Article

Isolated and Associated Use of the Nematophagous Fungi *Pochonia chlamydosporia* and *Duddingtonia flagrans* to Control *Taenia saginata* Eggs

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Abstract: The aim of this study was to evaluate the isolated and associated use of the nematophagous fungi *Pochonia chlamydosporia* (isolate VC4) as an ovicide and *Duddingtonia flagrans* (isolate AC001) as a predator in the control of *Taenia saginata* eggs. Viable *T. saginata* eggs were obtained by dissecting mature proglottids from a specimen. For the experimental assay, four groups were formed in microtubes, as follows: (G1—*T. saginata* eggs/control); (G2—100 *T. saginata* eggs + VC4); (G3—100 *T. saginata* eggs + AC001); (G4—100 *T. saginata* eggs + VC4 + AC001). All the groups were stored in a B.O.D. incubation chamber at a temperature of 27 °C for 15 days and then the contents of the microtubes were analyzed using an optical microscope with a 40x objective. At the end of the assay the treated groups (G2 to G4) showed ovicidal activity (destruction of eggs) compared to the control group (G1). The highest ovicidal percentage was observed in group G2 (eggs + VC4), with 43.3%. In groups G3 and G4 (combination of fungal isolates), the ovicidal percentages were 25.7% and 25.6%, respectively. The results of this study shed light on a new possibility for the combined use of different species of nematophagous fungi, which could be used in the future for environmental biological control of *T. saginata* eggs.

Keywords: helminths; isolate AC001; isolate VC4; biological control; biotechnology; one health

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1. Introduction

Taenia saginata, commonly known as the beef tapeworm, follows a complex life cycle involving both human and bovine hosts. The adult tapeworm resides in the human intestine, where it attaches to the intestinal wall and releases gravid proglottids filled with eggs [1]. These proglottids are expelled in human feces, contaminating the environment. Cattle become infected by ingesting the eggs or proglottids while grazing. Inside the bovine host, the eggs hatch into larvae, penetrate the intestinal wall, and migrate to muscle tissues, developing into cysticerci, a condition known as bovine cysticercosis. When humans consume undercooked or raw beef containing cysticerci, the larvae are released in the human intestine, where they mature into adult tapeworms, thus completing the cycle. Human cysticercosis, although rare with *T. saginata*, can occur if humans accidentally ingest eggs, leading to larvae migrating to various tissues and forming cysts [2].

Taenia saginata is widely distributed; however, the epidemiological data available are insufficient to estimate its sub-national spatial distribution, the prevalence, incidence, and intensity of infections [3–6]. *Taenia saginata* eggs can survive at temperatures between −10 °C to 17 °C and with high longevity in the environment, enabling a high risk of transmission [7,8]. In the past, Penfold et al. [9] already mentioned that eradicating *T. saginata* from a country would be a costly and difficult task. In fact, since then, *T. saginata* infection is still a serious problem, causing health and economic losses due to the condemnation, refrigeration, and declassification of carcasses infected by the larval stages (*Cysticercus bovis*), given the marketing of animal products for both consumption and export [5–7,10–13].

On the other hand, as in other parts of the world, in Brazil human teniasis is not a notifiable disease and therefore the incidence of this infection is generally estimated from the sale of tenicidal drugs [14]. However, bovine cysticercosis is still controlled through anatomopathological diagnosis during the post-mortem inspection of animals in slaughterhouses [11]. Given this scenario, it is clear that other alternatives and different strategies are still needed for effective parasite control of *T. saginata* and, specifically here, "old" experiences in biological control of eggs with nematophagous fungi will be discussed, but presenting a new approach.

Nematophagous fungi are a diverse group of fungi that prey on nematodes, playing a crucial role in natural soil ecosystems as biological control agents. These fungi employ various mechanisms to capture and kill parasites, including producing specialized structures like adhesive networks, constricting rings, and sticky spores that trap their prey [15,16]. Once a nematode is captured, the fungi penetrate its cuticle, secrete enzymes to break down its tissues, and absorb the nutrients. This parasitic relationship helps regulate parasite populations, making nematophagous fungi valuable in managing parasite-related problems in agriculture and livestock, reducing the reliance on chemical parasiticides. Their ability to control pathogenic parasites highlights their potential as eco-friendly alternatives in integrated pest management strategies [17].

Nematophagous fungi are classified into five groups: (a) predators, (b) ovicides, (c) endoparasites, (d) toxin producers, and (e) producers of special attack devices [18,19]. In the past, the in vitro activities of nematophagous fungi on *T. saginata* eggs were experimentally evaluated [20–22]. In those experiments, isolates of two species of ovicidal fungi, *Pochonia chlamydosporia* and *Paecilomyces lilacinus*, were used. The results were promising and determined the ovicidal destruction parameters, as well as establishing the best time period for ovicidal activity (egg destruction) which were used in various other experimental designs.

From the 2000s to the present day, research with nematophagous fungi has evolved and has always maintained its innovative approach, especially with new records and proof of their enzymatic production, the production of nanoparticles, and the diversity of their attack mechanism against the target organism [23–26]. Precisely on this last point, the literature has already presented new data, mainly on the species *P. chlamydosporia* (isolate VC4) and *Duddingtonia flagrans* (isolate AC001) [27–31]. On the other hand, the combined use of nematophagous fungi of different species (ovicides + predators) could be an innovative strategy for parasite control of potentially zoonotic helminths. However, in vitro experiments are still needed [18,22,25,29,32–35].

Thus, the aim of this study was to evaluate the isolated and associated use of the nematophagous fungi *P. chlamydosporia* (isolate VC4), an ovicidal species, and *D. flagrans* (isolate AC001), a predatory species, in the control of *T. saginata* eggs.

2. Results

At the end of the experimental assay (15 days) there was a significant reduction in *T. saginata* eggs ($p \leq 0.05$) in the treated groups (G2 to G4) compared to the control group (G1) (Table 1). The highest percentage of ovicidal reduction was observed in group G2 (eggs + *P. chlamydosporia*-VC4), clearly demonstrating its ovicidal activity. It was also observed that groups G3 (eggs + *D. flagrans*-AC001) and G4 (eggs + *P. chlamydosporia* VC4 + *D. flagrans*

AC001) also showed ovicidal action, with reductions of 25.7% and 25.6%, respectively. There was no statistical difference ($p > 0.05$) between the ovicidal activity of the treated groups (G2 to G4), only in relation to the control group.

Table 1. Averages of viable eggs and percentages of ovicidal reduction in the experimental groups, control group (G1); *T. saginata* eggs + *P. chlamydosporia* (G2); *T. saginata* eggs + *D. flagrans* (G3); *T. saginata* eggs + *P. chlamydosporia* + *D. flagrans* (G4), at the end of 15 days of the experimental assay.

Experimental Groups	Average Number of Viable Eggs	% Reduction
Control group (G1)	101.6a ± 54	-
Eggs + *P. chlamydosporia* (G2)	57.6b ± 36.0	43.3
Eggs + *D. flagrans* (G3)	75.4b ± 17.3	25.7
Eggs + *P. chlamydosporia* + *D. flagrans* (G4)	75.6b ± 30.5	25.6

Averages followed by the same lowercase letters in the columns show no difference ($p > 0.05$)—Tukey's test at 5% significance.

3. Discussion

The ovicidal activity of *P. chlamydosporia* VC4 observed in this study aligns with the findings of Araújo et al. [21], who evaluated its in vitro action on *T. saginata* eggs. These authors reported that after 15 days *P. chlamydosporia* VC4 showed ovicidal activity against *T. saginata* eggs compared to the control group, mainly through internal colonization of the eggs, with 8% destruction. This research aimed to acquire new knowledge to aid in the environmental decontamination of potentially zoonotic helminth eggs. In this context, the initial reports by Araújo et al. [22] demonstrated that the fungus *Paecilomyces lilacinus* (PL1) destroyed *T. saginata* eggs after 15 days of in vitro study. In the present work, the action of the fungal isolates, tested both individually and in combination, resulted in a reduction of egg viability: G2 (43.3%), G3 (25.7%), and G4 (25.6%), with group G2 (VC4-*P. chlamydosporia*) showing the highest ovicidal activity. In that study, the activity of *D. flagrans* AC001 was tested and in the end only a lytic effect was proven, with no morphological damage to the eggshell. In the present study, however, the results showed that *D. flagrans* AC001 was able to reduce eggs by 25.7%, which indicates new information on the approach and action of this fungus. On the other hand, the authors emphasize that the activity of *D. flagrans* is proven to be predatory, but as previously described, the elucidation of the production of hydrolytic enzymes must be a path to be explored in new designs.

In past records, Braga et al. [20] demonstrated that *D. flagras* AC001 was able to colonize the eggshell of the potentially zoonotic trematode *Fasciola hepatica*. In another record, these authors, in the same period, proposed another experimental assay with *P. chlamydosporia* and *D. flagrans* on *Schistosoma mansoni* trematode eggs and once again, on that occasion, only the adhesion of *D. flagrans* AC001 conidia on the eggshell was recorded [36]. Thus, from these first records, much has been elucidated about the "attack" characteristics of *D. flagrans* and, currently, research has converged towards a better understanding of its complex physical and chemical enzymatic mechanism (production of hydrolytic enzymes), which is produced on its target organism [37–40]. Over the years, the "possible" ovicidal activity of *D. flagrans* AC001 has continued to be tested and with promising results, however, always seeking to elucidate its enzymatic production on eggshell components and future association with other fungal isolates [41].

Specifically, in this study, the growth of *D. flagrans* AC001 on solid PDA medium suggests a hypothesis of the production, albeit discreet, of hydrolytic enzymes (such as proteases and chitinases), which may possibly have been extracted along with the conidia/chlamydospores from the Petri dishes of the experimental groups and thus contributed to the ovicidal activity. *Duddingtonia flagrans* is known to produce proteases, which play a crucial role in their ability to break down the structural proteins of parasite eggs, helping the fungi penetrate and digest nematode eggs [42]. The growth of AC001 on PDA medium could indicate enzymatic activity, as this medium supports fungal growth and

enzyme expression [40]. This fact is interesting and is in line with previous work by Araujo et al. [21,22] on *T. saginata* eggs, since on that occasion the authors only used 2% water agar. This proves that the evolution of research into nematophagous fungi is dynamic, but that much research is still needed.

Another extremely important point is the constitution of helminth eggs in general. The eggshell of these parasites is an extremely resistant biological structure; impermeable to most substances, with the exception of gases and lipid solvents, and its constituent chitin is probably the structural “wall”. If it were removed, the lipid layer would be easily subjected to mechanical damage, allowing harmful chemicals to enter [43].

In this study, the ovicidal activity of *D. flagrans* AC001 on *T. saginata* eggs was 25.7%, which, given its predatory nature, suggests that it produces enzymes that act directly in the digestion of proteins and chitin that are present in the eggshell of *T. saginata*. Proteases catalyze the hydrolysis of peptide bonds between the amino acid residues of proteins, while chitinases catalyze the hydrolysis of glycosidic bonds between each N-acetylglycosamine unit of the chitin polysaccharide [44]. This fact is interesting, since *T. saginata* eggs, for example, have a double layer made up of lipids and proteins, which can undoubtedly provide “a richer environment” with a greater stimulus for the production of extracellular enzymes [18,22]. In this sense, in a recent study, Calazans et al. [31] demonstrated promising results when using the fungus *D. flagrans* AC001 on strongylid nematode eggs obtained from positive feces, under laboratory conditions. In that study, the ovicidal percentage of strongylids was over 70%. However, attention should be drawn to the constitution of the shell of the different types of eggs (strongyloides), which generally have a single layer and their hatchability in the environment is faster, around 72 h. Perin et al. [45] evaluated the in vitro action of the fungus *D. flagrans* on *Toxocara canis* eggs in combination with chemical disinfectants. After 21 days, the group composed of *T. canis* eggs + *D. flagrans* AC001 showed a 48.2% reduction in eggs. According to Monteiro [46], *T. canis* eggs have a thick layer, just like *T. saginata* eggs, and are very resistant to the environment, maintaining their viability for several months, making it an important aspect to consider in relation to control.

In group G4 (*P. chlamydosporia*-VC4 + *D. flagrans*-AC001), there was an average of 75.6% viable eggs with 25.6% egg reduction (destruction), compared to group G2 with 43.3% reduction and G3 with 25.7% egg destruction. These results are important from the point of view of the “attack” mechanism of the fungal species evaluated. Even though *P. chlamydosporia* is an ovicidal fungus and *D. flagrans* a predatory fungus, when they were combined they showed destruction of the target organism (*T. saginata* eggs). However, synergistic or antagonistic activity in this study was only assessed by the percentage of egg reduction/destruction achieved by the two species (G4—25.6%). Therefore, it is necessary to use future tests to verify the compatibility of fungi, such as direct confrontation, antibiosis tests, and volatile metabolites [32]. The authors emphasize that, although the individual and combined results were observed and quantified, the effectiveness of the combination of VC4 with AC001 was not high. Therefore, they recommend further studies to elucidate the real potential of this combination.

To date, this is the first study to use the action of two fungi with completely different characteristics on *T. saginata* eggs and, in this way, given the uncertain scenario of helminth infections (teniasis cysticercosis complex), new studies can be generated that can contribute to environmental decontamination, boosting other research groups in biological control. On the other hand, the in vitro use of various species of nematophagous fungi can reduce potential failures when used alone and, conversely, can act as an enhancer of a certain action [32]. Eichenberger et al. [6] in their systematic review point out the lack of knowledge about the distribution of human tapeworm infections, especially in regions where there are different species of human tapeworms.

In this sense, the continuation of research aimed at environmental control with a greater focus on the eggs of this parasite is extremely important [22,41]. It is worth noting that *P. chlamydosporia* and *D. flagrans* are two fungi with proven different activities and

this would make their use a promising proposal in the control of *T. saginata* eggs (since there was ovicidal activity) and to be employed in the future to effectively interrupt the evolutionary cycle of *T. saginata*.

4. Materials and Methods

4.1. Obtaining Taenia saginata Eggs

Following parasitological confirmation (*T. saginata*) according to the identification key [47], the eggs were recovered by dissecting their mature proglottids. The proglottids were donated to the Experimental Parasitology and Biological Control Laboratory (LPECB) of the Vila Velha University (UVV), Espírito Santo, Brazil.

4.2. Obtaining the Fungal Isolates

The fungi *P. chlamydosporia* (isolate VC4) and *D. flagrans* (isolate AC001), originating from Brazilian soil, were used. They are maintained at the LPECB/UVV-Brazil through continuous subculturing in 9 cm diameter Petri dishes containing 2% water agar culture medium.

4.3. Experimental Assay

To carry out the experimental assay, the concentrations of viable *T. saginata* eggs and conidia/chlamydospores of *P. chlamydosporia* VC4 and *D. flagrans* AC001 fungi grown in the 2% potato dextrose agar (PDA) culture medium were first calculated. The groups were formed in 1.5 mL microtubes, with a final volume of 500 µL, in triplicates. The egg concentration was calculated by reading aliquots on glass slides using an optical microscope, in which a concentration of 25 eggs/5 µL of sterile distilled water was obtained. Next, the concentrations of *P. chlamydosporia* VC4 and *D. flagrans* AC001 conidia were obtained by counting them in a Neubauer chamber [20].

The material was distributed into four groups, in which the treated groups (G2, G3, and G4) received a specific ratio of 1:1, 100 eggs (20 µL) to 100 conidia (2 µL of *D. flagrans* AC01 and/or *P. chlamydosporia* VC4), according to Table 2.

Table 2. Division and composition of the experimental groups formed in microcubes: (G1—control); (G2—*Taenia saginata* eggs + *Pochonia chlamydosporia* conidia-VC4); (G3—*T. saginata eggs* + *Duddingtonia flagrans* conidia-AC001); (G4—*T. saginata* eggs + *P. chlamydosporia*-VC4 + *D. flagrans*-AC001).

Groups	Composition
G1 (control group)	20 µL (100 *T. saginata* eggs) + 480 µL distilled water
G2	20 µL (100 *T. saginata* eggs) + 2 µL VC4 (100 conidia) + 478 µL distilled water
G3	20 µL (100 *T. saginata* eggs) + 2 µL AC001 (100 conidia) + 478 µL distilled water
G4	20 µL (100 *T. saginata* eggs) + 1 µL VC4 (50 conidia) + 1 µL AC001 (50 conidia) + 478 µL distilled water

Subsequently, all the experimental groups were stored in a B.O.D. incubation chamber at a controlled temperature of 27 °C for 15 days and then analyzed using an optical microscope with a 40x objective, according to methodology described in Araújo et al. [22], and modified. Five replicates were carried out for each experimental group. After this period, the contents of all the microtubes in the experimental groups were read under a light microscope and the number of viable eggs (not destroyed) was counted to calculate the percentage reduction, using the following formula [48]:

$$\% \text{ reduction} = \frac{\text{average of control group} - \text{average of treatment group}}{\text{average of control group}} \times 100$$

4.4. Data Analysis

The results obtained were interpreted by analysis of variance (ANOVA) and the Tukey test, at the 5% probability level, using BioEstat 5.0 software [49].

5. Conclusions

In conclusion, the study demonstrated that the fungal isolates tested (VC4 and AC001), both individually and in combination, exhibit ovicidal activity against *T. saginata* eggs. The findings confirm the potential of *P. chlamydosporia* (VC4) as an effective ovicidal agent. Notably, *D. flagrans* (AC001) also showed significant ovicidal activity, suggesting enzymatic production and predatory behavior. Future studies should focus on understanding the compatibility and interaction mechanisms of these fungi to optimize their combined use in environmental decontamination and control of helminth infections.

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