Control of infective larvae of gastrointestinal nematodes in heifers using different isolates of nematophagous fungi

Controle de larvas infectantes de nematóides gastrintestinais de novilhas por diferentes isolados dos fungos nematófagos

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Abstract

The effect of different nematophagous fungi [*Duddingtonia flagrans* (AC001 and CG722) and *Monacrosporium thaumasium* (NF34)] with regard to controlling infective larvae (L_3) of nematodes after gastrointestinal transit in female cattle (3/4 Holstein × Zebu) was evaluated. A total of 24 pubescent female cattle were used, weighing approximately 320 kg each one. There were three treatment groups, each contained six animals that received 150 g of pellets (0.2 g of mycelium), orally in a single dose, in a sodium alginate matrix containing mycelial mass of the fungus *D. flagrans* (AC001 or CG722) or *M. thaumasium* (NF34); and one control group (without fungi). Fecal samples were collected from the animals at intervals of 12, 15, 18, 21, 24, 48, and 72 hours. At the end of 17 days, the L_3 not subjected to predation were recovered by means of the Baermann method. The fungal isolates tested were capable of destroying the L_3 after gastrointestinal transit. It was observed that within 72 hours, the isolates AC001, CG722, and NF34 showed a higher predatory activity (81.2%, 97.3%, and 98.3%, respectively). The results justify the need for studies in the field, and over longer intervals, in order to observe the efficiency of the fungus *D. flagrans*, or even *M. thaumasium*, for environmental control over nematodes in naturally infected cattle.

Keywords: Nematophagous fungi, *Duddingtonia flagrans*, *Monacrosporium thaumasium*, nematodes, cattle.

Resumo

No presente estudo, foi avaliado o efeito de diferentes fungos nematófagos [*Duddingtonia flagrans* (AC001 e CG722) e *Monacrosporium thaumasium* (NF34)] no controle de larvas infectantes (L_3) de nematóides após o trânsito gastrointestinal em fêmeas bovinas (3/4 Holandês x Zebu). Um total de 24 fêmeas bovinas pubescentes foram utilizadas, pesando aproximadamente 320 kg cada. Foram utilizados três grupos de tratamento; cada um contendo seis animais que receberam por via oral de 150 g de péletes (0,2g de micélio), em dose única, em uma matriz de alginato de sódio contendo massa micelial dos fungos *D. flagrans* (AC001 ou CG722), *M. thaumasium* (NF34), além de um grupo controle (sem fungo). Amostras de fezes foram colhidas dos animais em intervalos de 12, 15, 18, 21, 24, 48 e 72 horas. No final de 17 dias, as L_3 não predadas foram recuperadas pelo método de Baermann. Os isolados de fungos testados foram capazes de destruir as L_3 após trânsito gastrointestinal. Observou-se após 72 horas, os isolados AC001, CG722 e NF34 mostraram uma maior atividade predatória (81,2%, 97,3% e 98,3%, respectivamente). Estes resultados justificam a necessidade de estudos a campo e em intervalos mais longos, a fim de observar a eficácia dos fungos *D. flagrans* ou mesmo *M. thaumasium* no controle ambiental dos nematóides de bovinos naturalmente infectados.

Palavras-chave: Fungos nematófagos, *Duddingtonia flagrans*, *Monacrosporium thaumasium*, nematóides, bovinos.

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Introduction

Cattle, goats, and sheep represent one of the main sources of protein for the human population. However, one of the obstacles to their production worldwide is gastrointestinal helminthosis. This is the disease most often responsible for damage to livestock, with estimated costs around of 68 million dollars a year, since almost all animals produced in the field harbor one or more species of helminth (ANUALPEC, 2003; TORINA et al., 2004; AMARANTE, 2009).

The country with the largest commercial cattle herd in the world is Brazil. However, because cattle production most often takes place wholly or partly on pasture, there is constant infection due to parasites present in the grazing land (ANUALPEC, 2003). Because of this, gastrointestinal nematodes are a serious problem in ruminant production; once the animals have been exposed to high parasite loads they may succumb, especially younger individuals, which are more susceptible (AMARANTE, 2009).

In this context, nematodes, especially the genus *Haemonchus*, are responsible for large economic losses in livestock (URQUHART et al., 1996; AMARANTE, 2011). The conventional method for controlling such gastrointestinal parasites is to use synthetic anthelmintic drugs. However, over recent decades there has been increasing interest in developing new methods for controlling nematode parasites of livestock. The main reason for this has been the increasing development of anthelmintic resistance in several species of parasitic nematodes in ruminants, including anthelmintic resistance among nematode parasites in cattle (FIEL et al., 2001; ARAÚJO et al., 2004a; CONDI et al., 2009; SUTHERLAND; LEATHWICK, 2011).

Thus, use of nematophagous fungi, in particular of the species *Duddingtonia flagrans* and *Monacrosporium thaumasium*, as an alternative control has been constantly tested, with interesting results both in the field and under laboratory conditions (BRAGA et al., 2009, 2011a; SILVA et al., 2009; PAZ-SILVA et al., 2011; TAVELA et al., 2011). In addition, passage of different fungal structures, as well as of different isolates of the same species of fungus, through the gastrointestinal tract of different animal species has been the target of several studies (LARSEN, 1999; GRONVOLD et al., 1993, 1996). In this context, the challenge of these studies has been to investigate whether there are differences in viability and predatory activity of these fungi after suffering 'stress' during passage through the gastrointestinal tract of animals. The best fungal isolates, exhibiting the characteristics of potential biological controls while being marketable through production of chlamydospores (resistant structures) need to be identified.

The objective of the present study was to evaluate the effect of different nematophagous fungi in relation to controlling the infective larvae (L_3) of gastrointestinal nematodes, after gastrointestinal transit in female cattle (3/4 Holstein × Zebu).

Materials and Methods

1. Fungi

Two isolates of the nematophagous fungus *D. flagrans* (AC001 and CG722) and one isolate of the fungus *M. thaumasium*

(NF34) were used. These isolates were obtained from Brazilian agricultural soil, in the municipality of Vicosa, in the *Zona da Mata* region of the state of Minas Gerais. They were collected using the soil-sprinkling method of Duddington (1955).

2. Production of mycelial mass

To produce fungal mycelia of *D. flagrans* (AC001 and CG722) and *M. thaumasium* (NF34), culture discs of approximately 4 mm in diameter, in 2% water agar (2% WA) were transferred to 250 mL Erlenmeyer flasks containing 150 mL of the liquid medium GPY (glucose, sodium peptone and yeast extract). The flasks were continually stirred at 120 rpm, in the dark and at the temperature of 26 °C, for 10 days. After this period, the mycelia were removed, filtered, and weighed on an analytical balance. All the procedures followed the methodology of Braga et al. (2009).

3. Animals

A total of 24 pubescent female cattle (3/4 Holstein × Zebu) of approximate age 20 months were used. Their live weight was approximately 320 kg each one. Throughout the experiment the animals were kept in stalls in a cowshed and were fed with sugarcane plus urea and 16% protein concentrate. These animals were on the Santa Rita Experimental Farm, which is owned by the Agricultural Research Corporation of Minas Gerais (Empresa de Pesquisa Agropecuaria de Minas Gerais, EPAMIG).

4. Experimental assay

For the *in vivo* assay, the animals were kept in stalls and were dewormed using 1% ivermectin (Ivergen®, Biogenesis, Brasil 200 μg/kg body weight) 45 days before receiving the experimental diet. They were then separated into four groups of six animals each: one group was fed with alginate pellets containing the *D. flagrans* isolate AC001; one group received pellets containing *D. flagrans* CG722; and a third group received pellets containing *M. thaumasium* NF34. The fourth group remained served as control group and was fed pellets without fungi.

In the groups treated with AC001, CG722, and NF34, each animal received 150 g of pellets in a single dose containing mycelial mass of the respective fungus (0.2 g of mycelium). For this, each of the fungi was mixed in 500 g of feed for cattle with 16% protein. The animals in the control group received a single administration of 150 g of pellets without fungi plus 500 g of feed.

Later, after administration of the fungi, fecal samples were collected from the animals at intervals of 12, 15, 18, 21, 24, 48, and 72 hours afterwards. About 30 g of feces from each animal in each of the treated and control groups were homogenized to form a 'pool' for each experimental group. Next, from the fecal material collected (the 'pool') at the different times after fungus administration, coprocultures were performed in accordance with the technique described by Roberts and O'Sullivan (1952). At each set time (12, 15, 18, 21, 24, 48, and 72 hours), six repetitions were performed for each coproculture, for each of the treated and control groups, totaling 24 samples per time and a total of 168 coprocultures. These coprocultures were incubated

in a BOD chamber at 26 °C and were protected from light for 17 days. After this period, the L$_3$ that had not been subjected to predation were recovered in hemolysis tubes with the aid of the Baermann apparatus, and were quantified and identified in accordance with the criteria proposed by Keith (1953). For this, we used optical microscope objective lenses of 10× and 40×. The data obtained were subjected to analysis of variance (F test) and subsequent regression analysis. Means were compared using the Tukey test at the 5% level of probability (AYRES et al., 2003).

Results

In the present study, it was observed that the fungi *D. flagrans* (AC001 or CG722) and *M. thaumasium* (NF34) destroyed the L$_3$ of gastrointestinal nematodes after transit through the gastrointestinal tract of cattle. In the feces of the treated groups, conidia and chlamydospores of the fungal species tested (*D. flagrans* and *M. thaumasium*) were identified, and L$_3$ that had been subjected to predation could be viewed (Figures 1a-d). For each time studied, the fungi showed a percentage reduction in the number of L$_3$

recovered from the coprocultures using the Baermann method after 17 days that ranged from 87% to 81.2% for the AC001 isolate, from 88.4% to 97.3% for NF34 and from 98.2% to 98.3% for CG722 (Figure 2). Comparisons with the control group are shown in Figure 3.

On the other hand, over the sampling time, there was no difference ($p > 0.05$) in the action of the fungal isolates tested, and therefore in the recovery of L$_3$ at the end of 17 days. However, the CG722 isolate showed better performance than shown by AC001 and NF34. In relation to identification of the L$_3$ obtained from coprocultures, it was observed that the genus *Haemonchus* was the most prevalent, accounting for 69%, then *Oesophagostomum* with 26% and *Cooperia* with 5%.

Discussion

The fungi tested (AC001, NF34 and CG722) were able to remain viable following gastrointestinal transit through naturally infected cattle, and thus their predatory activity was demonstrated at the end of the experiment (Figures 1a-d and
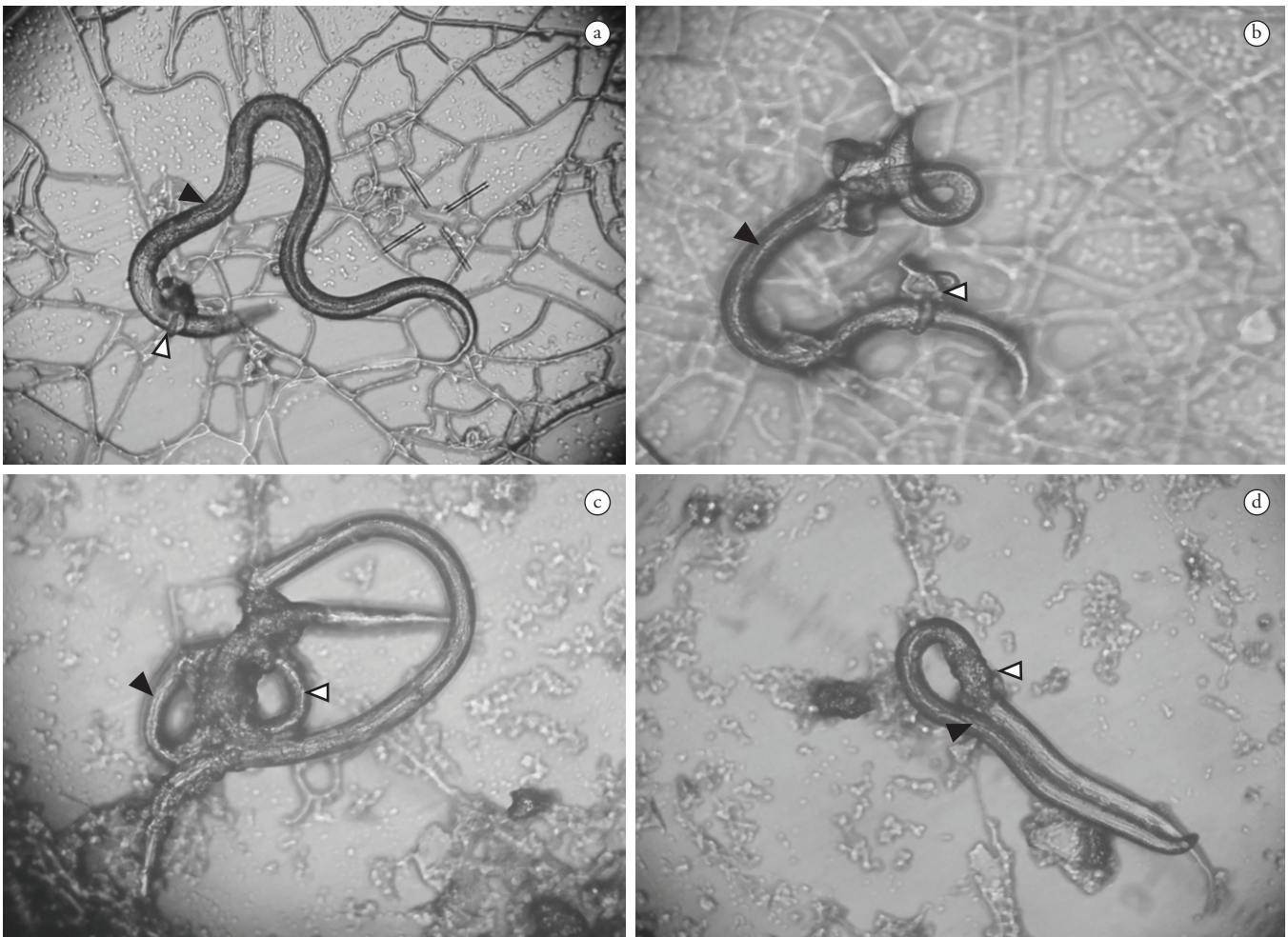

Figure 1. a-d) Infective larvae captured by nematophagous fungi [*Duddingtonia flagrans* (AC001 and CG722) and *Monacrosporium thaumasium* (NF34)] (white arrow) in Petri dishes containing 2% water-agar, and trap formation by the fungal isolates (black arrow). Magnification: (a) 10× and (d) 40× objective lens.

Figure 2). Therefore, they may be used in other studies in the field. It is worth remembering that in the present study, the genus *Haemonchus* was the most prevalent, confirming the studies confirming previous studies. Moreover, these results are compatible with other reports regarding the passage and predatory activity of nematophagous fungi in relation to *in vitro* and *in vivo* control of gastrointestinal nematodes in cattle (ARAÚJO et al., 2004b).

Use of nematophagous fungi for biologically controlling the gastrointestinal parasites of domestic animals reduces soil contamination, since these fungi act directly on L_3 present in the environment (LARSEN, 1999; ARAÚJO et al., 2004a). Studies conducted in several regions in Brazil have shown higher prevalence of the genera *Cooperia*, *Haemonchus*, *Oesophagostomum*, *Trichostrongylus*, *Trichuris*, and *Bunostomum* in cattle (FURLONG et al., 1985). This premise is concordant with Lima (1989), who stated that the L_3 of parasitic nematode species are available in pastures almost all year round, thus serving as a continuous source of infection for animals.
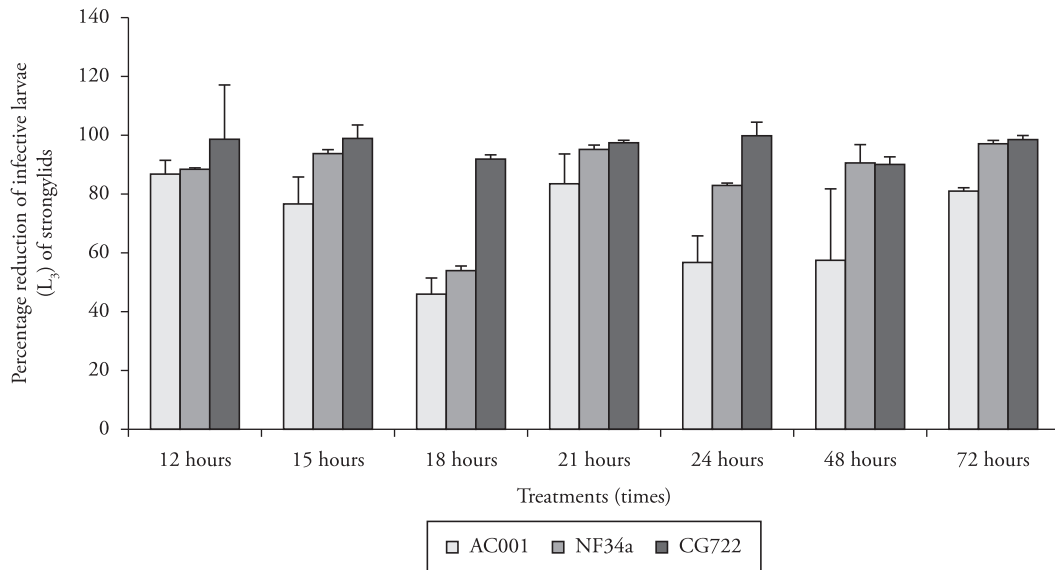
Figure 2. Mean percentage reduction of nematode infective larvae (L_3) recovered from coprocultures by means of the Baermann method after 17 days of incubation with the fungal isolates *Duddingtonia flagrans* (AC001 or CG722) and *Monacrosporium thaumasium* (NF34) obtained at the times of 12, 15, 18, 21, 24, 48, and 72 hours.
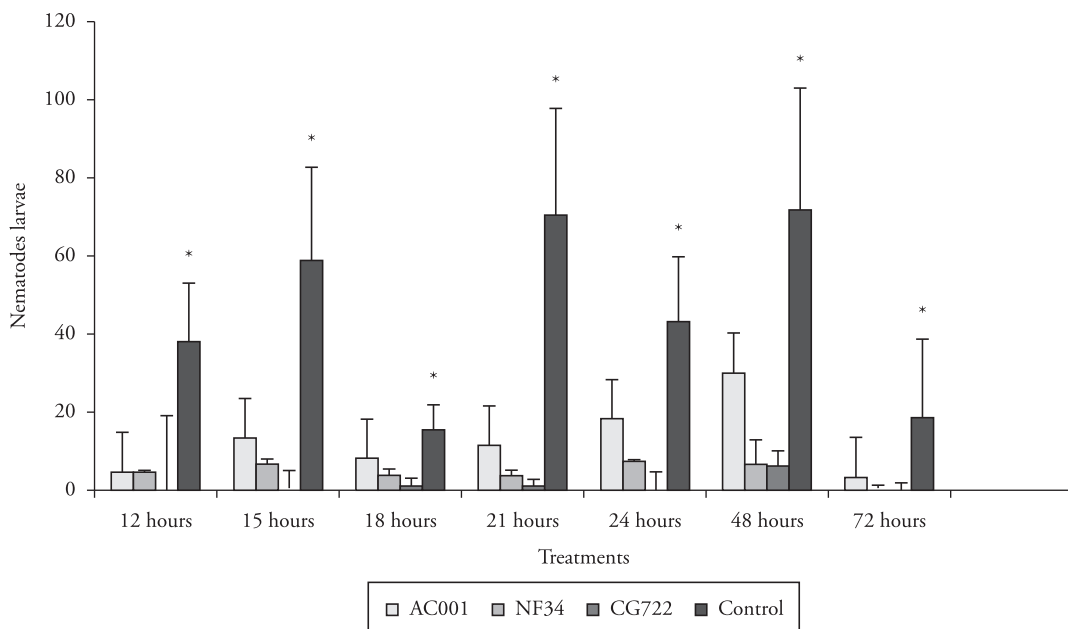
Figure 3. Mean numbers of infective larvae (L_3) recovered by means of the Baermann method from coprocultures after 17 days of treatment with the fungal isolates *Duddingtonia flagrans* (AC001 or CG722) or *Monacrosporium thaumasium* (NF34), and in the control group (without fungi). *Statistical difference ($p < 0.01$) among the isolates tested in relation to the control group at the sampling times.

Regarding the results relating to the degree of reduction in the quantities of L_3 recovered from coprocultures, it was found that the three isolates tested (AC001, NF34 and CG722) showed similarity in their predatory activity ($p > 0.05$), which was thus reflected in the high numbers of larvae destroyed. It should be noted that although this experimental assay was conducted in coprocultures, the same data could be extrapolated for future assays in the field, especially if a longer time interval were to be studied. Moreover, any of the isolates tested could be used, except for some peculiarities of each species (BRAGA et al., 2011b).

In the literature, it has been reported that the most practical way to use the fungi *D. flagrans* (AC001 or CG722) and *M. thaumasium*, or any other nematophagous fungus, is by means of oral administration of fungal material in the form of mycelium, conidia and/or chlamydospores. Inoculation of these organisms into a sodium alginate matrix, to form pellets, is a more recent method that has presented good results (LARSEN, 1992; ARAÚJO et al., 2004a; DIAS et al., 2007; TAVELA et al., 2011). On the other hand, there has been much discussion on the use of different isolates of the same species of fungus under the same conditions, in this case partly natural, which could present distinct results. In this regard, inter and intraspecific differences in the predatory activity of nematophagous fungi are common and have already been observed in experiments with other fungal isolates (MENDOZA-DE-GUIVES et al., 1999).

The species *D. flagrans* (isolates AC001 and CG722) has been tested both under laboratory conditions and in the field. In the past, Gronvold et al. (1993) observed a reduction in the levels of *Ostertagia ostertagi* L_3 in the fecal mass of calves, when these were fed with barley grain containing the fungus *D. flagrans*. In another study, Dias et al. (2007) demonstrated that treatment of young cattle with pellets containing the fungus *D. flagrans* (CG722) was effective in reducing the number of eggs per gram of feces and consequently in reducing the number of L_3 of gastrointestinal nematodes obtained from coprocultures. Those results are in agreement with the present study, in which the fungus was also observed to be an efficient predator. However, it is worth remembering that even though there was no difference ($p > 0.05$) in the reduction of L_3 recovered from coprocultures according to the isolate used, CG722 proved to be more efficient, with a percentage reduction of 98.3% after 72 hours, whereas the reduction with AC001 was 81.2%. However, there are few reports of comparisons between the isolates AC001 and CG722 of *D. flagrans*, used for controlling nematode parasites in naturally infected Holstein cattle.

Araújo et al. (2004b) evaluated the resistance of the nematophagous fungus *M. sinense* (isolate SF470) to passage through the gastrointestinal tract of cattle and its subsequent predatory ability on infective trichostrongylid larvae. In that study, a 61.3% difference in L_3 recovery from coprocultures at the end of the experiment was reported. Furthermore, it was observed that the number of L_3 of gastrointestinal nematodes recovered from the animals of the control group was higher ($p < 0.05$) than that of the treated group. In another study, Araújo and Ribeiro (2003) demonstrated that the isolates *M. appendiculatum* (CGI) and *M. sinense* (SF53) had the capacity to pass through the gastrointestinal tract of cattle and observed that conidia were

present after 14 days of incubation. Those results are in agreement with the present study, since the tested isolate of *M. thaumasium* (NF34) was able to cause a reduction (97.3%) in the number of L_3 recovered at the end of 17 days. In addition, the fungal structures were also observed to be in agreement with this genus.

Lastly, although use of nematophagous fungi for biologically controlling nematode parasites in ruminants has been widely studied, the present authors take the view that such control will only become possible after commercial organizations start to be involved in developing fungal formulations. On the other hand, the present results justify the need for studies in the field, over longer intervals, in order to observe the efficiency of the fungi *D. flagrans* (AC001 or CG722) or *M. thaumasium* (NF34) for environmental control over nematodes in naturally infected cattle. Through this, in the future, it will be possible to assess the best approach towards integrated control over bovine helminths.

Acknowledgements

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