ÍTALO STOUPA VIEIRA

FUNGOS NEMATÓFAGOS NO CONTROLE BIOLÓGICO DE NEMATOIDES PARASITAS GASTRINTESTINAIS DE BOVINOS

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.*

Orientador: Jackson Victor de Araújo

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Ítalo Stoupa Vieira

Autor

Jackson Victor de Araújo

Orientador

RESUMO

VIEIRA, Ítalo Stoupa, D.Sc., Universidade Federal de Viçosa, julho de 2019. **Fungos nematófagos no controle biológico de nematoides parasitas gastrintestinais de bovinos.** Orientador: Jackson Victor de Araújo.

Nematoides parasitas gastrintestinais são responsáveis por perdas significativas nos processos produtivos de ruminantes, principalmente em sistemas extensivos de produção de bovinos. A resistência dos helmintos a anti-helmínticos tem se tornado frequente e o controle biológico pela utilização de fungos nematófagos é uma promissora alternativa na profilaxia das helmintoses gastrintestinais de bovinos. Fungos nematófagos ovicidas e predadores possuem mecanismos de ação distintos, assim, quando utilizados em combinação, podem apresentar ação complementar e sinérgica no controle biológico de helmintos. Os objetivos do presente trabalho foram: avaliar os fungos *Arthrobotrys cladodes* (isolado CG719), *Duddingtonia flagrans* (isolado AC001) e *Pochonia chlamydosporia* (isolado VC4) em diversas condições de temperatura, quanto ao crescimento micelial, à produção de clamidósporos e à atividade nematicida sobre larvas infectantes (L3) de helmintos parasitas de bovinos; avaliar a compatibilidade de crescimento conjunto entre o fungo predador *A. cladodes* e o fungo ovicida *P. chlamydosporia*; avaliar a viabilidade e a atividade nematicida de *A. cladodes* e de *P chlamydosporia*, isoladamente e associados em matriz de alginato de sódio, após passagem pelo trato gastrintestinal de bovinos; e avaliar a ação conjunta e isolada de *A. cladodes* e *P chlamydosporia* na redução da carga parasitária de bovinos criados em pastagens. *A. cladodes, D. flagrans* e *P. chlamydosporia* apresentaram níveis variados de crescimento micelial, atividade nematicida e produção de clamidósporos nas temperaturas de 15, 20, 25, 30 e 35 ° C. Os testes de antagonismo em confrontação direta, de antibiose e do efeito de metabólitos voláteis entre *P. chlamydosporia* e *A. cladodes* indicaram a viabilidade de crescimento em conjunto destes fungos. As formulações peletizadas de alginato de sódio contendo *P. chlamydosporia* e *A. cladodes* resistiram à passagem pelo trato gastrintestinal de bovinos e os fungos mantiveram a viabilidade de crescimento e predação de helmintos. O uso combinado de *A. cladodes* e *P. chlamydosporia* mostrou-se eficaz no controle biológico de helmintos parasitas gastrintestinais de bovinos e apresentou maior atividade nematicida que os mesmos fungos utilizados isoladamente. A carga parasitária foi menor e o ganho de peso foi maior ($p \leq 0,05$) nos grupos de bovinos tratados com fungos nematófagos. Portanto, a utilização de *A. cladodes* e *P. chlamydosporia* mostrou-se promissora no controle de helmintoses em bovinos criados em pastagens.

Palavras-chave: Controle biológico. Helmintos. Bovinos. *Arthrobotrys cladodes*. *Duddingtonia flagrans*. *Pochonia chlamydosporia*.

ABSTRACT

VIEIRA, Ítalo Stoupa, D.Sc., Universidade Federal de Viçosa, July, 2019. **Nematophagous fungi in biological control of bovine gastrointestinal parasitic nematodes.** Orientador: Jackson Victor de Araújo.

Gastrointestinal parasitic nematodes are responsible for significant losses in ruminant production processes, especially in extensive cattle production systems. Helminth resistance to anthelmintics has become frequent and biological control by the use of nematophagous fungi is a promising alternative in the prophylaxis of bovine gastrointestinal helminthiasis. Ovicidal and predatory nematophagous fungi have distinct mechanisms of action, so when used in combination they may have complementary and synergistic action in the biological control of helminths. The objectives of the present work were: to evaluate the fungi *Arthrobotrys cladodes* (CG719 isolate), *Duddingtonia flagrans* (AC001 isolate) and *Pochonia chlamydosporia* (VC4 isolate) under different temperature conditions, for mycelial growth, chlamydospore production and nematicidal activity against bovine helminth infective larvae; to evaluate the compatibility of joint growth between the predatory fungus *A. cladodes* and the ovicidal fungus *P. chlamydosporia*; to evaluate the viability and nematicidal activity of *A. cladodes* and *P. chlamydosporia*, alone and associated in sodium alginate matrix, after passage through the gastrointestinal tract of cattle; and evaluate the joint and isolated action of *A. cladodes* and *P. chlamydosporia* in reducing the parasitic load of grazing cattle. *A. cladodes, D. flagrans* and *P. chlamydosporia* presented varying levels of mycelial growth, nematicidal activity and chlamydospore production at temperatures of 15, 20, 25, 30 and 35 ° C. Tests of direct confrontation antagonism, antibiosis and the effect of volatile metabolites between *P. chlamydosporia* and *A. cladodes* indicated the viability of these fungi to grow together. Pelletized sodium alginate formulations containing *P. chlamydosporia* and *A. cladodes* resisted passage through the bovine gastrointestinal tract and the fungi maintained the viability of growth and predation of helminths. The combined use of *A. cladodes* and *P. chlamydosporia* was effective in the biological control of bovine gastrointestinal parasitic helminths and showed higher nematicidal activity than the same fungi used alone. The parasite load was lower and the weight gain was higher ($p \leq 0.05$) in the groups of cattle treated with nematophagous fungi. Therefore, the use of *A. cladodes* and *P. chlamydosporia* was promising to control helminthiasis in grazing cattle.

Keywords: Biological control. Helminths. Cattle. *Arthrobotrys cladodes*. *Duddingtonia flagrans*. *Pochonia chlamydosporia*.

SUMÁRIO

1. INTRODUÇÃO GERAL

A agropecuária foi responsável por 5,1% de todos os bens e serviços finais produzidos no Brasil em 2018. Além disso, tal setor gerou aproximadamente 9,0 milhões de vagas no mercado de trabalho brasileiro em 2018. Os sistemas de produção de gado de corte e leite são importantes componentes da atividade agropecuária. Em 2018 foram abatidos 31,9 milhões de bovinos, resultando na produção de 7,68 milhões de toneladas de carcaças bovinas e os laticínios brasileiros captaram 24,45 bilhões de litros de leite (IBGE, 2019).

A criação de bovinos em sistemas extensivos baseados na utilização de pastagens é largamente adotada pelos pecuaristas brasileiros e tal fato favorece a ocorrência constante de parasitoses causadas por helmintos. As helmintoses gastrointestinais representam um dos entraves ao desenvolvimento da produção brasileira de bovinos, causando perdas financeiras em torno de $7,11 bilhões/ano, os quais são reflexos dos custos dos tratamentos veterinários, queda da produção, retardo no crescimento e morte de animais (Grisi et al., 2014).

Drogas anti-helmínticas representam a principal forma utilizada para o controle de helmintoses, entretanto, os resultados na diminuição da carga parasitária dos animais nem sempre são satisfatórios. Os relatos de desenvolvimento de resistência anti-helmíntica são frequentes e, assim, torna-se necessária a busca por métodos complementares no controle de helmintoses (Gasbarre, 2014; Fazzio et al., 2014).

O controle biológico pela utilização de fungos nematófagos é uma alternativa para complementar as estratégias de controle de helmintoses intestinais. A dispersão de estruturas fúngicas diretamente nas fezes, onde ocorre a eclosão de ovos e as larvas se tornam infectantes (L3), é uma das formas utilizadas para o estabelecimento do controle biológico de nematoides parasitas gastrintestinais de bovinos (Paz-Silva et al., 2011). Uma alternativa para a disseminação destes fungos no ambiente é incorporar estruturas fúngicas em matriz de alginato de sódio (Silva et al., 2014) e fornecê-las incorporadas à dieta dos bovinos. Após passarem pelo trato gastrintestinal, essas estruturas germinam nas fezes, formando uma rede de hifas que se diferenciam em armadilhas que capturam e destroem formas infectantes de vida livre dos helmintos parasitas (Braga e Araújo, 2014). Mudanças na temperatura ambiental e variações diárias na temperatura do bolo fecal contendo fungos nematófagos, após passarem pelo trato gastrintestinal de animais, podem afetar o crescimento, a produção de clamidósporos e a atividade nematicida destes fungos.

Duddingtonia flagrans, Arthrobotrys cladodes e *Pochonia chlamydosporia* são fungos nematófagos com grande potencial de utilização no controle biológico de helmintos parasitas de animais domésticos (Silva et al., 2011; Tavela et al., 2012; Braga et al., 2013; Oliveira et al., 2018a, 2018b). O fungo ovicida *P. chlamydosporia* parasita ovos de helmintos por meio de apressórios, desenvolvidos a partir de hifas indiferenciadas, que permitem a colonização da superfície do ovo e a penetração por ação mecânica e enzimática (Braga et al., 2008). Há relatos que *P. chlamydosporia*, além da ação ovicida, parasita fêmeas de nematoides do gênero *Meloidogyne*, as quais são colonizadas e digeridas completamente (Podestá et al., 2009; Zouhar et al., 2010). Os fungos predadores *D. flagrans* e *A. cladodes* aprisionam os helmintos em armadilhas formadas por redes tridimensionais de hifas, as quais penetram na cutícula do parasita, ocorrendo a digestão dos conteúdos internos da larva (Mota et al., 2003; Eslami et al., 2005; Oliveira et al., 2018a, 2018b).

Por possuírem mecanismos de ação distintos, fungos nematófagos ovicidas e predadores, quando utilizados conjuntamente, podem apresentar ação complementar e sinérgica no controle biológico de helmintos (Ayupe et al., 2016). Entretanto, o crescimento em conjunto de fungos nematófagos distintos pode ser inviável, assim, torna-se necessário conhecer a interação entre eles através de estudos de compatibilidade antes de utilizá-los a campo.

2. JUSTIFICATIVA E OBJETIVOS

O parasitismo gastrintestinal por nematoides é um significativo fator limitante nos sistemas de produção de bovinos. O uso de compostos anti-helmínticos visa a diminuição do número de larvas infectantes na pastagem por meio da diminuição da população de parasitos adultos nos animais. Entretanto, a utilização de anti-helmínticos possui limitações, tais como: resíduos de drogas em produtos animais, efeitos tóxicos em organismos não alvos, poluição do meio ambiente e resistência a compostos químicos anti-helmínticos. Com o intuito de minimizar o uso de anti-helmínticos em ruminantes, o controle biológico pelo uso de fungos nematófagos se faz uma alternativa eficiente e segura na redução da população de larvas infectantes de nematoides parasitas nas pastagens. A associação e compatibilidade entre fungos nematófagos são pouco estudadas. Assim, a associação de espécies fúngicos pode ocasionar sinergismo e potencialização das ações nematicidas ou suprir deficiências que possam existir quando se usa uma única espécie.

Objetivo geral: Avaliar a atividade nematicida dos fungos *P. chlamydosporia* e *A. cladodes*, isoladamente e em associação, sobre helmintos parasitas gastrointestinais de bovinos criados extensivamente em pastagens do sudeste do Brasil.

Objetivos específicos:

- Avaliar o crescimento, produção de clamidósporos e atividade nematicida dos fungos *P. chlamydosporia* (isolado VC4)*, D. flagrans* (isolado AC001) e *A. cladodes* (isolado CG719) em diferentes temperaturas;
- Verificar a compatibilidade entre os fungos *P. chlamydosporia* (isolado VC4) e *A. cladodes* (isolado CG719) através dos testes de confrontação direta, antibiose e de compostos voláteis;
- Avaliar as reduções do número de larvas infectantes (L3) recuperadas das coproculturas nos testes de eficácia *in vitro* e de passagem pelo trato gastrintestinal de bovinos dos fungos *P. chlamydosporia* (isolado VC4) e *A. cladodes* (isolado CG719);
- Avaliar as contagens de ovos de helmintos parasitas por grama de fezes (OPG) e o ganho de peso de bovinos criados em pastagens e tratados com os fungos *P. chlamydosporia* (isolado VC4) e *A. cladodes* (isolado CG719);
- Avaliar a carga parasitária de pastagens em que foram criados bovinos tratados com os fungos *P. chlamydosporia* (isolado VC4) e *A. cladodes* (isolado CG719).

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4. CAPÍTULO 1

EFFECT OF TEMPERATURE ON THE NEMATICIDAL ACTIVITY, MYCELIAL GROWTH AND CHLAMYDOSPORE PRODUCTION OF NEMATOPHAGOUS FUNGI

ABSTRACT

Variations in temperature can affect the development of nematophagous fungi, especially when they are used in the biological control of parasitic helminths in the pastures where cattle are reared. The mycelial growth, chlamydospore production and nematicidal activity of *Duddingtonia flagrans, Arthrobotrys cladodes* and *Pochonia chlamydosporia* on parasitic helminths were evaluated at 15, 20, 25, 30 and 35 ºC. The fungi showed higher growth at intermediate temperatures (20, 25 and 30 ºC) than at the extremes of 15 and 35 ºC. At 25 and 30 °C, *Duddingtonia flagrans* presented 96.8 and 94.5% nematicidal activity on bovine parasitic helminths, respectively. *Arthrobotrys cladodes* presented nematicidal activity of 85.3 and 83.5%, respectively, at 20 and 25 °C. At 20 and 30 °C, Pochonia chlamydosporia presented nematicidal activity, respectively, of 81.3 and 87.4%. The maximum chlamydospore production was reached at 20, 25 and 30 ºC for *Duddingtonia flagrans*, at 20 and 25 °C for *Arthrobotrys cladodes* and *Pochonia chlamydosporia. Arthrobotrys cladodes* presented the highest production of chlamydospores among the isolates. The tested fungi presented mycelial growth, chlamydospores production and nematicidal activity on parasitic helminths under all temperature conditions tested. Therefore, the use of these fungi as biological controllers of parasitic helminths is promising.

Keywords: *Duddingtonia flagrans*; *Arthrobotrys cladodes*; *Pochonia chlamydosporia*; temperature; biological control; helminths.

INTRODUCTION

The temperature of an organism or environment reflects the average kinetic energy of its particles and is important for understanding the processes that regulate all forms of life, including the simplest forms, such as fungi. Temperature is considered one of the most influential factors for fungi growth, spore production and maintenance of these microorganisms in the environment (Carrillo-Inungaray et al., 2014; Lasram et al., 2010; Li et al., 2009).

Gastrointestinal parasitic helminths cause productive and financial losses in bovine production systems (Grisi et al., 2014). The use of anthelmintic compounds aims to decrease helminth infecting larvae in the pasture by decreasing the population of adult parasites in the animals. However, the use of anthelmintics has limitations, such as: drug residues in animal products, toxic effects on non-target organisms, pollution of the environment and anthelmintic resistance (Gasbarre, 2014; Fazzio et al. al., 2014). In order to minimize the use of anthelmintics, biological control through the use of nematophagous fungi is a method to reduce parasitic nematode infecting larvae in pastures where cattle are raised.

A method for the dissemination of these fungi in the environment is the incorporation of fungal structures in the bovine diet. After passing through the gastrointestinal tract, these structures germinate in the feces, forming a network of hyphae that differentiate into traps that capture and destroy infectious forms of free-living parasitic helminths (Braga and Araújo, 2014). The dispersion of fungal structures directly into feces, where eggs hatch and larvae become infective (L3), is one of the forms used to establish biological control of gastrointestinal parasitic nematodes in cattle (Paz-Silva et al., 2011).

Duddingtonia flagrans, Arthrobotrys cladodes and *Pochonia chlamydosporia* are nematophagous fungi with great potential for use in the biological control of the parasitic helminths of cattle (Oliveira et al. 2018a, 2018b; Silva et al., 2011; Vieira et al., 2019). The fungi *A. cladodes* and *D. flagrans* produce traps that promote adhesion, immobilization, penetration and destruction of helminth larvae (Grønvold et al., 1996; Oliveira et al., 2018a). The fungus *P. chlamydosporia* parasites eggs of helminths through structures known as apressories, which promote egg penetration by mechanical and enzymatic action (Stroze et al., 2013; Zare et al., 2001) and presents larvicidal action on bovine parasitic helminths (Vieira et al., 2019).

Changes in environmental temperature and daily variations in the temperature of the fecal environment containing nematophagous fungi, after passing through the gastrointestinal

tract of animals, can affect the growth, chlamydospore production and nematicidal activity of these fungi, therefore, it is necessary to know how the temperature affects such characteristics of nematophagous fungi. In this study, the fungi *D. flagrans, A. cladodes* and *P. chlamydosporia* were evaluated under various temperature conditions for mycelial growth, chlamydospore production and nematicidal activity on the infective larvae (L3) of bovine parasitic helminths.

MATERIAL AND METHODS

The fungi *Duddingtonia flagrans* (AC001 isolate), *Arthrobotrys cladodes* var. *macroides* (CG719 isolate) and *Pochonia chlamydosporia* (VC4 isolate) used in this study are part of the collection of the Laboratory of Parasitology, Department of Veterinary, Federal University of Viçosa, where they are kept at 4 °C in the dark in test tubes containing 2% corn meal agar (2% CMA).

EVALUATION OF MYCELIAL GROWTH

Discs (1 cm in diameter) containing *P. chlamydosporia*, *D. flagrans* and *A. cladodes* mycelium were transferred separately to the center of 9-cm diameter Petri dishes containing 2% potato dextrose agar medium (2% PDA) and incubated at 15, 20, 25, 30 and 35 ºC. For each fungus and temperature condition, ten replicates were used.

The colonies were measured every 24 hours in the orthogonal position for 10 days, resulting in 10 readings. These values were used in the calculation of the mycelial growth rate index, according to the formula: MGRI = Σ (D - Da) / N, where MGRI: mycelial growth rate index; D: current mean diameter of the colony; Da: mean diameter of the colony from the previous day; N: number of days after inoculation.

EVALUATION OF CHLAMYDOSPORE PRODUCTION

Discs (1 cm in diameter) containing *P. chlamydosporia*, *D. flagrans* and *A. cladodes* mycelium were transferred separately to the center of 9-cm diameter Petri dishes containing 2% PDA and incubated at 15, 20, 25, 30 and 35 ºC for twenty-one days. After, 5 mL of distilled water was added to each plate and the surface of the culture medium was washed and scraped to obtain suspensions containing chlamydospores. Subsequently, the number of

chlamydospores in these suspensions was determined using a hemocytometer, obtaining the mean number of chlamydospores produced in each treatment.

EVALUATION OF NEMATICIDE ACTIVITY

Discs (1 cm in diameter) containing *P. chlamydosporia*, *D. flagrans* and *A. cladodes* mycelium were transferred separately to the center of 9-cm diameter Petri dishes containing 4% water agar (4% WA) and incubated at 15, 20, 25, 30 and 35 ºC. For each fungus and temperature condition, ten replicates were used.

Gastrointestinal nematode larvae were obtained from naturally contaminated bovine feces collected at a farm, with no history of antihelmintic resistance, in the city of Abre Campo, state of Minas Gerais, southeastern Brazil, latitude 20º 18'04 "S, longitude 42° 28'39 "W. Coprocultures were made with 20 g of feces from the animals mixed with vermiculite and incubated for 12 days. After this period, the infective larvae (L3) were recovered using the Baermann funnel technique, with water at 42°C for 6 hours and identified according to the criteria of Keith (1953).

One thousand bovine gastrointestinal parasitic nematode infective larvae (L3) were added to each plate containing 4% WA medium. Cultures remained incubated in the absence of light at the temperatures of 15, 20, 25, 30 and 35 ºC. The percentages of the L3 genera added to the plates were 70.05, 19.23 and 10.72%, respectively, for the genera *Haemonchus*, *Cooperia* and *Oesophagostomum*. Such genera harbor important species of gastrointestinal parasites of cattle of long occurrence in Brazil.

After 15 days, L3 not predated were recovered by the Baermann method. The recovered L3 were quantified and identified under a light microscope (objective of 10x), obtaining an average number of L3 not predated per plaque in each treatment. The percentage of the reduction of L3 in the groups treated with nematophagous fungi, in relation to the control without fungus, was calculated according to the formula: *% reduction = (mean control group larvae - mean group treated larvae) x 100 / mean of control group larvae.*

STATISTICAL ANALYSIS

The mean values for the Mycelial Growth Velocity Indices (MGRI), chlamydospore production and recovered L3, for each temperature condition, were submitted to the non-parametric Kruskal–Wallis statistical test, at a significance level of 5%. Quadratic polynomial

regression analysis was performed between the values of MGRI, the production of chlamydospores and number of L3 recovered as a function of the different temperature conditions (15, 20, 25, 30 and 35 °C) in which the fungi *P. chlamydosporia*, *D flagrans* and *A. cladodes* were grown.

RESULTS

The Mycelial Growth Rate Index (MGRI) of *D. flagrans, A. cladodes* and *P. chlamydosporia* fungi, at different temperatures, for 10 days in the 4% WA and 2% PDA culture media are presented in the Table 1.

Table 1: Means (standard errors) for the Mycelial Growth Rate Index (MGRI - mm/day) of the nematophagous fungi *Duddingtonia flagrans* (AC001), *Arthrobotrys cladodes* (CG719) and *Pochonia chlamydosporia* (VC4) grown for 10 days in 2% potato dextrose agar (PDA), under different temperature conditions.

T (ºC)	*Duddingtonia flagrans*	*Arthrobotrys cladodes*	*Pochonia chlamydosporia*
15	0.60^{bA} (0.006)	0.28^{cB} (0.003)	0.30^{bB} (0.005)
20	1.40^{aA} (0.012)	0.81^{bB} (0.007)	0.45^{abC} (0.007)
25	2.18^{aA} (0.023)	1.40^{aA} (0.011)	0.74^{aB} (0.010)
30	2.12^{aA} (0.021)	1.39^{aA} (0.010)	1.09^{aB} (0.019)
35	1.18^{aA} (0.012)	0.03^{dB} (0.001)	0.05^{cB} (0.002)

$^{a, b, c, d, e, A, B, C}$ Different capital letters in the same row and lower case letters in the same column indicate that there is statistical difference ($p \leq 0.05$) between the data.

In 2% PDA medium, *D. flagrans* showed minimal growth at 15 °C and presented MGRI values that were not significantly different at the temperatures of 20, 25, 30 and 35 °C. *A. cladodes* presented maximum growth at 25 and 30 ºC and minimum MGRI values at 35 ºC. *P. chlamydosporia* presented maximum MGRI values, without significant differences, at the temperatures of 20, 25 and 30 ºC and minimum mycelial growth at 35 ºC.

Compared with each other, *D. flagrans* had higher mycelial growth rate index than *A. cladodes* and *P. chlamydosporia* in 2% BDA medium at the temperatures of 15, 20 and 35 °C. *D. flagrans* and *A. cladodes* presented similar MGRI values, but greater MGRI values than *P. chlamydosporia*, in 2% BDA medium at the temperatures of 25 and 30 °C.

A. cladodes and *P. chlamydosporia,* in 2% BDA medium, at 15 and 35 °C, presented similar MGRI values and at the same temperatures *D. flagrans* had higher mycelial growth rate index than *A. cladodes* and *P. chlamydosporia.* The MGRI of *A. cladodes* was higher than the *P. chlamydosporia* MGRI in 2% BDA medium at 20 °C, which were both smaller than the *D. flagrans* MGRI under the same conditions (Table 1).

The mean values of the number of infective larvae (of the genera *Haemonchus*, *Cooperia* and *Oesophagostomum*) of bovine gastrointestinal parasitic nematode recovered from plaques with 4% WA medium in which the nematophagous fungi *D. flagrans, A. cladodes* and *P. chlamydosporia* were inoculated at different temperatures are presented in the Table 2. The values of the percentages of the reduction of L3 in relation to the control group, caused by the nematicidal action of *D. flagrans*, *A. cladodes* and *P. chlamydosporia* also are presented in the Table 2.

Table 2: Means (standard errors) for the number of recovered infective larvae (L3) of bovine parasitic helminths, and the percentages of the reduction of L3, after 15 days, on plates containing 4% water agar medium (WA), in which the nematophagous fungi *Duddingtonia flagrans* (AC001), Arthrobotrys cladodes (CG719) and *Pochonia chlamydosporia* (VC4) were added, under different temperature conditions.

	Duddingtonia flagrans		*Arthrobotrys cladodes*		*Pochonia chlamydosporia*		Control
T (°C)	Recovered L3	L3 Reduction (%)	Recovered L3	L3 Reduction (%)	Recovered L3	L3 Reduction (%)	Recovered L3
15	76.6^{Ba} (8.7)	61.9	71.4^{Ba} (2.5)	64.7	57.6^{Bbd} (4.4)	71.5	203.6^{Aa} (3.7)
20	24.2^{Bad} (4.7)	88.5	30.8^{Bb} (1.8)	85.3	39.2^{Bad} (2.6)	81.3	210.2^{Aa} (2.8)
25	6.4^{Bb} (1.4)	96.8	33.2^{BDbd}(1.2)	83.5	47.0^{Dbd}(0.7)	76.6	201.8^{Aa} (4.7)
30	11.4^{Bbd} (0.9)	94.5	60.4^{Dad} (2.2)	71.0	26.0^{BDa} (1.9)	87.4	208.4^{Aa} (3.4)
35	42.6^{Ba} (1.9)	80.2	68.4^{Da} (3.7)	68.1	43.2^{BDbd}(5.9)	79.7	215.2^{Aa} (3.6)

$^{a, b, d, A, B, D}$ Different capital letters in the same row and lower case letters in the same column indicate that there is statistical difference ($p \leq 0.05$) between the data.

The number of infective larvae (L3) recovered from the plates containing *D. flagrans, A. cladodes* and *P. chlamydosporia* were lower than the L3 values recovered from the control group without fungus at all tested temperature conditions. At 15 and 20 ºC, the three tested nematophagous fungi did not present significant differences in their nematicidal activity on the infective larvae (Table 2). The percentage of reduction in relation to the control group was 61.86% for *D. flagrans*, 64.71% for *A. cladodes* and 71.50% for *P. chlamydosporia* at 15 ºC.

At 20 ºC, the percentage of reduction in relation to the control group was 88.52% for *D. flagrans*, 85.29% for *A. cladodes* and 81.28% for *P. chlamydosporia*.

At a temperature of 25 ºC, the plaques containing the nematophagous fungus *D. flagrans* had a lower number of recovered L3 and a higher percentage of L3 reduction (96.81%) than the plates containing *P. chlamydosporia*, which presented a percentage of L3 reduction of 76.64%. The percentage of reduction (83.52%) and the number of L3 recovered from *A. cladodes*-containing plaques did not differ from the percentages of reduction and the number of L3 recovered from the plates containing *D. flagrans* and *P. chlamydosporia* grown at 25 °C (Table 2).

At 30 and 35 °C, the group treated with the fungus *D. flagrans* presented a lower value of recovered L3 than the group treated with the fungus *A. cladodes*. At the same temperatures, the group containing *P. chlamydosporia* presented a non-significantly different number of recovered L3 compared to the groups containing *D. flagrans* and *A. cladodes*. The reduction percentage in relation to the control group was 94.54% for *D. flagrans*, 71.04% for *A. cladodes* and 87.39% for *P. chlamydosporia* at 30 °C. At 35 °C, the reduction percentage in relation to the control group was 80.22% for *D. flagrans*, 68.08% for *A. cladodes* and 79.69% for *P. chlamydosporia*.

The maximum predatory capacity on the L3 parasites of cattle was reached at 25 and 30 °C for *D. flagrans*, at 20 and 25 °C for *A. cladodes*, and at 20 and 30 °C for *P. chlamydosporia* (Table 2).

The mean values for the number of chlamydospores produced by the nematophagous fungi *D. flagrans*, *A. cladodes* and *P. chlamydosporia* inoculated on plates containing 2% BDA medium, for 21 days at different temperatures are presented in the Table 3.

The fungus *D. flagrans* had maximum chlamydospore production at the temperatures of 20, 25 and 30 ºC, with no significant difference between the values at these temperatures. At 35 ºC, *D. flagrans* had lower chlamydospore production than at the temperatures of 20, 25 and 30 ºC. However, there was no significant difference between the chlamydospore production of the fungus *D. flagrans* at the temperatures of 15 and 35 ºC (Table 3).

A. cladodes had maximum chlamydospore production at 20 and 25 °C. At the temperatures of 20 and 30 ºC, there was no significant difference in the number of chlamydospores produced by the fungus *A. cladodes*. The minimal chlamydospore production by *A. cladodes* occurred at the temperatures of 15 and 35 ºC, with no significant difference between them (Table 3).

Table 3: Means (standard errors) for the number of chlamydospores (x 10^4) produced by the nematophagous fungi *Duddingtonia flagrans* (AC001), *Arthrobotrys cladodes* (CG719) and *Pochonia chlamydosporia* (VC4) under different temperature conditions, and grown in 2% potato dextrose agar (PDA) for 21 days.

T (°C)	*Duddingtonia flagrans*	*Arthrobotrys cladodes*	*Pochonia chlamydosporia*
15	10.54^{adA} (0.65)	11.62^{aA} (1.68)	5.71^{bB} (0.38)
20	24.29^{bA} (0.59)	76.17^{bdB} (4.19)	32.28^{dA} (2.38)
25	22.37^{bdA} (2.92)	119.70^{bB} (4.71)	7.73^{bdA} (1.18)
30	25.87^{bA} (3.23)	63.44^{adB} (1.54)	1.77^{aD} (0.03)
35	2.65^{aA} (0.39)	12.18^{aB} (1.19)	6.00^{abA} (0.70)

$^{a, b, d, A, B, D}$ Different lower case letters in the same column and capital letters on the same line indicate that there is statistical difference ($p \leq 0.05$) between the data.

The fungus *P. chlamydosporia* had maximum chlamydospore production at the temperatures of 20 and 25ºC. At the temperatures of 15, 25 and 35 ºC, there was no significant difference between the chlamydospores produced by *P. chlamydosporia*. The minimum chlamydospore production by *P. chlamydosporia* occurred at the temperatures of 30 and 35 °C (Table 3).

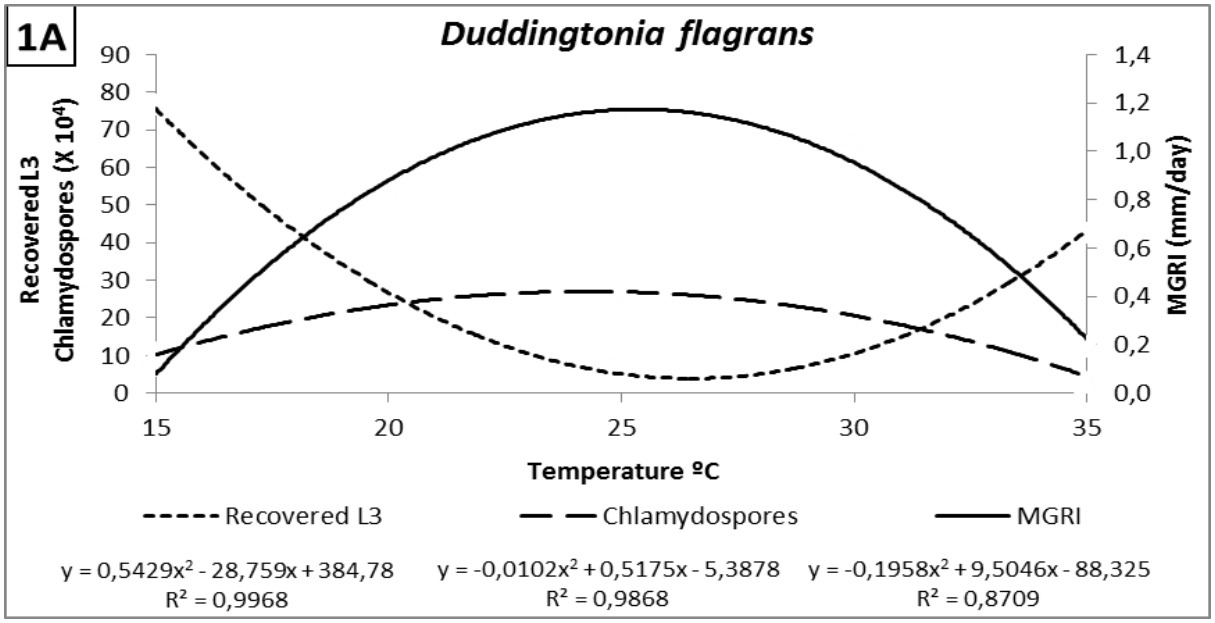
Comparing the production of chlamydospores by *D. flagrans* and *A. cladodes* at 15 ºC, there were no significant differences between the values; however, the production of chlamydospores by *P. chlamydosporia* was lower than that of *D. flagrans* and *A. cladodes*.

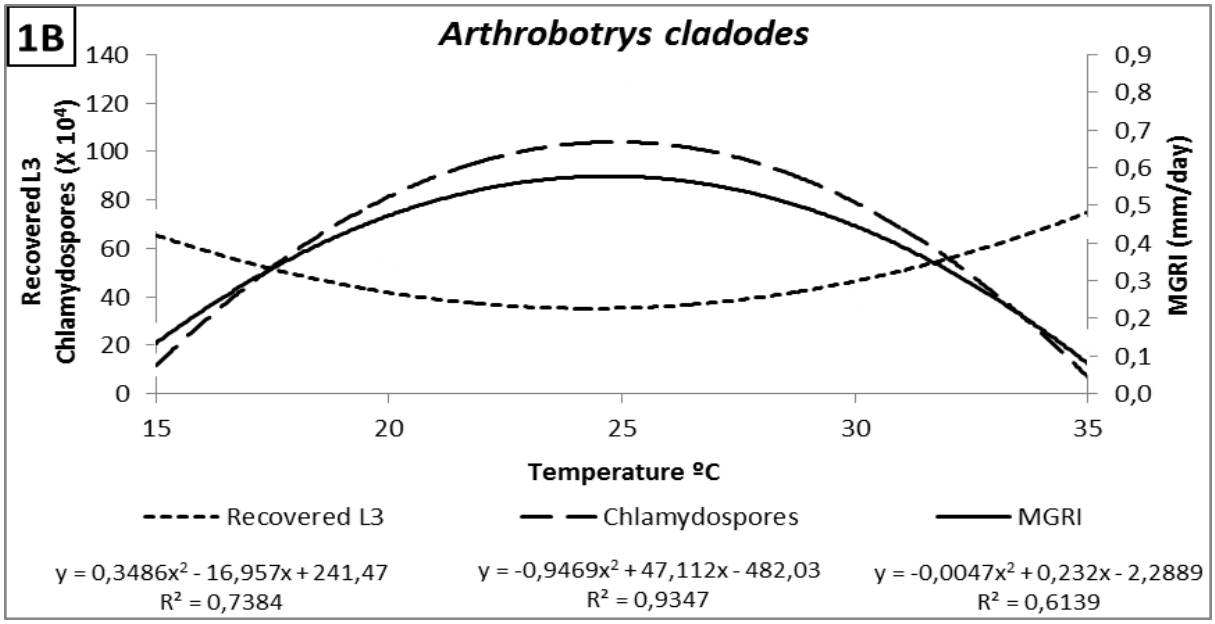
At the temperatures of 20 and 25 °C, *A. cladodes* produced a higher number of chlamydospores than *D. flagrans* and *P. chlamydosporia*, which were not significantly different in terms of the production of chlamydospores at these temperatures.

At 30 ºC, *A. cladodes* produced a higher number of chlamydospores than *D. flagrans* and *P. chlamydosporia*. *D. flagrans* produced more chlamydospores than *P. chlamydosporia*, when grown at 30 °C. At 35 ºC, *A. cladodes* had higher chlamydospore production than *D. flagrans* and *P. chlamydosporia*, with the last two fungi not differing in relation to the production of chlamydospores at 35 ºC (Table 3).

The polynomial regressions between the different temperature conditions and the MGRI values, the number of recovered infective larvae and the number of chlamydospores produced by the fungi *D. flagrans, A. cladodes* and *P. chlamydosporia*, are presented in the Figure 1. The regression equations and the values for the coefficients of determination (R^2)

are shown in the figure. Most of the regression models presented high R^2, indicating a high correlation between the real values observed for each variable and the predicted values by the regression equations. Nematicidal activity, Mycelial Growth Rate Index and chlamydospore production of the three fungal isolates tested were higher at intermediate temperatures (20, 25 and 30 ° C) than at temperature extremes (15 and 35 ° C).





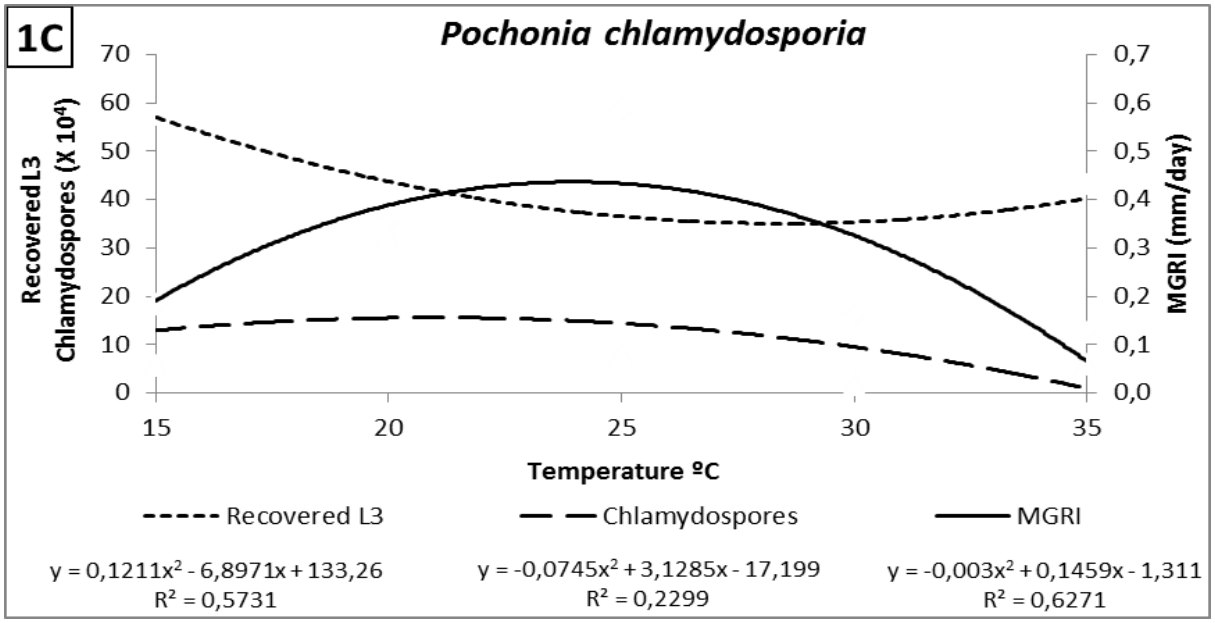


Figure 1: Quadratic polynomial regression of the Mycelial Growth Rate Index (MGRI), the number of recovered infective larvae (L3) of bovine parasitic helminths, and the number of chlamydospores produced (X 10^4) on plaques containing the nematophagous fungi *Duddingtonia flagrans* (a), *Arthrobotrys cladodes* (b) and *Pochonia chlamydosporia* (c), at different temperature conditions.

DISCUSSION

The development and activity of microorganisms in the environment can be affected by several biotic and abiotic factors. Considering that the site of nematophagous fungi used for biological control are the feces deposited in pastures, the fungal isolates that demonstrate greater resistance to the adversities found in the environment would be the most suitable for use in control strategies. The temperature of the environment is a factor that can determine the success of biological control of parasitic helminths of cattle by the use of nematophagous fungi, since daily temperature variations can affect the mycelial growth, chlamydospore production and nematicidal activity of these fungi.

The Mycelial Growth Rate Index results for *D. flagrans* were 1.40 and 2.12 mm/day at the temperatures of 20 and 30 °C. Grønvold et al. (1996) reported that *D. flagrans* presented a growth rate of 2.14 and 8.57 mm/day, respectively, at the temperatures of 20 and 30 °C, thus, our MGRI results for *D. flagrans* were lower than those of the reported by Grønvold et al. (1996). Isolates of *P. chlamydosporia*, described by Zare et al. (2001), presented colonies varying between 20–38 mm in diameter, after 10 days of growth, with an optimal growth

temperature between 24 and 30 °C, a minimum growth temperature of 10 °C. In the present study the mycelial growth results for *P. chlamydosporia* were different from those reported by Zare et al. (2001). The differences observed in growth rates and optimal temperatures for fungal growth may be due to the use of different fungal isolates or culture media, or to variations in experimental methodologies in each study.

The colonization of cattle feces by nematophagous fungi and subsequent formation of traps or metabolites with nematicidal action depends of the mycelial growth of these fungi. *D. flagrans* presented higher Mycelial Growth Rate Index than *A. cladodes* and *P. chlamydosporia* in most temperature conditions studied, which may explain the higher percentages of L3 reduction caused by *D. flagrans* than by *A. cladodes* and *P. chlamydosporia*.

In our experiment, *D. flagrans* had a higher predatory capacity against the L3 of parasitic helminths of cattle over the temperature range of 25 and 30 ºC, and the percentages of reduction were 96.81 at 25 ºC and 94.54 at 30 °C. Different predatory capacities of the *D. flagrans* fungus on helminths have been reported in other papers. Fernández et al. (1999) reported that *D. flagrans* reduced the number of *Cooperia oncophora* larvae by 70–96% at 15 °C and 63–98% at 20 °C. Santos et al. (2001) reported that *D. flagrans* caused 90% reductions in the number of cyathostomes L3 larvae at 25 and 30 °C. According to Buske et al. (2013), the best temperature for the nematicidal action of *D. flagrans* was 30 °C, in which the fungus was responsible for a 74.5% reduction of the L3 of *Haemonchus contortus*.

In the present study, *A. cladodes* had a higher predatory capacity against the L3 of parasitic helminths of cattle over the temperature range of 20 and 25 ºC, and the percentages of reduction were 85.3 at 20 ºC and 83.5 at 25 °C. Other studies report different predatory actions by *A. cladodes*. Oliveira et al. (2018a) reported a 68.7% reduction in the L3 of the parasitic helminths of cattle at 25 ºC due to the predatory action of *A. cladodes*. Ranjbar-Bahadori et al. (2010) reported that there was no significant difference in the predatory capacity of *A. cladodes* on *H. contortus* infective larvae at the temperatures of 15, 20 and 25 ºC, with percentages of reduction of 97.55, 96.71 and 95.93%, respectively.

P. chlamydosporia had maximum nematicidal activity over the L3 of bovine parasitic helminths between 20 and 30 °C (reduction percentages of 81.28 and 87.39%, respectively). Zouhar et al. (2010) evaluated the nematicidal activity of *P. chlamydosporia* in phytopathogenic species and the mortality rates due to the action of *P. chlamydosporia* on the nematodes *Globodera rostochiensis* and *Meloidogyne hapla* were, respectively, 20.0% and

39.0%. The *P. chlamydosporia* nematicidal activity described by Zouhar et al. (2010) was lower than that observed in the present study.

The differences observed in the nematicidal activity of nematophagous fungi described above may be due to the use of fungal isolates with different nematicidal potential, the use of different helminth species or variations in experimental methodologies in each study.

Extracellular enzymes (proteases and chitinases) produced by *P. chlamydosporia* are considered responsible for the destruction of helminth eggs (Yang et al., 2013; Van Ooij, 2011) and, according to Soares et al. (2013) and Braga et al. (2014), these enzymes are capable of causing cuticle hydrolysis and death of helminth larvae. Mukhtar and Pervaz (2003) reported that, in addition to enzymes, the fungus *P. chlamydosporia* produces toxins with nematicidal action. In the present study the ovicidal activity of *P. chlamydosporia* was not evaluated, however, larvicidal activity was observed on parasitic helminths of cattle, which may be due to the action of proteases and toxins produced by this fungus.

Most of the cited authors tested the influence of different temperatures on mycelial growth and nematicidal activity, but did not verify the production of chlamydospores by fungi. The establishment and permanence of most fungi in a given environment has a greater influence on spore production than on mycelial growth, as these spores serve as inoculum in the distribution and maintenance of the fungal species (Muller et al., 1956). The fungus *A. cladodes* presented, at 20, 25, 30 and 35 ºC, the highest production of chlamydospores among the isolates, this fact indicates a greater ability of *A. cladodes* to settle in the environment than *D. flagrans* and *P. chlamydosporia*.

The production of maximum chlamydospores by *D. flagrans* occurred over a wider range of temperatures than their maximum nematicidal activity. The maximum nematicidal activity and the maximum production of chlamydospores by *A. cladodes* occurred over the same temperature range. The maximum nematicidal activity of *P. chlamydosporia* was verified over a broader temperature range than that for the maximum production of chlamydospores. The maximum mycelial growth of nematophagous fungi was not closely associated with the production of chlamydospores and maximal nematicidal activities, since the temperature conditions of the medium influenced these characteristics in different ways.

Once the temperature influenced the development of distinct nematophagous fungi in different ways, the combined use of different nematophagous fungi may lead to improvements in the efficacy of the biological control of the parasitic helminths of bovines raised on pasture. Vieira et al. (2019) reported that the combined use of the fungi *A. cladodes* and *P. chlamydosporia* presented, under laboratory conditions, higher nematicidal activity over

bovine parasitic nematodes than *A. cladodes* and *P. chlamydosporia* used separately. It is necessary to carry out studies under natural conditions to evaluate the effect of combined use of nematophagous fungi on the biological control of bovine parasitic helminths, since the temperature and nutrient availability conditions of the environment vary during the year and do not meet the needs growth of all fungal isolates.

According to the National Institute of Meteorology of Brazil (INMET), the average compensated temperature varies between 24.01, 25.64, 26.55, 22.02 and 18.61 ºC, respectively, for the midwest, northeast, north, southeast and south of Brazil. The use of *D. flagrans, A. cladodes* and *P. chlamydosporia* for the biological control of helminths is unlikely to be limited by temperature variations, since mycelial growth, nematicidal activity and the production of satisfactory chlamydospores of the tested isolates are included in the common temperature ranges in countries with a tropical climate, such as Brazil.

In vitro experiments do not reliably replicate the conditions observed in the environment, since other biotic and abiotic factors that are not studied in the experimental trials may be present and influence the activity of the tested fungal isolates. However, laboratory conditions allow for greater control of the analyzed factors, constituting an important step in the initial analysis of the selection of potential candidates to be used in the development of biotechnological products for the biological control of gastrointestinal parasitic nematodes.

The temperature influenced the development of distinct nematophagous fungi in different ways, so the extrapolation of these results to other fungi is not feasible; it is necessary to perform specific tests for each fungal isolate. *D. flagrans, A. cladodes* and *P. chlamydosporia* presented mycelial growth, chlamydospores production and nematicidal activity over the L3 of bovine parasitic helminths under all temperature conditions tested. Therefore, the use of these fungi as biological controllers of parasitic helminths is promising.

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5. CAPÍTULO 2

ASSOCIATION AND PREDATORY CAPACITY OF FUNGI *POCHONIA CHLAMYDOSPORIA* AND *ARTHROBOTRYS CLADODES* IN THE BIOLOGICAL CONTROL OF PARASITIC HELMINTHS OF BOVINES

ABSTRACT

Nematophagous fungi are used in the biological control of the parasitic helminths of animals and plants. The association of ovicidal and predator nematophagous fungi may present a complementary and increased action on the biological control of helminths. Joint growth compatibility and predation tests were carried out on infective larvae of nematode parasites of bovines with the nematophagous fungus ovicide *Pochonia chlamydosporia* and the nematophagous fungus predator *Arthrobotrys cladodes*. The tests of antagonism in direct confrontation, antibiosis and the effect of volatile metabolites between the isolates of *Pochonia chlamydosporia* and *Arthrobotrys cladodes* indicated the viability of joint growth of these fungi. The association of the fungi *Pochonia chlamydosporia* and *Arthrobotrys cladodes* presented a higher predatory capacity of infective larvae of the parasitic nematodes of bovines when compared to the predation of the fungi used alone. Therefore, under laboratory conditions, the fungi studied presented growth compatibility and the association of these increased the nematicidal activity against parasitic helminths of cattle.

Keywords: antibiosis, volatile metabolites, direct confrontation, helminths, cattle, biological control.

INTRODUCTION

Gastrointestinal helminths are responsible for significant production losses in field breeding systems. Anti-helminthic drugs represent the main form used for the control of helminths, however, the results in the reduction in the parasitic load of the animals are not always satisfactory and these drugs can leave residues in products for human consumption. The reports of the development of anthelmintic resistance are very frequent and thus, it is necessary to search for complementary methods in the control of helminths (Fazzio et al., 2014; Gasbarre, 2014).

The biological control of parasitic helminths of animals through the use of nematophagous fungi presents satisfactory results in several studies (Silva et al., 2011; Tavela et al., 2012; Braga et al., 2013; Oliveira et al., 2018a,b). The ovicidal fungus *Pochonia chlamydosporia* parasites eggs of helminths through structures known as apressory, developed from undifferentiated hyphae, allow the colonisation of the egg surface and the penetration by a mechanical and enzymatic action (Braga et al., 2008). There are reports in the literature that *P. chlamydosporia*, in addition to the ovicidal action, parasite females of nematodes are colonised and fully digested (Podestá et al., 2009; Zouhar et al., 2010). The predatory fungus *Arthrobotrys cladodes* produces tridimensional traps that promote adhesion, immobilisation, penetration and consequently, the destruction of helminth larvae (Oliveira et al., 2018a).

Ovicidal and predator nematophagous fungi have distinct mechanisms of action, thus, when used together, they may present a complementary and synergistic action in the biological control of helminths. However, the joint growth of distinct nematophagous fungi may not be feasible, so it is necessary to know the interaction between them through compatibility studies (Ayupe et al., 2016). The objective of this work was to evaluate the growth compatibility of fungi *P. chlamydosporia* and *A. cladodes*, as well as to evaluate the predation capacity of these fungi associated and separately.

MATERIAL AND METHODS

The fungi *P. chlamydosporia* (VC4 isolate) and *A. cladodes* var *macroides* (CG719 isolate) used in the assays were maintained in the dark at 4°C, in test tubes containing 2% corn-meal-agar (2% CMA), in the Laboratory of Parasitology of the Department of Veterinary Medicine of the Federal University of Viçosa, Minas Gerais, Brazil.

TEST OF ANTAGONISM IN DIRECT CONFRONTATION

5 mm diameter discs containing *P. chlamydosporia* mycelium were placed at a distance of 1 cm from the border of the Petri dishes (9 cm in diameter) containing 2% potato-dextrose-agar medium (2% PDA). The plates were stored in the dark at 26°C, for 10 days. After this period, *A. cladodes* mycelial discs were placed on the plates opposite the *P. chlamydosporia* colony. For the control group, colonies of the same fungus were confronted. The colonies were incubated in the dark at 26°C, for eight days. For the evaluations, the adapted scale of notes, proposed by Bell et al. (1982) was used: 1 - complete colonisation of the plaque by *P. chlamydosporia*; 2 - colonisation of 2/3 of the plaque by *P. chlamydosporia*; 3 – colonisation of 50% of the plaque per fungus; 4 - colonisation of 2/3 of the plaque by *A. cladodes*; 5 - complete colonisation of the plaque by *A. cladodes*. Ten replicates were performed per treatment.

ANTIBIOSIS TEST

The antibiosis test followed the methodology described by Martins-Corder and Melo (1998), modified by the use of 2% PDA medium (Ferreira et al., 2008). On the surface of the 2% PDA culture medium, in 9 cm diameter Petri dishes, dialysis membrane discs (SIGMA®) were placed. Subsequently, disks of 5 mm in diameter of mycelium of *P. chlamydosporia* and *A. cladodes* were placed separately in the centre of distinct plates. Colonies were incubated in the dark at 26°C, for 96 hours. The growth area of the colonies was demarcated externally at the bottom of the plates and the dialysis membrane, along with the respective colony, were removed. Then, the plates were inverted and 1 ml of chloroform was added to the lower part in order to eliminate the possible structures of the fungus. After the evaporation of the product, the plates were left for 30 minutes under direct irradiation of ultraviolet light in a laminar flow chamber. Then, an aqueous suspension containing *P. chlamydosporia* mycelium was added on the surface of the culture medium on the plates where the fungus *A. cladodes* was grown and a suspension of *A. cladodes* where *P. chlamydosporia* was grown. These suspensions were obtained from colonies previously grown in 2% PDA culture medium in the dark at 26°C, for 10 days. The control group consisted of cultivation of *P. chlamydosporia* and a subsequent suspension culture containing mycelium of *P. chlamydosporia*, being equal for the control group of *A. cladodes*. The plates were kept in the dark at 26°C, for ten days

and after this period it was observed if there was formation of an inhibition halo in the growth of *P. chlamydosporia* formed by *A. cladodes*, or vice versa. Ten replicates were performed per treatment.

EFFECT OF VOLATILE COMPOUNDS

Petri dish covers of 9 cm in diameter, containing 2% PDA culture medium, were positioned one above the other according to the technique described by Bharat et al. (1980), modified by the use of 2% PDA medium (Ferreira et al., 2008). A 5 mm diameter disc containing *A. cladodes* mycelium was added to the lower plate and a 5 mm diameter disc containing *P. chlamydosporia* mycelium was added to the top plate. In another treatment, a 5 mm diameter disc containing *P. chlamydosporia* mycelium was added to the lower plate and a 5 mm diameter disc containing *A. cladodes* mycelium was added to the top plate. For the control group, *P. chlamydosporia* was grown in the lower and upper plaques, the same procedure was used to control *A. cladodes*. The plates were laterally sealed with a plastic membrane (Parafilm M®) and held in the dark at 26°C, for 15 days. For the evaluations, a measurement of the area of the colonies and comparison with the control was performed. Ten replicates were performed per treatment and the areas of the colonies were compared statistically by the Student's t-test at a significance level of 5%.

PREDATORY CAPACITY OF THE ASSOCIATION OF NEMATOPHAGOUS FUNGI ON INFECTIVE LARVAE

Gastrointestinal nematode larvae were obtained from naturally contaminated bovine faeces collected at a farm, with no history of antihelmintic resistance, in the city of Abre Campo, state of Minas Gerais, southeastern Brazil, latitude 20º 18'04 "S, longitude 42° 28'39 "W. Coprocultures were made with 20 g of feces from the animals mixed with vermiculite and kept in an oxygen demand chamber for 12 days. After this period, the infective larvae (L3) were recovered using the Baermann funnel technique, with water at 42°C for 6 hours and identified according to the criteria of Keith (1953). The percentages of the L3 recovered were 70.05%, 19.23% and 10.72%, respectively, for the genera *Haemonchus*, *Cooperia* and *Oesophagostomum*.

Forty Petri dishes with 9 cm in diameter containing 2% water-agar (WA 2%) were divided into four groups: "CG719" (*A. cladodes*), "VC4" (*P. chlamydosporia*), "CG719 +

VC4 "(*A. cladodes* and *P. chlamydosporia*, grown in association) and control (plaques containing only 2% WA). Each treated group consisted of 10 plaques, with the previously grown fungal isolates. 1000 L3 was poured into each plate. The control group consisted of 10 other plaques with 2% agar-water and 1000 L3. The plates were kept in an oxygen demand chamber oven in the dark at 25°C. On the seventh day, the L3 were recovered with the aid of the Baermann funnel, with water at 42-45°C and for 12 hours waiting for decantation. The L3 were counted and identified, following the criteria of Keith (1953), obtaining the average of the recovered larvae of each group.

The percentage reduction of larvae of the treated groups in relation to the control was calculated according to the formula: *% reduction = (mean control group larvae - mean group treated larvae) x 100 / mean of control group larvae.*

The L3 means recovered, the percentages of each L3 genus and the reduction percentages were transformed into log (x + 1) and compared by the Tukey test at a significance level of 5%.

RESULTS

Figure 1 shows the results of the direct confrontation test performed with the tested fungi. *P. chlamydosporia* and *A. cladodes* presented homogeneous growth in the direct confrontation tests, with no overlap of the colonies and none of the fungi confronted inhibited the growth of the other (Fig. 1c). In the scale of notes adapted, the test presented note "3" (colonisation of 50% of the plate for each fungus).

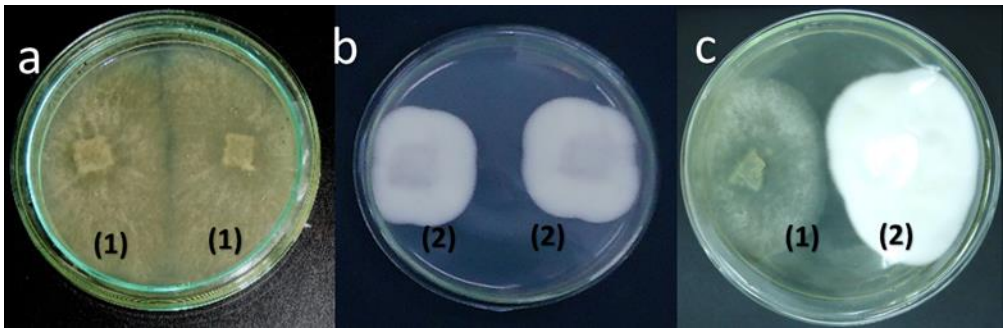

Figure 1. Direct confrontation test: a - *Arthrobotrys cladodes* (1) *versus A. cladodes* (1); b - *Pochonia chlamydosporia* (2) versus *P. chlamydosporia* (2); c - *A. cladodes* (1) versus *P. chlamydosporia* (2).

In the antibiosis tests there was no inhibition of halo formation among the nematophagous fungi, so *P. chlamydosporia* and *A. cladodes* did not produce any compounds and did not compete directly for nutrients to the point of inhibiting their growth together.

In Table 1, the mean values of the mycelial growth area in the upper parts of the plaques of the *P. chlamydosporia* and *A. cladodes* fungi submitted to the volatile metabolite effect test are presented. There was no difference in the mycelial growth area of any of the tested isolates ($p < 0.05$).

Table 1. The mean values and standard deviation of the mycelial growth area, in the upper Petri dishes, 9 cm in diameter, containing 2% potato-dextrose-agar (2% PDA), for 15 days, of the fungi *Pochonia chlamydosporia* (VC4 isolate) and *Arthrobotrys cladodes* (CG719 isolate) submitted to the test of the effect of volatile compounds.

Arthrobotrys cladodes		***Pochonia chlamydosporia***	
Treatments	**Area (cm^2)**	**Treatments**	**Area (cm^2)**
CG719 *versus* VC4	$35.49^{a} + 2.23$	VC4 *versus* CG719	$7.66^{b} + 0.75$
CG719 *versus* CG719	$37.35^{a} + 2.64$	VC4 *versus* VC4	$7.07^{b} + 0.53$

[a,b] Different letters in the same column indicate the difference between the data ($p < 0.05$).

Table 2 shows the mean values of the number of infective larvae (L3) of bovine gastrointestinal parasitic nematodes recovered from plaques with 2% WA medium, containing the association of the fungi *A. cladodes* and *P. chlamydosporia* (CG719 + VC4), the fungus *P. chlamydosporia* (VC4) and fungus *A. cladodes* (CG719), the latter isolated, as well as the percentage distribution of the recovered L3 genera. The three groups that contained the nematophagous fungi showed lower values of recovered L3 compared to the control group without fungus ($p < 0.05$). The group that contained the associated *A. cladodes* and *P. chlamydosporia* had the lowest L3 recovery (30.17) among the groups. The group containing *A. cladodes* alone presented a lower L3 recovery (75.17) compared to the group containing *P. chlamydosporia* 130.67).

Table 2. The average values and standard deviations of the number of infective larvae (L3) recovered from plaques with 2% water-agar medium (WA2%), after 7 days of interaction, containing the association of the nematophagous fungi *Arthrobotrys cladodes* and *Pochonia*

chlamydosporia (CG719 + VC4), *P. chlamydosporia* (VC4) and *A. cladodes* (CG719), as well as the percentage distribution of the recovered L3 genera.

Group	L3 Recovered	*Haemonchus %*	*Cooperia %*	*Oesophagostomum %*
CG719 + VC4	30.17^{a} (+14.70)	49.71^{a} (+11.17)	26.19^{a} (+10.79)	24.10^{a} (+12.11)
CG719	75.17^{b} (+33.09)	38.18^{b} (+10.32)	26.14^{a} (+12.56)	35.68^{b} (+7.65)
VC4	130.67^{c} (+38.84)	42.88^{ab} (+9.51)	21.59^{a} (+7.44)	35.53^{ab} (+13.43)
Control	411.50^{d} (+148.50)	71.54^{c} (+8.32)	17.95^{a} (+5.28)	10.51^{c} (+5.09)

^{a,b,c,d} Different letters in the same column indicate difference between the data ($p < 0.05$).

The percentage reduction in the L3 of the group containing the association of *A. cladodes* and *P. chlamydosporia* (92.67%) was higher than the percentage of the group containing *A. cladodes* (81.73%), which was greater than the percentage of the group containing *P. chlamydosporia* (68.25%), as shown in Fig. 2.

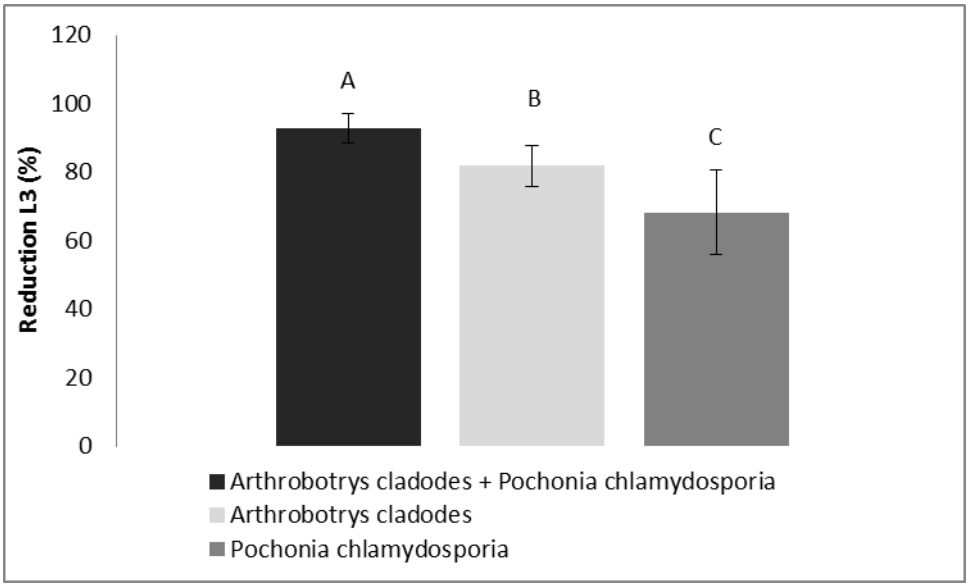

Figure 2. The percentage reduction of the infective larvae (L3) of gastrointestinal parasitic nematodes recovered from plaques with 2% water-agar medium (2%WA), after 7 days of interaction, with an association of the fungi *Arthrobotrys cladodes* and *Pochonia chlamydosporia* (CG719 + VC4), *P. chlamydosporia* (VC4) and *A. cladodes* (CG719). Different letters indicate the difference between the values ($p < 0.05$).

The percentage of the L3 of the genus *Haemonchus* was lower in the groups treated with nematophagous fungi than in the control group without the fungus (Table 2). The group

that contained the association between *A. cladodes* and *P. chlamydosporia* presented a higher percentage of the L3 of the genus *Haemonchus* (49.71%) than the group containing only *A. cladodes* (38.18%). However, the group containing only *P. chlamydosporia* showed no difference in the percentage of the L3 of the genus *Haemonchus* compared to the groups that contained the association of fungi nematófagos and the group containing only *A. cladodes*.

The percentages of the L3 of the genus *Cooperia* did not vary among the four groups. The percentage of the L3 of the genus *Oesophagostomum* was higher in the groups treated with nematophagous fungi than in the control group without the fungus (Table 2). The group that contained the fungi association had a lower percentage of the L3 of the genus *Oesophagostomum* (24.10%) than the group containing only *A. cladodes* (35.68%). However, the group containing only *P. chlamydosporia* showed no difference in the percentage of the L3 of the genus *Oesophagostomum* compared to the groups that contained the association of fungi and the group containing only *A. cladodes*.

DISCUSSION

The nematicidal activity of fungi of the genera *Arthrobotrys* and *Pochonia* on populations of parasitic helminths is demonstrated in several studies. In view of the good results with fungal isolates of these genera, the present study evaluated the compatibility of joint growth between *A. cladodes* and *P. chlamydosporia*, as well as the joint and isolated action of these fungi on L3 of bovine parasitic helminths.

The direct confrontation tests performed did not demonstrate joint growth incompatibility between *P. chlamydosporia* and *A. cladodes*. Compatibility tests with other isolates of nematophagous fungi obtained conflicting results. Ferreira et al. (2008) reported the joint growth compatibility between *P. chlamydosporia* and *Trichoderma* spp. submitted to the direct confrontation test. However, Ayupe et al. (2016), when evaluating the growth of *Arthrobotrys robusta* and *Duddingtonia flagrans* by the direct confrontation test, verified that *A. robusta* colonised approximately 2/3 of the plaque and *D. flagrans* colonized 1/3, suggesting competition and antagonism between them.

Ferreira et al. (2008) and Ayupe et al. (2016) performed antibiosis tests, respectively, between *P. chlamydosporia* and *Trichoderma* spp, and between *D. flagrans* and *A. robusta* and they verified that the fungi did not produce metabolites capable of inhibiting the growth of them together. In the present work we also did not verify the production of volatile metabolites by *P. chlamydosporia* and *A. cladodes* that inhibit the joint growth of these fungi.

In our study, the test of the effect of volatile metabotites did not demonstrate joint growth incompatibility between *P. chlamydosporia* and *A. cladodes*, however, Ferreira et al. (2008) and Ayupe et al. (2016) reported some kind of incompatibility among the fungi studied by them. Ferreira et al. (2008) did not observe the production of volatile compounds of *P. chlamydosporia* against *Trichoderma* spp., but reported that *Trichoderma* spp. reduced the growth of *P. chlamydosporia* by producing volatile compounds. In the work of Ayupe et al. (2016), the isolate of *A. robusta* reduced the growth of *D. flagrans* in the test of the effect of volatile metabolites but *D. flagrans* did not reduce the growth of *A. robusta*.

The results of the compatibility tests performed in this work, as well as the results of the other authors cited, show that it is important to evaluate the compatibility between specific isolates and that the extrapolation of the results to other isolates of the same species or associations would probably not be possible.

In the *in vitro* test of efficacy of the L3 predation, the three groups treated with nematophagous fungi showed lower values of the recovered L3 compared to the control group (without fungus), which indicates the efficacy in the predation of bovine gastrointestinal parasitic nematodes by the fungi tested.

According to Oliveira et al. (2018a) *A. cladodes* presents a mechanism of nematicidal action based on the production of traps that promote adhesion, penetration and destruction of larvae, which justifies the predatory capacity on L3 of bovine parasitic helminths verified in our study.

Braga et al. (2008) reported that *P. chlamydosporia* parasites eggs of helminths through the colonization of the egg surface and the penetration by a mechanical and enzymatic action, however, in our study we did not evaluate the ovicidal action of this fungus but its larvicidal activity. Our results indicate that *P. chlamydosporia* presents nematicidal action on larvae of parasitic helminths, as well as in the work of Podestá et al. (2009) and Zouhar et al. (2010). The mechanisms of the larvicidal action of *P. chlamydosporia* were not elucidated.

Ranjbar-Bahadori et al. (2010) reported that *A. cladodes* was effective in reducing the infective larvae of *Haemonchus contortus* and the percentage reduction in the L3 was 78.8%. Braga et al. (2013) reported that *A. cladodes* was efficient in predating the L3 of *Libyostrongylus douglassii* (ostrich gastrointestinal parasite nematode) and presented a reduction percentage of 89.2% in relation to the control group without the fungus. Oliveira et al. (2018a) reported an *in vitro* percentage of 68.7% reduction of the L3 of bovine gastrointestinal parasitic nematodes due to the predatory action of *A. cladodes*. In the present

study, the group containing only *A. cladodes* showed 81.73% reduction of the L3 parasites of bovines, being higher than the value found by Oliveira et al. (2018a) and similar to the values reported by Ranjbar-Bahadori et al. (2010) and Braga et al. (2013).

The group containing only *P. chlamydosporia* demonstrated a predatory capacity of 68.25% of the parasite L3 of cattle. Zouhar et al. (2010) evaluated the virulence of *P. chlamydosporia* in phytopathogenic species and the mortality rates due to the action of *P. chlamydosporia* on the nematodes *Globodera rostochiensis* and *Meloidogyne hapla* were, respectively, 20.0% and 39.0%, which were lower than the value found in our study. However, in the study by Silva et al. (2011), there was no predation of the L3 of *H. contortus* by *P. chlamydosporia* fungus.

There are no reports in the literature of works that evaluated the capacity of the predation of the L3 by the association of fungi *A. cladodes* and *P. chlamydosporia*. However, Tavela et al. (2012) also associated predatory (*D. flagrans*, AC001 isolate and *M. thaumasium*, NF34 isolate) and ovicidal (*P. chlamydosporia*, VC1 isolate) nematophagous fungi and evaluated their effects against eggs and the L3 of cyathostomes. The following percentage reductions compared to the control group were observed by such authors: AC001 + VC1, 86.8%; NF 34 + VCl, 77.3%. Although the fungus and the L3 were different species, the percentage of reduction of the association between *P. chlamydosporia* and *A. cladodes* (92.67%) in the present study was higher than that of the associations made by Tavela et al. (2012).

There is variation between nematophagous fungus larvae predation values when comparing the results obtained and those cited by other authors. Mendoza-de-Gives et al. (1999), Oliveira et al. (2018a) suggests that the differences in nematophagous fungi predation results are due to the particular structure and the composition characteristics of the nematode cuticle, or due to the antigenic variations in the different nematode species or to variations among nematode isolates species of fungus.

The lower percentages of L3 recovered from the genus *Haemonchus* in the fungus treated groups compared to the control group (without fungus), suggest a higher selectivity of the predation of larvae of the genus *Haemonchus* by the fungi used in the present experiment. However, Oliveira et al. (2018a), when evaluating the predatory capacity of the L3 parasites of cattle by the fungus *A. cladodes*, found no difference in the percentages of *Haemonchus* sp., *Cooperia* sp. and *Oesophagostomum* sp. recovered.

The results indicated that the growth of *P. chlamydosporia* and *A. cladodes* together is feasible, since none of the fungi caused inhibition or antagonism to the growth of the other.

The association of nematophagous fungi *A. cladodes* (CG719) and *P. chlamydosporia* (VC4) showed a higher percentage of L3 reduction than fungi used alone and proved to be effective in the biological control of gastrointestinal nematodes of bovines. Thus, work evaluating the effectiveness of this association of fungi on the biological control of helminthses under environmental conditions can be developed.

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6. CAPÍTULO 3

ARTHROBOTRYS CLADODES AND *POCHONIA CHLAMYDOSPORIA*: EFFECTS OF SINGLE AND COMBINED USE AGAINST GASTROINTESTINAL PARASITIC NEMATODES

ABSTRACT

Biological control of parasitic helminths using nematophagous fungi complements the control strategies for intestinal parasitic diseases in cattle. The incorporation of mycelium, conidia, and chlamydospores of nematophagous fungi in pelleted formulations of sodium alginate and the inclusion of this in the bovine diet is used for the dissemination of these fungi in the environment. After passing through the gastrointestinal tract, the fungi germinate in faeces, forming traps that capture and destroy infectious free-living forms of parasitic helminths. In this study, the viability and nematicidal activity of *Arthrobotrys cladodes* and *Pochonia chlamydosporia*, isolated and combined in pelleted formulations of sodium alginate, were evaluated after passage through the gastrointestinal tract of cattle. Pellets containing the nematophagous fungi resisted the passage through the gastrointestinal tract of cattle and the fungi were viable and able to grow and prey on helminths. The reduction in parasitic helminth infective larvae of bovines by the combined use of *Arthrobotrys cladodes* and *Pochonia chlamydosporia* was more than the reduction in infective larvae by *Arthrobotrys cladodes* or *Pochonia chlamydosporia* alone in the collections 24 and 36 h after giving the pellets to the animals, demonstrating that the association between these two fungi potentiated their predation on larvae.

Keywords: *Arthrobotrys cladodes*; *Pochonia chlamydosporia*; cattle; biological control; helminths.

INTRODUCTION

Brazil has a large cattle herd, often raised in extensive systems, in which the use of largely pasture-based feed leads to a constant occurrence of helminth infections (Charlier *et al*. 2016). Gastrointestinal helminths represent one of the obstacles to the full development of Brazilian cattle production, accounting for financial losses of approximately $ 7.11 billion per year, reflecting the cost of treatments, falling production rates, and the death of animals (Grisi *et al*. 2014).

The use of synthetic anthelmintic drugs is the main method used by breeders to control gastrointestinal helminths. However, there is a growing interest in the development of new methods to combat parasitic helminths, mainly due to the development of resistance to synthetic anthelmintics by parasitic nematodes and demands for the production of food free of chemical residues (Condi *et al*. 2009; Lopes *et al*. 2009; Soutello *et al*. 2010; Sutherland and Leathwick, 2011; Almeida *et al*. 2013).

Biological control using nematophagous fungi is a method to complement the chemical control strategies of intestinal parasites. The dispersion of fungal structures directly into faeces, where eggs hatch and larvae become infective (L3), is one of the forms used to establish biological control of gastrointestinal parasitic nematodes in cattle (Paz-Silva *et al*. 2011). A method for the dissemination of these fungi in the environment is the incorporation of fungal structures (mycelium, conidia, and chlamydospores) in a matrix of sodium alginate (Silva *et al*. 2014) which is then provided in the bovine diet. After passing through the gastrointestinal tract, these structures germinate in the faeces, forming a network of hyphae that differentiate into traps that capture and destroy infectious forms of free-living parasitic helminths (Braga and Araújo, 2014).

According to Mota *et al*. (2003), viability after passage through the gastrointestinal tract of animals is the main requirement for a fungus to be used in the biological control of helminths in the field, since the nematicidal action of nematophagous fungi occurs in the faecal environment. Another requirement is that the nematophagous fungi should be able to be reproduced on an industrial scale and under economically viable conditions.

The objective of this study was to evaluate the viability and nematicidal activity of the nematophagous predator fungus *Arthrobotrys cladodes* and the nematophagous ovicidal fungus *Pochonia chlamydosporia*, isolated and combined in a matrix of sodium alginate, after passage through the gastrointestinal tract of cattle.

MATERIAL AND METHODS

The fungi *Arthrobotrys cladodes* var. *macroides* (CG719 isolate) and *Pochonia chlamydosporia* (VC4 isolate) used in this study are part of the collection of the Laboratory of Parasitology, Department of Veterinary Medicine, Federal University of Viçosa, where they are kept at 4 °C, protected from light in test tubes containing 2 % corn-meal-agar (2 % CMA).

The mycelia of fungi grown in GPY (glucose, peptone, and yeast extract) liquid medium were used for the preparation of pellets in a sodium alginate matrix, according to the technique described by Walker and Connick (1983), modified by Lackey *et al*. (1993).

Twenty-four bovine females, crossbred dutch × zebu, with a mean age of nine months and a mean weight of 150 kg, were pretreated with 1 % anti-helminth ivermectin (1 mL/50 kg body weight) and albendazole (7.5 mg/kg body weight). After 21 days of anthelmintic treatment, the parasitic load of the animals was shown to be null using the egg counting technique in faeces and the animals were randomly divided into four groups, each with six animals. In the first group, each animal consumed 100 g of pellets (1.7 g of fungal mycelia) containing the fungus *P. chlamydosporia*, together with wheat bran. In the second group, each animal consumed 100 g of pellets (1.7 g of fungal mycelia) containing the fungus *A. cladodes*, along with wheat bran. In the third group, each animal consumed 100 g of pellets (1.7 g of fungal mycelia) containing both the fungi *P. chlamydosporia* and *A. cladodes* in the same pellet, together with wheat bran. In the control group, each animal consumed 100 g of pellets without fungus, along with wheat bran.

Faecal samples of 500 g were collected from each animal's rectum in all groups at 12, 24, 36, 48, 60, and 72 h after administration of the pelleted formulations. The samples from each group were homogenised and the pellets recovered from the faeces were added to Petri dishes 5 cm in diameter containing 2 % water-agar (2 % WA). The plates were stored at 25 °C in the dark for 24 h. Twelve replicates were performed by schedule for each group. Five hundred infective helminth larvae that parasitise cattle were added to these plaques, which were obtained from coprocultures of naturally contaminated bovine faeces.

The plates were analysed daily in order to detect the spores characteristic of the tested isolates, according to identification criteria proposed by Van Oorschot (1985) and Zare *et al*. (2001). On the 15th day, the L3 not predated were recovered by Baermann's method using water at 42–45 °C for 12 h before for decantation, quantified, and identified according to the criteria of Keith (1953) to obtain the average number of larvae not predated per plaque.

The percent reduction in the number of infective larvae from the treated group compared to the control was calculated according to the formula: % reduction = (mean larvae control - mean larvae treatment) × 100 / mean larvae control.

The L3 means recovered, the percentages of each L3 genus and the reduction percentages were transformed into log (x + 1) and compared by the Tukey test at a significance level of 5%.

RESULTS

The pellets supplied to the animals were recovered from the faeces at all collection times and, after being inoculated on plaques with 2 % WA medium and infective helminth larvae that parasitise bovines, *A. cladodes* and *P. chlamydosporia* fungi were able to prey on or cause the death of larvae and to produce characteristic spores of these species.

The mean values of the number of infective parasitic helminth larvae (L3) recovered from plates containing 2 % WA medium which were inoculated with pellets that had passed through the bovine gastrointestinal tract and that contained both of the nematophagous fungi *A. cladodes* and *P. chlamydosporia* or each of the fungi *P. chlamydosporia* and *A. cladodes* singularly are presented in the Table 1. The percentage of reduction in the number of L3 recovered from the groups treated with nematophagous fungi compared to the control group, without fungus, is presented in the Figure 1.

In the collection times of 12, 24, 36, 48, 60, and 72 h after pellet administration, treatments with fungi (*A. cladodes* and *P. chlamydosporia* combined or singularly) resulted in a decrease in the number of infective larvae (L3) recovered compared to the control group without fungus. At 12 h, the group containing the combination of fungi A. cladodes and *P. chlamydosporia* showed the lowest recovery and the highest percentage of L3 reduction (85.61 %) among the treatments, and comparison of the groups that received either *A. cladodes* or *P. chlamydosporia* alone revealed there was no significant difference in the number of L3 recovered and reduction percentages (76.12 and 70.00 %, respectively).

Table 1: Mean values (standard error) of the number of infective larvae of parasitic helminths recovered from plaques with 2 % WA medium in which pellets containing the combination of nematophagous fungi *Arthrobotrys cladodes* and *Pochonia chlamydosporia* (CG719 + VC4), the fungus *P. chlamydosporia* (VC4) alone, or the fungus *A. cladodes* (CG719) alone, were

inoculated after passage through the gastrointestinal tract of cattle and collection at 12 to 72 h after administration to cattle.

Time (h)	CG719 + VC4	CG719	VC4	Control
12	33.33^{bB} (3.42)	55.33^{dB} (1.91)	69.50^{dA} (2.17)	231.67^{aA} (15.09)
24	27.17^{bBD} (3.06)	45.67^{eBD} (1.91)	65.83^{dA} (2.79)	198.33^{aAB} (5.40)
36	34.33^{bBD} (2.57)	47.17^{eBD} (1.59)	61.00^{dAB} (1.10)	152.33^{aD} (5.30)
48	34.33^{bD} (1.72)	44.17^{bD} (3.68)	47.67^{bBD} (4.19)	149.33^{aD} (12.87)
60	38.50^{bBD} (3.31)	47.67^{bBD} (2.27)	45.50^{bD} (3.80)	163.00^{aBD} (7.98)
72	55.67^{bA} (5.35)	70.33^{bdA} (3.09)	87.83^{dA} (9.17)	185.33^{aABD} (11.69)

* Different capital letters in the same column and lower case letters on the same line indicate that there is statistical difference ($p \leq 0.05$) between the data.

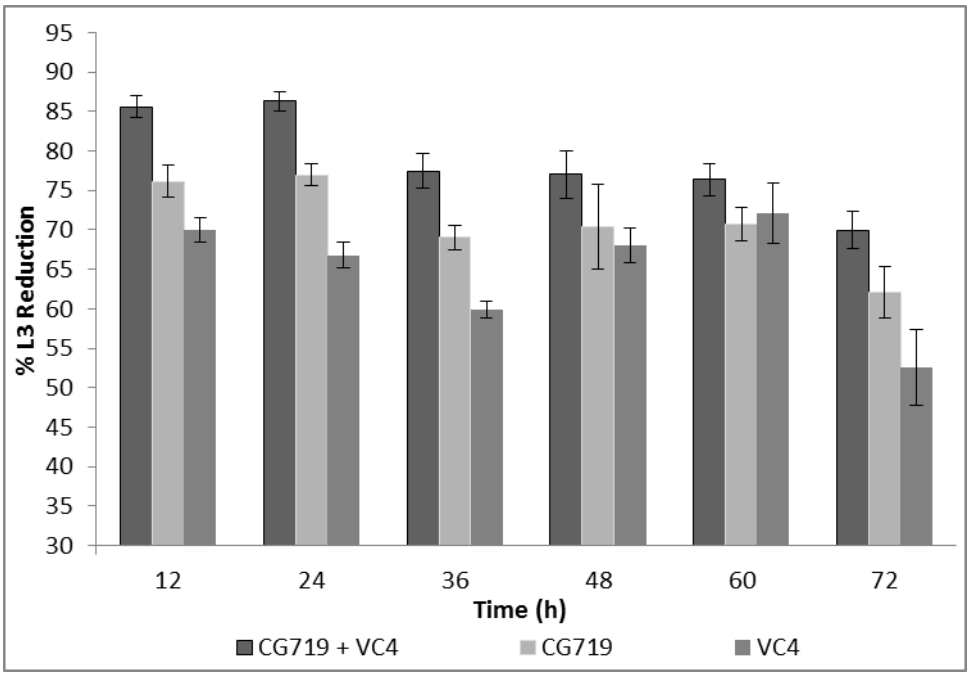

Figure 1: Percentage of reduction in the number of infective larvae (L3) of helminth gastrointestinal parasites recovered from plates containing 2 % water-agar medium (2 % WA), in which pellets that contained the combination of the nematophagous fungi *Arthrobotrys cladodes* and *Pochonia chlamydosporia* (CG719 + VC4), the fungus *P. chlamydosporia* (VC4) alone, or the fungus *A. cladodes* (CG719) alone, were inoculated, after passing through the gastrointestinal tract of cattle and being collected at 12 to 72 h after administration to cattle.

In the 24 and 36 h collections, the three groups treated with fungi differed as to the number of L3 recovered and percentages of reduction. The group that contained the combination of *A. cladodes* and *P. chlamydosporia* had the lowest number of L3 recovered and the highest reduction percentage (24 h: 86.30; 36 h: 77.46), followed by the group treated with *A. cladodes* alone, which presented a reduction of 76.97 % in the 24 h collection and of 69.03 % in the 36 h collection. The reduction percentage of the group containing *P. chlamydosporia* was 66.81 % in the 24 h collection and 59.96 % in the 36 h collection.

In the 48 and 60 h collections, the three treatments with nematophagous fungi did not significantly differ to each other ($p > 0.05$) in their recovered L3 numbers and reduction percentages.

At 72 h, the group containing the combination of the fungi *A. cladodes* and *P. chlamydosporia* and the group containing *A. cladodes* alone did not differ in the number of L3 recovered and their percentage of reduction, whereas the group treated with *P. chlamydosporia* alone presented a higher number of recovered L3 and a lower reduction percentage (52.61 %) than the other treatments (Figure 1).

The L3 recovery was lower than the other groups and did not present significant differences in the collections at 24, 36, 48, and 60 h from the groups containing the combination of fungi *A. cladodes* and *P. chlamydosporia* or *A. cladodes* alone. The group containing *P. chlamydosporia* alone had a lower number of L3 recovered at 48 and 60 h than the other groups (Table 1).

The percentages of the L3 genera added to the plaques were 22.55 %, 20.65 %, and 56.80%, respectively, for the genera *Haemonchus, Cooperia,* and *Oesophagostomum*. As shown in Table 2, in the samples collected 36 and 48 h after pellet administration, all the groups presented no significant difference in the percentage of L3 of the genus *Haemonchus* ($p > 0.05$).

Table 2: Mean values (standard error) of percentages of infective larvae (L3) of parasitic helminths (*Haemonchus, Cooperia,* and *Oesophagostomum*) recovered from plaques with 2 % WA medium in which pellets containing the nematophagous fungi *Arthrobotrys cladodes* and *Pochonia chlamydosporia* (CG719 + VC4), the fungus *P. chlamydosporia* (VC4) alone, or the fungus *A. cladodes* (CG719) alone, were inoculated after passing through the gastrointestinal tract of cattle and being collected at 12 to 72 h after administration to cattle.

Group	12 h	24 h	36 h	48 h	60 h	72 h
			Haemonchus			
Control	26.25^{ad} (4.03)	17.48^{a} (2.19)	21.66^{a} (3.40)	28.74^{a} (5.39)	21.64^{a} (1.48)	22.06^{a} (2.22)
CG719 + VC4	50.44^{b} (1.74)	47.44^{b} (0.95)	30.99^{a} (1.61)	36.11^{a} (2.30)	42.53^{b} (2.75)	34.79^{b} (2.27)
VC4	24.60^{d} (4.11)	36.75^{bd} (6.25)	22.10^{a} (3.95)	33.02^{a} (3.36)	9.10^{d} (1.04)	16.98^{a} (1.21)
CG719	39.95^{ab} (4.69)	27.53^{ad} (3.46)	29.94^{a} (4.02)	29.88^{a} (4.90)	37.62^{b} (3.28)	21.71^{a} (2.87)
			Cooperia			
Control	25.90^{ab} (1.00)	15.23^{a} (1.41)	18.02^{a} (2.29)	21.65^{a} (2.96)	19.03^{ab} (1.13)	20.84^{a} (1.97)
CG719 + VC4	33.37^{d} (2.44)	27.05^{b} (1.38)	27.23^{ab} (1.53)	22.93^{a} (2.12)	33.83^{d} (1.05)	27.03^{b} (1.07)
VC4	22.01^{a} (1.91)	23.25^{ab} (2.96)	24.04^{ab} (3.23)	27.30^{a} (1.38)	14.99^{a} (2.81)	15.31^{d} (1.20)
CG719	31.33^{bd} (2.23)	23.38^{ab} (2.94)	28.94^{b} (2.98)	23.33^{a} (1.83)	26.06^{bd} (3.04)	15.53^{d} (0.94)
			Oesophagostomum			
Control	47.85^{a} (4.61)	67.28^{a} (3.46)	60.33^{a} (5.53)	49.61^{a} (8.31)	59.32^{a} (2.40)	57.10^{a} (4.05)
CG719 + VC4	16.22^{b} (2.28)	25.51^{b} (2.15)	41.78^{a} (2.10)	40.96^{a} (3.95)	23.64^{b} (2.04)	38.18^{b} (2.28)
VC4	53.39^{a} (5.48)	40.00^{bd} (8.97)	53.87^{a} (6.82)	39.67^{a} (4.20)	75.91^{d} (3.48)	67.70^{a} (1.79)
CG719	28.72^{b} (5.14)	49.18^{ad} (6.26)	41.12^{a} (4.81)	46.78^{a} (6.55)	36.31^{b} (5.17)	62.77^{a} (3.22)

*Different lowercase letters in the same column and same genus indicate statistical difference ($p \leq 0.05$) between the data.

In the collection at 12 h, the group “CG719 + VC4” presented a greater percentage of L3 of the genus *Haemonchus* than the “VC4” group and the control group, however the control group did not differ from the “CG719” and “VC4” groups for the percentage of L3 of the genus *Haemonchus*. The group “CG719” did not differ from the group “CG719 + VC42” in its percentage of L3 of *Haemonchus*.

In the collection at 24 h, the group “CG719 + VC4” presented a higher percentage of L3 of the genus *Haemonchus* than the groups “CG719” and control, and the control did not differ from the group “CG719” in its percentage of L3 of the genus *Haemonchus*. The percentage of L3 of *Haemonchus* in group “VC4” did not differ from that in groups “CG719 + VC4” and “CG719”, however, “VC4” presented a higher percentage of L3 of *Haemonchus* than the control group.

The “CG719 + VC4” group had a higher percentage of L3 of the *Haemonchus* genus than the control and “VC4” groups, and the “CG719” group did not differ from the “CG719 + VC4” group in its percentage of *Haemonchus* L3, at 60 h. The group “CG719” presented a higher and the “VC4” group a lower percentage of L3 of *Haemonchus* than the control group at 60 h. In the collection at 72 h, the percentage of L3 of *Haemonchus* in the group “CG719 + VC4” was higher than that in the “CG719” group, “VC4” group, and the control group, and the last three presented no significant difference between them ($p > 0.05$).

As shown in Table 2, the percentage of L3 of the *Cooperia* genus did not differ between the four groups at 48 h after pellet delivery. At 12 and 60 h, the group “CG719 + VC4” had a higher percentage of L3 of *Cooperia* than the control, and “VC4” and “CG719” did not differ from group “CG719 + VC4” in terms of the percentage of *Cooperia* L3. The groups “CG719” and “VC4” did not differ from the control group in terms of the percentage of L3 of *Cooperia* at 12 and 60 h.

At 24 h, the percentage of L3 recovered from the *Cooperia* genus did not differ between the groups treated with fungi. However, the “CG719” group presented a higher percentage of L3 of *Cooperia* than the control group, which did not differ from the “CG719 + VC4” and “VC4” groups in its percentage of *Cooperia* L3.

At 36 h, the percentage of L3 recovered from the *Cooperia* genus did not differ between the groups treated with fungi. However, the group “CG719 + VC4” had a higher percentage of L3 of *Cooperia* than the control group, which did not differ from the “CG719” and “VC4” groups in its percentage of *Cooperia* L3.

At 72 h, the group “CG719 + VC4” presented a higher percentage of L3 of the genus *Cooperia* than the control, “CG719”, and “VC4” groups. The groups “CG719” and “VC4” did not differ as much in their percentage of L3 of *Cooperia*, although both had lower *Cooperia* L3 percentages than the control group.

As shown in Table 2, at 12 h, the *Oesophagostomum* percentages of the L3 of the groups “CG719 + VC4” and “CG719” did not differ from each other and were less than the percentages of the L3 of the “VC4” group and the control group, and the *Oesophagostomum* percentages of the L3 of the last two groups also did not differ statistically.

The “CG719 + VC4” group presented a lower percentage of L3 of the genus *Oesophagostomum* than the “CG719” group and the control group, and the control group did not differ from the “CG719” group in terms of its percentage of L3 of the genus *Oesophagostomum*. The percentage of L3 of *Oesophagostomum* in the group “VC4” did not differ from that of the groups “CG719 + VC4” and “CG719”, however it presented a lower percentage of *Oesophagostomum* L3 than the control group.

The percentages of L3 of the genus *Oesophagostomum* did not differ between the four groups at the collection times 36 and 48 h after pellet delivery. At 60 h, the group “CG719 + VC4” presented a lower percentage of L3 of *Oesophagostomum* than the control and “VC4” and “CG719” did not differ from group “CG719 + VC4” in terms of their *Oesophagostomum* L3 percentage. The group “CG719” presented a lower and the group “VC4” a higher percentage of L3 of *Oesophagostomum* than the control group in the collection at 60 h.

In the collection at 72 h, the percentage of L3 of *Oesophagostomum* in the group “CG719 + VC4” was lower than that of the groups “CG719”, “VC4” and the control, and the last three did not present a significant difference between their percentage of L3 of the genus *Oesophagostomum*.

DISCUSSION

Evaluation of the passage of the fungi *A. cladodes* and *P. chlamydosporia* through the gastrointestinal tract of cattle at 12, 24, 36, 48, 60, and 72 h revealed the three treatments with the fungi presented lower values of recovered infective larvae than the control group without fungus, thus demonstrating the nematicidal activity and the potential of these organisms to be used in the biological control of parasitic helminths in the field.

In order to use nematophagous fungi as field biological control agents, it is necessary for these organisms to be incorporated into formulations that protect them from the adverse conditions of the gastrointestinal tract of the animals, so that they can be dispersed along with the faecal cake and kept viable for multiplication and predation on parasitic helminths. As an example of a formulation, sodium alginate matrix pellets were successful in the passage through the gastrointestinal tract of cattle in the present study. In the faecal cake, nematophagous fungi find an environment rich in organic matter, which increases the supply of nutrients and allows the fungi to present nematicidal action and establish themselves in the environment.

According to Oliveira *et al*. (2018), *A. cladodes*, formulated in sodium alginate matrix, survives passage through the gastrointestinal tract of cattle and the administration of this fungus was associated with a reduction of 48.65 %, 53.33 %, and 45.16 %, respectively, in infective helminth larvae at 12, 24, and 36 h stool collection times after oral administration. In the present study, we observed L3 reduction percentages of 76.12 %, 76.97 %, and 69.03 %, respectively, at the collection times of 12, 24, and 36 h, which are higher than those cited by Oliveira *et al*. (2018).

Ranjbar-Bahadori *et al*. (2010) reported that *A. cladodes* resisted the passage through the gastrointestinal tract of sheep, presenting trap production and a reduction in the percentage of *Haemonchus contortus* L3 of 78.8 %. Our results showing a reduction in the percentage of L3 parasites of cattle by the fungus *A. cladodes* varied numerically between 62.05 % and 76.97 %, which are similar to the percentage reported by Ranjbar-Bahadori *et al*. (2010).

Although the fungus *P. chlamydosporia* is frequently evaluated in relation to the destruction of helminth eggs, there are few reports in the literature of the larvicidal activity of this fungus. Dias *et al*. (2012) evaluated the effect of the fungus *P. chlamydosporia* (VC1 isolate) in a sodium alginate matrix after passage through the gastrointestinal tract of cattle on *Fasciola hepatica* eggs and observed that the fungus was efficient at destroying the eggs in the faecal samples recovered at the collection times of 12, 18, 24, 48, 72, and 96 h. In our study, we did not evaluate the ovicidal capacity of *P. chlamydosporia*, however, we observed nematicidal activity against L3 parasites of cattle by this fungus and the percentage of reduction varied numerically between 52.61 % and 72.09 % at the different collection times.

There are other reports on the ability of other nematophagous fungi, in a sodium alginate matrix, to resist passage through the gastrointestinal tract of domestic animals. Silva *et al*. (2013) reported that 72 h after gastrointestinal transit in bovine females, *Duddingtonia*

flagrans (AC001 and CG722 isolates) and *Monacrosporium thaumasium* (NF34 isolate) showed a higher predatory activity on infective larvae of bovine parasitic helminths (81.2 %, 97.3 %, and 98.3 %, respectively) than in the others collection times. Araújo *et al.* (2012) demonstrated a higher percentage of reduction *of Strongyloides westeri* infective larvae by the nematophagous fungi *D. flagrans* (85.3 %) and *M. thaumasium* (92.2 %) after 72 h of gastrointestinal transit in donkeys than in the others collection times.

The co-administration of different nematophagous fungi might increase the efficacy of biological control of parasitic helminths present in pastures, since these fungi might present different mechanisms of nematicidal action or establishment in the environment.

Silveira *et al*. (2017) evaluated the fungi *Arthrobotrys robusta* (I31), *A. conoides* (I40), *D. flagrans* (AC001), and *M. thaumasium* (NF34) for use in the biological control of the nematode parasite of sheep and reported that the combinations of fungi AC001 + I31 and NF34 + I40 resisted the passage through the gastrointestinal tract of goats without losing their predatory ability. In comparison to the control group, the combination AC001 + I31 reduced the number of L3 recovered in 53 % and 68 %, respectively, after 12 and 48 h of passage through the gastrointestinal tract and the combination NF34 + I40 reduced the number of L3 in 56 %, 61 %, and 48 %, respectively, after 24, 48 and 72 h of passage through the gastrointestinal tract. The combinations evaluated by Silveira *et al*. (2017) had lower predatory capacities than the combination of nematophagous fungi assessed in our study (85.61 %, 86.30 %, 77.46 %, 77.01 %, 76.38 %, and 69.96 %, respectively, 12, 24, 36, 48, 60, and 72 h after oral administration to the animals).

In the study by Tavela *et al*. (2013), were evaluated the viability of the combination of the fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34) and its predatory activity on infective larvae (L3) of cyatostominae after passage through the gastrointestinal tract of horses. The aforementioned authors observed that the combination of fungi presented high predatory activity (with reduction percentages varying numerically between 74 % and 92 %) and no statistical difference was observed between the percentages of L3 reduction at the time intervals 12, 24, 36, 48, 60, and 72 h. The combination assessed by Tavela *et al*. (2013) presented a predatory capacity similar to the combination of nematophagous fungi tested in our study.

Tavela *et al*. (2012) evaluated the effects of the nematophagous fungi *D. flagrans* (AC001 isolate), *M. thaumasium* (NF34 isolate), and *P. chlamydosporia* (VC1 isolate), both singularly and combined, against cyathostomes, and the percentage reductions in L3,

compared to the control group, were: AC001, 61.6 %; NF34, 66.1 %; VC1, 73.2 %; AC001 + VC1, 86.8 %; NF34 + VC1, 77.3 %; AC001 + NF34, 92.4 %. “AC001 + VC1” and “NF34 + VC1” are also a combination of a predatory fungus (*D. flagrans*, AC001 isolate or *M. thaumasium*, NF34 isolate) and an ovicidal fungus (*P. chlamydosporia*, VC1 isolate), and such combinations presented a predatory capacity similar to the combination of *A. cladodes* and *P. chlamydosporia* described in the present study. In the evaluation of the nematicidal activity of *P. chlamydosporia* (isolated VC1), Tavela *et al*. (2012) reported a reduction percentage of L3 of 73.2 %, which is similar to that observed in our study (72.09 % at 60 h).

The results of the percentages of the L3 genera recovered from plaques corresponding to the various collection times were not uniform. Therefore, it is not possible to generalise whether there is more or less selectivity of predation or nematicidal activity of the fungi *A. cladodes* and *P. chlamydosporia* on the infective larvae of the genera *Haemonchus*, *Cooperia,* and *Oesophagostomum*.

Oliveira *et al*. (2018) did not find a difference in the percentage of the genera *Haemonchus*, *Cooperia,* and *Oesophagostomum* when evaluating the predatory capacity of the *A. cladodes* fungus on L3 parasites of cattle. Similarly to the present study, Oliveira *et al*. (2018) did not indicate the selectivity of predation on a particular genus of nematode by the fungus *A. cladodes*. However, Gomes *et al*. (1999), when evaluating the differences in pathogenicity of fungi of the genus *Monacrosporium* on phytonematodes, free-living nematodes, and bovine parasitic nematodes, reported that the free-living nematode *Panagrellus* spp, followed by the phytonematode *Meloidogyne incognita*, was the most susceptible to most of the *Monacrosporium* isolates studied, whereas the parasitic nematodes of bovine *Cooperia punctata* and *Haemonchus placei* were the least susceptible. The fungus *M. appendiculatum* was the most effective against *H. placei* and *C. punctata* (Gomes *et al*. 1999).

CONCLUSION

The reduction in infective larvae of parasitic helminths of bovines by the combination of the predatory fungus *A. cladodes* and the ovicidal fungus *P. chlamydosporia* was higher than the reduction in infective larvae by *A. cladodes* or *P. chlamydosporia* alone at collection times of 24 and 36 h after the pellets were supplied to the animals, demonstrating that this combination enhanced the predation of the fungi on larvae.

Pelleted formulations of sodium alginate resisted the passage through the gastrointestinal tract of cattle and the fungi maintained their ability to grow and prey on helminths, which justifies the need for field studies to evaluate the predatory capacity of the combination of *A. cladodes* and *P. chlamydosporia*.

ETHICAL CONSIDERATIONS

This study was previously approved by the Ethics Committee on the Use of Animals, Federal University of Viçosa, under protocol number 06/2017.

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7. CAPÍTULO 4

FUNGOS NEMATÓFAGOS NO CONTROLE BIOLÓGICO DE HELMINTOSES EM SISTEMA EXTENSIVO DE PRODUÇÃO DE BOVINOS

RESUMO

A produção de bovinos em sistemas extensivos favorece a ocorrência de helmintoses gastrintestinais e a utilização de fungos nematófagos é uma alternativa para complementar as estratégias de controle dessas helmintoses. Nosso objetivo foi avaliar a atividade nematicida dos fungos *Pochonia chlamydosporia* e *Arthrobotrys cladodes* sobre helmintos parasitas gastrointestinais de bovinos criados em pastagens. Vinte e quatro bezerras foram divididas aleatoriamente em quatro grupos e alocadas em piquetes independentes durante o período de Fevereiro de 2018 a Janeiro de 2019. No primeiro grupo os animais receberam peletes contendo *P. chlamydosporia.* No segundo grupo os animais receberam peletes contendo *A. cladodes*. No terceiro grupo os animais receberam peletes contendo a combinação dos fungos *A. cladodes* e *P. chlamydosporia*. No grupo controle os animais receberam peletes sem fungo. O uso combinado de *A. cladodes* e *P. chlamydosporia* apresentou maior eficácia no controle biológico de helmintos parasitas gastrintestinais de bovinos do que os mesmos fungos utilizados separadamente. A carga parasitária foi menor e o ganho de peso foi maior ($p \leq 0.05$) nos grupos de bovinos tratados com fungos nematófagos. Portanto, a utilização de *A. cladodes* e *P. chlamydosporia* mostrou-se promissora no controle biológico de helmintoses em bovinos.

Palavras-chave: *Arthrobotrys cladodes; Pochonia chlamydosporia;* bovinos; controle biológico; helmintos.

INTRODUÇÃO

A agropecuária é destaque na economia brasileira e foi responsável por 5,1% de todos os bens e serviços finais produzidos no Brasil em 2018. Além disso, tal setor apresenta grande impacto social, gerando aproximadamente 9,0 milhões de vagas no mercado de trabalho brasileiro. Os sistemas de produção de gado de corte e leite são importantes componentes da atividade agropecuária. Em 2018 foram abatidos 31,9 milhões de bovinos, resultando na produção de 7,68 milhões de toneladas de carcaças bovinas e os laticínios brasileiros captaram 24,45 bilhões de litros de leite (IBGE, 2019).

A criação de bovinos em sistemas extensivos baseados na utilização de pastagens é largamente adotada pelos pecuaristas brasileiros e tal fato favorece a ocorrência constante de parasitoses causadas por helmintos. As helmintoses gastrointestinais representam um dos entraves ao desenvolvimento da produção brasileira de bovinos, causando perdas financeiras em torno de $7,11 bilhões/ano, os quais são reflexos dos custos dos tratamentos veterinários, queda da produção, retardo no crescimento e morte de animais (Grisi et al., 2014).

O emprego em larga escala de anti-helmínticos sintéticos causou a redução da eficácia destes fármacos pela seleção de parasitas resistentes (Gasbarre, 2014; Fazzio et al., 2014). Assim, torna-se fundamental o desenvolvimento de métodos complementares para o controle de helmintos parasitas gastrointestinais de bovinos. A utilização de fungos nematófagos representa um método eficaz no controle de helmintos parasitas e possibilita que produtos de origem animal sejam livres de resíduos químicos indesejáveis.

O controle biológico pela utilização de fungos nematófagos é uma alternativa para complementar as estratégias de controle de helmintoses intestinais. A dispersão de estruturas fúngicas diretamente nas fezes, onde ocorre a eclosão de ovos e as larvas se tornam infectantes (L3), é uma das formas utilizadas para o estabelecimento do controle biológico de nematoides parasitas gastrintestinais de bovinos (Paz-Silva et al., 2011). Uma alternativa para a disseminação destes fungos no ambiente é incorporar estruturas fúngicas (micélio, conídios e clamidósporos) em matriz de alginato de sódio (Silva et al., 2014) e fornecê-las incorporadas à dieta dos bovinos. Após passarem pelo trato gastrintestinal, essas estruturas germinam nas fezes, formando armadilhas que capturam e destroem formas infectantes de vida livre dos helmintos parasitas (Braga e Araújo, 2014).

O fungo *Pochonia chlamydosporia* parasita ovos de helmintos por meio de estruturas conhecidas como apressórios, que promovem a penetração do ovo por ação mecânica e

enzimática (Braga et al., 2008) e apresenta ação larvicida sobre helmintos parasitas gastrointestinais de bovinos (Vieira et al., 2019). O fungo *Arthrobotrys cladodes* produz armadilhas que promovem a adesão, imobilização, penetração e destruição das larvas de helmintos (Oliveira et al., 2018a).

Os fungos nematófagos *P. chlamydosporia* e *A. cladodes,* quando cultivados em conjunto sob condições laboratoriais, não apresentam incompatibilidade de crescimento e o uso combinado destes fungos apresentou maior atividade predatória sobre larvas infectantes de helmintos do que quando utilizados isoladamente (Vieira et al., 2019). Entretanto, não existem relatos na literatura do uso combinado dos fungos *P. chlamydosporia* e *A. cladodes* no controle biológico de nematoides parasitas em condições naturais.

Quando utilizados no controle biológico de helmintos a campo, os fungos nematófagos podem sofrer influência das variações ambientais de temperatura, umidade, precipitação e disponibilidade de nutrientes. O objetivo deste estudo foi avaliar a atividade nematicida dos fungos *P. chlamydosporia* e *A. cladodes*, isoladamente e em associação, sobre helmintos parasitas gastrointestinais de bovinos criados extensivamente em pastagens do Brasil.

MATERIAL E MÉTODOS

FUNGOS

Os fungos nematófagos *A. cladodes* var. *macroides* (isolado CG719) e *P. chlamydosporia* (isolado VC4) utilizados neste estudo fazem parte da coleção do Laboratório de Parasitologia, do Departamento de Veterinária da Universidade Federal de Viçosa, onde são mantidos a 4°C, no escuro, em tubos de ensaio contendo corn-meal-ágar 2% (CMA 2%).

ENSAIO EXPERIMENTAL IN VIVO

O experimento foi realizado em uma fazenda localizada no município de Abre Campo, estado de Minas Gerais, sudeste do Brasil, latitude 20º 18' 04" S, longitude 42º 28' 39" W e teve duração de doze meses (Fevereiro de 2018 à Janeiro de 2019).

Vinte e quatro fêmeas bovinas, mestiças holandês x zebu, apresentando média de nove meses de idade, com peso médio de 150 kg, foram tratadas previamente com o anti-helmíntico albendazole (7,5 mg/Kg de peso vivo). Transcorridos 21 dias do tratamento com

anti-helmíntico, o número de ovos de helmintos por grama de fezes (OPG) dos animais mostrou-se nula pela técnica descrita por Gordon e Whithlock (1939) e modificada por Lima (1989). Os animais foram divididos aleatoriamente em quatro grupos de seis animais e alocados em piquetes independentes, cada qual apresentando área de 6,0 ha, com pastagem de *Brachiaria brizantha*, naturalmente infestados com larvas de helmintos pelo prévio histórico de pastejo de animais jovens e adultos.

No primeiro grupo cada animal foi tratado com 1g de peletes / 10 Kg de peso corporal (0,2 g de fungo / 10 Kg de peso corporal) contendo o fungo *P. chlamydosporia*, administrados duas vezes por semana, juntamente com farelo de trigo. No segundo grupo cada animal foi tratado com 1g de peletes / 10 Kg de peso corporal (0,2 g de fungo / 10 Kg de peso corporal) contendo *A. cladodes*, administrados duas vezes por semana juntamente com farelo de trigo. No terceiro grupo cada animal foi tratado com 1g de peletes / 10 Kg de peso corporal (0,2 g de fungo / 10 Kg de peso corporal) contendo a combinação dos fungos *A. cladodes* e *P. chlamydosporia*, administrados duas vezes por semana juntamente com farelo de trigo. No grupo controle cada animal recebeu peletes (1g / 10 Kg de peso corporal) sem fungo, duas vezes por semana juntamente com farelo de trigo.

FORMULAÇÕES PELETIZADAS CONTENDO FUNGOS NEMATÓFAGOS

Micélio dos fungos crescidos em meio líquido GPL (Glicose, Peptona e estrato de Levedura) foram utilizados para a confecção de formulações peletizadas de alginato de sódio, de acordo com a técnica descrita por Walker e Connick (1983), modificada por Lackey et al. (1993).

COLETA E PROCESSAMENTO DO MATERIAL FECAL

A cada 15 dias, após a introdução dos animais nos piquetes, amostras de fezes de todos os animais de cada grupo foram coletadas diretamente da ampola retal. Nessas amostras foram determinadas as contagens de ovos de helmintos parasitas gastrintestinais por grama de fezes (OPG). Em seguida, coproculturas foram confeccionadas com 20g de fezes misturadas a vermiculita e incubadas, a 25 °C, por 12 dias. Após esse período foi realizada a recuperação das larvas infectantes (L3) por meio da técnica do funil de Baermann, com água a 42-45 °C, durante 12 horas. A identificação das L3 foi realizada seguindo os critérios de Keith (1953).

As larvas infectantes obtidas das coproculturas foram agrupadas por gênero e os dados foram apresentados como percentuais de gêneros.

LARVAS INFECTANTES DA PASTAGEM

A cada 15 dias, duas amostras de pastagem (0–20 e 20–40 cm de distância do bolo fecal) foram coletadas dos piquetes de cada um dos grupos a partir de seis pontos alternados, de acordo com a técnica descrita por Raynaud & Gruner (1982). Todas as amostras foram constituídas de 500 g da parte aérea da pastagem e a partir destas foram recuperadas as larvas infectantes de helmintos parasitas de bovinos (L3), seguindo a metodologia descrita por Lima (1989) e identificadas segundo os critérios estabelecidos por Keith (1953).

Foi determinada a composição em matéria seca das amostras de pastagem em estufa a 100 °C. Os dados obtidos foram convertidos em número de larvas infectantes recuperadas por quilograma de matéria seca (L3 / Kg MS).

GANHO DE PESO

Os animais foram pesados mensalmente para determinação do ganho de peso diário médio através da fórmula: (peso bruto no mês atual - peso bruto no mês anterior) / número de dias transcorridos entre as pesagens.

DADOS CLIMÁTICOS

Os dados climáticos referentes às temperaturas mínimas, médias e máximas, bem como as precipitações pluviais mensais foram registrados em estação meteorológica especializada localizada no município de Abre Campo, Minas Gerais, Brasil.

ANÁLISES ESTATÍSTICAS

As médias de contagens de ovos por gramas de fezes (OPG) foram transformados em log (x+1) e submetidos ao teste estatístico não paramétrico de Kruskal-Wallis, ao nível de significância de 5%. Os dados de ganho de peso diário, percentuais de gêneros de L3 das

coproculturas e número de L3 recuperadas das pastagens foram submetidos à análise de variância (ANOVA) e teste F (Tukey), ao nível de significância de 5%.

RESULTADOS

As médias mensais do número de ovos por grama de fezes (OPG) nos três grupos tratados e no grupo controle durante o período de Fevereiro de 2018 a Janeiro de 2019 são apresentadas na Figura 1. No primeiro mês de tratamento (fevereiro de 2018), o baixo número de OPG foi resultado do tratamento anti-helmíntico administrado aos animais anteriormente ao início do experimento. Nos dois primeiros meses os valores de OPG não apresentaram diferenças significativas ($p \leq 0,05$) entre os quatro grupos.

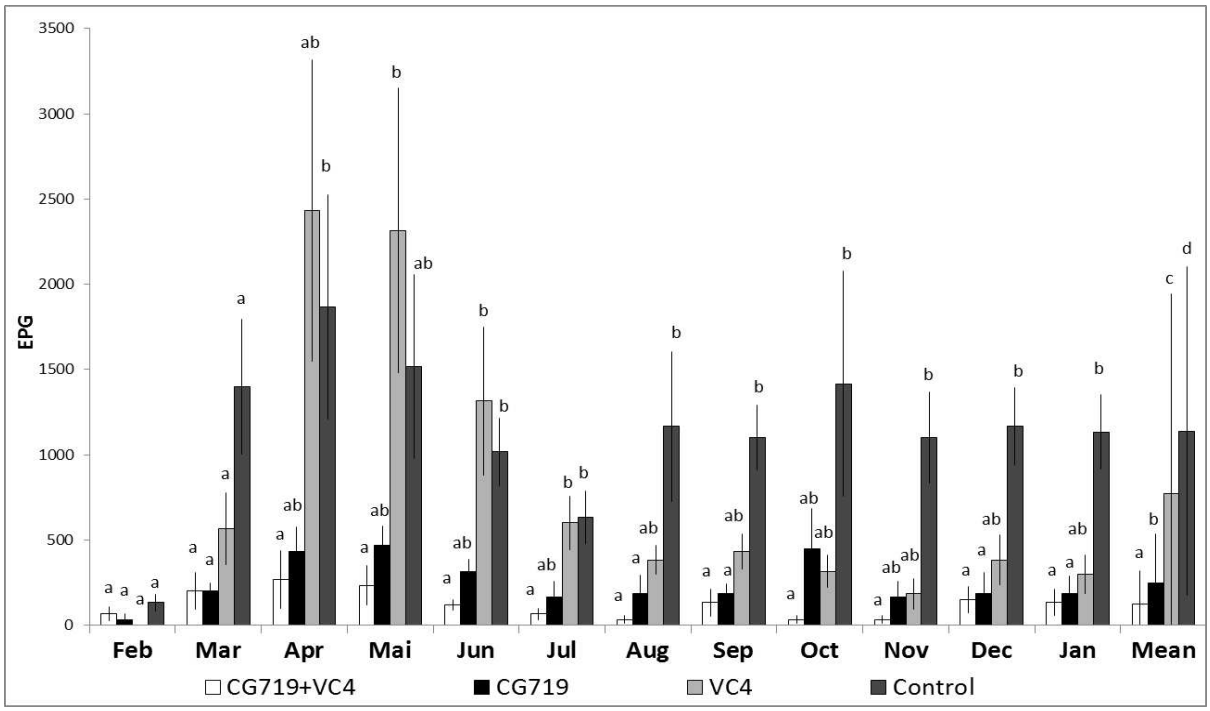

Figura 1: Médias mensais e erro padrão (barras) do número de ovos por grama de fezes (OPG) nos grupos tratados com *Arthrobotrys cladodes* (CG719), com *Pochonia chlamydosporia* (VC4), com a combinação de *Arthrobotrys cladodes* e *Pochonia chlamydosporia* (CG719+VC4) e no grupo controle durante o período de Fevereiro de 2018 a Janeiro de 2019, em Abre Campo, Minas Gerais, Brasil. Letras iguais no mesmo mês indicam não haver diferença significativa ($p > 0.05$) entre os dados.

Nos meses Abril, Outubro e Novembro de 2018 apenas o grupo tratado com a combinação dos fungos *A. cladodes* e *P. chlamydosporia* apresentou valor de OPG significativamente menor do que o grupo controle ($p \leq 0.05$). Os valores de OPG do grupo tratado com a combinação dos fungos foram 85,71; 97,65 e 96,97% menores do que os do grupo controle, respectivamente nos meses Abril, Outubro e Novembro. No mesmo período, a contagem de ovos nos grupos tratados unicamente com *A. cladodes* ou *P. chlamydosporia* não diferiu estatisticamente da contagem de ovos do grupo tratado com a combinação de fungos.

Em Maio de 2018 o grupo tratado com a combinação dos fungos *A. cladodes* e *P. chlamydosporia* apresentou valor de OPG menor do que o grupo tratado apenas com *P. chlamydosporia*, entretanto, entre os outros grupos não houve diferença significativa entre os valores de OPG.

No período de Junho e Julho de 2018 os animais tratados com a combinação dos fungos *A. cladodes* e *P. chlamydosporia* apresentaram valor de OPG significativamente menor do que os animais do grupo controle e do grupo tratado apenas com *P. chlamydosporia* ($p \leq 0.05$). Os valores de OPG do grupo tratado com a combinação dos fungos foram 88,52 e 89,47% menores do que os do grupo controle, respectivamente nos meses Junho e Julho. Entretanto, no mesmo período, a contagem de ovos no grupo tratado unicamente com *A. cladodes* não diferiu estatisticamente das contagens dos grupos controle e do grupo tratado unicamente com *P. chlamydosporia*.

Nos meses de Agosto, Setembro, Dezembro de 2018 e Janeiro de 2019 o grupo tratado com a combinação dos fungos *A. cladodes* e *P. chlamydosporia* e o grupo tratado com *A. cladodes* apresentaram valores de OPG sem diferenças entre si e significativamente menores do que o grupo controle, entretanto, a contagem de ovos no grupo tratado unicamente com *P. chlamydosporia* não diferiu estatisticamente da contagem do grupo controle. No mesmo período, o OPG do grupo tratado unicamente com *A. cladodes* não diferiu estatisticamente do OPG do grupo tratado unicamente com *P. chlamydosporia* (Figura 1).

Os valores de OPG do grupo tratado com a combinação dos fungos foram 97,14; 87,88; 87,14 e 88,24% menores do que os do grupo controle, respectivamente nos meses Agosto, Setembro, Dezembro de 2018 e Janeiro de 2019. Os valores de OPG do grupo tratado unicamente com *A. cladodes* foram 84,29; 83,33; 84,29 e 83,82% menores do que os do grupo controle, respectivamente nos meses Agosto, Setembro, Dezembro de 2018 e Janeiro de 2019.

As médias anuais de OPG apresentaram diferença significativa ($p \leq 0,05$) entre os quatro grupos, sendo que a maior contagem de ovos foi observada no grupo controle. O grupo

tratado com a combinação dos fungos *A. cladodes* e *P. chlamydosporia* apresentou média anual de OPG 89,3% menor que o grupo controle, enquanto os grupos tratados unicamente com *A. cladodes* ou *P. chlamydosporia* apresentaram, respectivamente, uma redução de 78,3 e 32,4% nos valores de OPG em relação ao grupo controle.

Os valores percentuais dos gêneros das L3 recuperadas de coproculturas realizadas com as fezes dos animais dos três grupos tratados e do grupo controle são apresentados na Tabela 1.

Tabela 1: Valores médios dos percentuais dos gêneros de larvas infectantes de *Haemonchus* (Haem)*, Cooperia* (Coop) e *Oesophagostomum* (Oeso) recuperadas de coproculturas dos grupos de animais tratados com *Arthrobotrys cladodes* (CG719), com *Pochonia chlamydosporia* (VC4)*,* com a combinação de *Arthrobotrys cladodes* e *Pochonia chlamydosporia* (CG719+VC4) e do grupo controle durante o período de Fevereiro de 2018 a Janeiro de 2019, em Abre Campo, Minas Gerais, Brasil.

	Control			**CG719+VC4**			**CG719**			**VC4**		
	Haem	***Coop***	***Oeso***	***Haem***	***Coop***	***Oeso***	***Haem***	***Coop***	***Oeso***	***Haem***	***Coop***	***Oeso***
Feb	55,0	42,8	2,2	38,5	57,4	4,1	63,5	34,8	1,7	89,2	10,8	0,0
Mar	65,7	24,3	10,0	62,1	26,3	11,6	53,2	45,1	1,7	65,2	34,1	0,7
Apr	58,6	39,1	2,3	40,7	36,9	22,4	54,4	42,6	3,0	52,9	44,7	2,4
Mai	50,2	20,2	29,6	31,3	26,8	41,9	49,4	35,3	15,3	45,3	39,5	15,2
Jun	30,2	15,5	54,3	24,5	21,8	53,7	44,5	16,5	39,0	40,8	20,0	39,2
Jul	19,3	14,0	66,7	11,7	24,9	63,4	37,0	13,1	49,9	32,8	27,2	40,0
Aug	53,8	22,7	23,5	42,1	18,0	39,9	31,7	11,5	56,8	19,1	5,4	75,5
Sep	33,2	27,9	38,9	25,8	26,7	47,5	22,3	13,9	63,8	32,9	24,4	42,7
Oct	36,5	33,3	30,2	58,0	16,2	25,8	26,3	28,8	44,9	22,6	17,8	59,6
Nov	36,2	29,0	34,8	47,2	28,2	24,6	20,3	20,0	59,7	23,2	22,7	54,1
Dec	38,3	26,1	35,6	26,2	32,7	41,1	12,6	9,7	77,7	25,2	25,2	49,6
Jan	33,8	27,2	39,0	30,0	32,2	37,8	6,5	10,0	83,5	23,3	25,8	50,9
Mean	42,6[A]	26,8[A]	30,6[A]	36,5[A]	29,0[A]	34,5[A]	35,2[A]	23,4[A]	41,4[A]	39,4[A]	24,8[A]	35,8[A]
EPM[B]	3,7	2,0	4,8	4,0	3,0	4,8	5,0	3,6	8,1	5,8	3,1	7,0

[A] Letras maiúsculas iguais indicam não haver diferença estatística ($p > 0,05$) entre os dados.

[B] Erro Padrão da Média.

Dentre os quatro grupos, a média anual dos percentuais dos gêneros *Haemonchus*, *Cooperia* e *Oesophagostomum* não diferiram significativamente ($p > 0,05$). Entretanto, nos

primeiros três meses do experimento o percentual do gênero *Oesophagostomum* foi menor que os percentuais de *Haemonchus* e *Cooperia.*

O número médio anual de L3 recuperadas das pastagens a distâncias de 0-20 cm e 20-40 cm do bolo fecal são apresentados na Figura 2. Embora numericamente diferentes, não houve diferença estatística significativa entre os valores anuais de L3 recuperadas, na mesma distância do bolo fecal, entre os quatro grupos durante o período experimental.

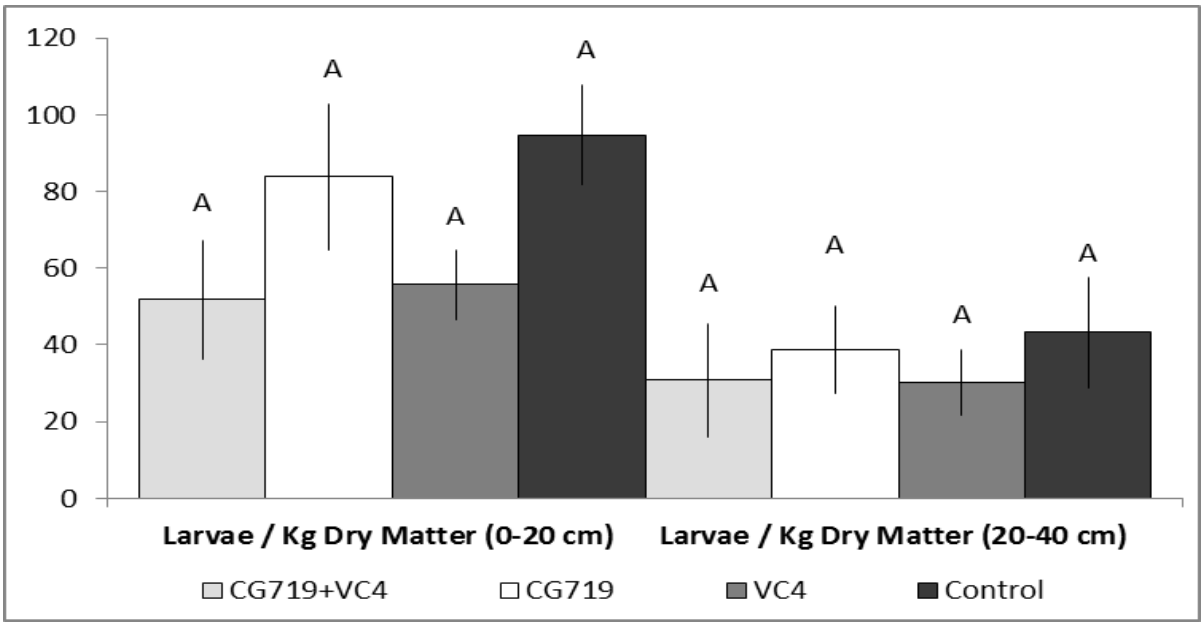

Figura 2: Número médio anual e erro padrão (barras) de larvas infectantes recuperadas por quilograma de matéria seca, a distâncias de 0-20 e 20-40 cm do bolo fecal, nos grupos tratados com *Arthrobotrys cladodes* (CG719), com *Pochonia chlamydosporia* (VC4), com a combinação de *Arthrobotrys cladodes* e *Pochonia chlamydosporia* (CG719+VC4) e no grupo controle durante o período de Fevereiro de 2018 a Janeiro de 2019, em Abre Campo, Minas Gerais, Brasil. Letras iguais indicam não haver diferença significativa entre os dados de larvas recuperadas à mesma distância do bolo fecal ($p > 0,05$).

Os valores do Ganho de Peso (Kg / dia) dos grupos tratados com *A. cladodes* (CG719), com *P. chlamydosporia* (VC4), com a combinação de *A. cladodes* e *P. chlamydosporia* (CG719+VC4) e no grupo controle durante o período experimental são apresentados na Figura 3. Na maioria dos meses (Fevereiro, Março, Abril, Setembro, Outubro, Novembro e Dezembro de 2018) não houve diferença significativa entre o ganho de peso dos animais nos quatro grupos estudados.

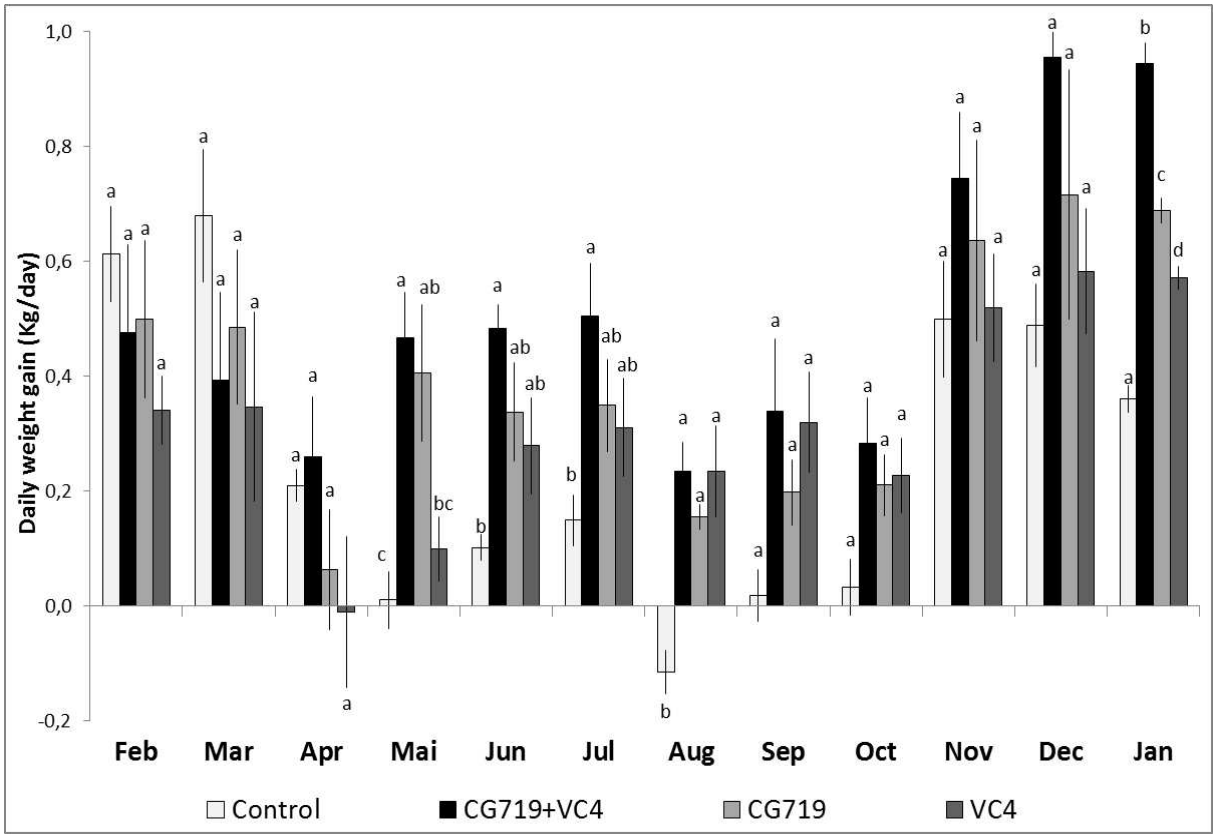

Figura 3: Ganho de Peso médio (Kg / dia) e erro padrão (barras) dos grupos tratados com *Arthrobotrys cladodes* (CG719), com *Pochonia chlamydosporia* (VC4), com a combinação de *Arthrobotrys cladodes* e *Pochonia chlamydosporia* (CG719+VC4) e no grupo controle durante o período de Fevereiro de 2018 a Janeiro de 2019, em Abre Campo, Minas Gerais, Brasil. Letras iguais no mesmo mês indicam não haver diferença significativa ($p \leq 0,05$) entre os dados.

No mês de Maio o grupo tratado unicamente com *A. cladodes* e o grupo tratado com a combinação de *A. cladodes* e *P.* chlamydosporia apresentaram maior ganho de peso que o grupo controle. Nos meses de Junho e Julho, apenas o grupo tratado com a combinação de *A. cladodes* e *P.* chlamydosporia apresentou maior ganho de peso que o grupo controle. Em agosto, os três grupos tratados apresentaram maior ganho de peso que o grupo controle, o qual apresentou redução do peso corporal. No último mês do experimento, todos os grupos tratados com fungos nematófagos apresentaram maior ganho de peso que o grupo controle ($p \leq 0,05$) e o grupo tratado com a combinação de *A. cladodes* e *P. chlamydosporia* apresentou maior ganho de peso que os grupos tratados com *A. cladodes* e com *P. chlamydosporia*.

Os dados climáticos referentes às temperaturas mensais mínimas, médias e máximas, bem como as precipitações pluviais mensais durante o período experimental na cidade de

Abre Campo, Minas Gerais, Brasil, são apresentados na Figura 4. Nos meses de Fevereiro, Março, Dezembro de 2018 e Janeiro de 2019 as temperaturas médias ambientais foram maiores e nos meses de Fevereiro, Março, Novembro e Dezembro de 2018 ocorreram os maiores índices de pluviosidade.

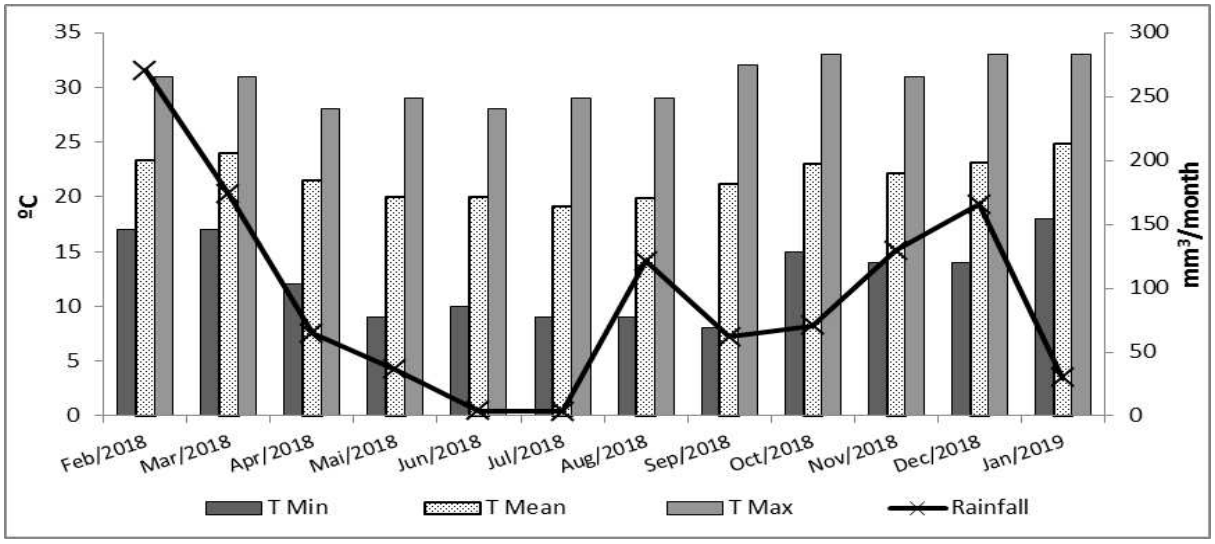

Figura 4: Temperatura Mínima (T Min), Média (T Mean), Máxima (T Max) e Precipitação (mm^3/mês) no período de Fevereiro de 2018 a Janeiro de 2019 no município de Abre Campo, Minas Gerais, Brasil.

As figuras 5, 6 e 7 representam, respectivamente, regressões polinomiais dos valores OPG e dos valores de L3 recuperadas no pasto a distâncias de 0 a 20 cm e 20 a 40 cm do bolo fecal em função do período experimental. Nas figuras são representados também os valores de temperatura média e precipitação mensal observados no município de Abre Campo no período de Fevereiro de 2018 a Janeiro de 2019.

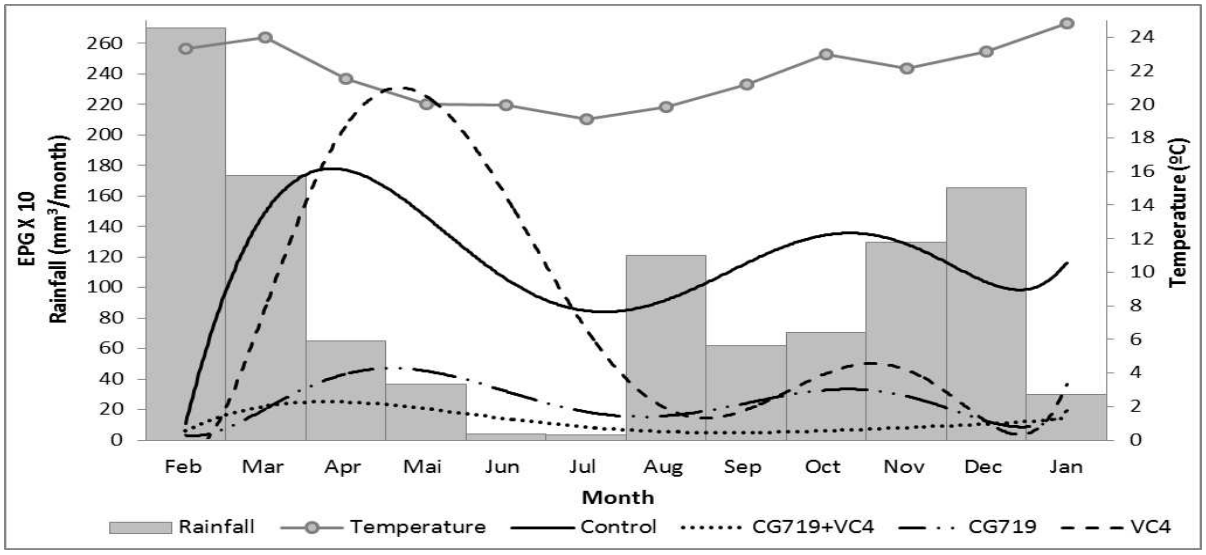

Figura 5: Regressão Polinomial da contagem de ovos por grama de fezes em função do período experimental nos grupos tratados com *Arthrobotrys cladodes* (CG719), com *Pochonia chlamydosporia* (VC4), com a combinação de *Arthrobotrys cladodes* e *Pochonia chlamydosporia* (CG719+VC4) e no grupo controle durante o período de Fevereiro de 2018 a Janeiro de 2019, no município de Abre Campo, Minas Gerais, Brasil.

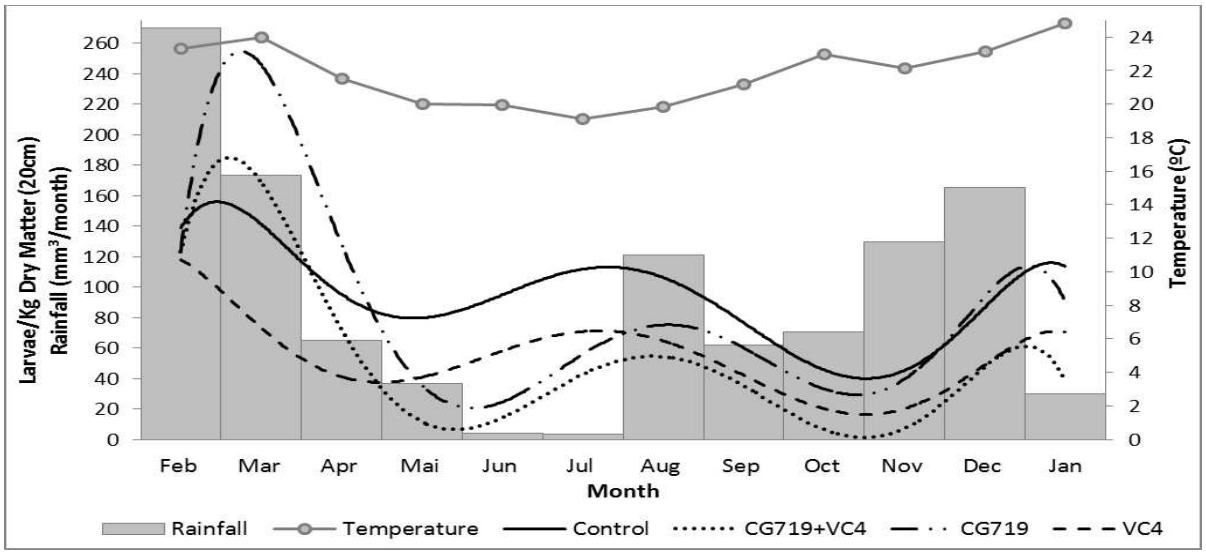

Figura 6: Regressão Polinomial entre o número de larvas infectantes recuperadas da pastagem a distância de 0-20 cm do bolo fecal em função do período experimental nos grupos tratados com *Arthrobotrys cladodes* (CG719), com *Pochonia chlamydosporia* (VC4), com a combinação de *Arthrobotrys cladodes* e *Pochonia chlamydosporia* (CG719+VC4) e no grupo controle durante o período de Fevereiro de 2018 a Janeiro de 2019, no município de Abre Campo, Minas Gerais, Brasil.

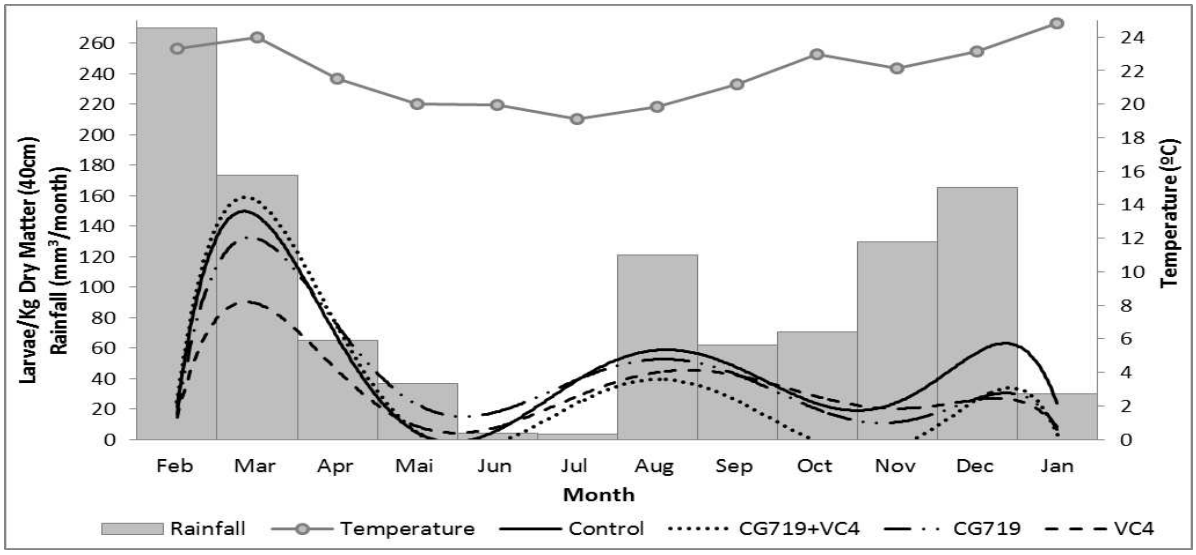

Figura 7: Regressão Polinomial entre o número de larvas infectantes recuperadas da pastagem a distância de 20-40 cm do bolo fecal em função do período experimental nos grupos tratados com *Arthrobotrys cladodes* (CG719), com *Pochonia chlamydosporia* (VC4), com a combinação de *Arthrobotrys cladodes* e *Pochonia chlamydosporia* (CG719+VC4) e no grupo controle durante o período de Fevereiro de 2018 a Janeiro de 2019, no município de Abre Campo, Minas Gerais, Brasil.

Os modelos de regressão envolvendo a contagem de OPG apresentaram coeficientes de determinação (R^2) de 0,91; 0,78; 0,82; e 0,92 respectivamente nos grupos controle, tratado com a combinação de *A. cladodes* e *P. chlamydosporia,* tratado unicamente com *A. cladodes* e tratado unicamente com *P. chlamydosporia* (Figura 5).

Os modelos de regressão envolvendo os valores de L3 recuperadas a distância de 0-20 cm do bolo fecal apresentaram coeficientes de determinação (R^2) de 0,46; 0,85; 0,88 e 0,74 respectivamente nos grupos controle, tratado com a combinação de *A. cladodes* e *P. chlamydosporia,* tratado unicamente com *A. cladodes* e tratado unicamente com *P. chlamydosporia* (Figura 6).

Os modelos de regressão envolvendo os valores de L3 recuperadas a distância de 20-40 cm do bolo fecal apresentaram coeficientes de determinação (R^2) de 0,60; 0,78; 0,61 e 0,79 respectivamente nos grupos controle, tratado com a combinação de *A. cladodes* e *P. chlamydosporia,* tratado unicamente com *A. cladodes* e tratado unicamente com *P. chlamydosporia* (Figura 7).

A contagem de OPG aumentou por influência das condições de temperatura e pluviosidade, as quais foram adequadas para o desenvolvimento dos nematoides parasitas de

bovinos. Tal comportamento foi mais evidente no grupo de animais que não recebeu tratamento com fungos nematófagos, em contrapartida a contagem de OPG do grupo de animais tratados com a combinação de fungos sofreu menor influência das condições climáticas (Figura 5). As Figuras 6 e 7 demonstram que aumentos na temperatura e precipitação determinaram os picos da carga parasitária nas pastagens.

DISCUSSÃO

Fungos nematófagos ovicidas e predadores possuem mecanismos de ação distintos, assim, quando utilizados em combinação, podem apresentar ação complementar e sinérgica no controle biológico de helmintos. Vieira et al. (2019) relataram que o uso combinado do fungo predador *A. cladodes* e do fungo ovicida *P. chlamydosporia* apresentou atividade nematicida *in vitro* de 92,67% sobre nematoides parasitas de bovinos, enquanto *A. cladodes* e *P. chlamydosporia ,* utilizados como únicos isolados, apresentaram capacidade predatória *in vitro* de 81,73 e 68,25%, respectivamente. No presente estudo foi comprovada a eficácia dos fungos *A. cladodes* e *P. chlamydosporia* na redução da carga parasitária de bovinos criados em pastagens.

Em estudo conduzido por nosso grupo de pesquisas (Capítulo 3 desta Tese), formulações peletizadas de alginato de sódio contendo a combinação dos fungos *A. cladodes* e *P. chlamydosporia*, bem como os fungos *A. cladodes* ou *P. chlamydosporia* como único isolado resistiram à passagem pelo trato gastrintestinal de bovinos e mantiveram a viabilidade de crescimento e predação *in vitro* de nematoides. A redução de larvas infectantes de helmintos parasitas de bovinos pelo uso combinado de *A. cladodes* e *P. chlamydosporia* foi maior que a redução de larvas infectantes por *A. cladodes* ou *P. chlamydosporia,* utilizados como únicos isolados, a 24 e 36 horas após o fornecimento dos péletes aos animais, demonstrando que tal associação potencializou a predação *in vitro* de larvas pelos fungos.

Estudos avaliando a utilização combinada de fungos nematófagos são escassos e o presente trabalho foi o primeiro a avaliar o uso combinado dos fungos *A. cladodes* e *P. chlamydosporia* em formulações peletizadas de alginato de sódio no controle biológico de helmintos parasitas em bovinos criados em sistema extensivo, cuja principal fonte de nutrientes para os animais é a pastagem. A contagem fecal de ovos observada em nosso estudo mostrou-se menor no grupo tratado com a combinação de *A. cladodes* e *P. chlamydosporia* do que no grupo controle a partir do terceiro mês do experimento, fato que

foi observado no grupo tratado unicamente com *A. cladodes* somente a partir do sétimo mês do experimento, portanto o uso combinado *A. cladodes* e *P. chlamydosporia* antecipou o efeito negativo destes fungos sobre helmintos parasitas gastrintestinais de bovinos em comparação à utilização isolada dos mesmos.

O grupo tratado unicamente com *P. chlamydosporia* não demonstrou diferença significativa das contagens mensais de OPG em comparação ao grupo controle, entretanto, os valores de OPG do grupo tratado unicamente com *P. chlamydosporia* foram numericamente menores que os do grupo controle na maioria dos meses. Este foi o primeiro estudo a avaliar os efeitos de *P. chlamydosporia,* utilizado como único isolado em formulações peletizadas de alginato de sódio, no controle biológico de helmintos parasitas em bovinos criados em sistema extensivo. Enzimas extracelulares (proteases e quitinases) produzidas por *P. chlamydosporia* são consideradas responsáveis pela destruição de ovos de helmintos, além de serem capazes de causar hidrólise da cutícula e morte de larvas de helmintos (Yang et al., 2013; Braga et al. 2014). Mukhtar e Pervaz (2003) relataram que, além das enzimas, o fungo *P. chlamydosporia* produz toxinas com ação nematicida.

Ao analisarmos os percentuais anuais de redução de OPG dos três grupos tratados em relação ao grupo controle, a combinação de *A. cladodes* e *P. chlamydosporia* demonstrou ser mais eficiente (OPG 89,3% menor em relação ao grupo controle) que o uso separado de *A. cladodes* (OPG 78,3% menor em relação ao grupo controle) ou *P. chlamydosporia* (OPG 32,4% menor em relação ao grupo controle). O fungo *P. chlamydosporia* parasita ovos de helmintos (Braga et al., 2008) e apresenta ação larvicida sobre helmintos parasitas gastrointestinais de bovinos (Vieira et al., 2019), por sua vez *A. cladodes* promove a adesão, penetração e destruição das larvas de helmintos (Oliveira et al., 2018a). Assim, a redução das contagens de OPG nos grupos tratados foi resultado da ação dos fungos *A. cladodes* e *P. chlamydosporia*, que, ao atuarem sobre as formas de vida livre dos nematoides, diminuiu a contaminação das pastagens e, consequentemente, o risco de reinfecção dos animais tratados com os fungos.

Há relatos de outros estudos, conduzidos em condições experimentais semelhantes ao do presente estudo, avaliando fungos nematófagos no controle biológico de helmintos parasitas gastrintestinais de bovinos. Dias et al. (2007) e Assis et al. (2012) relataram, respectivamente, que *Duddingtonia flagrans* foi responsável pela redução de 31,0 e 56,7% da contagem fecal de ovos em relação ao grupo controle. Assis et al. (2013) relataram que *D. flagrans* e *Monacrosporium thaumasium* reduziram as contagens de OPG em relação ao

grupo controle, respectivamente, em 56,7 e 47,8%. Contrariamente ao que foi observado no presente estudo, Oliveira et al. (2018b) relataram que bovinos tratados com *A. cladodes* não apresentaram valores de OPG significativamente menores que bovinos não tratados com o mesmo fungo.

Segundo Luns et al. (2018) os valores de OPG do grupo tratado unicamente com *D. flagrans* e do grupo tratado com a combinação de *D. flagrans* e *M. thaumasium*, foram, respectivamente, 96,4% e 93,8% inferiores ao OPG dos animais do grupo controle ao final do experimento, assim, o uso combinado de *D. flagrans* e *M. thaumasium* não demonstrou melhor eficiência de ação nematicida que o uso isolado de *D. flagrans*. Segundo o mesmo autor, animais tratados com a combinação de *D. flagrans* e *A. robusta*, assim como animais tratados com a combinação de *D. flagrans*, *M. thaumasium* e *A. robusta* apresentaram, respectivamente, reduções de 85,3% e 82,7% nos valores de OPG, quando comparados com os animais do grupo controle. Entretanto, Luns et al. (2018) não avaliaram os efeitos de *M. thaumasium* e *A. robusta* como únicos isolados em formulações peletizadas de alginato de sódio no controle biológico de helmintos parasitas de bovinos.

O gênero de helminto com maior ocorrência no parasitismo dos animais foi *Haemonchus,* seguido por *Oesophagostomum* e *Cooperia*, tal resultado também foi relatado por Dias et al. (2007). Os testes realizados com diferentes fungos nematófagos por Araújo et al. (2004), Assis et al. (2012), Assis et al. (2013), Assis et al. (2015) e Luns et al. (2018) demonstraram que os fungos não foram seletivos para gêneros particulares de helmintos, o que foi confirmado também para *A. cladodes* e *P. chlamydosporia* no presente estudo.

Nos primeiros meses do experimento o percentual do gênero *Oesophagostomum* foi menor que os percentuais de *Haemonchus* e *Cooperia.* Segundo Taylor (2007) o período pré-patente do gênero *Oesophagostomum* é de 35-49 dias, assim, o fato dos animais terem recebido tratamento anti-helmíntico anteriormente ao início do experimento fez com que os percentuais de *Oesophagostomum* fossem menores que os de *Haemonchus* (28 dias de período pré-patente) e *Cooperia* (14-21 dias de período pré-patente) nos primeiros meses do experimento.

Considerando que o local de atividade dos fungos nematófagos utilizados para o controle biológico é o bolo fecal depositado nas pastagens, variações ambientais de temperatura e pluviosidade afetam diretamente características importantes como crescimento, produção de clamidósporos e atividade nematicida destes fungos. Em estudo preliminar conduzido por nosso grupo de pesquisas (Capítulo 1 desta Tese) foi avaliado o efeito da

temperatura no desenvolvimento dos fungos nematófagos *A. cladodes* e *P. chlamydosporia* e estes apresentaram níveis variados de crescimento micelial, atividade nematicida e produção de clamidósporos nas temperaturas de 15, 20, 25, 30 e 35 º C. Assim, durante o período experimental do presente estudo, as condições ambientais de temperatura não foram desfavoráveis ao estabelecimento no meio ambiente e atividade nematicida dos fungos nematófagos *A. cladodes* e *P. chlamydosporia*.

As infestações das pastagens foram numericamente menores nos grupos tratados com os fungos nematófagos, entretanto não foram observadas diferenças estatísticas significativas nas cargas parasitárias das pastagens entre os grupos tratados e controle. Entretanto, Oliveira et al. (2018b) relataram que o número de larvas infectantes de helmintos parasitas de bovinos recuperadas tanto a distância de 0 a 20 cm, quanto a 20 a 40 cm dos bolos fecais foram menores no grupo tratado com *A. cladodes* do que no grupo controle, demonstrando a eficiência deste fungo na redução da carga parasitária das pastagens.

Maior número de larvas infectantes foi recuperado a distância de 0-20 cm dos bolos fecais comparando-se com o número de L3 recuperado a distancia de 20-40 cm. Do total de larvas recuperadas nos quatro grupos 66,2% foi proveniente de pastagens a distância de 0-20 cm. Tal resultado corrobora com os de Dias et al. (2007) e Assis et al. (2012) que relataram maior número de larvas recuperadas em amostras de 0-20 cm, confirmando que poucas larvas migram para a pastagem além de 0-20 cm do bolo fecal.

O fato do ganho de peso dos animais não apresentar diferença significativa entre os grupos tratados e o grupo controle em grande parte da estação chuvosa se deve a maior produção e valor nutritivo das pastagens neste período, o que minimizou os efeitos negativos das parasitoses gastrintestinais sobre o desempenho dos animais. Entretanto, em meses de menor pluviosidade o ganho de peso dos animais dos grupos tratados foi maior em comparação aos animais do grupo controle, tal fato se deve a ação nematicida dos fungos nematófagos nas pastagens, principalmente no grupo de animais tratados com a combinação de *A. cladodes* e *P. chlamydosporia.* No último mês do experimento todos os grupos tratados apresentaram maior ganho de peso que o grupo controle, tal resultado está de acordo com o relatado no estudo de Luns et al. (2018). Dias et al. (2007) e Assis et al. (2013) também relataram maior ganho de peso em bovinos tratados com fungos nematófagos em comparação a animais não tratados. Entretanto, segundo Oliveira et al. (2018b) a média de peso de bovinos tratados *A. cladodes* não foi diferente da média do grupo controle.

O uso combinado de *A. cladodes* e *P. chlamydosporia* apresentou maior eficácia no controle biológico de helmintos parasitas gastrintestinais de bovinos do que os mesmos fungos utilizados separadamente. A carga parasitária foi menor e o ganho de peso foi maior nos grupos de bovinos tratados com os fungos nematófagos, o que reflete em melhor desempenho produtivo dos animais. Portanto, a utilização de *A. cladodes* e *P. chlamydosporia* mostrou-se promissora no controle de helmintoses em bovinos.

CONSIDERAÇÕES ÉTICAS

Este estudo foi previamente aprovado pela Comissão de Ética no Uso de Animais da Universidade Federal de Viçosa (protocolo de número 06/2017). O ensaio experimental seguiu rigorosamente todos os procedimentos recomendados pelas normas de conduta para o uso de animais no ensino, pesquisa e extensão do Departamento de Veterinária da Universidade Federal de Viçosa.

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8. CONCLUSÃO GERAL

Duddingtonia flagrans, Arthrobotrys cladodes e *Pochonia chlamydosporia* apresentaram crescimento micelial, produção de clamidósporos e atividade nematicida sobre helmintos parasitas nas temperaturas de 15, 20, 25, 30 e 35 ºC. Os resultados deste estudo demonstraram que o uso de *D. flagrans, A. cladodes* e *P. chlamydosporia* no controle biológico de helmintos parasitas de bovinos não será limitado por variações na temperatura. Portanto, o uso desses fungos como controladores biológicos de helmintos parasitas é promissor.

Os testes de antagonismo em confrontação direta, antibiose e efeito de metabólitos voláteis entre os isolados de *P. chlamydosporia* e *A. cladodes* indicaram a viabilidade de crescimento em conjunto destes fungos. A associação *in vitro* dos fungos *P. chlamydosporia* e *A. cladodes* apresentou maior atividade nematicida sobre larvas infectantes de nematoides parasitas de bovinos quando comparada aos mesmos fungos utilizados isoladamente.

Formulações peletizadas em matriz de alginato de sódio resistiram à passagem pelo trato gastrintestinal de bovinos e mantiveram a viabilidade de crescimento e larvicida dos fungos *A. cladodes* e *P. chlamydosporia.* A redução de larvas infectantes de helmintos parasitas de bovinos pela associação do fungo predador *A. cladodes* e do fungo ovicida *P. chlamydosporia* foi maior que a redução de larvas infectantes, isoladamente, por *A. cladodes* e *P. chlamydosporia* nos horários de coleta 24 e 36 horas após o fornecimento dos fungos aos animais, demonstrando que tal associação potencializou a os efeitos larvicidas dos fungos.

O uso combinado de *A. cladodes* e *P. chlamydosporia* apresentou maior eficácia no controle biológico de helmintos parasitas gastrintestinais de bovinos criados a campo do que os mesmos fungos utilizados separadamente. A carga parasitária foi menor e o ganho de peso foi maior nos grupos de bovinos tratados com os fungos nematófagos, o que reflete em melhor desempenho produtivo dos animais. Portanto, a utilização de *A. cladodes* e *P. chlamydosporia* mostrou-se promissora no controle de helmintoses em bovinos.