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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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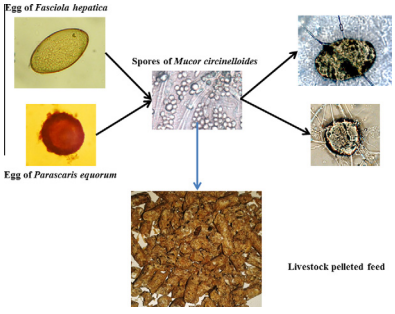
HIGHLIGHTS

- 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