ALEXANDRE DE OLIVEIRA TAVELA

AVALIAÇÃO DA EFICÁCIADA ASSOCIAÇÃO *in vitro* E *in vivo* DOS FUNGOS NEMATÓFAGOS *Duddingtonia flagrans, Monacrosporium Thaumasium* E *Pochonia chlamydosporia* SOBRE LARVAS INFECTANTES DE CIATOSTOMÍDEOS E TRICHOSTRONGILÍDEOS

Tese apresentada a Universidade Federal de Viçosa como parte das exigências do Programa de Pós-graduação em Medicina Veterinária, para obtenção do título de "DoctorScientiae"

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_____	_____
Ita de Oliveira e Silva	Walter dos Santos Lima
_____	_____
Fábio Ribeiro Braga (Coorientador)	Rogério Oliva Carvalho

_____

Jackson Victor de Araújo
(Orientador)

A minha família,

Aos meus amigos,

Aos meus colegas de trabalho.

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BIOGRAFIA

Alexandre de Oliveira Tavela, filho de Sebastião Alexandre Tavela e Maria Cristina de Oliveira Tavela, nasceu em 1985 em Juiz de Fora, MG.

Em março de 2004, ingressou na Universidade Federal de Viçosa, onde, em janeiro de 2009, obteve o título de Médico Veterinário.

Em março de 2009, iniciou o curso de Pós-Graduação em Medicina Veterinária, na Universidade Federal de Viçosa, concluindo o Mestrado em março de 2010.

Em agosto de 2010, iniciou o Doutorado em Medicina Veterinária, na Universidade Federal de Viçosa.

Em julho de 2012 iniciou seus trabalhos como professor de nível superior, ministrandoaulas de Parasitologia Veterinária e Doenças Parasitárias na Facastelo.

Em julho de 2013 foi nomeado professor Auxiliar I da Universidade Federal de Santa Catarina.

Normas de Conduta para o Uso de Animais no Ensino, Pesquisa e Extensão do DVT/UFV

Esse trabalho contou com a utilização de doze eqüinos e oito caprinos, os quaisparticiparam da experimentação segundo as normas de conduta para o uso de animais no ensino, pesquisa e extensão do DVT/UFV, vinculada ou projeto 168956 registrado em 03/01/2011.

Médico veterinário responsável: Jackson Victor de Araújo.

RESUMO

Tavela, Alexandre de Oliveira, D.Sc., Universidade Federal de Viçosa, julho de 2013. **Avaliação da eficácia da associação *in vitro* e *in vivo* dos fungos nematófagos *Duddingtonia flagrans, Monacrosporium thaumasium* e *Pochonia chlamydosporia* sobre larvas infectantes de Ciatostomídeos e Trichostrongilídeos.** Orientador: Jackson Victor de Araújo. Coorientadores: Fábio Ribeiro Braga e Laércio dos Anjos Benjamin.

O controle das verminoses de equinos e caprinos tem sido baseado na utilização de drogas antihelmínticas. No entanto, atualmente a resistência parasitária é um sério problema instalado em todo o mundo. Por outro lado, o controle biológico com fungos nematófagos tem sido estudado no combate alternativo de nematoides de animais domésticos, embora formulações contendo associações de dois ou mais gêneros diferentes de fungos ainda seja pouco explorada.O objetivo do presente trabalho foi avaliar a associação *in vitro* e *in vivo* dos fungos nematófagos *Duddingtonia flagrans*, *Monacrosporium thaumasium* e *Pochonia chlamydosporia* no controle das nematodioses de equinos e caprinos. O trabalho foi dividido em três ensaios experimentais denominados A, B e C, realizados em etapas distintas. No ensaio A foi testada a ação de três fungos nematófagos associados em condições laboratoriais utilizando placas de Petri contendo os fungos *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) e *Pochonia chlamydosporia* (VC1) no controle de ciatostomíneos parasitos de equinos. No ensaio B foi avaliada a associacão *in vivo* de péletes de alginato de sódio contendo os fungos *D. flagrans* e *M. thaumasium* no

controle de ciatostomíneos. No ensaio C, foi avaliada a associação dos fungos *D. flagrans* e *M. thaumasium* sobre larvas infectantes de tricostrongilídeos em condições laboratoriais e após a passagem pelo tubo digestivo de caprinos. A utilização dos fungos *D. flagans*,*M. thaumasium* e *P. chlamydosporia* associados no controle de ciatostomíneose trichostrongilídeos*in vitro* e *in vivo* apresentou resultados promissores. Contudo, devem ser realizados mais testes a campo, com a finalidade de observar a eficiência da associação desses fungos no controle ambiental de nematóides de equinos e caprinos.

ABSTRACT

Tavela, Alexandre de Oliveira, D.Sc., Universidade Federal de Viçosa, July, 2013. **Evaluation of the association *in vitro* and *in vivo* of nematophagous fungi *Duddingtonia flagrans, Monacrosporium thaumasium* and *Pochonia chlamydosporia* to control infective larvae of Cyathostomin and Trichostrongykids.** Adviser: Jackson Victor de Araújo. Co-advisers: Fábio Ribeiro Braga and Laércio dos Anjos Benjamin.

The control of nematode infections in horses and goats has been based on the use of anthelminthic drugs. However, the parasitic resistance is now a serious problem installed around the world. On the other hand, the biological control with nematophagous fungi has been studied as an alternative method in combating nematodes of domestic animals, although formulations containing combinations of two or more different genera of fungi are unexplored. Thiswork aimed to evaluate the association *in vitro* and *in vivo* of nematophagous fungi *Duddingtonia flagrans*, *Monacrosporium thaumasium* and *Pochonia chlamydosporia* to control nematodiosis of horses and goats. This work was divided into three experimental essays (A, B and C), performed in separate steps. In the essay Awas tested the action of three nematophagous fungi associated in laboratorial conditions using Petri dishes containing *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) and *Pochonia chlamydosporia* (VC1)fungi to control cyathostomin. In the essay B was evaluated *in vivo*the association of sodium alginate pellets containing fungi *D. flagrans* and *M. thaumasium* to control equine cyathostomin. In the essay C was evaluated the association of fungi *D. flagrans* and *M. thaumasium* on trichostrongylides infective larvae under laboratory conditions and after passage through the gastrointestinal tract of goats. The use of the fungi *D.*

flagrans, M. thaumasium and *P. chlamydosporia* associated to control cyathostomin and trichostrongylides*in vitro* and *in vivo* showed promising results. However, more tests should be performed in the field, in order to observe the effectiveness of the association of these fungi on environmental control of nematodes in horses and goats.

SUMÁRIO

1. INTRODUÇÃO

O Brasil possui o maior rebanho de equinos na América Latina e o terceiro mundial, atrás de China e Estados Unidos (MAPA, 2013). Somados aos muares (mulas) e asininos (asnos) são 8 milhões de cabeças, movimentando R$ 7,3 bilhões, somente com a produção de cavalos.O rebanho envolve mais de 30 segmentos, distribuídos entre insumos, criação e destinação final e compõe a base do chamado Complexo do Agronegócio Cavalo, responsável pela geração de 3,2 milhões de empregos diretos e indiretos (IBGE, 2010).Quanto a exportação de cavalos vivos, os números são significativos: a expansão alcançou 524% entre 2000 e 2013, passando de US$ 702,8 mil para US$ 4,4 milhões (IBGE, 2010). Além disso, o Brasil é o oitavo maior exportador de carne equina, sendo Bélgica, Holanda, Itália, Japão e França são os principais importadores da carne de cavalo brasileira (MAPA, 2013).

No entanto, no país ainda predominam as formas de criação pouco tecnificadas,o que favorece a grande incidência de infecçõesparasitárias, já nas primeiras semanas de vida dos animais (Molento, 2005). Por outro lado, os equinos são hospedeiros de uma grande variedade de nematoides, sendo que, dentre esses, os mais prevalentes e importantes economicamente são os ciatostomídeos, conhecidos também como pequenos estrongilídeos (Reinemeyer, 1986; Lyons et al., 1999; Braga et al., 2009a, 2009b; Tavela et al. 2011).

Reinemeyer (1986) classificou mais de 40 espécies de ciatostomíneos em equinos domésticos, sendo estas morfologicamente similares, especialmente na fase larval. Todas as espécies descritas têm ciclo direto, envolvendo um período de desenvolvimento externo, usualmente no pasto (Love et al., 1997). Os ovos postos pelas

fêmeas adultas, presentes no ceco e intestino grosso, são eliminados nas fezes do hospedeiro, e se desenvolvem no ambiente, passando por dois estágios larvais intermediários até atingir a fase infectante, ou L3. Ao ser ingerida, as L3 invadem a parede do intestino alojando-se na mucosa ou sub-mucosa, levando a um acúmulo de fibroblastos ao seu redor, tornando-se, portanto, encistadas. Uma vez encistada, a larva poderá se desenvolver em L4, também encistada. Abandonando, posteriormente, o cisto e evoluindo a L5/adultos machos e fêmeos no lúmen intestinal, acasalando-se e produzindo ovos, reiniciando assim o ciclo (Urquhartet al., 1996). As larvas encistadas podem ainda entrar em desenvolvimento retardado, ou hipobiose (Love et al., 1997). A persistência da larva L3 por um longo estágio inibindo a fase L4, quando hipobiótica, pode durar de 2 a 30 meses. Em situações normais, o ciclo evolutivo desse parasito é de aproximadamente 60 dias (Reinemeyer, 1986).

Os pequenos estrongilídeos, em moderadas ou altas cargas parasitárias podem causar anemia, emaciação, distúrbios intestinais como diarreia e má absorção, diminuição da resistência imunológica, episódios de cólicas e até a morte do hospedeiro (Assis e Araújo, 2003; Braga et al., 2009b). As lesões intestinais incluem enterite catarral ou fibrinosa no cólon maior e ceco, com numerosos focos de hemorragia, necrose ou formação de granulomas na mucosa e submucosa. Essas lesões são associadas a larvas de ciatostomíneos. Outras lesões intestinais incluem edema da parede intestinal e aumento de volume dos linfonodos mesentéricos (Pierezanet al., 2009).

Os equinos adquirem resistência aos pequenos estrongilídeos com a idade, sendo os mais jovens e os mais velhos mais susceptíveis (Urquhartet al., 19996; Anjos e Rodrigues, 2006). Isso pode ser verificado através da redução da carga parasitária e da

contagem de ovos nas fezes, porém esta resposta é lenta e inconsistente na maioria dos animais e não tem relação com a intensidade do contato parasitário anterior (Anjos e Rodrigues, 2006). Além disso, esses parasitos estão presentes ao longo de todo o ano nas pastagens, provocando constantemente a recidiva das infecções (Tavela et al., 2011).

Dentre os parasitos de pequenos ruminantes, destacam-se os nematoides estrongilídeos, os quais são responsáveis por grande impacto negativo na produção de carne e leite, além dos altos custosrelacionados às medidas de controle (WallereLarsen, 1993; Sykes, 1994; Torinaet al., 2004). Esses helmintospossuem distribuição cosmopolita, e dentre os gêneros de maior importância em países de clima tropical, destacam-se *Haemonchus* sp.,parasito do abomaso, *Trichostrongylus* sp., parasito do abomaso e do intestino delgado de ruminantes, além dos vermes pulmonares do gênero *Dictyocaulus* (Freitas, 1982; Urquhart et al., 1996).

O gênero *Haemonchus* possui várias espécies, sendo que *H. contortus* é a espécie dominante em termos de intensidade de infecção em pequenos ruminantes (Achiet al., 2003), pois estes animais mostram-se altamente susceptíveis, com alta taxa de estabelecimento da infecção e grande excreção de ovos pelas fêmeas (Jacquiet et al., 1998), em comparação com outras espécies de ruminantes.

A espécie *H. contortus* tem um ciclo evolutivo direto. As fêmeas são ovíparas e prolíferas. Os ovos são eliminados nas fezes e em condições ideais (18 a 26°C e 80 a 100% umidade) se desenvolvem na pastagem até o estádio larval infectante (L_3) em aproximadamente 5 dias. Em condições frias o desenvolvimento pode ser retardado por semanas ou meses. A temperatura ótima para a sobrevivência das larvas é de 18 a 26ºC (OnyaheArslan, 2005). Em baixas temperaturas as larvas sobrevivem por longos

períodos devido ao seu baixo metabolismo e reservas energéticas. A umidade é também um fator importante para a sobrevivência da larva, em condições secas, como no semi-árido brasileiro, as larvas podem não sobreviver ao longo de todo o ano (Arosemena et al., 1999).

A irrigação pode influenciar na disponibilidade de L3, sendo encontradas em grande número em pastagens irrigadas durante o verão com temperaturas em torno de 24°C (Kreceket al., 1991). Após a ingestão e desembainhamento no rúmen, as larvas sofrem duas mudas. Exatamente antes da muda final eles desenvolvem a lanceta perfurante que lhes permite a obtenção do sangue dos vasos da mucosa do abomaso, local de fixação do parasito. Quando adultos, movem-se livremente na superfície da mucosa. O período pré-patente é de duas a três semanas (Soulsby, 1987).

Em casos de severa infecção por *Haemonchus*, os sinais clínicos mais comuns apresentados pelos animais são a progressiva perda de peso e anemia, caracterizada pela queda do volume globular (Achiet al., 2003). Os animais podem apresentar-se posteriormente edemaciados devido à anemia que se torna cada vez mais grave. Assim sendo, o animal pode apresentar o edema submandibular e ascite (Achiet al., 2003).

Na infecção crônica, o animal apresenta uma baixa progressiva no volume globular e um pequeno ganho de peso, quando comparado com animais livres de parasitos. Durante a haemonchose hiperaguda o animal pode morrer subitamente como consequência de gastrite hemorrágica grave. Observa-se ainda hipoproteinemia e hipoalbunemia. Diarreia não é um sintoma comum em uma infecção por *H. contortus*(Soulsby, 1987). O impacto da patogenia da hemonchose sobre o hospedeiro pode ainda ser afetado pela dieta oferecida aos animais, àqueles que têm dietas pobres em proteína, apresentam sinais clínicos mais pronunciados, apesar de apresentar carga

parasitária semelhante àqueles que têm uma dieta rica em proteína (Abbott, et al., 1986), portanto a doença pode ser intensificada devido à baixa qualidade alimentar dos animais (Urquhart, 1996).

Dessa forma, as criações de animais com finalidade produtiva sofrem grandes perdas econômicas associadas ao parasitismo, sobretudo por nematoides parasitos do tubo digestivo. Os diversos prejuízos ocasionados por essas infecções estão relacionados com a queda na produtividade, retardo no crescimento dos animais, elevados custos com tratamentos veterinários, com os recursos terapêuticos a serem empregados e, em algumas situações, com os prejuízos advindos do óbito dos animais (Araújo et al., 2006).

O combate aos nematoides parasitos de animais domésticos tem sido feito ao longo de muitas décadas com a utilização de anti-helmínticos. Contudo, o emprego desse método de controle de maneira isolada nem sempre apresenta resultados satisfatórios (Kaplan, 2004). Esse fato se deve principalmente ao manejo inadequado dos animais e das propriedades e à resistência parasitária, já instalada em diversos países e relatada como grande problema em criações de ruminantes e equídeos (Drudgeet al., 1964; Kaplan, 2004). Além disso, problemas relacionados à ecotoxicidade enfatizam a necessidade de serem implantados programas integrados de controle parasitário que assegurem saúde e segurança dos organismos vivos, por meio de tratamentos estratégicos baseados na escolha das melhores épocas para vermifugação dos animais associado ao manejo ambiental(McKellar, 1997; Mota et al., 2003).

Algumas espécies de fungos, bactérias, vírus, protozoários, entre outros, são tidos como nematófagos, motivo pelo qual podem ser uma alternativa no controle de nematoides parasitas de animais(Mota et al., 2003). Dentre esses organismos, se

destacam os fungos nematófagos, pelo alto potencial biológico e de produção em escala industrial (Araújo, 1996, 1998; Mota et al., 2003).

O biocontrole com fungos nematófagos não substitui o controle químico que atua sobre nematóides adultos no trato gastrintestinal do animal, ele o integra, atuando sobre as formas evolutivas destes parasitas nas fezes liberadas no ambiente pelo animal portador (Waller e Larsen, 1993). Seus agentes raramente erradicam o organismo-alvo, mas reduzem sua população a níveis aceitáveis, mantendo um balanço entre patógeno e antagonista, permitindo assim a sobrevivência de um pequeno número que possibilite aos animais parasitados desenvolver imunidade, reduzindo a incidência de casos clínicos (Waller e Larsen, 1993). Este tipo de controle visa reduzir o número de ovos e estádios de vida livre no ambiente – larvas L1, L2 e L3 – interrompendo assim o processo no qual a contaminação no ambiente torna-se uma infecção no hospedeiro final (Gomes, 1998). Mas para ser efetivo o fungo deve estar presente e ativo nas fezes, solo e ambiente no mesmo tempo que as formas pré-parasitárias (Faedoet al., 2000).

Diferentes tipos de fungos interagem com os nematoides, sendo caracterizados como predadores, endoparasitas de nematoides e oportunistas ou parasitos de ovos. Eles são cosmopolitas, ocorrendo em solos naturais, solos agricultáveis e em todos os tipos de matéria orgânica em decomposição (Araújo et al., 1996).

As espécies de fungos predadores variam em sua capacidade de capturar os nematoides; são os organismos mais estudados e que apresentam maior potencial de serem comercializados, principalmente por sua facilidade de isolamento e cultivo em laboratório quando comparados às outras categorias (Mota et al., 2003). Os fungos predadores formam armadilhas ao longo das hifas, separadas por pequenos intervalos. Essas estruturas são produzidas em resposta à presença de nematoides ou de substâncias

por eles secretadas: dessa forma, as culturas puras apresentam dificuldade na produção ou não produzem armadilhas. A diferenciação de uma hifa pode ocorrer em 24 horas, onde numerosas estruturas de captura podem ser produzidas. Nesse grupo destacam-se as espécies *Monacrosporium thaumasium* e *Dudingtonia flagrans* como importantes controladores biológicos de nematóides parasitos gastrintestinais (Larsen, 1991; Larsenet al. 1992, 1994; Araújo et al., 1996, 1998, 1999; Alves et al., 2003; Dimander et al., 2003; Braga et al., 2009a, 2009b; Tavela et al., 2010, 2011).

A espécie *D. flagrans* é a mais estudada no controle das helmintoses gastrintestinais de animais domésticos, sendo considerada como uma das mais promissoras (Braga et al., 2009a, 2009b), predando nematoides por meio de hifas adesivas simples. Este fungo produz dois tipos de conídios: conídios com paredes delgadas em conidióforos eretos, em número limitado e numerosos conídios resistentes com paredes grossas – clamidósporos, intercalados em hifas maduras em condições de crescimento desfavoráveis (Gronvold et al., 1993). Os conídios da espécie *D. flagrans* são persistentes no ambiente permitindo seu uso como agente de controle biológico (Faedo et al., 2000).Este fungo cresce lentamente em temperaturas abaixo de 25 ºC, sendo o ótimo crescimento obtido a 30ºC além de produzir grande número de clamidósporos que são conídios altamente resistentes em condições adversas(Larsen, 1991).

As espécies do gênero *Monacrosporium* capturam nematoides por meio de nódulos e redes tridimensionais adesivas ou anéis constritores (Barron, 1977). A espécie *M. thaumasium* apresenta desempenho *in vitro* homogêneo nas temperaturas de 25, 28 e 30ºC, que são muito comuns nos trópicos, supondo-se que esta espécie também se adapte facilmente às condições brasileiras (Castro et al., 2000).

Os fungos endoparasitas persistem principalmente como esporos, liberados no solo a partir de nematóides desintegrados. Não há extensivo desenvolvimento de hifas para o exterior do corpo dos nematóides infectados, mas apenas prolongamento de tubos de liberação de esporos (Araújo et al., 1996).

O grupo de fungos parasitos de ovos é formado por indivíduos oportunistas eportanto, independem da presença de ovos dos nematoides no solo para sua sobrevivência. Dentre as espécies promissoras desse grupo destaca-se *Pochonia chlamydosporia*, como potencial agente de biocontrole (Braga et al., 2008a, 2008b, 2008c).

As espéciesde fungos predadores afetam predominantemente nematoides cujos ovos possuem curto estádio de desenvolvimento, pois enquanto as larvas residem no ovo, estes fungossão incapazes de capturá-las por meio de armadilhas e, consequentemente, digeri-las de maneira eficiente (Faedoet al., 2000). Associações de espécies predadoras e entre espécies predadoras e ovicidas podem ser promissoras em utilizações a campo, na medida em que cada espécie possui ótimas condições de crescimento e sobrevivência, além de diferentes estratégias de predação, podendo estas, se complementarem (Tavela et al., 2011).

Nesse contexto, para que o controle biológico com estes fungos seja incorporado em um sistema industrial de produção, o mesmo deve ser exaustivamente estudado, para que se tenha conhecimento sobre as principais características, a melhor formulação, a capacidade de passagem pelo tubo digestivo da espécie alvo, os melhores isolados fúngicos e a sua atividade predatória a campo sobre nematoides parasitos gastrintestinais (Araújo et al., 2004). No entanto, as formulações contendo associações

de dois ou mais fungos nematófagos no controle de nematodioses de animais domésticos ainda é pouco explorada.

Sendo assim, a presente tesepretende contribuir como uma ferramenta para o melhor conhecimento das interações de fungos associados *versus* nematoides gastrintestinais de equinos e caprinos.

2. OBJETIVOS

2.1. Objetivo geral

Avaliar a associação *in vitro* e *in vivo* dos fungos nematófagos *Duddingtonia flagrans*, *Monacrosporium thaumasium* e *Pochonia chlamydosporia* no controle das larvas infectantes denematoides de equinos e caprinos.

2.2. Objetivos específicos

- Avaliar *in vitro* a eficácia da associação de diferentes espécies de fungos nematófagos, *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) e *Pochonia chlamydosporia* (VC1) no controle de ciatostomíneos.
- Avaliar a viabilidade da associação de péletes em matriz de alginato de sódio contendo os fungos *D. flagrans* (AC001) e *M. thaumasium* (NF34) e sua atividade predatória *in vitro* sobre larvas infectantes (L_3) de ciatostomíneos e após a passagem pelo tubo digestivo de equinos (*in vivo*).
- Avaliar a associação dos fungos nematófagos *Duddingtonia flagrans* (AC001) e *Monacrosporium thaumasium* (NF34) sobre larvas infectantes de tricostrongilídeos *in vitro*e após a passagem pelo tubo digestivo de caprinos (*in vivo*).

3. HIPÓTESES

- As associações de fungos nematófagos estudadas serão eficientes na predação de larvas de ciatostomíneos de equinos e de tricostrongilídeos de caprinos.

- A manutenção *in vitro* e/ou a coadministração de diferentes espécies fúngicas aos animais pode potencializar ou inibir a predação das larvas de nematoides.

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5. CAPÍTULO 1

In vitro association of nematophagous fungi*Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) and *Pochonia chlamydosporia* (VC1) to control horse cyathostomin (Nematoda: Strongylidae)

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Alexandre de Oliveira Tavela[1]*; Jackson Victor de Araújo[1]; Fábio Ribeiro Braga[1]; Juliana Milani Araujo[1]; Layane Queiroz[1]; Wendeo Ferreira da Silveira[1]; Luana Alcântara Borges[1]

[1] Departamento de Veterinária, Universidade Federal de Viçosa, Rua PhRolfs s/n, Viçosa-MG, Cep: 36570-000, Brazil

*Corresponding author: alexandre.tavela@ufv.br

Abstract

In vitro effects of nematophagous fungi*Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) and *Pochonia chlamydosporia* (VC1) against eggs and third-stage infectivelarvae (L_3) of horse cyathostomin (Nematoda: Strongylidae) were evaluated.The followingpercentage reductions compared with the control group were observed after a 20 days exposure period:AC001,80%; NF34, 78%; VC1, 76%; group AC001+VC1,80%; NF34 +VC1,81%; AC001+ NF 80%. The results showedthat thefungal isolates(VC1, AC001andNF34), acting alone or in conjunction,were efficientin controlling horsecyathostomin under *invitro* conditions.

Key words: Nematophagousfungi, *Pochonia chlamydosporia*, *Duddingtonia flagrans*, *Monacrosporium thaumasium*, cyathostomin, horses.

Researchhas shownpromising resultsin combatingthegastrointestinalhelminthosisof horses usingnematophagous fungi(Braga etal., 2009; Tavelaetal., 2011). However, there are no studiesthat demonstrateapotential efficacy of theassociation ofdifferentspecies ofnematophagous fungiaspredators andovicidalfungiincyathostomincontrol, which may contribute asonemore toolin the controlof these nematodes.

This study aimed to evaluate *in vitro*the association of nematophagous fungi*Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) and *Pochonia chlamydosporia* (VC1) to control horse cyathostomin (Nematoda: Strongylidae).

Three brazilian soil isolates of the nematophagous fungus *D. flagrans* (AC001), *M. thaumasium* (NF34) and *P.chlamydosporia*(VC1) (Araújo et al., 2004) were used. Cyathostomineggswere obtainedbydissectingadultsobtained fromnaturally infectedhorses. Thesewere analyzedbylight microscopy, fortheir morphology andintegrity.The eggswere washed10 timesfor 5 minuteseach with the aid ofacentrifuge (1000x ***g***) in distilled water anddiscardingthe supernatantat the endof each cycle.

Fragments of approximately4 mmin diameter of eachfungus were removed from cultures andtransferred toPetri dishes,4.5cmin diameter, containing 2% water–agar. A total of seven groupswere formedcontaining thefungal isolates: group 1, AC001; group 2, NF34; group 3, VC1; group 4, AC001 + VC1 (association); group 5, NF34 + VC1 (association); group 6, AC001 + NF34 (association); group 7, control (no fungus). For each group,sixrepetitions weretested.

After 10days in an incubation chamber, 250cyathostomineggswere transferred onto each plate.The platesof the groups testedwere placed in the incubation chamberat 25°C for10 days. On the twentieth day, theL_3not predated by the contents of the Petri dishes were recoveredusing Baermannapparatuswith waterat 42°C.The means ofcyathostominL_3recovered werecalculated.Therecovered larvaewereidentified according toBevilacquaetal. (1993).

The dataobtained were transformed intolog (x+ 1)and submitted to analysis ofvariance (ANOVA) at significance levelsof 1 and5% probability. The efficiency of L_3predation, compared with control,was evaluatedby the Tukey testat 1% of probability.

Subsequently, theaveragepercentage reductionof $L_3$was calculated (Ayres etal., 2003):

$$\%\text{Reduction of } L_3: \frac{XC - XT}{XC} \times 100,$$

whereXCis the averageof the control group andXT the average of thetreated groups.

The fungiwere able to destroythe eggs andlarvae thathatchedin the Petri dishes during10 days. At the end of20days, there was lessrecovery ofL_3in groupscontaining thefungal isolatesseparatelyor in combination compared withthe control group($p<0.05$).On the other hand, there was no difference ($p>0.05$)in the recovery ofL_3between the treated groups(Fig.1).The followingpercentage reductions compared with the control group were observed:group1 (AC001),80%; group 2 (NF34), 78%; group 3 (VC1), 76%; group 4 (AC001+VC1),80%; group 5 (NF34 +VC1),81%; group 6 (AC001+ NF34), 80%.

Nematophagous fungi have shown efficacy in both laboratory and natural conditions for control of nematode parasites of horses (Braga et al., 2009, Braga et al., 2010a; Paz-Silva et al., 2011). In this study, it was demonstrated that predatory isolates (AC001 and NF34) as well as an ovicidal isolate (VC1) were able to reduce the numbers of L_3 after 10 days. It is possible that isolate VC1 destroyed some of the eggs present in the Petri plates, consequently reducing the number of L_3 recovered. On the other hand, both the AC001 and NF34 isolates destroyed a proportion of the hatched larvae. Other studies have shown that *D. flagrans* and *M. thaumasium* do not have ovicidal capacity; however, they can adhere to the egg shell without causing its

destruction (Braga et al., 2008). On the other hand, there are no reports that mention the association of these two species of fungi in the control of cyathostomin. Regarding the use of *P. chlamydosporia*, an ovicidal fungus, the present results depart from previous studies that investigated its ovicidal activity on various genera of helminths (Braga et al., 2010b, Ferreira et al., 2011); however, there is only one report mentioning the activity on cyathostomin eggs under laboratory conditions (Braga et al., 2009).

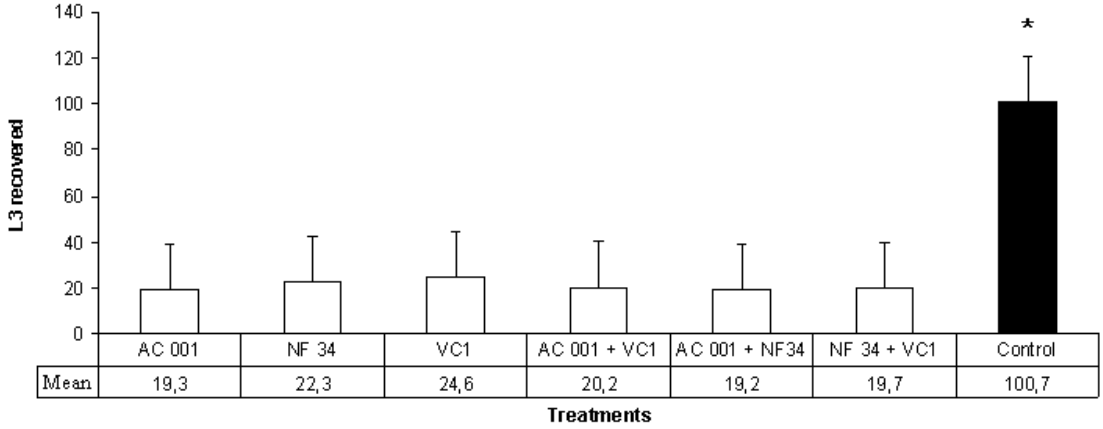

Fig. 1. Means and standard deviations (bars) of infective non-predated cyathostomin larvae recovered from 2% water–agar plates by the Baermann method on the seventh day of treatment with the following fungal isolates: *Pochonia chlamydosporia* (VC1), *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34), associations and control (without fungus). * Significant difference ($p<0.05$) between the fungus-treated group and the control (Tukey's test at the 5% probability level).

In the present study, where combinations of fungi were grown on Petri plates, a possible synergism between the ovicidal action of *P. chlamydosporia* and the predatory activity of *D. flagrans* and *M. thaumasium* was expected. However, the results showed

no greater efficacy of combinations regarding the use of the fungal isolates. Regarding the percentage reduction, there were no significant differences (p>0.05) in plates containing species of fungus or the plates with the associations. Regarding group 6 (AC001 + NF34), there was a higher percentage reduction (81%), although no difference (p>0.05) compared with the other groups.

Braga et al. (2009) and Tavela et al. (2011) showed that oral administration of pellets containing the fungus *M. thaumasium* (NF34) and *D. flagrans* (AC011) in horses naturally infected with cyathostomin was effective in southeastern Brazil. These reports suggest then that both fungi are effective in controlling cyathostomin. However, combining these two fungi in a single formulation might improve efficacy because these species have distinct characteristics. The present study showed that all three fungal isolates (VC1, AC001 and NF34) were efficient in controlling cyathostomin under *in vitro* conditions.

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6. CAPÍTULO 2

Coadministration of sodium alginate pellets containing the fungi *Duddingtonia flagrans* and *Monacrosporiumthaumasium* on cyathostomin infective larvae after passing through the gastrointestinal tract of horses

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Alexandre de Oliveira Tavela[1,2]*, Jackson Victor de Araújo[1], Fábio Ribeiro Braga[1,3], Wendeo Ferreira da Silveira[1], Vinicius Herold Dornelas e Silva[1], Moacir Carretta Júnior[1], Luana Alcântara Borges[1], Juliana Milani Araujo[1], Laércio dos Anjos Benjamin[1], Giovanni Ribeiro Carvalho[4], Alessandra Teixeira de Paula[1]

1 - Departamento de Veterinária, Universidade Federal de Viçosa. Av. P. H. Rolfs s/n, Viçosa, Minas Gerais, Brazil. 36570-000

2 – Faculdade de Castelo, Castelo, Espírito Santo, Brazil.

3 - Universidade Vila Velha, Vila Velha, Espírito Santo, Brazil.

4 - Departamento de Zootecnia, Universidade Federal de Viçosa. Av. P. H. Rolfs s/n, Viçosa, Minas Gerais, Brazil. 36570-000

*Corresponding author: alexandre.tavela@ufv.br

Abstract

The predatory nematophagous fungi have been used as an alternative control of gastrointestinal nematodes of domestic animals in natural and laboratory conditions. However, it is unclear if the association of some of these species could bring some kind of advantage, from a biological standpoint. In this context, this study consisted of two tests *in vitro*: in assay A, assessment of the viability of the association of pellets in sodium alginate matrix containing the fungus *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34) and its predatory activity on infective larvae (L_3) of cyathostomin after passing through the gastrointestinal tract of horses and assay B, assessment of the cyathostomin L_3 reduction percentage in coprocultures. Twelve crossbred horses, females, with a mean weight of 356 kg and previously dewormed were divided in three groups with four animals each: in the group one each animal received 50 g of pellets containing mycelial mass of the fungus *D. flagrans* and 50 g of pellets of the fungus *M. thaumasium*, associated and in a single oral dose; in the group two, 100 g of pellets containing *D. flagrans* and 100 g of pellets containing *M. thaumasium,* associated and in a single oral dose; group three, control. Fecal samples were collected from animals in the treated and control groups at time intervals of 12, 24, 36, 48, 60 and 72 hours after the administration of treatments and placed in Petri dishes containing 2% water-agar (assay A) and cups for coprocultures (assay B). Subsequently, 1,000 cyathostomin L_3 were added to each Petri dish (assay A) and 1,000 cyathostomin eggs were added to each coproculture (assay B) of fungi-treated and control groups. At the end of 15 days, there was observed that the two associations of pellets containing the fungi tested showed predatory activity after passing through the gastrointestinal tract of horses (assay A). In assay B, all the intervals studied showed reduction rate in the number of L_3 recovered from coprocultures exceeding 80%. However, no difference (p

> 0.01) was seen in recovery of not predated L_3 between the fungi-treated groups in the time intervals studied. The results obtained showed that the associations of pellets (50 g or 100 g of each fungal isolate) were viable after passage through the gastrointestinal tract in horses and could be used in natural conditions.

Keywords: Nematophagous fungi, *Duddingtonia flagrans*, *Monacrosporiumthaumasium*, cyathostomin, horses, biological control.

1. Introduction

Overall helminthic infections are an old problem and a serious barrier for cattle ranching. Typically, large portion of nematodes in fecal environment passes from egg to larval stages, reaching the infective form (Braga and Araújo, 2012). The use of predatory nematophagous fungi has been identified as an alternative control of gastrointestinal nematodes of domestic animals in natural and laboratory conditions (Larsen et al., 1992; Paz-Silva et al., 2011; Sagüés et al., 2011; Paraud et al., 2012). The combat of these parasites, and in particular to horse cyathostomin, has been realized with anthelmintics. However, it has not shown satisfactory results, due to the emergence of resistance to these compounds (Love et al., 1999).

In this context, a current challenge is the selection of organisms that can be produced industrially and economically viable. The species *Duddingtonia flagrans* and *Monacrosporium thaumasium* has been successfully used to combat nematodes of domestic ruminants and horses, when used separately under even in the field and controlled conditions (Paraud et al., 2012; Tavela et al., 2011). These species are most

studied and that presents a greater potential to be marketed. It is a species that produces resistant structures known as chlamydospores, and can pass through the gastrointestinal tract of domestic animals, and thus can withstand extreme conditions of "stress". However, there may be differences in the action of different isolates of this species, as some investigations have mentioned this fact in different regions (Araújo et al., 2004).

From this premise, as their use is individual, mostly from pellets of sodium alginate matrix, it is unclear if the association of these species could bring some kind of advantage, from a biological standpoint (Braga et al., 2009; Tavela et al., 2011). In recent work, Tavela et al. (2012) showed that the association of different nematophagous fungi in laboratory conditions were effective to control cyathostomin, however, these authors did not perform tests of passage through the gastrointestinal tract of domestic animals, which, according to Mota et al. (2003) is the main requirement that a fungus, or in this case the association of two or more fungi, may be used for biological control in the field, since its action occurs in the fecal environment.

This study aimed to evaluate the viability of the association of pellets in matrix of sodium alginate containing the fungus *D. flagrans* and *M. thaumasium* and its predatory activity *in vitro* on larvae of cyathostomin (Nematoda: Strongylidae) after passage through the gastrointestinal tract of horses.

2. Material and Methods

2.1. Fungi and production of mycelial mass

Two predatory fungi isolates were used: *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34). These isolates were obtained from Brazilian soils (Zona da Mata of Minas Gerais).

Fungal mycelia were obtained by transferring culture disks (approximately 4 mm diameter) of fungal isolates (*D. flagrans* and *M. thaumasium*) in 2% water-agar (2%WA) to 250 mL Erlenmeyer flasks with 150 mL of liquid GPY medium (glucose, sodium peptone and yeast extract), and incubated under agitation of 120 rpm in the dark at 26 °C, for 10 days. After this period, the mycelia were removed, filtered and weighed on an analytical balance. All procedures followed the methodology of Araujo et al. (2010).

2.2. Animals and experimental site

The experiment was conducted at the Department of Veterinary Medicine, Federal University of Viçosa, Brazil. At the beginning of the experiment, stool samples of the 12 female horses, with average weight of 356 kg and average age of 2.5 years, were collected directly from the rectum to be performed the initial egg account per gram of faeces (EPG) according Gordon and Withlock (1939) modified by Lima (1989). It was found that the initial mean of the EPG was 1400. Subsequently, these animals were treated with an oral dose of equine anthelmintic at a dose of 0,2 mg/kg of ivermectin and 6.6 mg/kg of pyrantel pamoate (Centurion Vallé ®, Montes-Claros, Minas Gerais, Brazil). The EPG was negative after the anthelmintic administration. Fourteen days after the anthelmintic treatment the animals received two different concentrations of pellets containing mycelial mass (0.2 mg of fungus per kg of body weight) of the isolates of *D. flagrans* (AC001) and *M. thaumasium* (NF34) associated and pellets without fungus (control).

2.3. In vitro assay

This study consisted of two *in vitro* experiments: the A assay, that evaluated the viability of the association of pellets of sodium alginate matrix containing the fungus *D.*

flagrans and *M. thaumasium* and its predatory activity on L_3 of cyathostomin (Nematoda: Strongylidae) after passing through the gastrointestinal tract of horses and the B assay, that evaluated the reduction percentage of cyathostomin larvae in coprocultures.

2.3.1 Assay A

In this assay, the horses were divided in three groups, each having 4 animals: group 1: each animal received 50 g of pellets containing the mycelial mass of the fungus *D. flagrans* and 50 g of pellets of the fungus *M. thaumasium*, associated and in a single oral dose; group 2: each animal received 100 g of pellets containing mycelial mass of *D. flagrans* and 100 g of pellets of *M. thaumasium* associated and in a single oral dose; and group 3, control, in which the animals received a single oral administration of 100 g of pellets without fungus. The pellets were mixed in 100 g of commercial ration for horses to facilitate the intake.

After administration of fungi, within the hours intervals (12, 24, 36, 48, 60 and 72), fecal samples were collected according to described by Araujo et al. (2010). Next, these samples were homogenized, and 4 g of faeces were placed in Petri dishes with 9 cm diameter containing 2% water agar (2% WA), placed in an incubator chamber at 25° C in the dark. To test the predatory activity and the viability of association of the fungi pellets tested (AC001 and NF34), in Petri dishes of the tested groups (treated and control) were added 1000 cyathostomin L_3. Then, were performed 12 replicates of each set time.

For the verification and identification of the passage of the fungi tested through the gastrointestinal tract, were adopted the keys of classification for fungal structures (conidia and/or chlamydospores) proposed by Van Oorschot (1985) and Liu and Zhang

(1994), where the visualization of dishes was performed daily. On the fifteenth day, the L_3 not predated were recovered from Petri dishes by Baermann apparatus (Araujo et al. 2010). The data were submitted to analysis of variance (F test). Subsequently, the means were compared using the Tukey test at the 1% level of probability, via software Biostat 3.0 (Ayres et al. 2003).

2.3.2 Assay B

Concomitantly to the assay A, fresh faeces from the 12 horses from this present study, collected at the same time intervals, were processed for the preparation of coprocultures, being mixed with industrialized fragmented and wetted vermiculite. Then, in each coproculture of the groups tested with the different doses of the association of fungi AC001 and NF34 and the control groups (from assay A) were added 1000 cyathostomin eggs constituting the treated and control groups. The cultures were incubated at 28ºC for 10 days. At the end of this period were obtained L3 by the method of Baermann, which were identified and quantified according Ueno and Gonçalves (1994) in an optical microscope and 10X objective.

3. Results

The two associations of pellets tested, group 1 (AC001 50g and 50g NF34) and group 2 (100g and 100g AC001 NF34) showed predatory activity after passing through the gastrointestinal tract of horses (Fig. 1). On the other hand, evidences of predation were also observed through the production of reproductive structures (conidia and/or chlamydospores) of fungi AC001 and NF34 during the experimental trials.

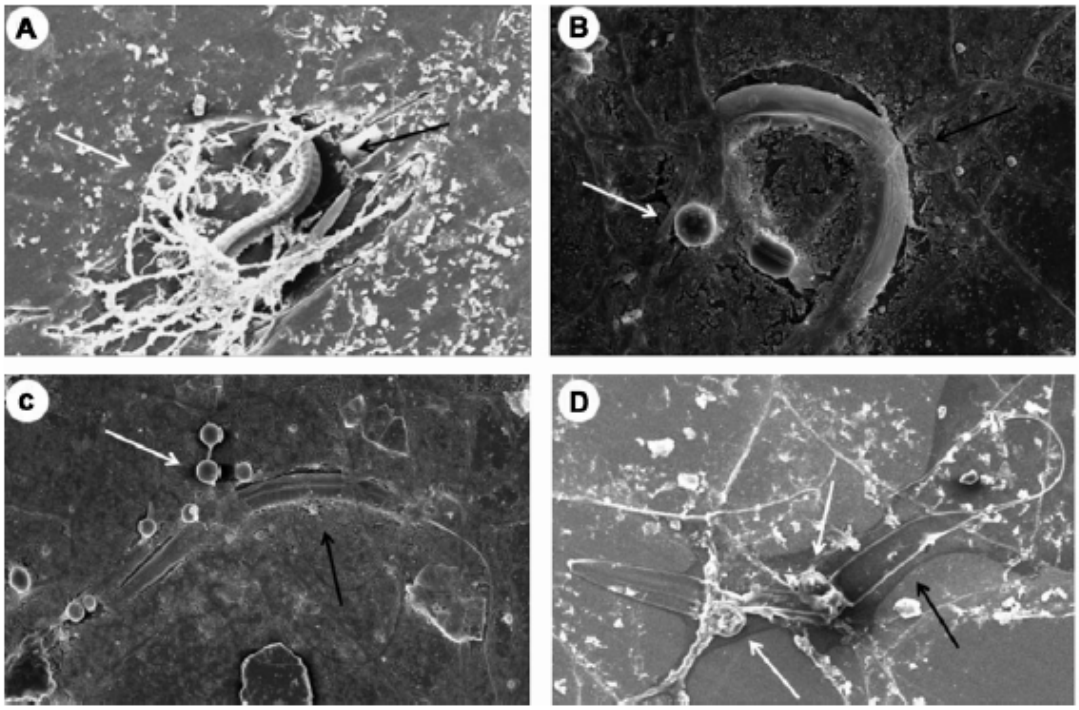
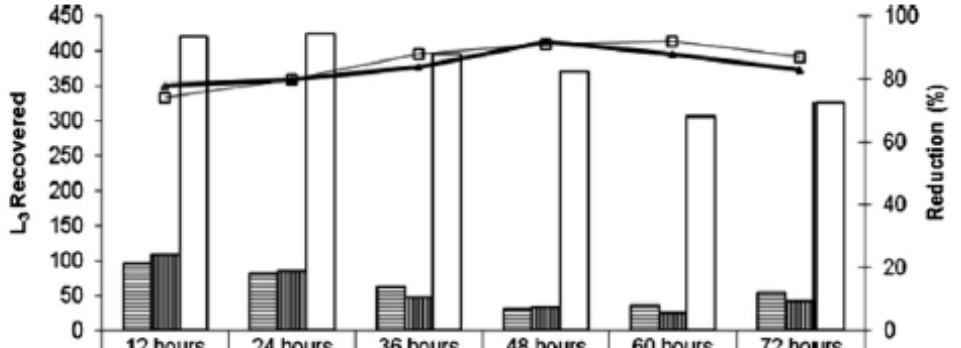


Fig. 1.(A) Infective larvae (L_3) of cyathostomin (black arrow) and hyphae of fungi *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34) (white arrow). (B) Fungal traps (white arrow). (C and D) Cyathostomin L_3(black arrow), chlamydospores of the fungus *D. flagrans* and formation of traps (consytiction rings) in Petri dishes containing 2% water-agar (2% WA).

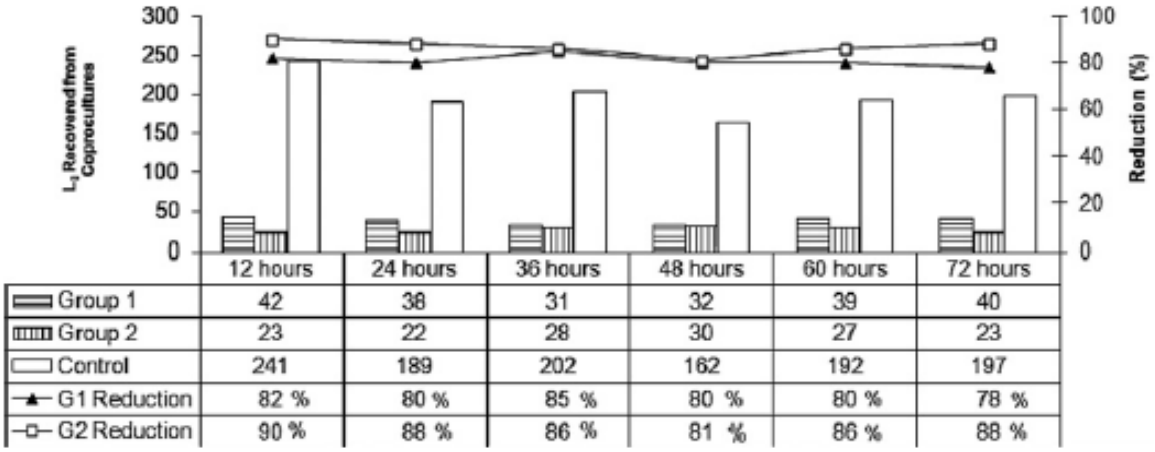
In assay A, the results for different time intervals studied (12-72 hours) showed no difference ($p > 0.01$) in the recovery of non-predated L_3 between the treated groups; however, differences were observed in fungus-treated groups in relation to the control group. Regarding the reduction percentage in group 1 (50 g of AC001 and 50 g of NF34) was observed that the interval of 48 hours demonstrated a lower recovery of L_3 and consequently a higher larvae percentage decrease (92%). In group 2 (100g of AC001 and 100g of NF34) was observed a lower L_3 recovery in the interval of 60 hours, reflecting a larvae reduction percentage of 92% (Fig. 2).



	12 hours	24 hours	36 hours	48 hours	60 hours	72 hours
Group 1	95	82	62	30	35	54
Group 2	108	84	47	32	24	41
Control	420	425	395	370	306	326
G1 Reduction	78 %	80 %	84 %	92 %	88 %	83 %
G2 Reduction	74 %	80 %	88 %	91 %	92 %	87 %

Fig. 2 – Mean of cyathostomin infective larvae (L_3) recovered from Petri dishes by Baermann apparatus and reduction percentage of fungi-treated groups: group 1 (50 g of pellets containing AC001 and 50 g of pellets containing NF34); group 2 (100 g of pellets containing AC001 and 100 g of pellets containing NF34) and control group, after 15 days of treatment with the fungal isolates *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34) at time intervals of 12, 24, 36, 48, 60 and 72 hours.

The assay B (larvae recovered from coprocultures) showed similar results to the assay A. In all intervals studied there was no difference ($p > 0.01$) between the groups treated with associations of pellets containing the fungi tested. Moreover, in group 1 the interval of 36 hours there was observed the highest larvae reduction percentage (85%). However, the other intervals studied (12, 24, 48, 60 and 72 hours) showed a percentage reduction in the number of L_3 recovered from coprocultures around 80%. In group 2 (100g of AC001 and 100g of NF34) the highest percentage of reduction was observed at the interval of 12 hours (90%), however, the other time intervals also showed a reduction rate exceeding 80% (Fig. 3).



	12 hours	24 hours	36 hours	48 hours	60 hours	72 hours
Group 1	42	38	31	32	39	40
Group 2	23	22	28	30	27	23
Control	241	189	202	162	192	197
G1 Reduction	82 %	80 %	85 %	80 %	80 %	78 %
G2 Reduction	90 %	88 %	86 %	81 %	86 %	88 %

Fig. 3 – Mean of cyathostomin infective larvae (L_3) recovered from coprocultures by Baermann apparatus and reduction percentage of fungi-treated groups: group 1 (50 g of pellets containing AC001 and 50 g of pellets containing NF34); group 2 (100 g of pellets containing AC001 and 100 g of pellets containing NF34) and control group, after 10 days of treatment with the fungal isolates *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34) at time intervals of 12, 24, 36, 48, 60 and 72 hours.

4. Discussion

Nematophagous fungi have been suggested as promising biological controllers to combat the infective forms of nematodes present in the environment (Paz-Silva et al., 2011; De et al., 2008; Tavela et al., 2011). Thus, much has been described about its predatory activity in laboratory and field conditions when used in pellets of sodium alginate matrix formulations (Silva et al., 2009; Vilela et al., 2012). On the other hand, the results showed in this work are promising since it represents the first report of the passage of different fungal species associated in a formulation of pellets containing *D. flagrans* and *M. thaumasium* separately and at the same time through the gastrointestinal tract of horses.

Fungal formulations should withstand the extreme environments of stress as the gastrointestinal tract of domestic animals to be used in programs to control nematodes of domestic animals (Mota et al., 2003; Araújo et al., 2004). In addition, it is necessary to study conditions of storage of these fungi that can be successfully used in the field (Sagués et al., 2011; Braga et al., 2011). Accordingly, the use of pellets containing micelia of nematophagous fungi in isolated formulations has been successfully tested (Braga et al., 2009; Tavela et al., 2011). However, there are no records of the use of these fungi in pellets containing associated formulations.

In the results presented in the assay A, it was observed that the two associations in different concentrations were effective in passage through the gastrointestinal tract of horses. In this context, the fungi were grown from the faeces, which suggest that these associations could possibly be used in the field, since the higher availability of cyathostomin L3 is dispersed in the environment (Tavela et al., 2011). Moreover, there is much discussion about the best dose of fungi that could potentially be more effective in field conditions. Thus, some studies have shown that the use of formulations based on sodium alginate pellets at the dose of 1g for each 10 Kg of corporal weight has been successful (Vilela et al., 2012). In this context, the use of two different concentrations of pellets corresponds the comparison between the half of the dose of pellets of each fungus (group 1: 50 g of pellets containing the mycelial mass of the fungi) and the full dose (group 2: 100 g of pellets containing the mycelial mass of the fungi) that was already successfully tested in other works (Araujo et al., 2010)

In this work, fecal samples were collected at intervals from 12 to 72 hours after administration of the associated pellets. According Assis and Araújo (2003) and Araujo

et al. (2010) these intervals have proved to be ideals for the passage of nematophagous fungi. This fact is interesting because when used as the formulation of pellets, individually, the fungi *D. flagrans* or *M. thaumasium* were recovered at time intervals until 72 hours and, in this case, the associations of these two fungi also were successfully recovered at the same time intervals. It suggests that the formulation sodium alginate pellets can tolerate the stress and confers resistance to this association of predators fungi through the passage through gastrintestinal tract and storage. Thus, the authors of the present study suggest that this formulation could be used in the field orally, at time intervals of 72 hours and keeping their predatory activity. Moreover, both the use of 50 g of pellets (group 1) of each fungus tested as 100 g (group 2) showed to be effective in *in vitro* control of L_3.

In test B, the results were similar, and the reduction of L_3 recovery from contaminated faeces by the fungi AC001 and NF34, associated, was always over 80%. Regarding the use of fungi in coprocultures, Bird and Herd (1995) evaluated the effect of the addition of spores of *A. oligospora* and *D. flagrans* in stool of horses, observing reduction rates of 95.8% and 93.9% respectively in the number of cyathostomin L_3. In another study, Castro et al. (1999) conducted an experimental assay, evaluating the predatory ability of *M. thaumasium* on pre-parasitic stages of horse cyathostomin, in which 1000 conidia of fungal isolate were added to coprocultures containing 1250 eggs per gram of faeces. In that work, the number of larvae in fungus-treated coprocultures was 97% lower than in control cultures. In the same way, Santos et al. (2001) reported reduction in the number of larvae recovered from coprocultures of cyathostomin horses treated with 500 spores of *D. flagrans* per gram of faeces above 90%. Thus, these

results corroborate the present work; however, it is valid to point out that the association of pellets containing fungi *D. flagrans* and *M. thaumasium* was able to pass through the gastrointestinal tract in horses, then these fungi were germinated in the faeces and after, were effective in reducing L_3 cyathostomin at the end of the experimental period.

In the present study were tested the associations of pellets of the fungi *D. flagrans* and *M. thaumasium* in different concentrations. Thus, through the results obtained, the authors suggest that these associations of pellets (with either 50 g or 100 g of each fungal isolate) were viable after passing through the gastrointestinal tract of horses and could be tested in field conditions in a higher range of time.

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7. CAPÍTULO 3

Association of different nematophagous fungi: effect on trichostrongylid infective larvae *in vitro* and after passage through the gastrointestinal tract of goats

Alexandre de Oliveira Tavela[1*], Jackson Victor Araújo[2], Fabio Ribeiro Braga[2,3], Wendeo Ferreira da Silveira[2], Filippe Elias Freitas Soares[4], Juliana Milani Araujo[2], Moacir Carretta Júnior[3], Vinicius Herold Dornelas e Silva[2], Alessandra Teixeira de Paula[2] and Laércio dos Anjos Benjamin[2]

1Centro de Ciências Rurais (CCR) - Universidade Federal de Santa Catarina-UFSC

Rod. Ulysses Gaboardi, Km 3, Curitibanos-SC, CEP: 89.520-000

2Departamento de Veterinária, Universidade Federal de Viçosa, Viçosa, MG.

3 Universidade Vila Velha, Vila Velha, ES

4 Departamento de Bioquímica e Biologia Molecular, Universidade Federal de Viçosa, Viçosa, MG

*Corresponding author: alexandre.tavela@ufsc.br

Abstract

The use of biological control using nematophagous fungi has shown good results in laboratory and field conditions. However, formulations with two or more fungi species have been little explored. The present work aimed to evaluate the effect of the nematophagous fungi *Duddingtonia flagrans* (AC001) and *Monacrosporiumthaumasium* (NF34) in association on trichostrongylid infective larvae under laboratory conditions (assay A) and after passing through the gastrointestinal tract of goats (assay B). For assay

A, four groups of Petri dishes with 2% water–agar (2% WA) were formed. In the treated groups each Petri dish contained 1000 trichostrongylid third-stage larvae (L_3) and three different concentrations of conidia of the fungi to be tested: group 1 (AC001 + NF34 – 500 conidia); group 2 (AC001 + NF34 – 1000 conidia); group 3 (AC001 + NF34 – 3000 conidia). The control group (without fungi) contained only 1000 L_3. For assay B, two groups (with four goats each) either received orally 50 g of pellets containing mycelia (0,2 mg/10 kg b.w.) of the fungus AC001 and 50 g of pellets of the fungus NF34 in association, or no fungus (control). Subsequently, samples of faeces of the animals were collected 12, 24, 36, 48, 60 and 72 hours after the inoculation. Faeces were poured into Petri dishes containing 2% WA and 1000 trichostrongylid L_3. In assay A, at the end of the experiment were observed reductions in L_3 of 93.2% to 94.9%. In assay B, in all the studied time intervals the average number of L_3 recovered from the plates of the treated group (combination of pellets) was lower (p<0.01) than for the control group, varying from 88.2% to 93.1%. The use of the fungi *D. flagrans* and *M. thaumasium* in association (*in vitro*) for the control of trichostrongylids showed promising results. Tests should be performed in the field, in order to observe the effectiveness of association of the fungi *D. flagrans* and *M. thaumasium* in the environmental control of nematodes of goats.

Keywords: Nematophagous fungi, trichostrongylids, *Duddingtonia flagrans*, *Monacrosporium thaumasium*, control of parasites, goats.

1. Introduction

Trichostrongylid nematodes are responsible for large economic losses of the farmers of small ruminants, due to the negative impact that they cause in the production of meat and milk, as well as the high costs of control measures (Waller and Larsen 1993; Sykes 1994; Torina et al. 2004). Trichostrongylids have a cosmopolitan distribution; among the most important genera in tropical countries are *Haemonchus* sp., parasite of the abomasum, and *Trichostrongylus* sp., parasite of the abomasum and intestine of ruminants (Urquhart et al. 1996).

Anthelmintics have been used over many decades to combat these nematodes. However, the use of this method of control alone does not always produce satisfactory results (Kaplan 2004). This is due, among other factors, to the parasitic resistance that has already been established worldwide (Drudge et al. 1964; Kaplan 2004). According to Torres-Acosta and Hoste (2008), the current situation of parasite resistance implies a need to change the concept of using anthelmintics, together with the search for alternative or complementary solutions to conventional chemical treatments.

In this sense, the rise of goat and sheep farming internationally has increased the demand for knowledge and technology that contribute to improving the efficiency of these activities (Cavalcante et al. 2009). Therefore, another approach would be to use biological control, mainly using nematophagous fungi along with chemical control, a strategy that in recent decades has shown good results in laboratory conditions and the field (Larsen 1999; Braga et al. 2009, 2011; Silva et al. 2009; Tavela et al. 2011). Biological control with nematophagous fungi can be incorporated in industrial production, but it must be thoroughly studied in order to gain knowledge about the main characteristics of the best formulation, the ability to pass through the gastrointestinal tract of target species, and the

best fungal isolates and their predatory activity in gastrointestinal nematode parasites in the field (Araújo et al. 2004b).

Diferent species of nematophagous fungi vary in their ability to capture nematodes (Mota et al. 2003). Predatory fungi form traps along the hyphae separated by small intervals. These structures are produced in response to the presence of nematodes or substances secreted by them thus pure cultures have difficulty in producing or not producing traps (Larsen et al. 1992, 1995). “Helmintophagous fungi” species predominantly affect nematodes whose eggs have short environmental development stage, because while the larvae live in the egg, these fungi are unable to capture them and digest them (Braga and Araújo, 2012). Associations between predator species may be promising uses in the field because the effect of reduction in the larvae population could be complementary, since each fungal species has optimal conditions for growth and survival, as well as different strategies of predation (Tavela et al. 2011).

However, to date, formulations containing combinations of two or more nematophagous fungi in the control of domestic animal nematodiosis have been little explored. The only report has been of the use of formulations of sodium alginate pellets (0.2 g of fungus/10 b. w.) from genus *Duddingtonia,Monacrosporium* and*Arthrobotrys*, which showed efficacy against infective third-stage larvae (L_3) of cyathostomin under laboratory conditions (Assis and Araújo 2003; Braga et al. 2009; Tavela et al. 2012).

In this context, the present work aimed to evaluate the effect of the nematophagous fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34) in association on trichostrongylid infective larvae under laboratory conditions and after passing through the gastrointestinal tract of goats.

2. Materials and methods

In the present work two experimental assays (A and B) were performed in distinct steps. In assay A, we evaluated the *in vitro* capacity of the predatory fungi *D. flagrans* and *M. thaumasium* at three different concentrations (500, 1000 and 3000) of fungal structures (conidia) on trichostrongylid L_3. In assay B, we evaluated the passage capacity of the fungi *D. flagrans* and *M. thaumasium* associated in pellets of sodium alginate matrix through the gastrointestinal tract of goats, regarding resistance and viability of this formulation, observing their predatory activity after passage on trichostrongylid L_3.

2.1. Experimental assay A

2.1.1.Conidia

One isolate of predatory fungus of the genus *Duddingtonia* (*D. flagrans* – AC001) and an isolate of fungus of the genus *Monacrosporium* (*M. thaumasium* – NF34) were used. These isolates were obtained from Brazilian soil using the soil sprinkling method (Duddington 1955) as modified by Santos et al. (1991). They were kept at 4°C, in the dark and in test tubes containing 2% CMA.

Culture discs of 4 mm in diameter were extracted from fungal isolates kept in test tubes containing 2% cornmeal–agar (2% CMA) and transferred to Petri dishes of 9.0 cm in diameter containing 20 ml of 2% potato dextrose–agar kept at 25°C in the dark for 10 days. After growth of the isolates, new culture discs of 4 mm in diameter were transferred to Petri dishes of 9.0 cm in diameter containing 20 ml of 2% water–agar (2% WA), where 1 ml of distilled water containing 1000 larvae of *Panagrellus* sp. was added daily for a period of 21 days to induce formation of conidia. When complete fungal development was

observed, 5 ml of distilled water were added to each Petri dish. Conidia and mycelial fragments were removed according to the technique described by Araújo et al. (1993).

2.1.2. Trichostrongylid larvae

Faeces of goats naturally infected with trichostrongylids were collected with the aid of collection bags made of cotton sacks. Faecal samples of 20 g were mixed with 20 g of sterilized vermiculite and 5 ml of distilled water and incubated for 10 days at 30°C in the dark. The larvae were recovered from coprocultures of haemolysis tubes, through the Baermann apparatus. The larvae were identified according to Ueno and Gonçalves (1994). The reading of Baermann showed the presence of trichostrongylid L_3.

2.1.3. *In vitro* experimental assay

In this study four groups in Petri dishes of 4.5 cm in diameter containing 10 ml of 2% WA were formed: three treated groups and one control group (six replicates per group). In the treated groups each Petri dish contained 1000 trichostrongylid L_3 and three different concentrations of conidia of tested fungi: group 1 (AC001 + NF34 – 500 conidia from each fungi); group 2 (AC001 + NF34 – 1000 conidia from each fungi); group 3 (AC001+ NF34 – 3000 conidia from each fungi) in 2% WA. The control group (without fungi) contained only 1000 L_3 in plates with 2% WA. The Petri dishes were kept at 27ºC, in the dark at ± 80% humidity.

For 6 days, every 24 hours, 10 random fields of 4 mm diameter on each plate of the treated and control groups were observed by light microscopy using objective 10x, counting the number of non-predated L_3 for each field. On the sixth day culture discs of 2 mm diameter were transferred to Petri dishes containing 2% water-agar (2% WA) of the

medium whose surface is covered with a dialysis membrane (SIGMA). Then, the plates were incubated in the dark at 25 °C for seven days (Nordbring-Hertz 1978). After 24 hours of observation of interaction were obtained cuts of 4x4 mm diameter in dialysis membrane with samples of L3 exposed to predation by fungi. The material was fixed in 2.5% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4 for 24 hours, washed six times in the same buffer, postfixed in 2% osmium tetroxide and dehydrated by serial passage of increasing concentrations of ethanol. The material was dried in a critical point dryer BALZERS, metallized and observed in a scanning electron microscope LEO 10-15 kV (Nordbring-Hertz 1978) at the Center for Electron Microscopy and microanalysis, located at the Federal University of Viçosa.

On the seventh day, the non-predated L_3 were recovered from the content of each Petri dish through the modified apparatus of Baermann with water at the starting temperature of 42°C. Then, the apparatus is placed over night at room temperature, during which the larvae migrate out of the faeces and through the sieve to sediment in the neck of the funnel (Kaufmann 1996). The genera *Haemonchus* sp. (70%), *Cooperia* sp. (20%) and *Trichostrongylus* sp. (10%) were identified.

2.1.4. Statistical analysis

In assay A, the average of trichostrongylid L_3 recovered from plates after 7 days was calculated. The data were interpreted statistically by analysis of variance (ANOVA), with significance levels of 1% probability (Ayres et al. 2003). The predation efficiency of L_3 in the treated groups compared with control was assessed by the Tukey test at 1% probability for each day of observation. Subsequently, the average percentage of reduction of L_3 was calculated according to the following formula:

$$\% \text{ Reduction} = \frac{(\text{Average of } L_3 \text{ recovered from control} - \text{average of } L_3 \text{ recovered from treatment})}{\text{Average of } L_3 \text{ recovered from control}} \times 100$$

2.2. Experimental assay B

2.2.1. Location of the experiment and animals

The experiment was conducted at the Department of Veterinary Medicine, Federal University of Viçosa, located in the city of Viçosa, Minas Gerais, Brazil (latitude 20° 45' 20"; longitude 42º 52' 40"). A total of eight goats were used, males and females, with an average weight of 30 kg and eight months old, stabled, previously orally dewormed at a dose of 5 μg albendazole per kg body weight and 10% cobalt sulphate (1.3 g/100 ml) (Aldazol 10co; Vallé®, Montes Claros-Minas Gerais, Brazil).

During the experimental period water and autoclaved grass were provided to animals *ad libitum*. Every day, all animals were supplemented with protein–energy concentrate at a concentration of 0.75%, with balanced mineral salts.

2.2.2. Mycelial mass and formulation in a matrix of sodium alginate

One isolate of predatory fungus of the genus *Duddingtonia* (*D. flagrans* – AC001) and an isolate of fungus of the genus *Monacrosporium* (*M. thaumasium* – NF34) were used. These isolates were obtained from Brazilian soil using the soil sprinkling method (Duddington 1955) as modified by Santos et al. (1991). They were kept at 4°C, in the dark and in test tubes containing 2% CMA.

To induce the formation of mycelia, fungal culture disks in 2% CMA of approximately 5 mm were transferred to 250 ml Erlenmeyer flasks, containing 150 ml of potato dextrose liquid medium (Difco), pH 6.5, under agitation at 120 rpm in the dark

and at a temperature of 26°C for 10 days. After this period, the mycelial mass was removed for the preparation of pellets, which were made in matrix of sodium alginate according to the technique described by Walker and Connick (1983) and modified by Lackey et al. (1993).

2.2.3. Passage test

The animals were randomly divided into two groups with four animals each (a group treated with the combination of fungi and a control group without fungus) and kept in separate pens.

In the treated group, each animal received simultaneously 50 g of pellets, in a single dose, containing mycelial mass (0.2 g) of the fungus *D. flagrans* and 50 g of pellets, in a single dose, containing mycelial mass (0.2 g of fungus) of *M. thaumasium* mixed in 100 g of commercial goat feed. The control group animals received a single administration of 100 g of sodium alginate pellets without fungus mixed in 100 g of commercial goat feed.

Faecal samples were collected from each animal at intervals of 12, 24, 36, 48, 60 and 72 hours after administration of treatment with the fungus and control, according to Assis and Araújo (2003). The collected samples were homogenized, and then 2 g were removed and placed in Petri dishes of 9 cm diameter containing 2% WA. Into each Petri dish of the treated and control groups 1000 trichostrongylid L_3 were poured. Then the plates were placed in an incubator at 25°C in the dark for 15 days. For each established time, 12 replicates for each group of animals were performed. Every day, the Petri dishes of the treated and control groups were observed for the detection of structures of the fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34), conidia, conidiophores and characteristic chlamydospores. For the proof and identification of the passage of the

tested fungi through the gastrointestinal tract, the keys for the classification of fungal structures (conidia and/or chlamydospores) proposed by Van Oorschot (1985) and Liu and Zhang (1994) were adopted.

On the fifteenth day, the non-predated L_3 were recovered from the Petri dishes by the Baermann technique.

2.2.4. Statistical analysis

Data obtained were submitted to analysis of variance (ANOVA). The averages were compared using Tukey's test at the 1% level of probability (Ayres et al. 2003).

3. Results

In assay A, the tested isolates of the predatory fungi of nematodes, *D. flagrans* (AC001) and *M. thaumasium* (NF34), in different concentrations of conidia, were effective in the *in vitro* destruction of the trichostrongylid L_3 (Fig. 1). In relation to this fact, evidence of predation was observed since the day 1 and by day 6 most larvae in the fungal-treated groups had been trapped. At the end of the experiment (seventh day), the following reduction percentages of L_3 were observed: group 1 (AC001 + NF34 – 500 conidia), 93.2%; group 2 (AC001 + NF34 – 1000 conidia), 93.8%; group 3 (AC001 + NF34 – 3000 conidia), 94.9%.

No significant difference ($p>0.01$) was observed between the predation of the isolates with different concentrations at the end of 7 days in the treated groups; however, a difference was noted ($p<0.01$) compared with the control group at all times of visualization.

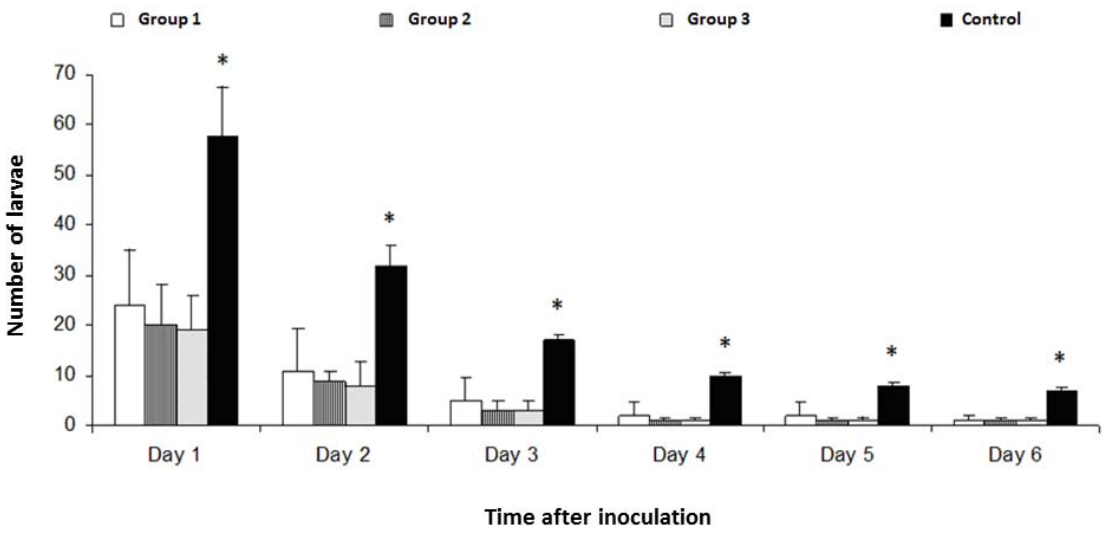

Fig. 1 – Mean of infective non-predated larvae of trichostrongylidsfor random fields of 4 mm diameter counted during the six days of observations in groups of different concentrations of conidia: group 1 (AC001 + NF34 - 500 conidia from each fungi); group 2 (AC001 + NF34 – 1000 conidia from each fungi); group 3 (AC001+ NF34 – 3000 conidia from each fungi) in 2%WA and the control group (without fungi). Asterisks mark statistical difference ($p<0.01$).

Every 24 hours, the predation and formation of traps were visualized in all plates of the treated groups with different concentrations of conidia of associated fungi in the Petri dishes (Fig. 2). However, neither the presence nor the formation of traps originating from nematophagous fungi in the control group was noticed.

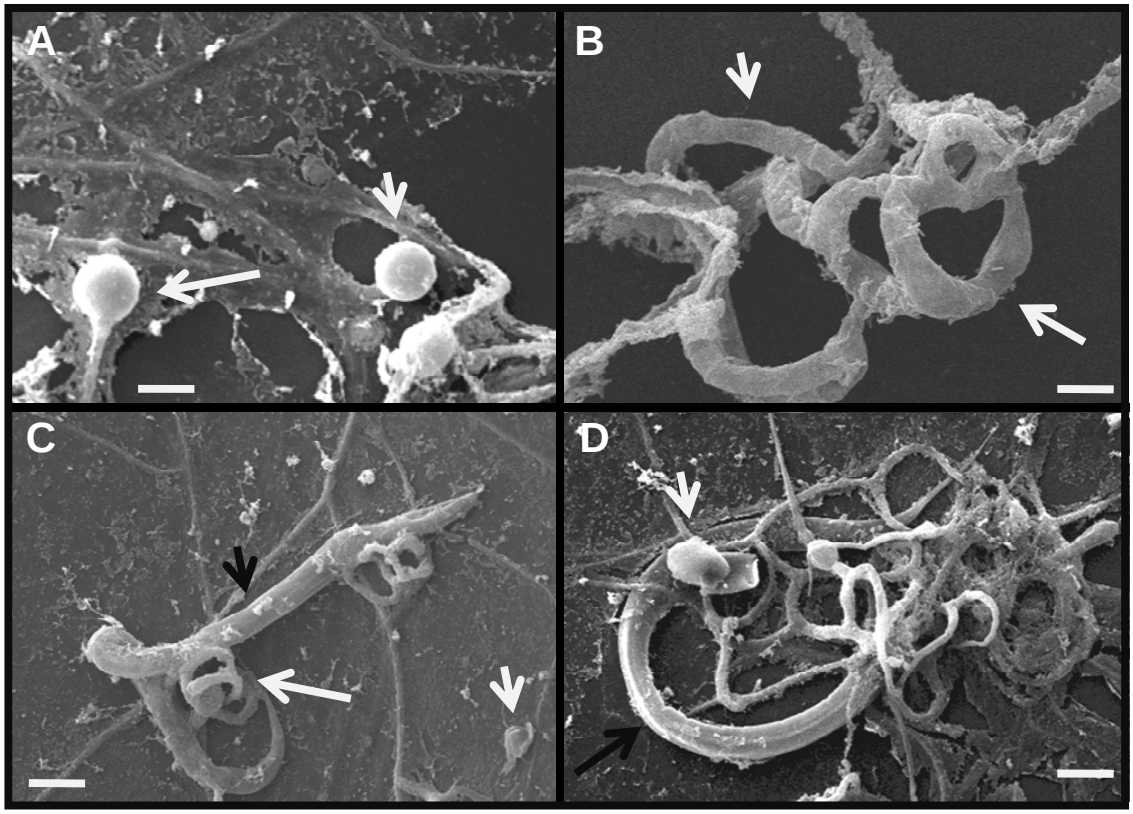

Fig. 2 – A-D: Scanning electron micrografs of fungal structures of tested nematophagous fungi *Duddingtonia flagrans* (AC001) and *Monacrosporium thaumasium* (NF34) (traps, chlamydospores and conidia - white arrow) and infective larvae of trichostrongylids (black arrows). A: fungal structures (chlamydospores). B - traps (constrictor rings). C and D: AC001 and NF34 hyphae and the interaction with trichostrongylid larvae. Bars: A – 2.5 µm; B – 20 µm; C – 50 µm and D – 50 µm.

In assay B, it was observed that the fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34) given orally (pellets) destroyed the trichostrongylid L_3 after passage through the gastrointestinal tract of goats. At the end of each studied time, the following percentages of reduction were found: 88.2% (12 hours); 91.4% (24 hours); 85.5% (36 hours); 93.1% (48 hours); 91.3% (60 hours); and 89.6% (72 hours) for the isolates AC001 and NF34, when compared with the control group. Thus, at all the times

studied the average of recovered larvae from plates of the treated group (association of pellets) was lower (p<0.01) than that of the control group(Fig. 3).

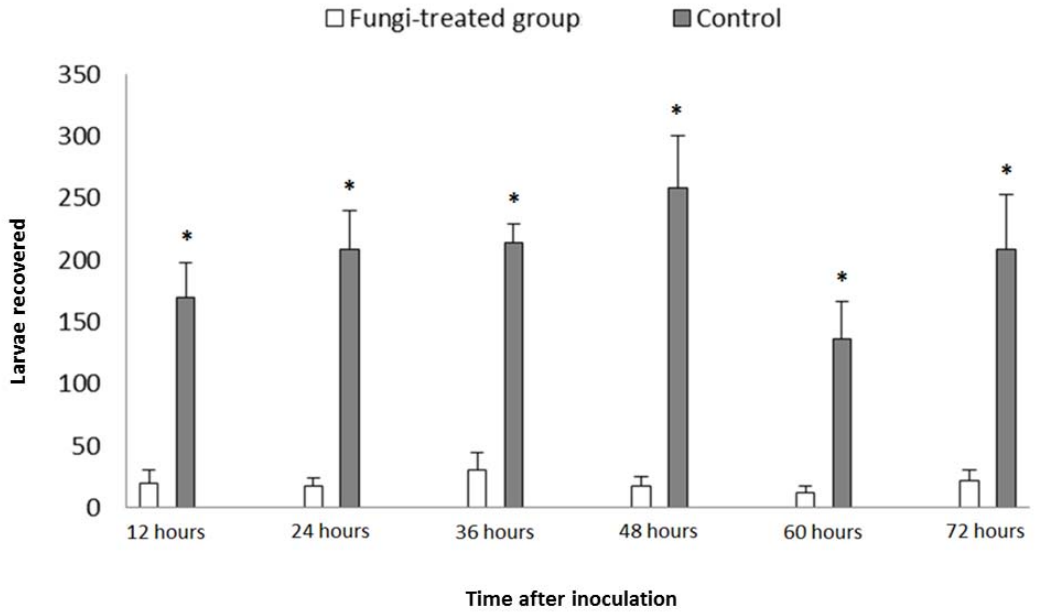

Fig. 3 – Mean of recovered infective larvae (L3) of trichostrongylids by the method of Baermann from Petri dishes containing faeces of the treated group (50 g of pellets with AC001 and 50 g of pellets with NF34) and the control group after 15 days of incubation with 1000 L3. Faecal samples were taken from both groups of animals at 12, 24, 36, 48, 60 and 72 hours post-treatment. Asterisk mark statistical difference (p<0.01).

Regarding the intervals studied, the 48-hour period after administration of the association of pellets containing mycelial mass of the isolates AC001 and NF34 showed a higher predatory activity and consequently a greater percentage of reduction in the recovered trichostrongylid L_3.

4. Discussion

Parasitic resistance due to the incorrect use of anthelmintics represents a major concern for the sector (Kaplan 2004; Papadopoulos 2008) and alternative measures of control must be constantly studied (Silva et al. 2009). Araújo et al. (2004b) mentioned

that the use of nematophagous fungi for the biological control of gastrointestinal parasites of domestic animals can reduce the contamination of pasture, acting directly on the infective larvae in the environment. On the other hand, cattle, goats and sheep in a general way harbour the same genera of gastrointestinal helminth parasites; however, there are insufficient studies of considerable data on the integrated control of helminth parasites of goats.

In assay A, it was observed that the association of different concentrations of nematophagous fungi destroyed trichostrongylid L_3 within 7 days. Although several authors have reported the efficacy of these organisms under laboratory conditions (Araújo et al. 1993; Larsen 1999), the association of nematophagous fungi in order to control nematode L_3 of small ruminants requires more studies. In this context, this work presents a new approach to *in vitro* control of trichostrongylids of goats.

Tavela et al. (2012) showed that the association of predatory and ovicidal nematophagous fungi was efficient in the destruction of eggs and cyathostomin L_3 of horses. However, these authors proposed that studies in the field with a combination of fungi should be performed. In this context, in assay A of the present study, the association of the isolates AC001 and NF34 was proposed in three different concentrations of conidia which increases the chances of these organisms of being used in the field.

Little is known about the effects of the association of fungal structures or isolates of nematophagous fungi on trichostrongylid L_3. Tavela et al. (2012) observed that the association of fungal isolates *D. flagrans* (AC001) and *M. thaumasium* (NF34) decreased, at the end of the experiment, 92.4% of cyathostomin L_3. These results are in agreementwith the present work, where we also observed the effectiveness of these

isolates with a reduction in the average of trichostrongylid L_3 recovered exceeding 90% at the end of assay A, at the three concentrations tested.

Araújo et al. (2004a) reported that fungi of the genera *Duddingtonia*, *Arthrobotrys* and *Monacrosporium* used separately or in association showed no variations in the predatory capacity among the tested fungi against trichostrongylid L_3 of cattle for 5 days of experimental trial. On the other hand, these authors reported that when there was an association of three or four fungi in Petri dishes, the results showed no efficacy (p>0.05) of the same and suggested that perhaps there was competition among the tested fungi in these situations.In the present work, the high percentage of L_3 reduction seen at the end of the assay "A" in the three treated groups suggests that the fungi*M. thaumasium* and *D. flagrans* maintained their efficacy when coadministrated.

In relation to the use of fungal structures (conidia, chlamydospores and mycelium), Campos et al. (2008) reported that, although chlamydospores have a high resistance when passing through the gastrointestinal tract of animals and maintain their predatory activity, their production requires a longer period of time when compared with the production of mycelia and conidia. Thus, the association of predatory fungal isolates *D. flagrans* (which produces conidia and chlamydospores) and *M. thaumasium* (which produces conidia) could be interesting for the treatment of animals in the field.

In assay B, it was demonstrated that the pellets in a matrix of sodium alginate containing the isolates of the fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34), when administered at the same time, were efficient in passage through the gastrointestinal tract of goats. In the present study predatory capacity against trichostrongylid L_3 at the end of 15 days was observed. The fungi were able to germinate from the faeces and produce reproductive structures (conidia and/or

chlamydospores) and traps. These results are consistent with several reports (Walker and Connick 1983; Fravel, et al. 1985; Salgado and Campos 1993; Melo and Sanhueza 1995; Araújoet al. 1999; Braga et al. 2009; Tavela et al. 2011). These previous studies have been conducted using pellets containing a single non-associated fungal isolate, however may serve as parameters for comparison with the present work.

Goat rearing is an activity of increasing socio-economic importance in the world. The raising of goats for meat and milk are alternatives, both for food production, as well as for the diversification of income from property and employment generation in the field (Silva 2003). Vilela et al. (2012) showed that a sodium alginate formulation (pellets) containing mycelial mass of *D. flagrans* (AC001) was effective in controlling gastrointestinal nematodes of goats in the semi-arid northeast region of Brazil.

In another context, Silva et al. (2009) reported that pellets containing mycelia of the fungus *M. thaumasium* (NF34) were effective in the prophylaxis of nematode parasites of sheep when administered in the ration for 6 months. On the other hand, the assays of the present work have showed that the fungi are effectively nematophagous under their laboratory conditions prior to being administered to the goats (Fig. 2). However, is the first report of pellets containing mycelial mass of these two isolates and the results obtained may serve as a tool for the use of this new formulation in the field.

Finally, the use of fungi of the genera *Duddingtonia* and *Monacrosporium* was tested through base formulations of sodium alginate. However, a new approach was described in the present work and with promising results. In this context, tests should be performed in the field, in order to observe the effectiveness of association of the fungi *D. flagrans* and *M. thaumasium* in the environmental control of nematodes of goats.

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8. Consideraçõesfinais

O presente estudo mostrou que os fungos nematófagos *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34) e *Pochonia chlamydosporia* (VC1) foram eficientes no controle ciatostomíneos em condições laboratoriais, sendo que todas as combinações testadas foram semelhantes quanto a redução de larvas recuperadas ao final do experimento.

Além disso, as associações de péletes em matriz de alginato de sódio contendo os fungos*D. flagrans* e *M. thaumasium*,em diferentes concentrações,se demonstraram viáveis após a passagem através do tubo digestivo de equinos, preservando a capacidade predatória dos fungos, com redução significativa das larvas infectantes de ciatostomíideos em todos os intervalos de tempo testados.

Da mesma forma, osresultados do presente trabalho demonstraram que os fungos*D. flagrans* e *M. thaumasium*possuem elevada capacidade predatória *in vitro* quando utilizados em conjunto. Além disso, os fungos testados mantiveram-se viáveis após a passagem pelo tubo digestivo de caprinos, quando coadministrados.

Neste contexto, os ensaios experimentais realizados contribuíram para um melhor compreendimento sobre a capacidade de predação relacionada a associação dos fungos nematófagos estudados *in vitro* e após a passagem pelo tubo digestivo de equinos e de ruminantes, independente da concentração de conídios testadas.

Sendo assim, mais testes devem ser realizados, a campo, a fim de observar a eficácia das associações entre os fungos e *M. thaumasium*e *D. flagrans* no controle ambiental dos nematoides parasitos de equinos e de caprinos.