Ciências Agrárias
SEMINA

DOI: 10.5433/1679-0359.2023v44n4p1587

Evaluation of the fungus *Duddingtonia flagrans* (Bioverm®) on *Ascaris suum* eggs and infective larvae of *Oesophagostomum* spp. and *Hyostrongylus rubidus* from swine

Avaliação do fungo *Duddingtonia flagrans* (Bioverm®) sobre ovos de *Ascaris suum* e larvas infectantes de *Oesophagostomum* spp. e *Hyostrongylus rubidus* de suínos

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Highlights

First report of the effect of Bioverm® on swine parasites.
No lytical effects types 2 and 3 observed on *A. suum* eggs.
High effect on infective larvae of *Oesophagostomum* spp. and *H. rubidus.*
Predation peak after 24 h of product administration.

Abstract

The aim of this study was to evaluate the action of a commercial formulation based on *Duddingtonia flagrans* (Bioverm®) on *Ascaris suum* eggs and infective larvae (L3) of *Oesophagostomum* spp. and *Hyostrongylus rubidus* from swine. Twelve male pigs were divided into two groups: treated, which received a single dose of 1 g/10 kg of body weight (105 chlamydospores of *D. flagrans*); and control, which remained untreated. Fecal samples (100g) were collected individually at 0, 12, 24, 36, 48, 60 and 72 hours after treatments. In the assay A, 2 g of feces and 1000 eggs of *A. suum* were added to petri dishes, and larval predation was assessed to classify the effects of predation. In the assay B, 2000 L3

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Received: Apr. 11, 2023 Approved: Sept. 15, 2023

of *Oesophagostomum* spp. and *H. rubidus* were added to the coprocultures, incubated for 10 days and recovered for quantification of non-predated L3. In the assay A, there was only a type 1 effect, with fungal growth in eggs, but no embryo damage. In the assay B, a reduction of L3 of *Oesophagostomum* spp. and *H. rubidus* of 73.9% ($p<0.01$), with predation peak 24 h after product administration. It was concluded that *D. flagrans* (Bioverm®) only had predatory efficacy on L3 of swine gastrointestinal strongyles.
Key words: Ascarides. Biological control. Gastrointestinal strongyles. Nematophagous fungi. Pigs.

Resumo

O objetivo deste estudo foi avaliar a ação de uma formulação comercial à base de *Duddingtonia flagrans* (Bioverm®) sobre os ovos de *Ascaris suum* e larvas infectantes (L3) de *Oesophagostomum* spp. e *Hyostrongylus rubidus* de suínos. Doze suínos machos foram divididos em dois grupos: tratado, que recebeu dose única de 1 g/10 kg de peso vivo (105 clamidósporos de *D. flagrans*); e controle, que permaneceram sem tratamento. Amostras fecais (100g) foram coletadas individualmente às 0, 12, 24, 36, 48, 60 e 72 horas após os tratamentos. No ensaio A, 2 g de fezes e 1000 ovos de *A. suum* foram adicionados a placas de Petri, e a predação larval foi avaliada para classificar os efeitos da predação. No ensaio B, 2000 L3 de *Oesophagostomum* spp. e *H. rubidus* foram adicionados às coproculturas, incubadas por 10 dias e recuperadas para quantificação das L3 não predadas. No ensaio A, houve apenas efeito tipo 1, com crescimento fúngico em ovos, mas sem ação lítica. No ensaio B, observou-se uma redução de L3 de *Oesophagostomum* spp. e *H. rubidus* de 73,9% ($p<0,01$), com pico de predação 24 h após a administração do produto. Concluiu-se que *D. flagrans* (Bioverm®) apenas teve eficácia predatória sobre L3 dos estrongilídeos gastrintestinais de suínos.
Palavras-chave: Ascarídeos. Controle biológico. Estrongilídeos gastrintestinais. Fungos nematófagos. Porcos.

Introduction

Gastrointestinal helminthiasis in swine has multiple etiological causes. Among the species of intestinal parasites commonly observed, *Ascaris suum* and gastrointestinal strongyles (*Oesophagostomum* spp. and *Hyostrongylus rubidus*) stand out, being responsible for causing economic losses in pig farming (Li et al., 2022). Aiming at controlling these helminthiasis, alternative measures have been studied, with the focus on reducing the use of anthelmintics and, thus, making livestock activity more sustainable (Araújo et al., 2021).

One of the alternatives to the use of conventional anthelmintics is the use of nematophagous fungi. Among the fungal species, *Pochonia chlamydosporia* stands out, acting in the destruction of parasites eggs, while *Duddingtonia flagrans* is considered the most effective with regard to predation of larvae (Araújo et al., 2021). Although *D. flagrans* is a species of larvicidal fungus, in vitro studies have been carried out in order to investigate its possible action on eggs of different parasites such as *Ascaris lumbricoides, Eurytrema coelomaticum* and *Toxocara canis* (Braga et al., 2008; Motta et al., 2022).

An integrated approach that combines biological control and chemical control allows for maximizing the benefits of both methods (Mendes et al., 2022). While chemical control can be used to treat acute and severe infections, biological control can be maintained as a long-term preventive measure. This can help reduce the dependency on chemical products and minimize the risks of residues in animal products (Vilela et al., 2018). A commercial product based on *D. flagrans* (Bioverm®) has been indicated for the control of gastrointestinal helminthiasis in ruminants, horses and swine. Experiments already carried out in cattle, horses and sheep have shown a reduction in the parasitic load in the environment and, consequently, in animals (Fausto et al., 2021; Oliveira et al., 2021; J. A. Rodrigues et al., 2022). However, there are no studies evaluating the action of this product (Bioverm®) on gastrointestinal nematodes in pigs; and its action on the eggs of parasites whose infection occurs by ingestion of their infective eggs, such as *A. suum,* has not yet been reported.

Faced with the need to study and describe alternative forms of control of gastrointestinal helminthiasis in swine, the objective of this work was to evaluate the in vitro action of a commercial product containing chlamydospores of *D. flagrans* on eggs of *A. suum* and on infective larvae of *Oesophagostomum* spp. and *H. rubidus* after passing through the gastrointestinal tract of pigs.

The research was approved by the Ethics Committee on the Use of Animals of the Instituto Federal de Educação, Ciência e Tecnologia da Paraíba (CEUA/IFPB), in accordance with current rules and regulations, under registration number 23000.000665.2020-87.

The experimental tests were carried out in the pig sector of the Laboratory of Veterinary Parasitology/ IFPB, in Sousa, Paraíba, Brazil. An antiparasitic product for veterinary use (Bioverm®; GhenVet Saúde Animal, Paulínia, SP, Brazil) was used, containing a *D. flagrans* chlamydospore concentration of 105/g. This product is sold in the form of fine-grained powder, packed in hermetically sealed polypropylene colorless bags.

Eggs of *A. suum* and gastrointestinal strongyles were collected through fecal recovery. Feces were collected from pigs that had been found to be positive for gastrointestinal parasites, through the technique for counting eggs per gram of feces (EPG) (Gordon & Whitlock, 1939), in the pig sector at IFPB. Only in positive samples for *A. suum,* approximately 15g of feces were washed ten times in distilled water and centrifuged at 1,000 rpm for 5 min each time. The supernatant was discarded at the end of each centrifugation cycle. The volume of the sediment containing the eggs was measured, and subsequently, five aliquots of 50 µl each were placed between a slide and a cover slip. They were then examined under an optical microscope using a 10x objective (100x magnification) to quantify the recovered eggs and placed in distilled water to form a suspension containing approximately 150,000 eggs. Only positive samples for gastrointestinal strongyles (*Oesophagostomum* spp. and *H. rubidus*) were used to perform coprocultures (Roberts & O' Sullivan, 1950) in order to obtain infective

third-stage larvae. The concentrated of larvae were recovered and placed in distilled water to form a suspension containing approximately 300,000 larvae (55% *H. rubidus* and 45% *Oesophagostomum* spp.).

To better infer the specific action on parasite eggs or larvae, samples positive for more than one parasite (for example: *A. suum* + *Oesophagostomum* spp. and *H. rubidus*) were discarded. The parasite eggs and larvae were identified based on structural and morphometric criteria (Taylor et al., 2017).

Twelve crossbred male pigs, aged four months, and weighing a mean of 70± 3kg, were kept in stalls in the pig farming sector of the IFPB campus in Sousa, Paraíba, and were fed specific commercial feed for pigs, with water available *ad libitum.* These animals had previously been treated with the anthelmintic levamisole hydrochloride 7.5% (Ripercol*L; Zoetis Indústria de Produtos Veterinários Ltda., Campinas, São Paulo, Brazil), at a dose of 1 mL/20 kg, subcutaneously. Ten days after this treatment, three EPG counts were performed. After confirmation that 0 EPG had been achieved, the animals were randomly divided into two groups of six pigs each (treated and control).

In the treated group, each animal individually received a single dose of 1 g per 10 kg of live weight (10^5 chlamydospores of *D. flagrans*) of the product Bioverm®, together with commercial feed. In the control group, each animal individually received, as placebo, 1 g of commercial feed per 10 kg of live weight. Subsequently, fecal samples weighing approximately 100 g were obtained from each animal in each group, at 0, 12, 24, 36, 48, 60 and 72 hours after product administration (J. A. Rodrigues et al., 2021).

In the assay A, approximately 1000 *A. suum* eggs were placed on 2% water-agar medium (2% WA) in Petri dishes of diameter 9.0 cm containing 2 g of swine feces at each collection time: 0 (before fungus administration) and 12, 24, 36, 48, 60 and 72 hours after product administration. The same procedure was performed in Petri dishes containing only 2% WA (without fungus), which constituted the control group. This assay allows visualization of fungal growth, trap production, and egg predation since the WA 2% is transparent, enabling these observations under an optical microscope (100x and 400x magnification). For each group, at each sampling time, 10 repetitions were performed. Subsequently, on days 7, 14 and 21 after product administration, approximately 100 eggs were removed from each plate. These eggs were then placed on glass slides with a drop of 1% Amman blue and were evaluated under an optical microscope in accordance with the parameters established by Lysek (1976): type 1, lytic effect without morphological damage to the eggshell, with hyphae adhered to the shell; type 2, lytic effect with morphological alteration of the eggshell and embryo, without penetration of hyphae through the eggshell; type 3, lytic effect with morphological alteration of the embryo and eggshell, along with penetration of hyphae and internal colonization.

In the assay B, the feces collected from the animals at each sampling time were homogenized and 15 g of these feces were added to each coproculture, along with expanded vermiculite. This was performed in triplicate for each sample. An aliquot of 300 µl of suspension containing 2000 L3 of the gastrointestinal strongyles was added (J.

A. Rodrigues et al., 2021). This assay allows for a better mimicking of the environmental conditions in which fungi and L3 cohabit in the faeces, stimulating trap production and larval predation. For each group, at each sampling time, 10 repetitions were performed. The fecal cultures were incubated at 26 °C in a BOD incubator for 10 days. After this period, the non-predated larvae were recovered using the Baermann technique. The contents were washed and centrifuged in test tubes three times at 1,500rpm for five minutes each time. After discarding the supernatant, the numbers of larvae were estimated.

The larval predation rate was estimated by comparing the mean numbers of larvae recovered in the treated and control groups:

$$Reduction\ (\%) = \frac{Mean\ L3\ recovered\ CG - Mean\ L3\ recovered\ TG}{Mean\ L3\ recovered\ CG} X\ 100$$

CG - control group; TG - treated group

The data were subjected to the Shapiro-Wilk normality test. From this, the samples were found to show normal distribution. The data were then subjected to the t-test for independent samples, at the 5% probability level. The results were analyzed in the GraphPad Prism 9.0 software.

In the assay A, between 24 and 48 hours after administration of the product, only a type 1 effect was observed on *A. suum* eggs, without damage to the eggshells or embryos. These results were observed after 7, 14 and 21 days of interaction. No type 2 or 3 effects that could alter egg viability were observed. The absence of any lytic effect types 2 and 3 on *A. suum* eggs was similar to that described by Araújo et al. (2008), evaluating the action of nematophagous fungi, including *D. flagrans,* on *A. suum* eggs. According to Lysek (1976), only fungi with a type 3 effect on nematode eggs should be considered ovicidal.Among the fungi examined in that study, only *P. chlamydosporia* (isolates VC 1 and VC 4) was classified as ovicidal.

In addition, after 21 days, the presence of larvae in the eggs was observed, which gave the eggs the infective status, that is, capable of infecting the animals (Figure 1). In Petri dishes, intense spontaneous production of traps was observed, consisting mainly of adhesive hyphae and conidia. These structures were attached around the eggs and were observed mainly at 24, 36 and 48 hours. On the other hand, Barron (1977) reported that predatory fungal hyphae may grow in nematode eggs, but in these cases the eggs are dead or non-viable before the attack and the relationship is a saprophytic rather than a parasite. Thus, as reported by Lysek and Sterba (1991), only a small number of fungi are specialized for parasitism in eggs, with emphasis on the ovicidal properties of *P. chlamydosporia.*

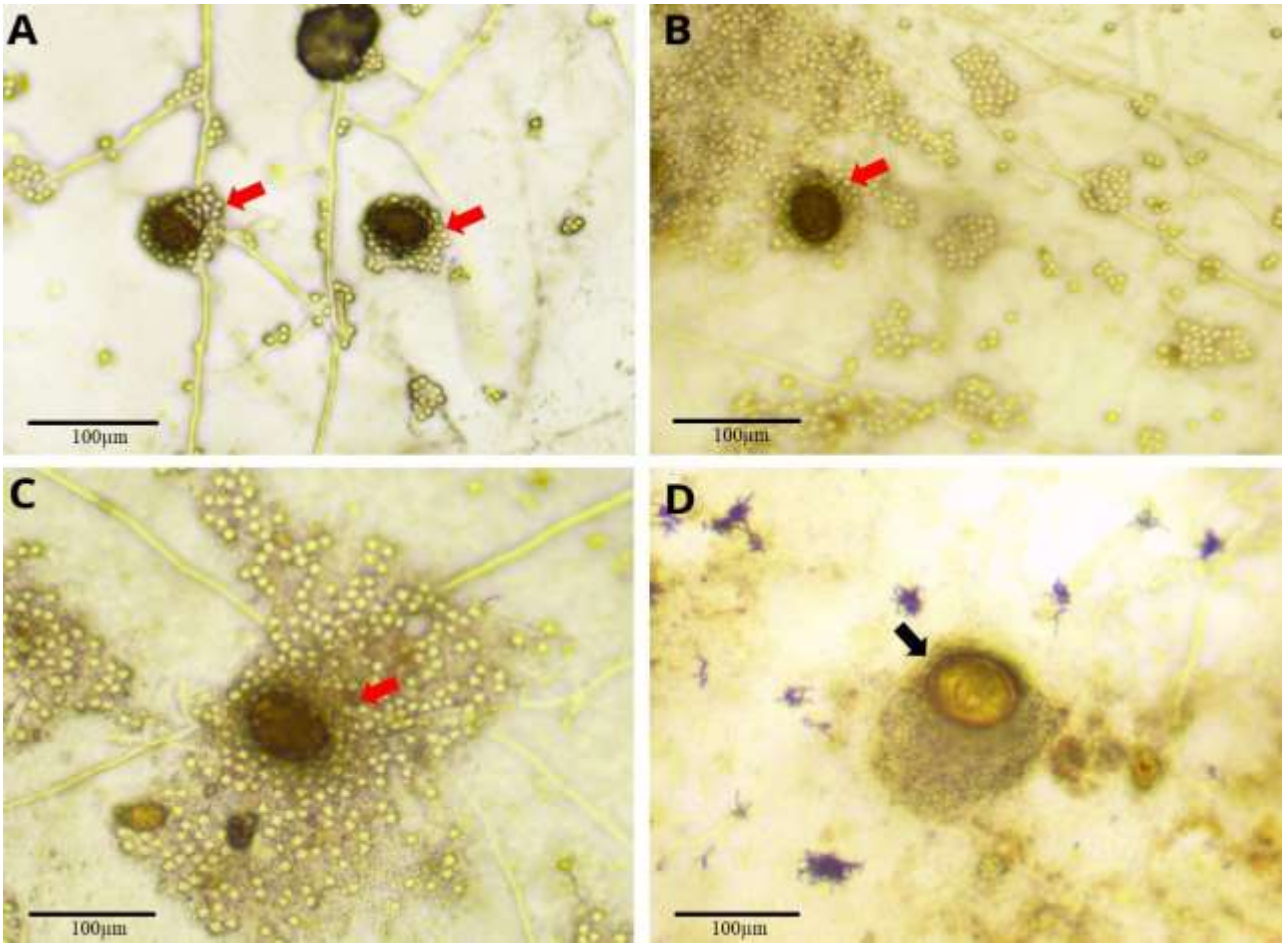
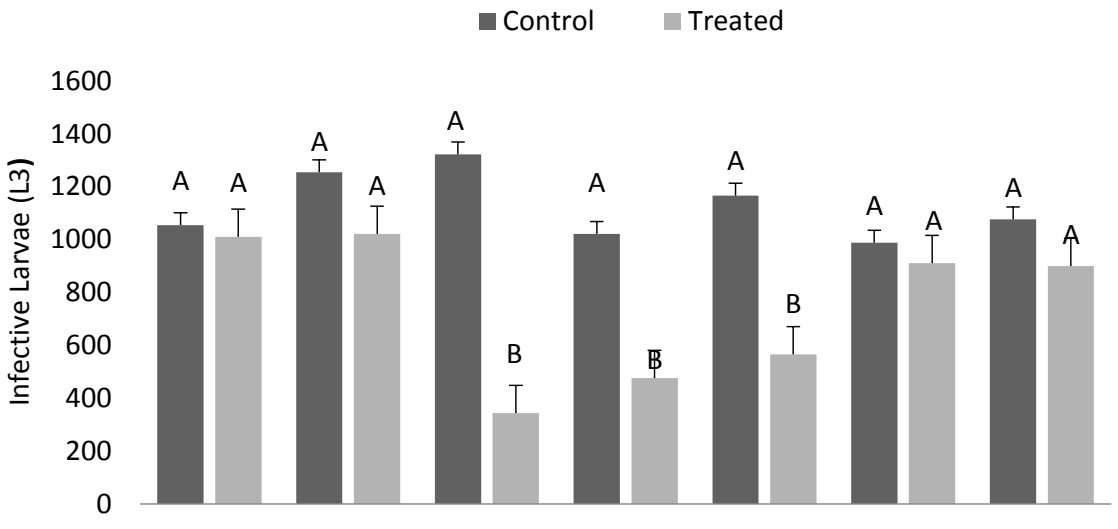


Figure 1. Performance of *Duddingtonia flagrans* in commercial product (Bioverm®), with trap production and interaction with eggs of *Ascaris suum* of pigs. A, B and C: *A. suum* eggs surrounded by *D. flagrans* conidia and hyphae, showing type 1 effect, in treated group (red arrows); D: Absence of fungal growth in *A. suum* eggs, in control group (black arrow). Optical microscopy (100x).

In the assay B, a reduction in the number of L3 of *Oesophagostomum* spp. and *H. rubidus* in the treated group, compared to the control group ($p<0.01$), at 24, 36 and 48 hours. The peak of action in larvae was observed 24 hours after product administration, with a 73.9% L3 reduction (Figure 2). J. V. F. Rodrigues et al. (2018) used *D. flagrans* formulated in rice bran to control *Oesophagostomum* spp. in pigs. After passing through the gastrointestinal tract, a reduction of L3 was observed throughout the experimental period, with greater efficacy observed in 24 hours, of 63%, as well. In both cases, the animals used were fed with commercial feed for pigs. It is important to emphasize that the time of passage through the gastrointestinal tract of swine, and the efficiency of digestion, is influenced by the degree of grinding of the food and the

composition of the nutrition provided (J. V. F. Rodrigues et al., 2018). In other species, the peak of action of Bioverm® varies according to the time spent in gastrointestinal transit. In cattle, J. A. Rodrigues et al. (2021) observed an efficacy of over 80% in larval predation after 48 hours. In buffaloes, however, Mendes et al. (2023) observed an efficacy of 92% after 60 hours).



	0 h	12 h	24 h	36 h	48 h	60 h	72 h
Control	1055	1255	1322	1022	1166	988	1077
Treated	1011	1022	344	477	566	911	900
Reduction	4.1%	18.5%	73.9%	41.2%	51.4%	7.8%	16.4%

Figure 2. Evaluation of the effect of a commercial product based on *Duddingtonia flagrans* on infective larvae of swine gastrointestinal strongyles, for up to 72 hours after oral administration, after 10 days of interaction in coprocultures. A and B indicate a statistical difference according to Tukey's test at 1% probability.

Additionally, the product (Bioverm®) demonstrated a larvicidal effect on parasites from other animal species, thus proving its viability after passing through the gastrointestinal tract. In sheep, an action of 91.5% on L3 of *Haemonchus contortus* and *Strongyloides papillosus* was observed (Braga et al., 2020). The formation of three-dimensional adhesive hyphae ensures the nematophagous activity of the fungus, by promoting a mechanical/enzymatic process of adherence, immobilization, penetration and destruction of the nematode (Araújo et al., 2021). The utilization of Bioverm® for biological control offers a viable strategy to diminish larval contamination rates, thereby

leading to reduced reinfection in animals. This approach fosters the development of innate immunity against helminths. Moreover, animals treated exhibit elevated rates of weight gain, increased levels of globular volume, and lowered EPG values in comparison to their non-treated counterparts (Fausto et al., 2021; Oliveira et al., 2021; J. A. Rodrigues et al., 2022).

It was concluded that the commercial product containing *D. flagrans* survives after passing through the gastrointestinal tract of swine and showed high larvicidal activity against *Oesophagostomum* spp. and H. rubidus. On the other hand, no ovicidal action was observed against *A. suum,* which is one of the most prevalent and pathogenic swine parasites. Thus, studies evaluating formulations composed of associations between *D. flagrans* and other fungi with ovicidal action, such as *P. chlamydosporia,* could be carried out in order to obtain an alternative for effective biological control for mixed gastrointestinal infections caused by parasites in swine.

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