an oven at 103 - 105° C; mineral matter (MM) - incinerated in a muffle furnace at 550 – 600 °C; crude protein (BP) - Dumas method (sample combustion); acid detergent fiber (ADF); neutral detergent fiber (NDF); and hemicellulose (Van Soest et al. 1991).

Modified devices for larval recovery

To count and identify infective and free-living larvae, specific devices were used, called "parallel vessels", which consisted of jars with capacity of 6 liters, with filtering sieves, hoses, and registers to collect the decanted liquid. All the forage collected from the paddocks was chopped with a knife mill and placed inside the jar, with completion of the volume with water heated to approximately 36 ºC, allowing the material to be washed while at rest. After 48 hours of rest, the liquid samples were collected in 50 ml Falcon tubes and left at rest for a further 24 hours. The entire supernatant was discarded at the end, leaving 3 ml for reading, identification and counting of the larvae, with the assistance of a digital counter.

Experiment 2

Sheep bedding containing manure was removed from the concrete floor of a barn and placed in piles for drying. After this procedure, it was collected and designated as manure. About 4 kg of this material was collected and placed in sterilized plastic bags (correctly dried before being collected for laboratorial procedure) and taken to the Parasitology Laboratory of the Animal Science Institute for further processing. The collected manure was separated and evaluated for the presence of larvae, in a different situation, compared to the experiment 1 (open space, pasture evaluation), in a controlled environment, simulating confinement space. The material was placed in aluminum containers with a capacity of 500g of material and divided into three treatments: 0.25g of commercial product containing chlamydospores of *D. flagrans* in 25 ml of distilled water; 0.50g of product with water; and only 25 ml water for control. This time, the amount of commercial product was divided in half, allowing to observe if the fungus could develop in such low dosages, but in a proper environment. The collected manure was evaluated and observed many infective genera of larvae present, as well free-living genera.

About 30 containers were stored at room temperature, away from lighting and with daily humidification, with one container being evaluated per day, the process of larval recovery used the same "parallel vessels" in experiment 1. After 3 weeks of readings, new containers were prepared with the same procedure previously described, but with placement in a biological oxygen demand (BOD) oven at temperature of 25 ºC.

The remaining material was stored in tubes, identified according to the paddock and treatment. Fifty 50 microliters was removed from each tube with the aid of a pipette and placed on a microscope slide together with Lugol. The material was observed under an optical microscope to identify infective larvae (*Haemonchus* spp, *Trichostrongylus* spp, *Strongyloides* spp, *Oesophagostomum* spp and *Cooperia* spp) and free-living larvae. All the numbering was carried out with the aid of a digital counter. In the second experiment, the procedure for recovery was the same, the only difference being that the material used was manure instead of forage.

Statistical analysis

For experiment 1, a randomized block design was used, while in experiment 2, a completely randomized design was used. Recoveries of larvae in pastures, recovery of larvae in manure (room temperature and BOD oven), eggs per gram of feces, weight, body score and Famacha™ score were analyzed by the MIXED procedure (SAS, Inst. Inc. Cary, NC, USA), considering repeated measures over time. The model included treatment effects (fungus treated/control and 0. 25g/0.50g/control) and time (collections 1 to 7, 1 to 10 and 1 to 4). weeks 1 to 10 and 1 to 4).

RESULTS

Experiment 1

Animal results

Animal results are shown in Table II. No difference was found between the treatments used, with animals receiving the fungus and the control having similar values ($p>0.05$) for the metrics initial and final weight, initial and final body score, initial and final hematocrit and initial and final Famacha™ score. Table III shows the values of eggs per gram of feces, with comparisons between treatments and collections, respectively.

There were differences between the treated and control groups regarding the mean values of the presence of Trichostrongylids and Strongilyds ($p<0.001$). More eggs of Trichostrongylids were counted in the group receiving supplementation with the fungus (2087 larvae ± 329 treated / 1356.56 larvae ± 244.10 control), while the opposite was observed for Strongylids (171.17larvae ± 41.11 treated / 253.12 larvae ± 50.30 control, respectively). Between collections, a difference was observed in all of them, with recurrent variations ($p<0.001$).

The average percentages of infectious genera recovered in the coprocultures differed significantly between treatments ($p<0.001$).

Hematology and biochemistry

Means of hematological and biochemical variables are shown in Table IV. Total leukocytes, neutrophils, lymphocytes, and eosinophils differed between treatments ($p<0.001$) and were within the reference standards for sheep according to Kaneko (1997). Only erythrocytes and monocytes showed no differences between treatments (p-values of 0.0670 and 0.07, respectively).

The results of the biochemical examination are reported as means and standard errors. Urea, albumin, total proteins, glucose, cholesterol, and creatinine showed no differences between treatments ($p>0.05$), while there were significant differences in GT and phosphatase ($p<0.001$). For the collections, urea, glucose and phosphatase were different ($p<0.001$), while the values of albumin, total protein, Gama GT, cholesterol and creatinine did not differ ($p>0.005$). The values were within the parameters for sheep, except for total proteins, which was slightly below the reference value, a possible consequence of parasitism (Meyer & Harvey 2004).

Table II. Weight (initial W0 and final WF). Body score (initial BS0 and final BSF). Hematocrit (initial Ht0 and final HtF) and Famacha™ (initial F0 and final FF) are available for the treatments used.

Treatment	W0	WF	BS0	BSF	Ht0	HtF	F0	FF
Treated	28.26 ± 2.35	40 ± 3.4	2.4 ± 0.23	2.33 ± 0.23	30 ± 1.4	26 ± 1.4	1.5 ± 0.24	1.83 ± 0.21
Control	29.07 ± 3.16	40.19 ± 3.1	2.10 ± 0.22	2.29 ± 0.21	30 ± 1.3	24 ± 1.27	± 0.21	1.4 ± 0.2
Pr > |t|	0.9349	0.7444	0.3591	0.9106	0.2415	0.4570	0.08	0.1112

Table III. EPG values, divided in Treated group and Control, with significant values. In sequence, the genera of infectant larvae are displaced, with group differences (treated and control).

Treat/ Collect/larval genera	Trichostrongylids/ Treated	Trichostrongylids/ Control	Strongylids/ Treated	Strongylids/ Control
EPG	2087 ± 329[b]	1536.56 ± 244.10[a]	171.17 ± 41.11[a]	253.12 ± 50.30[b]
Pr > |t|	<0.001	<0.001	<0.001	<0.001
-	-	-	-	-
1	506 ± 0.22[a]	410 ± 0.27[a]	89 ± 0.23[b]	124 ± 0.38[b]
2	1805 ± 0.42[f]	2354 ± 0.24[g]	374 ± 0.30[g]	434 ± 0.35[g]
3	2230 ± 0.23[d]	1637 ± 0.42[e]	195 ± 0.30d	180 ± 0.41[c]
4	1310 ± 0.26[d]	1192 ± 0.41[b]	190 ± 0.32[f]	350 ± 0.58[f]
5	2925 ± 0.20[e]	1304 ± 0.29[c]	135 ± 0.20[e]	342 ± 0.25[e]
6	3620 ± 0.19[g]	1914 ± 0.23[f]	145 ± 0.33[c]	196 ± 0.43[d]
7	2050 ± 0.22[c]	1810 ± 0.37[d]	50 ± 0.28[a]	109 ± 0.40[a]
Pr > |t|	<0.001	<0.001	<0.001	<0.001
-	-	-	-	-
	Treated (%)	Control (%)		Pr > |t|
Haemonchus spp	79.17[b]	68[a]		<0.001
Trichostrongylus spp	13.1[a]	18.21[b]		<0.001
Cooperia spp	0.4	0.2		0.09
Oesophagostomum spp	0.41	4		0.06
Strongyloides spp	6.37[a]	9.33[b]		<0.001

Means followed by distinct letters on the same line differ from each other by Tukey's test (p<0.05)

Pasture larval recovery

Table V reports the data on treatments and weeks for larval recovery in pastures. *Haemonchus* spp, *Trichostrongylus* spp and *Strongyloides* spp showed differences (p<0.001), while *Cooperia* spp, *Oesophagostomum* spp and free-living larvae (FL) did not (respective p-values of 0.60, 0.12 and 0.055). As for the weeks, only *Cooperia* spp and *Oesophagostomum* spp did not show differences (p-values of 0.8799 and 0.9975, respectively).

Experiment 2

Room temperature

Table VI shows the means and standard errors for larval recovery at room temperature. The genera *Haemonchus* spp and *Oesophagostomum* spp were the only ones without differences between treatments (p>0.05), with very small means (respectively 0.83 larvae ± 1.03 and 1.20 larvae ± 0.98). The other genera, *Trichostrongylus* spp, *Strongyloides* spp and FL (free-living larvae) showed differences between treatments (p<0.001). In the collections, only *Haemonchus* spp, *Oesophagostomum* spp and FL showed no differences (p>0.05).

BOD oven

In the samples kept in a BOD oven, the genera *Trichostrongylus* spp, *Strongyloides* spp and FL were the only ones to show differences between treatments (p>0.001), while *Haemonchus* spp and *Oesophagostomum* spp did not differ (p-values of 0.051 and 0.065, respectively).

In the weekly evaluations, only free-living larvae showed differences (p<0.001), unlike what was observed at room temperature, where FL, was not different(p<0.05).

Table IV. Means. standard errors and reference values of erythrocytes, leukocytes, neutrophils, lymphocytes, monocytes, and eosinophils of the animals evaluated in the experiment. Following the biochemical values: Urea, Albumin, Total protein, Glucose, Gama GT (GT), Phosphatase, Cholesterol and Creatine.

Treatment/ Collections	Erythrocytes (x10^6 /µL)	Total leukocytes (/mL)	Neutrophils (/mL)		Lymphocytes (/mL)	Monocytes (/µL)		Eosinophils (/mL)
Treated	9.81 ± 2.09	14445.81 ± 4262^b	4200.61 ± 2950^b		6031 ± 4035.26^b	332 ±286.22		847.50 ± 821.15^b
Control	9.86 ± 2.23	13516 ± 3432^a	3317±2441^a		5711 ± 3720^a	400 ± 329		722.16 ± 726.27^a
Pr > |t|	0.0670	<0.001	<0.001		<0.001	0.07		<0.001
-	Urea (mg/dL)	Albumin (g/dL)	Total protein (g/dL)	Glucose (mg/dL)	GT (µL)	Phosphatase (µL)	Cholesterol (mg/dL)	Creatinine (mg/dL)
Treated	36 ± 1.5	2.33 ± 0.63	5 ± 0.13	42.18 ± 1.8	23 ± 2^a	91 ± 7^a	52.5 ± 2	1.1 ± 0.2
Control	36 ± 1.54	2.4 ± 0.63	5.6 ± 0.13	43 ± 2	26.2 ± 2^b	72.7 ± 6.31^b	54 ± 1.64	1 ± 0.2
Pr > |t|	0.124	0.4578	0.4378	0.7035	0.3237	<0.001	0.3989	0.9990
-	-	-	-	-	-	-	-	-
1	48 ± 2.3^f	2.61 ± 0.3	5 ± 0.2	49.25 ± 1.6^f	40.46 ± 4.5	38.04 ± 4^a	50 ± 3.3	1.13 ± 0.1
2	36 ± 3^c	2.37 ± 0.7	5 ± 0.2	42.1 ± 4^d	21.03 ± 3.2	68.42 ± 12^c	49 ± 3.4	0.93 ± 0.2
3	30.20 ± 2^a	2.41 ± 0.6	4.63 ± 0.3	41.61 ± 3^c	20.13 ± 4	91.16 ± 12^e	49.25 ± 3.3	0.81 ± 0.2
4	32 ± 2.3^b	2.2 ± 0.7	4.33 ± 0.3	39.04 ± 3^b	20 ± 3.5	101.05 ± 14^f	48.28 ± 4	0.8 ± 0.3
5	41.51 ± 2.3^e	2.66 ± 0.5	5.42 ± 1	57.07 ± 2.2^g	34.63 ± 2.5	125 ± 15^g	66.5 ± 2	1 ± 0.1
6	54 ± 2.3^g	2 ± 0.7	5.16 ± 0.2	25 ± 3^a	12.34 ± 2.6	55.7 ± 8^b	54 ± 2.3	0.99 ± 0.2
7	40 ± 3^d	2.37 ± 0.6		43.51 ± 2.6^e			54.5 ± 4	
Pr > |t|	<0.001			<0.001			0.0621	

Means followed by distinct letters in the same column differ from each other by the Tukey test (p<0.05). Reference values: Erythrocytes: 9 to 15; Total leukocytes: 4000 to 12000; Neutrophils: 400 to 6100; Lymphocytes: 1600 to 9000; Monocytes: 0 to 750 and Eosinophils: 0 to 1000). Reference values: Urea: 17 to 43; Albumin: 2.4 to 4; Total proteins: 6 to 7.5; Glucose: 40 to 80; GT: 0 to 32; Phosphatase: 0 to 387; Cholesterol: 50 to 72 and Creatinine: 1 to 1.9.

Table V. Means and standard errors of the larval genera, found in 0.75m^2 and their respective treatments with collections 1 to 7.

Treat/ Collect	*Haemonchus* spp	*Trichostrongylus* spp	*Cooperia* spp	*Oesophagostomum* spp	*Strongyloides* spp	VL
Treated	91.07 ± 166.22^b	84.82 ± 153.55^a	9.5 ± 29	9.07 ± 35.40	85.53 ± 96.16^a	1509 ± 2029
Control	70 ± 97.07^a	98.64 ± 90.58^b	1.17 ± 5.67	13.21 ± 40.57	99.60 ± 176.47^b	1485 ± 1683
Pr > |t|	<0.001	<0.001	0.6011	0.1200	<0.001	0.055
-	-	-	-	-	-	-
1	6.5 ± 5.5^a	2.25 ± 2.37^a	0.4 ± 1	0.37 ± 1	4.25 ± 3.24^a	33 ± 20^a
2	283 ± 250^g	161 ± 97^f	23 ± 45	22 ± 63	93 ± 105^e	3028 ± 2230^g
3	74 ± 54^e	58 ± 70^c	10 ± 28	17 ± 42	63 ± 126^d	600 ± 363^b
4	41.65 ± 53.35^c	203 ± 244^g	0.6 ± 0.88	30 ± 64	29 ± 45^b	1148 ± 1200^d
5	52.55. ± 93.15^d	112 ± 69^e	0.8 ± 1	4 ± 12	153 ± 100^f	2237 ± 1856^e
6	82.75 ± 81.19^f	29 ± 32.76^b	4 ± 11	4 ± 11	49.25 ± 35^c	606 ± 189^c
7	19.12 ± 28.13^b	86 ± 76^d	0.4 ± 1.1	0	254 ± 255^g	2752.55 ± 2510^f
Pr > |t|	<0.001	<0.001	0.8788	0.9975	<0.001	<0.001

Means followed by distinct letters on the same line differ from each other by Tukey's test (p<0.05).

Table VI. Means and standard deviations for all larval genera, found at room temperature and their respective treatments, in sequence, B.O.D stove temperature.

Treat/ Week	*Haemonchus* spp	*Trichostrongylus* spp	*Oesophagostomum* spp	*Strongyloides* spp	VL
0.25g	0.40 ± 0.75	28.65 ± 21.73[a]	1.5 ± 5	51.50 ± 31.20[c]	12 ± 21.63[a]
0.50g	2 ± 3.95	24.05 ± 27.12[b]	2 ± 5.12	38 ± 27[b]	25 ± 24.16[b]
Control	0.10 ± 0.30	16.75 ± 18.15[b]	0.10 ± 0.44	24.25 ± 27.12[a]	31.70 ± 30.40[c]
Pr > | t|	0.894	<0.001	0.8390	<0.001	<0.001
-	-	-	-	-	-
1	0	39 ± 18d	0	16 ± 8.14[b]	15.3 ± 9.13
2	0	14.33 ± 15[b]	0	42 ± 36[f]	12.5 ± 15.5
3	0.16 ± 0.40	31 ± 30.17[i]	0.5 ± 0.83	83 ± 27[d]	14 ± 22.4
4	0	1 ± 1.21[a]	0	46 ± 33.53[h]	34.3 ± 34.18
5	0.33 ± 0.51	27 ± 25.63[h]	0	34.33± 25.46[d]	8.3 ± 9.41
6	0.33 ± 0.81	21 ± 20.26[d]	5.16 ± 8.7	20 ± 26[c]	21 ± 26
7	2.1 ± 5	26.33 ± 20[g]	4 ± 7.6	39 ± 28[e]	13 ± 16.47
8	1.16 ± 1	23 ± 22.75[f]	2 ± 4.4	15.16 ± 16[a]	8.33 ± 10
9	3.33 ± 5.3	17.16 ± 21[c]	0	49.33 ± 25[i]	44 ± 40.28
10	0.66 ± 1.63	22 ± 18.2e	0	44 ± 40[g]	58 ± 32
Pr > | t|	0.9911	<0.001	0.993	<0.001	0.0416
-	-	-	-	-	-
0.25g	0.75 ± 0.77	2.62 ± 2.82[b]	0.65 ± 0.74	1.62 ± 2.26[b]	5.37 ± 3.70[b]
0.50g	0.87 ± 1.12	2.75 ± 2.65[c]	1.2 ± 1.83	3.25 ± 2.86[c]	7.15 ± 4.91[c]
Control	0.75 ± 0.88	1.75 ± 2.65[a]	0.37 ± 0.88	1.25 ± 2.1[a]	2 ± 2.44[a]
Pr > | t|	0.051	<0.001	0.99	<0.001	<0.001
-	-	-	-	-	-
1	0.33 ± 0.81	3 ± 4	1.16 ± 1.94	3.16 ± 3.18	6.33 ± 5.16[c]
2	0.55 ± 0.5	3.33 ± 2.8	1 ± 1.09	6.16 ± 6.92	3.5 ± 2.36[b]
3	1.33 ± 0.81	2.33 ± 1.36	0.5 ± 0.83	1.55 ± 1.51	7.17 ± 5.15[d]
4	1 ± 1.1	0.83 ± 1.32	0.33 ± 0.51	0.16 ± 0.40	2.83 ± 2.5[a]
Pr > | t|	0.9900	0.3	0.99	0.089	<0.001

Means followed by distinct letters on the same line differ from each other by Tukey's test (p<0.05).

DISCUSSION

Experiment 1

In the present study, the lack of predation by the fungus *Duddingtonia flagrans* can be explained by several factors, the main one being the duration of action (Carvalho 2018). Predatory fungi need approximately 12 months for adequate soil colonization, especially considering the supply and ingestion by ruminants. Assays performed by other authors evaluated the use of a commercial product called BioWorma™ containing chlamydospores of *D. flagrans*, supplied to some species of ruminants (sheep, cattle, goats) as well as horses (Healey et al. 2018b).

A gradual and staggered observation of the fungal action time *in vivo* was estimated of at least 10 months for the colonization of a soil with adequate conditions, such as moisture and temperature (Besier et al. 2016). Is of great importance that the intention of this present study contrasts with other previously made work, since the doses used where very low and different of what is recommended by the producers.

The values reported in Table V (larvae recovered in pasture) differed between treatments for the genera *Haemonchus* spp, *Trichostrongylus* spp and *Strongyloides* spp ($p<0.001$). *Cooperia* spp, *Oesophagostomum* spp and free-living larvae did not differ ($p>0.05$). Similar results were observed for the collections, between January and July, in which *Cooperia* spp and *Oesophagostomum* spp were the only ones that did not show differences ($p>0.05$). The recoveries showed sudden drops and increases, without uniformity, indicating the lack of predation by the fungus *D. flagrans*.

According to Goettel et al. (2001), nematophagous fungi develop properly around 25 ºC. When ideal conditions are not present, growth stops partially or completely (Baron 1977). In our study, temperatures varied, mainly March and July, when the average fluctuated between 25.6 ºC and 16.6 ºC (CIIAGRO 2021). The low room temperature added to the prolonged drought possibly generated unfavorable situations for colonization.

Although it has been reported that the fungus *D. flagrans* is a poor soil colonizer (Knox et al. 2002), in this test, the presence of chlamydospores was observed, starting from the third month of the experiment. Chlamydospores began to be observed in all subsequent collections, with increasing numbers until the formation of larger groups. Such behavior is indicative of gradual fungal colonization of the soil although without larval predation (Figure 1).

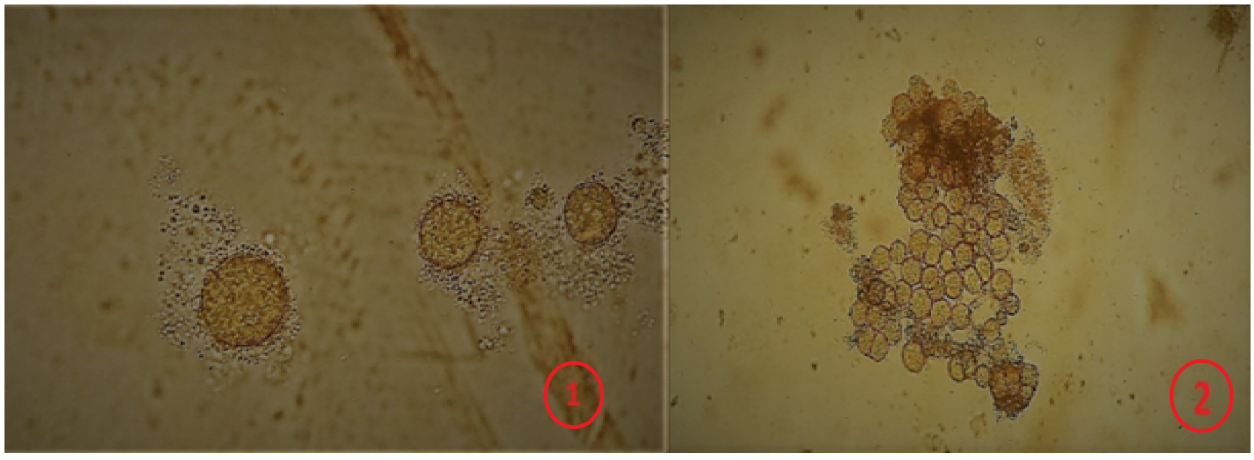

Figure 1. Evolution of chlamydospores in pasture (1 March and 2 July).

In the results for eggs per gram of feces (Table III), there were differences between treated and control groups for Trichostrongylids and Strongylids ($p<0.001$). A larger number of eggs of Trichostrongylids was observed in the treated group, while the control was higher in the case of Strongylids. According to the small sample size, this factor could serve as a reflection of sudden changes, where some animals in the treated group were possibly sensitive to worms, as also indicated by the laboratory results and clinical symptoms, with higher EPG counts than in the control group.

As for the hemogram results, all values reported in Table IV were within the normal range (Kaneko 1997). Total leukocytes, neutrophils, lymphocytes, eosinophils, and monocytes showed differences between treatments ($p<0.001$). Only erythrocytes and monocytes did not differ (p-value of 0.067). When studying sheep worms, hematological parameters are strong indicators of the severity of the problem, mainly in the case of parasitism by *Haemonchus* spp. However, in the present study, the animals used in the experiment did not show non-standard values.

In the biochemical results, a difference was observed for GT gamma and phosphatase ($p<0.001$), while urea, albumin, total proteins, glucose, cholesterol and creatine levels were not different ($p>0.05$). As for collections 1 to 7, urea, glucose and phosphatase differed ($p<0.001$) and albumin, total proteins, GT gamma, cholesterol and creatinine did not ($p>0.05$). All values were within the parameters for sheep (Meyer & Harvey 2004) except for total proteins, albumin and glucose, which were close to the reference values. Total proteins remained below the ideal (6 to 7.5 g/dL), possibly a consequence of worms affecting the animals in the experiment, due to hematophagy, directly affecting the total protein in blood samples.

The low concentrations of total serum proteins observed may have been mainly the consequence of blood loss due to the hematophagous activity of *Haemonchus* spp parasites. This has also been found to be associated with nutritional deficiencies and, various other impairments, all possibly causing a reduction in total proteins (Amarante et al. 2014). However, in this experiment, all animals received daily food supplementation to keep their immune responses prepared to overcome parasitic infection.

Experiment 2

Room temperature

The two genera that stood out the most were Strongyloides spp and Trichostrongylus spp, although at times, free-living larvae appeared to be more present (Table VI). The genera Trichostrongylus spp, Strongyloides spp and FL differed ($p<0.001$), while Haemonchus spp and Oesophagostomum spp showed no differences ($p>0.05$). During the 10 weeks of readings, only Trichostrongylus spp and Strongyloides spp varied ($p<0.001$). Haemonchus spp, Oesophagostomum spp and FL did not change in the weeks ($p>0.05$).

The genera *Strongyloides* spp and *Trichostrongylus* spp were observed most in the evaluations. The greater presence of *Strongyloides* spp in the samples can be explained by the cycle of infection in the hosts, involving skin penetration (Buzzulini et al. 2007, Cavalcante et al. 2016), unlike most other genera, which require a grazing environment to ingest L3 larvae.

There was no predation by the nematophagous fungus *Duddingtonia flagrans*, evident in the results between treatments, as well as during the weeks of evaluation. In the 0.25g and 0.50g treatments, no changes were

observed in the number of larvae recovered, maintaining unstable behavior. The presence of chlamydospores was also observed, with small clusters in the first weeks of the experiment (Figure 2a).

BOD oven

Strongyloides spp and *Trichostrongylus* spp larvae were predominant in this assay, like at room temperature. The predominant behavior of successive recoveries of these genera reinforced the fact that they develop better in confined environments (Ferraz et al. 2019). As indicated in Table VI, *Trichostrongylus* spp, *Strongyloides* spp and FL genera were the only ones to differ between treatments ($p<0.001$). *Haemonchus* spp (p-value of 0.051) and *Oesophagostomum* spp (p-value of 0.065) showed no differences between treatments. As for the evaluation weeks, only FL showed differences, with the opposite occurring for all other genera.

Unlike the test at room temperature, there were atypical observations in the structure of the chlamydospores of *D. flagrans*, with the presence of deformed structures with twisted cell walls. Another factor was the proliferation of microorganisms, causing a foul odor (Figure 2b). Contrary to experiment 1, in experiment 2 chlamydospore structures were seen from the first week (both at room temperature and BOD temperature). In the first experiment, this was observed only 3 months after inoculation.

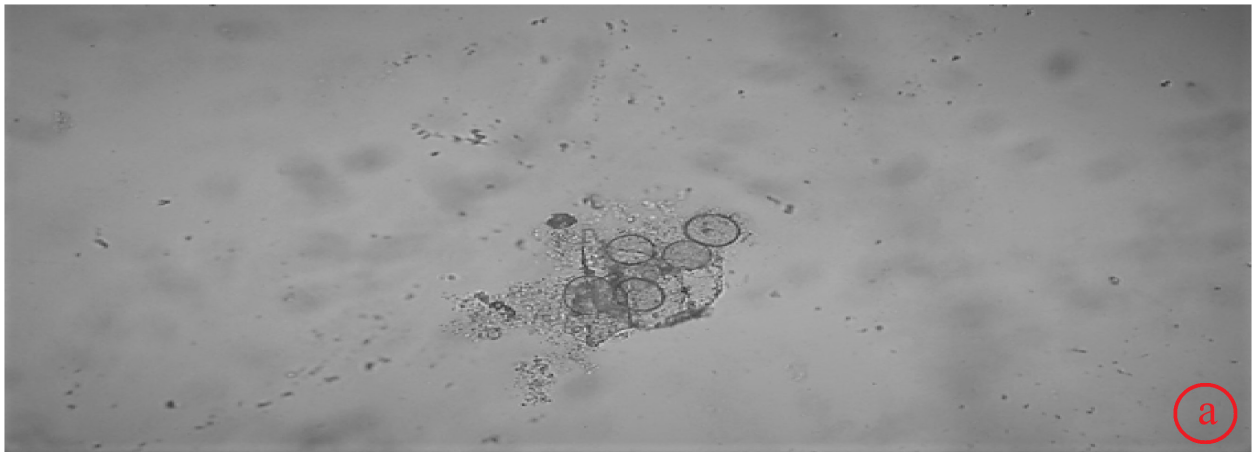

Figure 2a. Group of chlamydospores observed in manure, room temperature.

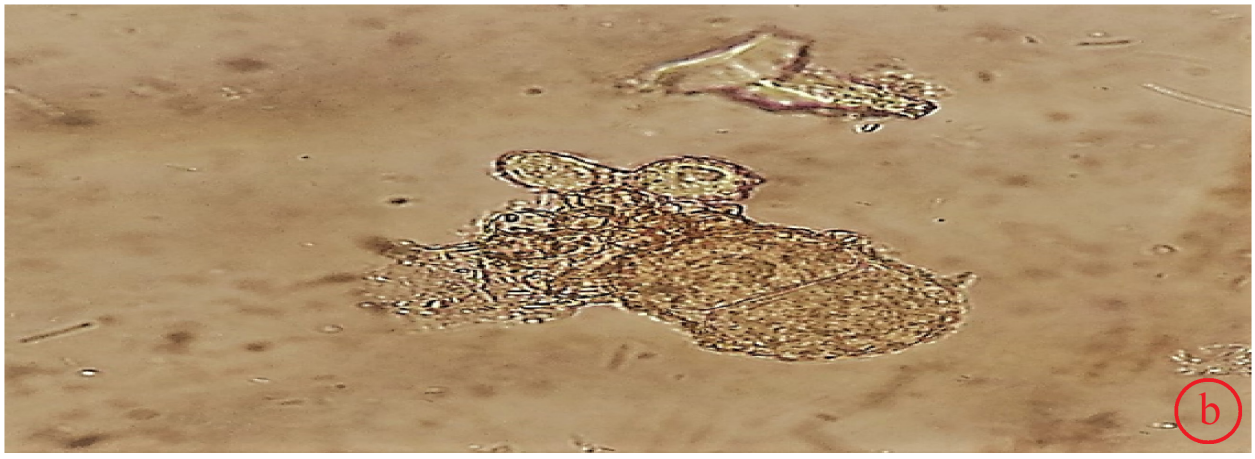

Figure 2b. Deformed chlamydospores in B.O.D stove.

It is known that microorganisms present in high density and quantity from decomposition can attack other structures during their colonization, possibly generating distorted shapes of chlamydospores (Sanyal et al. 2008). We noted the occurrence of fermentation due to the presence of layers with protein supplement along with the manure in the containers. Besides fungi, a considerable decrease in the presence of larvae was observed in comparison with the first test. Possibly both infective and free-living larvae suffered direct attack from the other decomposing microorganisms in the medium (Rösler et al. 2021).

CONCLUSION

There was no reduction of the number of infective larvae in pastures and in containers with manure, as well as no action of the nematophagous fungus *D. flagrans*, despite its presence in both experiments.

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