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Short note

A pilot study of the *in vitro* efficacy of different concentrations of *Duddingtonia flagrans* for the control of gastrointestinal nematodes of sheep

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ABSTRACT. *Duddingtonia flagrans* is a nematode trapping fungus used for the control of gastrointestinal nematodes in livestock. The quantity of chlamydospores of *D. flagrans* required for the reduction of third-stage larvae (L3) of sheep gastrointestinal nematodes (GIN) is largely unknown, and a matter of discussion. The aim of this experiment was to determine *in vitro* the nematophagous activity of four different concentrations of *D. flagrans* (1000, 3000, 6250, or 11000 chlamydospores/ml) in the presence of varying numbers of GIN third-stage larvae (L3) (500, 1000, 1500). Additionally, the study sought to evaluate the efficacy of this fungus on *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus colubriformis* and *Chabertia ovina*. The results showed that as fungal concentrations increased, so did the larval reduction of third-stage infective larvae in each test. L3s number was not a determining factor in the efficacy against GIN. The comparison between various concentrations of chlamydospores revealed significant differences, particularly between 1000 and 11000 chlamydospores ($P \leq 0.05$). Regarding the larval reduction of the GIN species considered, *D. flagrans* demonstrated the same effectiveness across all species tested. The results of the current study confirm the efficacy and underscore the importance of *D. flagrans* as an alternative for controlling of GIN.

Keywords: gastrointestinal nematodes, sheep, *Duddingtonia flagrans*, chlamydospores, biological control

Introduction

Sheep gastrointestinal nematodes (GIN) are recognized as being responsible for lowered productivity and significant economic losses [1,2]. The use of anthelmintic drugs has been a primary method of controlling parasites in the past [3]. However, the development and the worldwide extent of anthelmintic resistance (AR) to various drug classes, along with the emergence of multi-drug resistant isolates, poses a threat to the sustainability of their use [4–6]. Alternative or complementary methods, such as biological control, may provide opportunities to effectively maintain control. In this way, it is possible to reduce chemical usage, management costs, toxicity, and decrease chemical residues in meat, milk, and the environment [7]. Among all-natural antagonists studied so far, the nematode-trapping fungi have shown interesting results both in the field, and under laboratory conditions [8–11]. The overall effect of the fungus is to prevent the massive transmission of free-living, infective larvae from animal faeces to the surrounding herbage, thus decreasing pasture infectivity and the resulting intake of parasitic larvae by grazing livestock. Literature shows that, among all nematophagous fungi isolates, the *Duddingtonia flagrans* species is the most efficient in controlling ruminant nematodes because of its ability to survive the passage through the animal digestive tract and trap larvae in faeces [12–14]. Recent *in vitro* and *in vivo* experimental studies have shown that *D. flagrans* could reduce GIN by up to 100% [15]. Nevertheless, the quantity of

chlamydospores of *D. flagrans* required for the reduction of GIN third-stage larvae (L3) is largely unknown and is still a matter of discussion [16].

The aim of this experiment was, therefore, to determine, *in vitro,* the nematophagous activity of various *D. flagrans* concentrations in the presence of different numbers of L3, and to evaluate the efficacy of this fungus on different species of Trichostrongylidae.

Materials and Methods

Preparation of culture media and fungal suspension

Duddingtonia flagrans (Mycelia NV, Belgium) was grown for four weeks on enriched sabouraud agar with the addition of chloramphenicol to inhibit bacterial growth (2% CHF-WA) in Petri plates (9-cm-diameter) at 25°C [17]. Chlamydospores of *D. flagrans* were harvested by gently squirting distilled water over the mycelia and carefully scraping it from the agar surface. The mycelial suspension was mechanically blended, after which three 10 µl aliquots were extracted and diluted in distilled water, in order to count the number of chamydospores per millilitre using a haemocytometer (Neubauer chamber).

Preparation of infective larvae

Third-stage infective larvae (L3) of mixed gastrointestinal nematodes were cultured from a pool of fresh faeces of infected sheep (500 eggs per gram of faeces). Faeces were collected from a sheep farm in central Italy. The samples collected directly from the animals' rectum, were pooled and incubated for 10 days at 25°C in a large container to ensure good levels of oxygen and humidity (50–80%). At the end of the coproculture, larvae were recovered using the Baermann method, cleaned with a sucrose solution to remove contaminating detritus, and rinsed three times in sterile distilled water to eliminate the sucrose residues [18]. The L3 were counted and kept at 4°C. An aliquot of larvae was used for a morphological identification to the genus level [19]. Morphological identification was confirmed by molecular analysis [20]. The amplicons obtained by PCR were sequenced (Bio-Fab research, Rome, Italy,) and the sequences were aligned using Data Analysis in Molecular Biology and Evolution version 4.5.55 (DAMBE) and compared to the sequences available in GenBank database using Nucleotide BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Trial design

The growth of *D. flagrans* and its interaction with L3 were examined on 2% CHF-WA in Petri plates (9-cm-diameter). The plates were sown with 1 ml of fungal suspension containing 1000, 3000, 6250, or 11000 chlamydospores, respectively, and cultivated for 7 days at 25°C [16]. On the 7th day, 1 ml of culture suspension containing respectively 500, 1000, or 1500 L3 was poured into the center of each plate. Three parallel trials were performed for each chlamydospores concentration. Each trial included control plate to which 1 ml of distilled water, without fungi, was added. All plates were incubated at 25°C, in the dark, for a period of 7 days. On the 7th day, the agar from all trials was removed from the Petri plates and L3 were collected using the Baermann technique. The recovered larvae were counted and identified as described above [19,20].

Statistical analysis

The results obtained underwent statistical analysis using IBM SPSS 28 software. The non-parametric Kruskal-Wallis test was used to compare the numbers of recovered L3 among different chlamydospores concentrations and between each a fungus-treated group, and its respective control. In case of significance, Pairwise Tests were conducted using the Bonferroni correction for multiple comparison. The fungal efficacy for each fungal concentration was estimated using the formulae: Reduction % = 100-(mean L3 in fungus group × 100/mean L3 in control group) [12].

Results

Morphological and molecular identification

Morphological identification of the larvae obtained from larval culture showed that nematodes belonged to the genera *Haemonchus* (53%), *Teladorsagia* (29%), *Trichostrongylus* (10%), and *Chabertia* (8%). *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus colubriformis*, and *Chabertia ovina* were detected by molecular analysis. The nucleotide sequences obtained by PCR showed 99–100% identity with sequences currently available in GenBank (GenBank accession nos: KX829170.1; JF680984.1; JF680985.1; KC998758.1).

Statistical analysis

The statistical analysis of the results indicated that the larval reductions by *D. flagrans,* increasing

Table 1. The percentage reduction of the total nematode larvae and each species (L3) recovered from plate with the agar set up with different numbers of L3 and various concentrations of *Duddingtonia flagrans.* Different letters in the same column indicate a significative difference in the data comparison ($P \leq 0.05$).

	Duddingtonia flagrans	Third-stage larvae (L3)	*Haemonchus contortus*	*Teledorsagia circumcincta*	*Trichostrongylus colubriformis*	*Chabertia ovina*
	chlamydospores	Reduction %±SD	Reduction %±SD	Reduction %±SD	Reduction %±SD	Reduction %±SD
500 L3	1000	92.90^{a}±0.83	93.75^{a}±2.27	93.38^{a}±1.08	88.57^{a}±5.71	88.24^{abc}±5.88
	3000	98.53±0.30	98.44±0.52	98.58±0.00	98.10±1.65	100.00^{a}±0.00
	6250	99.39±0.15	99.65±0.60	99.05±1.08	99.05±1.65	100.00^{b}±0.00
	11000	100.00^{a}±0.00	100.00^{a}±0.00	100.00^{a}±0.00	100.00^{a}±0.00	100.00^{c}±0.00
1000 L3	1000	97.43^{a}±0.26	97.91^{a}±0.55	97.32^{b}±0.42	94.72±1.86	97.83±2.17
	3000	98.94±0.26	99.03±0.32	99.03±0.21	97.56±2.44	100.00±0.00
	6250	99.85±0.13	99.79±0.21	99.88^{b}±0.21	100.00±0.00	100.00±0.00
	11000	100.00^{a}±0.00	100.00^{a}±0.00	100.00±0.00	100.00±0.00	100.00±0.00
1500 L3	1000	98.81^{ab}±0.11	98.90^{ab}±0.00	98.48±0.73	98.98±0.44	99.33±1.17
	3000	99.35±0.07	99.45±0.14	98.96±0.37	99.49±0.88	100.00±0.00
	6250	100.00^{b}±0.00	100.00^{b}±0.00	100.00±0.00	100.00±0.00	100.00±0.00
	11000	100.00^{a}±0.00	100.00^{a}±0.00	100.00±0.00	100.00±0.00	100.00±0.00

order of fungal concentration, were 93%, 98.5%, 99% and 100% compared to the 500 L3-control group; 97%, 99%, 100% and 100% compared to the 1000 L3-control group; 99%, 99%, 100% and 100% compared to 1500 L3-control group. Comparison between different chlamydospore concentrations revealed significant differences between 1000 and 11000 chlamydospores for the culture with 500 L3 (P=0.012), 1000 L3 (P=0.019), and between concentrations of 1000 and 11000 chalmydospores (P=0.039), as well as between 1000 and 6250 chlamydospores for the culture with 1500 L3 (P=0.039). Additionally, significant differences in *H. contortus* larval reductions were observed between concentrations of 1000 and 11000 chlamydospores for the culture with 500 L3 (P=0.029), 1000 L3 (P=0.020 and 1500 L3 (P=0.037), as well as between 1000 and 6250 chlamydospores for the culture with 1500 L3 (P=0.037). There was a significant variation in *T. circumcincta* and *T. colubriformis* larval reduction between 1000 and 11000 chlamydospores in the culture with 500 L3 (P=0.018, P=0.037, respectively), while *T. circumcincta* showed a significant variation also between 1000 and 6250 chlamydospores for the culture with 1000 L3 (P=0.037). *Chabertia ovina* showed a significant variation in the larval reduction between 1000 and 3000 chlamydospores (P=0.045), 1000 and 6250 chlamydospores (P=0.045) and between 1000 and 11000 chlamydospores (P=0.045), only for the culture with 500 L3. The percentage reduction of the total GIN L3 and each species recovered from plate with agar set up with different numbers of L3 and different concentrations of *D. flagrans* is shown in Table 1.

Discussion

The results of this study have shown that as fungal concentrations increased, so did the larval reduction of third-stage infective larvae. The comparison between various concentrations of

chlamydospores revealed significant differences mainly between 1000 and 11000 chlamydospores in each trial. In relation to the results obtained, the number of L3 in trials does not appear to be a determining factor in the predatory capacity of *D. flagrans* and according to the present study, a minimum concentration of *D. flagrans* (1000 chlamydospores/ml) is able to determine a significant percentage reduction (92.9%) on 500 L3. The reduction effect on L3 obtained in the current research (92.90%–100%) is consistent with finding from previous studies [13,14,21–23]. However, it is difficult to compare the *in vitro* effectiveness results of *D. flagrans*, as the fungal dose and number of L3s used vary among these studies. It is known that predatory activity depends on the isolate's ability to produce traps and the number of traps developed on surface area unit. Most authors claim that *D. flagrans* produces traps only after stimulation by nematodes [24–27]. Nevertheless, the present research does not confirm that the high motility of L3 is a potent stimulus for fungal trap morphogenesis, and the results obtained are mainly in agreement with two studies carried out *in vitro* bovine faecal culture. In such studies, Zegbi et al. [16] and Gronvold et al. [28] showed that an increase in fungal concentrations resulted in increased predatory activity regardless of the number of eggs orlarvae in bovine faeces. On the contrary, Sagües et al. [29] described that the trapping efficacy of *D. flagrans* is positively influenced by higher number of eggs per gram of faeces (epg), suggesting that greater larval density stimulates the development of traps or nets, significantly reducing the number of emerging larvae compared to the control group. Therefore, it could be hypothesized that the predatory capacity of *D. flagrans* may depend on the different characteristics of the media or faeces used for the cultures, on the concentration of chlamydospores as well as on the strain of *D. flagrans*. In fact, it is known that different isolates of *D. flagrans* show varying trapping success [26,30,31]. It has been reported that even equal doses of the same isolates had different efficacy against the same parasite species in separate studies. For example, Danish isolate *D. flagrans* Troll A showed varying efficacy against L3 of *Cooperia oncophora* [28,32]. Different trapping potential also exists, as some isolates need a smaller inoculum size compared to others to achieve good trapping effect [33]. Regarding the larval reduction of the GIN species considered in this trial, *D. flagrans* showed the same effectiveness on all of them, although the L3 reduction could be influenced by the predominance of the nematode species present in the initial population used for the trials [22,34–36]. In the current study, the reduction percentage of *H. contortus* (93.75%–100%) and *T. circumcincta* (93.38%–100%) in fungi groups compared with control groups is similar, the difference has always been significant between 1000 and 11000 chlamydospores. Other reports showed similar results against the same GIN species [11,37,38]. In the present research, the reduction of *C. ovina* was off 100% already at a concentration of 3000 chlamydospores of *D. flagrans.* Only in a *vivo* study by Liu *et al*. [39] the effectiveness of *D. flagrans,* compared to *C. ovina* (71.4%), was reported.

The results of the current study confirm the efficacy of *D. flagrans* and highlight its importance as an alternative control method for GIN. This is especially crucial considering the increasing resistance of GIN to synthetic anthelmintics, which can lead to significant economic losses and impact food security [7,40]. However, further studies should be conducted to gather more information on the effectiveness of *D. flagrans*. Since environmental conditions seem to play a significant role in determining the level of induced predation, it would also be interesting to evaluate the efficacy of *D. flagrans* both on its own and in combination with other nematophagous fungi under natural grazing conditions.

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