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Evaluation of *Arthrobotrys flagrans* concentration, predatory activity, and efficacy in a commercial product

Maria Vitória Lamóglia Bastos Ferreira [a], Edna Barcelos Alves [a], Caroline Bittencourt Miranda [a], Vanessa Cola Thomazini [a], Ricardo Augusto Mendonça Vieira [b], Luis Fonseca Matos [c], Clóvis de Paula Santos [a,*]

[a] *Laboratory of Cell and Tissue Biology, Center for Biosciences and Biotechnology, State University of Northern Rio de Janeiro – Darcy Ribeiro, 28013-602, Campos dos Goytacazes, RJ, Brazil*

[b] *Laboratory of Animal Science and Nutrition, Center for Agricultural Sciences and Technologies, State University of Northern Rio de Janeiro – Darcy Ribeiro, Av. Alberto Lamego, 2000, Campos dos Goytacazes, RJ 28013-602, Brazil*

[c] *Laboratory of Animal Reproduction and Genetic Improvement, Center for Agricultural Sciences and Technologies, State University of Northern Rio de Janeiro – Darcy Ribeiro, Av. Alberto Lamego, 2000, Campos dos Goytacazes, RJ 28013-602, Brazil*

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ABSTRACT

Gastrointestinal nematodes pose a major threat to livestock health and productivity, and the growing inefficacy of commercial anthelmintics highlights the need for alternative control methods. This study evaluated BioVerm®, a Brazilian commercial product (BCP) based on the nematophagous fungus *Arthrobotrys flagrans*, using three criteria: chlamydospore count per gram (CCG), predatory activity, and efficacy in reducing infective larvae (L3). CCG was quantified using a Neubauer chamber. Predatory activity was assessed by inoculating *Panagrellus* spp. in Petri dishes. Efficacy was evaluated through coprocultures treated or not with (BCP), including post-gastrointestinal tract analysis using sheep feces. Three commercial batches were tested. The mean CCG ranged from 8333 to 12,500, significantly below the 5×10^5 spores per gram stated on the label. Fungal growth was observed in only one batch, and high contamination was found in another. No significant reduction in L3 counts was detected in either *in vitro* or *in vivo* assays. These results indicate low fungal viability and support the urgent need for quality control measures to ensure BCP efficacy.

1. Introduction

Gastrointestinal nematodes are a major constraint to livestock production worldwide. These parasites directly impact animal health, affecting morbidity and mortality rates, and indirectly through the increasingly ineffective treatment of anthelmintic resistance. The associated costs are high: the most recent estimates from 18 European countries indicate that around €38 million is spent annually on controlling anthelmintic-resistant nematodes in ruminants. When dictyocaulosis and fasciolosis are included, the total production and treatment costs amount to €1.8 billion (Charlier et al., 2022). In Brazil, the annual cost of these parasites to the sheep production industry, excluding treatment costs, is around US$107.52 million (Chagas et al., 2022).

Biological control using nematophagous fungi has emerged as a promising strategy for managing gastrointestinal nematodes in various domestic animal species. Over the past 30 years, several studies have demonstrated its potential efficacy (Larsen, 1999, 2000, 2006; Castro et al., 2003; Campos et al., 2007; Cruz et al., 2008; Braga et al., 2008; Sagües et al., 2011; Buzatti et al., 2015), leading to the development of commercial formulations. In 2017, BioWorma® (Healey et al., 2018) was launched and commercialized in Australia, New Zealand, and Europe. More recently, BioVerm® was licensed for use in Brazil by the Ministry of Agriculture, Livestock and Supply (MAPA). Both products are based on *A. flagrans* and are recommended for the control of gastrointestinal nematodes in ruminants, horses, and other species. However, only a few studies have evaluated the efficacy of the Brazilian commercial product (BCP) (Braga et al., 2020; Fausto et al., 2021; Oliveira et al., 2021; Rodrigues et al., 2021, 2022; Mendes et al., 2023; Nunes et al., 2023).

Therefore, it is crucial to conduct studies that validate BCP, especially those that analyze aspects that have not yet been examined. Furthermore, given the serious problems related to anthelmintics in

* Corresponding author.
E-mail address: cps@uenf.br (C. de Paula Santos).

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ruminants in Brazil (Cruz et al., 2010; Baiak et al. 2018; Salgado et al., 2019; Bassetto et al., 2024; Borges et al., 2024) and other countries (Papadopoulos et al., 2012; Torres-Acosta et al., 2012; Preston et al., 2019; Charlier et al., 2022), finding alternatives for controlling gastrointestinal nematodes is essential. In this study, the authors evaluated the concentration of chlamydospores per gram of the BCP or of feces, the predatory activity, and the efficacy of reducing infective larvae by the fungus *A. flagrans* of the BCP both '*in natura*' and after passage through the gastrointestinal tract of sheep.

2. Material and methods

2.1. Fungal material

Three batches of BioVerm® (manufactured by Ghenvet Animal Health in Paulínia, São Paulo) were used for the experiments: batch 000321 (manufactured 10/03/2021, expiry date 10/03/2022), batch 000821 (manufactured 24/08/2021, expiry date 24/08/2022) and batch 2915 (manufactured 27/11/2024, expiry date 27/11/2025). The analyses were performed within the products' validity periods. The manufacturer's recommendation on the product label is 1 g to 10 kg of live weight. Each gram should contain 5 × 105 chlamydospores of *A. flagrans*.

2.2. Evaluation of BCP 'in natura'

2.2.1. Chlamydospore counting

Chlamydospore quantification was conducted for each BCP batch by weighing 10 g of the product and suspending it in 100 ml of distilled water. The mixture was filtered through a 100-micron mesh and homogenized, after which a 1 ml aliquot was collected for counting in a Neubauer chamber. Counts were performed in triplicate for each batch and results expressed in chlamydospore/g of BCP.

2.2.2. BCP in a Petri dish

To determine fungal predatory activity one gram of BCP was dispersed in a cross configuration on each Petri dish containing 2 % water agar. Each plate was baited with *Panagrellus* spp. The plates were kept at room temperature (19–28 °C) and examined twice a week for two weeks to check for the presence of fungi. Isolated fungi were identified according to the descriptions of Cooke and Godfrey (1964), van Oorschot (1985), and Rubner (1996). Contaminating fungi were identified based on the morphological criteria described by Barnett and Hunter (1998) and Watanabe (2010). Eight, nine, and ten replicates were performed respectively for batches 000321, 000821, and 2915. Plates for batch 2915 were also prepared without the incorporation of *Panagrellus* spp to establish the origin of contamination fungi.

2.2.3. Efficacy

To determine the efficacy of BCP against gastrointestinal nematode larvae, samples of feces positive for gastrointestinal nematodes from sheep at the UENF were homogenized and used for coprocultures. One gram of BCP was added to each of ten 4 g fecal samples, while other ten samples were left untreated (control). Coprocultures were prepared by adding 4 g of each fecal sample into 50 ml disposable plastic cups, which were then placed in a plastic tray containing 100 ml of water. The tray was covered with a plastic film to prevent loss of moisture during the ten days incubation period. After the incubation, the cultures were filled with distilled water, and a Petri plate was added as a cover, which was then turned abruptly. Then, 10 ml of distilled water was added to the Petri plate. After four hours, the infective larvae (L3) that had migrated from the cup were pipetted out. Recovered L3 were preserved at 2º C for later quantification as L3 per gram of feces (LPG). Only batches 000821 and 2915 were analyzed. Specifically for this analysis, batch 000321 was not used as its expiration date had already passed.

2.3. Efficacy of BCP after passage through the gastrointestinal tract of sheep

2.3.1. Animals

Ten Santa Inês hairless sheep (both male and female), aged between 3 and 6 months, naturally infected with gastrointestinal nematodes and housed at the State University of Northern Rio de Janeiro (UENF, Campos dos Goytacazes, RJ, Brazil), were used for the administration of BCP batch 000321. The animals were allocated to two pens during the treatment period and received a standard sheep diet with free access to pasture and water (*ad libitum*). For the evaluation of batch 000821, eight crossbred sheep from a privately owned farm in the same municipality were used. These animals were also naturally infected with gastrointestinal nematodes. During the administration period, these animals were subjected to the same management and feeding procedures as those used at UENF.

2.3.2. Administration of BCP

The product was administered for four consecutive days in accordance with the manufacturer's recommendations, i.e, 1 g to 10 kg of live weight. The dose, calculated based on each animals' body weight, was thoroughly mixed into 2 kg of crushed corn per pen and offered to the sheep in the early morning to ensure complete ingestion.

2.3.3. Feces collection and processing

Feces were collected directly from the animals' rectum for 12 consecutive days, as follows:

Period 1, from day 01 to day 04 (pre-administration of BCP)
Period 2, from day 05 to day 08 (BCP administration)
Period 3, from day 09 to day 12 (post-administration of BCP)

Feces collected were set up: a) fecal egg counts (FEC), following the modified Gordon, Whitlock (1939), and expressing results in eggs per gram of feces (EPG); b) coprocultures as described in 2.2.3; c) quantification of chlamydospores, as proposed by Ojeda-Robertos et al. (2008) and expressing the results in chlamydospore per gram of feces (CPG); and d) establishing the presence of fungi in feces in Petri dishes.

Fecal plating to obtain fungal isolates was performed following the methodology described by Saumell et al. (1999, 2000). Feces collected from day 6 (24 h after the first administration) to day 12 (96 h after the last administration) were inoculated onto Petri dishes containing 2 % water agar. The dishes were maintained and monitored as previously described in 2.2.2 BCP in a Petri dish.

2.3.4. Statistical analysis

The response variables in this study (larval count and EPG) are count data and therefore require statistical modeling based on appropriate probability distributions. Time was included as a covariate in the model. Accordingly, the analysis followed the structure outlined by Stroup (2012) and Vonesh (2012):

$$\xi_{ijk} = \log\left(\mu_{ijk}\right) = \xi + \alpha_i + a_j + \tau_k + \alpha\tau_{ik}$$

Where ξ is a constant in the link function $\left(\xi_{ijk} = \log\left(\mu_{ijk}\right)\right)$ that transforms the mean μ_{ijk} into the natural scale to fit the negative binomial distribution (BN).

Thus $y_{ijk} \sim BN\left(\mu_{ijk}, \mu_{ijk} + \phi\mu_{ijk}^2\right)$, where $\sigma_{ijk}^2 = \mu_{ijk} + \phi\mu_{ijk}^2$ and ϕ is a scale parameter. In the link function, α_i is the fixed effect of the periods pre-, during and post-treatment with BCP, τ_k is the quantitative effect of days within each period, the respective interaction $\alpha\tau_{ik}$.The term a_j corresponds to the random effect of animal, assuming $a_j \sim N\left(0, \sigma_a^2\right)$, where σ_a^2 is the variance of animal.

The model was adjusted to the count values using the GLIMMIX procedure of the SAS statistical software (SAS/STAT software v.8, SAS on Demand for Academics, SAS Institute Inc., Cary, NC, USA).

The goodness-of-fit criterion for the listed effects was the production of a positive definite matrix at model convergence. The effects were declared significant considering the probability for type I error less than 0.05.

3. Results

3.1. Evaluation of BCP 'in natura'

The mean chlamydospore count per gram of BCP was 8333 (0; 0; 25,000) for batches 000321 and 000821, and 12,500 (0; 12,500; 25,000) for batch 2915.

The fungal plating assays showed that *A. flagrans* was detected only in 33.3 % of Petri dishes containing batch 000821. No plates from any of the other two batches tested positive for the presence of this fungus. Plates inoculated with batch 2915, including those without *Panagrellus spp.*, exhibited heavy contamination by fungal species unrelated to the experimental objectives (Fig. 1). Morphological analysis identified *Aspergillus spp.*, *Rhizopus spp.*, and *Verticillium spp.* and two yeast types (one yellowish, one reddish).

No predatory activity was recorded in coprocultures from BCP batches 000821 and 2915 as the mean LPG numbers recovered from them did not differ significantly ($P > 0.05$) from the control; non-fungal product cultures (Fig. 2). Macroscopic fungal growth was observed only in fecal cultures treated with batch 2915 (Fig. 3).

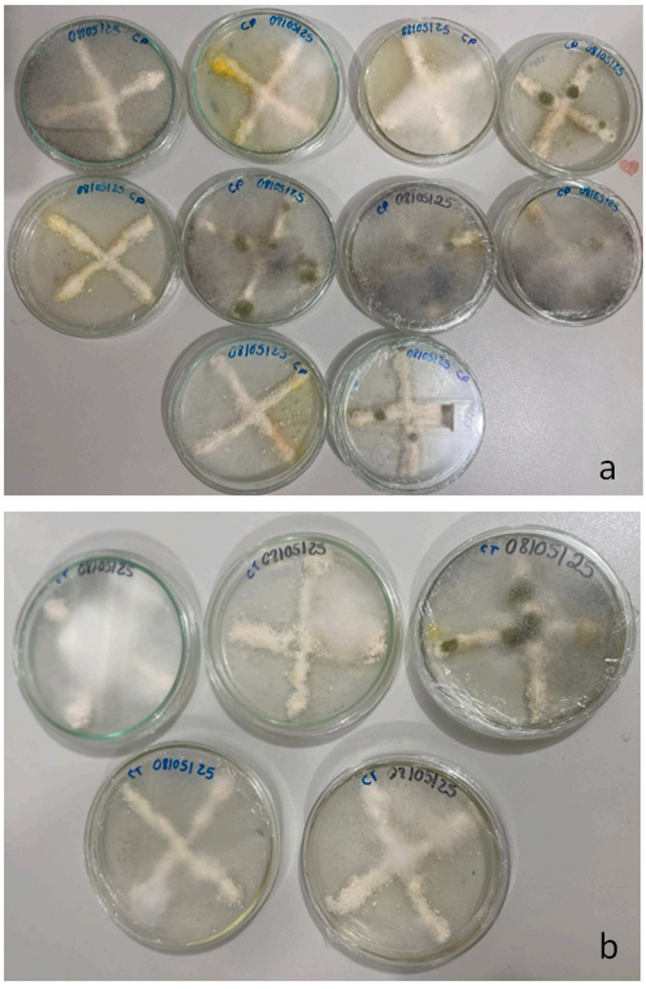

Fig. 1. Petri dishes inoculated with BCP Batch 2915. a) Petri dishes with *Panagrellus* spp. addition, b) Petri dishes without *Panagrellus* spp. addition. Both Petri dish groups showed high contamination by fungal species unrelated to the objectives of the study.

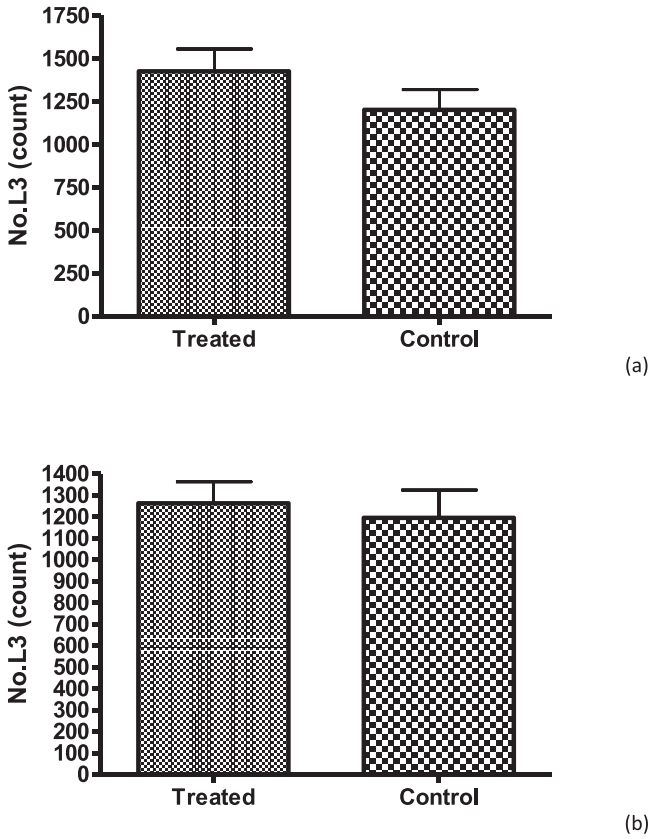

Fig. 2. Mean number of infective larvae (L3) per gram of ovine feces recovered in coprocultures treated and untreated with 1 g of BCP per coproculture. Coprocultures untreated (control) and treated with batch 000821 (a) and batch 2915 (b) of BCP.

3.2. Efficacy of BCP after passage through the gastrointestinal tract of sheep

The FEC and L3 values observed for BCP Batch 000321 (Fig. 4) and for batch 000821 (Fig. 5) in periods 2 and 3 (during and after administration of the product) showed variability within the predicted range for period 1 (pre-administration of the product). On day 1 of the period 1 there was a recorded count equal to 2700 EPG that was statistically an outlier (Fig. 4).

The variability exhibited as the confidence intervals for predicted counts (EPG and LPG) on periods 2 and 3 were high and encompassed the predicted variability for period 1, which reduced the likelihood for detectable differences among periods. The L3 counts were not zeroed or significantly reduced in any period, (Fig. 4 and Fig. 5).

Larval identification revealed that the genera of GIN present were *Haemonchus* (85 %), *Trichostrongylus* (13 %) and *Cooperia* (2 %). Chlamydospore quantification in feces was 0 CPG in both BCP batches during the seven days of analysis (days 06–12 of the experimental period).

The results from fecal plating (Table 1) showed that *A. flagrans* was present only in 4/10 and 1/10 plates at days 07 and 08, respectively, for batch 000321, and only in 1/8 and 4/8 plates at days 8 and 9, respectively, for batch 000821.

Interestingly, *Arthrobotrys musiformis* was isolated from one Petri dish from day 07 of the experimental period with batch 000821.

4. Discussion

Arthrobotrys flagrans is the most extensively studied nematophagous fungus and has been considered a promising candidate for the biocontrol of gastrointestinal nematodes in livestock since the early 1990s (Larsen

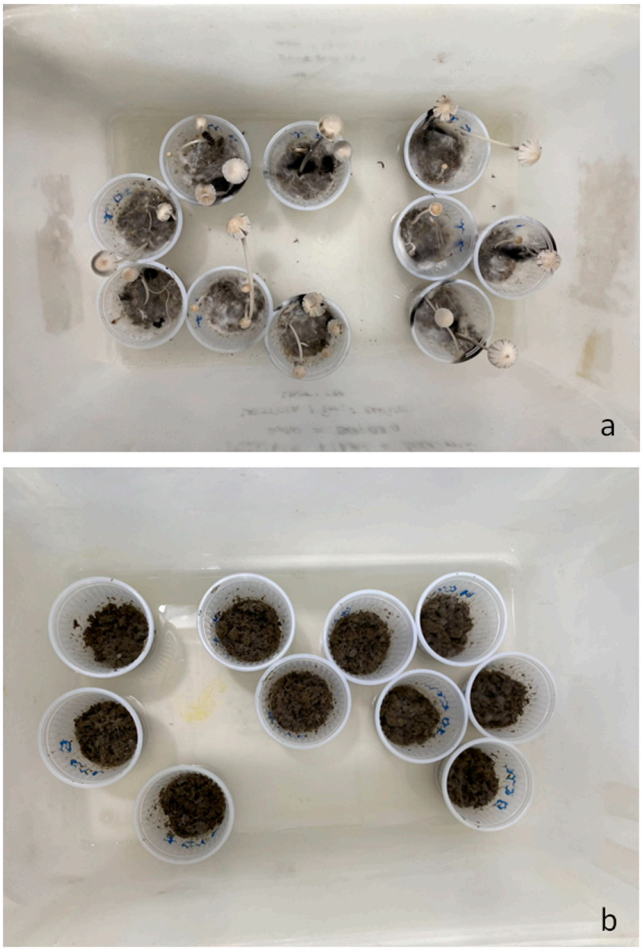

Fig. 3. (a) Coprocultures treated with BCP Batch 2915 showing macroscopic fungal growth. (b) Untreated, control coprocultures.

et al., 1991, 1992; Peloille, 1991). Numerous subsequent studies have reinforced its potential (Larsen, 2000, 2006; Braga and Araujo, 2014; Junco et al., 2023), leading to the development of two commercial products based on this fungus: BioVerm® (*A. flagrans* AC001), marketed in Brazil, and BioWorma® (*A. flagrans* NCIMB 30336), available in Australia, New Zealand, and Europe. In the present study, BioVerm® was not effective in controlling gastrointestinal nematodes in sheep, either after gastrointestinal transit or when tested *in natura*. These findings may be associated with quality control issues in the product formulation.

The survival of fungal formulations after passage through the gastrointestinal tract is a critical feature, as oral administration is the most practical route for delivering such products to animals (Waller and Larsen, 1993; Braga and Araújo, 2014). Chlamydospore concentration in the final product is also directly related to predatory efficacy and must therefore be considered during evaluation (Bird and Herd, 1995; Peña et al., 2002; Waghorn et al., 2003; Terrill et al., 2004; Paraud et al., 2005; Ojeda-Robertos et al., 2008; Mendoza-de-Gives et al., 2018; Sagués et al., 2011).

Prior studies have shown that a concentration of 5×10^5 chlamydospores per gram of *A. flagrans* body weight is effective even after gastrointestinal stress (Peña et al., 2002; Terrill et al., 2004). However, previous studies in sheep indicate a clear dose–response relationship for *A. flagrans,* with doses around or below 1×10^5 chlamydospores/kg body weight resulting in reduced or inconsistent efficacy compared with higher daily doses (Larsen et al., 1998; Larsen and Wolstrup, 1999). BCP is a commercially licensed product containing 5×10^5 chlamydospores per gram of product to treat 10 kg of body weight. The findings of this

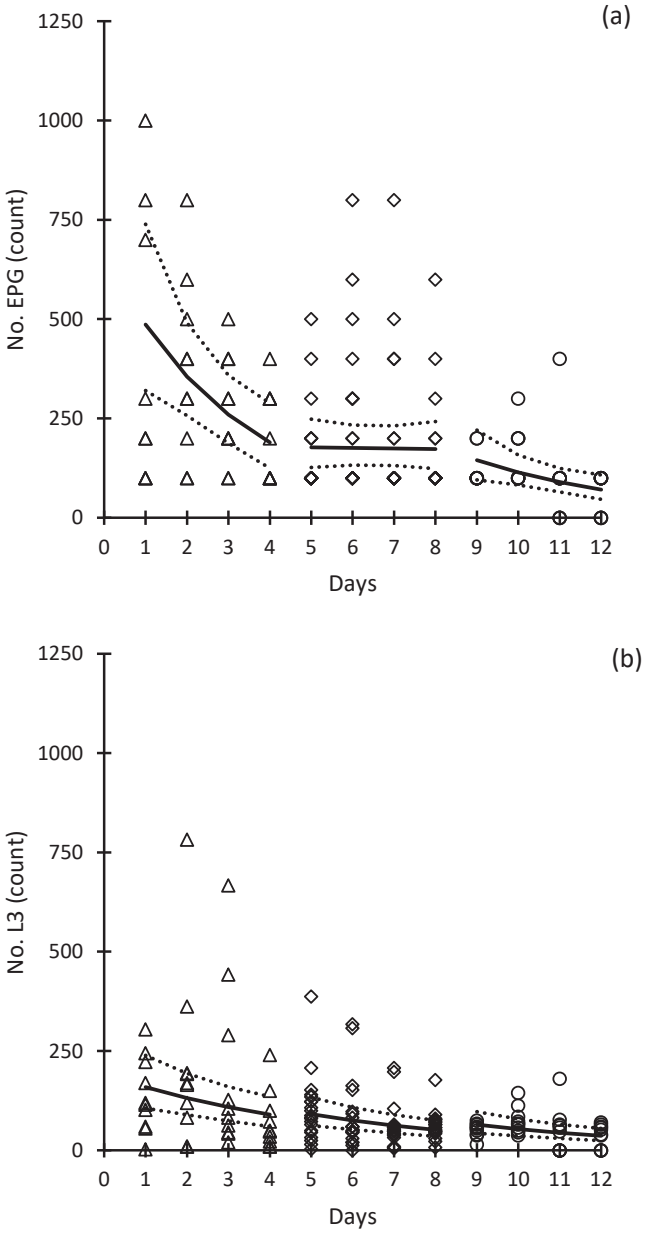

Fig. 4. Fecal egg and larval counts of batch 000321 as a function of the days within experimental periods, namely period 1 (from day one to day four), period 2 (from day five to day eight), and period 3 (from day nine to day 12). a) Fecal egg counts expressed as EPG b) fecal L3 counts expressed as LPG. Solid line: expected mean counts; dotted lines above and below solid lines: 0.95 confidence upper and lower limits, respectively. (triangle shape), (diamond shape) and (circle shape): observed data for the first, second and third experimental periods, respectively.

study contradict previously published data with BCP (Braga et al., 2020; Oliveira et al., 2021; Rodrigues et al., 2021, 2022; Fausto et al., 2021, Mendes et al., 2023; Nunes et al.2023). The three evaluated batches contained substantially lower amounts of chlamydospores than the manufacturer's stated specification, which likely contributed to the very poor performance of the product.

These findings contrast with previous studies reporting the efficacy of the BCP fungal formulation against gastrointestinal nematodes in ruminants (Braga et al., 2020; Oliveira et al., 2021; Rodrigues et al., 2021, 2022) horses (Fausto et al., 2021, Nunes et al. 2023), and buffaloes (Mendes et al. 2023). Notably, Braga et al. (2020), Rodrigues et al. (2021) and Mendes et al. (2023) also assessed the product's performance

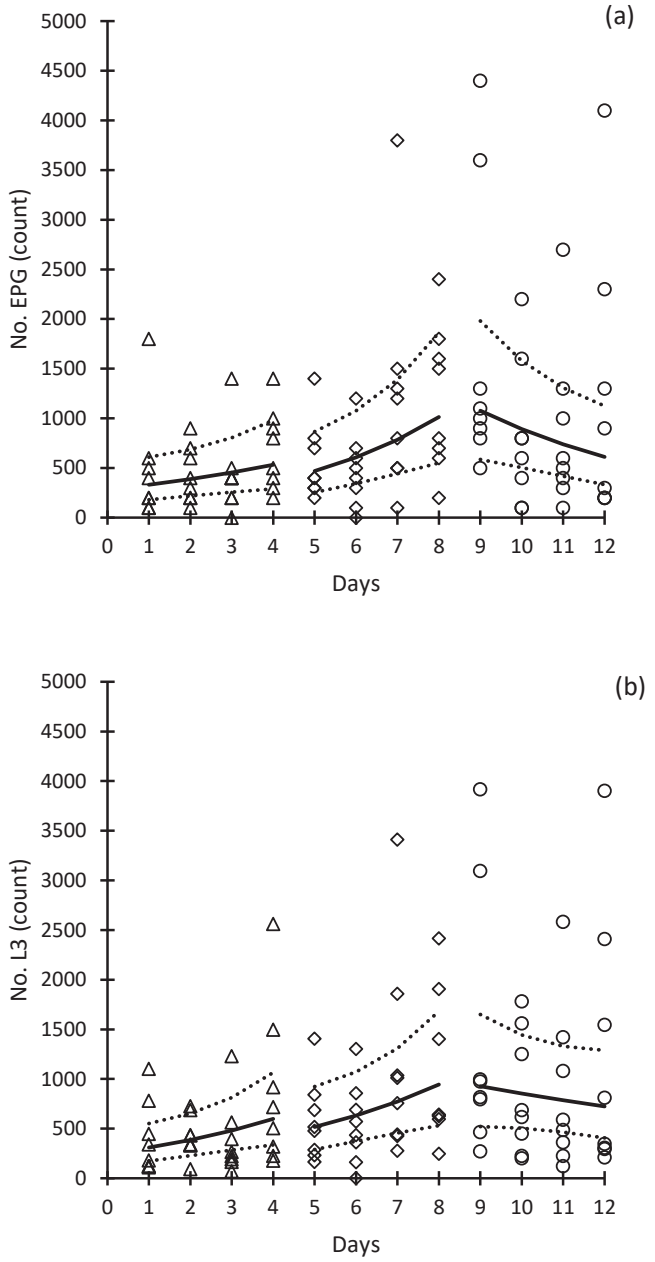

Fig. 5. Fecal egg and larval counts of batch 000821 as a function of the days within experimental periods, namely period 1 (from day one to day four), period 2 (from day five to day eight), and period 3 (from day nine to day 12). a) Fecal egg counts expressed as EPG b) fecal L3 counts expressed as LPG. Solid line: expected mean counts; dotted lines above and below solid lines: 0.95 confidence upper and lower limits, respectively. (triangle shape), (diamond shape) and (circle shape): observed data for the first, second and third experimental periods, respectively.

after gastrointestinal passage. In those studies, however, only a single dose was administered, whereas in the present study, treatment was carried out over four consecutive days. Additionally, a broader range of methods was used here to detect the fungus and evaluate its effects. Although the research objectives were similar, differences in outcomes may be attributable to batch variability or methodological differences.

In this study, BCP did not demonstrate the expected efficacy following passage through the gastrointestinal tract of sheep, as no significant differences were observed in L3 recovery before, during, or after treatment. Additionally, the absence of *A. flagrans* chlamydospores in fecal samples suggests that the fungus did not survive digestive transit due to a low concentration in the formulation and potential losses during ruminal passage. Nevertheless, *A. flagrans* was detected in Petri dish cultures from batches 000821 and 000321 in 50 % and 40 % of the samples, respectively, but only on a single day of observation. This indicates that while some fungal elements survived, their quantity was insufficient to significantly reduce larval counts.

The *in natura* evaluation of BCP supported the findings from the gastrointestinal passage tests. The batches contained much lower numbers of chlamydospores than the expected 5×10^5 spores per gram stated on the label, while *A. flagrans* growth was observed in only 33.3 % of the dishes inoculated with batch 000821, no growth was detected in batches 000321 and 2915. Notably, batch 2915 showed an unusually high level of fungal contamination, which had not been observed in previous isolations of nematophagous fungi from soil or feces. Such contamination may have negatively affected the development of *A. flagrans*, potentially through the release of inhibitory metabolites.

No significant reduction in L3 was observed in coprocultures treated with batches 000821 and 2915 compared to controls. Interestingly, Silva et al. (2011) achieved substantial larval reductions using much lower fungal concentrations. Thus, if *A. flagrans* were present at the levels claimed on the BCP label, larval counts should have approached zero.

From an animal health perspective, fungal contamination of the BCP raises important concerns. Certain *Aspergillus* species, such as *A. flavus* and *A. parasiticus*, are known to produce aflatoxins as secondary metabolites (Yagudayev and Ray, 2024). Aflatoxins are mycotoxins frequently detected in food and feed. In addition, the symbiotic bacterium *Mycetohabitans endofungorum*, associated with the fungus *Rhizopus microsporus*, produces the mycotoxins rhizonin A and B (Partida-Martinez et al., 2007). These compounds have been linked to hepatotoxic, nephrotoxic, carcinogenic, and other adverse effects (Wilson et al., 1984; Pickova et al., 2021; Sui et al., 2022). Genetic analyses aimed at identifying the contaminating fungal species are currently underway and will be presented in a separate publication.

The detection of the nematophagous fungus *Arthrobotrys musiformis* in the feces of one sheep represents a rare finding. Larsen et al. (1994) reported nematophagous fungi in only 3 % of 1742 fresh fecal samples collected from grazing animals. Given the amount of material ingested, the likelihood of animals consuming gut-resistant fungal propagules under natural conditions is considered extremely low.

Although *A. flagrans* is an effective nematophagous fungus for the control of gastrointestinal nematodes, the present results indicate that chlamydospore concentrations in the tested BCP batches were insufficient to reduce L3 counts despite using the recommended dose as stated on the product label and consistent with prior studies. As the therapeutic credibility of any product is intrinsically linked to its efficacy, the quality control measures applied to BCP should be critically reviewed to ensure product consistency and reliable performance in commercial applications.

5. Conclusion

The BCP batches analyzed in this study exhibited chlamydospore concentrations far below the levels stated on the product label, which likely explains the lack of significant larval reduction observed in the coprocultures. These findings underscore the need to implement rigorous quality control measures to prevent the commercialization of batches with inconsistent or substandard fungal content.

CRediT authorship contribution statement

Santos Clovis de Paula: Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Conceptualization. **Maria Vitória Lamóglia Bastos Ferreira:** Writing – review & editing, Investigation. **Edna Barcelos Alves:** Investigation. **Ricardo Augusto Mendonça Vieira:** Formal analysis. **Luis Fonseca Matos:** Investigation. **Caroline Bittencourt Miranda:** Investigation. **Vanessa**

Table 1
Detection of *A. flagrans* in Petri dishes containing water agar inoculated with fecal samples from sheep treated with BioVerm® between days 5 and 8 of the experimental period.

	Experimental days analyzed													
	06		07		08		09		10		11		12	
Petri dish	1	2	1	2	1	2	1	2	1	2	1	2	1	2
1	-	-	+	-	-	+	-	-	-	-	-	-	-	-
2	-	-	+	-	-	-	-	+	-	-	-	-	-	-
3	-	-	+	-	-	-	-	+	-	-	-	-	-	-
4	-	-	+	-	-	-	-	+	-	-	-	-	-	-
5	-	-	-	-	+	-	-	+	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	-		-		-		-		-		-		-	
10	-		-		-		-		-		-		-	

1 = BioVerm® batch 000321; 2 = BioVerm® batch 000821; (–) = negative; (+) = positive. Each plate corresponds to feces from a single animal. Fecal samples were collected from 24 h after the start of treatment until 96 h after its conclusion.

Cola Thomazini: Investigation.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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