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Research Article

Biological control of nematodes by nematode-trapping fungi *Duddingtonia flagrans* in naturally infected sheep in southern Brazil

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Abstract

The aim of this study was to evaluate the anti-helminthic effect of a commercial formulation Bioverm® (*Duddingtonia flagrans*) in 28 sheep naturally infected with gastrointestinal nematodes. Animals were classified into two groups: group 1 (G1, n=14) treated with nematophagous fungi and group 2 (G2, n=14) untreated control. The efficacy of the anti-helminthic drug was assessed based on the egg count per gram of feces (EPG) of strongyles, larval culture, hemogram, leukogram, plasma protein levels, animals body weight, and anemia using FAMACHA© at days 0, 30, 60, 90, 120, 150, and 180. Additionally, the nematode larvae were quantified in the dry matter of the pastures of both groups. Results showed that the EPG was significantly decreased in animals receiving nematophagous fungi from D30 until the end of experiment. The most common nematode genus was *Haemonchus* (63%), followed by *Cooperia* (23%) and *Trichostrongylus* (15%). Based on the fecal egg count reduction test (FECRT), treated animals showed a reduction in fecal egg count of 58.9%, 8.6%, 92.8%, 96.4%, and 96.2%, at D30, D60, D90, D120, and D180, respectively. The absolute values of red blood cells and leukocytes counts were significantly increased at D60 and D90, respectively. A significant weight gain was observed in the treated ewes at the end of the experiment; however, there was no correlation between the EPGs values and hematocrit with the FAMACHA© degrees of animals in both experimental groups. The mean EPG of both groups and the number of infectious larvae in the pastures were not directly proportional. In conclusion, nematophagous fungi contributed to decreased parasitic load in sheep, and consequently, improved animal performance; they can be a suitable alternative to reduce problems associated with nematode infections.

Keywords: *Duddingtonia flagrans*, small ruminants, gastrointestinal parasites, pasture

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Introduction

In Brazil, sheep are bred for the production of wool, milk, and meat. The State of Paraná has 588,996 heads of sheep, corresponding to 14.9% of sheep in the south and 3.0% of the Brazilian sheep herds (IBGE, 2019). The greatest interest is in breeding lambs for slaughter, originating from small and medium-sized breeds with a small number of matrices. It is usually a secondary activity to the exploitation of other animal species, especially bovine species. Small ruminants are affected by various sanitary problems, with gastrointestinal nematodes (GIN) being the main barrier against their development. The GIN causes significant losses in animal production due to subclinical infections, high costs of treatments, elevated herd mortality rates, which resulting in losses to the national economy (Rashid et al., 2019). Parasitism caused by GIN species is mainly due to *Haemonchus contortus* which is belonging to family *Trichostrongylidae* (Echevarria et al., 1996; Arosemena et al., 1999).

Deworming in sheep is performed preferably by using synthetic anti-helminthians, however, little or no emphasis on controlling the stages of parasites in surrounding pasture is considered. This method creates a selection of populations of resistant worms, making parasite control ineffective in the long term on the property, as this resistance can be transmitted to the next generations (Molento and Prichard, 1999). Thus, a single measure for parasites control is not sustainable, and a combination of strategies, such as integrated parasite management (Waller et al., 2006; Hoste and Torres-Acosta, 2011), employs good pasture management; the strategic use of anti-parasitic (synthetic or herbal) and biological control (Burke and Miller, 2020) should be adopted.

Biological control is defined as any activity of

a species that reduces the adverse effect of another species (Kwenti, 2017) and aims to reduce the number of stages of free life in the pasture. However, the lack of a method for biological control of parasites will have the same effect as an anti-helminth drug, which postponed developing these biocontrol methods (Szewc et al., 2021). Although still emerging, the recent sustainable food production trends and the search for biological alternatives in the control of GIN seem to have triggered a renewed interest in the field of agrobiology.

There are several proposals for alternatives to control GIN , such as the selection of resistant breeds (Estrada-Reyes et al., 2019), integration with other animal species, phytotherapy, biological therapy (Holsback et al., 2013), the use of nematophagous fungi (Vilela et al., 2020), vaccination against nematodes (Meeusen, 1996), and fodder containing tannins (Butter et al., 2000). Several researchers have already tested nematophagous fungi that currently represent an important alternative in the biological control of GIN in sheep (Faessler et al., 2007; Liu et al., 2020), goats (Araujo et al., 2006; Vilela et al., 2012, 2020), cattle (Assis et al., 2012). In equines, the efficacy of the nematophagous fungi was assessed *in-vitro* (Tavela et al., 2013; Braga et al., 2015) and *in-vivo* in 2-8 years-old Australian stock horses (Healey et al., 2018).

This study aimed to evaluate the anti-helminthic effect of a commercial formulation containing nematophagous fungi orally administered to sheep naturally infected with GIN, in addition to assessing the hematological and serum changes in animals treated with this product. The study also aimed to evaluate the weight gain and contamination of pasture by infectious larvae.

Materials and Methods

This project was approved by the "Comitê de Ética do Uso de Animais" (CEUA), Universidade Estadual do Norte do Paraná, Brazil with reference number (CEUA 2598/11).

Location, groups, and experimental periods

The experiment was conducted at the Farm-School of the State University of Northern Paraná, Campus Luiz Meneghel, in the Municipality of Bandeirantes, Paraná, Brazil; Geographical Coordinates 23°06'23.2"s, 50°21'37.6' ' (Figure 1). From 72 adult sheep, 28 with an egg count per gram of feces (EPG) mean greater than 150 were selected, which corresponds to the minimum established EPG for resistance and anti-helminth efficiency tests in sheep (Coles et al., 2006). Mixed-breed sheep that are naturally infected with GIN and duly identified, were randomized according to their EPG using the technique described by Gordon and Whitlock (1939) at a dilution of 1:25. Animals were classified into two groups: G1 (treated group), comprised of 14 healthy, non-pregnant (3.5 years old with a mean weight of 45 kg) ewes, and G2 (untreated control group), comprised of 14 healthy, non-pregnant (3.5 years old with a mean weight of 48 kg) ewes. Animals kept in G1 received mineral salt mixed with nematophagous fungi throughout the experimental period. Thirty-two kilograms of commercial product Bioverm® (*Duddingtonia flagrans*-AC001, GHEVET) containing 10^5 chlamydospores per gram was administered to the G1 group *ad libitum* for six consecutive months (December to May 2013).

The animals in the untreated control group (G2) also received mineral salt *ad libitum* but without the fungal product for the same period. The estimated daily mean consumption of the mineral salt was 30 gm/animal/day resembling the routinely provided quantities to the animals. After defining the animals in each group, both were introduced in the pasture of Panicum maximum cultivars Mombaça and Aruana.

Day 0 (D0) was defined as the first day of treatment followed by six experimental periods at D30, D60, D90, D120, D150, and D180. During these periods, animals were weighed, and stool harvests were carried out for EPG counting and culture of larvae. To identify severe anemia, the mucosal coloration was verified using the FAMACHA© method (Kaplan et al., 2004). FAMACHA© is a selective treatment method that indicates deworming of only anemic animals. Anemia was detected in the ocular mucosa of sheep by correlating the color of the ocular conjunctiva of small ruminants with the five ranges of anemia indicated by blood tests measuring the red cell percentage. The same evaluator examined the mucosa of the animals throughout the experimental period. The arithmetic mean of EPG was calculated for each group and on every testing day. To identify the genera of nematodes, larval culture (coproculture) was carried out following the methodology described by Roberts and O'Sullivan (1950), with the L3 larvae recovered and identified using the criteria given by Keith (1953). From these values, the percentage of fecal egg count reduction of nematodes of each identified genus in the culture of the larvae was calculated using the fecal egg count reduction test (FECRT) formula (Wood et al., 1995).

FECRT=EPG mean of the treated on day-x/EPG mean of the control on day-x x 100

In the present study, FECRT was not used to estimate the resistance of the helminths to treatment but to verify the percentage of reduction in the parasitic charge of animals and to identify the GIN species showing more substantial decrease due to biological therapy.

Hematology

Blood samples were collected from each animal by puncturing the jugular vein for hemograms, leukograms, and plasma protein levels. Total leukocyte and differential counts were done using a hemocytometer after getting the relative count measured from the absolute count of leukocytes (Hewitt, 1984). The total blood cell count was performed using the hemocytometer. The hemoglobin concentration was calculated using the cyanmethemoglobin method and the globular volume was determined using the microhaematocrit. The biuret reactive was used to measure the total protein level, and the albumin concentration was determined using the bromocresol green reactive (Doumas

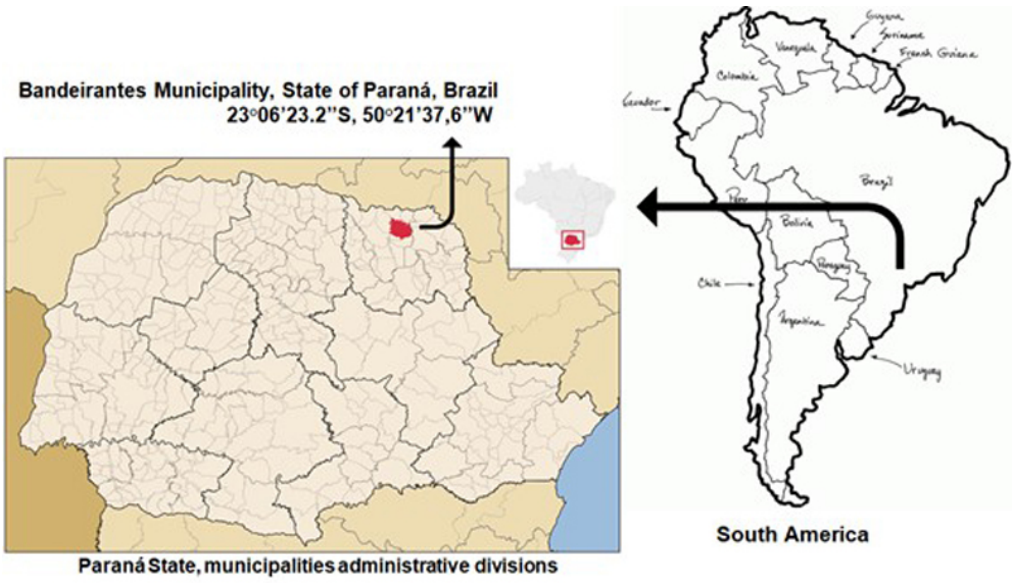

Figure 1: Map of Brazil and localization of Bandeirantes municipality in State of Paraná (IBGE, 2019, adapted. Image: Parana MesoMicroMunicip.svg, own work, CC BY 2.5. Map South America produced by Hicks, P.https://br.pinterest.com/pin/698480223437205248/.

et al., 1971). The concentration of total globulins was calculated as the difference between the concentration of total proteins and albumin concentration.

Pasture samples and meteorological data

Two samples of the pasture's aerial (0–20 and 20–40 cm fecal cake) of 500 gm each were collected from six alternate points at D30, D60, D90, D120, D150, and D180, according to the technique described by Raynaud and Gruner (1982). The larvae were recovered from the grass samples, quantified according to the methodology described by Carneiro and Amarante (2008) and identified according to the criteria established by Keith (1953). The obtained data were converted to the number of infectious larvae recovered per kilogram of dry matter (L3/kg-DM). Meteorological data such as temperature and precipitation intensity indices were obtained from the Universidade Estadual do Norte do Paraná-UENP agrometeorological station present at the same location of the present study.

Statistical analysis

Statistical tests were performed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Wilcoxon test was used to compare the means of the EPGs of the treated and control groups. The comparison between the means of EPGs under the genera identified after the growth of larvae was performed using Mann–Whitney test. Differences between the mean values of cell count obtained by hemograms, leukograms, and plasma protein dosages were analyzed using paired t-test. The correlations between the FAMACHA© degrees, hematocrit, and EPG were obtained using the Pearson correlation coefficient (r). Differences were considered significant at $p<0.05$

Results and Discussion

The mean EPG of both groups (G1 and G2) decreased significantly 30 days after the start of the treatment (Figure 2); however, this decrease can be explained by the change in paddocks when they left degraded pastures and were placed in a good pasture. The good nutritional and physiological status of animals influences the immune response against pathogens and, consequently, contributes to confronting parasitism, limiting the establishment of infectious larvae (Bishop and Stear, 2003).

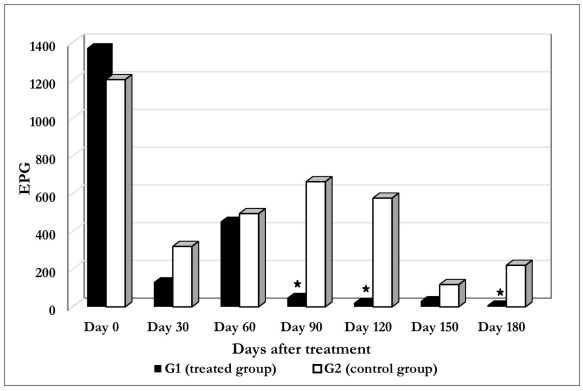

Figure 2: Mean values of the EPGs of the animals in G1 (treated with nematophagous fungi) and G2 (untreated control group) at the beginning of treatment (D0) and 30, 60, 90, 120, 150, and 180 days later. *Statistical differences ($p<0.05$) between the EPG of G1 and G2 on the same experimental day.

In addition to the decrease in EPG on D30, there was a significant decrease ($p<0.05$) of EPG in the feces of the nematophagous fungi-treated animals throughout the experimental period. A significant decrease was observed in the mean EPG of the animals in the control group, only 150 days ($p= 0.0085$) from the beginning of the experiment, which lasted until D180 ($p= 0.004$). However, on the last day, the animals of the treated

Table 1: Mean ± standard error (σx) of infectious larvae per kg of dry matter (L3/kg-DM) recovered from pastures where the animals of the G1 (treated with nematophagous fungi) and G2 (untreated control group) groups remained on days 30, 60, 90, 120, 150, and 180.

Groups	L3/Kg-DM ± SEM (σx)						
	D30	D60	D90	D120	D150	D180	Mean
G1 (Treated)	38.2±17.2	40.6±22.3	52.3±19.6	48.9±25.1	162.6±56.1	101.3±41.2	73.9±22.1
G2 (Untreated control)	57.1±25.8	68.8±39.7	30.4±19.6	34.1±11.4	151.8±49.2	262.0±144.2	100.7±42.9

group had 96% less eggs (p= 0.031) in the feces (mean 8.3±6.4 EPG) than those of the control group (mean 220±148.6 EPG). Decreased EPG in animals of both groups from D150 probably occurred because of the pasture management (mowing pastures) at the end of March (D120), which may have contributed to the decrease in infectious larvae.

The pasture mowing exposes the pasture plant bases to the solar rays that reduce the number of larvae (van Dijk et al., 2009). However, as shown in Figure 2, from D90, the animals treated with nematophagous fungi maintained low EPG, while high EPGs were observed in the control group animals. Reduced EPG counts were identified in the animals of the treated group compared to the control group on all experimental days; however, these differences were significant only on D90 (p= 0.037) and D180 (p= 0.031) (Figure 2).

These results confirm the reduction rates (FECRT) found in the animals of the group treated throughout the period, with the least reduction rates observed on D30 (58.9%) and D60 (8.6%), and the largest on D90, D120, and D180 (92.8, 96.4, and 96.2%, respectively). Environmental changes and pasture mowing may have influenced the decrease in the EPG of the treated animals because at the end of March. In the same experimental period (D120/ March), a simultaneous reduction in the mean daily temperatures (Figure 3) and the EPG of the control group (50% to 72% reduction) was observed. However, the EPG count reductions in the treated animals in the same period were 73.5% to 96.4%, demonstrating that the treatment was efficient in controlling nematodes in animals and climate influence.

The main climate factor that regulates the GIN cycle in tropical and subtropical climates is pluviometry and directly proportional to the increase in the availability of infectious larvae in the pasture (Souza et al., 2000). Minguetto et al. (2021) also observed the influence of climatic factors on GINs. They described that temperature and humidity were the most critical factors determining the nematode population dynamics in the environment and animals. A low L3/kg-DM count was found on D120 (41.5 L3/kg-DM) (Table 1), but the EPG means of both groups and the count of infectious larvae in pastures in the last periods of the experiment did not correspond. Despite low mean EPGs at D150 and D180, high amounts of larvae in pastures were found (Figure 4 A&B). Similarly, Carneiro and Amarante (2008) recovered only 1% of larvae in pastures compared to the number of eggs deposited. Levine and Todd (1975) asserted that more than 50% of eggs develop into infectious larvae under controlled laboratory conditions, but only a small percentage produce viable larvae in pastures.

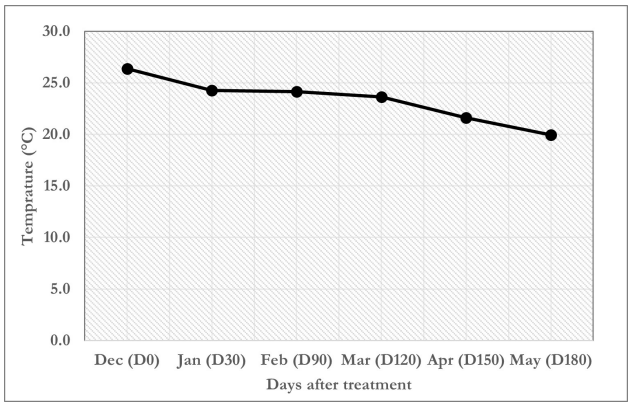

Figure 3: Mean daily temperatures recorded by the Universidade Estadual do Norte do Paraná-UENP agrometeorological station during the six months of the experiment.

No significant differences were observed between the means of L3/kg-DM recovered from the pastures of treated sheep compared to the control group. Other researchers have also reported this inconsistency (Faessler et al., 2007; Liu et al., 2020). We observed that in the months with high temperatures and high rainfall indices (which, in our experiment, coincided with D30 and D60), there was less recovery of infectious larvae in pastures. Although this climate favors egg hatching and larval development, we believe that it predisposes to higher mortality of these immature forms (Barger et al., 1994). The genus *Haemonchus* (Figure 5A) was the most frequently found in larval cultures, followed by *Cooperia* (Figure 5B) and *Trichostrongylus* (Figure 5C). FECRT of all genera identified in coproculture on all experimental days is shown in Table 2. The genus *Oesophagostomum* was not found on any of the experimental days. The lowest reductions in *Haemonchus* egg count in the feces of the treated animals occurred on D60 (FECRT 0%) and D150 (FECRT 36.2%), and the highest on D120 (FECRT 97%) and D180 (FECRT 96, 4%), supporting the *in-vivo* findings of Araujo et al. (2006) & (Molento et al., 2018), and the *in-vitro* findings of Braga et al. (2020). Minguetto et al. (2021) also observed that animals that received nematophagous fungus experienced

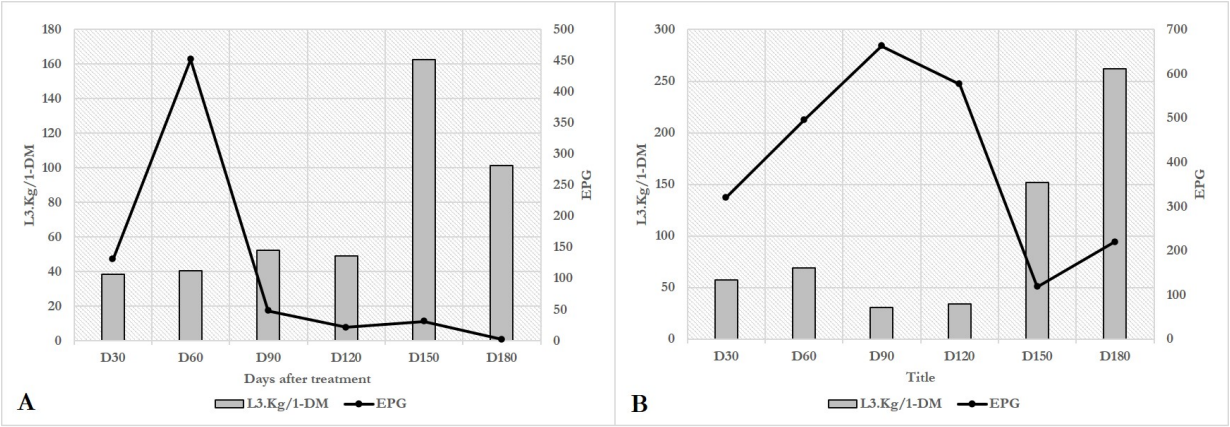

Figure 4: Infective larvae (L3/kg-DM) of the pastures and means of the EPGs of the treated group G1 **(A)** and untreated group G2 **(B)**.

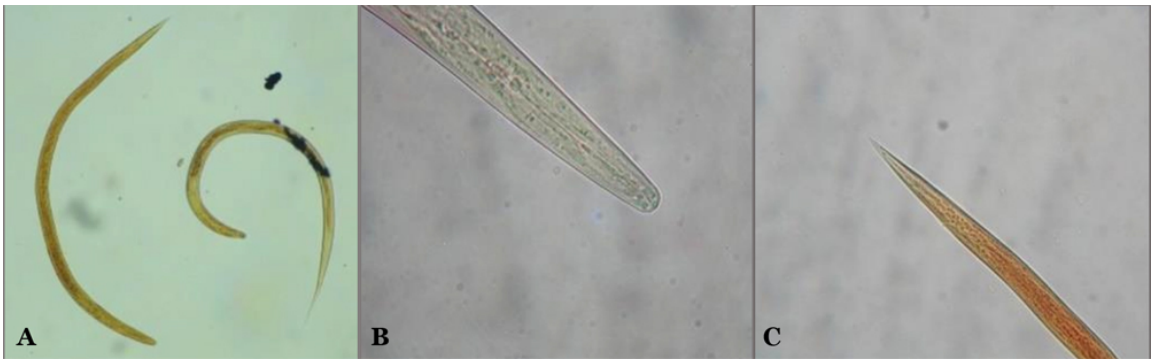

Figure 5: A. Third-stage larvae of *Haemonchus* (right) and *Trichostrongylus* (left), **B.** Third-stage larvae of *Cooperia*, and **C.** Third-stage larvae of *Trichostrongylus* (note nil filament as one of the characteristics of this genus).

a late reduction in the *Haemonchus* spp.

Table 2: Fecal egg count reduction test of *Haemonchus*, *Cooperia*, *Trichostrongylus*, and *Strongyloides* (FECRT%) in animals treated with nematophagous fungi at days 30, 60, 90, 120,150, and 180 after treatment.

Parasite	Days after treatment					
	D30	D60	D90	D120	D150	D180
Haemonchus	80.2%	0	93.4%	97.0%	36.2%	96.4%
Cooperia	0	0	69.9%	97.9%	83.1%	96.8%
Trichostrongylus	0	24.0%	94.6%	93.1%	96.5%	94.4%
Strongyloides	50.0%	100%	100%	0	0	0

The results presented in this study differ from those of others (Eysker et al., 2006) who evaluated the animals for shorter periods or in distinct seasons. In addition, previous studies also reported climatic interference, such as heavy rains, flooding, frequent movement of animals, and high stocking rates in pastures. Biological control using *D. flagrans* in short-term experiments is unlikely to be successful because the fungus remains on the fecal plate where it is deposited and does not spread horizontally across the pasture. The dissemination of fungi between the different soil layers is thought to be due to the dispersal of chlamydospores by coprophilic fauna and not by mycelial growth (Knox et al., 2002). Therefore, this dissemination and consequent action against the forms of the free life of nematodes would require more extended analysis periods.

Although Silva et al. (2010) performed their study for a period similar to ours (six months), they did not find satisfactory results in preventing GIN in sheep. However, the authors reported lower temperatures (minimum 16.2°C and maximum 20.2°C) than those observed in the present study (minimum 19.9°C and maximum of 26.4°C). Moreover, in our study, we started the experiment at a high temperature season (December) and finished at the end of the autumn season (May); however, Silva et al. (2010) started their experiment in June and finished in November. The higher contamination of pasture may have contributed to increased infection levels detected in animals. *D. flagrans* grows best in moist feces and the increased intensity of light (during sunny summer) reduces its effectiveness (Grønvold et al., 1993; Bilotto et al., 2018).

A study that includes all months of the year with diverse groups, including a group of non-treated animals, is necessary to understand better the effect of actual climatic conditions on the nematocidal activity of *D. flagrans*. Although there are many divergent results

Table 3: Mean ± standard error (σx) of the absolute number of red blood cells and leukocytes, globular volume (GV), and total plasma protein (TPP) of the animals of the treated (G1) and control (G2) groups, before treatment (D0) with fungi nematophagous and at D30, D60, D90, D120, D150, and D180.

Group	Days	Red blood cells ($9\text{-}15x10^6$)	GV (%) (28-40)	Leucocytes ($4\text{-}12x10^3$)	TPP (5.4-9.0)
G1 treated group	D0	6.8±0.15	25±0.90	7.921±0.599	6.0±0.12
	D30	7.3±0.27	30±0.96	6.300±0.271	6.7±0.17
	D60	9.4*±0.38	30±0.73	7.971±1.125	6.8±0.20
	D90	8.2±0.51	29±1.11	11.170*±0.755	7.0±0.18
	D120	7.9±0.44	28±0.79	9.300*±0.703	7.0±0.15
	D150	10.3*±0.75	32±0.94	9.275±0.776	7.2±0.20
	D180	7.2±0.39	28±0.92	8.200±0.515	7.2±0.19
G2 untreated control group	D0	7.7±0.28	28±1.29	7.554±0.669	5.9±0.18
	D30	7.8±0.27	33±0.99	5.881±0.305	6.8±0.16
	D60	8.8±0.39	32±1.23	11.071±3.867	6.7± 0.17
	D90	7.4±0.42	28±1.29	10.468±1.097	6.7±0.20
	D120	7.5±0.74	26±2.00	9.481±0.450	6.6±0.36
	D150	8.6±0.57	31±1.49	11.467±0.867	7.4±0.26
	D180	8.1±0.39	29±0.90	8.050±0.971	7.1±0.12

regarding the effectiveness of this biological treatment, there is more evidence to suggest its efficacy than other approaches (Canhão-Dias et al., 2020).

Hematological analyses demonstrated that the animals of the two groups remained anemic (Kramer, 2000) during almost the whole experimental period, except for the treated group on D60 and D150 (Table 3). It is known that the genus *Haemonchus* causes severe anemia in animals due to its hematophagous nature; however, this observation was not associated with a greater or lesser EPG count of *Haemonchus* since both the groups had similar EPG means after growing of larvae. The EPG means were 389±315.9 4σx and 379±160.4σx in the treated and control groups, respectively. The EPG means on D150 were 17±8.8 4σx and EPG 27±11σx) in the treated and control groups, respectively (data not shown).

According to Amarante et al. (2004), animals with high parasitic loads showed reduced GV and TPP values due to the destruction produced by the parasites. This reduction was not observed in our experiment, likely due to the high and daily supply of pasture fodder. Haemoglobin, neutrophil, monocyte, and lymphocyte counts remained normal in both groups. The treated sheep group showed an increase in eosinophil count (that elevated the total leukocyte means) (Table 3) on D90 and D120, but animals did not present eosinophilia. Eosinophils are essential cells in the elimination of helminths (Kindt et al., 2006). The association between the increased eosinophil count and protection against nematodes was proposed by (Wassom and Gleich, 1979). In addition, studies have demonstrated that *H. contortus* larvae incubated with anti-*H. contortus* antibodies exposure to eosinophils leads to larval death after 24 hrs (Rainbird et al., 1998).

On the same experimental days (D90 and D120), the mean *Haemonchus* EPG in the treated group was 93.4% and 96.9%, respectively, lower than those in the control group (Figure 6). The association between the reduced EPG in animals treated with nematophagous fungi and increased eosinophils cannot be elucidated due to the lack of studies investigating the possible action of *D. flagrans* on the immunological mechanisms in treated animals.

There was a significant difference in the weight gain of the animals in either group for up to 90 days from the start of the experiment. This parameter was expected since the experimental ewes were kept in good pastures. However, a significant weight gain by the end of the experiment was observed only in the treated ewes (Figure 7). The treated group ewes were 8.07 kg (p= 0.0118) heavier than the control group ewes, which were 0.55 kg (p= 0.76) lighter at the end of the experiment. Our findings corroborate those of Vilela et al. (2012, 2020), who observed an increased weight gain in male goats (10 months old) for six months and female goats (four months old) for four months.
According to a classification by Evans (1995), there was a very weak negative correlation (r= -0.060) and a weak positive correlation (r= 0.394) between the hematocrit of all animals and FAMACHA© in the treated and control groups, respectively. The mean of FAMACHA© of the treated group was three on every experimental day, except D120 and D150; the mean was four. Overall, the evaluation period, the mean FAMACHA© of the treated group was three. Comparing the EPG and FAMACHA© revealed a weak negative correlation (r= -0.064) of the treated animals and a weak positive cor-

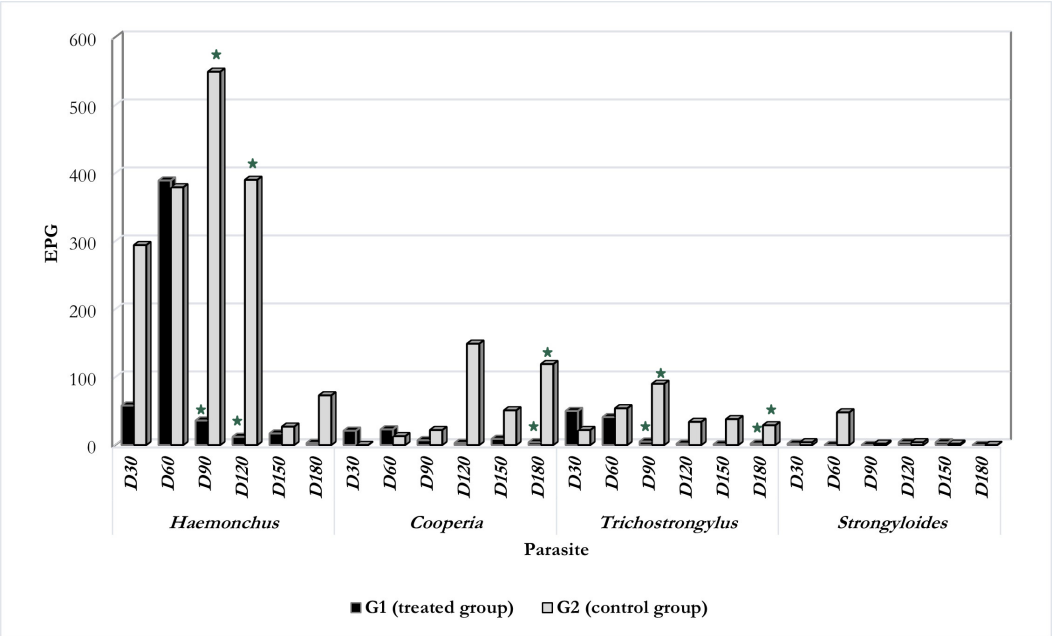

Figure 6: EPG of *Haemonchus*, *Cooperia*, and *Trichostrongylus* obtained after identification of coproculture, and *Strongyloides* EPG obtained at the egg counting (Gordon and Whitlock, 1939) on days 30, 60, 90, 120, 150, and 180 of the animals of the treated (G1) and untreated control (G2) groups. *Statistical differences ($p<0.05$) between the EPG media of G1 x G2 on the same experimental day.

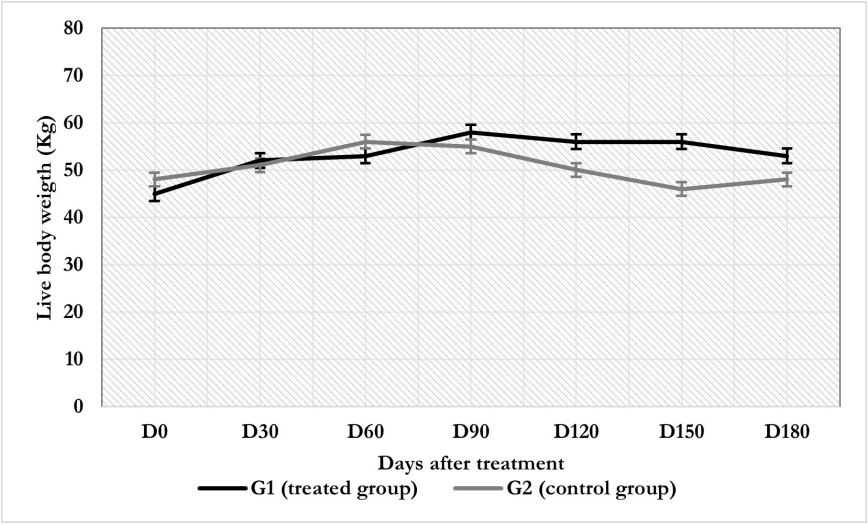

Figure 7: Mean ± standard error (σx) of the live weight of the treated (G1) and untreated control (G2) groups, before treatment (D0) with nematophagous fungi and at D30, D60, D90, D120, D150, and D180.

relation (r= 0.08) in the control group. These findings demonstrate no proportional relationship between the coloring of the sheep mucosa and the EPG levels or the hematocrit value. In the control group, the highest concordance between FAMACHA© and hematocrit was observed on D0 (28.6% hit) and D120 (21.4% hit). Simultaneously, the *Haemonchus* percentages in these animals were 62% and 68%, respectively. However, 85% of *Haemonchus* was detected in the treated group on D60, and 93% was detected in the control group on D30 with a null assertive rate (0%).

Although the same evaluator was assigned throughout the study, human error may have occurred while reading the mucous membranes as the evaluator had been trained shortly before starting this experiment. This problem has previously been reported by Niciura et al. (2009). Furthermore, the low number of animals (n=14) in each group associated with a reading error of two or three animals (11–14%) may have influenced results. Despite these findings, the inefficiency of the method cannot be affirmed.

Conclusions

Nematophagous fungi contributed to the decrease in the parasitic burden of sheep and consequently improved the hematological parameters of the myeloid and lymphoid series. This biological method could be an excellent alternative to control parasitic infections in sheep. We believe that the careful long-

term use of nematophagous fungi, in association with good health and nutritional management practices, provides an economical and efficient control measure of GIN in sheep raised extensively in tropical areas.

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