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Short Communication

In vitro efficacy of *Duddingtonia flagrans* against nematodes of sheep based on *in vivo* calculations

Eficácia *in vitro* de *Duddingtonia flagrans* contra nematoides de ovinos com base em cálculo *in vivo*

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Abstract

Duddingtonia flagrans has been tested as an alternative parasite control, but data from *in vitro* experiments based on *in vivo* calculations describing nematophagous fungi predation in nematodes are restricted. The objective of this work was to determine the efficacy of *D. flagrans* against sheep nematode larvae *in vitro* using *in vivo* calculations. Fecal samples were introduced to fungi in different concentrations: 0.0/control; 0.05; 0.1; 0.2; 0.4; 0.8; 1.6; 3.2; and 6.4 g corresponding, respectively, to 583.000; 1.166.000; 2.332.000; 4.664.000; 9.328.000; 18.656.000; 37.312.000 and 74.624.000 chlamydospores/kg of body weight. The material was incubated for 14 days, before the larvae recovery (Assay 1). Assay 2 was carried out with the doses of 0.00625; 0.0125; and 0.025 g. The results showed a negative correlation between fungal concentrations and larval numbers for both assays. The fungus demonstrated an efficacy above 89% in both assays. Thus, we consider that the data from *in vitro* studies based on *in vivo* calculations may optimize the fungi quantities for field experiments.

Keywords: *Duddingtonia flagrans*, predatory action, larvae, sheep, gastrointestinal nematodes.

Resumo

Duddingtonia flagrans tem sido testado como uma alternativa no controle de parasitos, entretanto, trabalhos *in vitro* da predação de nematoides por fungos nematófagos correlacionados com cálculos baseados para testes *in vivo* são restritos. O objetivo deste trabalho foi determinar a eficácia *in vitro* de *D. flagrans* contra larvas de nematoides de ovinos tendo como base cálculos *in vivo*. Amostras fecais receberam a adição do fungo em diferentes concentrações: 0.0/controle; 0,05; 0,1; 0,2; 0,4; 0,8; 1,6; 3,2 e 6,4 gramas correspondendo, respectivamente, às seguintes dosagens: 583.000; 1.166.000; 2.332.000; 4.664.000; 9.328.000; 18.656.000; 37.312.000 e 74.624.000 clamidósporos/Kg de peso vivo animal. O material foi incubado por 14 dias, para recuperação das larvas (Ensaio 1). O Ensaio 2 foi realizado com concentrações de 0,00625; 0,0125 e 0,025 g. Foi observada correlação negativa entre a concentração fúngica e o número de larvas, nos dois ensaios. O fungo demonstrou eficácia acima de 89% em ambos os ensaios. A partir destes dados, acreditamos que ensaios *in vitro* baseados em cálculos *in vivo* podem aprimorar as dosagens para a realização de experimentos a campo.

Palavras-chave: *Duddingtonia flagrans*, atividade predatória, larvas, ovinos, nematoides gastrointestinais.

Introduction

Endoparasites have become a serious problem to small ruminant farming (TARIQ, 2015). In Brazil, infective larvae of the main nematodes species are available on pasture practically throughout the year, becoming the source of a continuous infection (MOLENTO et al., 2016). The high economical losses in lambs, due to gastrointestinal parasitism (TAYLOR, 2012) was estimated to be of hundreds of millions of dollars worldwide (ROEBER et al., 2013).

The excessive use of anthelmintics to control parasite infections has brought up a sequence of undesirable consequences, such as, the lack of farmer assistance, ecotoxicity and the selection of resistant parasites to different drug classes (MOLENTO, 2004). However,

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other prophylactic measurements may reduce the frequency of treatments and can be used in combination, diminishing the dependence of these treatments (MOLENTO et al., 2013). Alternative control methods include biological control using nematophagous fungi (FITZ-ARANDA et al., 2015). The fungi are microorganisms that are able to reduce the population of parasites by acting on the free-life stages, being harmless to the host animals and to the environment (BUZATTI et al., 2015; SAUMELL et al., 2016).

D. flagrans is considered the most promising species for biological control of animal endoparasites (SAHOO & KHAN, 2016). From the beginning of the 90's until today, researchers have reported its effectiveness in the control of immature stages of parasites of cattle (LARSEN et al., 1995; SILVA et al., 2013), sheep, and goats (LARSEN et al., 1998; WAGHORN et al., 2003; OJEDA-ROBERTOS et al., 2008; OJEDA-ROBERTOS et al., 2015; FITZ-ARANDA et al., 2015). Although this fungus is one of the most studied organism for parasite control, data from *in vitro* experiments based *in vivo* calculations, describing their nematode predation, are restricted. As this information constitutes the basis for *in vivo* assays, the *in vivo* results cannot be related to *in vitro* data. The objective of this study was to determine the *in vitro* efficacy of the fungus *D. flagrans*, using *in vivo* calculation, at different concentrations against gastrointestinal nematodes of naturally infected sheep.

Material and Methods

Two experiments were carried out using 20 naturally infected female sheep, of mixed breed, of one to two-years old as feces donor. During the trial, the animals were allocated in native pasture and supplemented with corn silage *ad libitum*. No antiparasitic treatments were given during the experimental period. However, animals were clinically monitored by the FAMACHA (FMC) method and for body condition score (BCS) (BATH & VAN WYK, 2009).

D. flagrans (strain CG 768) was cultured on ground corn as a medium for growth at temperature between 23 and 27 °C in the absence of light for 21 days. Subsequently, the material was conditioned for drying in a stove at 25 °C. After drying, corn and fungus cultivation was homogenized and 10 gram aliquots were removed for quantification of chlamydospores in a Newbauer chamber. The corn employed to the treatments presented 350.000 chlamydospores per gram of corn.

For Assay 1, fecal egg count (FEC) was determined according to Gordon & Whitlock (1939). The fecal material was thoroughly mixed and randomly distributed into 10 cm diameter Petri dishes over filter paper discs. Each experimental replica received 10g of feces. The fungal treatment (mix of ground corn and chlamydospores of *D. flagrans*) was added to the feces in nine different concentrations: 0.0/control; 0.05; 0.1; 0.2; 0.4; 0.8; 1.6; 3.2 and 6.4g corresponding, respectively to 583.000; 1.166.000; 2.332.000; 4.664.000; 9.328.000; 18.656.000; 37.312.000 and 74.624.000 chlamydospores/kilogram body weight (Assay 1). The doses were obtained from the following calculation: the average of the animals' live weight from each group, were multiplied by the fungal treatment (g) correspondent to each evaluated dose and divided by the average amount of feces eliminated daily (1.2 kg) by the animals. All the concentrations were used in triplicates. The Petri dishes were incubated at 28 °C and 80% of relative air humidity for 14 days. Afterwards, the Baermann technique was performed (CORT et al., 1922) for recovering and identification of the genus of the third stage larvae (UENO & GONÇALVES, 1988). The same procedure was adopted for Assay 2, although, three values below of the lowest concentration were also added (0.00625; 0.0125 and 0.025g) corresponding to 2.187; 4.375 and 8.750 chlamydospores/kg live weight, respectively. The larval count was done in a 100 µl solution under microscope.

The comparison between treatments was tested by the Tukey test at 95% of probability ($P<0.05$) after One-Way ANOVA. The data was analyzed using the statistical program SPSS 17 and Assistat 7.5.

Results and Discussion

The mean FEC for Assay 1 was 11.658, showing an intense infection; and the FMC revealed a mean of 3.4, reflecting clinical anemia (MOLENTO et al., 2004). The percentage of the genus before Assay 1, were 46.9; 43.9; 3.3; 3.3; and 1.6% for *Trichostrongylus* sp., *Haemonchus* sp., *Cooperia* sp., *Bunostomum* sp. and *Chabertia* sp., respectively. However, after the addition of the fungus, the percentages changed to: 76.4; 19; 2.2; 1.5 and 0.9% for *Trichostrongylus* sp, *Haemonchus* sp., *Cooperia* sp., *Chabertia* sp. and *Bunostomum* sp., respectively. The results are different from Araújo et al. (2004) who demonstrated that *D. flagrans* was not selective for a particular genus. In the present work, we found a significant difference in the predatory activity related to *Haemonchus* sp. ($P<0.05$). However, more studies are required to confirm this statement.

It was determined a high mean larval count (3.120) on the control group compared to the treated groups on Assay 1. The reduction on larval count can be observed in Table 1, as all the doses had a significant reduction ($P<0.01$) in larval count when compared to the Control group. The results are very important, not just for obtaining a practical biological control, but also to avoid unnecessary administration of the product.

Data from Assay 2 had a correlation of -0.855 ($P<0.001$), determining that the higher the fungal concentration used the lower the amount larvae were recovered from the fecal cultures (data not shown). Some other studies reported that *in vitro* data were used to test the predatory action of *D. flagrans* sp. on infective larvae of *H. contortus* of sheep, finding a significant reduction of larvae, comparing treated and control groups (FITZ-ARANDA et al., 2015; OJEDA-ROBERTOS et al., 2015).

On Assay 2, the mean FEC was 9.560, demonstrating high egg elimination, and 3.6 average when performing FMC. The percentages of the larvae found in the fecal culture before treatment was: 73.9% of *Trichostrongylus* sp., 22.7% of *Haemonchus* sp., 3.0% of *Bunostomum* sp., 0.3% of *Chabertia* sp. ($P<0.05$), and 0.1% of *Cooperia sp*. However, after the addition of the fungus preparation, the percentages had a significant change to: 37.7% of *Trichostrongylus* sp. ($P<0.05$), 50.8% of *Haemonchus* sp. ($P<0.05$), 7.2% of

Table 1. Mean and standard deviation (SD) and reduction (%) of larvae of sheep nematodes for Assay 1 and 2, using different doses of *Duddingtonia flagrans* (chlamydospores/kilogram/live weight – Chla/kg/LW).

Chla/kg/LW	Larvae (SD)	Reduction (%)	Larvae (SD)	Reduction (%)
	Assay 1		Assay 2	
0.0	3,120.0 a (2650.3)	---	2,426.7 a (523.6)	---
2,187 (0.00625 g)	---	---	50.0 b (23.7)	97.94
4,375 (0.0125 g)	---	---	130.0 b (77.5)	94.73
8,750 (0.025 g)	---	---	253.3 b (299.6)	89.73
583,000 (0.05 g)	26.7 b (15.5)	99.07	30.0 b (15.5)	98.79
1,166,000 (0.1 g)	23.3 b (13.7)	99.17	26.7 b (10.3)	98.92
2,332,000 (0.2 g)	26.7 b (10.3)	99.07	26.7 b (5.2)	98.92
4,664,000 (0.4 g)	23.3 b (18.6)	99.18	30.0 b (23.7)	98.79
9,328,000 (0.8 g)	3.3 b (5.2)	99.98	6.7 b (10.3)	99.73
18,656,000 (1.6 g)	6.7 b (5.2)	99.77	0.0 b (0.0)	100
37,312,000 (3.2 g)	0.0 b (0.0)	100	3.3 b (5.2)	99.87
74,624,000 (6.4 g)	3.3 b (5.8)	100	0.0 b (0.0)	100

Different letters in the same column are significantly different by Tukey test at 5% after One-Way ANOVA.

Bunostomum sp. and 1.8% for *Cooperia* sp. and *Chabertia* sp. Therefore, this data corroborates with Assay 1, showing predatory difference of *D. flagrans* for different nematode genera.

The data from Assay 2 corroborates with the first one, where the concentrations of chlamydospores were able to significantly reduce the numbers of larvae, compared to the control group ($P<0.05$). Demonstrating that, not only the first dosage of 583.000 chlamydospores was efficient in the larval predatory activity, but also the three lower dosages used, equally showing values of great amplitude in comparison to the control group (Table 1). This effect reaffirms the premise that the higher the fungal dosage used, the greater the predatory activity of *D. flagrans*. It can also be assumed that the difference found in the number of larvae from the smallest dose, was significantly different from the Control group.

Although higher, different fungal doses were used by other authors with excellent percentages of reductions. In Malaysia, Chandrawathani et al. (2002) tested *D. flagrans* at dosage of 10.000.000 chlamydospores/animal/day and observed a reduction of up to 90% in the number of infected *H. contortus* larvae in fecal cultures. It must be considered that *in vitro* assays, such as the present study, require very low dosages of the fungus. This is the case, as the fungus does not have to pass over the gut barrier, as the *in vivo* condition. It is also evident from our data that when testing *D. flagrans* in animals, the fungal concentrations must be superior to the doses used *in vitro*. This is mainly due to adversities found by chlamydospores from ingestion to elimination by the animals - ruminal pH, intestinal peristalsis, and competition with the natural microbiota. Sahoo & Khan (2016) emphasized that results drawn from *in vitro* studies were encouraging, and suggested the use of fungi to control nematode larvae in the environment.

Table 1 shows the comparison of the percentage of reduction for both assays, and we observe that above 0.8g, which corresponds to 4.664.000 chlamydospores, there was a statistically significant decrease ($P<0.001$) of the number of larvae when compared to the previous concentrations in both assays, similarly to the work of Gives et al. (1998). These authors observed 88% of reduction in the number of larvae of *H. contortus* in fecal cultures of sheep, after the oral administration of 11.350.000 chlamydospores of *D. flagrans*.

We suggest that the dose of 0.05g of fungal substrate (583.000 chlamydospores) be the initial dose to be administered to sheep, even without killing all available larvae.

Although there has been proof of the decrease of the number of larvae by increasing the number of chlamydospores, the dose titration of the fungal substrate administrated to the animals must be considered to allow product optimization. Nevertheless, there is a consensus that the eradication of parasites is not recommended, thus keeping them in a level that does not cause any harm to the animals (MOLENTO, 2009; PARK et al., 2015).

Conclusions

The objective to correlate the *in vitro* assays with oral doses, based on live weight of the animal has proved to be a useful methodology to be employed before *in vivo* experiments. All evaluated dosages have demonstrated to be efficient (above 99% on Assay 1 and 89% on Assay 2) and the dosage of 0.05g (lowest on Assay 1) is recommended, as it obtained high efficacy in both assays.

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References

Araújo JV, Assis RCL, Campos AK, Mota MM. Atividade *in vitro* dos fungos nematófagos dos gêneros *Arthrobotrys, Duddingtonia* e *Monacrosporium* sobre nematóides trichostrongilídeos (Nematoda: Trichostrongyloidea) parasitos gastrintestinais de bovinos. *Rev Bras Parasitol Vet* 2004; 13(2): 65-71.

Bath GF, Van Wyk JA. The Five Point Check for targeted selective treatment of internal parasites in small ruminants. *Small Rumin Res* 2009; 86(1-3): 6-13. http://dx.doi.org/10.1016/j.smallrumres.2009.09.009.

Buzatti A, Santos CP, Fernandes MAM, Yoshitani UY, Sprenger LK, Santos CD, et al. *Duddingtonia flagrans* in the control of gastrointestinal nematodes of horses. *Exp Parasitol* 2015; 159: 1-4. PMid:26208781. http://dx.doi.org/10.1016/j.exppara.2015.07.006.

Chandrawathani P, Jamnah O, Waller PJ, Höglund J, Larsen M, Zahari WM. Nematophagous fungi as a biological control agent for nematode parasites of small ruminants in Malaysia: a special emphasis on *Duddingtonia flagrans. Vet Res* 2002; 33(6): 685-696. PMid:12498569. http://dx.doi.org/10.1051/vetres:2002049.

Cort WW, Ackert JE, Augustine DL, Payne FK. Investigations on the Control of Hookworm Disease. II. The Description of an Apparatus for Isolating Infective Hookworm Larvae from Soil. *Am J Hyg* 1922; 2(1): 1-16.

Fitz-Aranda J, Mendoza PM, Torres-Acosta J, Liébano-Hernández E, López-Arellano M, Sandoval-Castro C, et al. *Duddingtonia flagrans* chlamydospores in nutritional pellets: effect of storage time and conditions on the trapping ability against *Haemonchus contortus* larvae. *J Helminthol* 2015; 89(1): 13-18. PMid:23953994. http://dx.doi.org/10.1017/S0022149X13000539.

Gordon HM, Whitlock H. A new technique for counting nematode eggs in sheep faeces. *J Counc Sci Ind Res* 1939; 12(1): 50-52.

Larsen M, Faedo M, Waller PJ, Hennessy DR. The potential of nematophagous fungi to control the free-living stages of nematode parasites of sheep: Studies with *Duddingtonia flagrans. Vet Parasitol* 1998; 76(1-2): 121-128. PMid:9653996. http://dx.doi.org/10.1016/S0304-4017(97)00056-3.

Larsen M, Nansen P, Wolstrup J, Grønvold J, Henriksen SA, Zorn A. Biological control of trichostrongyles in calves by the fungus *Duddingtonia flagrans* fed to animals under natural grazing conditions. *Vet Parasitol* 1995; 60(3-4): 321-330. PMid:8747915. http://dx.doi.org/10.1016/0304-4017(95)00791-6.

Gives PM, Crespo JF, Rodriguez DH, Prats VV, Hernandez EL, Fernandez GO. Biological control of *Haemonchus contortus* infective larvae in ovine faeces by administering an oral suspension of *Duddingtonia flagrans* chlamydospores to sheep. *J Helminthol* 1998; 72(4): 343-347. PMid:9858632. http://dx.doi.org/10.1017/S0022149X00016710.

Molento MB, Buzatti A, Sprenger LK. Pasture larval count as a supporting method for parasite epidemiology, population dynamic and control in ruminants. *Livest Sci* 2016; 192: 48-54. http://dx.doi.org/10.1016/j.livsci.2016.08.013.

Molento MB, Tasca C, Gallo A, Ferreira M, Bononi R, Stecca E. Método Famacha como parâmetro clínico individual de infecção por *Haemonchus contortus* em pequenos ruminantes. *Cienc Rural* 2004; 34(4): 1139-1145. http://dx.doi.org/10.1590/S0103-84782004000400027.

Molento MB, Verissimo CJ, Amarante AT, Van Wyk JA, Chagas ACS, Araujo JV, et al. Alternative techniques for the control of gastrointestinal nematodes in small ruminants. *Arq Inst Biol* 2013; 80(2): 253-263. http://dx.doi.org/10.1590/S1808-16572013000200018.

Molento MB. Multidrug resistance in *Haemonchus contortus* associated with suppressive treatment and rapid drug alternation. *Rev Bras Parasitol Vet* 2004; 13(S1): 272.

Molento MB. Parasite control in the age of drug resistance and changing agricultural practices. *Vet Parasitol* 2009; 163(3): 229-234. PMid:19560869. http://dx.doi.org/10.1016/j.vetpar.2009.06.007.

Ojeda-Robertos NF, Torres-Acosta JF, Aguilar-Caballero AJ, Ayala-Burgos A, Cob-Galera LA, Sandoval-Castro CA, et al. Assessing the efficacy of *Duddingtonia flagrans* chlamydospores per gram of faeces to control *Haemonchus contortus* larvae. *Vet Parasitol* 2008; 158(4): 329-335. PMid:19028012. http://dx.doi.org/10.1016/j.vetpar.2008.08.022.

Ojeda-Robertos NF, Torres-Acosta JF, Mendoza-de-Gives P, Gonzalez-Garduño R, Valero-Coss RO, Liébano-Hernández E, et al. Optimizing the use of *Duddingtonia flagrans* chlamydospores against *Haemonchus contortus* in feces of sheep. *Trop Subtrop Agroecosyst* 2015; 18: 259-265.

Park AW, Haven J, Kaplan R, Gandon S. Refugia and the evolutionary epidemiology of drug resistance. *Biol Lett* 2015; 11(11): 20150783. PMid:26601680. http://dx.doi.org/10.1098/rsbl.2015.0783.

Roeber F, Jex AR, Gasser RB. Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance - an Australian perspective. *Parasit Vectors* 2013; 6(1): 153. PMid:23711194. http://dx.doi.org/10.1186/1756-3305-6-153.

Sahoo A, Khan F. Nutritional and biological control synergism against gastrointestinal nematodes in small ruminants. *J Vet Sci Anim Husb* 2016; 4(1): 104.

Saumell CA, Fernández AS, Echevarria F, Gonçalves I, Iglesias L, Sagües MF, et al. Lack of negative effects of the biological control agent *Duddingtonia flagrans* on soil nematodes and other nematophagous fungi. *J Helminthol* 2016; 90(6): 706-711. PMid:26620321. http://dx.doi.org/10.1017/S0022149X1500098X.

Silva ME, Araújo JV, Braga FR, Soares FE, Rodrigues DS. Control of infective larvae of gastrointestinal nematodes in heifers using different isolates of nematophagous fungi. *Rev Bras Parasitol Vet* 2013; 22(1): 78-83. PMid:23538499. http://dx.doi.org/10.1590/S1984-29612013005000012.

Tariq KA. A review of the epidemiology and control of gastrointestinal nematode infections of small ruminants. *Proc Natl Acad Sci, India, Sect B Biol Sci* 2015; 85(2): 693-703. http://dx.doi.org/10.1007/s40011-014-0385-9.

Taylor MA. SCOPS and COWS - 'worming it out of UK farmers'. *Vet Parasitol* 2012; 186(1-2): 65-69. PMid:22222010. http://dx.doi.org/10.1016/j.vetpar.2011.11.047.

Ueno H, Gonçalves PC. *Manual para diagnóstico das helmintoses de ruminantes.* Tokyo: Japan International Cooperation Agency; 1988.

Waghorn TS, Leathwick DM, Chen LY, Skipp RA. Efficacy of the nematode-trapping fungus *Duddingtonia flagrans* against three species of gastro-intestinal nematodes in laboratory faecal cultures from sheep and goats. *Vet Parasitol* 2003; 118(3-4): 227-234. PMid:14729170. http://dx.doi.org/10.1016/j.vetpar.2003.09.018.