JULIANA MILANI ARAUJO

AVALIAÇÃO DE FUNGOS NEMATÓFAGOS SOBRE LARVAS INFECTANTES DE *Strongyloides westeri* E OVOS DE *Toxocara canis*.

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-graduação em Medicina Veterinária, para obtenção do título de *Doctor Scientiae*.

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APROVADA: 17 DE JULHO DE 2012.

Eraldo Rodrigues de Lima

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(Orientador)

A Deus por mais essa conquista,
e aos meus pais, Otacílio e Ana,
pelo amor incondicional,
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BIOGRAFIA

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RESUMO

ARAUJO, Juliana Milani, D.Sc., Universidade Federal de Viçosa, julho de 2012. **Avaliação de fungos nematófagos sobre larvas infectantes de *Strongyloides westeri* e ovos de *Toxocara canis*.** Orientador: Jackson Victor de Araújo. Coorientadores: Laércio dos Anjos Benjamin e Giovanni Ribeiro Carvalho.

O objetivo deste trabalho foi avaliar a ação de três isolados de fungos predadores *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) e *Arthrobotrys robusta* (I-31) em teste *in vitro* quanto à capacidade de predar larvas infectantes (L_3) de *Strongyloides westeri* e, avaliar três concentrações de clamidósporos (1.000, 10.000 e 100.000) do fungo ovicida *Pochonia chlamydosporia* (isolados VC1 e VC4) na destruição de ovos de *Toxocara canis*. Em relação aos fungos predadores, quando comparado ao grupo controle, pode-se observar que houve uma redução significativa (P<0,01) de 80,4%, 67,9%, 72,8%, nas médias de larvas infectantes de *S. westeri* recuperadas dos tratamentos com os isolados AC001, NF34 e I-31, respectivamente. Todos os isolados testados foram eficientes na captura de *S. westeri* (P>0,01) no teste *in vitro*. No teste da avaliação ovicida com o fungo *P. chlamydosporia* sobre ovos de *T. canis*, cada placa de Petri continha mil ovos de *T. canis* com apenas uma das concentrações de clamidósporos de VC1 ou de VC4, em ágar-água 2% (AA 2%) e, mil ovos em AA 2% nos grupos controle. No intervalo de 15 dias, cem ovos foram retirados de cada placa de Petri e a atividade ovicida foi avaliada quanto as alterações morfológicas. A maior destruição dos ovos (efeito do tipo 3) foi observada na concentração de 100.000 clamidósporos para ambos os isolados. Após avaliação *in vitro*, os fungos predadores e ovicida, foram avaliados *in vivo*, quanto à sua capacidade de suportar a passagem pelo trato gastrintestinal de eqüídeos (AC001 e NF34) e predar L_3 de *S. westeri*, e pelo trato gastrintestinal cães (VC4) quanto sua ação ovicida sobre ovos de *T. canis*. Os fungos predadores sobreviveram à passagem pelo trato gastrintestinal dos eqüídeos e foram eficientes em predar as L_3 de *S. westeri* desde a primeira coleta (12h) (P<0,01) em relação ao grupo controle (sem fungo). O fungo ovicida também sobreviveu a passagem pelo trato gastrintestinal de cães mantendo sua atividade ovicida sobre os ovos de *T. canis*. Esses resultados demonstraram que os fungos *D. flagrans* e *M.*

thaumasium se mostraram promissores para serem utilizados no controle biológico de *S. westeri* assim como, resultados obtidos sugere-se que o fungo *P. chlamydosporia* (VC4) poderia ser utilizado como uma ferramenta no controle biológico de ovos de *T. canis*.

ABSTRACT

ARAUJO, Juliana Milani, D.Sc., Universidade Federal de Viçosa, July, 2012. **Evaluation of nematophagous fungi on infective larvae of the *Strongyloides westeri* and *Toxocara canis* eggs.** Adviser: Jackson Victor de Araújo. Co-advisores: Laércio dos Anjos Benjamin and Giovanni Ribeiro Carvalho.

The objective of this work was to evaluate the action of three isolates of predatory fungi *Duddingtonia flagrans* (AC001*), Monacrosporium thaumasium* (NF34) and *Arthrobotrys robusta* (I-31) in *in vitro* assay regarding their capacity to prey infective larvae (L_3) of *Strongyloides westeri* and, to evaluate *in vitro* three concentrations of chlamydospores (1,000, 10,000 and 100,000) of the nematophagous fungus *Pochonia chlamydosporia* (isolates VC1 and VC4) in the destruction of *Toxocara canis* eggs. In relation to the fungi predators when compared to the control group, one can observe that there was a significant reduction (P<0.01) of 80.4%, 67.9%, 72.8%, in the means of recovered infective larvae of *S. westeri* from treatments with the isolates AC001, NF34 and I-31, respectively. All tested isolates were efficient at capturing *S. westeri* (P>0.01) when tested *in vitro*. In the test of ovicidal evaluation with the fungus *P. chlamydosporia* on eggs of *T. canis*, each Petri dish contained thousand eggs of *T. canis* with only one concentration of chlamydospores of VC1 or VC4 in water-agar 2% (WA 2%), and only one thousand eggs in WA 2% in the control groups. Within 15 days, one hundred eggs were removed from each Petri dish and the ovicidal effect was evaluated according to morphological alterations. The higher percentage to effect of the type 3 was observed in concentration of 100,000 chlamydospores to both isolates. After *in vitro* evaluation, predatory and ovicidal fungi were evaluated *in vivo* regarding their capacity to withstand the passage through the gastrointestinal tract of horses (*Duddingtonia flagrans* and *Monacrosporium thaumasium*) on *S. westeri* and dogs (*Pochonia chlamydosporia*) on *T. canis* eggs. The predatory fungi survived the passage through the gastrointestinal tract of horses and were efficient in preying upon the L_3 since the first collection (12h) (P <0.01) compared to control (without fungi). The ovicidal fungus also survived the passage through the gastrointestinal tract of dogs keeping its ovicidal activity on *T. canis* eggs. These results demonstrated that the fungi *D. flagrans* and *M. thaumasium* were

promising to be used for biological control of *S. westeri* as well as from the results obtained suggest that the fungus *P chlamydosporia* (VC4) could be used as a tool for biological control of *T. canis* eggs.

1. INTRODUÇÃO GERAL

Os estudos sobre parasitismo em animais de estimação vêm despertando crescente interesse, frente à associação restrita e íntima entre o homem e os animais e sua conseqüência em saúde pública (Vasconcellos et al., 2006). Além disso, as helmintoses gastrintestinais influenciam no desenvolvimento da criação de animais domésticos de companhia e também daqueles com interesse zootécnico, em todo o mundo. Os prejuízos causados por essas infecções envolvem queda da produção, retardo no crescimento do animal, recursos terapêuticos, custos com tratamentos e em muitas vezes a morte dos animais (Araújo et al., 2004).

Dentre os grupos de animais domésticos, os cães e os equídeos têm relevante importância em todo território brasileiro. Contudo, em relação às helmintoses gastrintestinais existem diferenças na especificidade parasitária.

Os eqüídeos é um grupo muito prevalente no Brasil, apresentando a terceira maior criação no mundo (Bezerra et al., 2007). Além disso, representam uma importante espécie entre os animais domésticos em virtude da utilização destes como meio de transporte, tração, entretenimento, esporte, na recuperação de crianças especiais ou mesmo para o consumo humano de produtos e subprodutos cárneos. Em relação a forma de criação, esta pode ser extensiva ou intensiva, no entanto, sabe-se que as mesmas favorecem a grande incidência de infecções parasitárias já nas primeiras semanas de vida (Braga et al., 2009). Estes animais são hospedeiros de uma vasta quantidade de helmintos, onde os de importância mais relevada são os nematóides (Assis & Araújo, 2003; Braga et al., 2011). Por outro, a fauna parasitária é vasta e destacam-se vários parasitos dentre os quais, o *Strongyloides westeri*.

O nematóide *S. westeri* pertence a ordem Rhabditida, Superfamília Rhabditoidea, Família Strongyloididae (Monteiro, 2010). É um parasito que afeta principalmente os animais jovens. Contudo, alguns autores mencionam que a infecção é limitada a estes animais e, que o hospedeiro se torna resistente a re-infecção. São parasitos que vivem embebidos no epitélio glandular da mucosa do intestino delgado, particularmente do duodeno e do jejuno, onde realizam a ovipostura (Bowman, 2010; Monteiro, 2010).

O gênero *Strongyloides* possui morfologia diferenciada com o esôfago da fêmea quase cilíndrico e com comprimento de pelo menos um quarto do

comprimento total do corpo. As fêmeas são partenogênicas e, é o único gênero entre os parasitos de animais domésticos com geração parasitária e de vida livre alternadas. De maneira resumida, o ciclo evolutivo deste parasito, comporta-se da seguinte forma: as fêmeas parasitárias filariformes (encontradas no intestino do hospedeiro) produzem ovos por partenogênese mitótica que são eliminados com as fezes no solo, e as larvas desses ovos são chamadas de geração homogônica para diferenciá-las da geração heterogônica.(vida livre – sexuada). As larvas no ambiente podem se desenvolver para larvas infectantes (L_3) ou em machos e fêmeas de vida livre. Se a larva infectante encontra o hospedeiro adequado, esta penetra através da pele e se tornam adultas no intestino do hospedeiro. Caso contrário, as larvas vão se desenvolver através de mudas e se transformar em adultos no solo. Estes copulam e produzem larvas heterogônicas que podem desenvolver em larvas infectantes (Bowman, 2010; Monteiro, 2010).

Os ovos embrionados, a larva rabditiforme e a larva infectante filariforme de terceiro estágio são os estágios mais importantes nos procedimentos diagnósticos (Bowman, 2010; Monteiro, 2010). Os ovos são encontrados quase que exclusivamente em potros lactentes e recém-desmamados. A eliminação dos ovos nas fezes começa a partir de 10 dias a duas semanas após o nascimento (Bowman, 2010). A principal via de transmissão do *S. westeri* é a transmamária, onde, após uma infecção inicial ter se estabelecido, larvas adicionais tendem a migrar para os tecidos corporais mais profundos, onde infectam os recém-nascidos através do leite. Também pode ocorrer a infecção através da pele ou membranas da mucosa oral, podendo causar infecções percutâneas (Bowman, 2010).

Outro grupo de animais domésticos que desempenham um papel importante na sociedade em todo o mundo são os cães. Nenhuma outra espécie animal ocupa tantos e tão diversos papéis na sociedade, trazendo benefícios inestimáveis para a melhoria das condições fisiológicas, emocionais e sociais em especial para crianças e idosos (Carvalho et al., 2010). Entretanto, o aumento crescente desses animais, principalmente nos centros urbanos vem aumentando a exposição humana a agentes zoonóticos (Carvalho et al., 2010), entre os quais estão algumas espécies de helmintos.

Nesse contexto, um parasito que se destaca é da ordem Ascaridida, Superfamília Ascaridoidea, Família Ascarididae, Subfamília Toxocarinae, do

gênero *Toxocara*, particularmente a espécie *Toxocara canis* (Monteiro, 2010). A infecção por *T. canis* pode ser adquirida pelo homem através da ingestão de ovos larvados presente no ambiente onde, cães infectados por *T. canis* eliminam os ovos do parasito nas fezes que, em condições ambientais apropriadas, tornam-se infectantes e podem permanecer viáveis por longo período no ambiente (Rey, 2008). Nos humanos o *T. canis* não atinge a maturidade, mas as larvas permanecem vivas migrando erraticamente nos órgãos internos, produzindo uma patologia denominada Larva Migrans Visceral (LMV - onde os órgãos mais afetados são pulmão, fígado e cérebro), ou invadindo o globo ocular e causando a Larva Migrans Ocular (LMO) (Rey, 2008).

Os parasitos adultos vivem no intestino delgado de cães e gatos. Os ovos de *T. canis* são eliminados nas fezes, sendo as fêmeas muito fecundas. As fêmeas podem produzir 2 milhões de ovos por dia, no período mais fértil de sua existência (entre a sétima e a 28ª semana), caindo para uma média de 200.000 ovos, até o oitavo mês (Rey, 2008).

Apenas ovos embrionados são infectantes, e quando ingeridos pelos cães, esses eclodem no intestino, invadem a mucosa e através da circulação porta fazem o ciclo fígado – coração – pulmão, regressando ao tubo digestivo via brônquios, traquéia e esôfago. O tempo mínimo para o ciclo se completar é de 30 dias, e em seguida, começam a aparecer ovos nas fezes (Rey, 2008). Contudo, a principal via de infecção nestes animais ocorre por migração transplacentária e, pode ocorrer também a infecção transmamária de larvas pelo colostro ou pelo leite, ingestão destas em estádio avançado de evolução, e em menor escala, mediante parasitas imaturos eliminados pelas fezes ou vômito de cães (Rey, 2008; Bowman, 2010).

Em cães, o controle desse parasito é baseado em tratamento precoce com anti-helmínticos, tentando evitar dessa forma a contaminação ambiental pelos ovos, e deve ser iniciado aos 14 dias de vida e pode ser repetido em intervalos até os três meses de vida. No entanto, a literatura menciona a resistência à algumas drogas utilizadas no controle da infecção nestes animais, com destaque para o pirantel (Koop et al., 2008). Existem ainda, outras medidas que consistem em controle de cães vadios e cuidados higiênicos com o ambiente, e proteção de parques e espaços destinados ao lazer (Rey, 2008).

Em relação ao controle das verminoses em equídeos, este é feito por anti-helmínticos, onde estes não tem sido de total eficácia devido à ação restrita aos parasitos adultos (Assis & Araújo, 2003). Por outro lado, de uma maneira geral ainda existem outras desvantagens como resíduos na carne, no leite, a possibilidade do impacto ambiental e também o desenvolvimento eminente de resistência dos parasitos (Braga et al., 2011).

Os anti-helmínticos são a principal forma de controle de verminoses em animais domésticos, em especial aqui de cães e equídeos. Por outro lado, é cada vez mais crescente a "corrente" da sociedade de se tentar minimizar o uso destas drogas que podem interferir em um aspecto global na contaminação ambiental e riscos para a saúde (Araújo et al., 2004; Braga et al., 2010). Dessa forma, a utilização de alternativas em conjunto ao controle químico, como o controle biológico utilizando fungos nematófagos, pode vir a ser uma ferramenta promissora para a diminuição dos impactos ambientais e mesmo da saúde de animais e humanos (Braga et al., 2011).

O controle biológico realizado com fungos nematófagos aparece como uma alternativa promissora e viável, que tem por finalidade o sinergismo com o controle químico (Araujo et al, 2010; Braga et al., 2010). Estes fungos vêm se destacando entre os mais variados antagonistas de nematóides, apresentando melhores desempenhos em pesquisa de controle biológico de nematóides (Araújo et al, 2004; Braga et al., 2010). De acordo com Gronvold et al., (1996), os fungos são mais promissores para o controle biológico em relação a outros organismos, pois estão em abundância no solo e podem utilizar vários substratos orgânicos e micro-habitats que promovem oportunidades para a interação com nematóides.

Na prática, o controle biológico não atua sobre estágios internos do parasito. Os fungos nematófagos atuam sobre os hospedeiros intermediários, paratênico, vetores e estágios larvais de vida livre diminuindo assim, a fonte de infecção aos hospedeiros finais, além de causar menos efeitos negativos no ambiente que os métodos químicos (Araújo et al., 2004; Braga et al., 2010). Esses fungos são antagonistas naturais de nematóides, capazes de promover a captura, a morte ou mesmo a destruição desses organismos (Braga et al., 2009).

Os fungos nematófagos são classificados como predadores, endoparasitas e oportunistas. Esses fungos são cosmopolitas que ocorrem

naturalmente no solo, e em material orgânico em decomposição. No grupo dos predadores os gêneros *Arthrobotrys, Duddingtonia e Monacrosporium* se destacam pelo controle de larvas de nematóides no ambiente. Estes produzem estruturas em forma de anéis constritores e não constritores, hifas, botões e redes tridimensionais adesivas ao longo do micélio. Após o aprisionamento pela armadilha, ocorre a penetração das hifas na cutícula do nematóide, seguido de crescimento das hifas no interior do nematóide e digestão dos conteúdos internos (Araújo et al., 2004). No grupo dos endoparasitos, esses fungos são capazes de infectar os nematóides através de esporos, que uma vez ingeridos, desenvolvem hifas responsáveis pela absorção do conteúdo interno do nematóide. Estes não produzem hifas vegetativas fora do corpo do hospedeiro, mas hifas férteis ou conidióforos contendo esporos (Araújo et a., 2004). Já no grupo dos fungos oportunistas vem se destacando o fungo *Pochonia chlamydosporia* e têm ação nos ovos de helmintos. A ação deste fungo é baseada na formação de um apressório que se desenvolve por uma hifa não diferenciada. Este fungo coloniza a superfície dos ovos e penetram no interior destes por meio de ação enzimática de proteases, lípases e quitinases, bem como a ação mecânica (Araujo et al, 2009; Braga et al., 2011).

No entanto, um requisito essencial para que qualquer isolado fúngico nematófago seja possivelmente explorado como um controlador biológico de parasitas gastrintestinais, é que ele possua a habilidade de suportar a passagem pelo trato gastrintestinal dos animais após administração oral, ou seja, suportar condições de estress e, uma vez presente nas fezes seja capaz de germinar, colonizar o bolo fecal e capturar as larvas de parasitas recém eclodidas dos ovos antes que elas migrem para a pastagem (Braga et al., 2010). Além disso, os microorganismos selecionados para atuarem como controladores biológicos devem possuir especificidade de ação, alta capacidade reprodutiva e suportar condições ambientais no local onde o controle é realizado (Braga et al., 2009).

2. OBJETIVOS GERAIS

- Avaliar a ação *in vitro* dos isolados fúngicos de *Duddingtonia flagrans, Monacrosporium thaumasium* e *Arthrobotrys robusta* sobre larvas infectantes de *Strongyloides westeri*.

- Avaliar a ação *in vitro* de dois isolados do fungo de *Pochonia chlamydosporia* (VC1 e VC4) sobre ovos de *Toxocara canis*.

- Avaliar após a passagem pelo trato gastrintestinal de eqüinos e jumentas, a viabilidade e ação dos fungos *Duddingtonia flagrans* e *Monacrosporium thaumasium*, quanto a sua atividade predatória sobre larvas de *S. westeri* em diversos intervalos de tempo, e se há diferença de predação entre esses isolados.

- Avaliar se o fungo *P. chlamydosporia* (isolado VC4) resiste a passagem pelo trato gastrintestinal de cães e quanto a sua atividade ovicida sobre ovos de *T. canis* em diversos intervalos de tempo.

CAPÍTULO I

IN VITRO PREDATORY ACTIVITY OF NEMATOPHAGOUS FUNGI AND AFTER PASSING THROUGH GASTROINTESTINAL TRACT OF EQUINE ON INFECTIVE LARVAE OF *Strongyloides westeri*

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IN VITRO PREDATORY ACTIVITY OF NEMATOPHAGOUS FUNGI AND AFTER PASSING THROUGH GASTROINTESTINAL TRACT OF EQUINE ON INFECTIVE LARVAE OF *Strongyloides westeri*

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Abstract

Three isolates of predator fungi *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) and *Arthrobotrys robusta* (I-31) were assessed *in vitro* test regarding the capacity of prey infective larvae (L_3) *Strongyloides westeri*. Comparing to control, without fungus, there was a significant decrease (P<0.01) of 80.4%, 67.9%, 72.8% in means of infective larvae *S. westeri* recovered from treatments with isolates AC001, NF34 and I-31, respectively. All tested isolates were efficient in the capture of *S. westeri* (P>0.01) *in vitro* test. Linear regression coefficients of treated and control groups were -0.21 for control, -0.32 for *D. flagrans*, -0.34 for *M. thaumasium* and -0.22 for *A. robusta*. Following, isolates AC001 and NF34 were assessed *in vivo* regarding the capacity of supporting the passage through equine gastrointestinal tract, without loss of ability of preying infective larvae *S. westeri*. Fungal isolates survived the passage and were efficient in preying L_3 since the first 12h of collection (P<0.01) in relation to control group (without fungus). Comparing to control, there was a significant decrease (P<0.01) of 76.4% and 76.7% (12 hours), 86.4% and 85.9% (24 hours), 88.3% and 87.7% (48 hours), 89.9% and 87.2% (72 hours) in means of infective larvae *S. westeri* recovered from treatments with isolates AC001 and NF34, respectively. Linear regression coefficients of L_3 of recovered *S. westeri* regarding the collections due to time were 1.93 for control, -3.52 for AC001 and -2.64 for NF34. Fungi *D. flagrans* and *M. thaumasium* (NF34) have demonstrated to be promising for use in biological control of equine parasite *S. westeri*.

KEY WORDS: Nematophagous fungi*, Duddingtonia flagrans*, *Monacrosporium thaumasium*, *Arthrobotrys robusta*, *Strongyloides westeri,* equine.

1. Introduction

According to Bezerra et al. (2007), equine raising in Brazil represents the third greatest in the world, with about 36 million of animals. Those animals are hosts of a wide quantity of gastrointestinal parasite helminthes (Assis and Araújo 2003). Strongyles nematodes are common in equine, representing a group of large importance, since great part of the cattle is infected.

Strongyloides westeri is the nematode of largest prevalence among foals with age up to four months. It causes lesions in the small intestine and has been considered as possible cause of diarrhea of the rut of foal (Fenger 2000; Melo et al. 2007).

Matthews et al. (2004) and Kaplan (2002) report that the control of worms in equines usually is performed using anti-helminthic, which have not been totally effective for control of these nematodes due to its restricted action to adult parasites and occurrence of resistance.

However, the integration of other forms of parasite control that aim at decreasing the number of infective larvae in pastures, providing the decrease of parasite load in animals and consequently decrease in number of treatments with anti-helminthic are welcome (Araújo et al. 2004; Braga et al. 2009a).

In this way, the application of biological control with nematophagous fungi has became a viable alternative, and has presenting promising results *in vitro* and *in vivo* (Larsen 1999, Braga et al. 2009a). Nematophagous fungi are saprophytic organisms worldly studied, with capacity of preying nematodes. In predator fungi group, the species *Duddingtonia flagrans* detaches as the most promising for control of gastrointestinal nematodiosis of domestic animals (Terril et al. 2004; Dias et al. 2007). Species *Arthrobotrys robusta* and *Monacrosporium thaumasium* have predatory activity against larvae of gastrointestinal helminthes of domestic animals (Assis and Araújo 2003; Castro et al. 2003).

However, for a nematophagous fungus to be employed as agent of biological control of nematodes, it is necessary that it mainly has skills for capturing nematodes and that resists to the passage through gastrointestinal tract (Araújo t al. 2004).

Sodium alginate-based formulations have been experimentally assessed in the control of nematode parasites of animals by some research laboratories.

These formulations have demonstrated good results on field and lab conditions (Araújo and Sampaio 2000; Araújo et al. 2000).

The objective of this study was to assess the *in vitro* predatory capacity of isolates nematophagous fungi *D. flagrans* (AC001), *M. thaumasium* (NF34) and *A. robusta* (I-31) about infective larvae (L_3) of *S. westeri*, equine parasites. Also, to assess the capacity of AC001 and NF34 after the passage through equines gastrointestinal tract, without loss of predatory efficacy on L_3 of *S. westeri* in feces.

2. Material and Methods

2.1. Experimental test A - *In vitro* efficacy of nematophagous fungi on infective larvae (L_3) of *Strongyloides westeri*.

Panagrellus sp. (free nematodes) were maintained on Petri dishes with oats medium, moistened and mashed. These nematodes were extracted from culture medium by immersion of small quantities of oat in distilled water in Baermann apparatus and collected in hemolysis tubes after six hours of decantation.

Brazilian isolates of predatory nematode fungi of *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34), and *Arthrobotrys robusta* (I-31) were kept in assay tubes with 2% corn-meal-agar (2% CMA), at 4^{o}C and in dark.

To induce the production and collection of conidia by fungal isolates, culture discs of approximately 4 mm of diameter of each fungus were transferred to 8.5 cm diameter Petri dishes with 2% water-agar (2% WA), kept at 25^{o}C and in dark. To these plates, 1 mL of suspension with 1000 *Panagrellus* sp., every three days for 21 days, was added, until complete growth of fungal mycelium in the plates was done, and all nematodes were preyed. After this period, 5 mL of distilled water were added to each Petri dish, and with the help of brush conidia mycelium fragments were removed and stored at 4^{o}C.

L_3 of *S. westeri* were obtained from feces of naturally infected foals, through stool culture in vermiculite, for 15 days, and posterior use of Baermann funnel, with water at 42-45^{o}C and decantation time of 12 h.

To study the predatory activity of fungi over L_3 of *S. westeri* a treated group was formed, for each fungal species, constituted by 1,000 conidia and

1,000 L_3, and a control group with 1,000 L_3, being 10 repetitions performed for each group.

Fungi with L_3 were deposited on 9.0cm diameter Petri dishes, with 2% water-agar (2% WA) medium kept in stove at 25^{o}C and in dark. Daily, for a period of 7 days, plates were observed at light optical microscope (10x objective), randomly choosing 10 field per plate, being taken the number of L_3 free from predation by fungi, obtaining the mean of L_3 per field.

On the seventh day, L_3 were recovered with the aid of Baermann funnel, with water at 42-45^{o}C and waiting 12 hours for decantation. L_3 were counted, obtaining the mean of recovered larvae in the control group, and means of not preyed larvae of treated groups.

2.2. Statistic analysis

Data were submitted to variance analysis (F test) and Tukey test at levels of 1 or 5% of probability and linear regression with levels of 5% of probability. The estimation of L_3 mean decrease percentage in treated groups was performed by the equation:

$$\text{Reduction (\%)} = \left(\frac{\text{average recovered from control} - \text{average recovered from treatment}}{\text{average recovered from control}} \right) \times 100$$

2.3. Experimental test B- Efficacy test about L_3 of *Strongyloides westeri* after passage through equine gastrointestinal tract.

Fungal isolates *D. flagrans* (AC001) and *M. thaumasium* (NF34) were tested regarding quantity of passage of each isolate by equine gastrointestinal tract.

The experiment was developed in the Veterinary Department of Universidade Federal de Viçosa, located in the city of Viçosa, Minas Gerais, Brazil, latitude 20º45'20" and longitude 42º52'40".

Twelve crossbred, male equines were used, with average weight of 316.6 kg (±3.92), in stall, previously dewormed with one oral dose of vermifuge for equines and in the dose of 200µg per kilogram of alive weight of ivermectin 1% and 6.6mg/kg of alive weight of pyrantel pamoate (Centurion Vallé®, Montes Claros-Minas Gerais, Brazil) 14 days before receiving pellets with

mycelia mass of isolates from *D. flagrans* (AC001) and *M. thaumasium* (NF34) and pellets without fungus. In the treated group, each animal received 100 grams of pellets, single dose, with mycelia mass of mixed fungi in 100 grams of commercial food for equine. Animals of control group received a single administration of 100 grams of sodium alginate pellets without fungus.

In order to induce the formation of fungal mycelium, culture discs approximately 4 mm of diameter, in 2% WA, were transferred to 250 mL Erlenmeyer's flasks, with 150ml of GPY liquid medium (glucose, sodium peptone and yeast extract), under 120 rpm stirring, in dark and temperature of 26^{o}C for 10 days. After this period, mycelia were removed, filtered and heavy in analytical balance (Walker and Connick, 1983).

Fecal samples from animals were collect at 12, 24, 48 and 72 hours after the administration of fungi. Samples of each group were homogenized, and 4 g of feces were removed and placed in 9 cm diameter Petri dishes with 2% WA, stored in stove at 25^{o}C and in dark. In these plates, 1,000 L_3 of *S. westeri* were spread. From each established time, five repetitions were performed for each treatment group and for control. Daily, plates were observed for research of conidia and conidiophores characteristic of tested isolates, analyzing according to classification-keys proposed by Van Oorschot (1985) and Liu and Zang (1994). In the 15th day, L_3 not preyed were recovered from the Petri dishes by Baermann technique, obtaining the mean of not preyed larvae per plate.

2.4. Statistic analysis

Data were submitted for variance and regression analysis. Means were compared by using the Tukey test in level of 1 or 5% of probability (Ayres et al. 2003.

3. Results

3.1. Experimental test A

Isolates of predatory fungi of tested nematodes, *D. flagrans* (AC001), *M. thaumasium* (NF34) and *A. robusta* (I-31) were able to prey L_3 of *S. westeri* in the *in vitro* experimental test. Comparing the capture and destruction of L_3 of *S. westeri* in treated groups with the isolates *D. flagrans* (AC001), *M. thaumasium*

(NF34), and *A. robusta* (I-31) during the experimental test, no difference (P>0.01) was observed, Table 1.

Regarding the decrease percentage of L_3 of *S. westeri* at the end of the experimental test, the following decrease percentage was recorded: 80.4% (AC001), 67.9% (NF34), 72.8% (I-31).

Difference (p<0.01) was observed among means of L_3 of not preyed *S. westeri* per field of 4 mm diameter of the control group plates in relation to means of L_3 recorded in the plates of groups treated with fungi during the experimental test.

In the plates of the control group, no presence of nematophagous fungi during the experiment was observed. Evidence of predation was verified by the means of recovered L_3 of *S. westeri* in the seventh day by Baermann method, at the end of the experiment (Figure 1).

Linear regression coefficients calculated through analyses of mean L_3 per field of 4 mm diameter of the treated and control groups were -0.21 for control, -0.32 for *D. flagrans* (AC001), -0.34 for *M. thaumasium* (NF34), and -0.22 for *A. robusta* (I-31) (Figure 2).

3.2. Experimental test B

Fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34) had capacity of preying infective larvae of equines *S. westeri*, after the passage through gastrointestinal tract, not losing its viability and were efficient on preying L_3. Regarding the decrease percentage, results demonstrated by AC001 and NF34 were 76.4% and 76.7% (12 hours), 86.4% and 85.9% (24 hours), 88.3% e 87.7% (48 hours), 89.9% and 87.2% (72 hours) of L_3 (P<0.01), respectively, when compared to control group.

In the plates of control group the presence of nematophagous fungi was not detected, only observing the same saprophytic fungi of treated groups.

Throughout the experiment, the mean number of L_3 of control group was larger in relation to that of treated (P<0.01). Mean values of the infective larvae number of recovered *S. westeri* of Petri dishes incubated with four grams of feces from animals of treated groups (with isolates AC001 and NF34), and animals of control group (did not receive fungus) are represented in Table 2.

The growth of saprophytic fungal, not predator, species made visualization of conidia difficult, which could only be identified in the 12th day of observation, in plates referring to collections of 12 and 48 hours for all tested isolates. In plates referring to collections of 24 and 72 hours conidia were visualized from 11th day on. For AC001 it was observed that the 48-hour period was that in which this isolate had a lesser mean number of recovered L_3, meaning a higher predatory activity. On the other hand, for the isolate NF34 the best period was 72 hours.

Coefficients of linear regression curves of L_3 of *S. westeri* recovered from Petri dishes regarding the collections due to time were 1.93 for control, -3.52 for *D. flagrans* (AC001) and -2.64 for *M. thaumasium* (NF34) (Figure 3).

4. Discussion

Nematophagous fungi have predatory capacity over L_3 of gastrointestinal nematode parasites in domestic animals, with a highlight for genus *Duddingtonia*, *Monacrosporium* and *Artrobotrys* (Araújo et al. 2004; Campos et al. 2008; Silva et al. 2009). These genus are acknowledged just as predators, and have in the species *D. flagrans* and *M. thaumasium* and *A. robusta* its predatory capacity already discussed and proved in control of L_3 of gastrointestinal nematode parasites of ruminants (Chandrawathani et al. 2003; Castro et al. 2003; Dias et al. 2007). However, the predatory capacity of these species was never tested on L_3 of *S. westeri.* Few works have mentioned the *in vitro* and *in vivo* predatory activity of different nematophagous fungi on nematode larvae parasites of equines (Castro et al. 2003; Braga et al. 2009a).

Braga et al. (2009b), working with species *D. flagrans* (AC001), *M. thaumasium* (NF34) and *A. robusta* (I-31) recorded its efficacy on infective larvae of gastrointestinal nematode *Angiostrongylus vasorum* at the end of experimental test, at the end of seven days. In that work, although no difference ($P>0.01$) among tested isolates was observed, AC001 had larger efficacy in the predation of larvae (80.3%). In the current work, at the end of seven days, all isolates were effective in predation of larvae of *S. westeri* ($p>0.01$), but the isolate AC001 (80.4%) was more effective.

According to Scholler and Rubner (1994) and Gronvold et al. (1996), traps were formed in response to the presence of nematodes or derived

substances; still occur in consequence of limiting nutritional conditions and/or water scarcity. Also, according to Nansen et al. (1998), the higher is the mobility of nematodes, the higher is the stimulus to fungus for producing traps. In the present work, the presence of L_3 of *S. westeri* in Petri dishes with 2% WA was fundamental to formation of traps by fungal isolates, in a poor nutrient medium as 2% water-agar.

Regarding the results obtained *in vitro* test on Petri dishes (assay A), for linear regression coefficients, it is noted that negative values indicate a descending behavior of regression curves of treatments, due to a decrease of not preyed L_3 means per field of 4 mm diameter throughout time, by capture of L_3 in fungal traps. On the other hand, in the control group, the decrease of means of L_3 per field of 4 mm diameter throughout the seven days of observation is due to the fact of migration of part of larvae to the periphery of plates, where there is more humidity. These results are consistent with the works of Carvalho et al. (2009a, b) about infective larvae of *Ancylostoma* spp.

In the *in vivo* test (assay B), results of regression curves, represented in Figure 3, demonstrated that two tested fungal isolates (AC001 and NF34) had negative linear correlation coefficient. According to Carvalho et al. (2009a), the negative value indicates the existence of reverse correlation between variables, what proves the viability of predatory capacity of fungal isolates after passage through gastrointestinal tract of domestic animals. This information is consistent with the present work, where a decrease of number of infective larvae recovered from equine feces in the studied timelines was observed (12, 24, 48 and 72 hours).

The use of nematophagous fungi in biological control of animals parasitized by helminthes can reduce soil contamination, directly acting in the infective larvae present in the environment. In recent work, Braga et al. (2009a) demonstrated the efficacy of fungus *D. flagrans* (AC001) by using in pellets of sodium alginate matrix through the passage by gastrointestinal tract of equines. In that work, the authors registered a mean decrease in larvae recovered from stool culture of 40.9% at the end of the experiment for animals of treated group versus the animals of control group. This information is consistent with results demonstrated in the present work for the isolate AC001 over L_3 of *S. westeri.*

In other experiments, Rédua (2002) and Assis and Araújo (2003) proved that isolates of *Monacrosporium* resisted to the passage through

gastrointestinal tract of equines without loss of viability to prey infective larvae of cyathostomes. These results are also consistent with the present work.

5. Conclusion

All or tested isolates (AC001, NF34 and I-31) had *in vitro* action against L_3 of *S. westeri*. Fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34) have shown to be promising to be used in the biological control of *S. westeri* after the passage through gastrointestinal tract of equines without loss of its viability.

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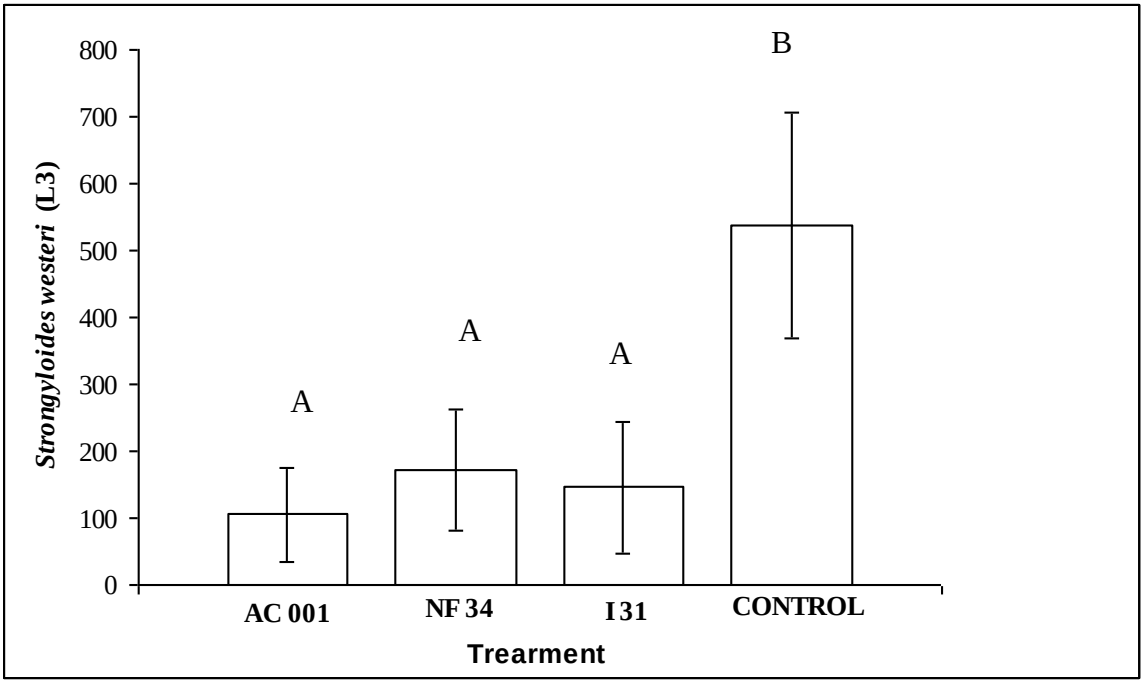
Table 1- Daily means of infective not preyed larvae of L_3 of *Strongyloides westeri* per field of 4 mm diameter in 2% water-agar (2% WA) medium during a period of seven days in treatments with isolates *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) and *Artrobotrys robusta* (I-31) and in control group (without fungus).

Time	Treatments (infective not preyed larvae)			
Days	AC001	NF34a	I-31	Control
1	$1.97^{A} \pm 1,28$	$2.48^{A} \pm 1,24$	$2.43^{A} \pm 1.58$	$5.55^{B} \pm 2.61$
2	$2.28^{A} \pm 1,52$	$1.47^{A} \pm 1,28$	$2.08^{A} \pm 1.27$	$8.15^{B} \pm 3.95$
3	$1.17^{A} \pm 1,12$	$1.22^{A} \pm 1,01$	$1.28^{A} \pm 1.25$	$8.15^{B} \pm 4.07$
4	$1.32^{A} \pm 1,30$	$0.92^{A} \pm 1,00$	$2.15^{A} \pm 1.58$	$5.07^{B} \pm 2.77$
5	$1.10^{A} \pm 1,39$	$1.10^{A} \pm 0,93$	$1.83^{A} \pm 1.88$	$5.33^{B} \pm 3.38$
6	$0.35^{A} \pm 0,58$	$0.18^{A} \pm 0,47$	$0.60^{A} \pm 0.92$	$4.42^{B} \pm 2.54$
7	$0.22^{A} \pm 0,49$	$0.15^{A} \pm 0,36$	$1.15^{A} \pm 1.26$	$6.93^{B} \pm 3.33$

Means followed by at least one letter, capital letter, in line, not differing among them at level of 1% of probability by Tukey test.

Fig. 1-Means and standard deviations of L_3 of *Strongyloides westeri*, not preyed, recovered from 2% water-agar (2% WA) medium by Baermann method after seven day of treatments with the isolates *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) and *Arthrobotrys robusta* (I-31) and in control group (without fungus).



Means followed by at least one letter, capital letter, in line, not differing among them at level of 1% of probability by Tukey test.

Fig. 2-Linear regression curves of infective larvae (L_3) of *Strongyloides westeri* recovered from Petri dishes in treatments with the isolate *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) and *Arthrobotrys robusta* (I-31) and control (without fungus) due to time.

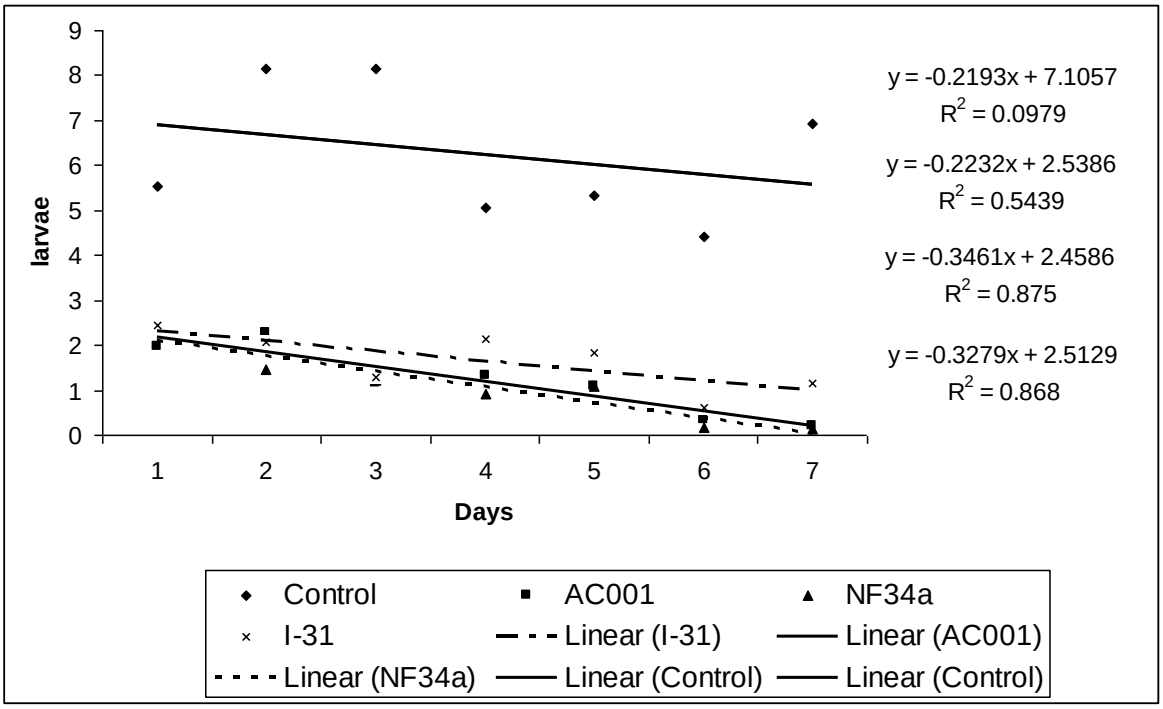
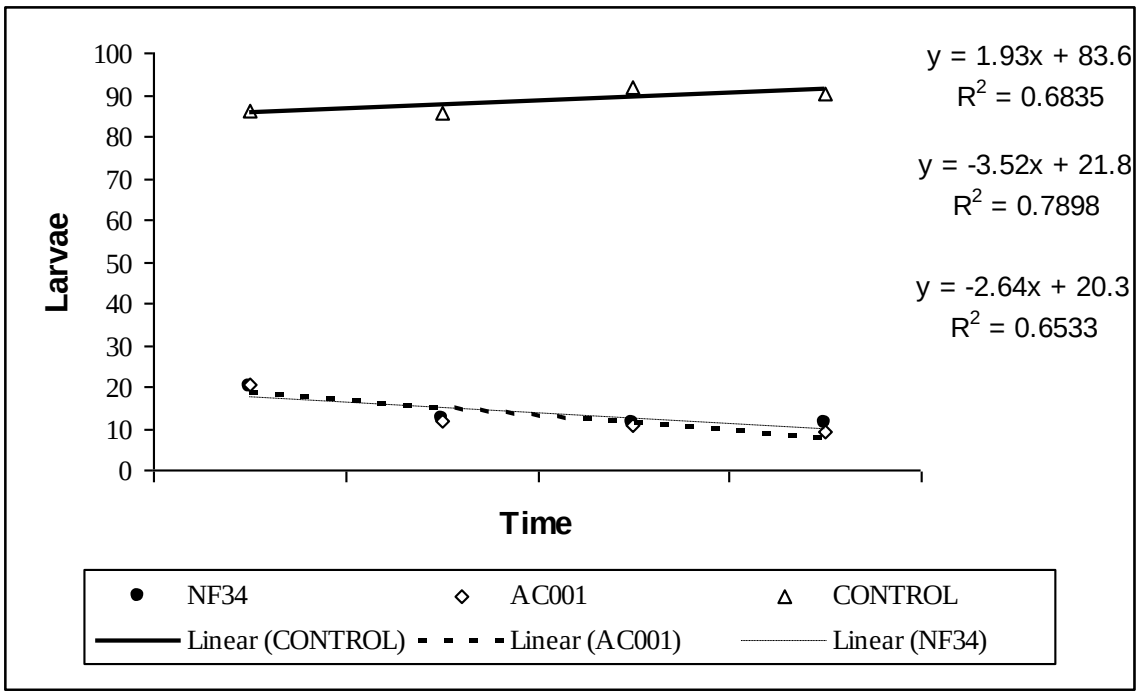


Table 2- Mean values of infective larvae number of *Strongyloides westeri* recovered from Petri dishes, filled with equine feces, sampled in timelines 12, 24, 48, 72 hours after the treatment with isolates *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34), and in control (without fungi).

Isolates	Time			
	12 h.	**24 h.**	**48 h.**	**72 h.**
AC001	20.0_A	12.1_A	11.2_A	11.5_A
NF34	20.5_A	11.7_A	10.7_A	9.2_A
Control	85.9_B	85.9_B	91.6_B	90.4_B

Means followed by at least one letter, capital letter, in line, not differing among them at level of 1% of probability by Tukey test.

Fig. 3-Linear regression curves of infective larvae (L_3) of *Strongyloides westeri* recovered from Petri dishes regarding collections (12 h, 24 h, 48 h and 72 h) in treatments with the isolate *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) and control (without fungus) due to time.



CAPÍTULO II

CONTROL OF *Strongyloides westeri* LARVAE BY NEMATOPHAGOUS FUNGI AFTER PASSAGE THROUGH THE GASTROINTESTINAL TRACT OF DONKEYS

Revista Brasileira de Parasitologia Veterinária v.21, n.2, p.156-160, 2012

RESEARCH NOTE

CONTROL OF *Strongyloides westeri* LARVAE BY NEMATOPHAGOUS FUNGI AFTER PASSAGE THROUGH THE GASTROINTESTINAL TRACT OF DONKEYS

CONTROLE DE LARVAS DE *Strongyloides westeri* POR FUNGOS NEMATÓFAGOS APÓS TRÂNSITO GASTRINTESTINAL EM JUMENTAS

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ABSTRACT

Strongyloides westeri is the most prevalent nematode among equines aged up to four months and causes gastrointestinal disorders. The objective of this study was to observe the control of infective *S. westeri* larvae (L_3) by the nematophagous fungi *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34) after passage through the gastrointestinal tract of in donkeys. Twelve dewormed donkeys that were kept in stables were used. Two treatment groups each comprising four animals received orally 100 g of pellets made of sodium alginate matrix containing a mycelial mass of either *D. flagrans* (AC001) or *M. thaumasium* (NF34). The control group consisted of four animals that received pellets without fungus. Feces samples were then collected from the animal groups at different times (after 12, 24, 48 and 72 hours). These feces were placed in Petri dishes containing 2% water-agar medium and 1000 L_3 of *S. westeri*. AC001 and NF34 isolates showed the ability to destroy the L_3, after gastrointestinal transit, thus demonstrating their viability and predatory activity.

KEY WORDS: Nematophagous fungi, *Duddingtonia flagrans*, *Monacrosporium thaumasium*, *Strongyloides westeri,* Donkeys.

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RESUMO

O *Strongyloides westeri* é o nematóide de maior prevalência entre equideos com idade até quatro meses, causando distúrbios gastrintestinais. O objetivo do presente trabalho foi observar o controle de larvas infectantes (L_3) de *Strongyloides westeri* pelos fungos nematófagos *Duddingtonia flagrans* (AC001) e *Monacrosporium thaumasium* (NF34) após trânsito gastrintestinal em jumentas. Foram utilizados 12 jumentas, estabulados e previamente vermifugados. A seguir dois grupos tratados contendo cada um 4 animais receberam por via oral 100 g de péletes em matriz de alginato de sódio contendo massa miceliana dos fungos *D. flagrans* (AC001) ou *M. thaumasium* (NF34). O grupo controle foi constituído de 4 animais que receberam péletes sem fungo. A seguir, amostras de fezes dos grupos de animais foram coletadas em distintos intervalos de horas (12, 24, 48 e 72). Estas fezes foram vertidas em placas de Petri contendo meio sólido ágar-água 2% e 1000 L_3 de *S. westeri.* Os isolados AC001 e NF34 apresentaram capacidade de destruir as L_3, após o trânsito, demonstrando sua viabilidade e atividade predatória.
PALAVRAS-CHAVES: Fungos nematófagos, *Duddingtonia flagrans*, *Monacrosporium thaumasium*, *Strongyloides westeri,* jumentas.

According to FAO (2008), as cited by Morrow et al. (2011), there are approximately 43 million donkeys (*Equus asinus*) in the world, serving mainly as a transportation resource in developing countries (PRITCHARD et al., 2005). Getachew et al. (2010) reported that many helminth species are found in these animals and cause direct damage to their health. Nevertheless, there are insufficient studies with data on strategic control of gastrointestinal helminthic parasites that affect this equine species (MATTHEE et al., 2002; VENEZIANO et al., 2011). Within this context, *Strongyloides westeri* is a relatively frequent nematode in young animals that may cause gastrointestinal tract disorders (URQUHART et al., 1998). Although equines generally develop satisfactory immunity to this infection between the ages of 15 and 23 weeks (SOULSBY, 1982), animals older than six months may still acquire the infection, such that 30% of them are infected (WELLS et al., 1998). Helminthic parasitism in equines, as well as in other domestic animals, is combated through using anthelmintics. However, these drugs may not present a satisfactory effect, given that parasitic resistance is already widespread around the world (BRAGA et al., 2009).

Thus, integrated control of helminthoses can be seen as a new approach, since biological control can be used synergistically with chemical control (BRAGA et al., 2009). Biological control is centered on using nematophagous fungi that can act as predators, ovicidal agents and endoparasites. These fungi, which are mainly predators, have been shown to be effective for reducing nematode populations, both in laboratories and under field conditions. *Duddingtonia flagrans* and *Monacrosporium thaumasium* are predators and have been used worldwide to combat the infective larvae of nematode parasites of productive domestic animals (PAZ-SILVA et al., 2011; BRAGA et al., 2011).

The objective of the present study was to evaluate the control of infective *Strongyloides westeri* larvae (L_3) by nematophagous fungi after gastrointestinal transit in donkeys (*Equus asinus*).

To obtain *S. westeri* L_3, fecal cultures were performed on positive feces from naturally infected young donkeys. Next, these larvae were classified in accordance with the criteria established by Soulsby et al. (1982). In order to induce formation of fungal mycelia of *D. flagrans* (AC001) and *M. thaumasium* (NF34), culture discs of approximately 4 mm in diameter, in 2% water-agar, were transferred to 250 ml Erlenmeyer flasks containing 150 ml of liquid YPG medium (glucose, peptone and sodium yeast extract), and were kept under continual stirring at 120 rpm in the dark and at a temperature of 26 °C for 10 days. After this period, the mycelia were removed, filtered and weighed on an analytical balance.

In the *in vivo* assay, 12 female donkeys were used, with a mean weight of 240 kg. They had previously been dewormed by means of vermifuge for equines at an oral dose of 200 µg/kg live weight of 1% ivermectin and 6.6 mg/kg live weight of pyrantel pamoate (Centurion Vallé®, Montes Claros, Minas Gerais, Brazil). This was done 14 days before they received pellets containing a mycelial mass of isolates of *D. flagrans* (AC001) or *M. thaumasium* (NF34), or pellets without fungus (control).

The animals were separated into three groups (one group treated with the isolated AC001, one group treated with the isolate NF34 and a control group), with four animals in each group. In the groups treated with AC001 and NF34, each animal received 100 g of pellets in a single dose containing a mycelial mass of fungus. For this, each of the fungi was mixed into 100 g of

commercial feed for horses. The control group received a single administration of 100 g of pellets without fungus.

After administration of the fungi, fecal samples were collected from the animal groups at different times (after 12, 24, 48 and 72 hours). These samples were then homogenized, and 4 g aliquots of feces were placed in Petri dishes of 9 cm in diameter, containing 2% water-agar (2% WA). These dishes were placed in an incubator at 25 °C, in the dark. Each Petri dish of the groups tested (treated and control groups) contained 1,000 L_3 of *S. westeri*. At each collection time, five repetitions were performed. To prove that the fungi tested actually passed through the gastrointestinal tract, and to identify them, the classification keys for fungal structures (conidia and/or chlamydospores) proposed by Van Oorschot (1985) and Liu and Zhang (1994) were used. The Petri dishes were viewed every day. Subsequently, on the fifteenth day, the L_3 that had not been destroyed by the fungus were recovered from the Petri dishes by means of the Baermann technique. The data obtained were subjected to analysis of variance (F test) and then regression analysis. The means were compared using the Tukey test at the 1% level of probability.

It was observed that the fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34) destroyed the *S. westeri* L_3, after passage through the gastrointestinal tract of the donkeys. At each of the collection times studied, the following percentage reductions were found: 81.2% and 81.1% (12 hours); 62.7% and 87.2% (24 hours); 78.6% and 76.7% (48 hours); and 85.3% and 92.2% (72 hours), for the isolates AC001 and NF34, respectively, in comparison with the control group (Figure 1). At the collection time of 72 hours, both isolates (AC001 and NF34) showed higher predatory activity and, consequently higher percentage reductions in the L_3 recovered from *S. westeri*.

On the other hand, the coefficients of the linear regression curves for *S. westeri* L_3 recovered from Petri dishes, relating to the collections as a function of time were: 5.54 for the controls, -1.25 for *D. flagrans* (AC001) and -1.06 for *M. thaumasium* (NF34). In the feces of the treated groups, conidia and chlamydospores were identified according to the fungal species tested (*D. flagrans* and *M. thaumasium*).

Araújo et al. (2004) mentioned that using nematophagous fungi for biological control of gastrointestinal parasites of domestic animals may reduce the soil contamination by acting directly on the infective larvae present in the

environment. On the other hand, although horses and donkeys generally harbor the same genera of gastrointestinal helminthic parasites, there are no studies with enough data regarding strategic control of helminths in donkeys. In this context, Araujo et al. (2010) reported that three fungal genera (*Arthrobotrys*, *Duddingtonia* and *Monacrosporium*) were efficient at destroying *S. westeri* L_3 in an *in vitro* assay. In this paper, the results showed that there was no difference ($p > 0.05$) in predatory activity between the fungi tested and therefore either of the isolates could be used in *in vivo* tests. This premise provided the justification for conducting the present study, and showed the need for knowledge regarding alternative approaches towards control of helminth parasites in donkeys. In addition, Araujo et al. (2010) used horses for the in vivo test, thus showing that the fungi *D. flagrans* and *M. thaumasium* have also been effective in passing through the gastrointestinal tract of these animals. Nonetheless, despite being in the same group of animals (equines), donkeys are a different species and thus, studies that can demonstrate alternative control methods for endoparasites, in particular in relation to *S. westeri*, are important.

Several studies have been conducted with regard to biological control of nematode parasites of horses, both *in vitro* and under natural conditions, which once again denotes the need to extrapolate these studies to other species of equines, such as donkeys. Tavela et al. (2011) studied the fungus *M. thaumasium* (NF34) in the field, administered to horses, and demonstrated that it was effective on the larvae of cyathostomins through decreasing the recurrence of helminth infections. Similar results, thereby confirming the action of this fungus, were also found in the present work, which proved that this isolate remained viable after passage through the gastrointestinal tract of donkeys.

The efficacy of the fungus *D. flagrans* (AC001) was also demonstrated in a study by Braga et al. (2009) that consisted of a six-month field test using weekly doses of pellets containing this fungus, among horses. A difference in parasite loads ($p < 0.05$) was recorded between the animals in the treated group and those in the control group. This result is also in accordance with the findings from the present study relating to the action of the isolate AC001 on *S. westeri* L_3.

The regression curve results demonstrated that the two fungal isolates tested had a negative linear correlation coefficient. This inverse correlation

(negative) between the variables proved that the fungal isolates continued to have a viable predatory capacity after passage through the gastrointestinal tract of these domestic animals. This information is in agreement with the study on horses by Assis and Araujo (2003), who found a regression curve with a negative value.

The results presented here justify the need to conduct studies in the field, with longer intervals, in order to observe the efficiency of the fungus *D. flagrans*, or even *M. thaumasium*, for environmental control of nematodes in donkeys, which may contribute towards a better and more integrated approach to control of helminths in this species of domestic animal.

Acknowledgements

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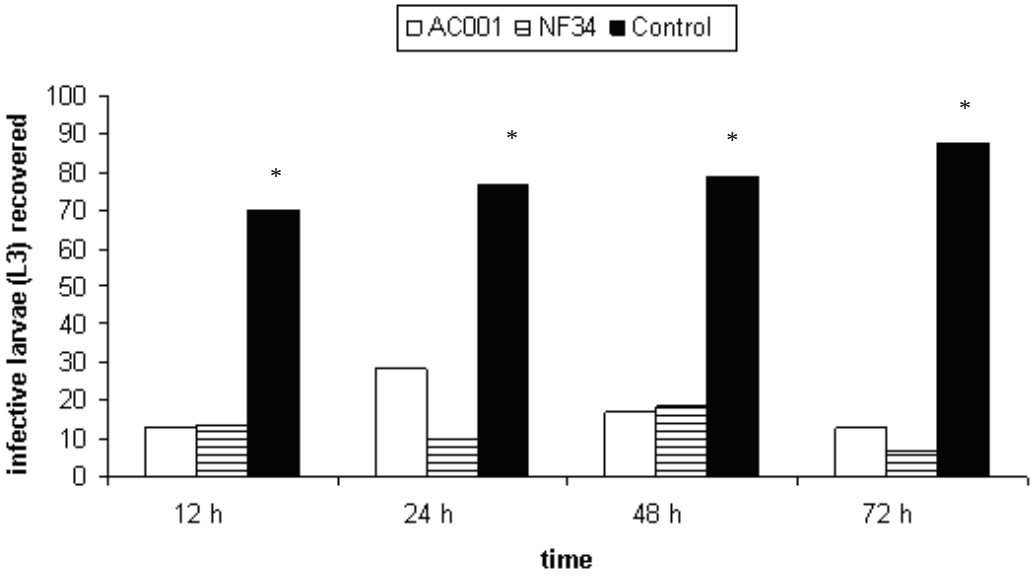

Figure 1. Mean numbers of infective larvae (L_3) of *Strongyloides westeri* that were recovered from 2% water-agar medium by means of the Baermann method on the fifteenth day of treatment with the fungal isolates *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34), and in the control group (without fungus). Asterisk denotes difference ($p < 0.01$) between the isolates tested and the control group at the times studied.

CAPÍTULO III

PREDATORY ACTIVITY OF CHLAMYDOSPORES OF THE FUNGUS *Pochonia chlamydosporia* ON *Toxocara canis* EGGS IN LABORATORY CONDITIONS

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RESEARCH NOTE

PREDATORY ACTIVITY OF CHLAMYDOSPORES OF THE FUNGUS *Pochonia chlamydosporia* ON *Toxocara canis* EGGS IN LABORATORY CONDITIONS

ATIVIDADE PREDATÓRIA DE CLAMIDÓSPOROS DO FUNGO *Pochonia chlamydosporia* SOBRE OVOS DE *Toxocara canis* EM CONDIÇÕES LABORATORIAIS.

Juliana Milani Araujo[1a]; Jackson Victor de Araújo[1]; Fabio Ribeiro Braga[1]; Sebastião Rodrigo Ferreira[1]; Alexandre de Oliveira Tavela[1]

ABSTRACT

This work assessed the predatory activity of chlamydospores of *Pochonia chlamydosporia* (isolates VC1 and VC4) against *Toxocara canis* eggs in a 15-day *in vitro* assay. A total of 1,000 eggs of *T. canis* were placed in Petri dishes containing 2% water-agar medium with different concentrations of chlamydospores (1,000, 10,000 and 100,000) from each fungal isolate of *P. chlamydosporia* (treated groups) and 1,000 eggs in Petri dishes without fungus (control group). Egg counts determined ovicidal activity, which was classified as type 1, type 2 and type 3. Significant differences (P<0.01) were found for egg destruction when compared with the control. The highest percentage of egg destruction was found in plates containing 100,000 chlamydospores (68.5% for VC1 and 70.5% for VC4). Chlamydospores of *P. chlamydosporia* effectively destroyed *T. canis* eggs and can be used as an alternative method to control this parasite.

KEY WORDS: Nematophagous fungi, *Pochonia chlamydosporia*, *Toxocara canis*, nematode eggs, chlamydospores.

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RESUMO

O objetivo deste trabalho foi utilizar clamidósporos do fungo *Pochonia chlamydosporia* (VC1 e VC4) na destruição de ovos de *Toxocara canis*. No ensaio *in vitro*, realizado no intervalo de 15 dias, em cada placa de Petri foram vertidos 1.000 ovos de *T. canis* em 1.000, 10.000 ou 100.000 clamidósporos do fungo (grupos tratados). Foram realizadas as contagens para verificar a atividade ovicida em três níveis de efeito, tipo 1; tipo 2 e tipo 3. Os resultados demonstraram que houve diferença ($P<0,01$) na destruição dos ovos em relação aos ovos observados nas placas do grupo controle. O maior percentual de ovos destruídos foi observado nas placas contendo 100.000 clamidósporos (VC1 e VC4). Clamidósporos do fungo *P. chlamydosporia* destruíram os ovos de *T. canis* podendo contribuir no futuro para o combate aos ovos deste parasito.

PALAVRAS-CHAVE: Fungos nematófagos, *Pochonia chlamydosporia, Toxocara canis*, ovos de nematoides, clamidósporos.

Toxocara canis is the causative agent of diseases known as visceral larva migrans and ocular larva migrans in humans (REY, 2008). In addition, the close association between men and domestic animals is considered a public health hazard (VASCONCELLOS et al., 2006). The increasing number of pets has led to a narrowing of contact with humans, increasing the risk of exposure to zoonosis (GENNARI et al., 1999). These authors also reported that children, especially in preschool age, are commonly infected by the ingestion of embryonated eggs. Dogs acquire *T. canis* by ingesting eggs that contain L_3 or by preying upon rodents, reptiles and birds that may be infected and act as paratenic hosts. Transplacental and lactogenic migration also account for infection (ALDAWEK et al., 2002; LEITE et al., 2004; MONTEIRO, 2010; BOWMAN, 2010). The use of anthelmintic drugs is the common approach to control adult parasites in hosts. However, the literature mentions resistance to some drugs used to control the infection in these animals, especially the pyrantel (KOOP et al., 2008). There are no reports, however, on the control of infective forms, larval and eggs, in the environment (REY, 2008).

Nematophagous fungi have been studied in laboratory conditions as an alternative for the control of *T. canis* eggs and other potentially zoonotic helminths (ARAUJO et al., 2009; CARVALHO et al., 2010). *Pochonia* is a

chlamydospore-producing genus capable of withstanding such conditions. There are, however, few studies on the use of chlamydospore structures for the destruction of helminth eggs *in vitro* (BRAGA et al., 2011). These studies demonstrated that species of the genus *Pochonia* are inoffensive to animals and humans and are able to destroy helminth eggs in a short time using special pressing organs called appresoria.

The aim of this study was to assess *in vitro* ovicidal activity of different concentrations of chlamydospores from each fungal isolate of *Pochonia chlamydosporia* on *T. canis* eggs.

Toxocara canis eggs were dissected from adult specimens expelled from naturally infected dogs and morphologically analysed by light microscopy (10x objective) according to Urquhart et al. (1998). The eggs were washed in distilled water, centrifuged (1,000 ***g*** for 5 minutes) and incubated at 25°C for 14 days in a solution containing 0.005% streptomycin sulphate and 0.01% chloramphenicol. This procedure was used to obtain embryonated eggs. This assay was based on the protocol described by Araujo et al. (1995).

Chlamydospores were obtained from two *P. chlamydosporia* isolates (VC1 and VC4). This fungus has been maintained in continuous culture at the laboratory of Parasitology of the Veterinary Medicine Department, Federal University of Viçosa-MG, Brazil. The fungal isolates were grown in Petri dishes containing 20 ml of YPSSA medium (4 g yeast extract; 1 g K_2HPO_4; 0.5 g $MgSO_4$; 20 g soluble starch; 20 g agar; water to complete 1 litre of solution) at 25°C in the dark for 10 days. Dish surfaces were rinsed (10 ml of distilled water) using a paintbrush. The suspension obtained was sieved into a plastic container to remove mycelium fragments. Chlamydospores were recovered and quantified using a Neubauer chamber following the identification methods proposed by Gams and Zare (2001).

The following protocol established by Braga et al. (2011) was used. The experiment comprised six treatments consisting of 1,000 eggs of *T. canis* plated on 9 cm Petri dishes containing 2% water–agar (2% WA) medium with the different concentrations of chlamydospores (1,000, 10,000 and 100,000) from each fungal isolate (VC1 or VC4). A control group, containing 2% WA consisting of 1,000 eggs of *T. canis*, was established for each concentration of chlamydospores, with six repetitions.

At the end of the experimental period (15 days) 100 eggs of *T. canis* were removed from each dish (treated and control dishes) and examined for ovicidal activity (LYSEK et al., 1982): type 1, lytic effect without morphological damage to eggshell, with hyphae adhered to the shell; type 2, lytic effect with morphological alteration of embryo and eggshell, without hyphal penetration through the eggshell; and type 3, lytic effect with morphological alteration of embryo and eggshell, as well as hyphal penetration and internal colonization. Eggs were collected from each plate containing the isolate and from the control without fungus, as described by Araújo et al. (1995), placed in glass slides with a drop of 1% Amam blue and evaluated under a 40x lens. Data were analysed by the Friedman non-parametric test at the 1% probability level (AYRES et al., 2003).

Pochonia chlamydosporia chlamydospores were effective in destroying *T. canis* eggs. There was significant difference ($P<0.01$) in egg destruction at the end of the experiment between the treatment and control groups (Fig. 1A–F).

For concentrations of 1,000 chlamydospores, percentage egg destruction was 48.0% (VC1) and 50.0% (VC4); for concentrations of 10,000 chlamydospores percentage egg destruction was 66.0% (VC1) and 69.0% (VC4). The highest percentages of egg destruction were found with the concentration of 100,000 chlamydospores of both isolates (68.5% for VC1 and 70.5% for VC4) at the end of 15 days (Table 1).

Chlamydospores of the isolates VC1 and VC4 used in the three concentrations (1,000, 10,000 and 100,000) destroyed parasite eggs effectively. The use of chlamydospores against gastrointestinal helminth parasites has been reported for many years with positive results of effective destruction (LARSEN, 1999). There is, however, a lack of studies concerning ovicidal activity of chlamydospores under controlled conditions (BRAGA et al., 2011). The use of nematophagous fungi as biological control agents is promising, although contamination during chlamydospore production is still a serious obstacle (ARAÚJO et al., 2004).

Comparing the results found with those reported by Braga et al. (2011), it can be affirmed that: (1) both studies used increasing concentrations of chlamydospores that effectively destroyed eggs *in vitro*; (2) in this study, eggs of an ascarid parasite were used that were morphologically different from the

eggs of cestoda used by Braga et al. (2011), justifying the need for further studies to identify the possible obstacles to the use of *P. chlamydosporia*, based on the fact that geohelminths are present in large quantities in the environment; (3) 2% WA medium used in the *in vitro* test of both studies shows that *P. chlamydosporia* has low nutritional requirements, although, as Costa et al. (2001) pointed out, it does not reflect the natural environmental conditions; (4) finally, we confirmed that the ovicidal activity of *P. chlamydosporia* remains over long time periods, as reported by Braga et al. (2011). This is an important finding, because, in general, helminth eggs have completely different developmental stages. In this study, the decision to use only one time interval (15 days) was based on the prepatent period of 15 to 21 days for *T. canis*. The results suggest that the *P. chlamydosporia* isolates VC1 and VC4 can be used in the biological control of ascarid eggs.

Eggs of *T. canis* were most effectively destroyed at the highest concentration of chlamydospores. This result was already expected, since Braga et al. (2011) also reported that eggs of *Taenia taeniaeformis* were most effectively destroyed at the concentration of 20,000 chlamydospores. On the other hand, in a richer culture medium, such as 2% cornmeal–agar (BRAGA et al., 2011), chlamydospore production may be better observed, suggesting perhaps a greater ovicidal activity.

Various reports have shown the efficacy of the isolates VC1 and VC4 with no difference in egg destruction. There are, however, few studies on the effectiveness of chlamydospores from these fungal isolates. Carvalho et al. (2010) showed that *P. chlamydosporia* isolates grown in 2% WA were effective in destroying *T. canis* eggs using time intervals of 7, 14 and 21 days, with ovicidal activity of 20.3% (VC1) and 21.7% (VC4) at the end of the experiment. Frassy et al. (2010) using those same isolates of *P. chlamydosporia* fungus on *T. canis* eggs registered percentage values for type 3 effect of 43.3% and 47.3%, respectively, after 15 days. However, these studies were performed with the fungal isolates grown in Petri dishes containing the 2% WA medium. However, it is important to report that in the present work, the fungal isolates (VC1 and VC4) were evaluated on the form of chlamydospores, vegetative structures of resistance, which germinated on the plates, and destroyed eggs of *T. canis* eggs. Hence, one suggests that the use of chlamydospores of *P.*

chlamydosporia fungus could present better results in the biological control of that nematode.

This is the first report on using different concentrations of chlamydospores of *P. chlamydosporia* in the destruction of *T. canis* eggs. All the concentrations of chlamydospores of isolated VC1 and VC4 were efficient in the destruction of the eggs, but further studies are required for the future use of this fungus in the biological control of *T. canis* eggs.

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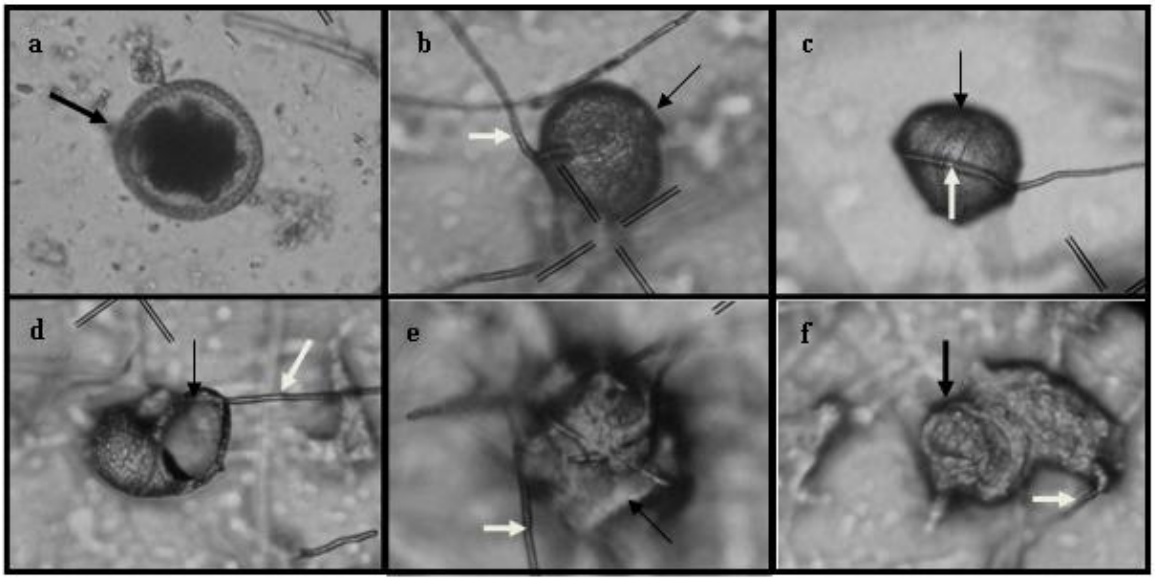

Fig. 1. (A) *Toxocara canis* eggs (black arrow), control. (B) Hyphae of the fungus *Pochonia chlamydosporia* (white arrow) attached to the eggshell, a type 1 effect. (C) *P. chlamydosporia* hyphae of the fungus (white arrow) causing deformity in the *T. canis* egg (black arrow), a type 2 effect. (D)–(F) *T. canis* eggs (black arrow) and hyphae of *P. chlamydosporia* destroying the eggs (white arrow), a type 3 effect.

Table 1 – Percentages and standard deviation for types 1, 2 and 3 effects of ovicidal activity against *Toxocara canis* eggs of *P. chlamydosporia* (VC1 and VC4) at concentrations of 1000, 10000 and 100000 chlamydospores and control group in 2% water-agar (2% WA), after 15 days of interaction.

Clamydospores	Fungi	Effect at 15 days		
		Type 1*	Type 2**	Type 3***
1000	VC1	12.0^{A}±18.3	40.0^{A} ±10.5	48.0^{A}±19.8
	VC4	16.0^{A}±19.5	34.0^{A} ±13.4	50.0^{A}±25.8
	Control	0^{B}±0	0^{B}±0	0^{B}±0
10000	VC1	0.8^{A}±1.7	34.2^{A}±17.9	66.0^{A}±11.1
	VC4	1.1^{A}±2.4	29.9^{A}±16.4	69.0 ^{A}±14.6
	Control	0^{B}±0	0^{B}±0	0^{B}±0
100000	VC1	0.5^{A}±1.5	31.0^{A}±18.9	68.5^{A}±19.3
	VC4	0.9^{A}±2.8.	28.6^{A}±18.0	70.5^{A}±16.8
	Control	0^{B}±0	0^{B}±0	0^{B}±0

Percentages followed by same letter in the same column are not significantly different (P>0.01) by the Friedman test.
*Physiological and biochemical effect, without morphological damage to the eggshell, with hyphae adhered to the shell.
**Lithic effect with morphological change of the eggshell and the embryo, without hypha penetration through the shell.
***Lithic effect with morphological change of the shell and the embryo, with hyphal penetration and internal colonization of the egg.

CAPÍTULO IV

SURVIVAL OF *Pochonia chlamydosporia* IN THE GASTROINTESTINAL TRACT OF EXPERIMENTALLY TREATED DOGS

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Survival of *Pochonia chlamydosporia* in the gastrointestinal tract of experimentally treated dogs

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Abstract

The predatory capacity of the nematophagous fungus *Pochonia chlamydosporia* (isolate VC4) after passage through the gastrointestinal tract of dogs was assessed *in vivo* against *Toxocara canis* eggs. Twelve dogs previously wormed were divided into two groups of six animals and caged. The treatments consisted of a fungus-treated group (VC4) and a control group without fungus. Each dog of the fungus-treated group received a single 4g dose of mycelial mass of *P. chlamydosporia* (VC4). Fecal samples from animals of both groups (treated and control) were collected at five different times (6, 12, 24, 36, and 48 hours) after fungal administration, and placed in Petri dishes. Each Petri dish of both groups for each studied time interval received approximately 1000 *T. canis* eggs. Thirty days after the fecal samples were collected, approximately one hundred eggs were removed from each Petri dish of each studied time interval and evaluated by light microscopy (LM) and Scanning Electron Microscopy (SEM). Microscopy examination of plates inoculated with the fungus showed that the isolate VC4 was able to destroy the *T. canis* eggs with destruction percentages of 28.6% (6 hours), 29.1% (12 hours), 32.0% (24 hours), 31.7% (36 hours), and 37.2% (48 hours). These results suggest that *P. chlamydosporia* can be used as a tool for the biological control of *T. canis* eggs in feces of contaminated dogs.

Keywords: Nematophagous fungus, *Pochonia chlamydosporia, Toxocara canis*, biological control.

1. Introduction

Soil-transmitted helminths (STH) are commonly found in areas of hot and humid climate, especially where hygienic conditions are poor. Helminthosis, in general, represent a major public health problem (OMS/WHO, 2006; Bóia et al., 2006). The literature mentions that *Toxocara* sp. (ascarids) and hookworms are the most common species among the animal population, but they also bring serious risks to the human health because of the close relationship between humans and animals. The presence of *Toxocara* sp. eggs in public squares represents a public health problem, since the species is the causal agent of Visceral Larva Migrans (VLM). Human infection by *T. canis* occurs primarily by ingestion of eggs or larvae present in contaminated soils, and hands contaminated with feces of infected animals (Robertson et al., 2000; Rey, 2008).

Moist and shaded soils provide a favorable environment for the prevalence and development of embryonic eggs, which, under favorable conditions, remain infective in the soil for several months. There are a number of measures to control helminthosis, but the main strategy is to interrupt the parasite's life cycle (Rey, 2008). Studies on the destruction of STH eggs using natural processes are still in early stages, but represent a viable alternative that can help to control this epidemiological important species, if used with other preventive measures (Araujo et al., 2009a).

Natural antagonists in the soil, such as ovicidal fungi, can destroy STH eggs (Lysek et al., 1982). These fungi have been tested for the control of infectious forms of helminths eggs, including gastrointestinal parasites of domestic animals and humans. However, their mechanism of action has not been fully elucidated (Braga et al., 2011). *P. chlamydosporia* is an ovicidal fungus, which has been studied in a variety of interactions with helminth eggs in laboratorial conditions (Araujo et al., 2009b; Carvalho et al., 2010). By this reasoning, several of these studies produced encouraging results, but there is general agreement that much remains to be investigated before the effective use of this fungus for the biological control of STH eggs. On the other hand, as Gronvold et al. (1996) discussed, a fungus must be able to withstand the passage through the gastrointestinal tract of domestic animals to be considered as a potential biological control. *Pochonia chlamydosporia* has been already

reported of being capable of destroying *T. canis* eggs when grown *in vitro* on solid medium (Frassy et al., 2010; Carvalho et al., 2010). In addition, *Pochonia chlamydosporia* maintained its ovicidal capacity even after passage through the gastrointestinal tract of dogs.

This work aimed to examine the predatory capacity of the nematophagous fungus *Pochonia chlamydosporia* (VC4) against *Toxocara canis* eggs after passage through gastrointestinal tract of experimentally treated dogs.

2. Material and methods

2.1. Fungus

An isolate of *P. chlamydosporia* (VC4) originated from a Brazilian soil was kept in test tubes with 2% corn-meal-agar (2% CMA), at 4°C, in the dark. This isolate has been deposited at the mycology collection of the Parasitology Laboratory of Veterinary Department, Federal University of Viçosa, State of Minas Gerais, Brazil. Culture disks (approximately 5 mm) of fungal isolates kept on 2% CMA were transferred to Petri dishes containing 2% water-agar (2% WA) and incubated at 28°C in the dark for seven days.

After the fungal growth on the dishes, new culture disks (approximately 5 mm) were transferred into 250 mL Erlenmeyer flasks containing 150 ml of liquid potato dextrose (Difco), pH 6.5, under agitation (120 rpm), in the dark at 26^{o}C for 10 days. After this period, the mycelial mass was removed, according to the technique described by Carvalho et al. (2009).

2.2. Obtaining eggs of Toxocara canis and in vivo assay

T. canis eggs were obtained from the dissection of adult females collected from feces of infected dogs, analyzed for their morphology and identified according to the parameters described by Urquhart et al. (1998). The eggs were washed in distilled water (ten times) and at each time centrifuged at 1000 X g for five minutes. The supernatant was discarded after each centrifugation. The eggs were then incubated at 25°C for 14 days with a solution containing 0.005% streptomycin sulfate and 0.01% chloramphenicol as described by Araújo et al. (1995).

In the experimental assay, 12 adult mongrel male dogs, average weight 12 kg (10-15kg) were used in the experiment. The dogs were treated with pyrantel pamoate anthelmintic (14.4 mg/kg), Febantel (15mg/kg), and Praziquantel (5mg/kg) - (Drontal® Plus1 Bayer, São Paulo-SP, Brazil) about 15 days before receiving the fungal mycelium containing the isolate (VC4) of *P. chlamydosporia.* Then, the dogs and divided into two groups of six animals and caged. The treatments consisted of a fungus-treated group (VC4) and a control group without fungus. The dogs received water and commercial dog food (18% crude protein, 6% ethereal extract, 6.5% fiber and 12% moisture) *ad libidum.*

In the assay *in vivo*, each animal in the treated group received 4 grams of fungal mycelium containing the isolate VC4 (*P. chlamydosporia*) mixed in equal proportions to canned wet commercial dog food (8% crude protein, 4% ethereal extract, 1.5% fiber, and 80% moisture). Dogs of the control group received oral portions of wet commercial dog food without fungal mycelia.

Fecal samples were collected at intervals of six, 12, 24, 36, and 48 hours after treatment administration from each dog of both groups and homogenized. All the procedures performed with the animals were done according to the rules established by the Ethic Committee of Veterinary Department, Federal University of Viçosa. For the *in vitro* assay, about 2 grams of faeces were taken from each sample and placed in petri dishes (5 cm diameter) containing 2% WA. Each Petri dish of both groups, and for each studied time interval, was inoculated with approximately 1000 *T. canis* eggs to evaluate the ovicidal activity after passage through the gastrointestinal tract of the dogs. The dishes of both groups and time intervals were incubated in an oven at 25^{o}C in the dark for ten days, according to methodology described by Carvalho et al. (2009). Six replicates were performed per group for each established time. Petri dishes of the treated and control groups were observed daily for detection of *P. chlamydosporia* (VC4) fungal structures such as conidia, conidiophores, and chlamydospores, according to the classification key by Gams and Zare (2001).

Thirty days after feces collection, about 100 eggs were removed from each Petri dish for each time interval, as described by Araújo et al. (1995), and evaluated by light microscopy (LM) and Scanning Electron Microscopy (SEM) according to the parameters established by Lysek et al. (1982): effect of type 1; type 2 and type 3 (egg destruction). Data were analyzed using the nonparametric statistical test of Friedman at 5% probability (Ayres et al., 2003).

3. Results

Table 1 shows the results recorded for the ovicidal activity (effects 1, 2, and 3) of *P. chlamydosporia* (VC4) in the studied intervals of the fecal collection (6, 12, 24, 36 and 48 hours) for the treated group ($p<0.01$) compared with the control group. For the type 3 (egg destruction), the percentages found throughout the collection intervals were: 28.6% (6h), 29.1% (12h), 32.0% (24h), 31.7% (36h), and 37.2% (48h). No destroyed eggs were observed in the control group. The highest destruction percentages were observed in the collection intervals of 24 and 48 hours after mycelial mass administration to the treated group.

It was also observed that *P. chlamydosporia* (VC4) produced chlamydospores in the Petri dishes related to the fecal samples of the treated group. However, the same fact was not observed in the dishes of the control group. From the third day after oral administration of mycelium, some structures (typical conidia) were observed, which confirmed the presence of *P. chlamydosporia* in Petri dishes from the treated group, referring to samples collected 6 and 24 hours after mycelial mass administration. Conidia were observed after 6 days in the plates of the intervals 24, 36 and 48 hours. Scanning electron microscopy showed the fungus (VC4) hyphae colonizing the surface and the inside of the eggs of *T. canis,* with subsequent destruction (Fig. 2A-D).

4. Discussion

There are many reports in the literature on studies that demonstrate the use of nematophagous fungi as a possible alternative in the control of STH eggs, since these fungi reduce the soil contamination, acting directly in the infective forms (Carvalho et al., 2009; Braga et al., 2011). However, the search for biological control agents is done before the evaluation of their ability to withstand environments unfit for survival, being the gastrointestinal tract of domestic animals a particular case (Larsen, 1999; Braga et al., 2010). In this study, the isolate of *P. chlamydosporia* (VC4) survived the passage through the gastrointestinal tract of dogs, germinated in the faeces, produced chlamydospores, and kept its ovicidal ability against *T. canis* eggs (Fig.1), confirming its potential for biological control.

The ovicidal activity of *Pochonia chlamydosporia* on STH egg has been widely studied in tests with 2% WA. In a *in vitro* study, Frassy et al. (2010) observed that the isolate VC4 grown in 2% WA showed a mean destruction percentage of 47.3% on *T. canis* eggs (type 3 effect) after fifteen days. However, Costa et al. (2001) reported that the potential of nematophagous fungi in 2% WA does not represent the conditions existing in soils with their complex habitats. In this context, the authors of this paper point out the mean destruction percentage of 31.7% on *T. canis* eggs in an environment similar to that found in nature, in this case with the fecal samples. There is only one report in the literature on the passage of *P. chlamydosporia* fungus through the gastrointestinal tract of horses (Braga et al., 2010).

However, it is important to highlight that, in the study of Braga et al. (2010), the fungus *P. chlamydosporia* (VC4) was administered orally and successfully in horses in form of pellets. In the present work, VC4 was applied successfully as mycelium mixed to moist dog food with a mean percentage of 31.7% (type 3). Regarding the results of these collection intervals, they were similar. Braga et al. (2010) reported mean percentage of 29.1% for type 3 effect. These comparisons suggest that *P. chlamydosporia* could be used in the form of mycelium and/or pellets with similar results for the ovicidal activity. Moreover, we should not forget that the physiology of the gastrointestinal transit of horses and dogs are completely different.

Rey (2008) reported that *T. canis* eggs can survive for a long time in the environment, surviving very well in any environmental conditions. Therefore, an alternative measure of control of these eggs in the environment could help to decrease human contamination, especially children at preschool age. The results shown in this study may be useful in future research aimed at the control of STH, based on the principle that the fungus *P. chlamydosporia* will be act in the fecal environment and thus contribute to the reduction of recurrent infections.

This is the first report of the passage of the fungus *P. chlamydosporia* through the gastrointestinal tract of dogs with subsequent destruction of *T. canis* eggs. These findings suggest the viability of using *P. chlamydosporia* as a biological control alternative for eggs of *T. canis* present in the feces of infected dogs.

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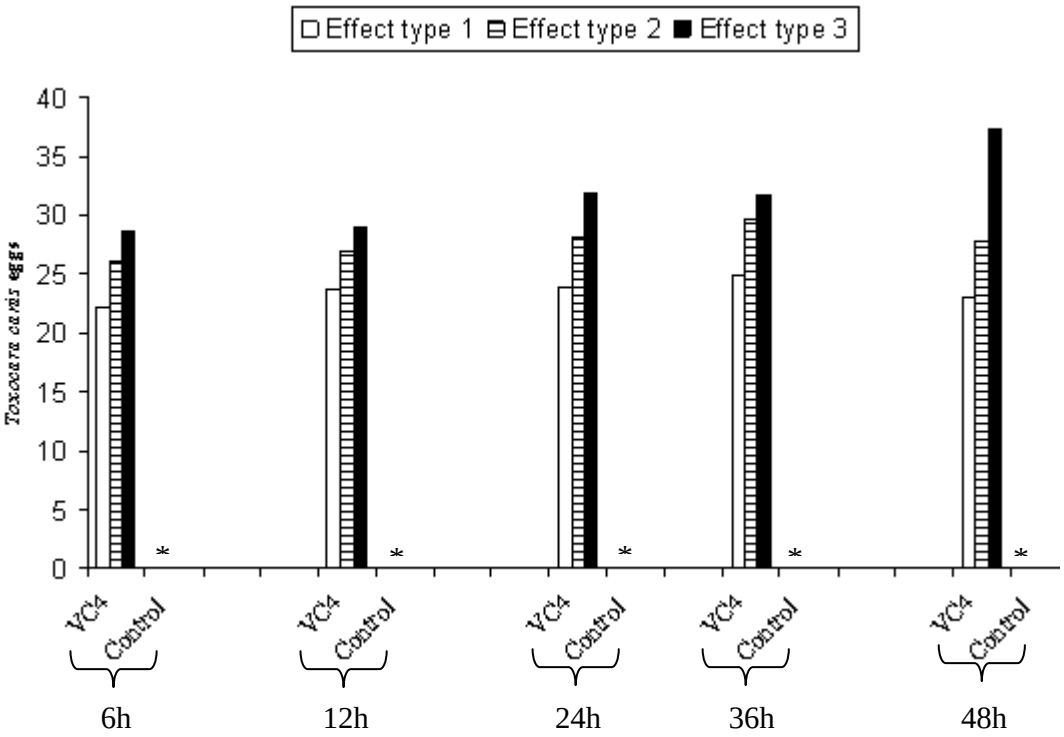

Fig.1 – Means of ovicidal activity for the nematophagous fungus *Pochonia chlamydosporia* (VC4) and the control group against eggs of *Toxocara canis* at feces collection times 6, 12, 24, 36 and 48 hours after 30 interaction days. Asterisk denote differences statistical ($p<0.01$).

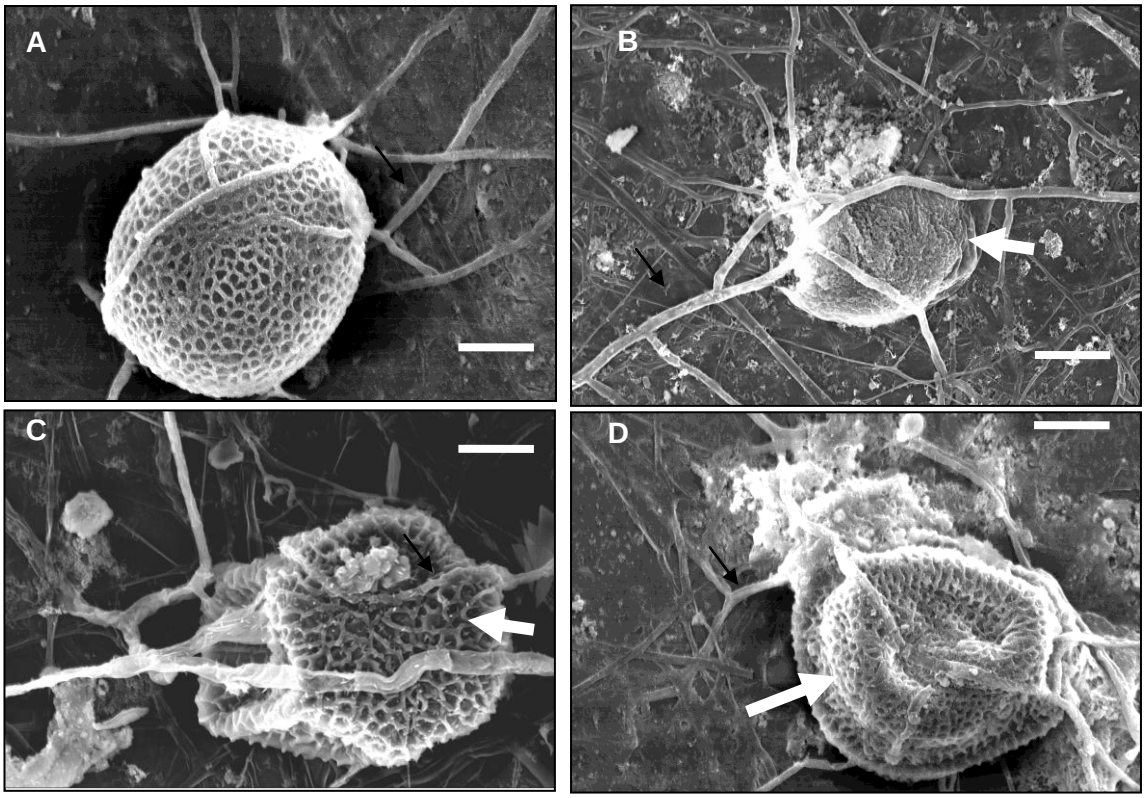

Fig.2A-D. Progressive destruction by hyphae from the nematophagous *P. chlamydosporia* fungus (VC4), B-D (black arrows) on the surface and inside and subsequent *Toxocara canis* eggs destruction, A-D (white arrows). Bars: A- 17.7 µm; B- 26.6 µm; C- 16 µm and D- 17.7 µm.

3. CONCLUSÕES GERAIS

- Os isolados AC001 do fungo *Duddingtonia flagrans*, NF34 do fungo *Monacrosporium thaumasium* e I-31 do fungo *Arthrobotrys robusta* são eficazes *in vitro*, na captura e destruição de larvas de *Strongyloides westeri*, após um período de 7 dias de interação, podendo ser utilizados em futuros testes a campo.

- Os isolados VC1 e VC4 do fungo *Pochonia chlamydosporia* destroem os ovos de *Toxocara canis* durante o intervalo de 15 dias de interação, podendo ser considerado uma alternativa futura para o controle integrado deste nematóide.

- Os isolados AC001 e NF34 resistem a passagem pelo trato gastrintestinal de equinos e jumentas, e destruíram as larvas de *Strongyloides westeri*, após os períodos de 12 a 72 horas, demonstrando uma nova abordagem no controle desse nematóide dessas duas espécies de equídeos.

- O isolado VC4 resiste à passagem pelo trato gastrintestinal de cães, mantendo sua atividade ovicida sobre ovos de *Toxocara canis*, após os períodos de 6 a 48 horas demonstrando uma nova abordagem no controle desse nematóide de cães.