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The capability of the fungus *Mucor circinelloides* to maintain parasiticidal activity after the industrial feed pelleting enhances the possibilities of biological control of livestock parasites

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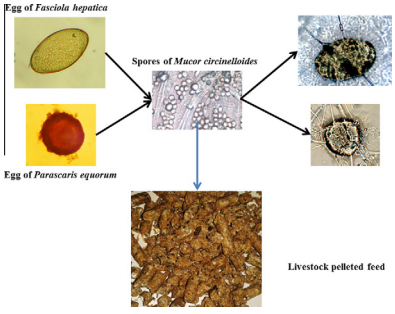
HIGHLIGHTS

- We investigate one possibility for the biological control of livestock parasites.
- We manufacture pelleted feed with spores of the fungus *Mucor circinelloides*.
- Biological development of the fungus did not reduce in the pellets.
- Viability of *Fasciola hepatica* decreased by 55% and *Parascaris equorum* by 65%.

GRAPHICAL ABSTRACT



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ABSTRACT

The ability of the spores of the ovicide fungus *Mucor circinelloides* to resist the industrial manufacturing of pelleted feed and retain their biological and parasitological activities has been tested. Firstly, survival of *M. circinelloides* spores at elevated temperatures was in vitro assayed. In a second assay, the spores of *M. circinelloides* were added in the mixing phase of the industrial pelleting of livestock (calves and horses) feed. The biological development (mycelium growth rates and sporogenesis) and the ovicidal activity on eggs of the parasites *Fasciola hepatica* and *Parascaris equorum* eggs were measured in plates.

In the in vitro assay, a similar level of biological development in all the conditions except by heating the spores at 72 °C for 10 min were observed. Viability of *F. hepatica* eggs reduced to 55–60%, and 56–70% that of *P. equorum* eggs.

After the addition of the spores to the meal previous to the pelletization phase, percentages of reduction of 54–58% viability *F. hepatica* eggs and 61–67% *P. equorum* eggs were recorded.

It is concluded that the spores of *M. circinelloides* maintain their antagonistic effect against eggs of the parasites *F. hepatica* and *P. equorum* in industrially manufactured pellets, providing thus a very helpful tool to prevent infection by trematodes or ascarids among pasturing livestock.

1. Introduction

Certain parasitic infections such as fasciolosis or paramphistomosis are transmitted by ingestion of motionless free-living stages present in the soil or herbage, the metacercarial infective stages. Infected animals shed eggs in the feces, then an embryo called miracidium develops inside, exits off and swims actively to find the intermediate snail host (*Lymnaea* spp.). Once the stages of sporocyst, redia and cercaria are attained, the cercariae leave the snail, swim to herbage and encyst to transform into metacercariae (Rojo-Vázquez et al., 2012). This external phase might take about

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2–4 weeks according to the environmental temperature and moisture (Andrews, 1999).

Eggs of ascarids are passed in the feces of infected animals, and after 3–6 weeks depending on the temperature and moisture, embryonation occurs in the soil and the infective second stage larva is developed. Ascarioses occur when infective eggs (containing the second stage larva) are ingested (Cruz et al., 2012). Because of their outer shell, eggs of ascarids are highly resistant to damage and desiccation in the soil, and can remain viable and infectious for many years (Kim et al., 2012).

Prevention of these parasitoses has involved different strategies. The control of the vector snail population using molluscicides is a well-recognized method for reducing the risk of fasciolosis (Hanif and Singh, 2013). Avoidance of livestock access to snail-infected pasture is frequently advised, but impractical because of the cost of fencing risky areas (Arias et al., 2010). Pasture rotation is recommended for trying to reduce the risk of ascariosis among grazing animals. More recently, the usefulness of some soil fungi as *Pochonia chlamydosporia* against eggs of trematodes (*Fasciola hepatica*) and ascarids (*Toxocara canis*, *Toxocara vitulorum*, *Ascaris suum*) has been reported (Frassy et al., 2010; Carvalho et al., 2010; Ferreira et al., 2011).

Pelleted feed is frequently given to the animals due to this presentation ensures that they receive a well-balanced diet by preventing the selective intake of ingredients. By applying appropriate conditions of moisture, heat and pressure, feed ingredients achieve a certain degree of gelatinization, which allows animals to better utilize the nutrients, and as a consequence feed conversion indexes result significantly improved. Other notable advantage relies on the enhancement of shipping and handling conditions, as well as storage capabilities. With the aim to enhance the fungal distribution in cattle feces, homemade pellets added *Pochonia* mycelium have been successfully tested (Dias et al., 2012).

Mucor circinelloides is a filamentous soil fungus with proven activity against the eggs of certain helminths. In the presence of the eggs of trematodes (*Calicophoron daubneyi*) and/or ascarids (*Baylisascaris procyonis*, *T. canis*), the spores develop a mycelium which adhere to the eggshell, penetrate and eliminate the embryo (Arias et al., 2013a; Cazapal-Monteiro et al., 2015).

Herein it is described an approach to incorporate spores of *M. circinelloides* to pelleted feed during the factory manufacturing. The objective is to provide a helpful tool to ensure the presence of the spores in the feces of cattle passing eggs of *F. hepatica* as well as in the feces of horses shedding *Parascaris equorum* eggs, to reduce their viability and as a consequence their ability to reach the infective stages.

2. Material and methods

2.1. Fungal culture

The current investigation was developed between June 2012 and July 2013. According to Arias et al. (2013a), the isolate CECT20824 of *M. circinelloides* was cultured in a submerged medium (COPFr) (patent Nr PCT/ES2014/070110) for 1.5–2 months at room temperature, until a concentration higher than 1×10^8 spores/L of medium was achieved. The numbers of spores were calculated by means of a cell-counting hematocytometer (Neubauer chamber) and a light microscope.

2.2. Experimental design

Two experiments were conducted in the current investigation. Firstly, resistance of the spores to the temperature conditions of the pellet manufacturing was in vitro assayed, by measuring the biological and parasiticide properties of spores of *M. circinelloides* heated at 72 °C for different intervals. Secondly, the elaboration of pellets containing spores was performed, and then the biological and parasiticide activities of the spores were analyzed.

2.3. Thermal stability of spores (assay 1)

To assess if the elevated temperatures recorded during the industrial pellet manufacturing process could affect the viability of *M. circinelloides* spores, 1 mL aliquots of a water solution containing 2×10^6/L were delivered in 2 mL eppendorf tubes and incubated at 72 °C in a Thermoblock for 10 s, 30 s, 1 min, 2 min, 5 min and 10 min.

After the exposition of the spores to 72 °C during different time intervals (assay 1), they were let to reach room temperature and then poured on one side of Petri dishes with water agar medium (Arias et al., 2013a). The number of replicates (all with the same quantity of spores) for every temperature and time interval was 20, as well as the plates with non-heated *M. circinelloides* spores (Time = 0 s) which served as controls. The dishes were incubated at room temperature in the dark during 20 days.

Mycelial growth was measured every 4 days, by examining 3 plates of each group under the microscope at 40–100× magnification. At the same time, eight 2-cm^2 square circles were drawn on the bottom of each plate for estimating the numbers of spores (Arias et al., 2013b).

The parasiticide activity of the spores of *M. circinelloides* was individually assayed on eggs of *F. hepatica* and *P. equorum*. For the collection of *F. hepatica* eggs, gall-bladders were taken from cattle slaughtered at a local abattoir and then opened in the laboratory. For obtaining *P. equorum* eggs, feces of horses passing 2350 eggs per gram were repeatedly processed by the flotation technique with saline solution until obtaining a clean solution (de Carvalho et al., 2014).

Aliquots containing spores previously heated at 72 °C were put in the center of water agar plates, and then 200 eggs (*F. hepatica* or *P. equorum*) simultaneously added. Six replicates were observed for each temperature and time interval.

According to Lỳsek et al. (1982) ovicidal activity is classified into type 1 (hyphae attached to the eggshell without morphological damage); type 2 (hyphal penetration and morphological alteration of embryo) and type 3 (destruction of the eggs). After the observation under a microscope at a 40× magnification, in the current study non-viable eggs were considered those presenting type 2 or type 3 ovicidal effects (Fig. 1).

2.4. Pellet manufacturing (assay 2)

Pelleted feed commercially available for raising calves (*Recria18*®, Nanta, Padrón, Spain) and for horses maintenance (*Forequus*®, Nanta, Padrón, Spain) containing cereal grains and byproducts, oil seeds and derivatives, sugar cane processing byproducts, minerals, forages and amino acids, were utilized in the current investigation.

The analytical composition of the calves feed comprises crude protein (18.5%), crude fat (3.3%), crude fibre (8.6%), crude ash (7.4%), Calcium (0.8%), Phosphorus (0.48%), Sodium (0.34%), Magnesium (0.54%), vitamin A (20,000 UI/kg), vitamin D3 (2750 UI/kg) and vitamin E (45 UI/kg).

The horse feedstuff was composed by crude protein (14%), crude fat (2.9%), crude fibre (12.5%), Calcium (1.5%), Phosphorus (0.65%), Sodium (0.53%), Magnesium (0.54%), vitamin A (10,000 UI/kg), vitamin D3 (1500 UI/kg) and vitamin E (42 UI/kg).

In each case, one batch of concentrate was elaborated with fungal spores. After milling the feed ingredients, a total volume

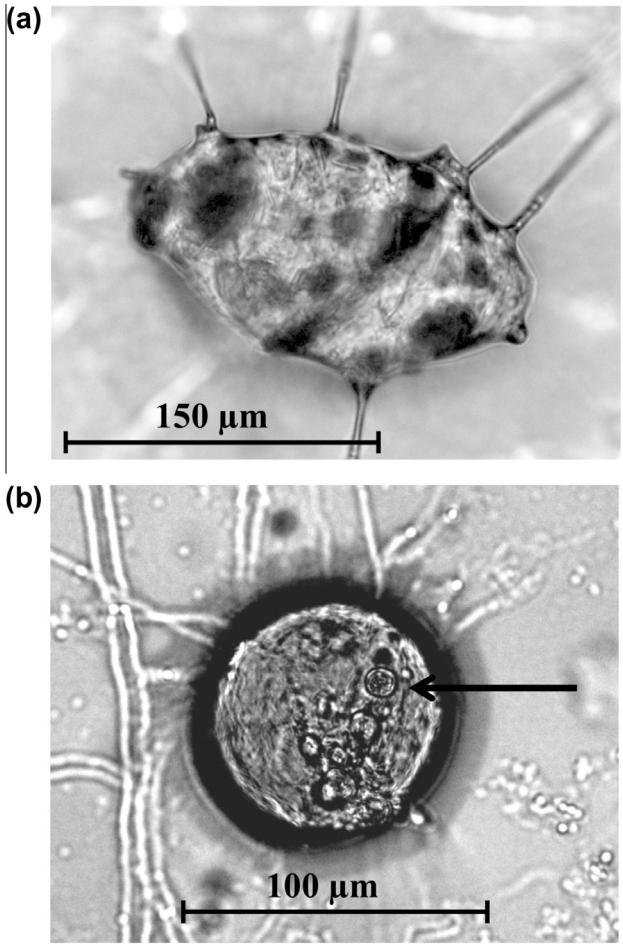

Fig. 1. Non-viable eggs of parasites. (a) *Fasciola hepatica*; (b) *Parascaris equorum*. Black arrow points the presence of spores inside the egg.

of 15–20 L fungal medium was added per ton of meal in the feed mixer to obtain a final concentration of 2×10^6 spores/kg. The complete blend was conditioned by injecting steam (70–75 °C for 90 s) and then put through the pelletizer. The final product was cooled, dried, packed into 30 bags of 40 kg for the calves and 45 bags of 25 kg for the horses, and finally stored in a pile in a farm avoiding sunlight. All the bags were exposed to the same conditions. Due to the assay was developed between August and January, storage temperatures ranging from 28 °C to −4 °C were recorded.

The biological activity of the pellets containing *M. circinelloides* spores was assessed by conducting a spore viability test. One gram of pellets with spores was grinded with 10 mL water in a mixer until their disintegration, and then 1 mL of this solution spread on water-agar plates. A total of 24 plates were prepared and incubated at room temperature in the dark. Every 4 days for a total of 20 days, mycelial growth and sporogenesis (production of spores) were measured as previously explained.

The parasiticide activity of the spores enclosed in the pellets was tested by spreading 1 mL of a pellet/water solution (see above) into water–agar plates. Twenty-four replicates were performed, 12 were added 200 eggs of *F. hepatica* and the other 12 were placed 200 eggs of *P. equorum*. One lot of 12 Petri dishes was put 1 mL water solution with pellets without spores, and then eggs of parasites were also added. Other set of 24 plates without spores was also placed 200 eggs/plate as controls for each parasite.

By taking into account that quality of pelleted feeds is guaranteed for 3 months by the manufacturer, the biological and parasiticide activity of the fungal spores included in the pellets was measured the day of their elaboration (Time = 0, controls), 1, 3 and 6 months after. Twenty-four replicates were observed for each interval.

2.5. Determination of efficacy

After a period of incubation of 28 days at room temperature and darkness, the content of the plates was harvested by means of the sedimentation technique (plates added *F. hepatica* eggs) and flotation test (plates added *P. equorum* eggs).

The efficacy of *M. circinelloides* against the parasite eggs was measured by comparing the numbers of viable eggs recovered from plates with and without fungal spores, as follows:

$$\%\text{Viability reduction} = [1 - (\text{mean viable eggs}_{\text{day0}} / \text{mean viable eggs}_{\text{day28}})] \times 100$$

2.6. Stability of pelleted feed

The physical stability of the pellets fabricated with *M. circinelloides* spores was established by visual examination looking for signs of degradation (brittleness, fragmentation, chipping) or unusual appearance (surface hyphal growth, abnormal coloration, odor) (Thomas and van der Poel, 1996).

2.7. Statistical analysis

After running the Kolmogorov–Smirnov test, it was ascertained that data do not have a normal distribution ($P < 0.05$). Thus, the non-parametric Kruskal–Wallis and Mann–Whitney *U* tests were performed at a significance level of $P < 0.05$. The Levene's test was applied for demonstrating the homogeneity of variances ($P < 0.05$).

All tests were performed with the statistical package SPSS, version 20 (IBM SPSS Inc., Chicago, IL, USA).

Data in the figures were presented as the mean and the standard deviation for making possible the discussion with other investigations.

3. Results

3.1. In vitro exposure of M. circinelloides spores to different temperatures

3.1.1. Biological activity

In the control plates and in those added spores previously heated for ⩽5 min, hyphal growth was observed after 4 days, and sporogenesis from the 8th day (Figs. 2 and 3). By opposite, mycelial growth was slower and irregular in the plates added spores heated during 10 min, and *M. circinelloides* spores were barely produced (Fig. 3).

Significant differences in the hyphal development and in the numbers of spores were observed among all the groups and that containing spores heated during 10 min at 72 °C only ($\chi^2 = 28.765$, $P = 0.001$, and $\chi^2 = 31.036$, $P = 0.001$, respectively).

3.1.2. Parasiticide activity

Table 1 summarizes data obtained on *Fasciola* eggs. In the assay 1, the percentages of viable eggs reduced between 63% and 21%. Significantly lower values both in the controls and in the plates spread spores maintained at 72 °C for 10 min were achieved.

In respect to the viability of *P. equorum*-eggs (Table 1), the percentages of reduction ranged from 66% to 11%. Viability of *P. equorum*-eggs was statistically higher in the control plates and in that cultured with spores maintained at 72 °C for 10 min.

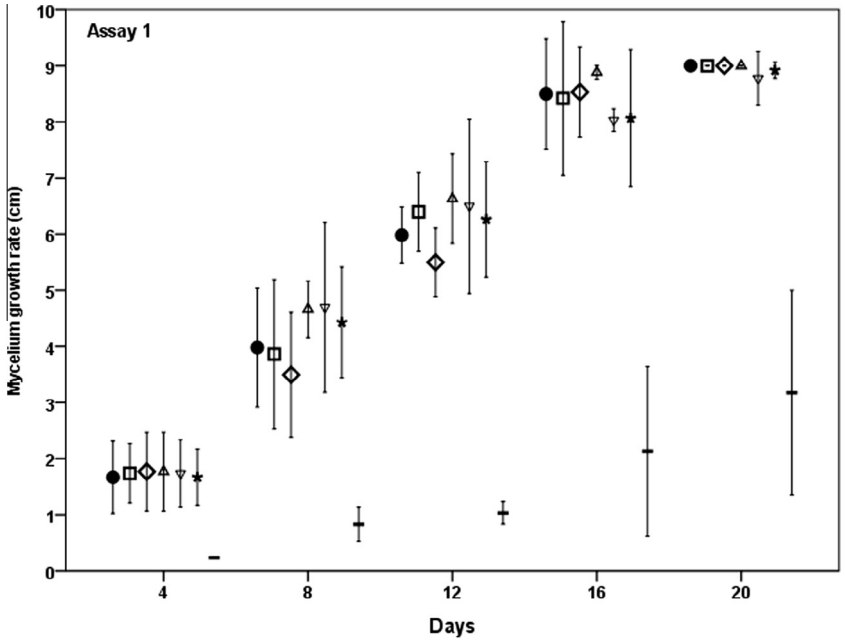

Fig. 2. Mycelial growth rates of *Mucor circinelloides* (in cm) in plates by using spores obtained in a submerged culture. Results are the mean plus 2 SD. (●): controls (0 s); (□): exposed to 72 °C for 10 s; (◇): 30 s; (△): 1 min; (▽): 2 min; (★): 5 min; (—): 10 min.

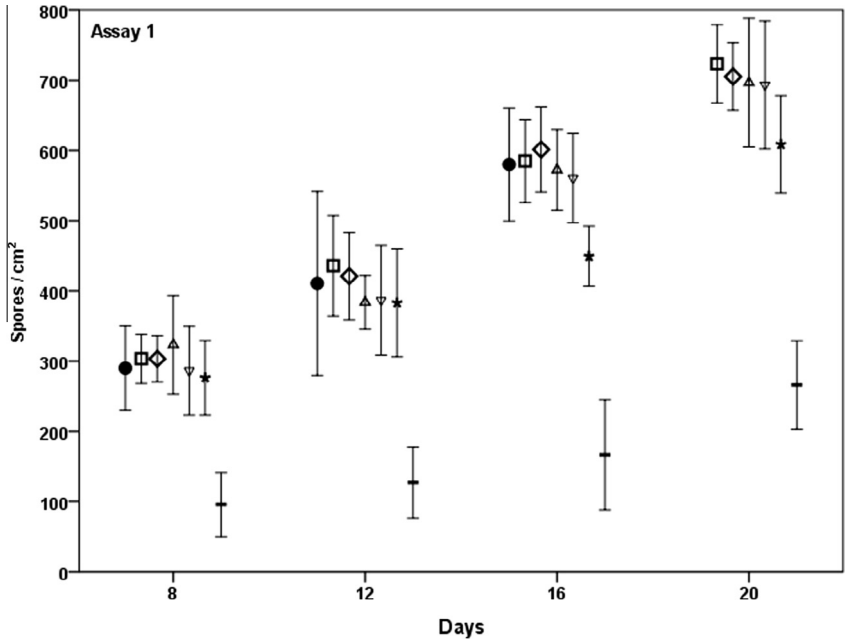

Fig. 3. Sporogenesis (production of spores) in plates added spores of *Mucor circinelloides* obtained in a submerged culture. Results are the mean plus 2 SD. (●): controls (0 s); (□): exposed to 72 °C for 10 s; (◇): 30 s; (△): 1 min; (▽): 2 min; (★):5 min; (—): 10 min.

3.2. Pellets containing spores of M. circinelloides

3.2.1. Biological activity

As shown in Fig. 4, hyphal growth was observed in the plates 4 days after placing pellets added *M. circinelloides* spores, and a significant increment until the 20th day was recorded. Sporogenesis was recorded from the 8th day (Fig. 5). No mycelium growth or production of spores was detected in the control plates receiving pellets without fungal spores.

No statistical differences were demonstrated in the growth rates ($\chi^2 = 1.485$, $P = 0.686$) or the production of spores ($\chi^2 = 0.060$, $P = 0.996$) regarding the storage period.

3.2.2. Parasiticide activity

Reduction of viability of *F. hepatica*-eggs in the assay 2 was 54–58% (Table 1).

The viability of *P. equorum*-eggs reduced to 61–67% (Table 1). No statistical differences were demonstrated in the percentages of reduction of viable eggs of *F. hepatica* or *P. equorum* ($P < 0.05$) regarding the length of storage.

3.2.3. Stability of pelleted feed

The evaluation of the pellets (with and without *M. circinelloides* spores) for 6 months after their manufacturing revealed no evidence of damaged consistency or abnormal appearance.

Table 1
Ovicidal activity of the fungus *Mucor circinelloides* on the eggs of *F. hepatica* and *P. equorum* 28 days after using spores obtained in a submerged culture (assay 1) or added in pellets (assay 2).

Time at 72 °C	Fasciola hepatica viable eggs			Parascaris equorum viable eggs		
	Count	% Reduction	95% Confidence interval	Count	% Reduction	95% Confidence interval
Assay 1: Spores of Mucor circinelloides in a submerged culture						
0 s	77	62	55, 68	68	66	60, 73
10 s	75	63	56, 69	69	65	59, 72
30 s	90	55	48, 62	75	62	55, 69
1 min	85	58	51, 64	71	64	58, 71
2 min	84	58	51, 65	76	62	55, 69
5 min	84	58	51, 65	78	61	54, 68
10 min	159	21	15, 26	178	11	7, 16
Controls without spores	180	10	10, 19	184	8	4, 12
Assay 2: Spores of Mucor circinelloides in pelleted feed						
0 months	85	58	51, 64	66	67	60, 74
3 months	91	55	48, 61	74	63	56, 70
6 months	93	54	47, 60	79	61	54, 67
Pellets without spores	178	11	14, 24	188	6	3, 9

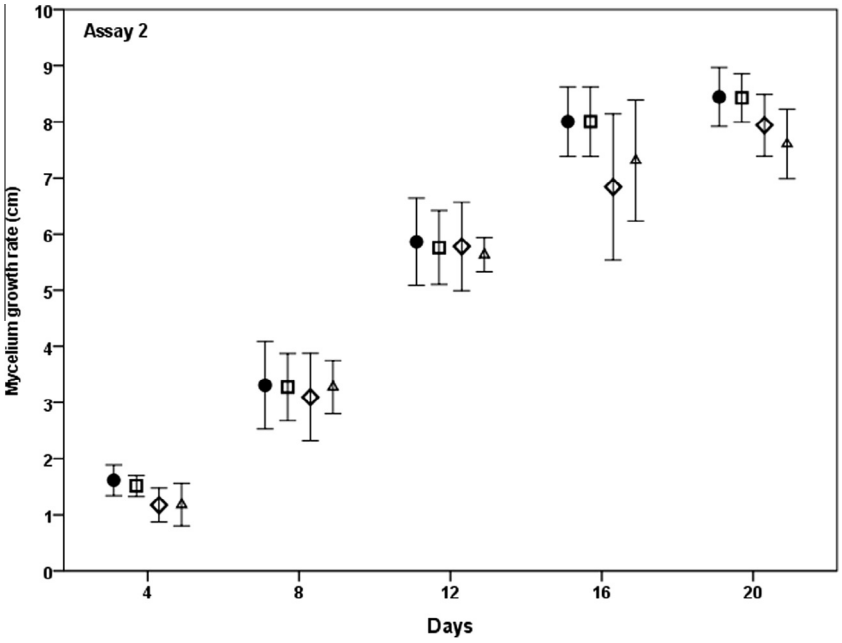

Fig. 4. Mycelial growth rates of *Mucor circinelloides* (in cm) in plates by using pellets manufactured with spores. Results are the mean plus 2 SD. (●) (controls): the day of manufacturing; (□): 1 month after; (◇): 3 months after; (△): 6 months after.

4. Discussion

The possibility of the industrial manufacturing of pellet feed for cattle and horses which contain spores of *M. circinelloides* has been demonstrated. The assays performed in the current research showed the spores enclosed in the pellets retained their biological characteristics, developed their mycelia and reduced the viability of *F. hepatica* eggs by 54–58%, and of *P. equorum* eggs by 61–67%. There are few studies about the factory manufacturing of pellets with spores of parasiticide fungus. Recently, Arias et al. (2015) demonstrated that this procedure does not affect the survival of the chlamydospores of the nematophagous fungus *Duddingtonia flagrans*, and their parasiticidal properties against nematode larvae were maintained.

Prior investigations involving the handmade manufacturing of pellets with fungal spores and/or biomass have been conducted against vegetal or animal parasites. By means of pellets prepared with isolates of *Trichoderma* spp. and *Gliocladium virens*, Lewis and Papavizas (1985) pointed a reduction in the survival of *Rhizoctonia solani* in infested beet seed in natural soil. There are no previous studies about *M. circinelloides*, but Fitz-Aranda et al. (2013) showed a reduction of 67–82% *Haemonchus contortus* larvae by enclosing the chlamydospores of *D. flagrans* into freshly handmade pellets.

Failure of anthelmintics efficacy, together with the search for healthy food, is increasing the farmers awareness for applying preventive measures to livestock rearing. Prevention of infection by *F. hepatica* is currently focused on the direct action on the snails serving as intermediate hosts (*Lymnaea* spp.), or on the ground for limiting their survival possibilities. Some biological molluscicides with notable activity have been reported (Hanif and Singh, 2013), but there is no agreement in the appropriate way for their distribution. Other point needing further consideration is that most of these substances do not provide a selective effect, and both parasitized and non-parasitized snails can be eliminated. On the other hand, eggs of ascarids can remain viable and infective for months and even years under suitable conditions, thus most common actions to avoid livestock infection rely on adequate hygiene and frequent deworming to reduce the parasitic burden in the soil.

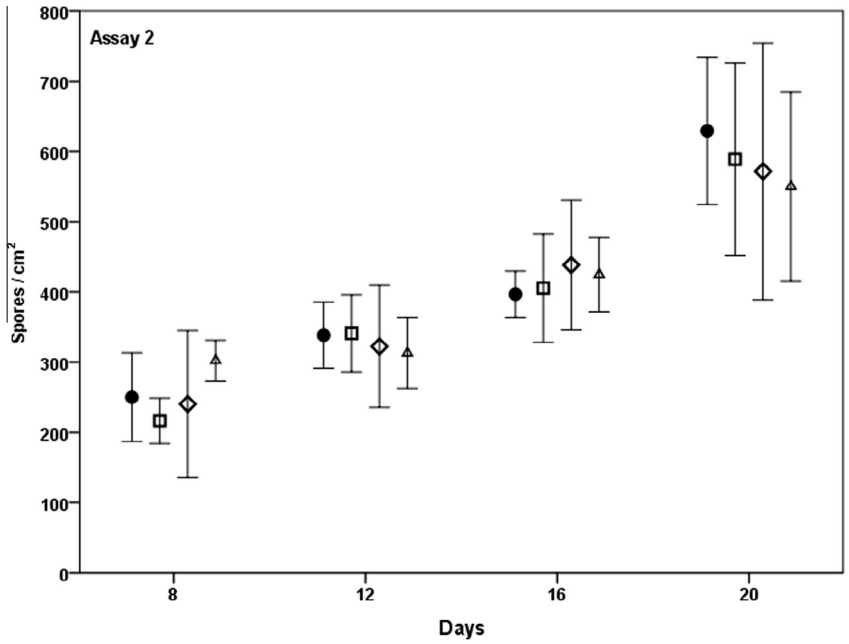

Fig. 5. Sporogenesis (production of spores) in plates by using pellets manufactured with spores. Results are the mean plus 2 SD. (●) (controls): the day of manufacturing; (□): 1 month after; (◇): 3 months after; (△): 6 months after.

One interesting approach insufficiently investigated for preventing infection by helminths as ascarids or trematodes could be to reduce the viability of their eggs, inhibiting thus the appearance of the infective stages and in this way the risk of infection. Depending on climatic conditions, the eggs of *P. equorum* excreted in the feces of horses develop to the infective stage after a period of 3–6 weeks. In the present research, eggs of *P. equorum* exposed during 4 weeks (28 days) to spores of *M. circinelloides* involved in pellets lessened their viability by 61–67%.

The eggs of *F. hepatica* passed in the feces are undeveloped and embryonation takes 2–4 weeks at 23–26 °C or more if lower temperatures occur (Andrews, 1999). In the current investigation, viability of *F. hepatica* eggs diminished by 60% after their exposure for 4 weeks to *M. circinelloides* spores contained in industrially manufactured pellets. These results agree with data collected by means of handmade pellets added mycelium of the ovicide fungus *P. chlamydosporia* (Dias et al., 2013).

Despite the undeniable usefulness of the elaboration of handmade pellets with fungal spores to improve the distribution of parasicide fungi, certain disadvantages can be identified. Firstly, it seems problematic to ensure the homogeneity of the final product, which could lead to unexpected results and then to a misinterpretation of the results achieved. Secondly, the manual elaboration of pellets increases the routine tasks of the animal keepers, limiting their practical utilization. Other point (third) to take into account consists of preservation of the feedstuff. Previous studies performed with *D. flagrans* provided no differences when chlamydospores were exposed to indoors, outdoors or refrigeration conditions for 2 months (Fitz-Aranda et al., 2013). Nevertheless, spore germination and fungal growth can occur in handmade pellets due to an important level of residual moisture remains after being sun dried. The presence of hyphae around the pellets might lead to farmers to think about a possibly dangerous contamination, limiting their administration (Chandrawathani et al., 2003).

In view of the achieved results, it is determined that industrial manufacturing of pelleted feed with spores of *M. circinelloides* provides a helpful solution to the aforementioned problems due to (a) the spores are mechanically mixed with the meal and thus an homogeneous product is obtained; (b) no additional job is required to the animal keepers, which ensures their utilization; (c) industrially manufactured pellets contain a residual moisture below 12%. The visual examination of these pellets did not show any sign of degradation, alteration, abnormal odor or fungal growth. Furthermore, in the current research no differences regarding the storage interval of pellet feed containing *M. circinelloides* spores have been recorded for up 6 months (October–March). It is striking to point that most of manufacturers warrant the quality of the final product for 3 months.

5. Conclusions

This is the first analysis of the capability of an ovicide fungus to maintain its parasitic activity after the industrial manufacturing of pelleted feed. Notable percentages of viability reduction of eggs of trematodes (*F. hepatica*) and ascarids (*P. equorum*) have been demonstrated. The absence of adverse influence of routinely storage conditions has also been verified. Further studies are in progress to determine the efficacy of this procedure under field conditions.

6. Conflict of interest

The final article has been approved by all authors, whose assert the absence of any financial or personal interests that could improperly influence the present paper.

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References

Andrews, S.J., 1999. The life cycle of *Fasciola hepatica*. In: Dalton, J.P. (Ed.), Fasciolosis. CABI Publishing, Wallingford (UK), pp. 1–29.

Arias, M., Piñeiro, P., Hillyer, G.V., Suárez, J.L., Francisco, I., Cortiñas, F.J., Díez-Baños, P., Morrondo, P., Sánchez-Andrade, R., Paz-Silva, A., 2010. An approach of the laboratory to the field: assessment of the influence of cattle management on the seroprevalence of fascioliasis by using polyclonal- and recombinant-based ELISAs. J. Parasitol. 96, 626–631.

Arias, M.S., Suárez, J., Cazapal-Monteiro, C.F., Francisco, I., López-Arellano, M.E., Piñeiro, P., Suárez, J.L., Sánchez-Andrade, R., Mendoza de Gives, P., Paz-Silva, A., 2013a. Trematodes enhance the development of the nematode-trapping fungus *Arthrobotrys* (*Duddingtonia*) *flagrans*. Fungal Biol. 117, 540–544.

Arias, M.S., Cazapal-Monteiro, C.F., Suárez, J., Miguélez, S., Francisco, I., Arroyo, F.L., Suárez, J.L., Paz-Silva, A., Sánchez-Andrade, R., Mendoza de Gives, P., 2013b. Mixed production of filamentous fungal spores for preventing soil-transmitted helminth zoonoses: a preliminary analysis. Biomed. Res. Int. http://dx.doi.org/10.1155/2013/567876.

Arias, M.S., Arroyo, F.L., Cazapal-Monteiro, C., Hernández, J.A., Suárez, J., Francisco, I., López-Arellano, M.E., Sánchez-Andrade, R., Mendoza de Gives, P., Paz-Silva, A., 2015. Formulating *Duddingtonia flagrans* in nutritional pellets for the sustainable control of equine strongyles. J. Sci. Technol. Environ. 5, 1–16.

Carvalho, R.O., Araújo, J.V., Braga, F.R., Araújo, J.M., Alves, C.D., 2010. Ovicidal activity of *Pochonia chlamydosporia* and *Paecilomyces lilacinus* on *Toxocara canis* eggs. Vet. Parasitol. 169, 123–127.

de Carvalho, L.M., Braga, F.R., Domingues, R.R., Araujo, J.M., Lelis, R.T., de Paula, A.T., da Silveira, W.F., de Araújo, J.V., 2014. Interaction of the nematophagous fungus *Pochonia chlamydosporia* and *Parascaris equorum* eggs in different culture media. J. Basic Microbiol. 54 (Suppl 1), S109–S114.

Cazapal-Monteiro, C.F., Hernández, J.A., Arroyo, F.L., Miguélez, S., Romasanta, Á., Paz-Silva, A., Sánchez-Andrade, R., Arias, M.S., 2015. Analysis of the effect of soil saprophytic fungi on the eggs of *Baylisascaris procyonis*. Parasitol. Res. 114, 2443–2450.

Chandrawathani, P., Jamnah, O., Adnan, M., Waller, P.J., Larsen, M., Gillespie, A.T., 2003. Field studies on the biological control of nematode parasites of sheep in the tropics, using the microfungus *Duddingtonia flagrans*. Vet. Parasitol. 120, 177–187.

Cruz, L.M., Allanson, M., Kwa, B., Azizan, A., Izurieta, R., 2012. Morphological changes of *Ascaris* spp. eggs during their development outside the host. J. Parasitol. 98, 63–68.

Dias, A.S., Araújo, J.V., Braga, F.R., Araújo, J.M., Puppin, A.C., Fernandes, F.M., Ramos, R.F., Bertonceli, R.M., da Silva, R.G., Perboni, W.R., 2012. Biological control of *Fasciola hepatica* eggs with the *Pochonia chlamydosporia* fungus after passing through the cattle gastrointestinal tract. Parasitol. Res. 110, 663–667.

Dias, A.S., Araújo, J.V., Braga, F.R., Puppin, A.C., Perboni, W.R., 2013. *Pochonia chlamydosporia* in the biological control of *Fasciola hepatica* in cattle in Southeastern Brazil. Parasitol. Res. 112, 2131–2136.

Ferreira, S.R., Araújo, J.V., Braga, F.R., Araújo, J.M., Carvalho, R.O., Silva, A.R., Frassy, L. N., Freitas, L.G., 2011. Ovicidal activity of seven *Pochonia chlamydosporia* fungal isolates on *Ascaris suum* eggs. Trop. Anim. Health Prod. 43, 639–642.

Fitz-Aranda, J.A., Mendoza-de-Gives, P., Torres-Acosta, J.F., Liébano-Hernández, E., López-Arellano, M.E., Sandoval-Castro, C.A., Quiroz-Romero, H., 2013. *Duddingtonia flagrans* chlamydospores in nutritional pellets: effect of storage time and conditions on the trapping ability against *Haemonchus contortus* larvae. J. Helminthol. 16, 1–6.

Frassy, L.N., Braga, F.R., Silva, A.R., Araújo, J.V., Ferreira, S.R., Freitas, L.G., 2010. Destruction of *Toxocara canis* eggs by the nematophagous fungus *Pochonia chlamydosporia*. Rev. Soc. Bras. Med. Trop. 43, 102–104 (Portuguese).

Hanif, F., Singh, D.K., 2013. Binary Combination of *Carica papaya*, *Areca catechu* and *Myristica fragrans* with Piperonyl Butoxide/MGK-264 against Freshwater Snail *Lymnaea acuminata*. Trop. Life Sci. Res. 24, 1–11.

Kim, J.S., Oh, D.S., Ahn, K.S., Shin, S.S., 2012. Effects of kimchi extract and temperature on embryostasis of *Ascaris suum* eggs. Korean J. Parasitol. 50, 83–87.

Lewis, J.A., Papavizas, G.C., 1985. Characteristics of alginate pellets formulated with *Trichoderma* and *Gliocladium* and their effect on the proliferation of the fungi in soil. Plant. Pathol. 34, 571–577.

Lýsek, H., Fassatiová, O., Cuervo Pineda, N., Lorenzo Hernández, N., 1982. Ovicidal fungi in soils of Cuba. Folia Parasitol. (Praha) 29, 265–270.

Rojo-Vázquez, F.A., Meana, A., Valcárcel, F., Martínez-Valladares, M., 2012. Update on trematode infections in sheep. Vet. Parasitol. 189, 15–38.

Thomas, M., van der Poel, A.F.B., 1996. Physical quality of pelleted animal feeds. 1. Criteria for pellet quality. Anim. Feed Sci. Technol. 61, 89–112.