

# Proteolytic activity of extracellular products from *Arthrobotrys musiformis* and their effect in vitro against *Haemonchus contortus* infective larvae

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## ABSTRACT

*Arthrobotrys musiformis* is a nematophagous fungus with potential for the biological control of *Haemonchus contortus* larvae. This study aimed to identify and demonstrate the proteolytic activity of extracellular products from *A musiformis* cultured in a liquid medium against *H contortus* infective larvae.

*A musiformis* was cultured on a solid medium and further grown in a liquid medium, which was then processed through ion exchange and hydrophobic interaction chromatography. The proteolytic activity of the purified fraction was assayed with either gelatin or bovine serum albumin as substrate. Optimum proteolytic activity was observed at pH 8 and a temperature of 37°C. Results obtained with specific inhibitors suggest the enzyme belongs to the serine-dependent protease family. The purified fraction concentrate from *A musiformis* was tested against *H contortus* infective larvae. A time-dependent effect was observed with 77 per cent immobility after 48 hours incubation, with alteration of the sheath. It is concluded that *A musiformis* is a potential candidate for biological control because of its resistant structures and also because of its excretion of extracellular products such as proteases. The present study contributes to the identification of one of the in vitro mechanisms of action of *Amusiformis*, namely the extracellular production of proteases against *H contortus* infective larvae. More investigations should be undertaken into how these products could be used to decrease the nematode population in sheep flocks under field conditions, thereby improving animal health while simultaneously diminishing the human and environmental impact of chemical-based drugs.

## INTRODUCTION

Gastrointestinal nematode infection in sheep is a major cause of economic loss. In tropical and subtropical regions of the world, parasitic infections have negative effects on livestock

production, causing direct economic losses (death of young animals) and indirect losses (persistent diarrhoea, anaemia, malnutrition, stunting and low production of meat and milk). Exact details are unknown, but estimates of costs for treating internal parasites in ruminants are in the region of US\$1.7 billion annually, with overall losses estimated at up to US\$4 billion annually (Rodríguez-Vivas and others 2011). Currently their control is dependent on the use of anthelmintics; however, resistance to benzimidazoles, imidazothiazoles and macrocyclic lactones has been reported in *Haemonchus contortus* (Campos and others 1992, Figueroa and others 2000, Coop and Kyriazakis 2001, Torres-Acosta and others 2012). Furthermore, some synthetic anthelmintics are toxic to animals, adding an indirect economic cost related to meat and milk withdrawal periods, as well as causing damage to the terrestrial and aquatic environment and thus constituting a public health problem (Kolar and others 2008, Martínez and Cruz 2009, Beynon 2012, Yang 2012).

The challenge lies in finding control strategies that allow a rational use of anthelmintics combined with an alternative biological control strategy such as the use of nematophagous fungi. Nematophagous fungi are characterised by their ability to capture and use nematodes as the main or complementary source of food for their saprophytic existence. This type of predatory fungi produces trapping devices in the mycelium (adhesive rings or networks), which are used to capture and immobilise nematodes, penetrating their body and eventually consuming their contents (Waller and Larsen 1993). Invasion of nematodes by nematophagous fungi involves

the breakdown of the outer layer of the nematode, either by mechanical or enzymatic strategies. The complexity of the cuticle penetration suggests a synergic mechanism that requires several different enzymes (Huang and others 2004). In the first stage of nematode infection by a nematophagous fungus, penetration of the nematode surface (cuticle) results from the combination of mechanical and hydrolytic enzyme activity. There are also extracellular enzymes that degrade collagen. Proteases from other nematophagous fungi are known (Tunlid and Jansson 1991, Tunlid and others 1994, Zhao and others 2004, Wang and others 2006, Yang and others 2007); however, these studies did not assess the effect of such enzymes against parasitic nematode larvae.

The saprophytic fungus *Arthrobotrys musiformis* is a facultative predator of nematodes, and therefore has the potential to be an indirect biological control agent of animal parasitic nematodes. This species has demonstrated its ability to survive after passing through the gastrointestinal tract of sheep and to conserve its predatory activity against infective larvae of gastrointestinal parasitic nematodes, reducing the number of larvae on pasture (Gronvold and others 1996, Graminha and others 2005). *A. musiformis* develops three-dimensional adhesive nets which capture nematodes. To date, there has been no description of the extracellular enzymes produced by *A. musiformis* and their action against *H. contortus* infective larvae, which is important for understanding the nematocidal action of this species. The aims of this study were to identify and demonstrate the proteolytic activity of these extracellular products from *A. musiformis* cultured in a liquid medium against *H. contortus* infective larvae.

## MATERIALS AND METHODS

### Culture of *A. musiformis*

*A. musiformis* was isolated from soil samples collected in Mexico (Acevedo-Ramirez and others 2011) and maintained at room temperature (20–25°C) in 90 mm diameter Petri dishes with potato dextrose agar (PDA) medium for two weeks. In order to obtain fungal extracellular products, the fungus was grown in liquid culture medium as follows. The PDA plates containing *A. musiformis* were cut into 1 cm<sup>2</sup> fragments, placed in flasks (capacity 4 litres) at half capacity containing liquid medium (sucrose 30 g/l, potassium chloride [KCl] 0.5 g/l, potassium dihydrogen phosphate [KH<sub>2</sub>PO<sub>4</sub>] 1 g/l, magnesium sulphate heptahydrate [MgSO<sub>4</sub>·7H<sub>2</sub>O] 0.5 g/l, chloramphenicol 0.5 g/l), and incubated at room temperature with continuous stirring for 14 days. The mycelium was then filtered through gauze placed in a funnel over a flask and the filtered medium was used for processing.

### Partial purification of extracellular proteases

The filtered medium was adsorbed onto a DEAE-Sephacel ion exchange chromatography column previously equilibrated in 50 mM Tris-hydrochloric acid (Tris-HCl) buffer,

pH 8. After washing thoroughly, the adsorbed protein was eluted with a sodium chloride linear gradient (0–0.3 M). The fractions with proteolytic activity were concentrated in 10 kDa Amicon Ultra tubes by centrifuging at 2500 g. The concentrate was dialysed overnight against 1.5 M ammonium sulfate dissolved in 50 mM Tris-HCl buffer, pH 8. The concentrate was then adsorbed onto a phenyl-sepharose hydrophobic column previously equilibrated with the buffer used to dialyse the sample. The protein was eluted with an ammonium sulfate decreasing linear gradient (1.5–0 M). The fractions were dialysed overnight against 50 mM Tris-HCl buffer, pH 8. The fractions with proteolytic activity were concentrated as above and stored at –10°C.

### Collection and maintenance of the *H. contortus* infective larvae

Faecal samples collected from a lamb previously infected with *H. contortus* were used to obtain *H. contortus* nematode eggs. The faeces were used to develop a coproculture and incubated for 10–12 days at 27°C. The infective larvae (L3) were then collected using the Baermann technique (Hendrix 2012), washed using the density gradient technique with a 40 per cent sucrose solution, and stored refrigerated at 4°C.

### Effect of the liquid culture medium of *A. musiformis* on *H. contortus* infective larvae

The potential nematocidal effect of the extracellular products in the liquid culture was evaluated by exposing *H. contortus* infective L3 larvae according to the following random design with four treatments: (1) liquid medium prepared and maintained in the absence of the fungus (control); (2) filtered non-concentrated liquid medium obtained after growing *A. musiformis*; (3) the concentrate fraction containing proteolytic activity obtained from ion exchange chromatography; (4) the concentrate fraction containing proteolytic activity obtained from hydrophobic interaction chromatography. In addition, infective L3 larvae were exposed to phosphate buffer solution (PBS) and to the fraction obtained from the hydrophobic interaction chromatography added to the protease inhibitor phenyl-methyl-sulphonyl fluoride (PMSF). Experiments were carried out in ELISA plates and previous preliminary bioassays (non-published) were performed with different concentrations/volumes of each treatment, which led to the selection of 200 µl as the best activity to use in the present study. Approximately 50 infective L3 larvae in each well were exposed to 200 µl of the corresponding test medium. The plates were incubated at 25°C, and the numbers of viable L3 larvae were counted at 0, 24 and 48 hours to calculate the immobility rate.

### Statistical analysis

Data were analysed using a generalised linear model. The distribution of the variable used was the negative binomial with the natural logarithm link (Wilson and Grenfell 1997). The non-parametric Kruskal–Wallis test

was used (Conover 1980) to compare treatments at 0, 24 and 48 hours.

### Identification and characterisation of the proteolytic activity of *A musiformis*

To detect proteolytic activity in the filtered culture medium, as well as throughout the purification process, a 9 per cent polyacrylamide gel copolymerised with 1 per cent gelatin as the protease substrate (GS-PAGE) was prepared (Tunlid and Jansson 1991). A small aliquot of the corresponding fraction was loaded on the top of the gel and electrophoresis was carried out at 4°C under constant current (15 mA). After the run, the gel was stained with Coomassie Blue R-250 and destained with methanol (40 per cent) and acetic acid (6 per cent) (Heussen and Dowdle 1980, Naggie and others 1997).

To characterise the proteolytic activity, enzyme assays were carried out following the protocol published by Wang and others (2006) using bovine serum albumin (BSA) as a substrate. An aliquot of filtrate was added to a BSA solution prepared in 50 mM Tris-HCl, pH 8, and the mixture was incubated at 37°C. At certain time intervals (0, 4, 8, 24, 48 and 72 hours) a small aliquot was withdrawn and the reaction was stopped by adding 5 per cent trichloroacetic acid (TCA). Undigested proteins were precipitated by centrifugation at 14,000 rpm for five minutes. A 300 µl aliquot of supernatant was mixed with 2.5 ml of 0.55 M sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>) and 200 µl Folin phenol reagent, and incubated at 30°C. After 15 minutes incubation the resulting absorbance of the supernatant was read at 680 nm.

To obtain the kinetic parameters Km and Vm of the proteolytic activity, full time courses were carried out at different BSA micromolar concentrations (50, 100, 250, 500, 750, 1000, 1500 and 2000 µM). For each BSA concentration, the initial velocity was obtained from the corresponding absorbance versus time plot by determining the initial slope of the trace. Each experiment was carried out in triplicate. From the dependence of the initial velocity on BSA concentration, both Km and Vm were obtained by fitting the Michaelis–Menten rate equation through non-linear regression using Sigma plot software. A unit of proteolytic activity was defined as the increase in absorbance at 680 nm/µl/hour, according to Tunlid and others (1994).

The dependence of the proteolytic activity on both pH and temperature was determined employing 500 µM BSA as substrate. For the determination of the optimum pH, buffers with pKa values in the pH range 3–10 were used and the full time courses of enzyme activity were performed at 37°C. The effect of temperature was analysed in the range 4–60°C. For each pH or temperature experiment, a full time course was obtained and the corresponding initial velocity was determined as described above.

The effects of the protease inhibitors PMSF and EDTA at a final concentration of 10 mM were assessed in the presence of BSA (500 µM, pH 8, 37°C), and the reaction

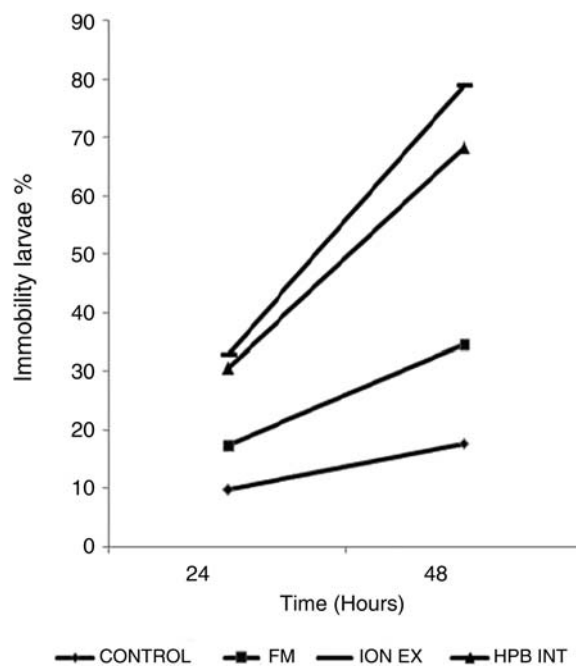
was initiated by adding an aliquot of protease. Assays were carried out in triplicate. The protein content was determined by the Bradford method (Bradford 1976).

## RESULTS

### Effect of the culture liquid medium of *A musiformis* on *H contortus* infective larvae

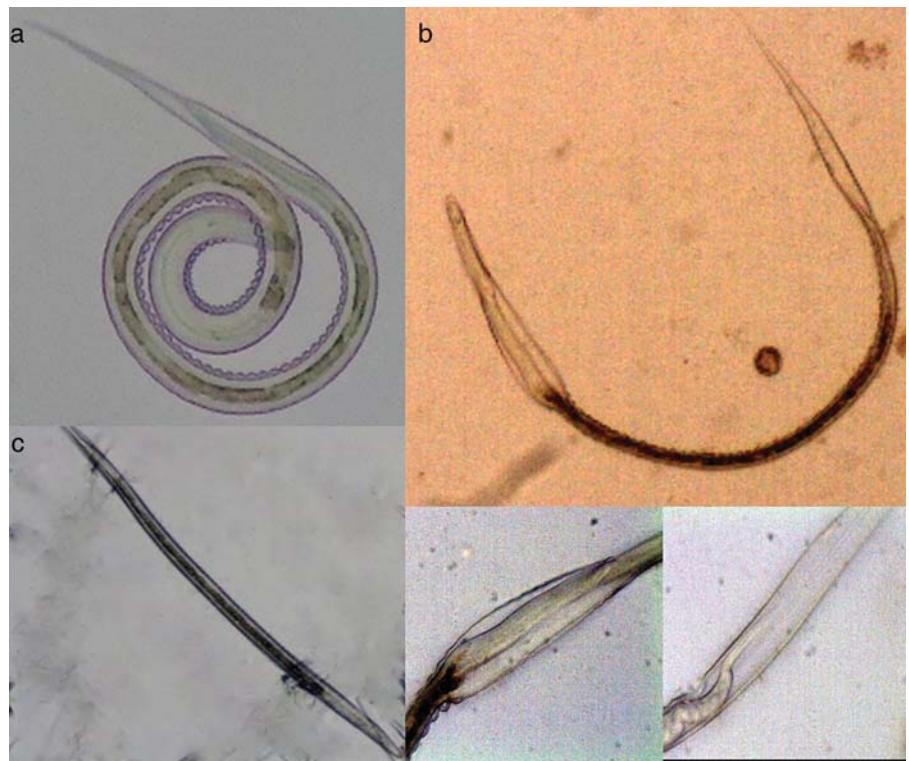
The infective L3 larvae exposed to the liquid medium without *A musiformis* remained viable, as indicated by rapid undulating movements. In contrast, the infective L3 larvae incubated in the filtered culture medium in which the fungus was grown were immobile, and remained rigid even when exposed to light or heat. An immobility rate of 38 per cent was recorded with the filtered medium after 48 hours exposure. When the infective L3 larvae were exposed to the fractions with proteolytic activity from either ionic exchange or hydrophobic interaction chromatography for 48 hours, the immobility rate was significantly increased (85 per cent and 77 per cent, respectively) as compared with both the control and the filtered medium obtained in the absence of the fungus (Fig 1).

In addition to the differences observed among concentrates, a time-dependent effect with both concentrates was found (P<0.01). Interestingly, when the



**FIG 1:** Effect of *Arthrotrys musiformis* treatments against *Haemonchus contortus* infective larvae: CONTROL: liquid medium prepared and maintained in the absence of the fungus; FM, filtered liquid medium; ION EX, concentrate from ionic exchange chromatography; HPB INT, concentrate from hydrophobic interaction chromatography. There was a significant difference (P<0.01) between control and the filtered unconcentrated medium and the concentrates obtained from ionic exchange and hydrophobic interaction chromatography after 24 and 48 hours incubation

**FIG 2:** *Haemonchus contortus* infective larvae exposed for 48 hours to: (a) phosphate buffer solution (PBS) (10×); (b) concentrate from the hydrophobic interaction chromatography (10×); (b1) anterior region (40×); (b2) posterior region (40×); (c) concentrate from the hydrophobic interaction chromatography in the presence of 10 mM phenyl-methyl-sulphonyl fluoride (PMSF) (10×). In panels a and c the sheath was not altered



infective L3 larvae were exposed to the fraction obtained by hydrophobic interaction chromatography, damage to the outer sheath of the immobilised worms at both the anterior and posterior regions of the body was evident, as revealed by microscopic analysis. This effect was not observed in infective L3 larvae exposed to PBS or to the medium obtained in the absence of the fungus (Fig 2).

#### Identification of proteolytic activity in the extracellular products of *A musiformis*

Proteolytic activity was detected in the *A musiformis* liquid culture medium even when the fungus was grown in the absence of nematodes. By contrast, no activity was recorded in the culture medium maintained in the absence of the fungus. Thus, it can be concluded that *A musiformis* is able to secrete proteins with proteolytic activity that could be involved in the digestion of the nematode cuticle. Proteolytic activity was enriched after its purification through ionic exchange and hydrophobic interaction chromatography. As shown in Table 1, a gradual increase in proteolytic activity was achieved. It is worth noting that two peaks with proteolytic activity were obtained with hydrophobic interaction chromatography. Based on the enzyme activity recorded in the electrophoretic experiments the more active fraction was chosen (line 5 in Fig 3) to characterise the proteolytic activity.

The purified extract showed saturation kinetics when assayed with BSA as substrate (Fig 4). The non-linear regression analysis yielded a  $K_m$  of  $100 \pm 34 \mu M$ .

Proteolytic activity was maximal at  $37^\circ C$  and pH 8. In the presence of PMSF the activity was 6 per cent and 100 per

cent lower after 4 and 24 hours incubation, respectively, than that observed in the sample without an inhibitor. This result suggests the extracellular protease from *A musiformis* belongs to the serine proteases family. After 4 hours incubation, enzyme activity with EDTA was 6 per cent higher than that found in the sample without inhibitor. However, the proteolytic activity after 24 hours incubation was similar to that found in the sample without inhibitor.

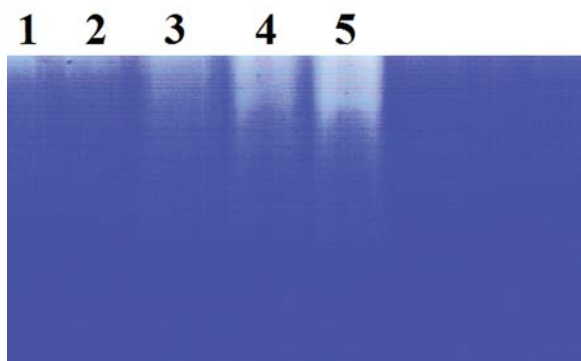
#### DISCUSSION AND CONCLUSIONS

Although *A musiformis* has been the subject of this study, because of its resistance to adverse conditions and its action against ovine gastrointestinal nematodes (Graminha and others 2005, Saumell and others 2008), information concerning its culture and production, as well as its extracellular products, is scarce. Researchers have used liquid media enriched with proteins,

**TABLE 1:** Purification of the proteolytic activity of the extracellular products of *Arthrobotrys musiformis* with BSA as substrate (500  $\mu M$ )

	Total protein ( $\mu g$ )	PU ( $\Delta A_{680} \mu l/h$ ) $\times 10^{-3}$
Medium without fungus (control)	0	0
Filtered medium	1440	0.019
Ion exchange chromatography concentrate	9.9	3.41
Hydrophobic interaction chromatography concentrate	14.4	3.51

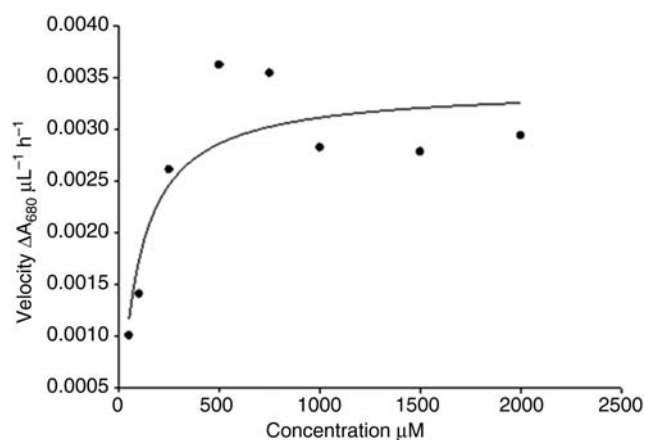
BSA, bovine serum albumin; PU, proteolytic units



**FIG 3:** Identification of proteolytic activity of extracellular products of *Arthrobotrys musiformis* cultured in liquid medium. The proteolytic activity was recorded with gelatin as substrate (gelatin substrate-polyacrylamide electrophoresis: GS-PAGE). Line 1: liquid medium without fungus (control); line 2: filtered liquid medium; line 3: concentrate from the ion exchange chromatography; lines 4–5: fractions from hydrophobic interaction chromatography

including the addition of nematodes, as protease inducers (Zhao and others 2004, Wang and others 2006). In the present work, we obtained successful growth of *A. musiformis* in a modified Czapek–Dox liquid medium that was not supplemented with proteins. Persson and Friman (1993) stated that the extracellular products will be dependent on the culture medium in which the fungus is grown. In the present study we showed, as did Mendoza and others (2003), that nematophagous fungi may be lethal to infective larvae even when the fungus is cultured in the absence of nematodes.

In a previous study it was shown that *A. musiformis* had a 44 per cent trapping rate of *H. contortus* infective larvae (Acevedo-Ramirez and others 2011); in this work cultivation of *A. musiformis* was achieved in a liquid medium, which identified some of the products in the extracellular medium that could be causing damage suffered by



**FIG 4:** Saturation kinetics of the proteolytic activity of *Arthrobotrys musiformis* purified from the liquid culture medium. Bovine serum albumin (BSA) was used as a substrate, and the enzyme assays were carried out at pH 8 and 37°C

the nematodes coming into contact with the nematophagous fungus. In the present study, it was possible to identify the proteolytic activity in the extracellular medium, indicating that there are proteases that may be involved in breaking the cuticle of the larvae.

The biochemical characterisation of the proteolytic activity revealed that it is capable of hydrolysing substrates such as albumin and gelatin. The conditions of activity were similar to some proteases obtained from other nematophagous fungi (Tunlid and others 1994, Zhao and others 2004, Wang and others 2006, Yang and others 2007, 2012). Proteolytic activity was observed with albumin and gelatin as substrate. The highest activity was recorded at 37°C. Maximum activity was observed in the alkaline zone and was maximal at a pH close to 8. The inhibitory effect of PMSF suggested that the proteolytic secretions of *A. musiformis* are dependent on a serine residue, as stated by Braga and others (2011). Proteases have only a few hours of activity when exposed to ambient temperatures, as reported by Tunlid and others (1994) for proteases obtained from *Arthrobotrys oligospora*. In this case, the concentrate obtained from *A. musiformis* demonstrated that the proteolytic activity retains activity up to 72 hours with albumin at 37°C, which makes a stable protease.

Studies of proteases obtained from nematophagous fungi have been carried out, but their nematocidal effect have been evaluated only in free-living nematodes, such as *Caenorhabditis* and *Panagrellus* species. In the present study, we report for the first time the effect of extracellular proteases on the larvae of a parasitic worm. The body of *H. contortus* infective L3 larvae is covered with a sheath that was damaged by the extracellular proteases. Hence the proteases secreted by *A. musiformis* may be involved in the immobilisation mechanism of the fungus. The production of proteases in the fungi is not dependent on the presence of nematodes.

In this work, cultivation of *A. musiformis* was achieved in a liquid medium which identified some of the products in the extracellular medium that could be causing the initial damage suffered by nematodes when first coming into contact with the nematophagous fungus. In this study, it was possible to identify proteolytic activity in the extracellular medium, indicating that there are proteases that may be involved in damaging the cuticle of the larvae, which is the first step toward breaking the body of the larva, enabling the fungus to invade it and start internal digestion.

*A. musiformis* is a potential candidate for a biological control agent because of its ability to survive passage through the digestive tract of sheep and also because of its excretion of extracellular products such as proteases. This study provided evidence of an enzymic mechanism of action by *A. musiformis* against *H. contortus* infective larvae. The fungus was shown to produce and release nematocidal substances in the extracellular medium, in which there is at least one protease. The production of proteases is a natural mechanism of fungi, and it does not depend on the presence of nematodes. This report recorded

immobility of the larvae, but as time passed larval death was also recorded. This could result from the presence of other compounds in the extracellular medium having nematocidal activity; thus, other substances, such as secondary metabolites, may act synergistically with the proteases. Further investigations are required into the substances produced by these nematophagous fungi, their optimal conditions of activity, how they interact with each other, and how they work at the field level for possible application in decreasing the parasite population while simultaneously having fewer negative consequences for animal, human, and environmental health.

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