FÁBIO DIAS LUNS

AVALIAÇÃO DA INTERAÇÃO ENTRE OS ISOLADOS FUNGICOS *Duddingtonia flagrans*, *Monacrosporium thaumasium* E *Arthrobotrys robusta* NO CONTROLE BIOLÓGICO DE NEMATÓIDES GASTRINTESTINAIS DE BOVINOS LEITEIROS A CAMPO

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

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APROVADA: 25 de julho de 2013.

_____ _____

Prof[a]. Márcia Cristina Cury

Prof[a]. Isabele da Costa Angelo

_____

Prof. Jackson Victor de Araújo
(Orientador)

A Deus, que me permitiu chegar até aqui.

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SUMÁRIO

Capítulo 3. Association of the fungi *Duddingtonia flagrans, Arthrobothyrs robusta* and *Monacrosporium thaumasium* on biologic control of dairy cattle nematodiasis

LISTA DE FIGURAS E TABELAS

Capítulo 1

Capítulo 2

Capítulo 3

RESUMO

LUNS, Fábio Dias, M.Sc., Universidade Federal de Viçosa, julho de 2013. **Avaliação da interação entre os isolados fúngicos *Duddingtonia flagrans, Monacrosporium thaumasium* E *Arthrobotrys robusta* no controle biológico de nematóides gastrintestinais de bovinos leiteiros a campo.** Orientador: Jackson Victor de Araújo. Coorientador: Fábio Ribeiro Braga.

O parasitismo gastrintestinal por nematóides constitui relevante problema nos rebanhos bovinos mundiais. O controle biológico desses parasitos com fungos nematófagos é alternativa eficaz para o controle das parasitoses de bovinos a campo. O objetivo deste estudo foi avaliar a interação entre os fungos predadores de nematóides *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34A) e *Arthrobotrys robusta* (I31) no controle de parasitos gastrintestinais em bovinos de leite a campo. Quarenta bezerras Girolando, fêmeas, entre seis e 12 meses de idade, foram divididas em cinco grupos de 08 animais cada e durante seis meses, cada grupo recebeu os seguintes tratamentos: Grupo 1: 1g/10 kg de peso vivo (PV) de pellets de alginato de sódio (0,2 g de fungo/ 10 kg de PV) contendo os fungos *D. flagrans* e *M. thaumasium*, duas vezes por semana; Grupo 2: 1g/10 kg de PV de pellets contendo os fungos *D. flagrans* e *A. robusta*, duas vezes por semana; Grupo 3: 1g/10 kg de PV de pellets contendo os fungos *M. thaumasium*, *A. robusta* e *D. flagrans*; Grupo 4: controle negativo, não tratado (pellets sem fungos) e Grupo 5: controle positivo: 1g/10 kg de PV de pellets contendo o fungo *D. flagrans*. As percentagens de redução do OPG obtidos neste estudo foram de 78% (Grupo 1), 78% (Grupo 2), 80% (Grupo 3) e 73% (Controle +). No último mês do estudo, houve uma redução de OPG de 67% (Grupo 1), 74% (Grupo 2), 70% (Grupo 3) e 74% (controle +). Não houve diferença significativa na redução de OPG entre os grupos tratados com a associação de dois (G1, G2) ou três (G3) isolados fúngicos e o grupo tratado com *D. flagrans* apenas ($p>0,05$). Conclui-se que não houve sinergismo entre os isolados fúngicos *M. thaumasium* (NF34A), *A. robusta* (I31) e *D. flagrans* (AC001), quando utilizados associados em formulação de pellets de alginato de sódio no controle de nematóides de bovinos de leite a campo.

ABSTRACT

LUNS, Fábio Dias. M.Sc., Universidade Federal de Viçosa, July, 2013. **Evaluation of the interaction between the fungal isolates Duddingtonia flagrans Monacrosporium thaumasium And Arthrobotrys robusta in biological control of gastrointestinal nematodes of dairy cattle in the field.** Adviser: Jackson Victor de Araújo. Co-adviser: Fábio Ribeiro Braga.

The gastrointestinal parasitism by nematodes is a relevant problem in cattle around the world. Biological control of nematodes with nematophagous fungi is an effective alternative for the control of parasitic diseases of cattle in the field. The aim of this study was to evaluate the interaction between nematophagous fungi *Duddingtonia flagrans* (AC001), *Monacrosporium thaumasium* (NF34A) and *Arthrobotrys robusta* (I31) in the control of gastrointestinal nematodes in cattle under field conditions. Forty heifers Girolando females, aged between six and 12 old-months were divided into five groups of 08 animals for a period of six months and each group received the following treatments: Group 1: 1g/10 kg body weight (b.w.) of pellets sodium alginate (0.2 g of fungus/ 10 kg b.w.) containing fungi *D. flagrans* and *M. thaumasium* twice a week, Group 2: 1g/10 kg b.w. of pellets containing fungi *D. flagrans* and *A. robusta*, twice a week, Group 3: 1g/10 kg b.w. of pellets containing fungi *M. thaumasium, A. robusta* and *D. flagrans*, Group 4: negative control, untreated; and Group 5: positive control: 1g/10 kg b.w. of pellets containing the fungus *D. flagrans* only. The EPG reduction percentages obtained in this study were 78% (Group 1), 78% (group 2), 80% (Group 3) and 73% (Control +). In the last month of the study, there was a reduction of 67% (Group 1), 74% (Group 2), 70% (Group 3) and 74% (Control +). There was no significant difference in EPG between the groups treated with association by two fungal isolates (G1 and G2), three fungal isolates (G3) and the group treated with pellets of *D. flagrans* only ($p>0.05$). It was concluded that there was no synergy between the fungal isolates *M. thaumasium* (NF34A), *A. robusta* (I31) and *D. flagrans* (AC001) when used in associated on the formulation of pellets sodium alginate to control nematodes of dairy cattle in the field.

1. INTRODUÇÃO GERAL

A bovinocultura é um dos principais destaques do agronegócio brasileiro no cenário mundial. O rebanho bovino brasileiro proporciona o desenvolvimento de dois segmentos lucrativos: as cadeias produtivas da carne e leite. O valor bruto da produção desses dois segmentos, estimado em R$ 67 bilhões, aliado à presença da atividade em todos os estados brasileiros, evidenciam a importância econômica da bovinocultura em nosso país (MAPA, 2012).

O clima tropical e a extensão territorial do Brasil contribuem para esse resultado, uma vez que permitem a criação do gado em pastagens, mas, por outro lado, estas condições favorecem infecções por parasitos presentes nas pastagens, o que gera impacto na produção e altos custos nas medidas de controle, acarretando perda econômica estimada em milhões de dólares (ANUALPEC, 2003).

As helmintoses gastrintestinais de ruminantes produz impacto na bovinocultura de leite. Os prejuízos causados por essas infecções envolvem queda da produção, retardo no crescimento do animal, custos com tratamentos e até a morte dos animais (MOTA et al., 2003).

O alto custo do tratamento e o surgimento da resistência aos anti-helmínticos prevêem novas alternativas de controle dessas infecções (MOTA et al, 2003).

Segundo Campos et al. (1998), na bovinocultura leiteira, o sistema de cria e recria de bezerras não tem a importância merecida, visto que nessa fase não há lucro direto para o produtor. As novilhas representam cerca de 15 a 20% dos custos de produção da atividade leiteira e especial importância na recria deveria ser dada ao manejo sanitário e as instalações dos animais, já que um correto desenvolvimento das novilhas contribuiria significativamente na produção leiteira (SANTOS et al., 2002).

Nas pesquisas de novas alternativas para o controle das helmintoses de bovinos destacam-se o desenvolvimento de vacinas, manejo de pastagens, seleção de animais geneticamente resistentes e o controle biológico, onde antagonistas naturais atuam na redução de uma população de pragas que causam perdas econômicas significativas (ARAÚJO & RIBEIRO, 2003).

Dentre os mais variados antagonistas nematóides, encontram-se organismos como fungos, bactérias, protozoários, vírus, entre outros. Os fungos nematófagos são aqueles que apresentam melhores desempenhos em pesquisas de controle biológico de nematóides (MACIEL et al., 2006), destacando-se os fungos predadores dos gêneros *Arthrobotrys*, *Duddingtonia* e *Monacrosporium* (ARAÚJO et al., 2004).

Os fungos nematófagos são organismos saprófitas mundialmente estudados, com capacidade de predar nematóides, produzindo armadilhas ao longo das hifas e exibindo redução efetiva na população de nematóides em experimentos laboratoriais e a campo (ARAÚJO et al., 1998).

Esses fungos pertencem a um grupo heterogêneo que utilizam nematóides como fonte principal ou adicional de nutrientes. São encontrados em todo o mundo em diferentes habitats, sendo frequentemente encontrados em ambientes ricos em material orgânico a temperaturas que podem variar de 20 a 30°C (LARSEN, 2000).

São conhecidos como fungos destruidores de nematóides e estão catalogados em mais de 150 espécies. São divididos em três grupos: endoparasitas, predadores e oportunistas, mas a maioria das espécies é classificada como fungos predadores de nematóides (BARRON, 1977).

Esses fungos produzem estruturas em forma de anéis constritores e não constritores, hifas, botões e redes tridimensionais adesivas ao longo do micélio. Depois do nematóide aprisionado, segue-se a penetração das hifas na cutícula do nematóide, onde ocorre o crescimento das hifas e digestão dos conteúdos internos (LARSEN et al, 1999; ARAÚJO et al., 2004).

Para que um fungo seja considerado como agente promissor no controle biológico é necessário habilidade de passar pelo trato gastrintestinal do ruminante para ser disseminado nas fezes. Alguns exemplos são os fungos das espécies *Arthrobotrys robusta* e *Monacrosporium thaumasium* que tem atividade predatória contra larvas de helmintos gastrintestinais de bovinos, sendo capazes de resistir à passagem gastrintestinal destes (ARAÚJO et al., 1999; RODRIGUES et al., 2001; MOTA et al., 2003), assim como o fungo *Duddingtonia flagrans* (GRONVOLD et al., 1993 e 2004; LARSEN et al., 1995).

Em alguns laboratórios de pesquisa, formulações a base de alginato de sódio tem sido avaliadas, demonstrando bons resultados em condições laboratoriais e a campo (ARAÚJO et al., 2000).

Na Nova Zelândia, a combinação *in vitro* de fungos nematófagos dos gêneros *Duddingtonia*, *Monacrosporium* e *Harposporium* foi testada por Waghorn et al. (2006) sobre estádios de vida livre de *Ostertagia circuncicta* e apresentou resultados promissores.

Testes *in vitro* utilizando associações entre fungos dos gêneros *Arthrobotrys*, *Duddingtonia* e *Monacrosporium* foram feitos por Araújo et al. (2004) e obtiveram redução no número de larvas infectantes de nematóides de bovinos. No entanto, essas associações ainda não foram testadas *in vivo*.

2. OBJETIVOS

2.1. Objetivo geral

- Avaliar a interação entre os fungos predadores de nematóides *Duddingtonia flagrans*, *Monacrosporium thaumasium* e *Arthrobotrys robusta*, em formulação de pellets de alginato de sódio, no controle das nematodioses gastrintestinais de bovinos de leite da raça Girolando criados a campo.

2.2. Objetivos específicos

- Verificar a contagem de ovos por grama de fezes (OPG) e o número de larvas recuperadas em pastagem;

- Comparar os resultados entre o grupo controle e os grupos tratados com diferentes associações fungicas;

- Aferir o peso dos animais;

- Avaliar qual dos tratamentos foi o mais promissor no controle das nematodioses gastrintestinais de bovinos de leite Girolando, na fase de recria a campo.

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CAPÍTULO 1 – *Veterinary Parasitology* (submetido e em revisão)

F. D. LUNS [a *]; R. C. L. ASSIS [a]; J. V. ARAÚJO [a]; F. R. BRAGA [a]

Association of the fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium* on biologic control of dairy cattle nematodiasis

[a] Federal University of Viçosa, Department of Veterinary Medicine - Laboratory of Parasitology, Av. P.H. Rolphs - Viçosa Campus. Zip code 36570-000. Viçosa - Minas Gerais - Brazil.

* Corresponding author: P.H. Rolphs Avenue, Viçosa Campus, Department of Veterinary Medicine - Laboratory of Parasitology, Viçosa, Minas Gerais, Brazil. Zip code: 36570-000, telephone: +55 31 3899-1458 Fax: +55 31 3899-1457, e-mail: fabio.luns@ufv.br

Abstract

The viability of the association of the fungi *D. flagrans* and *M. thaumasium* was tested for the biological control of dairy cattle nematodiasis in Brazil. Twenty-four 6-month-old female Girolando heifers were separated into three groups of 8 heifers each. The heifers were allocated to three 10ha paddocks of *Brachiaria decumbens* naturally infested with gastrointestinal parasitic helminths. Each animal of the group treated with the fungal association (group 1) received 1g of pellets (0.2g of fungal mycelium) for each 10kg of body weight (b.w.) containing *D. flagrans* and *M. thaumasium* mycelia. Animals of the positive control group received 1g of pellets containing the fungus *D. flagrans*, while the animals of the negative control group received 1g of fungus-free pellets for 5 months. The EPG reduction percentage in this study was 78% (group 1) and 73% (+ control). The reduction percentages of L3 in pasture in the distances up to 20 and 20-40 cm from the faecal pats were 39 and 34% (group 1) and 48 and 35% (+ control) respectively. There was no significant difference between the group treated with the fungal association and the group treated with *D. flagrans* only in the EPG and L3 recovered from the herbage samples, i.e., the use of the fungal association was not more efficient than the use of *D. flagrans* only. These results are promising because they show for the first time the passage of different fungal species associated in a pellet formulation containing *D. flagrans* and *M. thaumasium* through the gastrointestinal tract of dairy cattle monitoring the reduction of larvae in pasture.

KEYWORDS: Cattle-Nematoda; Biological Control; *D. flagrans*; *M. thaumasium;* bovine

INTRODUCTION

Anthelmintic resistance in cattle nematode parasites has not been investigated as extensively as that in small ruminants (Coles et al., 2006). This scarcity of studies might be due to the almost always subclinical effects of nematodiasis in cattle. Until recently, anthelmintic resistance in cattle nematode was not considered a serious problem, but today it appears to be increasing (AHVLA, 2010). Moreover, the increasing demand by consumers for agricultural products that should not contain chemical residues and have less potential for environmental contamination has shown the need to invest in new alternative parasite control in livestock. The use of predatory nematophagous fungi has been described as an alternative control of gastrointestinal nematodes of domestic animals in natural and laboratory conditions (Larsen et al., 1992, 1995; Paz-Silva et al., 2011; Tavela et al., 2012; Assis et al., 2013). Several studies have evaluated the effect of fungi of the genus *Duddingtonia* in vitro and in pasture, mainly on sheep (Waller et al., 2001; Waghorn et al., 2003), lambs (Githigia et al., 2001) and foals (Larsen et al., 1996) including the associated chemical compounds (Burke et al., 2005). But few studies have assessed the action of this fungus to control parasites of dairy cattle (Larsen et al., 1995). On the other hand, the fungus *Monacrosporium* sp. has been successfully used to combat nematodes of cattle in Brazil (Alves et al., 2004; Assis et al., 2004; Assis et al., 2013). The use of the association of nematode trapping fungi has also been investigated in vivo shortly. Tavela et al. (2012) evaluated the *in vitro* action of Brazilian isolates of nematophagous fungi *D. flagrans* and *M. thaumasium* on cyathostomin. This association provided a synergistic effect, achieving better results than when a single isolate was applied, however the increased competition between the fungi for nematode larvae need to be evaluated in these associations *in vivo*. This study aimed to evaluate the viability of the fungal association of *D. flagrans* and *M. thaumasium* in biologic control of dairy cattle nematodiasis in Brazil.

MATERIAL AND METHODS

Fungi and production of mycelial pellets

Two isolates of the predatory fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34A) were kept in test tubes containing corn meal agar 2% (2% CMA, Difco®, USA), at 4°C in the dark. These isolates came from a Brazilian soil and belonged to the mycology collection of the Federal University of Viçosa, Brazil. To induce the formation of the fungal mycelium, culture discs of 5 mm in diameter of isolates in 2% water-agar (2% WA) were transferred to 250 mL Erlenmeyer flasks with 150 mL of liquid GPY

medium (glucose, sodium peptone and yeast extract), pH 6.5, under the agitation of 120 rpm, in the dark, 26°C, for 10 days. After this period, the mycelia were harvested with a platinum loop and weighed in an analytic scale for the future production of sodium alginate pellets, according to Walker and Connick (1983) and modified by Lackey et al. (1993).

Animals and experimental site

The experiment was carried out in the private farm located in the municipality of Ouro Branco, state of Minas Gerais, southeast region of Brazil, 43°41'31" South latitude and 20°31'15" West longitude, from April to September 2012. The topography is hilly, with average elevation of 1000m and the native vegetation is Atlantic rain forest-cerrado transition zone. The climate is tropical with a dry season (Rating Köppen-Geiger climate: Aw), annual average maximum temperature of 71.60°F and minimum of 44.60°F. At the beginning of the experiment, twenty-four 6-month old female Girolando calves, with average body weight of 130kg, were treated with oral application of 10% albendazole (Mogivet Lab®, Brazil), at the dose of 7.5ml/10kg of b.w. Fifteen days after the anthelmintic treatment, the animals were separated into three groups of 8 calves each, based on the average weight. The calves were allocated to three 10ha paddocks of *Brachiaria decumbens*, naturally infected with gastrointestinal parasitic helminths from the previous grazing by young and adult animals. Each group was allocated to only one paddock without rotational grazing between the groups during the experiment. Each animal of the treated group received 1g of pellets (0.2g of fungal mycelium) per 10kg of b.w. containing the associated fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34a) in a single oral dose. In the positive control group, each animal received 1g of pellets (0.2g of fungal mycelium) per 10kg of b.w. containing the fungus *D. flagrans* (AC001). The animals of the negative control group received 1g of fungus-free pellets per 10kg of b.w. All animals received the pellets orally, twice a week, mixed in balanced dairy cattle ration (18% of total protein – Total®, Brazil), and water *ad libitum* during 6 months, starting from April 2012. Faecal samples were collected weekly directly from the rectum to determine egg account per gram of faeces (EPG) according Gordon and Whitlock (1939). Simultaneously to the EPG exam, coprocultures were carried out for each animal. The identification of the infective larvae in the coprocultures was performed according to Keith (1953).

Every 15 days, herbage samples were collected from each paddock of treated and control groups, in a zigzag pattern from alternated points, 20cm and 20–40cm far from

the faecal pats, according to Amarante et al. (1996). Then, a 500g herbage sample was weighed and parasitic nematode larvae were recovered following the procedure of Lima (1989). The samples were incubated in a drying oven at 100°C for 3 days to determine the dry matter content. Data were transformed into larvae per kg of dry matter.

The data were submitted to analysis of variance (ANOVA) and means were compared using the Tukey test at the 5% level of probability.

RESULTS

In the first month of the experiment (April, 2012) no statistical difference was observed ($p<0.05$) between the groups treated with fungi and the control group. The EPG was higher in the negative control group than in the treated animals of both groups during the months of May, June, August and September of 2012 ($p<0.05$). The reduction percentages obtained in this study were 78% (group 1) and 73% (+ control). In the last month of the study, there was a reduction of 67% and 74%. The largest absolute difference of EPG (2313 and 2417 eggs) was found in August 2012. The largest percentage difference also occurred in August 2012, reaching 92% and 96%. There was no significant difference between the group treated with pellets made with the two fungal isolates (G1) and the group treated with pellets of *D. flagrans* only ($p<0.05$). The monthly averages of the EPG are shown in Figure 1.

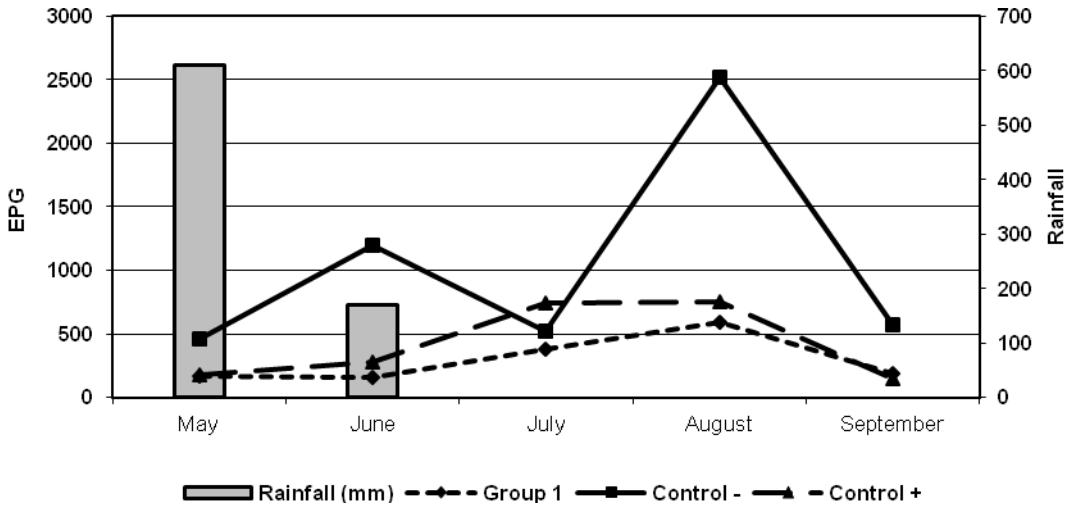

Fig. 1. Monthly means (±SD) of eggs per gram of feces of *D. flagrans* associated to *M. thaumasium* (Group 1), *D. flagrans* only (control +) and control animals (control -) collected from May 2012 to September 2012, in Ouro Branco, MG, southeastern Brazil.

In the analysis of the genera of larvae recovered from the coprocultures, there was no difference between the groups treated with the fungal association (*D. flagrans* and *M.*

thaumasium) and *D. flagrans* only. In the end of five months of study, the percentage of nematodes found in groups 1, + control and - control were respectively: *Cooperia* sp. 49%, 45% and 44%, *Haemonchus* sp. 43%, 46% and 46% and *Oesophagostomum* sp. 8%, 9% and 10%. Other species found in smaller quantities in all groups were *Bunostomum* sp. and *Strongyloides* sp., whose percentages did not reach 1%.

Table 1 shows the reduction percentages corresponding to the infective larvae (L3) recovered from the coprocultures of the groups treated with the association of the nematophagous fungi *D. flagrans* and *M. thaumasium* (Group 1) and the positive control group (*D. flagrans* only) in relation to the negative control. The larval numbers of *Cooperia*, *Haemonchus* and *Oesophagostomum* of the group treated with the association reduced by 31, 41 and 23%. In the group treated with *D. flagrans* the reduction was 27, 43 and 46% respectively ($p<0.05$), in relation to the negative control. The L3 total reduction was 47 and 35% for the Group 1 and positive control; respectively. The association provided similar reduction percentage of infective larvae in relation to D. *flagrans* and there was no significant difference in the L3 reduction percentage between the groups.

Table 1. Percentage of reduction of infective larvae (L3) recovered from the coprocultures of the groups treated with the association of the nematophagous fungi *D. flagrans* and *M. thaumasium* (Group 1) and the positive control group (*D. flagrans* only) in relation to the negative control from May 2012 to September 2012, Ouro Branco, MG, Brazil.

	Group 1				Control +			
	Coop	**Haem**	**Oeso**	**Total**	**Coop**	**Haem**	**Oeso**	**Total**
May	9	19	6	37	3	42	17	15
June	20	2	36	43	5	49	39	22
July	53	75	41	47	34	30	48	34
August	39	30	23	35	45	18	24	37
September	36	78	9	73	50	77	100	65
Average	31	41	23	47	27	43	46	35
S.D.	17	34	16	15	22	22	33	19

Table 2 shows the absolute values of L3 per kg of dry matter obtained from pastures grazed by the three groups of calves. In pasture, larvae were found in the same distribution pattern as the stool. The genus *Cooperia* was the most prevalent, followed by *Haemonchus* and *Oesophagostomum*. The L3 reduction percentage in relation to the - control in the distances up to 20 and 20-40 cm from the faecal pats was 39 % and 34 %, for Group 1. The + control showed reduction percentages of 48% and

35%, in the same distances. The largest reduction was found in the last month of the study (September) with 65 and 64% for Group 1 and + control, respectively, however, there was no significant difference between the group treated with the association and the group treated with *D. flagrans* only.

Table 2 Absolute values of L3 per kg of dry matter obtained from pastures grazed by the calves treated with the association of the nematophagous fungi *D. flagrans* and *M. thaumasium* (Group 1) , *D. flagrans* only (control +) and without fungus (control-) from May 2012 to September 2012, Ouro Branco, MG, Brazil.

0-20 cm	Group 1				Control -				Control +			
	Coop	Haem	Oeso	Total	Coop	Haem	Oeso	Total	Coop	Haem	Oeso	Total
May	167	75	20	262	260	38	28	326	128	35	10	173
June	83	34	12	129	153	25	28	206	80	22	38	140
July	41	14	14	69	50	33	8	91	40	21	10	71
August	141	64	38	244	320	67	50	437	143	29	29	200
September	71	14	6	91	170	67	10	247	76	21	0	97
Average	101	40	18	159	191	46	25	261	93	26	17	136
S.D.	52	28	12	89	104	20	17	130	42	6	16	53

20-40 cm	Group 1				Control -				Control +			
	Coop	Haem	Oeso	Total	Coop	Haem	Oeso	Total	Coop	Haem	Oeso	Total
May	184	86	33	303	173	92	22	287	228	59	34	321
June	107	38	21	166	159	65	19	243	100	25	18	143
July	37	22	15	75	48	60	12	120	49	20	20	88
August	93	33	27	153	98	53	16	168	86	29	17	132
September	0	167	0	167	150	300	50	500	43	130	0	174
Average	44	81	14	173	83	111	22	264	126	88	26	172
S.D.	47	76	13	82	76	165	25	147	138	60	29	89

Figure 2 shows the mean weight gain of animals of the three groups. The weight gain of the animals of the treated groups differed from those of the control group in the end of experiment ($p<0.05$). There was no significant difference for animal weight during the first 2 months of the experiment (May, June) between the three groups. However, in the last three months of the experiment (July, August and September) significant differences of 7, 9 and 12% were found for the weight between Group 1 and non-treated animals. The positive control group showed differences of 6.25, 7.41 and 10.1% in the same period compared to the untreated group.

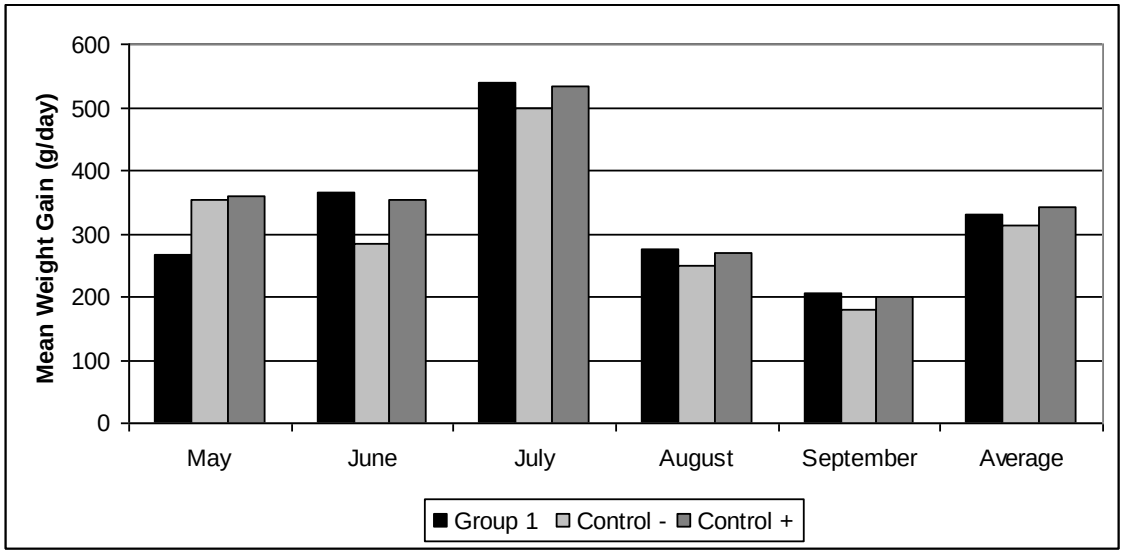

Fig. 2. Mean weight gain (kg/day) for calves treated with the association of the nematophagous fungi *D. flagrans* and *M. thaumasium* (Group 1) , *D. flagrans* only (control +) and without fungus (control-) from May 2012 to September 2012, Ouro Branco, MG, Brazil.

DISCUSSION

Various reports have described the predatory activity of nematophagous fungi in laboratory and field conditions when used in sodium alginate pellets (Alves et al., 2004; Silva et al., 2009; Assis et al., 2012). However, there are no records of the use of these fungi in pellets containing associated formulations. Tavela et al. (2012) showed that the association of different nematophagous fungi in laboratory conditions was effective to control cyathostomin. The results of this work are the first report of *in vivo* association of *D. flagrans* and *M. thaumasium* in a pellet formulation through the gastrointestinal tract of dairy cattle with accompanying reduction of parasite load during five months of application of the formulation.

A number of studies on *D. flagrans* using horses and ruminants reported lower average monthly EPG counts for treated animals than for non-treated animals (Baudena et al., 2000; Knox and Faedo, 2001; Dimander et al., 2003; Fontenot et al., 2003; Araujo et al., 2004; Paraud et al., 2007). These findings are in agreement with results obtained in the present work, confirming that the fungus acts on the infective forms in the fecal environment, with consequently decrease in EPG. However, an important new finding from this study is that the EPG counts of animals treated with *D. flagrans* and *M. thaumasium* in association were not significantly lower than those of the animals treated with *D. flagrans* alone. This result suggests that there was no synergism between these

fungi. Assis et al. (2013) in a comparative study of the action of the same fungal isolates in beef cattle found EPG reduction of 56.7% and 47.8% for isolates *D. flagrans* (AC001) and *M. thaumasium* (NF34A), separately. In this study, with dairy cattle, we found EPG reductions greater than 70% in both treated groups.

Regarding the percentage of infective larvae found in stool, the findings of this study concur with those of Dias et al. (2007) and Assis et al. (2012) who obtained similar results in studies with cattle in Southeastern Brazil. It is already known that the genera *Cooperia* sp., *Haemonchus* sp. and *Oesophagostomum* sp. are the most prevalent nematodes in southeastern Brazil (Lima, 1989).

Tavela et al. (2012) reported that the reduction of L3 recovery from contaminated faeces by the isolates AC001 and NF34, in association, was always over 80%. Araújo et al. (2004) found reduction of 59.3 % of L3 recovery from coprocultures in goat treated with pellets containing *M. thaumasium*.

Thus, these results corroborate the present work, in which the reduction of L3 in both treated groups was 47 and 35%. This finding confirmed that the association of pellets containing the fungi *D. flagrans* and *M. thaumasium* was able to pass through the gastrointestinal tract of dairy cattle, germinated in the faeces and effectively reduced L3 trichostrongylid larvae.

In this study, the results suggest that there was a direct action of *D. flagrans* on infective trichostrongylid larvae present in the pasture and a consequent lower parasitic infection of fungus-treated animals. However, the association of the isolate of *M. thaumasium* to *D. flagrans* did not enhance the reduction of larvae on pasture. Therefore, we concluded that there was no synergism between the isolates tested.

The number of larvae recovered in the distances 0-20 and 20-40 cm from fecal pats is probably related with the use of nematophagous fungi that act directly on the L3 present in pastures, confirming that *D. flagrans* accounted for the satisfactory reduction of environmental contamination (Araújo et al., 2004). Assis et al. (2013) reported reduction in L3 recovered from pasture in the distances 0-20 and 20–40 cm from the fecal pats of 64.5% and 73%, respectively for *D. flagrans*, while *M. thaumasium* showed percentage reductions of 47.3% and 58% in the same distances.

The difference ($p<0.01$) found in weight gain of treated animals compared to the control group was probably caused by a lower parasite load in animals that received pellets containing *D. flagrans* mycelia, which may have contributed to a better food conversion of treated animals. These results are similar to those found by Dias et al. (2007) on

weight gain of cattle treated with pellets containing *D. flagrans* mycelia and Braga et al (2009) on weight gain of horses also treated with the fungus. Assis et al. (2013) also reported better weight gain in bulls treated with isolates of *D. flagrans* and *M. thaumasium* separately administered to animals.

The results of this work are promising because they show for the first time the passage of different fungal species associated in a pellet formulation containing *D. flagrans* and *M. thaumasium* through the gastrointestinal tract of dairy cattle monitoring the reduction of larvae in pasture.

CONCLUSION

The treatment of dairy cattle with sodium alginate pellets containing the mycelial mass of nematophagous fungi *D. flagrans* alone or in association with *M. thamasium*, twice a week for five months, decreased the EPG of the animals by more of 70%. However, the association of the fungi *D. flagrans* (AC001) and *M. thaumasium* (NF34A) showed no synergism, because there was no significant difference when compared with the group receiving the isolate *D. flagrans* alone.

Acknowledgements

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CAPÍTULO 2 – Tropical Animal Health and Production (submetido)

F. D. LUNS [a] *; R. C. L. ASSIS [a]; J. V. ARAÚJO [a]; F. R. BRAGA [a]

Association of the fungi *Duddingtonia flagrans* and *Arthrobotrys robusta* on biologic control of dairy cattle nematodiasis

[a] Federal University of Viçosa, Department of Veterinary Medicine - Laboratory of Parasitology, Av. P.H. Rolphs - Viçosa Campus. Zip code 36570-000. Viçosa - Minas Gerais - Brazil.

* Corresponding author: P.H. Rolphs Avenue, Viçosa Campus, Department of Veterinary Medicine - Laboratory of Parasitology, Viçosa, Minas Gerais, Brazil. Zip code: 36570-000, telephone: +55 31 3899-1458 Fax: +55 31 3899-1457, e-mail: fabio.luns@ufv.br

Abstract

The viability of the association the fungi *D. flagrans* and *A. robusta* was tested in biologic control of dairy cattle nematodiasis in Brazil. 24 6-month old female Girolando heifers were separated into three groups of 8 heifers each. The heifers were allocated to three 10ha paddocks of *Brachiaria decumbens* and each animal of group treated with association (group tested) received 1g of pellets (0.2g of fungal mycelium) for each 10kg of body weight (b.w.) containing the fungi *D. flagrans* and *A. robusta*. In group control positive, each animal received 1g of pellets containing the fungus *D. flagrans*. The animals of group control negative received 1g of fungus-free pellets, during 5 months. The EPG percentage of reduction in this study was 78% (group tested) and 73% (+ control). The percentage of reduction of L3 in pasture in the distances of up to 20 and 20-40 cm from the fecal pat was 43 and 55% (group tested) and 48 and 35% (control +). There was no significant difference between the group treated with association and the group treated with *D. flagrans* only in the EPG and L3 recovered of pasture. Concluded that the association has not been more effective than the isolate *D. flagrans* only. The results showed in this work are promising since it represents the first report of the passage of different fungal species of *D. flagrans* and *A. robusta* associated in a formulation at the same time through the gastrointestinal tract of cattle monitoring the reduction of larvae on pasture.

Keywords: control biologic, parasites, dairy cattle

Introduction

Nematophagous fungi, especially the genera *Duddingtonia*, *Monacrosporium* and *Arthrobotrys* have predatory capacity on infective larvae (L3) of gastrointestinal nematode parasites of domestic animals (Araújo et al., 1998, 2004; Campos et al., 2007). Among predatory fungi, the genus *Arthrobotrys* has been proven to be a potential biological control agent of parasitic nematodes in domestic animals (Araújo et al., 1998; Braga et al., 2009). *Arthrobotrys robusta* is a nematode predator fungus which develops an adhesive network of tridimensional loops in which the nematodes are caught and then destroyed (Barron, 1977; Gives et al., 1992). The species *A. robusta* presents erect conidiophores, sometimes branched, about 300mm in length, having the extremity increased in size, usually carrying six ovoid-shaped conidia, hyaline, sectioned closely to the intermediate region, 18-27mm long and 8-12mm wide, and capable of producing chlamydospores and adhesive networks for preying on nematodes (Araújo et al., 1998, 2000). This fungus is considered a good predator of some plant-parasitic nematodes. In France it has been marketed as a commercial product (Royal 300 and Royal 350) to control the mycophagous nematode *D. myceliophagus* which destroys commercial mushrooms (Cayrol et al., 1978). According to Mota et al. (2003) this product out of the market due to problems with the formulation made grain rye.

This study aimed to test a formulation of nematophagous fungi to sodium alginate which has been highly promising *in vivo* tests (Araújo et al., 1998; Mota et al., 2003; Assis et al., 2012; Assis et al., 2013) the application of two different fungi in association. The use of association of nematode trapping fungi was also investigated in vivo shortly. This association can provide a synergistic effect, achieving better results than a single isolated applied, however the increased competition between the fungi for nematode larvae need to be evaluated in these associations *in vivo*. This study aimed to evaluate the viability of the association the fungi *D. flagrans* and *A. robusta* in biologic control of dairy cattle nematodiasis in Brazil.

2. Material and methods

2.1. Fungi and production of mycelial pellets

Two isolates of the predatory fungi *D. flagrans* (AC001) and *A. robusta* (I31) were kept in test tubes containing corn meal agar 2% (2% CMA, Difco®, USA), at 4°C in the dark. These isolates came from a Brazilian soil and belonged to the mycology collection of the Federal University of Viçosa, Brazil. To induce the formation of the fungal mycelium, culture discs of 5 mm in diameter in 2% water-agar (2% WA) were

transferred to 250 mL Erlenmeyer flasks with 150 mL of liquid GPY medium (glucose, sodium peptone and yeast extract), pH 6.5, under the agitation of 120 rpm, in the dark, 26°C, for 10 days. After this period, the mycelia were harvested with a platinum loop, and weighed in an analytic scale for the future production of the pellets, which were made in a sodium alginate matrix, according to Walker and Connick (1983) and modified by Lackey et al. (1993).

2.2. Animals and experimental site

The experiment was carried out in a private farm located in the municipality of Ouro Branco, state of Minas Gerais, southeast region of Brazil, 43°41'31" South latitude and 20°31'15" West longitude, from April to September 2012. The topography is hilly, with an average elevation height of 1000m and the native vegetation is Atlantic rain forest-cerrado transition zone. The climate is tropical with a dry season (Rating Köppen-Geiger climate: Aw), annual average maximum temperature of 71.60°F and minimum of 44.60°F. In the beginning of the experiment, 24 6-month old female Girolando calves, with average body weight of 130kg were previously treated with 10% albendazole (Mogivet Lab®, Brazil), at an oral dose of 7.5ml/10kg of b.w. Fifteen days after the anthelmintic treatment, the animals were separated into three groups of 8 calves each, based on the average weight. The calves were allocated to three 10ha paddocks of *Brachiaria decumbens*, naturally infested with gastrointestinal parasite helminths, due to the previous grazing by young and adult animals. Each group was allocated to only one paddock without rotational grazing between the groups during the experimental period. Each animal of group treated received 1g of pellets (0.2g of fungal mycelium) for each 10kg of b.w. containing the fungi *D. flagrans* (AC001) and *A. robusta* (I31) associated and in a single oral dose. In group control positive, each animal received 1g of pellets (0.2g of fungal mycelium) for each 10kg of b.w. containing the fungus *D. flagrans* (AC001). The animals of group control negative received 1g of fungus-free pellets for each 10kg of b.w. All the animals received the pellets orally, twice a week, mixed in concentrated and balanced ration provided for dairy cattle (18% of total protein – Total®, Brazil), and water *ad libitum* during 6 months, starting from April 2012. Once time for week, stool samples were collected directly from the rectum to be performed the egg account per gram of faeces (EPG) according Gordon and Whitlock (1939). Simultaneously to the EPG exam, coprocultures were carried out, for each animal. The identification of the infectant larvae in the coprocultures was performed according to Keith (1953).

Every 15 days, herbage samples were collected in each paddock of groups, in a zigzag pattern from alternated points, 20cm and 20–40cm far from the fecal pats, according to Amarante et al. (1996). Then, a 500g herbage sample was weighed, and parasitic nematode larvae were recovered following the procedure of Lima (1989). The samples were incubated in a drying oven at 100°C for 3 days to determine the dry matter content. Data were transformed into larvae per kg of dry matter.

The data were submitted to analysis of variance (ANOVA). Subsequently, the means were compared using the Tukey test at the 5% level of probability.

This study was approved by the Ethics Committee of the Federal University of Viçosa, protocol 66/2012.

3. Results

In the first month of the experiment (April, 2012) no statistical difference was observed ($p<0.05$) between the groups treated with fungi and the control group. The EPG was higher in the negative control group than in the treated animals of both groups during the months of May, June, August and September of 2012 ($p<0.05$). In July the tested group (two fungi) was EPG lower than groups with only a fungus and control without fungus, but no significant difference. The percentage of reduction in this study was 78% (group tested) and 73% (+ control). In the last month of the study was a reduction of 74% and 74%. The largest absolute difference of EPG (2334 and 2417 eggs) was found in August 2012. The largest percentage difference also occurred in August 2012, reaching 93% and 96%. There was no significant difference between the group treated with pellets made with the two fungal isolates (G1) and the group treated with pellets of *D. flagrans* only ($p<0.05$). The monthly averages of the EPG are shown in Figure 1.

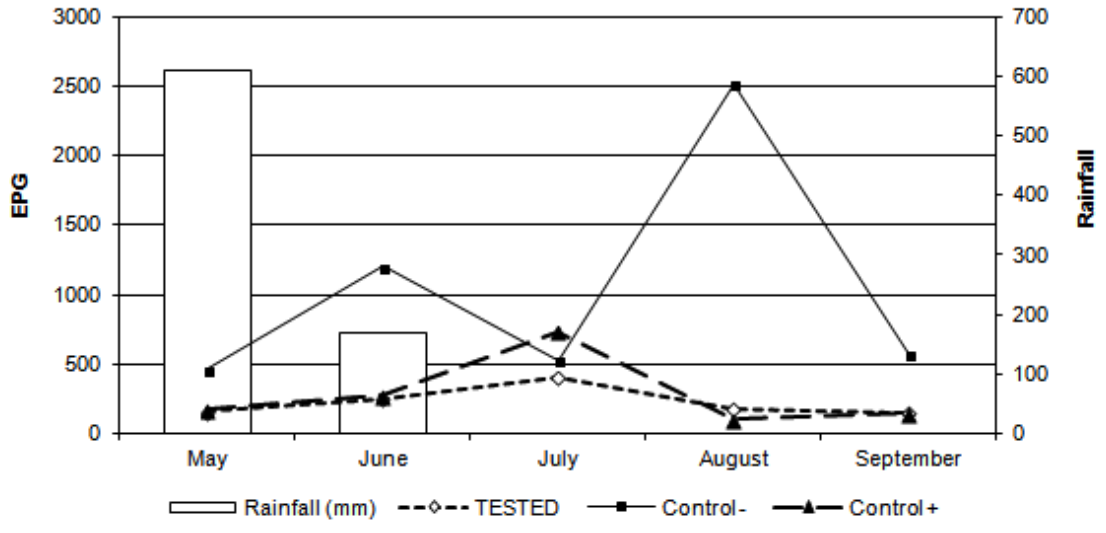

Fig. 1. Monthly averages of the countings of eggs per gram of feces (EPG) of the animals in the groups treated with the nematophagous fungi D. *flagrans* and *A. robusta* association (1 g of fungus/10 kg of b. w.) (Group 1), the – control group (without treatment) and the + control group (*D. flagrans* only), collected from May 2012 to September 2012, Ouro Branco, Minas Gerais, Brazil.

Table 1 shows the analysis of genera larvae recovered from coprocultures. There was no difference between the groups treated with association of fungi (*D. flagrans* and *A. robusta*) and only *D. flagrans*. In the end of five months of study, the percentage of nematodes found in tested group, control- and control+ were, respectively: *Cooperia* sp. 48%, 45% and 44%, *Haemonchus* sp. 44%, 46% and 46% and *Oesophagostomum* sp. 8%, 9% and 10%. Other species found in smaller quantities in all groups were *Bunostomum* sp. and *Strongyloides* sp. whose percentages did not reach 1%.

Tab. 1. Percentage values corresponding to the infectant larvae recovered from the coprocultures of the groups treated with the nematophagous fungi *D. flagrans* and *A. robusta* association (Group 1) and the + control group (*D. flagrans* only) in relation with the - control group (without treatment) collected from May 2012 to September 2012, Ouro Branco, Minas Gerais, Brazil.

	Group 1			Control -			Control +		
	Coop	Haem	Oeso	Coop	Haem	Oeso	Coop	Haem	Oeso
April	54	41	5	44	50	6	10	49	16
May	49	45	6	48	41	11	48	42	10
June	59	33	8	54	37	9	77	21	2
July	56	32	12	52	40	8	48	41	12
August	63	30	6	59	30	9	52	38	10
September	15	76	9	13	80	7	7	86	8
Average	49	43	8	45	46	9	44	46	10
S.D.	18	17	3	16	18	2	27	22	5

Table 2 shows the absolute values of L3 per kg of dry matter obtained from pastures grazed by the three groups of heifers. In pasture, larvae were found in the same distribution pattern of the stool. The genus *Cooperia* was the most prevalent, followed by *Haemonchus* and *Oesophagostomum*. The percentage of reduction of L3 in relation to the group control - in the distances of up to 20 and 20-40 cm from the fecal pat was 43% and 55% for group tested with two fungi. The group control + showed percentage reductions of 48% and 35%, in the same distances. The largest reduction was found in the last month of the study (September) with 88 and 64% for group tested and control +, respectively. There was significant difference between the group treated with association and the group treated with *D. flagrans* only.

Tab. 2. Values of L3 per kg of dry matter obtained from pastures (0-20 cm and 20-40cm, respectively) grazed by the groups treated with the nematophagous fungi *D. flagrans* and *A. robusta* association (Group Tested), the - control group (without treatment) and the + control group (*D. flagrans* only), collected from May 2012 to September 2012, Ouro Branco, Minas Gerais, Brazil.

0-20 cm	Group Tested			Control -			Control +		
	Coop	Haem	Oeso	Coop	Haem	Oeso	Coop	Haem	Oeso
May	51	103	13	260	38	28	128	35	10
June	67	60	15	153	25	28	80	22	38
July	77	26	26	41	14	14	39	20	10
August	139	56	28	320	67	50	143	29	29
September	50	38	0	17	67	0	87	217	0
Average	77	56	16	158	42	24	95	65	17
S.D.	37	29	11	133	24	19	41	86	16

20-40 cm	Group Tested			Control -			Control +		
	Coop	Haem	Oeso	Coop	Haem	Oeso	Coop	Haem	Oeso
May	136	82	10	173	92	22	228	59	34
June	128	63	9	159	65	19	100	25	18
July	15	10	5	0	0	0	49	20	20
August	81	27	27	98	33	16	286	114	57
September	0	0	0	150	300	50	43	130	0
Average	72	36	10	83	111	22	126	88	26
S.D.	63	35	10	76	165	25	138	60	29

Fig. 2 shows the mean weight gain of animals of the three groups. The weight gain of the animals of the treated groups differed from those of the control group in the end of experiment ($p<0.05$). There was no significant difference for animal weight during the first 2 months of the experiment (May, June) between the three groups. However, in the last two months of the experiment (August and September) significant differences of 22 and 20% were found for the weight between group tested and non-treated animals. The positive control group got differences of 7.41 and 10.1% in the same period compared to the untreated group.

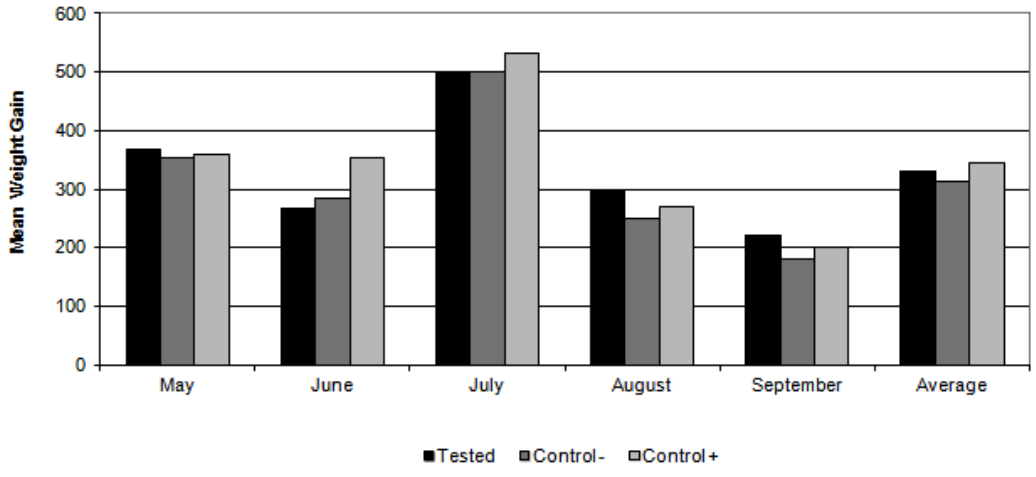

Fig 2. Mean weight gains (kg/day) of the groups treated with the nematophagous fungi *D. flagrans* and *A. robusta* association (Tested), the – control group (without treatment) and the + control group (*D. flagrans* only), collected from May 2012 to September 2012, Ouro Branco, Minas Gerais, Brazil.

4. Discussion

The results showed in this work are the first report of *in vivo* association of *D. flagrans* and *A. robusta* in a pellet formulation through the gastrointestinal tract of cattle with accompanying reduction of parasite load during five months of application of the formulation. A number of studies on only *D. flagrans* using bovines recorded average monthly EPG counts lower for treated animals than for non-treated groups (Assis et al, 2012; Assis et al., 2013). These findings are in agreement with results obtained in the present work, confirming that the fungus acts on the infective forms in the fecal environment, with consequently decrease in EPG. The fungus *D. flagrans* is most widely used in biologic control because it produces chlamydospores, structures highly resistant to the passage intestinal tract of animals (Mota et al., 2003). Araujo et al. (1998) tested the only isolated *A. robusta* (I31) in the biological control of bovine gastrointestinal nematodes parasites with two million of conidias and obtained reduction of 54% in the EPG. However, new data found in this study, was that the EPG counts of animals treated with *D. flagrans* and *A. robusta* in association were not significantly lower than those of the animals treated with *D. flagrans* alone. This finding suggests that there was no synergism between these fungi.

Regarding the percentage of infective larvae found in stool, the findings of this study concur with those of Dias et al. (2007) and Assis et al. (2012) which obtained similar results in studies with cattle in Southeastern Brasil. That is already known *Cooperia* sp., *Haemonchus* sp. and *Oesophagostomum* sp. genera of nematodes are most prevalent in southeastern Brazil (Lima et al., 1989). Araújo et al. (1998) tested the isolated *A. robusta* (I31) in the biological control and obtained 70% of reduction on number of worms recovered at necropsy of calves. Castro et al. (2003) tested different isolate of *Arthrobotrys* on cyathostomes and obtained reduction 86% to I-35 and 86.3% to I31. Braga et al. (2009) there was in vitro a significant reduction 71.8% in the means of *A. vasorum* L1 recovered from treatments with isolate I31, respectively, compared to the control without fungi. There are no previous studies which report the association of nematophagous fungus *in vivo* with reduction larvae in pasture. In this study was observed that the association of pellets containing fungi *D. flagrans* and *A. robusta* was

able to pass through the gastrointestinal tract in cattle, and then these fungi were germinated in the faeces and after, were effective in reducing L3 trichostrongyles. However, the association of the isolate of *A. robusta* to *D. flagrans* not enhanced the reduction of larvae on pasture. So we concluded that there was no synergism between the isolates tested. The number of larvae recovered in the distances 0-20 and 20-40 cm from fecal pats is likely to be directly related with the use of nematophagous fungi that act directly on the L3 present in pastures, confirming that nematophagous fungi was responsible for the satisfactory reduction of environmental contamination (Araújo et al., 2004). Assis et al. (2013) related a reduction of L3 recovered by pasture in the distances of up to 20 and 20–40 cm from the fecal pat for isolated AC001 corroborate this study. The difference found in weight gain of treated animals compared to the control group may have been caused by a lower parasite load in animals that received pellets containing fungus mycelia, which may have contributed to a better food conversion of treated animals. These results are similar to those found by Dias et al. (2007) and Assis et al. (2013) on weight gain of cattle treated with pellets containing *D. flagrans* mycelia. The results showed in this work are promising since it represents the first report of the passage of different fungal species associated in a formulation of pellets containing *D. flagrans* and *M. thaumasium* at the same time through the gastrointestinal tract of cattle monitoring the reduction of larvae on pasture.

Conclusion

The treatment of dairy cattle with sodium alginate pellets containing the mycelial mass of nematophagous fungi *D. flagrans* alone or in association with *A. robusta*, twice a week for five months, decreased the EPG of the animals in more of 70%. However, the association of the fungi *D. flagrans* (AC001) and *A. robusta* (I31) showed no synergism, because there was no significant difference when compared with the group receiving the isolated *D. flagrans* alone.

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CAPITULO 3

F. D. LUNS [a] *; R. C. L. ASSIS [a]; J. V. ARAÚJO [a]; F. R. BRAGA [a]

Association of the fungi *Arthrobotrys robusta, Duddingtonia flagrans* and *Monacrosporium thaumasium* on biologic control of dairy cattle nematodiasis

[a] Federal University of Viçosa, Department of Veterinary Medicine - Laboratory of Parasitology, Av. P.H. Rolphs - Viçosa Campus. Zip code 36570-000. Viçosa - Minas Gerais - Brazil.

* Corresponding author: P.H. Rolphs Avenue, Viçosa Campus, Department of Veterinary Medicine - Laboratory of Parasitology, Viçosa, Minas Gerais, Brazil. Zip code: 36570-000, telephone: +55 31 3899-11458 Fax: +55 31 3899-1457, e-mail: fabio.luns@ufv.br

Abstract

The viability of the association the fungi *D. flagrans*, *M. thaumasium* and *A. robusta* was tested in biologic control of dairy cattle nematodiasis in Brazil. 24 6-month old female Girolando heifers were separated into three groups of 8 heifers each. The heifers were allocated to three 10ha paddocks of *Brachiaria decumbens*, naturally infested with gastrointestinal parasite helminths. Each animal of group treated with association (group tested) received 1g of pellets (0.2g of fungal mycelium) for each 10kg of body weight (b.w.) containing the fungi *D. flagrans*, *M. thaumasium* and *A. robusta* in association. In group control positive, each animal received 1g of pellets/10kg of b.w. containing the fungus *D. flagrans* only. The heifers of group control negative received 1g of fungus-free pellets, during 5 months. The EPG percentage of reduction in this study was 80% (group tested) and 73% (+ control). The percentage of reduction of L3 in pasture in the distances of up to 20 and 20-40 cm from the fecal pat was 57 and 43% (group tested) and 48 and 35% (control +). There was no significant difference ($p<0.05$) between the group treated with association and the group treated with *D. flagrans* only in the EPG and L3 recovered of pasture. Concluded that the association has not been more effective than the isolate *D. flagrans* only. The results showed in this work are promising since it represents the first report of the passage of three different fungal species associated in a formulation at the same time through the gastrointestinal tract of cattle monitoring the reduction of larvae on pasture.

1. Introduction

Among the nematophagous fungi, those of the genera *Duddingtonia*, *Monacrosporium* and *Arthrobotrys* have been widely studied in the control of gastrointestinal nematode parasites of domestic animals (Araújo et al., 2004, Braga et al., 2009). The genus *Duddingtonia* was evaluated *in vitro* and in pasture, on sheep (Waller et al., 2001; Waghorn et al., 2003), lambs (Githigia et al., 2001) and bulls (Assis et al., 2012). The *Monacrosporium* sp. also has been successfully used to combat nematodes of cattle in Brazil (Alves et al., 2004; Assis et al., 2013). As the genus *Duddingtonia* and *Monacroscoporium*, the *Arthrobotrys* has been proven to be a potential biological control agent of parasitic nematodes in domestic animals (Castro et al., 2003; Braga et al., 2009). Thus, it is observed that there are several studies showing the effectiveness of these fungi to pass through the gastrointestinal tract of animals and kill infective larvae when used alone, but no study has examined the association of the three fungi. This association can provide a synergistic effect, achieving better results than a single isolated applied, however can also the increased competition between the fungi for nematode larvae need to be evaluated in these associations *in vivo*. This study aimed to evaluate the viability of the association the fungi *D. flagrans, M. thaumasium* and *A. robusta* in biologic control of dairy cattle nematodiasis in Brazil.

2. Material and methods

2.1. Fungi and production of mycelial pellets

Three isolates of the predatory fungi *D. flagrans* (AC001), *M. thaumasium* (NF34) and *A. robusta* (I31) were kept in test tubes containing corn meal agar 2% (2% CMA, Difco®), at 4°C in the dark. These isolates came from a Brazilian soil and belonged to the mycology collection of the Federal University of Viçosa, Brazil. To induce the formation of the fungal mycelium, culture discs of 5 mm in diameter in 2% water-agar (2% WA) were transferred to 250 mL Erlenmeyer flasks with 150 mL of liquid GPY medium (glucose, sodium peptone and yeast extract), pH 6.5, under the agitation of 120 rpm, in the dark, 26°C, for 10 days. After this period, the mycelia were harvested with a platinum loop, and weighed in an analytic scale for the future production of the pellets, which were made in a sodium alginate matrix, according to Walker and Connick (1983) and modified by Lackey et al. (1993).

2.2. Animals and experimental site

The experiment was carried out in a private farm located in the municipality of Ouro Branco, state of Minas Gerais, southeast region of Brazil, 43°41'31" South latitude and 20°31'15" West longitude, from April to September 2012. The topography is hilly, with an average elevation height of 1000m and the native vegetation is Atlantic rain forest-cerrado transition zone. The climate is tropical with a dry season (Rating Köppen-Geiger climate: Aw), annual average maximum temperature of 71.60°F and minimum of 44.60°F. In the beginning of the experiment, 24 6-month old female Girolando calves, with average body weight of 130kg were previously treated with 10% albendazole at an oral dose of 7.5mL/10kg of b.w. Fifteen days after the anthelmintic treatment, the animals were separated into three groups of 8 calves each, based on the average weight. The calves were allocated to three 10ha paddocks of *Brachiaria decumbens*, naturally infested with gastrointestinal parasite helminths, due to the previous grazing by young and adult animals. Each group was allocated to only one paddock without rotational grazing between the groups during the experimental period. Each animal of group treated received 1g of pellets (0.2g of fungal mycelium) for each 10kg of b.w. containing the fungi *D. flagrans* (AC001), *M. thaumasium* (NF34) and *A. robusta* (I31) associated and in a single oral dose. In group control positive, each animal received 1g of pellets (0.2g of fungal mycelium) for each 10kg of b.w. containing the fungus *D. flagrans* (AC001). The animals of group control negative received 1g of fungus-free pellets for each 10kg of b.w. All the animals received the pellets orally, twice a week, mixed in concentrated and balanced ration provided for dairy cattle (18% of total protein – Total®, Brazil), and water *ad libitum* during 6 months, starting from April 2012. Once time for week, stool samples were collected directly from the rectum to be performed the egg account per gram of faeces (EPG) according Gordon and Whitlock (1939). Simultaneously to the EPG exam, coprocultures were carried out, for each animal, according to the methodology described by Roberts et al. (1952). The identification of the infectant larvae in the coprocultures was performed according to Keith (1953).

Every 15 days, herbage samples were collected in each paddock of groups, in a zigzag pattern from alternated points, 20cm and 20–40cm far from the fecal pats, according to Amarante et al. (1996). Then, a 500g herbage sample was weighed, and parasitic nematode larvae were recovered following the procedure of Lima (1989). The samples were incubated in a drying oven at 100°C for 3 days to determine the dry matter content. Data were transformed into larvae per kg of dry matter.

The data were submitted to analysis of variance (ANOVA). Subsequently, the means were compared using the Tukey test at the 5% level of probability.

3. Results

In the first month of the experiment (April, 2012) no statistical difference was observed ($p<0.05$) between the groups treated with fungi and the control group. The EPG was higher in the negative control group than in the treated animals of both groups during the months of May, June, August and September of 2012 ($p<0.05$). In July the tested group (three fungi) presented EPG lower than control groups, but no significant difference. The percentage of reduction in this study was 80% (group tested) and 73% (+ control). In the last month of the study was a reduction of 70% and 74%. The largest absolute difference of EPG (1917 and 2417 eggs) was found in August 2012. The largest percentage difference occurred in June (89%) for tested group and August 2012, (96%) for the group treated with only *D. flagrans* (Control +). There was no significant difference between the group treated with pellets made with the three fungal isolates (G1) and the group treated with pellets of *D. flagrans* only ($p<0.05$). The monthly averages of the EPG are shown in Figure 1.

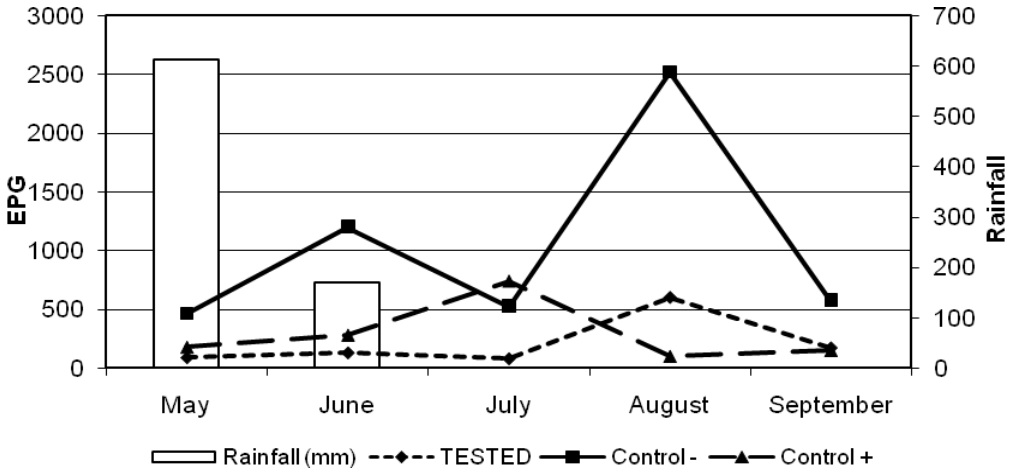

Fig. 1. Monthly averages of the countings of eggs per gram of feces (EPG) of the animals in the groups treated with the nematophagous fungi D. *flagrans, M. thaumasium* and *A. robusta* association (1 g of fungus/10 kg of b.w.) (Tested), the – control group (without treatment) and the + control group (*D. flagrans* only), collected from May 2012mto September 2012, Ouro Branco, Minas Gerais, Brazil.

Table 1 shows the analysis of genera larvae recovered from coprocultures. There was no difference between the groups treated with association of fungi (*D. flagrans, M. thaumasium* and *A. robusta*) and only *D. flagrans*. In the end of five months of study, the percentage of nematodes found in tested group, control- and control+ were, respectively: *Cooperia* sp. 48%, 45% and 44%, *Haemonchus* sp. 44%, 46% and 46%

and *Oesophagostomum* sp. 8%, 9% and 10%. Other species found in smaller quantities in all groups were *Bunostomum* sp. and *Strongyloides* sp. whose percentages did not reach 1%.

Tab. 1. Percentage values corresponding to the infectant larvae recovered from the coprocultures of the groups treated with the nematophagous fungi *D. flagran*, *M thaumasiums* and *A. robusta* association (Tested Group) and the + control group (*D. flagrans* only) in relation with the - control group (without treatment) collected from May 2012 to September 2012, Ouro Branco, Minas Gerais, Brazil.

Data	Tested Group			Control -			Control +		
	Cooperia	Haem.	Oesoph.	Cooperia	Haem.	Oesoph.	Cooperia	Haem.	Oesoph.
April	35	57	8	44	50	6	10	49	16
May	43	46	11	48	41	11	48	42	10
June	56	30	3	54	37	9	77	21	2
July	61	32	6	52	40	8	48	41	12
August	82	16	3	59	30	9	52	38	10
September	12	84	4	13	80	7	7	86	8
Average	48	44	6	45	46	8	40	46	10
S.D.	24	24	3	16	18	2	27	22	5

Table 2 shows the absolute values of L3 per kg of dry matter obtained from pastures grazed by the three groups of heifers. In pasture, larvae were found in the same distribution pattern of the stool. The genus *Cooperia* was the most prevalent, followed by *Haemonchus* and *Oesophagostomum*. The percentage of reduction of L3 in relation to the group control - in the distances of up to 20 and 20-40 cm from the fecal pat was 57% and 43% for Tested Group. The group control + showed percentage reductions of 48% and 35% in the same distances. The largest reduction was found in the last month of the study (September) with 63 and 64% for Tested Group and control +, respectively.

Tab. 2. Values of L3 per kg of dry matter obtained from pastures (0-20 cm and 20-40cm, respectively) grazed by the groups treated with the nematophagous fungi *D. flagrans* and *A. robusta* association (Tested Group), the - control group (without treatment) and the + control group (*D. flagrans* only), collected from May 2012 to September 2012, Ouro Branco, Minas Gerais, Brazil.

0-20 cm	Tested Group				Control -				Control +			
	Coop	Haem	Oeso	Total	Coop	Haem	Oeso	Total	Coop	Haem	Oeso	Total
May	42	33	8	83	260	38	28	326	128	35	10	173

June	74	28	17	119	153	25	28	206	80	22	38	140
July	38	19	7	64	50	33	8	91	40	21	10	71
August	147	28	15	190	320	67	50	437	143	29	29	200
September	80	25	5	110	170	67	10	247	76	21	0	97
Average	76	27	10	113	191	46	25	261	93	26	17	136
S.D.	44	5	5	48	104	20	17	130	42	6	16	53

20-40 cm	**Tested Group**				**Control -**				**Control +**			
	Coop	**Haem**	**Oeso**	**Total**	**Coop**	**Haem**	**Oeso**	**Total**	**Coop**	**Haem**	**Oeso**	**Total**
May	230	28	18	276	173	92	22	287	228	59	34	321
June	51	30	5	86	159	65	19	243	100	25	18	143
July	39	35	11	85	48	60	12	120	49	20	20	88
August	79	29	21	129	98	53	16	168	86	29	17	132
September	54	98	17	169	150	300	50	500	43	130	0	174
Average	44	81	14	149	83	111	22	264	126	88	26	172
S.D.	47	76	13	79	76	165	25	147	138	60	29	89

Fig. 2 shows the mean weight gain of animals of the three groups. The weight gain of the animals of the treated groups differed from those of the control group in the end of experiment ($p<0.05$). There was no significant difference for animal weight during the first 2 months of the experiment (May, June) between the three groups. However, in the last two months of the experiment (August and September) significant differences of 12 and 14% were found for the weight between Tested Group and non-treated animals. The positive control group got differences of 7.41 and 10.1% in the same period compared to the untreated group.

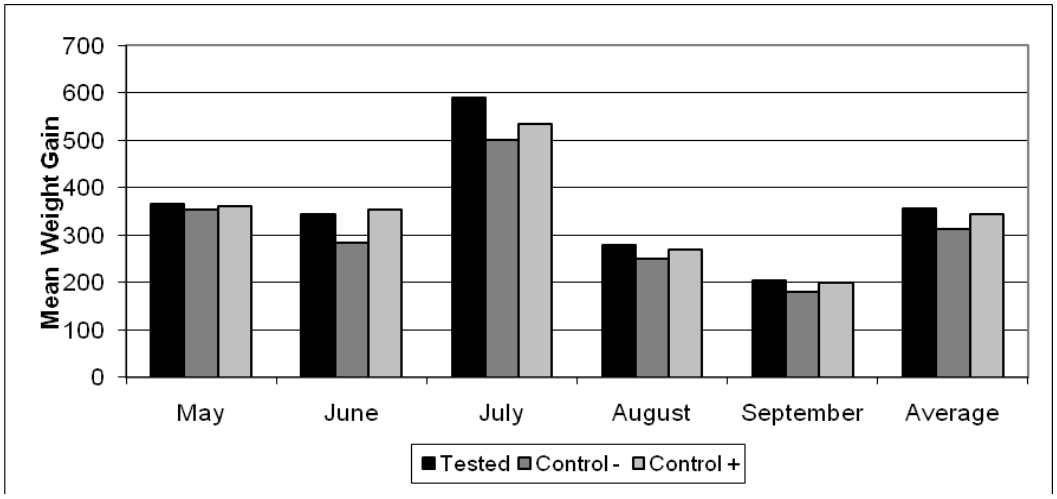

Fig 2. Mean weight gains (kg/day) of the groups treated with the nematophagous fungi *D. flagrans,M* thaumasium and *A. robusta* association (Tested), the – control group (without treatment) and the + control group (*D. flagrans* only), collected from May 2012 to September 2012, Ouro Branco, Minas Gerais, Brazil.

4. Discussion

The results showed in this work are the first report of *in vivo* association of *D. flagrans M. thaumasium* and *A. robusta* in a pellet formulation through the gastrointestinal tract of dairy cattle with accompanying reduction of parasite load during five months of application of the formulation. A number of studies on *D. flagrans* using horses and ruminants recorded average monthly EPG counts lower for treated animals than for non-treated groups (Baudena et al., 2000; Knox and Faedo, 2001; Dimander et al., 2003; Fontenot et al., 2003; Araújo et al., 2004; Paraud et al., 2007). These findings are in agreement with results obtained in the present work, confirming that the fungus acts on the infective forms in the fecal environment, with consequently decrease in EPG. Araújo et al. (1998) tested the isolate *A. robusta* (I31) in the biological control of bovine gastrointestinal nematodes parasites with two million of conidias and result in reduction of 54% in the EPG. However, the new found in this study, was that the EPG counts of animals treated with *D. flagrans, M. thaumasium* and *A. robusta* in association were not significantly lower than those of the animals treated with *D. flagrans* alone. This finding suggests that there was no synergism between these fungi.

In relation to the percentage of infective larvae found in stool, the findings of this study agree with those of Dias et al. (2007) and Assis et al. (2012) which obtained similar results in studies with cattle in Southeastern Brasil. That is already known *Cooperia* sp.,

Haemonchus sp. and *Oesophagostomum* sp. genera of nematodes are most prevalent in southeastern Brazil (Lima, 1989).

About the reduction of larvae by fungus *A. robusta*, used alone, there are several reports of high efficiency literature. In a in vitro trial Mendoza-de-Guives (1992) observed the nematode-destroying fungus *Arthrobotrys robusta* preyed 92.33% of L3 *Haemonchus contortus* after seven days of incubation. Araújo et al. (1998) tested the *A. robusta* (I31) in the biological control and obtained 70% of reduction on number of worms recovered at necropsy of calves. Castro et al. (2003) tested on various isolates of *Arthrobotrys* on cyathostomes and obtained 86% reduction for the I-35, 86.3% for I31 and 86.7% for *A. musiformis*. Braga et al. (2009) comparing the ability of predatory nematophagous fungi *D. flagrans* (AC001), *M. thaumasium* (NF34) and *A. robusta* (I-31) on infective larvae of *Strongyloides stercoralis* in laboratory conditions found a rate reduction of 83.7% (AC001), 75.5% (NF34) and 73.2% (I-31). However, there were no previous studies which report the association of isolates of the genus nematophagous fungus as was observed in this study is that the association of pellets containing fungi *D. flagrans M. thaumasium* and *A. robusta* was able to pass through the gastrointestinal tract in dairy cattle, then these fungi were germinated in the faeces and after, were effective in reducing L3 trichostrongyles but was not more effective in reducing the larvae isolated than *D. flagrans* used alone. So we concluded that there was no synergism between the isolates tested.

The number of larvae recovered in the distances 0-20 and 20-40 cm from fecal pats is likely to be directly related with the use of nematophagous fungi that act directly on the L3 present in pastures, confirming that nemathofagous fungi was responsible for the satisfactory reduction of environmental contamination (Araújo et al., 2004). Assis et al. (2013) related a reduction of L3 recovered by pasture in the distances of up to 20 and 20–40 cm from the fecal pat was 64.5% and 73%, respectively for *D. flagrans*, while *M. thaumasium* showed percentage reductions of 47.3% and 58%, in the same distances. In this study, the percentage reductions of 48% and 35%, in the same distances, with the same isolate of *D. flagrans* (AC001).

The difference ($p<0.01$) found in weight gain of treated animals compared to the control group may have been caused by a lower parasite load in animals that received pellets containing fungus mycelia, which may have contributed to a better food conversion of treated animals. These results are similar to those found by Dias et al. (2007) on weight gain of cattle treated with pellets containing *D. flagrans* mycelia and Braga et al (2009)

on weight gain of horses treated with also fungus. Assis et al. (2013) also had better weight gain in bulls treated with the same isolates of *D. flagrans* and *M. thaumasium* but administered single to animals.

The results showed in this work are promising since it represents the first report of the passage of different fungal species associated in a formulation of pellets containing *A. robusta*, *D. flagrans* and *M. thaumasium* at the same time through the gastrointestinal tract of dairy cattle monitoring the reduction of larvae on pasture.

Conclusion

The treatment of dairy cattle with sodium alginate pellets containing the mycelial mass of nematophagous fungi *D. flagrans* alone or in association with *M. thamasium* and *A. robusta* twice a week for five months decreased the EPG of the animals in more of 70%. However, the association of the different isolates fungi *D. flagrans*, *M. thaumasium* and *A. robusta* showed no synergism in association, because there was no difference in EPG and L3 recovered on pasture when compared with the group receiving the isolated *D. flagrans* alone.

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