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TESE DE DOUTORAMENTO

COMMERCIAL FORMULATIONS FOR THE PARASITICIDE FUNGUS *Mucor circinelloides*

JOSÉ ÁNGEL HERNÁNDEZ MALAGÓN

ESCOLA DE DOUTORAMENTO INTERNACIONAL

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USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

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RESEARCH STAY

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A QUIEN PUEDA INTERESAR

Los abajo firmantes, Docentes de Enfermedades Parasitarias en la Facultad de Medicina Veterinaria y Zootecnia de la Universidad de Ciencias Aplicadas y Ambientales (U.D.C.A., Bogotá, Colombia)

INFORMAN

Que D. José Ángel Hernández Malagón, alumno de Doctorado de la Universidad de Santiago de Compostela (USC, España) ha realizado bajo nuestra supervisión una estancia de investigación de **3 meses** de duración (1 abril a 30 de junio de 2015), con el tema "DESARROLLO DE UN BIOCIDA PARA EL CONTROL DE ZOONOSIS". Durante este periodo, el doctorando ha tenido oportunidad de familiarizarse con los procedimientos rutinarios de identificación de garrapatas, inducción a la ovoposición y diseño de ensayos para la evaluar la eficacia de una estrategia de Control Biológico basada en el empleo de hongos saprofitos con actividad parasiticida.

En todo momento, el grado de aprovechamiento ha sido notable, así como el interés en participar en todas las tareas que le han sido encomendadas, y que ha finalizado de forma satisfactoria. Es interesante destacar además que la permanencia del doctorando en nuestra Universidad ha contribuido a estrechar los lazos de colaboración entre ambas instituciones académicas.

Por todo ello firmamos la presente, para que surta los efectos oportunos, en Bogotá (Colombia), a 1 de julio de 2015.

Jorge Alexander León González	Rodrigo Bonilla Quintero
Docente Instructor	Profesor

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FIGURES AND TABLES

All figures and tables included in this PhD dissertation are originals and belong to the author and the COPAR Research Group (GI-2120).

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Con la finalización de esta Tesis Doctoral, se completa una etapa que sirve de punto de partida para explorar otras opciones, plantearse nuevos retos que nos lleven a nuevos proyectos y aventuras. Espero teneros a mi lado en todos ellos, porque siempre será sinónimo de ÉXITO.

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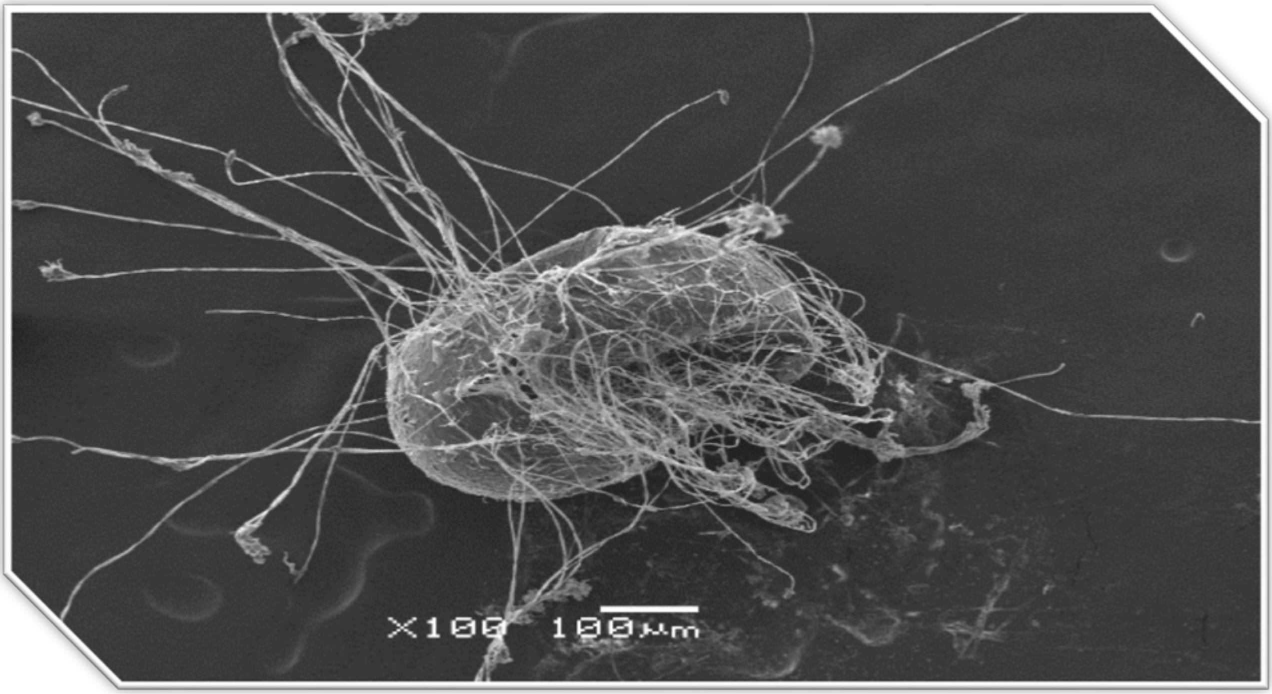
ABBREVIATIONS

EPG	Eggs per gram
ERP	Egg reappearance period
JMP	Joint Monitoring Program
STHs	Soil-Transmitted Helminths
WHO	World Health Organization

1. SUMMARY / RESUMEN / RESUMO



1. SUMMARY / RESUMEN / RESUMO

1.1. SUMMARY

Control of parasites based exclusively on the administration of pharmacological treatments has resulted insufficient for decades. Preventive actions are increasingly required to limit the risk of infection and make it possible to reduce the application of pharmacological therapies to those cases strictly necessary, that is, when health of animals is affected, or their productivity does not reach the minimum levels expected. In addition, there are numerous infections that can be transmitted from animals to humans or vice versa (zoonoses), and because of their involvement in public health, the need for greater control to prevent animals from reaching high intensities of parasitization that pose a risk to people is enhanced.

Over time, different measures emerged to reduce infection in herbivores, such as the management of pastures in which certain animal species with different sensitivity to parasitic infections are interspersed, or the periodic and manual collection of feces, difficult to apply on large parcels. In the case of pets, the tendency consisted of the preventive administration of antiparasitic drugs, together with the development of vaccines that stimulate the immune response against certain parasitic agents. In both cases, the results have not been as expected, and there is currently a need to apply treatments with high frequency, leading to the appearance of strains of resistant parasites in a significant number of cases.

For a long time, it is known the existence of biological agents found naturally in the soil, able to develop antagonistic activity against certain stages of different parasites, so they could be used to carry out preventive actions. These agents promoted the research of

so-called biological control strategies. Some of these procedures are based on the use of bacteria, viruses, mites, nematodes or saprophytic fungi, and their practical application involves overcoming a number of aspects, among which should be noted a selective antiparasitic efficacy, easiness of propagation in affordable culture media and under standard conditions, comfortable and simple distribution, innocuousness over environmental components and marketing possibilities.

To try to respond to these requirements, a research based on the study of the possibilities of application and commercialization of a saprophytic fungus with ovicidal activity, *Mucor circinelloides*, was assembled around five trials. In the first, ***Isolation of ovicidal fungi from faecal samples of captive animals maintained in a zoological park***, deepens in the knowledge that certain saprophytic fungi commonly identified in the soil have antagonistic effects on some parasites that affect animals and humans, can resist the transit through the digestive system of animals and are expelled with feces along with aforementioned parasites. The main objective was to provide information about practical methods for the isolation of parasiticidal fungi, with the aim of describing a simple, assumable and practical procedure. The reason for choosing the fecal matter to select the fungi was based on the fact that, in this way, it could be possible to ensure that they did not undergo alterations when crossing the gastrointestinal tract, and that they did not disturb the animals. Samples of captive wild animals in the Zoological Park "Marcelle Natureza" (Outeiro de Rei, Lugo, Spain) were considered based on the observation that some species of animals were parasitized only by strongyles, gastrointestinal nematodes whose transmission occurs by the ingestion of third instar larvae, mobile phases therefore, while others only showed infection by ascarids or whipworms, which are transmitted by the ingestion of eggs with a larva inside them, that is, immobile phases. A total of 60 soil samples were collected from different plots with vegetation and serially cultured in Petri dishes containing different media, with the aim of obtaining monoclonal cultures that allow isolation and identification of fungi present in those feces.

The fecal samples were analyzed with flotation and sedimentation techniques, and thus it was determined that a total of thirteen animals shed strongyles eggs. Seven fungi with ovicidal activity that are frequently found in the soil were isolated and identified. Later, their antagonistic activity on the eggs of *Calicophoron daubneyi* (trematode) and *Parascaris equorum* (nematode) was analyzed under laboratory conditions. Accordingly, it was proved the ability of the species *Clonostachys* (*Gliocladium*) and *Penicillium* to develop hyphae that adhered to the eggshell without penetrating or affecting it, keeping the parasitic embryo intact and viable, therefore, they were classified as ovicidal fungi type 1. The other five specimens of fungi were identified at the genus level as *Fusarium*, *Lecanicillium*, *Mucor*, *Trichoderma* and *Verticillium*; their hyphae were able to attach to the eggshell of the helminths, and through the formation of an organ known *haustorio*, penetrate and destroy the internal embryo, and consequently they were classified as type 3 ovicidal fungi. From these results it was concluded that isolation of fungi from animal feces is a very useful, practical and affordable method that makes it possible to identify species of interest to develop strategies for the biological control of parasitic forms affecting animals and people.

In experiences of biological control carried out until a few years ago, ovicidal fungi such as *Pochonia chlamydosporium* (= *Verticillium chlamydosporia*) and also larvicides, such as *Duddingtonia flagrans*, have been described. Although there is abundant information about their usefulness, few studies have been performed to test their effectiveness under field conditions, or to get information about interesting and applicable formulations. For this reason, in the second trial ***A combined effort to avoid strongyle infection in horses in an oceanic climate region: rotational grazing and parasiticidal fungi***, the application of a mixture of fungal spores that develop different parasiticidal activity as a feed additive was planned. To achieve this, in a company dedicated to the production of animal products, pelleted feed for horses was added spores of *Mucor circinelloides* and *Duddingtonia flagrans* during the mixing phase. The objective was to know the effect of this formulation on the health condition of horses maintained in rotating pastures, and on their parasitic infection status.

With the formulation of pellets with spores, it was sought to ensure a continuous administration of the fungi together with adequate nutrition. Twenty-two Spanish Sports Horses infected naturally by strongyles were used in this research, so that at the beginning of the investigation they received a pour on treatment of ivermectin, then divided into three groups; group G-1 (6 horses) remained stabled for most of the day and received pellets without spores; G-2 (8 individuals) remained in rotational grazing and received pellets without spores, and G-3 (8 equines) also in rotational grazing and fed pellets with spores. Monitoring of the antiparasitic strategy was carried out through the analysis of feces collected monthly for 13 months, by means of the flotation technique with saturated saline solution. Fecal samples were kept at 22-25ºC for 19 days to determine the genera of nematodes that infected equines.

Specimens of *Cyathostomum* nematodes (*sensu lato*) type A (63%), type C (14%) and type D (15%), *Gyalocephalus capitatus* (1%), *Triodontophorus serratus* (2%), *Poteriosthomum* spp. (2%), *Strongylus vulgaris* (2%) and *S. edentatus* (1%) were identified in the feces. The coprological analyses showed the presence of spores of the fungi in the G-3 two days after starting the administration. The initial deworming was effective in all three groups, but the counts of strongyles eggs increased gradually in the G-1 and in the G-2, showing values higher than 300 eggs per gram of feces (epg) by 3 months post-treatment. In G-3 horses, a progressive increment in fecal egg counts was noted, which remained around 200 epg throughout the study, except for one individual. These results seem to indicate that challenge of equines takes place in 1-1.5 months, due to the ingestion of free-living third stage larvae (L3, infective stages), which originate from strongyle eggs passed by feces. In the group of rotating equines that received pellets without spores (G-2), elimination of eggs of strongyles in feces decreased by 7% at 6 months, by 50% in those individuals that ingested spores of fungi parasiticides (G-3). At the completion of the 12-month rotation, the reduction reached 59%. No signs of adverse effects on respiratory, digestive or reproductive function were detected among horses receiving spores (G-3), and no skin alterations were observed. These results show the innocuousness of the continued ingestion of spores in nutritional pellets, as well as its beneficial effect in the prevention of infection by gastrointestinal nematodes (strongyles) in equines kept in rotational grazing, increasing the utility of this procedure.

In areas where climatic conditions enhance that enough forage is available almost all year, certain livestock species are maintained in small or medium size plots to maintain an acceptable level of brush cleaning, unwanted vegetation, etc. Among the domestic species that take advantage of these meadows, horses, donkeys and sheep are mainly considered. The absence of rotation leads many times to the high contamination of the soil by parasitic forms, thus it is necessary to repeat the pharmacological treatments with excessive frequency, but in many cases the expected effect is not reached. This problem is also observed in zoos, where for reasons of stress, the possibility of alternating different plots is not considered. In order to know the effectiveness of the ovicide fungi *M. circinelloides*, *Verticillium* sp. and *Trichoderma atrobrunneum*, their spores were directly sprayed on the feces of wild animals in captivity (Marcelle Natureza Zoological Park), in a third test entitled ***Biological control of soil transmitted helminths (STHs) in a zoological park by using saprophytic fungi***. In the aforementioned park Marcelle Natureza, feces of lynx (*Lynx lynx*) and dromedaries (*Camelus dromedarius*) were collected and analyzed with the techniques common in these cases of saline flotation, sedimentation and larval migration, evidencing the presence of eggs of *Toxascaris leonina* and *Trichuris* sp. Next, solutions were sprayed with a known concentration of spores of the aforementioned fungi on fecal samples that had tested positive for the presence of the mentioned helminths. The feces treated with spores were divided into two batches, one was placed in Petri dishes under laboratory conditions suitable for the embryonic development of the eggs, and the other in plastic containers that were kept under natural conditions in the field. Thirty days later, under the microscope, a total of 120 eggs were examined in the plates and it was shown that the viability of *T. leonina* eggs exposed to *M. circinelloides* was reduced to less than half, and to one third in contact with *Verticillium* sp. The viability of the eggs of *Trichuris* sp. decreased by half in the presence of *M.*

circinelloides or *T. atrobrunneum*. A striking result was the observation of broken eggshells of *T. leonina*, as well as larvae L2 outside the eggs, which become totally unviable. In addition to the alterations mentioned, it was also found that in the presence of *M. circinelloides, T. atrobrunneum* and *Verticillium* sp., a delay in the development of the eggs of the nematodes was detected, as well as the appearance of malformations inside the eggs. Data collected indicate that spraying of aqueous solutions of spores of ovicidal filamentous fungi is a very useful and efficient tool to reduce the viability and development of helminth eggs in feces of animals in captivity, providing a strategy environmentally respectful. Moreover, it is also an applicable measure in the control of possible zoonoses in parks and recreational areas, which would consist in spraying spores just after the removal of the feces.

In the line of preventing the transmission of diseases from animals to people, in groups that have close contact with them there is a greater risk, because that people are responsible for their care, or because they enjoy the care of companion species. Therefore, it is necessary to monitor and dispose of measures aimed at ensuring the absence of diseases in animals and preventing their transmission. One measure that seems very successful is the prevention of infection in animals, so that the risk is considerably limited between people. Propagation of fungi used in biological control has generated great interest to determine the methods that combine greater effectiveness and comfort for the user. Initially in laboratories, these fungi were cultured in solid media (based on bacterial agar, or rice), which require large spaces to obtain good results. Recently, the COPAR research group of the University of Santiago de Compostela has incorporated submerged (liquid) COPFr culture media (patent number PCT / ES2014 / 070110), which facilitates the production in large quantities and diversifies the use options, making possible the distribution of fungi by spraying, premixing with food or by incorporation into commercial feed. For this reason, a fourth essay entitled ***Potential usefulness of filamentous fungi to prevent zoonotic soil-transmitted helminths (STHs)*** was proposed, in which the usefulness of the administration of spores of *M. circinelloides* and *D.*

flagrans as a *feed additive* was analyzed. For this purpose, a feed premix was prepared by adding an aqueous solution of fungal spores to the commercial pelletized feed that received a group of puppies Grifón Azul de Gascuña and Gascón Saintongeois (G-2) kept in an authorized dog breeder, and another group (G-1) was fed with pellets without spores. The initial coprological examination using the saline flotation technique revealed the presence of eggs of *Toxocara canis*, *Toxascaris leonina*, *Trichuris vulpis* and *Ancylostoma caninum*, parasites with zoonotic potential that are included in the so-called STHs. To establish the viability of the eggs of the detected nematodes, a portion of the feces obtained daily for 13 days was introduced in plastic boxes with holes to facilitate oxygenation; these boxes were maintained in field conditions, in a meadow with shaded trees.

The feces of the group that received the premix with spores (G-2) were processed and analyzed from the first day under the microscope, in search of spores of the two fungi, confirming that a significant portion of the spores of *M. circinelloides* and *D. flagrans* are able to resist the passage through the intestinal transit, since spores were already detected in the feces of this group one day after starting the trial. The viability of *T. canis* eggs reduced to 57%, *T. leonina* to 61% and *T. vulpis* to 53%, with respect to the viability of the samples of the puppies that received the concentrate without spores (87 %, 92% and 85%, respectively). On the other hand, the number of third-stage larvae (L3) of *A. caninum* reduced by 59% in the group that was fed with feed with spores. Morphological alterations were observed under the microscope, which consisted in the adherence of hyphae to the eggshells, penetration and destruction. The disappearance of the polar plugs of eggs of *Trichuris* sp. was also detected, as well as vacuole formation in the interior. The study allowed to evaluate the absence of adverse effects in the puppies, as well as to glimpse the high potential of using filamentous fungi for the prevention and control of these parasites. These data confirm the suitability of strategies based on the distribution of spores to minimize the risk of infection in the feces of pets, and thus the environment in which people and other animals live together.

It is very common and widespread to understand the concept of STHs or soil-transmitted helminths, and yet it is not always taken into consideration that other very common parasites also develop part of their biological cycle in the soil. Ectoparasites such as fleas or ticks, in addition to causing alterations in the hosts, can transmit different diseases that aggravate clinical conditions. In the fifth trial entitled ***Advantageous fungi against parasites transmitted through soil***, the analysis of the effect of *M. circinelloides* on different parasitic stages found in the soil was proposed. Specifically, it was determined how varied the viability of the eggs of nematodes responsible for zoonosis (*Toxocara canis* and *Baylisascaris procyonis*), and those of the ixodid *Rhipicephalus microplus*. It is important to bear in mind that after fertilization and last ingestion of blood, adult female ticks fall to the ground for oviposition. In this phase, they look for humid, dark and vegetated areas to deposit the eggs, protecting them from the direct action of sunlight. When these optimal conditions occur, the percentage of effectiveness of the eggs is very high.

After spraying *M. circinelloides* spores on the eggs of the ascarids, it was demonstrated that at 30 days the viability decreased to 50% in the case of *T. canis*, and 34% *B. procyonis*. It shoud be considered that these are two parasites that pose a great risk to the human population, given that *T. canis* is found in most puppies when they are born, while *B. procyonis* affects mostly raccoons but can also do it to other carnivores, and a worldwide distribution it has been described. In both cases, these are parasites that do not complete the cycle in people, but that make erratic migrations leading to the presence of second instar larvae (L2) in the eye, or even in the central nervous system.

After contacting with *M. circinelloides*, the viability of the eggs of *R. microplus* decreased to 38%, while only 15% hatched. These data reveal the great utility of using this ovicidal fungus to reduce the possibilities of propagation of these ectoparasites, and in this way the risk of disease transmission. It is concluded that the dispersion of ovicidal fungi such as *M. circinelloides* provides a very effective tool to reduce the chances of infection of people and animals by parasitic forms found in the soil, and its application is recommended.

The data collected in the present investigation leads us to consider that in the feces of animal species that feed on meadows, areas with vegetation, etc, it is quite simple to isolate and characterize saprophytic fungi with parasiticidal activity; of the presence in the feces establishes its capacity to survive the passage through the gastrointestinal tract without undergoing alterations, and also without causing damage to the animal that ingests them. In order to ensure its distribution in the feces of animals to achieve a suitable concentration, it is possible to go to approaches characterized by the use of spores as a food additive. Since *M. circinelloides* can be cultured in submerged media, it is easier to obtain high amounts of spores, which can even be distributed by spraying in the soil, thus providing a useful tool to prevent infections by parasites that are they transmit in the middle. I would like to highlight the activity of *M. circinelloides* as endo- and ectoparasiticide, because of its ability to wound the eggshells of these parasites, and destroy the internal embryo. From a commercial point of view, it seems that two approaches could be successful, on the one hand under aqueous solutions to pour on the ground, and on the other, as a feed additive.

1.2. RESUMEN

El control parasitario basado únicamente en la administración de tratamientos farmacológicos se muestra insuficiente desde hace décadas. Cada vez son más necesarias acciones preventivas que limiten el riesgo de infección y hagan posible reducir la aplicación de terapias farmacológicas a aquellos casos estrictamente necesarios, es decir, cuando la salud de los animales se encuentre afectada, o su productividad no alcanza los niveles mínimos esperados. Además, existen numerosas infecciones que pueden transmitirse de los animales al ser humano o viceversa (zoonosis), y por su implicación en la salud pública incrementan la necesidad de un mayor control para evitar que los animales alcancen elevadas intensidades de parasitación que supongan un riesgo para las personas.

A lo largo del tiempo han ido surgiendo diferentes medidas con objeto de reducir la infección en herbívoros, como el manejo de pastos

en el que se intercalan ciertas especies animales con diferente sensibilidad a las infecciones parasitarias, o la recogida periódica y manual de heces, difícil de aplicar en extensiones grandes de terreno. En el caso de mascotas, la tendencia seguida ha consistido en la administración *preventiva* de antiparasitarios, unida al desarrollo de vacunas que estimulen la respuesta inmunitaria frente a ciertos agentes parasitarios. En ambos casos, los resultados no han sido los esperados, y en la actualidad persiste la necesidad de aplicar tratamientos con elevada frecuencia, que en un importante número de casos ha conducido a la aparición de cepas de parásitos resistentes.

Desde hace mucho tiempo, se conoce la existencia de agentes biológicos que se encuentran de forma natural en el suelo, y que son capaces de desarrollar actividad antagonista frente a ciertas formas de diferentes parásitos, por lo que podrían ser utilizados para llevar a cabo acciones preventivas. Estos agentes han impulsado la investigación de estrategias denominadas de *control biológico*. Dentro de estos procedimientos se encuentran algunos basados en el empleo de bacterias, virus, ácaros, nematodos o también hongos saprofitos, cuya aplicación práctica conlleva la superación de una serie de aspectos, entre los que conviene destacar una eficacia antiparasitaria selectiva, facilidad de propagación en medios de cultivo asequibles y bajo condiciones estándares, comodidad y sencillez en su distribución, inocuidad sobre componentes del medio y posibilidades de comercialización.

Para intentar dar respuesta a estos requisitos, se planteó una investigación basada en el estudio de las posibilidades de aplicación y comercialización de un hongo saprofito con actividad ovicida, *Mucor circinelloides*, que se ensambló alrededor de cinco ensayos. En el **primero**, ***Isolation of ovicidal fungi from faecal samples of captive animals maintained in a zoological park***, se profundiza en el conocimiento de que ciertos hongos saprófitos comunes en el suelo tienen efectos antagonistas sobre algunos parásitos que afectan a animales y seres humanos, resisten el tránsito a través del sistema digestivo de los animales y son expulsados a través de las heces junto con dichos parásitos. El principal objetivo consistió en aportar información acerca de métodos prácticos para el aislamiento de hongos parasiticidas, con la pretensión de describir un procedimiento sencillo, asumible y práctico. La razón de elegir la materia fecal para seleccionar los hongos se fundamentó en que de este modo se podía asegurar que no experimentaban alteraciones al atravesar el tracto gastrointestinal, y que no provocaban alteraciones en los animales. La explicación al empleo de muestras de animales salvajes cautivos en el Parque Zoolóxico "Marcelle Natureza" (Outeiro de Rei, Lugo, España) se debió a la observación de que algunas especies de animales estaban parasitados solamente por estrongilados, nematodos gastrointestinales cuya transmisión se produce por la ingestión de larvas de tercer estadio, *fases móviles* por lo tanto, mientras que otros solo mostraban infección por ascáridos o tricúridos, que se transmiten por la ingesta de huevos con una larva en su interior, es decir, *fases inmóviles*. Para ello, se recogieron 60 muestras de heces del suelo de diferentes parcelas con vegetación, que se cultivaron en serie en placas Petri que contenían diferentes medios, con el objetivo de obtener cultivos monoclonales que permitieran aislar e identificar los hongos presentes en esas heces.

Las muestras fecales se analizaron con las técnicas de flotación y sedimentación, y de este modo se determinó que un total de trece animales eliminaban huevos de estrongilados, y a partir de estas muestras se aislaron siete hongos con actividad ovicida que se encuentran con frecuencia en el suelo. Tras el aislamiento, identificación y multiplicación, estos hongos se enfrentaron a los huevos de *Calicophoron daubneyi* y *Parascaris equorum* en condiciones de laboratorio, para conocer su capacidad para desarrollarse y actividad antagonista. Se comprobó que las especies *Clonostachys* (*Gliocladium*) y *Penicillium* fueron capaces de desarrollar hifas que se adhieren a la cubierta del huevo sin llegar a traspasarla ni afectarla, manteniéndose el embrión parasitario intacto y viable, por lo tanto, se clasificaron como hongos ovicidas de tipo 1. Los otros cinco especímenes de hongos se identificaron a nivel de género como *Fusarium*, *Lecanicillium*, *Mucor*, *Trichoderma* y *Verticillium*; sus hifas fueron capaces de adherirse a la cubierta de huevos de los helmintos, y a partir de la formación del *haustorio* penetrar y destruir el embrión interno, y en consecuencia se clasificaron como hongos ovicidas de tipo 3. De estos resultados se concluyó que el aislamiento de hongos a partir de heces de animales constituye un método muy útil, práctico y asequible que hace posible la identificación de especies de interés para desarrollar pautas de control biológico de formas parasitarias que afectan a animales y personas.

En las experiencias de control biológico realizadas hasta hace pocos años se han descrito hongos ovicidas como *Pochonia chlamydosporium* (= *Verticillium chlamydosporia*) y también larvicidas, como *Duddingtonia flagrans*. A pesar de que existe abundante información acerca de su utilidad, escasean los estudios que muestren su eficacia en condiciones de campo, o que ofrezcan formulaciones interesantes y aplicables. Por este motivo, en el **segundo ensayo *A combined effort to avoid strongyle infection in horses in an oceanic climate region: rotational grazing and parasiticidal fungi***, se proyectó la aplicación de una mezcla de esporas de hongos que desarrollan diferente actividad parasiticida en forma de *aditivo alimentario*. Para conseguirlo, se procedió a la fabricación de alimento pelletizado para caballos en una empresa dedicada a la elaboración de productos para animales, al que se añadieron en la fase de mezcla esporas de *Mucor circinelloides* y *Duddingtonia. flagrans*. El objetivo fue conocer el efecto de esta formulación en la condición sanitaria de caballos que se mantenían en pastos en rotación, y sobre su estado de infección parasitaria. Con la formulación de los pellets con esporas se buscaba asegurar una administración continuada de los hongos y una nutrición adecuada. Se emplearon 22 Caballos de Deporte Español infectados de forma natural por estrongílidos, de modo que al inicio de la investigación recibieron un tratamiento *pour on* de ivermectina, y a continuación, los equinos se dividieron en tres grupos; el G-1 (6 caballos) se mantuvo estabulado la mayor parte del día y recibieron pellets sin esporas; el G-2 (8 individuos) se mantuvo en pastoreo rotacional y recibieron pellets sin esporas, y el G-3 (8 equinos) también en pastoreo rotacional y alimentado con pellets con esporas. El seguimiento de la estrategia antiparasitaria se realizó mediante el análisis de heces que se recogieron con periodicidad mensual durante

13 meses, empleando la técnica de flotación con solución salina saturada. Se mantuvieron muestras de heces a 22-25ºC durante 19 días para determinar los géneros de nematodos que infectaban a los equinos.

En las heces se identificaron ejemplares de nematodos *Cyathostomum* (*sensu lato*) tipo A (63%), tipo C (14%) y tipo D (15%); *Gyalocephalus capitatus* (1%), *Triodontophorus serratus* (2%), *Poteriosthomum* spp. (2%), *Strongylus vulgaris* (2%) y *S. edentatus* (1%). Los análisis coprológicos mostraron la presencia de esporas de los hongos en el G-3 a los dos días de iniciar la administración. El tratamiento inicial fue eficaz en los tres grupos, pero los recuentos de huevos de estrongílidos aumentaron de forma gradual en el G-1 y en el G-2, observándose valores superiores a los 300 huevos por gramo de heces (hpg) a partir de los 3 meses post-tratamiento. En los caballos del G-3 se observó un incremento progresivo de los recuentos fecales de huevos, que se mantuvieron alrededor de 200 hpg durante todo el estudio, a excepción de un individuo. Estos resultados parecen indicar que la reinfección de los equinos tiene lugar en 1-1,5 meses, debido a la ingestión de larvas de tercer estadio (L3, fases infectivas) libres en el ambiente, que se originan de huevos de estrongílidos eliminados a través de las heces. En el grupo de equinos en rotación que recibieron pellets sin esporas (G-2), la eliminación de huevos de estrongílidos en heces descendió un 7% a los 6 meses, por un 50% en aquellos individuos que ingerían esporas de los hongos parasiticidas (G-3). Al completar la rotación de 12 meses la reducción alcanzó el 59%. En los caballos que recibieron esporas (G-3), no se detectaron signos de efectos adversos sobre la función respiratoria, digestiva o reproductora, y tampoco se observaron alteraciones cutáneas. Estos resultados ponen de manifiesto la inocuidad de la ingestión continuada de esporas vehiculadas en pellets nutricionales, así como su efecto beneficioso en la prevención de infección por nematodos gastrointestinales (estrongílidos) en equinos que se mantienen en pastoreo rotacional, incrementando la utilidad de este procedimiento.

El mantenimiento de animales en parcelas de tamaño pequeño o mediano resulta muy frecuente para mantener un nivel aceptable de

limpieza de matorral, vegetación indeseada, etc. Entre las especies domésticas que aprovechan estas praderas se encuentran caballos, burros y ovejas, principalmente. La ausencia de rotación conduce muchas a veces a la contaminación elevada del suelo por formas parasitarias, provocando que sea necesario repetir los tratamientos farmacológicos con excesiva frecuencia, y que en muchos casos no se alcance el efecto esperado. Este problema también se observa en parques zoológicos, en los que por razones sobre todo de estrés, no se contempla la posibilidad de alternar diferentes parcelas. Con la finalidad de conocer la eficacia de una formulación como pulverizado de esporas de *M. circinelloides*, *Verticillium* sp. y *Trichoderma atrobrunneum*, sobre las formas parasitarias excretadas en las heces de animales salvajes en cautividad (Parque Zoolóxico Marcelle Natureza), se llevó a cabo un **tercer ensayo titulado *Biological control of soil transmitted helminths (STHs) in a zoological park by using saprophytic fungi***. En el mencionado parque *Marcelle Natureza* se recogieron heces de linces (*Lynx lynx*) y de dromedarios (*Camelus dromedarius*), que se analizaron con las técnicas comunes en estos casos de flotación salina, sedimentación y migración larvaria, poniendo en evidencia la presencia de huevos de *Toxascaris leonina* y de *Trichuris* sp. A continuación, se pulverizaron soluciones con una concentración conocida de esporas de los hongos mencionados sobre muestras de heces que habían resultado positivas a la presencia de los helmintos mencionados. Las heces tratadas con esporas se dividieron en dos lotes, uno se colocó en placas Petri bajo condiciones de laboratorio idóneas para el desarrollo embrionario de los huevos, y el otro en envases de plástico que se mantuvieron bajo condiciones naturales en el campo. Treinta días más tarde, bajo el microscopio se examinaron 120 huevos en las placas y se demostró que la viabilidad de los huevos de *T. leonina* expuestos a *M. circinelloides* se redujo a menos de la mitad, y a un tercio en contacto con *Verticillium* sp. La viabilidad de los huevos de *Trichuris* sp. disminuyó a la mitad en presencia de *M. circinelloides* o *T. atrobrunneum*. Un resultado llamativo consistió en la observación de cubiertas rotas de huevos de *T. leonina*, así como de larvas L2 fuera de los huevos, que se vuelven totalmente inviables. Además de las alteraciones mencionadas, también se constató que en presencia de *M. circinelloides*, *T. atrobrunneum* y *Verticillium* sp., se produjo un retraso en el desarrollo de los huevos de los nematodos señalados, y la aparición de malformaciones en el interior de los huevos. Los datos recabados señalan que la pulverización de soluciones acuosas de esporas de hongos filamentosos ovicidas constituye una herramienta muy útil y eficiente para reducir la viabilidad y desarrollo de los huevos de helmintos en heces de animales en cautividad, proporcionando una estrategia respetuosa con el medio. Además, también supone una medida aplicable en el control de posibles zoonosis en parques y lugares de recreo, que consistiría en la pulverización de esporas justo después de la retirada de las heces.

En la línea de prevenir la transmisión de enfermedades de animales a personas, existe un mayor riesgo en grupos que tienen un contacto estrecho con los mismos, porque se encargan de su cuidado, o porque disfrutan de la atención de especies de compañía. Por ello, se hace necesario vigilar y disponer de medidas encaminadas a asegurar la ausencia de enfermedades en los animales y evitar su transmisión. Una medida que parece muy acertada consiste en la prevención de infección en los animales, de modo que se limite considerablemente el riesgo entre personas. La propagación de hongos usados en el control biológico ha generado gran interés para determinar cuál son los métodos que combinan una mayor efectividad y comodidad para el usuario. Inicialmente en los laboratorios, para la reproducción de estos hongos se empleaban medios sólidos (a base de agar bacteriano, o de arroz), que requieren de grandes espacios para obtener buenos resultados. Recientemente, el grupo de investigación COPAR de la Universidad de Santiago de Compostela ha incorporado cultivos sumergidos (líquidos) COPFr (patente Número PCT/ES2014/070110), que facilita la producción en grandes cantidades y diversifica las opciones de uso, haciendo posible la distribución de hongos mediante pulverización, premezcla con el alimento o mediante la incorporación a piensos comerciales. Por este motivo, se planteó un **cuarto ensayo** titulado ***Potential usefulness of filamentous fungi to prevent zoonotic soil-transmitted helminths (STHs)***, en el que se analizó la utilidad de la administración de una formulación de esporas de *M. circinelloides* y

D. flagrans como *aditivo alimentario*. Para ello, se preparó una *premezcla alimentaria* al añadir una solución acuosa de esporas de los hongos al alimento pelletizado comercial que recibió un grupo de cachorros Grifón Azul de Gascuña y Gascón Saintongeois (G-2) mantenidos en un criadero autorizado, y otro grupo (G-1) se alimentó con pellets sin esporas. El examen coprológico inicial mediante la técnica de flotación con solución salina reveló la presencia de huevos de *Toxocara canis*, *Toxascaris leonina*, *Trichuris vulpis* y *Ancylostoma caninum*, parásitos con potencial zoonótico que se engloban dentro de las conocidas como STHs. Para establecer la viabilidad de los huevos de los nematodos detectados, una porción de las heces obtenidas diariamente durante 13 días se introdujo en cajas de plástico con orificios para facilitar su oxigenación; estas cajas se mantuvieron en condiciones de campo, en una pradera con sombra arbolada.

Las heces del grupo que recibió la premezcla con esporas (G-2) se procesaron y analizaron desde el primer día bajo el microscopio, en búsqueda de la presencia de esporas de los dos hongos, confirmando que una porción significativa de las esporas de *M. circinelloides* y *D. flagrans* son capaces de resistir el trayecto a través del tránsito intestinal, puesto que un día después de iniciar el ensayo, ya se detectaron esporas en las heces de este grupo. La viabilidad de los huevos *T. canis* se redujo a un 57%, de *T. leonina* un 61% y de *T. vulpis* un 53%, respecto a la viabilidad de las muestras de los cachorros que recibieron el concentrado sin esporas que fue 87%, 92% y 85%, respectivamente. Por otro lado, el número de larvas L3 de *A. caninum* se redujo un 59% en el grupo que se alimentaba con pienso con esporas. Al microscopio se vislumbraron alteraciones morfológicas que consistieron en la adherencia de hifas a la cubierta de los huevos, penetración y destrucción. También se detectó la desaparición de los tapones polares de los huevos de *Trichuris* sp., así como la formación de vacuolas en el interior. El estudio permitió evaluar la ausencia de efectos adversos en los cachorros, así como vislumbrar el alto potencial de uso de hongos filamentosos para la prevención y control de estos parásitos. Estos datos confirman la idoneidad de estrategias basadas en la distribución de esporas para minimizar el riesgo de infección en las heces de animales de compañía, y con ello del medio en el que conviven personas y otros animales.

Resulta muy común y extendido comprender el concepto de STHs o helmintos transmitidos por el suelo, y sin embargo no siempre se tiene en consideración que otros parásitos muy comunes también desarrollan parte de su ciclo biológico en el suelo, entre los que se encuentran ectoparásitos como las pulgas o las garrapatas, que además de provocar alteraciones en los hospedadores, pueden transmitir diferentes enfermedades que agravan los cuadros clínicos. En el **quinto ensayo** titulado ***Advantageous fungi against parasites transmitted through soil***, se planteó el análisis del efecto de *M. circinelloides* sobre diferentes estadios parasitarios que se encuentran en el suelo. En concreto, se determinó cómo variaba la viabilidad de huevos de nematodos ascáridos reponsables de zoonosis (*Toxocara canis* y *Baylisascaris procyonis*), y también de huevos de garrapatas del ixódido *Rhipicephalus microplus*. Es importante tener en cuenta que las garrapatas hembras adultas tras la fecundación y última ingestión de sangre, caen al suelo para la ovoposición. En esta fase, las garrapatas buscan zonas húmedas, sombrías y con vegetación para depositar los huevos, protegiéndolos de la acción directa de la luz solar. Cuando se dan estas condiciones óptimas, el porcentaje de efectividad de eclosión de los huevos es muy alto.

Tras la pulverización de esporas de *M. circinelloides* sobre los huevos de los ascáridos, se demostró que a los 30 días la viabilidad descendía al 50% en el caso de *T. canis*, y al 34% para *B. procyonis*. Hay que considerar que se trata de dos parásitos que entrañan un gran riesgo para la población humana, dado que *T. canis* se encuentra en la mayoría de los cachorros cuando nacen, mientras que *B. procyonis*, que afecta sobre todo a mapaches pero también puede hacerlo a otros carnívoros, y se ha descrito una distribución mundial. En ambos casos, se trata de parásitos que no completan el ciclo en personas, pero que realizan migraciones erráticas que conducen a la presencia de larvas de segundo estadio (L2) en el ojo, o incluso en el sistema nervioso central.

Tras el contacto con *M. circinelloides* la viabilidad de los huevos de *R. microplus* descendió hasta un 38%, mientras que solo el 15% llegaron a eclosionar. Estos datos revelan la gran utilidad que supondría el empleo de este hongo ovicida para reducir las posibilidades de propagación de estos ectoparásitos, y de este modo el riesgo de transmisión de enfermedades. Se concluye que la dispersión de hongos ovicidas como *M. circinelloides* proporciona una herramienta muy eficaz para disminuir las posibilidades de infección de personas y animales por formas parasitarias que se encuentran en el suelo, y se recomienda su aplicación.

Los datos recogidos en la presente investigación llevan a considerar que en las heces de especies animales que se alimentan en praderas, zonas con vegetación, etc, es bastante sencillo aislar y caracterizar hongos saprofitos con actividad parasiticida; de la presencia en las heces se establece su capacidad para sobrevivir al paso por el tracto gastrointestinal sin experimentar alteraciones, y también sin provocar daños al animal que los ingiere. Con el propósito de asegurar su distribución en las heces de animales hasta conseguir una concentración idónea, es posible acudir a planteamientos caracterizados por el empleo de esporas como aditivo alimentario. Puesto que *M. circinelloides* puede cultivarse en medios líquidos, se hace más fácil la obtención de elevadas cantidades de esporas, que se pueden incluso distribuir mediante la pulverización en el suelo, proporcionado de este modo una herramienta útil para evitar las infecciones por parásitos que se transmiten en medio. Compre destacar la actividad de *M. circinelloides* como endo- y ectoparasiticida, por su capacidad para herir la cubierta de los huevos de estos parásitos, y destruir el embrión interno. Desde un punto de vista comercial, parece que dos planteamientos podrían tener éxito, de un lado bajo soluciones acuosas para verter en el suelo, y de otro, como aditivo alimentario.

1.3. RESUMO

O control parasitario baseado unicamente na administración de tratamentos farmacolóxicos móstrase insuficiente desde hai décadas.

Cada vez son máis necesarias accións preventivas que limiten o risco de infección e fagan posible reducir a aplicación de terapias farmacolóxicas a aqueles casos estritamente necesarios, é dicir, cando a saúde dos animais atópese afectada, ou a súa produtividade non alcanza os niveis mínimos esperados. Ademais, existen numerosas infeccións que poden transmitirse dos animais ó ser humano ou viceversa (zoonoses), e pola súa implicación na saúde pública incrementan a necesidade dun maior control para evitar que os animais alcancen elevadas intensidades de parasitación que supoñan un risco para as persoas.

Ó longo do tempo foron xurdindo diferentes medidas con obxecto de reducir a infección en herbívoros, como o manexo de pastos no que se intercalan certas especies animais con diferente sensibilidade ás infeccións parasitarias, ou a recollida periódica e manual de feces, difícil de aplicar en extensións grandes de terreo. No caso de mascotas, a tendencia seguida ha consistido na administración preventiva de antiparasitarios, unida ó desenvolvemento de vacinas que estimulen a resposta inmunitaria fronte a certos axentes parasitarios. En ambos os casos, os resultados non foron os esperados, e na actualidade persiste a necesidade de aplicar tratamentos con elevada frecuencia, que nun importante número de casos conduciu á aparición de cepas de parasitos resistentes.

Desde hai moito tempo, coñécese a existencia de axentes biolóxicos que se atopan de forma natural no chan, e que son capaces de desenvolver actividade antagonista fronte a certas formas de diferentes parasitos, polo que poderían ser utilizados para levar a cabo acciones preventivas. Estes axentes impulsaron a investigación de estratexias denominadas de control biolóxico. Dentro destes procedementos atópanse algúns baseados no emprego de bacterias, virus, ácaros, nematodos ou tamén fungos saprofitos, cuxa aplicación práctica conleva a superación dunha serie de aspectos, entre os que convén destacar unha eficacia antiparasitaria selectiva, facilidade de propagación en medios de cultivo alcanzables e baixo condicións estándares, comodidade e sinxeleza na súa distribución, inocuidad sobre compoñentes do medio e posibilidades de comercialización.

Para tentar dar resposta a estes requisitos, expúxose unha investigación baseada no estudo das posibilidades de aplicación e comercialización dun fungo saprofito con actividade ovicida, *Mucor circinelloides*, que artellouse ó redor de cinco ensaios. No primeiro, ***Isolation of ovicidal fungi from faecal samples of captive animals maintained in a zoological park***, profúndase no coñecemento de que certos fungos saprófitos comúns no chan teñen efectos antagonistas sobre algúns parasitos que afectan a animais e seres humanos, resisten o tránsito a través do sistema dixestivo dos animais e son expulsados a través das feces xunto con devanditos parasitos. O principal obxectivo consistiu en achegar información acerca de métodos prácticos para o illamento de fungos parasiticidas, coa pretensión de describir un procedemento sinxelo, asumible e práctico. A razón de elixir a materia fecal para seleccionar os fungos fundamentouse en que deste xeito se podía asegurar que non experimentaban alteracións logo de atravesar o tracto gastrointestinal, e que non provocaban alteracións nos animais. A explicación ó emprego de mostras de animais salvaxes cativos no Parque Zoolóxico "Marcelle Natureza" (Outeiro de Rei, Lugo, España) debeuse á observación de que algunhas especies de animais estaban parasitados soamente por estronxílidos, nematodos gastrointestinales cuxa transmisión se produce pola inxestión de larvas de terceiro estadio, fases móbiles por tanto, mentres que outros só mostraban infección por ascáridos ou tricúridos, que se transmiten pola inxesta de ovos cunha larva no seu interior, é dicir, fases inmóbiles. Para iso, recolléronse 60 mostras de feces do chan de diferentes parcelas con vexetación, que se cultivaron en serie en placas Petri que contiñan diferentes medios, co obxectivo de obter cultivos monoclonales que permitisen illar e identificar os fungos presentes nesas feces.

As mostras fecais analizáronse coas técnicas de flotación e sedimentación, e deste xeito determinouse que un total de trece animais eliminaban ovos de estronxílidos, e a partir destas mostras illáronse sete fungos con actividade ovicida que se atopan con frecuencia no chan. Tras o illamento, identificación e multiplicación, estes fungos enfrontáronse ós ovos de *Calicophoron daubneyi* e *Parascaris equorum* en condicións de laboratorio, para coñecer a súa capacidade para desenvolverse e actividade antagonista. Comprobouse que as especies *Clonostachys* (*Gliocladium*) e *Penicillium* foron capaces de desenvolver hifas que se adhiren á cuberta do ovo sen chegar a traspasala nin afectala, manténdose o embrión parasitario intacto e viable, por tanto, clasificáronse como fungos ovicidas de tipo 1. Os outros cinco especímenes de fungos identificáronse a nivel de xénero como *Fusarium*, *Lecanicillium*, *Mucor*, *Trichoderma* e *Verticillium*; as súas hifas foron capaces de adherirse á cuberta de ovos dos helmintos, e a partir da formación do haustorio penetrar e destruír o embrión interno, e en consecuencia clasificáronse como fungos ovicidas de tipo 3. Destes resultados concluíuse que o illamento de fungos a partir de feces de animais constitúe un método moi útil, práctico e alcanzable que fai posible a identificación de especies de interese para desenvolver pautas de control biolóxico de formas parasitarias que afectan a animais e persoas. Nas experiencias de control biolóxico realizadas ata hai poucos anos describíronse fungos ovicidas como Pochonia chlamydosporium (= *Verticillium chlamydosporium*) e tamén larvicidas, como *Duddingtonia flagrans*.

A pesar de que existe abundante información acerca da súa utilidade, escasean os estudos que mostren a súa eficacia en condicións de campo, ou que ofrezan formulacións interesantes e aplicables. Por este motivo, no segundo ensaio ***A combined effort to avoid strongyle infection in horses in an oceanic climate region: rotational grazing and parasiticidal fungi***, proxectouse a aplicación dunha mestura de esporas de fungos con diferente actividade parasiticida en forma de aditivo alimentario. Para conseguilo, procedeuse á fabricación de alimento pelletizado para cabalos nunha empresa adicada á elaboración de produtos para animais, engadíndose na fase de mestura esporas de *Mucor circinelloides* e *Duddingtonia. flagrans*. O obxectivo foi coñecer o efecto desta formulación na condición sanitaria de cabalos que se mantiñan en pastos en rotación, e sobre o seu estado de infección parasitaria. Coa formulación dos pellets con esporas buscábase asegurar unha administración continuada dos fungos e unha nutrición adecuada. Empregáronse 22 Cabalos de Deporte Español infectados de forma natural por estronxílidos, de modo que ó comezo da investigación recibiron un tratamento pour on de ivermectina, e a continuación, os equinos dividíronse en tres grupos; o G-1 (6 cabalos) mantívose estabulado a maior parte do día e recibiron pellets sen esporas; o G-2 (8 individuos) mantívose en pastoreo rotacional e recibiron pellets sen esporas, e o G-3 (8 equinos) tamén en pastoreo rotacional e alimentado con pellets con esporas. O seguimento da estratexia antiparasitaria realizouse mediante a análise de feces que se recolleron con periodicidade mensual durante 13 meses, empregando a técnica de flotación con solución salina saturada. Mantivéronse mostras de feces a 22-25ºC durante 19 días para determinar os xéneros de nematodos que infectaban ós equinos.

Nas feces identificáronse exemplares de nematodos *Cyathostomum* (*sensu lato*) tipo A (63%), tipo C (14%) e tipo D (15%); *Gyalocephalus capitatus* (1%), *Triodontophorus serratus* (2%), *Poteriosthomum* spp. (2%), *Strongylus vulgaris* (2%) e *S. edentatus* (1%). As análises coprolóxicas amosaron a presenza de esporas dos fungos no G-3 nos dous días de iniciar a administración. O tratamento inicial foi eficaz nos tres grupos, pero os recontos de ovos de estronxílidos aumentaron de forma gradual no G-1 e no G-2, observándose valores superiores a 300 ovos por gramo de feces (opg) a partir dos 3 meses post-tratamento. Nos cabalos do G-3 detectouse un incremento progresivo dos recontos fecais de ovos, que se mantiveron arredor de 200 opg durante todo o estudo, fóra dun individuo. Estes resultados parecen indicar que a reinfección dos equinos ten lugar en 1-1,5 meses, debido á inxestión de larvas de terceiro estadio (L3, fases infectantes) libres no ambiente, que se orixinan de ovos de estronxílidos eliminados nas feces. No grupo de equinos en rotación que recibiron pellets sen esporas (G-2), a eliminación de ovos de estronxílidos en feces descendeu un 7% ós 6 meses, por un 50% naqueles individuos que inxerían esporas dos fungos parasiticidas (G-3). Ó completar a rotación de 12 meses a redución alcanzou o 59%. Nos cabalos que recibiron esporas (G-3), non se detectaron signos de efectos adversos sobre a función respiratoria, dixestiva ou reprodutora, e tampouco se observaron alteracións cutáneas. Estes resultados poñen de manifesto a inocuidade da inxestión continuada de esporas vehiculadas en pellets nutricionais, así coma o seu efecto beneficioso na prevención de infección por nematodos gastrointestinais (estronxílidos) en equinos en pastoreo rotacional, incrementando a utilidade deste procedemento.

O mantemento de animais en parcelas de tamaño pequeno ou mediano resulta moi frecuente para obter un nivel aceptable de limpeza de matogueira, vexetación indesexada, etc. Entre as especies domésticas que aproveitan estas praderías atópanse cabalos, burros e ovellas, principalmente. A ausencia de rotación conduce moitas ás veces á contaminación elevada do chan por formas parasitarias, provocando que sexa necesario repetir os tratamentos farmacolóxicos con excesiva frecuencia, e que en moitos casos non se alcance o efecto esperado. Este problema tamén se observa en parques zoolóxicos, nos que por razóns sobre todo de estrés, non se contempla a posibilidade de alternar diferentes parcelas. Coa finalidade de coñecer a eficacia dunha formulación como pulverizado de esporas de *M. circinelloides*, *Verticillium* sp. e *Trichoderma atrobrunneum*, sobre as formas parasitarias excretadas nas feces de animais salvaxes en catividade (Parque Zoolóxico Marcelle Natureza), levou a cabo un terceiro ensaio titulado ***Biological control of soil transmitted helminths (STHs) in a zoological park by using saprophytic fungi***. No mencionado parque recolléronse feces de linces (*Lynx lynx*) e de dromedarios (*Camelus dromedarius*), que analizaronse coas técnicas comúns nestes casos de flotación salina, sedimentación e migración larvaria, poñendo en evidencia a presenza de ovos de *Toxascaris leonina* e de *Trichuris* sp. A continuación, pulverizaronse solucións cunha concentración coñecida de esporas dos fungos mencionados sobre mostras de feces que resultaran positivas á presenza dos helmintos mencionados. As feces tratadas con esporas dividíronse en dous lotes, un colocouse en placas Petri baixo condicións de laboratorio idóneas para o desenvolvemento embrionario dos ovos, e o outro en envases de plástico que se mantiveron baixo condicións naturais no campo. Trinta días máis tarde, baixo o microscopio examináronse 120 ovos nas placas e demostrouse que a viabilidade dos ovos de *T. leonina* expostos a *M. circinelloides* reduciuse a menos da metade, e a un terzo en contacto con *Verticillium* sp. A viabilidade dos ovos de *Trichuris* sp. diminuíu á metade en presenza de *M.*

circinelloides ou *T. atrobrunneum*. Un resultado rechamante consistiu na observación de cubertas rotas de ovos de *T. leonina*, así como de larvas L2 fose dos ovos, que se volven totalmente inviables. Ademais das alteracións mencionadas, tamén se constatou que en presenza de *M. circinelloides, T. atrobrunneum* e *Verticillium* sp., produciuse un atraso no desenvolvemento dos ovos dos nematodos sinalados, e a aparición de malformacións no interior dos ovos. Estes achádegos sinalan que a pulverización de solucións acuosas de esporas de fungos filamentosos ovicidas constitúe unha ferramenta moi útil e eficiente para reducir a viabilidade e desenvolvemento dos ovos de helmintos en feces de animais en catividade, proporcionando unha estratexia respectuosa co medio. Ademais, tamén supón unha medida aplicable no control de posibles zoonoses en parques e lugares de lecer, que consistiría na pulverización de esporas xunto coa retirada das feces.

Na liña de previr a transmisión de enfermidades de animais a persoas, existe un maior risco en grupos cun contacto estreito cos mesmos, porque se encargan do seu coidado ou porque gozan da atención de especies de compañía. Por iso, faise necesario vixiar e dispoñer de medidas encamiñadas a asegurar a ausencia de enfermidades nos animais e evitar a súa transmisión. Unha medida que parece moi acertada consiste na prevención de infección nos animais, de modo que se limite considerablemente o risco entre persoas. A propagación de fungos usados no control biolóxico xerou grande interese para determinar os métodos que combinan unha maior efectividade e comodidade para o usuario. Inicialmente nos laboratorios, para a reprodución destes fungos empregábanse medios sólidos (a base de agar bacteriano, ou de arroz), que requiren de grandes espazos para obter bos resultados. Recentemente, o grupo de investigación COPAR da Universidade de Santiago de Compostela incorporou un cultivo mergullado (líquido) COPFr (patente Número PCT/ES2014/070110), que facilita a produción en grandes cantidades e diversifica as opcións de uso, facendo posible a distribución de fungos mediante pulverización, premezcla co alimento ou mediante a incorporación a concentrados alimentarios comerciais. Por este motivo, desenrolouse un cuarto ensaio titulado ***Potential usefulness of filamentous fungi to prevent zoonotic soil- transmitted helminths***

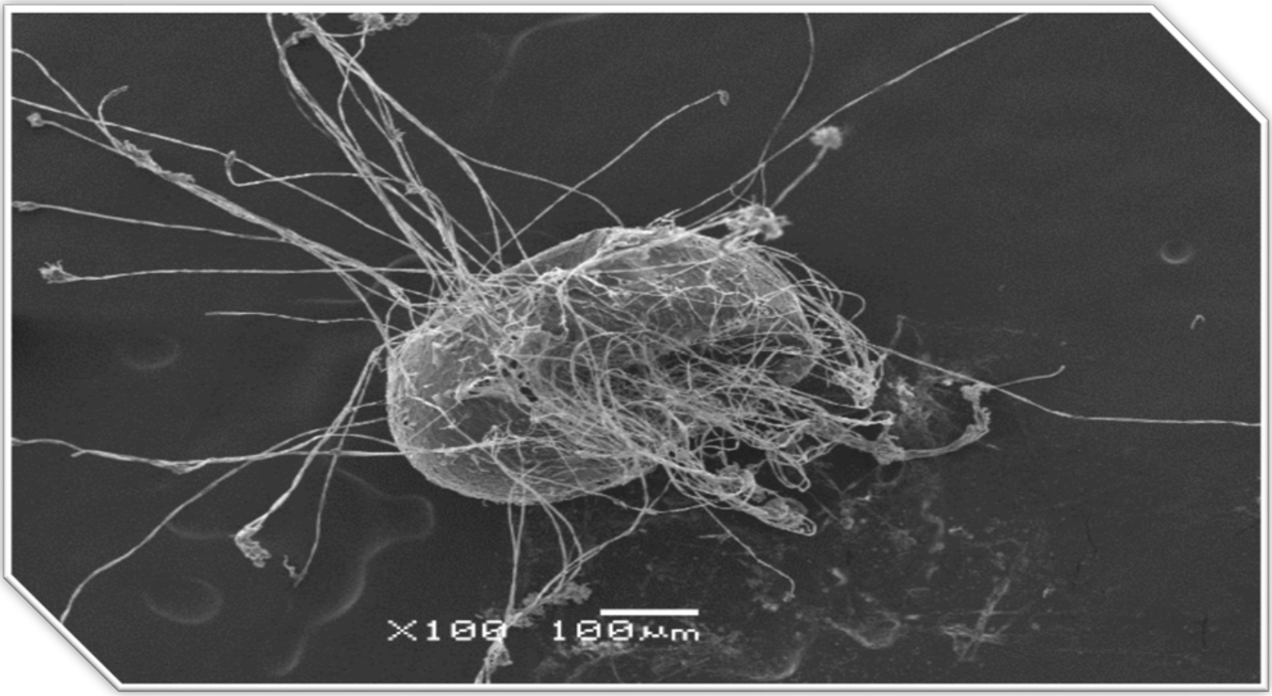
(STHs), no que analizouse a utilidade da administración dunha formulación de esporas de *M. circinelloides* e *D. flagrans* como aditivo alimentario. Para iso, preparouse unha premezcla alimentaria logo de engadir unha solución acuosa de esporas dos fungos ó alimento pelletizado comercial que recibiu un grupo de cachorros Grifón Azul de Gascuña e Gascón Saintongeois (G-2) mantidos nun criadeiro autorizado, e outro grupo (G-1) alimentouse con pellets sen esporas. A exame coprolóxica inicial revelou a presenza de ovos de *Toxocara canis*, *Toxascaris leonina*, *Trichuris vulpis* e *Ancylostoma caninum*, parasitos con potencial zoonótico que se engloban dentro das coñecidas como STHs. Para establecer a viabilidade dos ovos dos nematodos detectados, unha porción das feces obtidas diariamente durante 13 días introduciuse en caixas de plástico con orificios para facilitar a súa osixenación; estas caixas mantivéronse en condicións de campo, nunha pradaría con sombra arborizada.

As feces do grupo que recibiu a premezcla con esporas (G-2) procesáronse e analizaron desde o primeiro día baixo o microscopio, en procura de esporas dos dous fungos, confirmando que unha porción significativa das esporas de *M. circinelloides* e *D. flagrans* son capaces de resistir o traxecto a través do tránsito intestinal, posto que un día despois de iniciar o ensaio, xa se detectaron esporas nas feces deste grupo. A viabilidade dos ovos *T. canis* reduciuse a un 57%, de *T. leonina* un 61% e de *T. vulpis* un 53%, respecto a a viabilidade das mostras dos cachorros que recibiron o concentrado sen esporas que foi 87%, 92% e 85%, respectivamente. Doutra banda, o número de larvas L3 de *A. caninum* reduciuse un 59% no grupo que se alimentaba con concentrado con esporas. No microscopio albiscáronse alteracións morfolóxicas que consistiron na adherencia de hifas á cuberta dos ovos, penetración e destrución. Tamén se detectou a desaparición dos tapóns polares dos ovos de *Trichuris* sp., así como a formación de vacuolas no interior. O estudo permitiu avaliar a ausencia de efectos adversos nos cachorros, así como albiscar o alto potencial de uso de fungos filamentosos para a prevención e control destes parasitos. Estes datos confirman a idoneidade de estratexias baseadas na distribución de esporas para minimizar o risco de infección nas feces de animais de compañía, e con iso do medio no que conviven persoas e outros animais.

Resulta moi común e estendido comprender o concepto de STHs ou helmintos transmitidos polo chan, e con todo non sempre se ten en consideración que outros parasitos moi comúns tamén desenvolven parte do seu ciclo biolóxico no chan, entre os que se atopan ectoparásitos como as pulgas ou as carrapatas, que ademais de provocar alteracións nos hospedadores, poden transmitir diferentes enfermidades que agravan os cadros clínicos. No quinto ensaio titulado ***Advantageous fungi against parasites transmitted through soil***, expúxose a análise do efecto de *M. circinelloides* sobre diferentes estadios parasitarios que se atopan no chan. En concreto, determinouse cómo variaba a viabilidade de ovos de nematodos ascáridos reponsables de zoonosis (*Toxocara canis* e *Baylisascaris procyonis*), e tamén de ovos de carrachas do ixódido *Rhipicephalus microplus*. É importante ter en conta que as carrapatas femias adultas tra-la fecundación e última inxestión de sangue, caen ó chan para a ovoposición, e por iso buscan zonas húmidas, sombrías e con vexetación nas que depositar os ovos, protexéndoos da acción directa da luz solar. Cando se dan estas condicións óptimas, a porcentaxe de efectividade de eclosión dos ovos é moi alto. Tra-la pulverización de esporas de *M. circinelloides* sobre os ovos dos ascáridos, demostrouse que ós 30 días a viabilidade descendía até o 50% no caso de *T. canis*, e ó 34% para *B. procyonis*. Hai que considerar que se trata de dous parasitos que entrañan un grande risco para a poboación humana, dado que *T. canis* atópase na maioría dos cachorros cando nacen, mentres que *B. procyonis*, que afecta sobre todo a mapaches pero tamén pode facelo a outros carnívoros, e describiuse unha distribución mundial. En ambos os casos, trátase de parasitos que non completan o ciclo en persoas, pero que realizan migracións erráticas que conducen á presenza de larvas de segundo estadio (L2) no ollo, ou mesmo no sistema nervioso central. Logo do contacto con *M. circinelloides*, a viabilidade dos ovos de *R. microplus* descendeu ata un 38%, mentres que só o 15% chegaron a eclosionar. Estes datos revelan a grande utilidade que supoñería o emprego deste fungo ovicida para reducir as posibilidades de propagación destes ectoparásitos, e deste xeito o risco de transmisión de enfermidades. Conclúese que a dispersión de fungos ovicidas como *M. circinelloides* proporciona unha ferramenta moi eficaz para diminuír as posibilidades de infección de persoas e animais por formas parasitarias que se atopan no chan, e recoméndase a súa aplicación.

Os datos recollidos na presente investigación levan a considerar que nas feces de especies animais que se alimentan en pradarías, zonas con vexetación, etc., é razoadamente sinxelo illar e caracterizar fungos saprofitos con actividade parasiticida; da presenza nas feces establécese a súa capacidade para sobrevivir ó paso polo tracto gastrointestinal sen experimentar alteracións, e tamén sen provocar danos ó animal que os inxere. Co galo de asegurar a súa distribución nas feces de animais ata acadar unha concentración axeitada, é posible acodir a formulacións caracterizadas polo emprego de esporas coma aditivo alimentario. Posto que *M. circinelloides* pode cultivarse en medios líquidos, faise máis doada a obtención de elevadas cantidades de esporas, que se poden incluso distribuir mediante a pulverización no solo, proporcionado deste xeito unha ferramenta útil para evitar as infeccións por parásitos que se transmiten no medio. Compre salientar a actividade de *M. circinelloides* coma endo- e ectoparasiticida, pola súa capacidade para ferir a cuberta dos ovos destes parásitos, e destruir o embrión interno. Dende un punto de vista comercial, asemella que dúas formulacións poderían ter éxito, dun lado baixo solucións acuosas para verquer no solo, e doutro, coma aditivo alimentario.

2. BACKGROUND



2. BACKGROUND

2.1. THE IMPORTANCE OF SOIL IN THE TRANSMISSION OF DISEASES

The Earth and Life Science define ***soil*** as the external part of the Earth crust, biologically active, which tends to develop on the surface of rocks emerged due to the action of weather and living beings. Soil structure and composition is largely determined by the biological activity supported by the interaction between biota of microorganisms (parasites, bacteria, fungi), plants and small animals constituting the *edaphon*.

In recent decades, concern to preserve soil biodiversity has been growing. The balance in the biological interactions between the edaphon species and the microbiota results in the health of all animals, including humans (Bardgett and van der Putten 2014). Most of biological activity takes place on the Earth surface, limited by climatic conditions as temperature, humidity, solar radiation… Man is responsible for the global changes destroying soil biodiversity, mainly those related to land use and development, agriculture and deforestation.

At the end of the 20th century, at the Rio Summit (United Nations 1992), sustainable development was defined as The right to development must be fulfilled so as to equitably meet developmental and environmental needs of present and future generations.

Forecasts indicate that around the year 2050, two thirds of the world population will reside in cities and their relationship with the soil will be limited to areas without biodiversity. The remaining third may be devoted to agricultural activities whose interests seem incompatible with the balance of ecosystems.

Some studies have indicated problems due to the worsening of the environmental balance. Hanski et al. 2012 found that when the exposure to soil microorganism decreases the prevalence of allergic diseases increases. Other researches highlight the increase of bacteria resistant to most known antibiotics, and the same happens with some anthelmintics.

The use of soil microbiota species, such as certain types of fungi, has not yet been considered a solution to restore the imbalances caused by the aforementioned problems. Since the 80s of last century, **ivermectin**, an antiparasitic derivative of the abamectin product of the fungus *Streptomyces avermectilis*, has been used recurrently; until the 60s most of antibiotics were obtained by cultivating microorganisms of soil, effective for example against *Mycobacterium tuberculosis* (Ling et al. 2015).

Special mention should be made of the use of some fungal species for the control of certain endoparasites. Once in the soil, these parasites complete a series of phases to reach the infective stage. Some filamentous species as *Mucor circinelloides* or *Duddingtonia flagrans*, in contact with eggs or larvae of certain helminths, have the ability to destroy them or limit their viability (Arias et al. 2013b; Hernández et al. 2016). In this way it is possible to reduce the risk of infection in people, and also among grazing animals. In recent years, very important achievements have been made in the large-scale production of saprophyte fungal spores (Arias et al. 2013a).

2.2. PATHOGENIC SOIL ORGANISMS

Most microorganisms of the soil microbiota do not pose a public health problem, nevertheless attention should be drawn to the fact that some species are opportunistic (*Pseudomona*, *Enterobacter*). These species are capable of completing their life cycles in the soil and only infect immunocompromised individuals, or those exposed to large doses. Many of these opportunistic species are antagonists of pathogens that affect the roots of vegetables, growth promoters of some plants and decomposers of organic matter (Berg et al. 2005).

Obligate pathogens cannot complete their life cycle in the soil, although the phase through this medium is necessary to become infective for the next host (Wall et al. 2015). In the soil, these species can survive for long periods as spores, eggs or larvae. During the passage through the environment, some of them move from one host to another and almost always undergo some type of transformation. For example, the fly larvae have to bury themselves to form the puparium and become adults (Brevik and Sauer 2015).

Table 1. Pathogens that can be found in the soil

Organism	Pathogenic eudaphic organisms	Soil Transmitted Pathogens
Bacteria	*Bacillus anthracis* *Campylobacter jejuni* *Leptospira interrogans* *Yersinia entereocolitica* *Clostridium* spp *Listeria monocytogenes* *Francisella tulariensis*	*Escherichia coli* *Salmonella* spp *Shigella* spp *Pseudomona aeruginosa* *Entamoeba histolytica* *Coxiella burnetti* *Borrelia* spp
Viruses		Hantavirus
Protozoa		*Cryptosporidium* spp *Giardia duodenalis* *Balatidium coli* *Isospora* spp *Toxoplasma gondii*
Fungi	*Actinomyces israeli* *Aspergillus* spp *Blastomyces dermatidis* *Coccidiodes immitis* *Histoplasma capsulatum* *Rhizopus* spp	
Ectoparasites		Fleas Ticks
Helminths	*Strongyloides* spp	*Fasciola hepatica* *Trichuris* spp Strongylids *Ancylostoma spp* Ascarids Tapeworms

2.2.1. Parasite forms

Parasitology analyses parasitic-host relationships and places them in the environment, hence it is considered a part of Ecology. Previously, soil has been mentioned as the place where numerous parasitic forms can be found; it also lies in the fact that here some of them undergo changes until reaching the infective stages (Table 2), which can be found as immobile (oocysts, cysts, eggs) or mobile phases (larvae, nymphs). Furthermore, some transformations can carry out in small animals of the edaphon that intervene as intermediate hosts (snails, ants).

It is necessary to know the ecology of the interactions between soil, hosts and parasites to determine why some are prevalent and the conditions that allow them to resist. For example, *Haemonchus* larvae need adequate climatic conditions and a specific diet based on certain bacteria to survive.

Table 2. Stages of parasites that can be found in the soil.

	Soil-parasites	Phase
Digestive tract Protozoa	*Cryptosporidium* spp	Oocysts
	Giardia duodenalis	Cysts
	Balatidium coli	Cysts
	Coccidia	Oocysts
	Toxoplasma gondii	Oocysts
Digestive tract Helminths	*Fasciola hepatica*	Egg/Miracidium/ Cercariae/ Metacercariae
	Trichuris spp	Egg
	Strongylids	Egg/L1/L2/L3
	Ancylostoma spp	Egg/L1/L2/L3
	Ascarids	Egg
	Tapeworms	Egg
Ectoparasites	Ticks	Egg/Larva/Nymph/Adult
	Fleas	Egg/Larva/Pupa /Adult

2.2.1.1. Protozoa

Intestinal protozoa passed in feces can be infective, but in most cases infectivity is acquired in the environment after a short time under adequate humidity and temperature conditions (Table 3). Direct biological cycles are associated with overcrowding livestock, low frequency of beds renewal and winter weather also, for being seasons in which the animals remain stabled for longer. In kennels, protozoa are especially prevalent, but soil contamination by cocidia reduces when the concentration of animals decreases.

Table 3. Evolution of protozoan oocysts in the soil.

Protozoa	Sporulation time
Eimeria spp	1-4 days
Cystoisospora sp.	3-4 days
Toxoplasma gondii	1-2 days
Neospora caninum	3-4 days

In general, digestive tract infections by protozoa in people are endemic in countries where a correct management of waste water is not applied. In the 2014 World Health Organization (WHO) meeting, one of the main objectives for 2030 was to resolve the issues derived to open defecation.

The WHO and UNICEF (United Nations International Children's Emergency Fund) typically updates the Joint Monitoring Programme (JMP) (WHO and UNICEF 2017) establishing indicators and baseline estimates for the drinking water, sanitation and hygiene targets (WASH) within the Sustainable Development Goals (SDGs). The report includes data hygiene estimates for 70 countries and introduces the indicators of safely managed drinking water and sanitation services. Furthermore, the JMP also publishes updated report summaries through the Press Centre.

2.2.1.2. Helminths

Ascarids, tricurids and strongyles are the most frequently nematodes contaminating the soil (Szwabe and Blaszkowska 2017; Campos et al. 2018; Yawson et al. 2018).

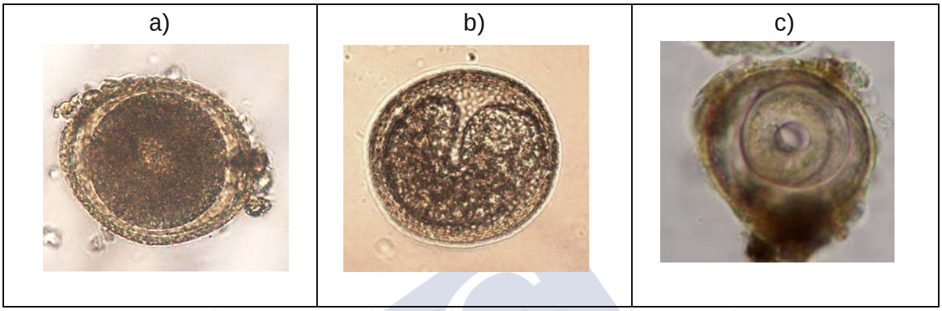


Figure 1. Eggs of ascarids are shed unembryonated.

a) Ascarids

Non-embryonated eggs are passed in the faeces of infected individuals to the soil (Figure 1a), and after a period of 5-21 days in which cell divisions occur (Figure 1b), one larva 1 (L1) is formed (Bojar and Kłapeć 2012). It is necessary a period between 2-6 weeks or even months for larva L2 develops (Figure 1c), and in this way, the egg becomes infective for definitive and paratenic hosts (rodents, humans) (Mejer and Roepstorff 2006).

Once ingested infective eggs, certain conditions of the intestine (body temperature, carbon dioxide, pH 6 and nonspecific reducing conditions) enhance that larvae are released. Some researchers suggest that two transformations or molts occur inside the egg, and therefore the infective stage is that which contains a L3 (Table 4).

Eggs of ascarids are extremely resistant in soil, even being able to survive at -2°C for 6 weeks, or to the action of different chemical disinfectants (Morrondo et al. 2006; Azam et al. 2012).

Table 4. Eggs of different species of ascarids need different periods to reach the infective stage in the soil.

Ascarids	Infective eggs
Toxocara canis	2-6 weeks
Toxascaris leonina	1-3 weeks
Ascaris suum	2-3 weeks
Toxocara vitulorum	2-3 weeks
Parascaris equorum	1-3 weeks
Bailysascaris procyonis	2-5 weeks

Though Ascarids only complete the cycle in their specific host, recent studies shown that *Ascaris lumbricoides* and *Ascaris suum* can do it in both pigs and humans in certain circumstances. Cross-transmission took place both in developed and developing countries, depending on the epidemiological potential and local phylogeography (Betson et al. 2014).

A few specific Ascarids affecting other animal species are capable of infecting people also, even without completing their life cycle. *T. canis* and *T. cati* were considered the only zoonotic species, but some researchers have shown that *A. suum* and *B. procyonis* can also be zoonotic (Samson et al. 2012; Zhou et al. 2012; Figueiredo et al. 2016), and the same applies to the potentially zoonotic *Toxascaris leonina* (Paquet-Durand et al. 2007; Soriano et al. 2010). Recent studies suggest that 75% raccoons of Central Park (New York City, USA) are infected by *B. procyonis* (Rainwater et al. 2017), whereas population of European raccons is uncontrolled (Salgado 2018).

b) *Trichuridae*

Species of *Trichuris* depend on their corresponding host to reach sexual maturity in the large intestine. Unembryonated eggs lemon shaped with two polar plugs (Figure 2) are passed in the faeces to the soil. When temperature and humidity conditions are adequate, the first-stage larva (L1, infective stage) develops inside for one-two months (Palomero et al. 2018).

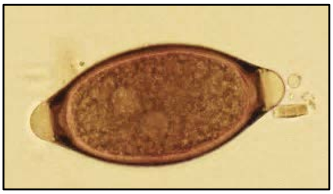


Figure 2. Eggs of *Trichuris* have two polar plugs.

If temperature is cold or very hot, although the embryonic period may differ (Borchert and Cordero del Campillo 1981) (Table 5), eggs can survive, especially in kennels or stables with clay or clay soil (Beugnet et al. 2018).

Table 5. Evolution of *Trichuridae* in the soil.

Temperature	Infective stage in eggs
15ºC	120 days
22-24ºC	54 days
35-37ºC	18 days

The epidemiology of trichuriosis is similar to that of ascarids. Faecal contamination of the soil, physicochemical characteristics, environmental humidity and elevated rainfall and temperature favour the maintenance and spread of these infections (Apt Baruch 2013).

c) Strongylids

Virtually, all species included into the order Strongylida have a direct biological cycle (monoxenous), with an exogenous phase characterized by hosts passing eggs by faeces (Figure 3a). In a few days, depending on temperature and humidity conditions, larva 1 (L1) is formed, the egg hatch and the larva emerges and feeds on organic matter present in the faeces. Once the L2 is attained, it leaves the faecal matter and finally transforms into L3 (infective stage) that no longer feeds (Figure 3b). This process takes several weeks below 10°C, 15-24 days at 10°C, and 3 days at 25-35°C (Mfitilodze and Hutchinson 1987). When the temperature drops below 6°C, eggs do not evolve but maintain their viability (Smith 2015).

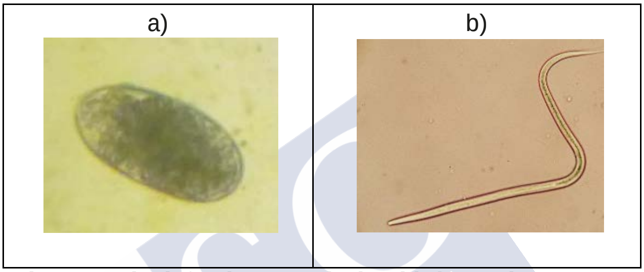


Figure 3. The infective stage is the third state larve or L3.

Table 6. Evolution of Strongylids in the soil.

Genus	Infective stages in eggs
Cyathostomum	2-6 weeks
Strongylus	1-3 weeks
Oesophagostomum *Chabertia* *Haemonchus* *Cooperia*	2-3 weeks
Ancylostoma	1-3 weeks

Eggs of nematodes develop easily in sandy soils, such as in river beaches or recreational areas, which entails a significant risk of percutaneous infection by *Ancylostoma* spp. known as *Cutaneous Larvae Migrans syndrome*. Traversa et al. 2014; Rocha et al. 2018 reported some of most frequently detected strongylid species in the soil (Table 6). Filariform larvae are able to survive for weeks in humid, shadowy areas and with an abundance of organic detritus that increase at high temperatures (Apt Baruch 2013).

2.2.1.3. Ectoparasites

a) Ticks

From a medical point of view, the most important tick species belong to the Ixodidae family, also known as *hard ticks*. These are large mites which during their development go through four phases, egg, larvae, nymph and adult. In some species the evolution from larvae to nymph and adult takes place on the same host, but in others requires two or even three hosts. To complete each phase, ticks need to ingest blood (Anderson and Magnarelli 2008). The infected and engorged adult females fall to the ground and seek out shady and humid places to perform oviposition (Greenfield 2011). After 28-61 days, eggs hatch and larvae need to feed blood (Figure 4).

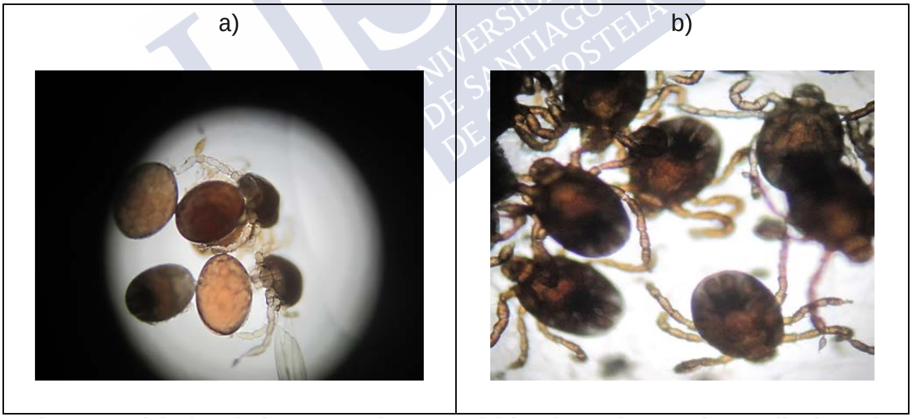


Figure 4. Ticks lay their eggs on the ground (a), where they evolve until a larva is formed inside and finally emerges (b).

Troughton and Levin 2007 analysed the life cycles of seven species of ixodid ticks in laboratory conditions and reported that larvae need between 16-50 days to become nymphs after ingesting blood. Nymphs need to feed again for 30-49 days to become into adult ticks. Under temperatures between 22-24ºC the complete cycle of the studied species ranged between 177 and 229 days. In nature, in addition to climatic conditions (temperature, precipitation, etc), ecological factors (vegetation, alternative hosts, etc) have a significant influence on the development of tick populations (Drugueri 2004).

Despite their hematophagous habits, it should be highlighted that about 95% of the life cycle of hard ticks occurs in humid niches of the surface of the ground, and only 5% on the host (Alekseev 2006). In addition to damage caused by the loss of blood, the transmission of different agents during the process of sucking the blood can cause important pathogenesis, as summarized in Table 7. These diseases are part of the those known as *vector-borne diseases.*

Table 7. Diseases transmitted by *Ixodes* spp.

Agent	Transmitted pathogen	Disease
Parasite	*Babesi a divergens* *B. microti*	Babesiosis
	Theileria spp	Theileriosis
Infectious	*Anaplasma* spp	Anaplasmosis
	Ehrlichia spp	Ehrlichiosis
	Nairovirus	Crimea-Congo
	Rickettsia spp	Mediterranean spotted fever
	Borrelia burgdorferi	Lyme
	Flavivirus	Encephalitis
	Francisella tulariensis	Tularemia

b) Fleas

During the adult phase, that usually lasts between 15-30 days, fleas can lay about 25 eggs / day that develop in the ground. Under optimal conditions of temperature and humidity conditions (29ºC and 85% RH), the larvae (which are a few millimeters in length) appear vermiform with long bristles, completing their development (three larvae stages) in less than a week by feeding on organic matter and the faeces of adult fleas. These stages are very sensitive to dryness and to ultraviolet light.

Once completed, fleas form a puparium, undergo the metamorphosis and turn into adults in almost 10 days when ideal conditions concur, but pupae remain quiescent for several months at temperatures <17-19ºC or in the absence of sound stimuli (Beugnet et al. 2018). Fleas can act as vectors transmitting diseases (Table 8).

Table 8. Diseases transmitted by fleas.

Agent	Transmitted pathogen	Disease
Infectious	*Pasteurella* or *Yersinia pestis*	Plague
	Rickettsia typhi	Typhus
	Rickettsia felis	Flea borne spotted fever
	Bartonella henselae	Hemobartonellosis
Parasitic	*Dipylidium caninum*	Teniosis
	Hymenolepis diminuta	Teniosis

2.2.2. Beneficial organisms for parasitic control

In addition to the harmful organisms or pathogens found in the soil, it is important to highlight the presence of beneficial agents, generally classified into three large groups according to their size (Table 9). It is undeniable their importance in the process of oxygenation of the deep layers of the soil, to take advantage of other organisms, etc.

In the mesobiota live nematodes and mites essential in the food pyramid. Their roles include to metabolise and transform nutrients that serve as food to other organisms, or be part of other animal diets. Finally, in the microbiota there are organisms that degrade organic matter to elements that can be absorbed by different living beings, such as plants, so they establish a bridge between the primary decomposers and the rest of soil organisms.

Table 9. Beneficial soil organisms for parasitic control.

Macrobiota	Mesobiota	Microbiota
Worms	Nematodes	Algae
Ants	Mites	Bacteria
Slugs		Fungi
Insects		Protozoa
Arachnids		Viruses

2.2.3. Self-cleaning of the soil

As already explained, a great biodiversity of organisms coexists in equilibrium in the environment, some harmful and others beneficial. The balance can be altered by biotic (weather) and abiotic factors (Arias et al. 2011b). In natural conditions harmful organisms are controlled by some components of the telluric microbiota (bacteria, fungi, mites), which prevent their proliferation and uncontrolled propagation, avoiding thus alteration of the macrobiota. When climatic alterations occur, especially increments in temperature and humidity, certain pathogenic organisms multiplicate and proliferate. But the most unbalancing situations are usually associated to abiotic factors in which human beings participate, altering the enviroment through deforestation or different agricultural operations. Some of these activities, such as plowing, used to oxygenate the land, change the composition of the soil cover, and as a result, biodiversity become damaged.

The use of manure to fertilize the pastures can be also somewhat controversial in spite of the advantages derived from its use as an organic fertilizer. Some studies showed that pathogens affecting animals may be distributed during manure fertilization (Table 10). However, it has also been shown that manure fertilization is a stimulus for the development of some beneficial organisms, such as the *Arthrobotrys oligospora*, the model organism for interactions between fungi and nematodes, which has been associated with the development of plant species and the reduction of parasites infecting them (Niezen et al. 1996; Widmer and Abawi 2000; Wachira et al. 2009).

Table 10. Pathogens distributed with manure fertilization that affect animals.

Bacteria	Protozoa	Viruses	Helminths
Listeria monocytogenes	*Cryptosporidium parvum*	*Coronavirus*	*Fasciola hepatica*
E. coli O157	*Giardia* spp.	*VBD*	*Calicophoron daubneyi*
Salmonella spp.	*Eimeria* spp.		*Ascaris Strongylids*
Mycobacterium paratuberculosis	*Ballantidium coli*		

Certain soil microorganisms produce metabolites that favour the purification of the environment (Kotze et al. 2005), for example, the fungus *Penicillium chrysogenum* produces penicillin and some *Bacillus* species synthetise antifungal agents. Other example is *B. thuringiensis* that forms spores with protein crystals. When larvae and adults of *Trichostrongylus colubriformis* and some insects ingest them, they dissolve in the intestine vacuolizing the enterocytes, causing death (Jouzani et al. 2008). It has also been shown to develop antagonistic activity against the free stages of the nematode *Ancylostoma ceylanicum* (Hu et al. 2013). This property of *B. thuringiensis* has been used against crop pests. Some plant species (corn, cotton) have been genetically modified to express the genes of the bacillus that encode the production of biotoxins against lepidoptera (Shelton et al. 2002).

Several species of *Streptomyces* secrete metabolites with antibiotic and antifungal activity in the soil (Procópio et al. 2012; Zhang et al. 2013). It should be noted that some of the most commonly used antibiotics (streptomycin, chloramphenicol, terramycin, cyclohexanide) originate from large-scale cultures of *Streptomyces*.

More than 1,600 viruses affect some 1,100 species of insects and mites, most of them belonging to the family Baculoviridae, Polyadnviridae and Ascoviridae. Infection occurs by ingestion, then virions bind to receptors in the intestine and penetrate the epithelial cells of arthropods (Chandler et al. 2011).

Entomopathogenic nematodes (Steirnomastoids and Heterorhabditis) used in agriculture carry in their intestine symbiotic bacteria of the genus *Xenorhabdus* and *Photorhabdus*. When breaking the cuticle of the insects, the larvae of the helminths introduce the bacteria and easily spread causing death by septicemia. Then, the nematodes feed on the interior of the insect, attain the adult stage and reproduce, giving rise to new generations of nematodes (Hajek 2004).

In the 1950's the possibility of using some fungi found in the soil against nematodes that infected plants was considered (Barron 1977, 1992). This idea comes from the observation that certain filamentous fungal species had the ability to develop mechanisms to limit the viability of parasitic larvae such as *Meloydogine* spp., *Globodera* spp. or *Heterodera* spp., which affect forage plants and vegetables of great importance in crops (Olthof and Estey 1963; Mankau 1980; Persson and Jansson 1999; Hussain et al. 2016). It was found in later years that some fungi such as *Beauveria bassiana* or *Metarhizium anisopliae* were highly useful to control parasitic insects of animals and plants (Bittencourt 2000). Since then, properties of saprophytic fungi (*Arthrobotrys*, *Duddingtonia*, *Monacrosporium*) with trapping activity have been discovered (Mendoza de Gives et al. 1998; Braga et al. 2007; Fitz-Aranda et al. 2015) and others that act on parasitic helminth eggs (da Silva et al. 2013; Arroyo et al. 2016).

2.3. Fungi parasiticides of the soil

It is estimated that the Fungi Kingdom encompasses 1.5-5 million eukaryotic microorganisms living in both terrestrial and aquatic ecosystems (Blackwell 2011). To date, only about 100,000 species have been described, representing a minimal proportion. These organisms have a high tendency to interact closely with other groups such as algae (21%) or plants (8%) (Araújo and Hughes 2016).

As eukaryotic organisms lacking chloroplasts (cellular organelles responsible for performing the fontosynthesis in eukaryotic organisms) fungi are not able to synthesize organic matter, so they have heterotrophic nutrition. Almost all the soil species are saprophytes or symbionts (lichens). Saprophytes feed on decomposing organic matter and perform external digestion through secretion / releasing of enzymes and subsequent absorption of the resulting molecules; this becomes into a crutial function because they are involved in decomposition of organic matter to inorganic molecules (Gadd 2007).

A small percentage (less than 0.5%) of the saprophytic fungi can also feed on living organisms, due to the development of structures specialized in trapping and digesting nematodes, rhizopods and rotifers (McInnes 2003). The mechanism by which some species of fungi can pass from a saprophyte to another predator has aroused great scientific interest, postulating that the lack of carbon and nitrogen in the environment could serve as a stimulus, while for others researchers the change is induced by the presence of fecal matter (Dackman and Nordbring-Hertz 1992; Anan'ko and Teplyakova 2011). More recent studies point to the intervention of some bacteria participating in *marking* of certain nematodes. (Wang et al. 2014) indicated that some nematodes ingest soil bacteria which release urea, acting as an inducer of the transformation of the filamentous fungus *Arthrobotrys oligospora* to its predatory form of gastrointestinal larvae found in the soil (L2 and L3 stages).

The switching of saprophytic and predatory behaviour could originate when living beings were extinguished en masse, a process that resulted in soils with high carbon content and low in nitrogen, hence the possibility of taking nitrogen directly from living organisms would provide a competitive advantage over strict saprophytic fungi (Yang et al. 2012).

During the predatory phase these fungal species develop different elements or structures, such as constrictor rings or adhesive traps in the case of trapping-nematodes (Figure 5). It has been reported the need of nematodes are alive for fungi form the traps, due to it is movement and products excreted by the nematodes seemed to serve as stimulati (Nordbring-Hertz 1977; Liu et al. 2012). By contrast, *Duddingtonia flagrans* forms traps and ellaborates chlamydospores in contact with dead helminths (trematodes, ascarids and oxyurids), and even with the only presence of their metabolic products (Arias et al. 2013a).

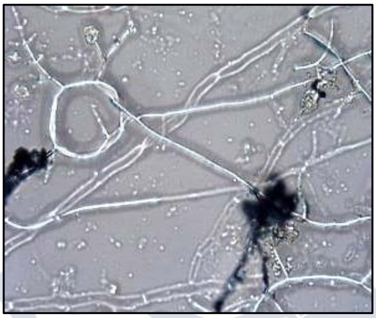


Figure 5. Constrictor rings created by trapping-nematodes.

The concept of *nemina* has been described as a set of substances found in the cuticle of parasitic nematodes which constitute a signal for the production of traps by filamentous fungi (Pramer and Stoll 1959). At first, some researchers established that *nemina* could be composed by some amino acids or a small polypeptides of parasitic nematodes (Pramer and Kuyama 1963), but Arias et al. 2013a showed that the presence of adult trematodes, or metabolic extracts (excretion / secretion antigens) of *Fasciola hepatica* and *Calicophoron daubneyi*, stimulated the development of *D. flagrans*. Furthermore, some larval species that under natural conditions would never be in contact with these fungi such as *Anisakis spp.*, have proven to serve as a stimulus for the development of mycelium and the production of traps. Hsu et al. 2015 concluded that different amino acids, as well as nematode extracts, can promote the creation of traps by nematophagous fungi. In this regard, the role of **ascarosides**, a family of signaling molecules synthesized by nematodes, has been analyzed in the induction of trapping (Choe et al. 2012; Hsueh et al. 2013).

2.3.1. Species of saprophytic fungi with parasiticidal activity

It is important to consider that the taxonomy of fungi has undergone many changes in recent years, mainly due to the advancement of molecular techniques. The ability of access fungi DNA sequences makes it difficult to reach a unanimous classification (Guarro 2012).

Although in this Doctoral dissertation the division of Fungi kingdom is maintained in different sub-kingdoms, at present some researchers highlight the existence of two sub-kingdoms, *Dykarya*, which includes *Ascomycetes* and *Basidiomycetes*, and *Basal fungi*, comprising *Zygomycetes* or *Zygomycota*.

Most of the saprophytic species with entomopathogenic or helminthic activity belong to *Ascomycetes* and *Zygomycetes*, and approximately 750 insects and mite antagonists (entomopathogenic) and about 700 nematicide species are described (Zhang et al. 2011).

The Entomophthorales encompassed *Zygomycota*, among the genera with greater entomopathogenic capacity are *Pandora*, *Entomophtora* and *Conidiobolus*. Entomophthorales can virtually infect all mites and insects.

The best known fungal genera of interest in agriculture are *Purpureocillium* (formerly *Paecilomyces*), *Pochonia*, *Hirsutella*, *Nemtophtora*, *Arthrobotrys*, *Drechmeria*, *Fusarium* and *Dactylellina* (Siddiqui and Mahmood 1996), while in livestock, *Duddingtonia*, *Monacrosporium*, *Verticillium*, *Mucor* and *Trichoderma* have widely been investigated (Braga et al. 2007; Arias et al. 2011a; Hernández Malagón 2014; Fitz-Aranda et al. 2015).

Oomycetes (Oomycetes or Oomycota) are a group of filamentous protists considered to be pseudo-fungi and also aquatic fungi, because most of them proliferate in fresh water (Scholte et al. 2004; Diaz et al. 2006). Oomycetes include both species saprophytes and parasites. Some studies analyse the control of mosquito larvae by oomycetes but the fact that these fungi affected larvae with lesions instead of healthy larvae indicates that in general the entomopathogenic oomycetes are opportunistic. Although different species of mosquitoes proved to be susceptible, Clark et al. 1968 concluded that the conditions under which it could become an important control agent would be difficult and unpractical to achieve.

Action mechanisms

Depending on the mechanism of action, fungi are divided into four groups (Figure 6). **Endoparasite** fungal species use spores to infect the host. Penetration is passive because of spores need to be ingested to develop their activity (Li et al. 2015). Species with the ability to make traps are called **trapping-nematodes** or **predators**. Traps are intercalated in the mycelium and the nematode larvae are retained, immobilized and finally digested. Taking into account their parasiticidal activity they are subdivided into: a) non-spontaneous trap formers, mainly saprophytes, and b) trap formers, which greatly depend on the presence of nematodes for their survival (Cazapal Monteiro 2016).

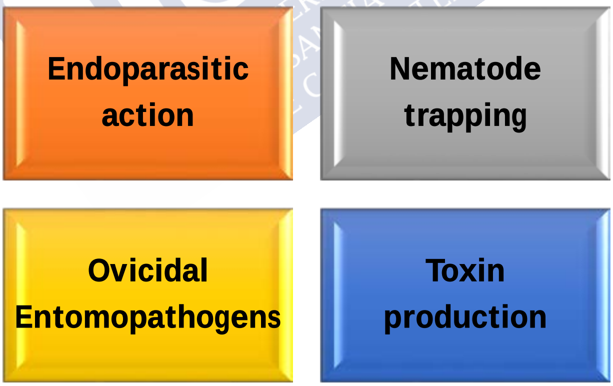


Figure 6. Action mechanism of parasiticidal fungi.

Ovicidal fungi and some **entomopathogens** are characterized by the development of specialized structures to penetrate target organisms, called *appressorio* and *haustorio*. **Three possibilities are established:**

First: the metabolites, produced by the fungus, inhibit embryonic development without affecting its morphology or integrity. This process is known as ovistasis (Ciarmela et al. 2002).

Second: the fungus releases metabolites that crack the outer shell of the egg. The ovicidal action is considered to be direct if the metabolites cause the death, or indirect, if the harmful substances in the surrounding environment diffuse through the shell gaps causing death (Hernández et al. 2017).

Third: the hyphae of the fungus penetrate inside the host, and destroy it completely in a sequence of different phases, similar to those previously indicated.

Toxin-producing species typically release different products for the immobilization of the antagonists, facilitating the penetration of the hyphae through the cuticle and the subsequent destruction (Li et al. 2015).

a) Ascomycetes with parasiticide activity

Ascomycetes are the largest group in the Fungi Kingdom, with about 64,000 species described (Araújo and Hughes 2016). Most of these fungi are filamentous with septate hyphae (Figure 7a). Asexual reproduction that gives rise to spores (Figure 7b) is the most common type of reproduction, and sexual reproduction (ascospores) takes place in bag-type structures (***disgust*** in Greek) (Suárez 2017).

They are species with nematicidal and entomopathogen activity. Most are hemibiotrophic, colonize the target organisms, kill them and ingest the inner contents, and then begin to reproduce forming spores (Roy et al. 2016).

Table 11 summarizes some of the most well-known ascomycetes species used in the control of helminth, insect and arthropod pathogens.

Table 11. Ascomycete fungi used against pathogens

Name	Activity	Target organism
Beauveria bassiana	Entomopathogen	*Xylotrechus arvicola* *Acanthoscelides obtectus* *Rhipicephalus sanguineus* *Rhipicephalus microplus* *Amblyomma cajenennse*
Metarhizium brunneum	Entomopathogen	*Rhipicephalus* spp. *Amblyomma* spp. *Ixodes scapularis* *Hyalomma* spp. *Varroa destructor*
Lecanicillium lecanii	Entomopathogen	*Matsucoccus matsumurae* *Rhipicephalus microplus*
Pochonia chlamydosporia	Helminticide / Ovicide	*Fasciola hepatica* *Calicophoron daubneyi* *Ascaris suum* *Toxocara canis* *Toxascaris leonina* *Toxocara vitulorum* *Trichuris* spp. *Ixodes scapularis* *Rhipicephalus sanguineus*
Purpureocillium lilacinum	Helminthicide / Ovicide	*Toxocara canis* *Toxascaris leonina*
Trichoderma spp.	Helminthicide / Ovicide	*Toxocara canis* *Toxascaris leonina* *Baylisascaris procyonis*
Duddingtonia flagrans	Helminthicide / Larvicide	Trichostrongyles *Ancylostoma*
Monacrosporium spp.	Helminthicide / Larvicida	Trichostrongyles *Ancylostoma*
Arthrobotrys spp.	Helminthicide / Larvicide	Trichostrongyles *Ancylostoma*

A large number of applications have been described against parasites that affect both plant and animal species. No differences have been reported in the mechanisms of action between zygomycetes and entomopathogenic ascomycetes. It is important to note the presence, among ascomycetes with helminthicide activity, of species that destroy eggs, and other species that destroy larvae from traps. These *trapping-nematodes* elaborate traps in the mycelium in order to trap the larvae of strongyles and feed on them (Mendoza-De Gives et al. 2006).

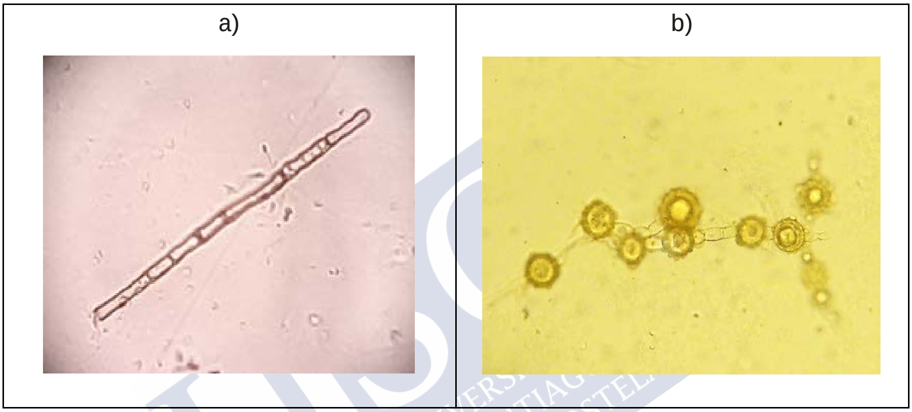


Figure 7. Ascomycetes fungi have septate hyphae (a), in which spores produced asexually (b) are interspersed.

b) Zygomycota or Zygomycetes with parasiticidal activity

From a structural point of view, Zygomycetes are characterized by having hyphae without partitions and thick-walled spores (zygosporas) although important morphological variations may appear depending on the culture medium. In adverse conditions, sexual reproduction is triggered, known as gametangia, and forms the zygosporangium by the fusion of two differentiated hyphae (Figure 8) (Suárez 2017).

Best-known species of zygomycetes have entomopathogenic activity, such as *Beauveria bassiana*, *Metarhizium anisopliae* or *Lecanicillium lecanii* (López Lastra and Scorsetti 2007; Araújo and Hughes 2016) (Table 12). At the moment, only one genus with helminthicide activity has been described, *Mucor* (Arias et al. 2011a; Cazapal Monteiro 2016; Arroyo Balán 2017).

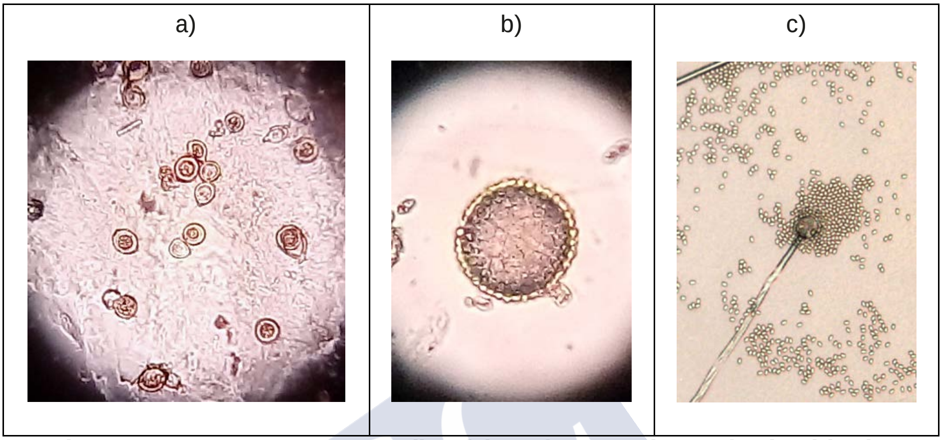


Figure 8. Zygomycete spores usually originate by asexual reproduction (a). The result of sexual reproduction is the zygosporangium (b), which, when broken, releases the spores inside it (c).

Table 12. Zygomycete fungi used against pathogens.

Name	Activity	Target organism
Pandora neoaphidis	Entomopathogen	*Acyrthosiphon pisum* *Musca domestica*
Entomophtora spp.	Entomopathogen	*Aphididae* *Formica polyctena*
Conidiobolus spp.	Entomopathogen	*Calliphora* spp. *Musca domestica*
Mucor circinelloides	Helminthicide / Entomopathogen	*Fasciola hepatica* *Calicophoron daubneyi* *Ascaris suum* *Toxocara canis* *Toxascaris leonina* *Toxocara vitulorum* *Trichuris* spp. *Ixodes scapularis* *Rhipicephalus sanguineus*

In any case, zygomycetes are usually **hemibiotrophic organisms** that colonize their hosts during the **biotrophic** phase (while they are alive), kill them (**necrotrophic**) and then start the production of spores (Münch et al. 2008).

The activity of these fungi is associated with the target organism. Mechanism of action against helminth eggs has been described in four stages (Figure 9), taking into account previous research on the action of *Verticillium chlamydosporia* (ascomycete) on eggs of *Ascaris lumbricoides* (Lýsek and Stěrba 1991).

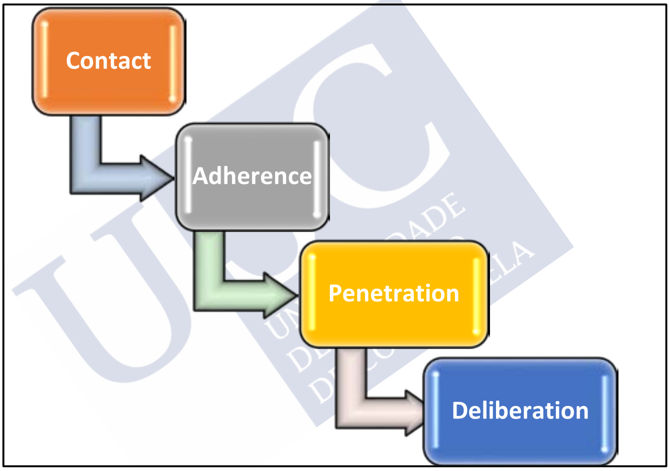


Figure 9. The ovicidal effect includes four stages.

When the presence of the parasite is detected, the fungi develop hyphae that come into contact and adhere to the surface of the egg. The penetration inside the egg without breaking the shell is produced through releasing various proteolytic enzymes (Lopez-Llorca et al. 2010), together with the pressure exerted by the end of the hyphae (appresorio) that contact at a 90° angle. Once inside through the haustorio, the entire internal content of the egg is digested. Then in the propagation phase, the fungus colonizes the interior and produces spores. In the final phase, zygomycetes leave the egg in search of more eggs (Arroyo Balán 2017). So far, fundamental application of entomopathogenic zygomycetes is carried out in the control of plant parasites (Table 12) (Figure 10).

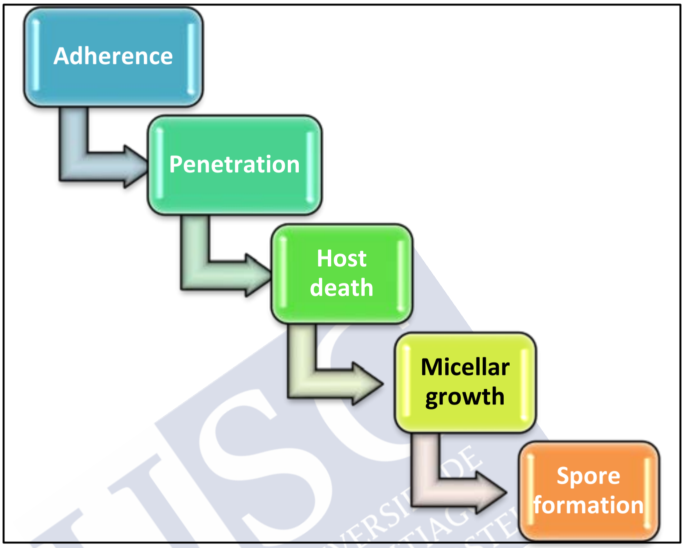


Figure 10. Zygomycetes act on arthropods through different phases.

Figure 11. Zygomycetes detect the presence of *Ixodes* eggs and develop hyphae.

As seen in Figure 11, the mechanism of action of zygomycetes is practically analogous against helminth and arthropod eggs. The size of the target organism makes it possible for the formation of hyphae from spores that adhere to the surface of larger eggs (Figures 12a, b y c).

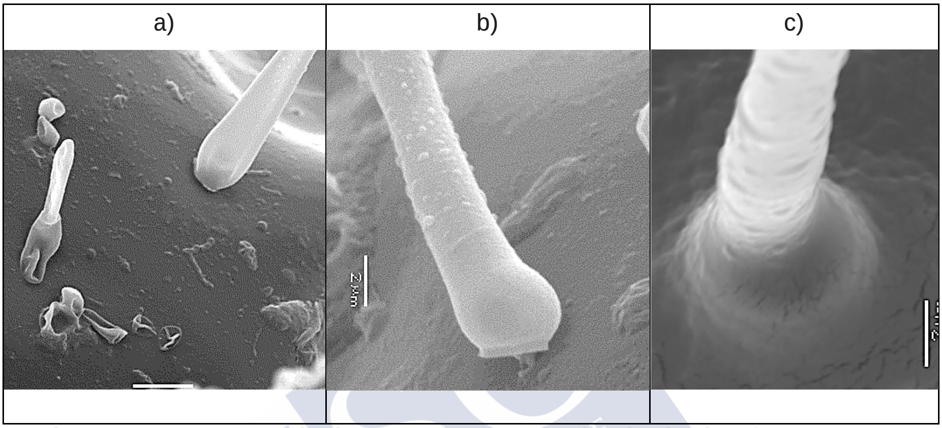


Figure 12. Zygomycetes in the presence of *Ixodes* develop hyphae from spores or mycelium, which adhere to the eggshell (a), form the appressorium (b) and penetrate without destroying it (c).

2.3.2. Mucorales: *Mucor circinelloides*

As detailed above, the order Mucorales encompasses 13 families, 56 genera and about 300 species (Table 13).

Table 13. *Mucor spp* taxonomic classification.

Kingdom	Fungi
Phylum	*Zygomicota*
Class	*ZygomicetesMucor*
Subclass	*Incertae Sedis*
Order	*Mucorales*
Family	*Mucoraceae*
Genus	*Mucor*

These are fast-growing species, many of them saprophytes (they grow in fruits, soil, manure, wood ...). Some are parasite antagonists that affect animals and people, as is the case of *Mucor circinelloides* (Arias et al. 2011b).

As it has been explained, they can present sexual or asexual reproduction. *Mucor* grows rapidly forming colonies of flocose texture and ochraceous brown color (Figure 13a). Development is enhanced at temperatures below 25ºC, although at higher temperatures it grows slowly. Under the microscope, the formation of non-septate mycelium and rhizoids (substrate fixation structures) is evident (Figure 13b).

Figure 13. ***Mucor circinelloides*** **quickly gives rise to brown colonies (a), and under the microscope it is verified that the hyphae do not have septa (b).**

Sporangiophores (support of structures of sporangia) branch sympodially (the lateral branches grow more than the main axis) (Figure 14a). Formation of asexual spores is observed in high numbers, arranged in sporangia (Figure 14b), spherical to subspherical, without apophysis (Figure 14c). Spores adopt an ovoid to ellipsoidal shape, and are smooth.

This fungus has been the focus of interest in research studies for over 35 years, due to its dimorphism, displaying a filamentous phase (micellar phase) or a yeast-like phase, depending on the environment.

In the presence of oxygen, *M. circinelloides* develop a mycelium (**filamentous phase**), whereas in anoxic environments they develop yeast (**yeast-like phase**).

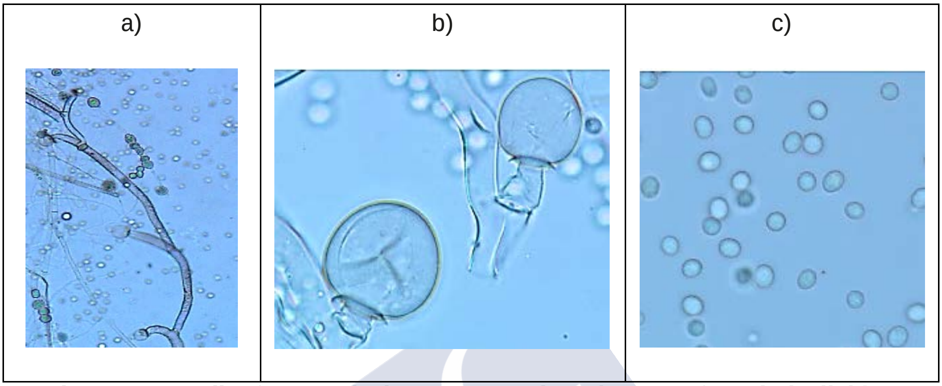


Figure 14. Details of sporangiophores, sporangia and spores of *M. circinelloides*.

Great attraction for biotechnological applications of this fungus is based on the aerobic / anaerobic ethanol production capacity, thanks to the manipulation of its genome (Mitra et al. 2012). Noteworthy is the production of carotenes and lipids due to their high capacity to accumulate these products in their mycelium, a phenomenon favored by the large production of biomass in submerged (liquid) crops. Discovery that the lipids obtained can easily be transformed into biodiesel has led us to consider the production of biomass from M. circinelloides as a reliable alternative to oil plants (Vicente et al. 2009). For all these reasons, great progress has been made in the field of industrial bioreactor culture (Nitayavardhana and Khanal 2012).

On the other hand, ecological and sustainable applications of this species, oriented to wastewater treatment (Bhanja et al. 2014), or to production of biosurfactants from oils used in frying foods (Hasanizadeh et al. 2017), make *M. circinelloides* a potential candidate for resolving situations of environmental contamination by oils and derivatives.

Recently, the use of *M. circinelloides* for the biological control of parasites is being evaluated, but there are still not many studies that reveal their activity against helminth eggs or ticks (Table 14). Occasionally it has been used together with the trapping-nematode species *D. flagrans*, since Arias et al. 2013a developed a liquid culture medium for the simultaneous production of *M. circinelloides* and *D. flagrans* spores in order to have a mixture of fungal stages that allowed the control of parasitic forms transmitted through the ingestion of eggs or larvae, respectively. Preliminary trials have shown that *M. circinelloides* presents ovicidal activity against trematodes, ascarids or tricruids (Cazapal-Monteiro et al. 2015; Cortiñas et al. 2015; Arroyo et al. 2016), and also against eggs of ixodids (Hernández et al. 2018). However, references about this possible activity for the control of flies that affect horticultural species practically do not exist.

Table 14. Activity of *M. circinelloides* against certain parasites.

Parasite	Viability reduction	Animal species	Reference
Calicophoron daubneyi		Cow	(Arroyo Balán 2017)
Toxocara canis	66%	Dog	(Arias et al. 2013b)
Toxascaris leonina	64-67%	Lynx	(Hernández Malagón 2014)
Baylisascaris procyonis	51-74%	Raccoon Coati	(Cazapal-Monteiro et al. 2015)
Ascaris suum	53%	Swine	(Cortiñas et al. 2015)
Parascaris equorum	68%	Equine	(Arroyo et al. 2016)
Trichuris spp	51%	Dromedary	(Palomero et al. 2018)
Rhipicephalus spp	80%		(Hernández Malagón et al. 2018)

2.3.3. Safety of soil saprophyte fungi

a) On the environment

The idea of using certain species of saprophyte fungi for the biological control of pathogenic forms of plant and animal species is based on the observation that under natural conditions in the environment, a balance between different organisms is reached.

As result, hosts do not reach high levels of infection (Arias et al. 2011b). Regarding veterinary medicine, some fungal stages, mainly spores, were observed in the faeces of grazing animals, leading to consider that these species could be innocuous for these animals. This fact was a remarkable basis for the development of a series of strategies based on the oral administration of fungal structures (spores). This way of administration would ensure the presence of these structures in the fecal material along with forms of propagation of different pathogens (Braga et al. 2011).

By favoring the contact between the pathogen and antagonist in an environment with remarkable humidity and temperature, the development of the mycelium is promoted. Therefore, the final result will depend, to some extent, on the amount of spores that resist passage through the gastrointestinal tract (Paz Silva 2016).

Some studies have analyzed the possible adverse effect of saprophytic fungi with high potential for the biological control of parasites, such as *D. flagrans*, on populations of non-parasitic nematodes in the soil. In France, Paraud et al. 2012 conducted an assay in which sheep were administered very high doses of spores of this fungus ($5 \cdot 10^6$ chlamydospores / Kg live weight.). No negative effect was demonstrated on the population of nematodes in the soil nor on the degradation of the faeces during a minimum period of 20 weeks. In Argentina, Saumell et al. 2016 conducted experiments where spores of the fungus trapnematodes *Duddingtonia flagrans* were placed in the soil of vegetated plots, and verified that no-effect on larvae of free-living nematodes (not parasites) were observed. Also, it was noted that the persistence of the fungus in the soil did not exceed two months, this fact is explained because fungus serves as food to other soil organisms, finding therefore the balance. This reinforces the need for periodic administration of fungal stages to maintain successful results. In this regard, it should also be noted that for some decades, it has been established that in the cover of parasitic nematodes (strongyles) a set of proteins that are called nemina constitute the target of the fungal hyphae trapping activity, as previously mentioned (Barron 1977).

b) *On mammals*

Existence of numerous non-inocuous species of fungi, especially at skin level, results in some disagreement in respect of the use of some filamentous species to prevent possible infections by protozoa, helminths and even ectoparasites.

Table 15. Infection by Mucorales can result in different clinical diseases.

Faeohifomycoses	***Hialohifomycoses***
Acrophialophora fusispora	*Acremonium*
Alternaria	*Aphanoascus fulvescens*
Arthrinium phaeospermum	*Beauveria bassiana*
Aureobasidium pullulans	*Cephaliophora irregularis*
Bipolaris	*Chrysonilia sitophila*
Cephaliophora irregularis	*Cylindrocarpon*
Chaetomium	*Engyodontium album*
Cladophialophora	*Fusarium*
Cladosporium	*Lecythophora*
Colletotrichum	*Puerperocillium (Paecilomyces)*
Curvularia	*Penicillium*
Drechslera biseptata	*Phialemonium*
Exophiala	*Schizophyllum commune*
Exserohilum	*Scopulariopsis*
Fonsecaea pedrosoi	*Scytalidium hyalinum*
Lecytophora	*Scedosporium apiospermum*
Microascus	*Trichoderma*
Nattrassia mangiferae	*Verticillium serrae*
Phialemonium	
Phoma	
Rhinocladiella	
Scedosporium prolificans	
Wangiella dermatitidis	

Traditionally in human medicine great importance has been given to infections by *Aspergillus* or mucorales. However, in recent years the *emergence* of filamentous fungi has been defined. With the purpose of clarifying some clinical pictures, *faeohifomicosis* was defined as those infections by filamentous fungi that contain melanin in their walls, while *hialohifomicosis* encompasses filamentous fungal infections with non-pigmented hyaline hyphae (San Juan et al. 2003) (Table 15).

Only one bibliographic citation about the infection of animal species by *M. circinelloides* exists, a Vietnamese pig which showed clinical signs of pneumonia (Evans et al. 2018). However, since 2010, COPAR Research Group (GI-2120; USC) has been developing numerous trials administering *M. circinelloides* to dogs, horses and cows, confirming the absence of alterations in them and in the people involved in their preparation and distribution. PRG horses received food pellets for 64 weeks with *M. circinelloides* and *D. flagrans* spores. Veterinarians carried out analysis of the respiratory, digestive and reproductive systems, skin and red and white blood series, and no clinical signs of animal health deterioration were observed (Hernández et al. 2016). Identical results have been obtained in dogs (Rodríguez Rodríguez 2018), calves (Arroyo-Balán et al. 2015) and piglets (Cortiñas et al. 2015).

2.4. Parasitic control

2.4.1. Pharmacotherapy

Over the last two decades and on numerous occasions, different strategies have been proposed to control parasitic infections in animals and people. However, the truth is that no real progress has been made and the administration of drugs for preventive and curative purposes is the most common course of action (Charlier et al. 2018).

The most remarkable issues originated from the use of drugs as therapy are summarised in Table 16. It is interesting to note that amongst the different options proposed to avoid these negative effects, the complete avoidance of antiparasitic drugs is not contemplated, since they are required under certain regimes of animal and vegetable production. A sensical usage based on criteria of health, productivity or risk of transmission between animals and people is recommended (Nielsen 2012).

Table 16. Main disadvantages of conventional antiparasitic treatment

Need for repeated administrations
Remarkable economic cost
Decrease in efficiencies can lead to resistance
Observation of suppression periods
Ecotoxicity

Despite the fact that many families of antiparasitic agents have been developed over time, whose efficacy is still present, the problem of the reduction of their effectiveness has led to the consideration of new formulations. This problem can worsen by the appearance of strains of *resistant* parasites. Some individuals can present genetic hereditary mutations (=transmissible) to survive the antiparasitic treatment (Nielsen et al. 2010). The high economic cost involved in the synthesis of new molecules, evaluation of the effect and legal registration, has led to the preparation of mixtures of known antiparasitic agents, in order to increase the efficacy and spectrum of action (Bloemhoff et al. 2014). In this way, it is possible to find products based on combinations of different active ingredients, for example against tramatodes, such as *Fasicomectina plus*® (Triclabendazole + Ivermectin + Oxiclozanide) or *Nitromec*® (Nitroxynil + Clorsulon + Ivermectin) for gastrointestinal nematodes (Bonilla 2016).

Although it is widely known that a significant number of parasitic forms develop a part of their cycle in the environment (soil), possible actions against these free life phases have not been given sufficient importance, although they could potentially reduce the likelyhood of infection by limiting the number and viability of infectious forms. If this aspect is not further explored the problems of conventional antiparasitic treatments will continue to exist.

2.4.2. Activities on the environment

a) Pets (dogs)

Numerous parasitic forms can survive in the environment for prolonged periods of time, which highlights the need to put measures in place to prevent their presence, or at least to prevent reaching the infective stage.

The European Scientific Counsel Companion Animal Parasites (ESCCAP) issued a *Guide for the control of vermes in dogs and cat* (ESCCAP 2017) in which pet owners are urged to show responsible behavior that includes the collection of faeces (especially in public spaces), along with proper cleaning and frequent disinfection of animal resting places.

In the United States, the *Companion Animal Parasite Council (CAPC)* offers information to pet owners, among which it is worth noting the usefulness of covering sandbuds when they are not used, as well as immediately removing the faeces of dogs from public areas. It is also specified that in order to prevent contact with animals without parasitic control or their faeces, pets should be kept on a leash or behind a fence (CAPC 2016). The *Association of Shelter Veterinarians* (ASV) and the *International Companion Animal Management Coalition* (ICAM coalition) are organizations concerned with the welfare of dogs and cats, which they pursue through a series of measures, designed especially for kennels or animal shelters. These include, among others, to indicate the proper location of these centers, the construction materials that must be used (wood, cement ...), the cleaning and disinfection routines, or deworming protocols, to maintain adequate levels of health and well-being of individuals (ICAM 2007; Newbury et al. 2010).

b) Livestock animals

Figure 15 shows activities related to parasitic control in livestock animals. Of all of them, only pasture management is focused on the reduction of infectious forms in the environment.

The economic global crisis unleashed a few years ago has caused a strict adjustment of inputs and outputs in livestock farms, and aiming to reduce food costs, some proffesionals have returned to use pasture management systems. Another important reason has been to satisfy the changes observed in consumers, who demand food obtained under welfare conditions and with specific organoleptic characteristics.

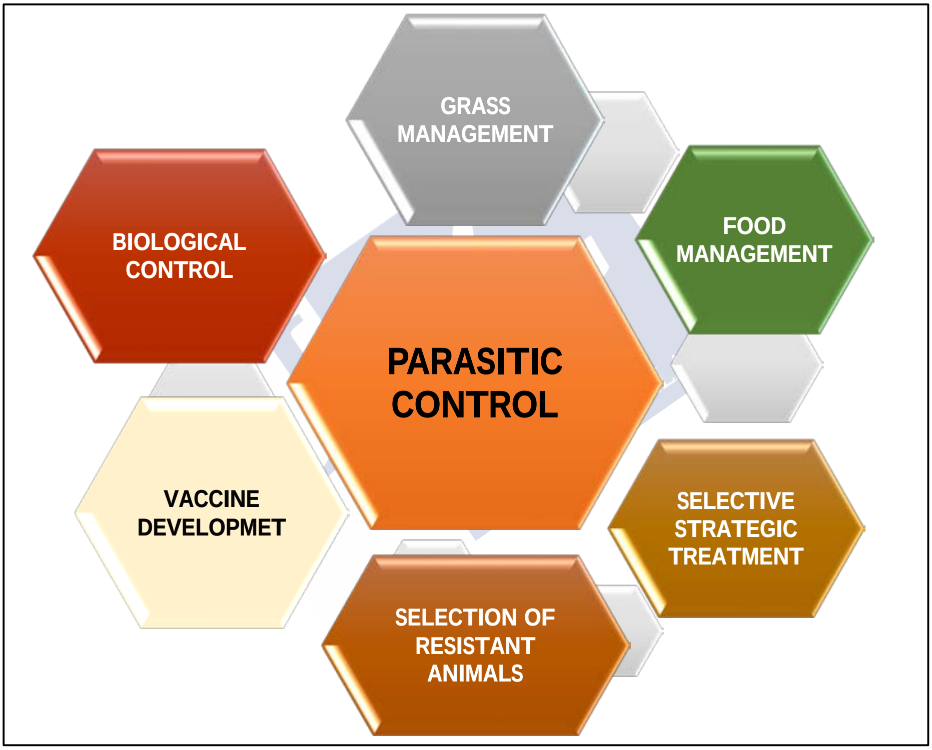


Figure 15. Parasitic control possibilities in livestock.

Based on the temporary effect of conventional systematic treatment, various actions could contribute to parasitic control, making it possible to administer antiparasitic treatments only in really necessary situations. One of the possibilities consists in the management of pastures, such as for example rotational pastures, whose objective would be that an appropriate period of time elapsed so that the parasitic forms do not survive in the environment. This procedure requires a large number of meadows, to avoid that when returning (completing the rotation), there is a high risk of re-infection (Singer et al. 2002; Colvin et al. 2008). Other actions could consider **plowing land** to destroy the habitat of parasitic forms.

Occasionally the addition of calcium cyanamide has been recommended due to its molluscicidal effect, which would be useful to interrupt the exogenous phase of some trematodoses (Cringoli and Rinaldi 2014). However, extracts from some plants have provided encouraging results (Jaiswal and Singh 2009), which have even been formulated in bait form pellets (Hanif and Singh 2013).

c) Species in captivity

Parasitic control of animal species in captivity has been understudied, in general it is based on the administration of formulations destined to livestock species; as the adequate doses are unknown, as well as the possible side effects, they tend to be underdosed (López Hidalgo 2018). It is necessary to take into account that livestock are individuals that are permanently in the same plot, and therefore in continuous risk of infection. Rotation of plots would be remarkably complicated to implement, for example, due to the stress that could be inflicted on the animals, which could decrease their immune response and thus increase the chances of developing parasitic infections (Gracenea et al. 2002; Citino 2003).

2.4.3. Saprophytic parasiticide fungi and Biological Control

Biological Control or Biocontrol is understood as the phenomenon of regulation of the number of plants and animals by means of natural enemies (parasites, predators and pathogens, and more specifically in relation to parasitic control, it is defined as the action of natural enemies that maintain a parasitic population in the host in lower levels than those that would exist in their absence

(Waller and Faedo 1996). From these definitions it could be inferred that the activity would be directed towards the hosts, but as it will be pointed out later this is not the most widespread application at present, precisely because of the presence of free life forms in the soil (Cortiñas et al. 2015).

First references to biological control indicate the use of ants as a measure to limit the presence of caterpillars in citrus fruits, because these organisms produce very appetizing molasses (Badii and Abreu 2006a). In the industrial age, different microorganisms were discovered that could be useful as biological control agents. Thus, in 1889 a beetle was used for the first time to control an insect that threatened the citrus industry (Badii and Abreu 2006a).

Natural biological control take place when it is developed by natural enemies that normally exist in the environment, while in *applied biological control* the intervention of the man is necessary. Natural biological control does not achieves a great impact on parasites, due to intensive farming practices that influence the composition of the soil, and which have caused an imbalance in favor of the parasites (Waller and Faedo 1996). As a remedy, applied biological control is postulated, which can be implemented in three different ways, or through combinations of them (Figure 16).

Conservative modality would be the most desirable, but for economic reasons, the return to past farming practices, based on the use of animals for traction or plowing, procedures not harmful to the environment, etc it is not contemplated nowadays. Another strategy could be to rotate pastures, but is it not a measure that most farmers are willing to assume correctly either. Nowadays it is carried out to ensure the correct nutrition of the animals, and not as a strategy of parasitic control that avoids infection getting rid of the external phase of the biological cycles of the parasites (Singer et al. 2002; Colvin et al. 2008).

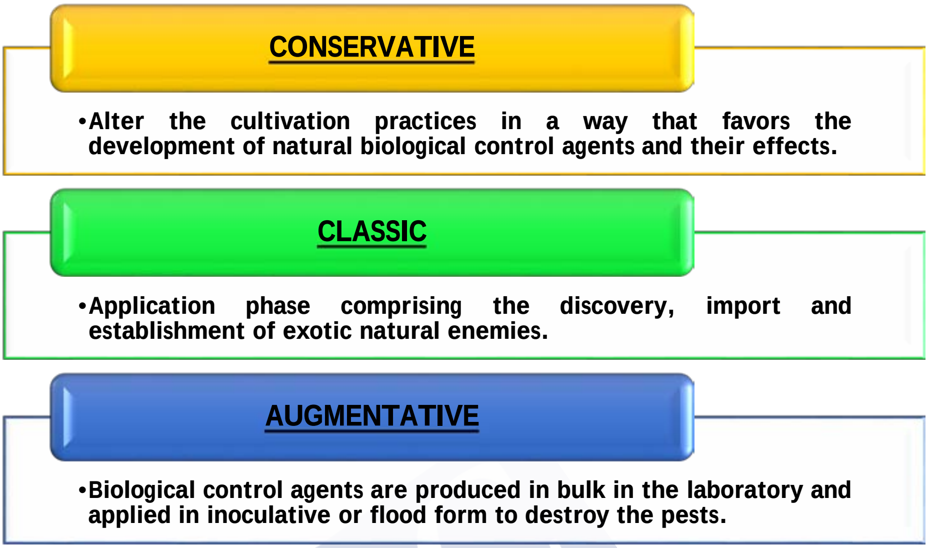


Figure 16. Procedures of biological control of parasitic stages.

In relation to the use of *exotic* antagonists, it is also not considered in the medium-long term, because the introduction of unknown species in a habitat could have much more harmful consequences than the presence of the parasites. Another drawback lies in the characteristics of the organisms used in biological control, which may not meet the previously established expectations, or they may not even not develop due to the climatic conditions of the area to be controlled.

Considering this background, *augmentative biological control* is postulated as the most appropriate option and with the greatest projection. It should be emphasised that that biological control is free of the side effects associated with broad spectrum products, when Biological Control is applied by specialists, under established principles, it is safe and have no adverses effects on the ecosystem. Several safety reasons, but above all they are based on their presence naturally in the environment, as well as that of other organisms that are responsible for regulating it (Arias et al. 2011b).

Some species of saprophytic fungi feed on decaying organic matter in the soil but if they are close to parasitic forms such as eggs, larvae, etc., certain species are able to use them as a source of nitrogen and carbon, feeding on these parasitic structures (Barron 1992; Askary 2015; Cazapal Monteiro 2015). For this reason, some biological control strategies are based on the use of certain species of saprophytic fungi naturally present in the soil.

a) Integrated biological control

Biological Control term was coined in the early nineteenth century (Badii and Abreu 2006c) when naturalists from different countries recalled the important role of entomophagous organisms in nature. With the use of biological control, an attempt is made to restore the altered ecological balance, through the use of living organisms or their metabolites, and to eliminate or reduce the damage caused by harmful organisms.

In most insect groups there are entomophagous species, which feed on other insects as predators or as parasites (Badii and Abreu 2006b).

In recent years, the natural evolution of agrarian production systems has led to methods of control of pests and diseases that are more rational and respectful to the environment. These techniques are consistent with the philosophy of sustainable development (Badii and Abreu 2006a, 2006c) and the notion of resource conservation, biodiversity and environmental ethics. They have also been used to control weeds, certain vertebrates, red spiders, etc... These new techniques have led to the concept and development of Integrated Production, as a strategy to solve some disadvantages posed by the exclusive use of chemical substances:

* The emergence of new resistance to insecticides in pests.

* The resurgence of the populations treated.

* Residues, risks and legal complications.

* Destruction of beneficial species.

* Cost of fumigants, equipment, labor and material.

The concept of *Integrated Control* is established as a method of controlling pests and diseases in which chemical products, useful organisms and cultural practices are used together. The main objective when Integrated Control is applied to livestock is the rational and effective control of pests and diseases, reducing the amount of waste in the products obtained (milk and meat) by using organisms with antagonist activity of certain pathogens. It is interesting to bear in mind that the approach would be primarily **preventive**, and with the intention of limiting the use of chemical products to those situations when they are totally necessary, these measures would help to prolong the efficacy of the parasiticide active ingredients.

b) Applications of fungi filamentous saprophytes to biological control / integrated control

It should noted that the main aim is to achive a preventive effect, therefore it is important to intially treat the hosts to eliminate or at least significantly reduce their parasitic load. Table 17 summarizes some of the trials in which the deworming of the animals was supplemented with the administration of spores of fungal parasiticides.

Most of field tests of biological control with fungi have been carried out by administering clamidospores of *D. flagrans* to sheep by oral route (Ojeda-Robertos et al. 2008b; Saumell et al. 2016), and in much less proportion to horses, cows and goats (Paraud et al. 2012; Hernández et al. 2016; Ortiz Pérez et al. 2017; Healey et al. 2018).

In recent years some innovations have been introduced. For instance, Arias et al. 2013a included spores of fungi parasiticides (*Duddingtonia flagrans*) in food premix, to limit the risk of infection with strongyles in equines (zebra, domestic donkey and African donkey) of Zoological Park "Marcelle Natureza" (Outeiro de Rei, Lugo, Spain). After administering an ivermectin-based treatment, and providing the premix with spores, egg rates in the faeces were maintained at around 200 eggs per gram of faeces, which meant that it was not necessary to deworm for another year. Later, the efficacy of a formulation consisting of making nutritional pellets with spores of *Mucor circinelloides* + *D. flagrans* was tested, and the results were very similar (López Hidalgo 2018). The use of *M. circinelloides* for the control of *Trichuris* has also been very successful in this park (Palomero et al. 2018).

The possibility of combining nematophagous fungi species with different activity, allows for the possibility to act against the different parasitic infective forms in the environment (Table 17). Arias et al. 2013b showed that *D. flagrans* and M. circinelloides are able to grow together in the same culture medium, without altering their growth capacity and their larvicidal and ovicidal activity respectively. The advantages of the combination of different fungal species in biological control have also been demonstrated, acting both against larvae and eggs, or using different species with the same purpose (da Silveira et al. 2017).

Table 17. Combination of fungi developing complementary parasiticide activity.

Parasite	Antiparasitic	Fungi	Assay
Toxocara canis	Fenbendazole	*M. circinelloides* + *D. flagrans*	(Balsa Vázquez 2018; Rodríguez Rodríguez 2018)
Toxascaris leonina	Fenbendazole	*M. circinelloides* + *D. flagrans*	(Teixeira Alves 2016)
Strongylidae	Ivermectin	*D. flagrans*	(Dimander et al. 2003)
Strongylidae	Ivermectin	*D. flagrans*	(Braga et al. 2011)
Strongylidae	Ivermectin	*M. circinelloides* + *D. flagrans*	(Arias et al. 2011b; Palomero et al. 2018)
Cyathostomins	Ivermectin	*M. circinelloides* + *D. flagrans*	(Almendros Sobrado 2016; Hernández et al. 2016; Vilá Pena 2018)
Trichuris spp.	Ivermectin	*M. circinelloides*	(Paz Silva 2016)

The possibility of displacement the microflora and mesofauna from the environment has been suggested by the incorporation into the environment of large quantities of these fungi, which could cause an imbalance in the ecosystems. *D. flagrans* has been shown not to affect the population of fungi present in the soil, neither to free-living nematodes, nor to microarthropods (Saumell et al. 2016). Its effect disappears or diminishes over time influenced by environmental factors and by the relocation of the spores in deeper layers on the soil due to the action of rain or certain microorganisms (Knox et al. 2002). Additionally, the lack of adverse effects on the animals that consume them has been demonstrated, based on the lack of immunological response in a study conducted by Hernández et al. 2016 in horses, which did not develop antibodies against the spores of *M. circinelloides* and *D. flagrans* incorporated during the industrial manufacture of nutritional pellets.

2.5. DISTRIBUTION OF FUNGI PARASITICIDES IN THE SOIL

An important question is how to distribute these fungi in the soil. Liquid media have been chosen for the reproduction of the fungi, as a consequence of the sort of advantages they offer over solid (homogeneity of the nutrients in the culture medium). Therefore, spraying or pulverization of particles should be easy to implement. Nevertheless, a large amount of spores are lost due to runoff and as a result, uniform distribution is not achieved, which reduces their accuracy.

Previous studies confirmed the ability of the spores of *D. flagrans* and *M. circinelloides* to survive the passage through the gastrointestinal tract of different species of herbivores and carnivores, and for this reason the incorporation of fungi into the food was considered. This formulation would guarantee the presence of fungi directly in the faeces, and therefore direct contact with the parasitic forms eliminated by the infected animals.

Amongst the different ways of oral administration, it might be mentioned the incorporation of submerged medium directly on the food (food premix) (Carvalho et al. 2009; Arias et al. 2013a; Cortiñas et al. 2015), formulations as handmade food concentrates (Longo Ribeiro Vilela et al. 2016; Silveira et al. 2017) or commercially manufactured (Hernández et al. 2016; Arroyo Balán 2017), or lyophilized (Carvalho et al. 2011). These studies have also proved spore resistance to processes such as pelletization or lyophilization. These procedures are convenient since they do not interrupt usual farming practices. Another advantage is that the spores contained in pellets can be stored for prolonged periods of time without altering their viability, facilitating handling, transport and storage (Berny and Hennebert 1991).

2.5.1. Biocides

Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. The term 'biocidal product' is defined as (European Parliament and Council of the European Union 2012):

> *"Any substance or mixture, in the form in which it is supplied to the user, consisting of, containing or generating one or more active substances, with the intention of destroying, deterring, rendering harmless, preventing the action of, or otherwise exerting a controlling effect on, any harmful organism by any means other than mere physical or mechanical action".*

Highlighted is the importance of biocides for the control of organisms harmful to human or animal health and control of organisms harmful to natural or manufactured materials; but it is also warned that biocides may involve risks for people, animals and the environment, due to their intrinsic properties and corresponding patterns of use. Therefore Regulation (EU) No 528/2012 (European Parliament and Council of the European Union 2012) harmonizes the European

Union's legislation with regards to the sale and use of biocides, while ensuring a high level of protection of human and animal health and the environment.

All biocides and their active ingredients must be approved by the European Union, for which a country or "rapporteur" is appointed, which will be responsible for preparing an assessment report on which all EU countries discuss with a view to reach a decision at EU level to approve, or not, the inclusion of the substance for a period of time not exceeding ten years, in the list of approved active substances that can be consulted on the website of the European Chemicals Agency (ECHA).

In order to be authorized, biocides must meet the conditions set forth in Article 19 of Regulation (EU) No. 528/2012 (European Parliament and Council of the European Union 2012), under certain premises (sic):

- realistic worst case conditions under which the biocidal product may be used;
- the way in which treated articles treated with the biocidal product or containing the biocidal product may be used;
- the consequences of use and disposal of the biocidal product;
- cumulative effects;
- synergistic effects.

These conditions include that the biocide at the time of authorization must guarantee that, if it is used in the manner and for the authorized purposes and according to current scientific and technical knowledge (European Parliament and Council of the European Union 2012) (sic):

- the biocidal product is sufficiently effective
- the biocidal product has no unacceptable effects on the target organisms, in particular unacceptable resistance or cross-resistance or unnecessary suffering and pain for vertebrates;

- the biocidal product has no immediate or delayed unacceptable effects itself, or as a result of its residues, on the health of humans, including that of vulnerable groups, or animals, directly or through drinking water, food, feed, air, or through other indirect effects;
- the biocidal product has no unacceptable effects itself, or as a result of its residues, on the environment, having particular regard to the following considerations:
- their physical and chemical properties have been assessed, and are considered acceptable for the use and transportation of the product.
- if necessary, maximum residue limits have been established in food and feed
- and in the case of using nanomaterials, the risk to human and animal health and the environment has been specifically evaluated

A biocidal product shall not receive marketing authorisation for use by the general public if the active ingredients meet the criteria to be considered carcinogenic, mutagenic or toxic for reproduction ((European Parliament and Council of the European Union 2008)No. 1272/2008), persistent, bioaccumulable and toxic (PBT), or very persistent and very bioaccumulative (vPvB) (according to Annex XIII of Regulation (EC) No. 1907/2006), be endocrine disrupters or have neurotoxic or immunotoxic effects on development.

There may be exceptions in which the authorization is justified by the disproportionate negative effect that the decision of not authorizing them would have in society in comparison with the risks derived from its use.

To expedite and facilitate administrative procedures, auxiliary measures are created, such as:

- Simplified authorization procedures for biocidal products with profiles more favorable for the environment or human or animal health, provided that they meet the conditions set out in Article 25 of Regulation (EU) No 528/2012.

- Offer advice and complementary assistance, operational guidance documents and other means of advice and assistance provided by the Agency, to help applicants, and especially small and medium-sized enterprises (SMEs), to comply with the requirements of the Regulation (EU) No 528/2012.

2.5.2. Food Additives

Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition defines "additive for animal feed" means substances, micro-organisms or preparations, other than feed material and premixtures,which are intentionally added to feed or water in order to perform, in particular, one or more of the functions (paragraph 3, article 5 of Regulation (EC) No 1831/2003 (European Parliament and Council of the European Union 2003)) (sic):

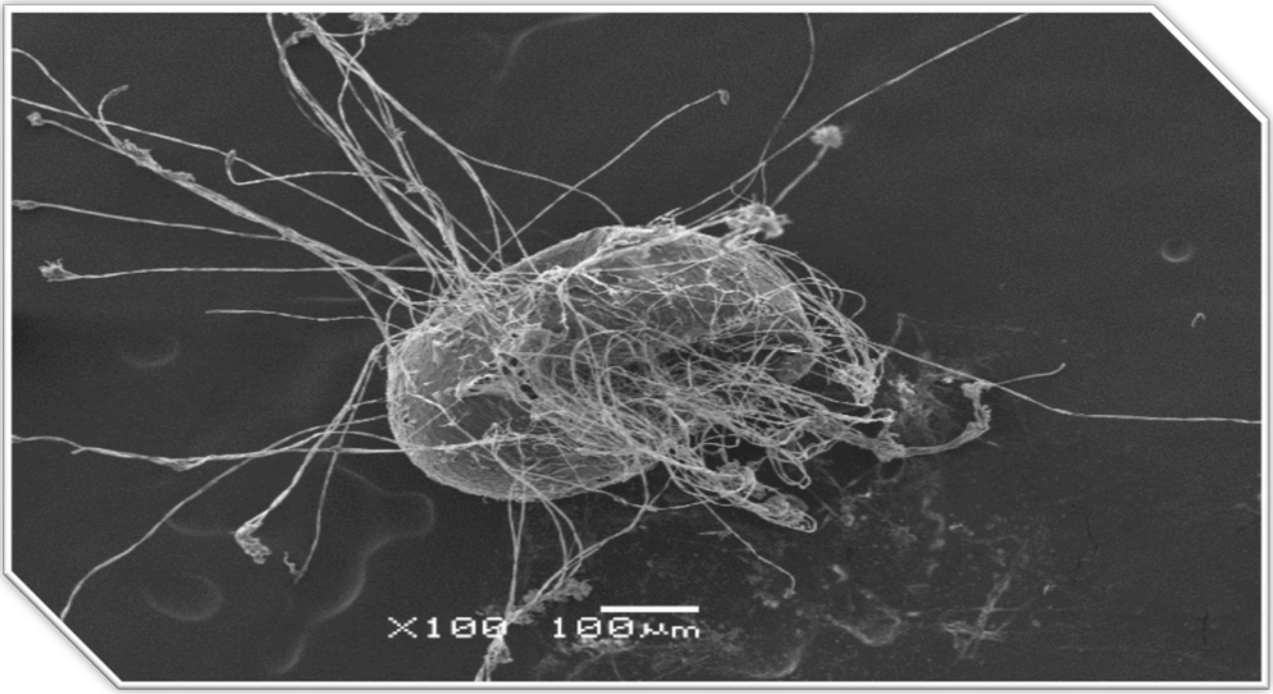
- favourably affect the characteristics of feed,
- favourably affect the characteristics of animal products,
- favourably affect the colour of ornamental fish and birds,
- satisfy the nutritional needs of animals,
- favourably affect the environmental consequences of animal production,
- favourably affect animal production, performance or welfare, particularly by affecting the gastro-intestinal flora or digestibility of feedingstuffs, or
- have a coccidiostatic or histomonostatic effect.

Through this Regulation, the European Union (EU) harmonizes and streamlines the rules on the evaluation and authorization of additives intended for animal feed, which must receive a favorable opinion from the European Food Safety Authority (EFSA) before obtaining a authorization for its commercialization and use. In addition, it prevents measures relating to the labeling of such additives and stricter rules on some substances as well as provisions regarding the use for research purposes of unauthorized additives and provisions on the use of certain additives (in particular, those produced from of genetically modified organisms).

The applicant must prove that the additive meets certain conditions (section 2, article 5 of Regulation (EC) No 1831/2003 (European Parliament and Council of the European Union 2003)) (sic):

- Under no circumstances should the additive have an adverse effect on animal health, human health or the environment;
- the additive must not compromise consumer safety by altering the distinctive characteristics of products of animal origin;
- the presentation of the product should not be presented in a way that could mislead the consumer.

3. OBJECTIVES



3. OBJECTIVES

Based on the background described in the previous section, a study divided into five Assays was proposed, in order to achieve the following **OBJECTIVES**:

1.- To develop a protocol for the isolation of fungi antagonistic of eggs of parasites.

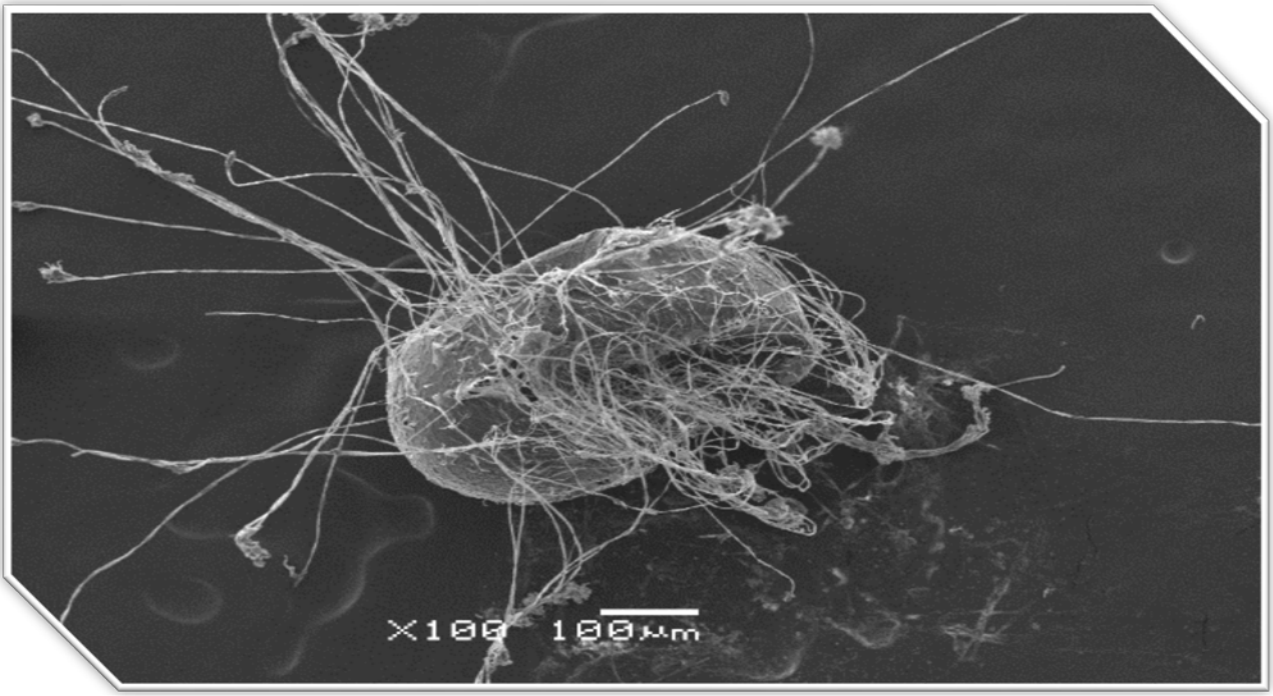
2.- To evaluate the possibilities of formulating *Mucor circinelloides* as a food additive.

3.- To analyze the prospects of developing formulations with *Mucor circinelloides* as a biocide.

4.- To study the possible effect of *M. circinelloides* on tick eggs.

5.- To establish which formulation of *M. circinelloides* can be viable from a commercial point of view.

4. THEMATIC UNIT



4. THEMATIC UNIT

This Memoir PhD Thesis is presented as an article-based thesis or ***Compendium of Publications,*** which have been chosen for their important contribution in the determination of which pharmaceutical formulations containing the fungus *Mucor circinelloides* may be of commercial interest.

When questioning the usage of parasiticidal saprophytic fungi, different questions arise, such as which species may be used, how to obtain them, how to design an assessment of their potential activity. On some occasions, already marketed species are purchased, but as an inconvenient, it can occur that this particular species may not be part of the regular soil biota of a given area, and as a result, we could be introducing *alien* species not properly adapted and with potential negative or unforeseen effects on other soil organisms. As a consequence, in the first publication ***Isolation of ovicidal fungi from faecal samples of captive animals maintained in a zoological park***, an online free access magazine was selected (***Journal of Fungi***), expecting a large information spread and also a greater isolation of parasiticidal saprophytic fungi in different parts of the globe. This activity consisted in the collection of 60 faeces samples from different wild animal species kept in the zoological park “Marcelle Natureza” (Outeiro de Rei, Lugo, Spain), and their culture in different media to obtain 11 monoconodial cultures. Consecutively, they were tested against trematodes and ascarids with the intention of determining the degree of ovicidal activity.

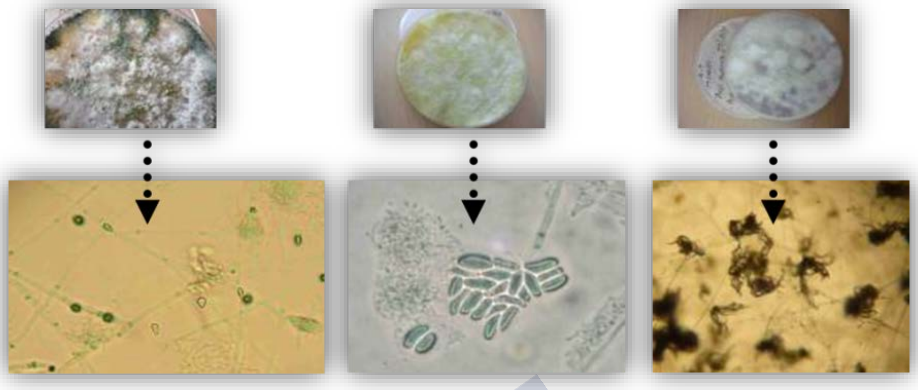
Over the last years, great interest has been placed on the need and suitability of parasites *biological control*, particularly in pastures. However, this is still a pending subject, since there is little or no available means to prevent infections. In relation to equines, different procedures such as a frequent manual collection and removal of stools or pastures rotation are advised, although they are not always successful. In the knowledge that a combination of different strategies could offer a preventive and sustainable solution, a second activity was designed (***A combined effort to avoid strongyle infection in horses in an oceanic climate region: rotational grazing and parasiticidal fungi*; Parasites & Vectors (2018) 11: 240; JCR Impact Factor: 3.163**); it was then carried out an assessment of the combination of pastures rotation and industrial feed pellets containing spores of certain fungi (*Mucor circinelloides* and *Duddingtonia flagrans)*. A different objective in this study was to analyse the potential marketing of this fungal pharmaceutical formulation. In order to achieve this objective, 22 sport Spanish breed horses were organised in three different groups. The effect was measured with the estimation of the percentage in the reduction of eggs in faeces and the percentage of positive individuals to faecal flotation tests, as well as the period of time that lapsed before the new detection of egg in stools.

Parasite control is considered to be of paramount importance in the livestock farming sector in order to lowering losses and increase profitability. Animal species kept in captivity in zoological parks have undergone notable changes in relation to their housing; generally speaking, those conditions found in the wild are reproduced including the maintenance of areas with typical vegetation. This could lead to situations where parasitic infections are favoured, especially those caused by helminths. Taking into account that the prevention of the infection is not possible though pastures rotation or the manual collection and removal of stools, in the third publication a new assay is proposed (***Biological control of soil transmitted helminths (STHs) in a zoological park by using saprophytic fungi*; Biological control (2018) 122: 24-30; JCR IF: 2.112**); it consisted in the analysis of the antagonist effect of two saprophytic fungi, *Mucor circinelloides* y *Verticillium* sp. on *Toxascaris leonina* eggs eliminated by lynxes (*Lynx lynx*), as well as the effect of *M. circinelloides* y *Trichoderma atrobrunneum* on *Trichuris* sp. eggs in dromedary faeces (*Camelus* *dromedarius*). For this purpose, a new pharmaceutical formulation with potential marketing interest was designed: the pulverization of water based solutions which contained fungal spores over faecal matter. It should be highlighted that *T. leonina* y *Trichuris* sp. are soil transmitted helminth infections and could potentially affect different species, and even human beings.

Soil can offer adequate habitat conditions for the survival and development of a large number of helminths, and as a result, the term *Soil-Transmitted Helminths* (STHs) was coined. An important group is composed by those helminths for which the definitive host are dogs, because the narrow contact between pets and humans may cause zoonotic infections. In the fourth publication (***Potential usefulness of filamentous fungi to prevent zoonotic soil-transmitted helminths (STHs);*** **Vector Borne and Zoonotic diseases (2018) JCR IF: 2.171**) the possible usefulness of a strategy of biological control of those STHs with zoonotic potential was studied. Two different groups of puppies which eliminated *Toxocara canis, Toxascaris leonina*, *Trichuris vulpis* and *Ancylostoma caninum* eggs in faeces were part of the case study. Individuals from one group were fed a mixture of feed pellets and a pharmaceutical formulation containing spores of fungi *Mucor circinelloides* y *Duddingtonia flagrans* (*premix*).

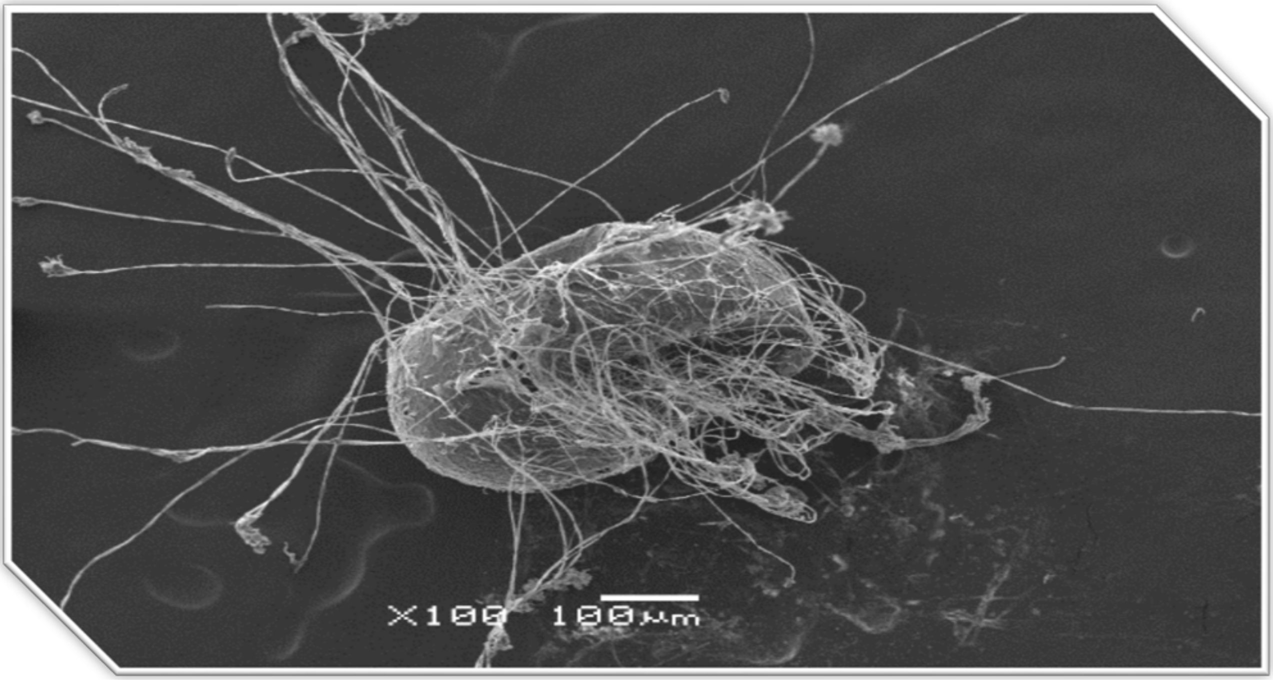
The concept of STHs has been gathering more importance in recent times. However, other non helminth parasitic forms also complete their life cycle in the soil and are equally important for their zoonotic potential and their ability to function as pathogens vehicles. That is the case of, for instance, ticks or fleas. The fifth contribution in this Memoir PhD Thesis was focused on the revision and analysis of the usefulness of the use of spores of saprophytic fungi to limit the presence of soil helminths and ticks. The publication of this article was presented as a Chapter in an Open Access Book (***Advantageous fungi against parasites transmitted through soil***; **InTechOpen Book Chapter**): The article reported the different precedents in the use of *Mucor circinelloides* and *Duddingtonia flagrans* spores to reduce the presence and viability of helminth eggs and larvae respectively. Data related to the activity of *M. circinelloides* on *Rhipicephalus microplus* (ixodid tick that, apart from an intrinsic pathogenic activity, may also act as a pathogen vehicle of, for example, babesiosis) was also discussed.

This publication is part of a larger collection initially focused on describing the most important diseases caused by fungi; however, our participation was required and we deemed it necessary to highlight the potential beneficial effect of some saprophytic species.



5. PAPER Nº 1:

ISOLATION OF OVICIDAL FUNGI FROM FECAL SAMPLES OF CAPTIVE ANIMALS MANTAINED IN A ZOOLOGICAL PARK



Journal of *Fungi*

MDPI

Article

Isolation of Ovicidal Fungi from Fecal Samples of Captive Animals Maintained in a Zoological Park

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Abstract: There are certain saprophytic fungi in the soil able to develop an antagonistic effect against eggs of parasites. Some of these fungal species are ingested by animals during grazing, and survive in their feces after passing through the digestive tract. To identify and isolate ovicidal fungi in the feces of wild captive animals, a total of 60 fecal samples were taken from different wild animals kept captive in the Marcelle Natureza Zoological Park (Lugo, Spain). After the serial culture of the feces onto Petri dishes with different media, their parasicitide activity was assayed against eggs of trematodes (*Calicophoron daubneyi*) and ascarids (*Parascaris equorum*). Seven fungal genera were identified in the feces. Isolates from *Fusarium*, *Lecanicillium*, *Mucor*, *Trichoderma*, and *Verticillium* showed an ovicidal effect classified as type 3, because of their ability to adhere to the eggshell, penetrate, and damage permanently the inner embryo. *Penicillium* and *Gliocladium* developed a type 1 effect (hyphae attach to the eggshell but morphological damage was not provoked). These results provide very interesting and useful information about fungi susceptible for being used in biological control procedures against parasites.

Keywords: ovicidal fungi; zoological park; biological control; sustainability

1. Introduction

Adult stages of certain helminths affecting animals release eggs that are passed out in the feces. Once in the soil, different phases are accomplished to attain the infective stage, and the life-cycle is completed when animals feed on pastures [1]. Some of these helminths are zoonotic agents because they can infect humans also [2].

In the soil, there are several possibilities for the transmission of helminths through eggs shed in feces: (1) a larva originates inside the egg in the soil, but the larva does not exit from the egg until it is ingested by the host and excysts at the gut level (nematodes: ascarids, trichurids); (2) the larva originates in the egg, leaves it, and molts in the environment until the infective stage is reached (nematodes: strongylids, ancylostomids), or (3) the larva abandons the egg and infects an intermediate host to reach the infective stage (trematodes, cestodes) [3,4]. In terms of moving capability, parasites remain immobile and confined in the eggs until they are ingested (ascarids, trichurids), or leave actively the eggs and scroll in the environment (trematodes; nematodes: strongylids, ancylostomids).

As occurs with domestic species, wild captive animals maintained always in the same paddock (continuous grazing) can be at risk of infection by certain helminths, because they are constantly shedding eggs to the environment. Despite the administration of successful therapy based on anthelmintics, these animals infect again because of the ingestion of infective stages when feeding on grass [5].

Under natural conditions, the presence of some saprophytic fungi in soil that can develop antagonistic effects on the eggs of parasites, with the aim to take nutrients as C or N, has been reported [6]. The ability of some of these fungi to pass through the gastrointestinal tract of different animal species and survive in their feces has been previously reported, concerning mainly *Duddingtonia flagrans*, *Pochonia chlamydosporia*, or *Mucor circinelloides* [2,7–9]. Hence, their employment has been notably advised in the last decades as a contribution to the control of parasites affecting livestock.

Studies performed on different countries demonstrated the presence of nematophagous fungi in fecal samples from domestic animal species [4,10–17].

Most known species with ovicidal activity are *Verticillium* spp., *Pochonia chlamydosporia*, *Paecilomyces lilacinus*, *Trichoderma* spp., or *Mucor circinelloides* [18–20]. By developing the phases of adhesion, colonization, penetration, and deliberation, these fungi develop an ovicidal activity [21,22]. Recently, the role of *Trichoderma* spp. in the biological control of insects pest such us *Xylotrechus arvicola* and *Acanthoscelides obtectus* has been described [23,24]. The objective of this study was to evaluate the presence of fungi with ovicidal activity in the feces of wild animals maintained captive at the "Marcelle Natureza" zoological park (NW Spain).

2. Material and Methods

2.1. Marcelle Natureza Zoological Park

The current investigation was conducted in "Marcelle Natureza", a 20 ha zoological park located in NW Spain (Outeiro de Rei, Lugo) (43°4′4.71″ N, 7°37′53.50″ W). Collection animals live in fenced semi-free ranging parcels of various sizes. The animals are routinely dewormed in spring and autumn by adding granulated anthelmintic preparations to their diet. Removal of fecal material is performed daily in the paddocks by the keepers, before the visitors arrive.

2.2. Collection and Analysis of Fecal Samples

Freshly deposited feces were taken in the morning from a total of 60 paddocks, then put into plastic flasks, and finally brought to the lab. Each fecal sample was analyzed by the flotation test to determine the presence of coccidian cysts/oocysts, eggs of cestodes and nematodes [25]. Briefly, 3 g of feces were emulsified in 42 mL of water, stirred shortly, and passed through a 150 μm mesh. The filtered solution was collected into two 15 mL tubes and centrifuged at 2500 rpm for 10 min. The supernatant was discarded and 10 mL of saturated NaCl solution (ϱ = 1.2 g/cm^3) was added to each tube. After 2 min, aliquots of 300 μL were taken and observed in a McMaster chamber under a light microscope (4–10×) (Leica DM 2500, Barcelona, Spain).

The existence of eggs of trematodes in feces was investigated by means of the sedimentation probe [1]. Five grams of feces were blended with water, filtered through a 150 μm sieve, and passed to a 1 L conic cup. After decanting three times for 15 min, the content was reduced to 50 mL. Finally, aliquots of 300 μL were collected to fill a McMaster chamber, and then observed under an optical microscope (4–10×) (Leica DM 2500).

2.3. Isolation of Fungi from the Feces

By means of the flotation test, captive animals passing eggs of strongyles in their feces were identified. One gram of each fecal sample was placed onto a Petri dish containing water agar with chloramphenicol (WA) and incubated at 25 °C for 15 days [26]. Four replicates were considered for each sample.

Once fungal growth was recorded, fungal isolates were subcultured twice in malt extract agar (MEA; Drogallega, A Coruña, Spain) and corn meal agar (CMA, Sigma, MO, USA) for purification and subsequent identification, following standard protocols [27,28].

Monosporic cultures were obtained on potato glucose agar (PGA, Drogallega) for morphometric and cultural characterization. In some cases, subcultures were made on wheat extract agar with chloramphenicol (AT). Plates were incubated at 18–22 °C in the dark.

2.4. Identification of Fungal Species

The microscopic characterization of the fungal isolates consisted of measurements of 40 conidia, conidiophores, spores/chlamydospores, and sporanges by using an optical microscope (Olympus CX23LEDRFS1, Ashburton, New Zealand) equipped with a digital camera. Measurements were performed with an eyepiece micrometer scale. Identification of the fungal isolates was based on morphological features from pure cultures fungi, by means of keys and species descriptions [29–33].

2.5. Obtaining Parasites

Feces of cattle and horses with previous records of infection by parasites were collected and analyzed by using coprological probes. After the observation of eggs of *Calicophoron daubneyi* (gastric fluke) in bovine feces by using the sedimentation test, eggs were concentrated to 800 eggs/mL.

By applying the flotation probe, eggs of roundworms (*Parascaris equorum*) were identified in the feces of horses, then purified [21], and finally kept at a concentration of 800 eggs/mL.

2.6. Parasiticide Activity Testing Assays

Two assays were developed by using CMA plates. For each fungus isolated, two sets of plates were prepared; Set 1 received 400 eggs of *C. daubneyi*, and Set 2 received 400 eggs of *P. equorum*. Ten replicates were carried out for each fungus and parasite.

Ten plates without fungi were provided with 400 eggs of *C. daubneyi* as controls, and the same was done with eggs of *P. equorum*.

2.7. Evaluation of the Fungal Parasiticide Activity

Twenty-two days after placing the parasites, the CMA plates were observed under an optical microscope (Leica DM2500) for recording the changes in the eggs in comparison with their respective controls. Assessment of fungal damage on eggs was carried out according to the following alterations [34]:

-Type 0: Eggs are viable and damage or alterations are not observed.
-Type 1: Hyphae attached to the eggshell but morphological damage was not provoked.
-Type 2: The eggshell and embryo show damage without penetration.
-Type 3: Fungal hyphae enter the egg, grow, and destroy the embryo.

3. Results

A total of 13 captive animals passing eggs of strongyles in their feces were detected by means of the flotation test, thus these fecal samples were cultured and subcultured in search of fungal species with activity against parasite eggs.

In all the isolates, a mycelium was developed in the presence of eggs of *C. daubneyi* and *P. equorum*, and hyphae attached to the eggshell (type 1 activity) (Figure 1). Isolates identified as *Gliocladium* (two fecal samples) and *Penicillium* (n = 3) displayed only a type 1 ovicidal effect. The contact area between the hypha of fungi and the egg surface is smooth at the first stage. No damage of superficial structures of eggshell can be observed during this period. During the interaction with the egg, some hyphae of these fungi formed a lentiform penetration organ (*appresorium*) on the undeveloped egg surface. This is considered an important organ involved in the mechanism of penetration of the fungi through the solid eggshell.

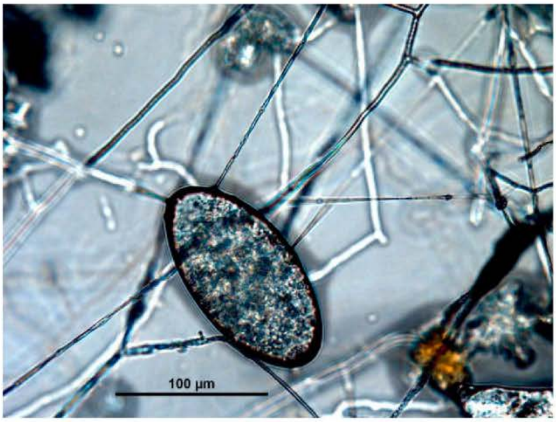

Figure 1. Hyphae of *Trichoderma* isolated from feces of captive wild animals developed in the presence of eggs of *C. daubneyi*.

Nine of the fungal isolates were also able to penetrate inside the eggshell after 6–10 days when the penetration organ (*haustorium*) started to damage the superficial structures of the chitin-protein layer of the envelope. As soon as the fungus has penetrated into the egg, it starts to form branches, and the formation of new hyphae was observed (Figure 2).

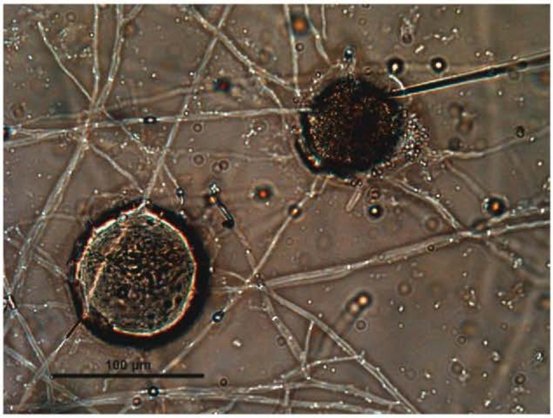

Figure 2. Eggshell of *Parascaris equorum* is penetrated by hyphae of *Mucor* spp.

Finally, after attaching to the eggshells and penetrating them, the interior was colonized and, the inner embryo was destroyed (Figures 3 and 4), so this ovicidal activity was classified as type 3. The consumption stage of the process begins here. The branching fungus starts to gradually liquidate the egg contents irrespective of the developmental stage of the embryo. The layer of the eggshell is already deformed.

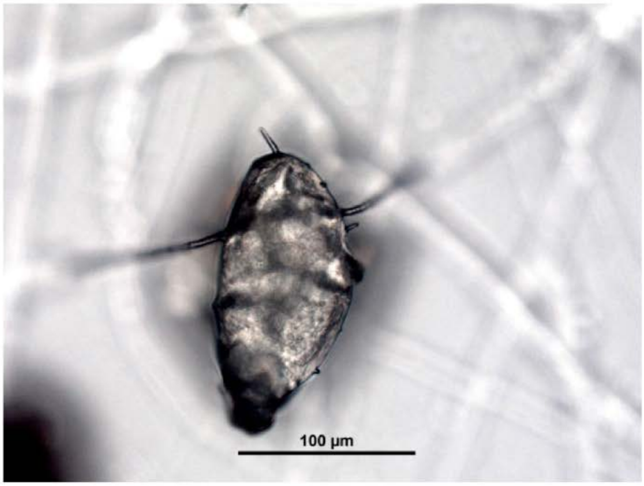

Figure 3. Destruction of the embryo inside egg of *C. dauneyi* by the action of *Trichoderma*.

Figure 4. Destruction of the embryo inside egg of *P. equorum* exposed to *Verticillium*.

The last stage of the ovicidal process begins, when the ovicidal fungus leaves the liquidated and dead remnants of the nematode egg. In some cases, spores were also observed within the eggs (Figure 5). These isolates were identified as *Fusarium*, *Lecanicillium*, *Mucor*, *Trichoderma*, and *Verticillium*.

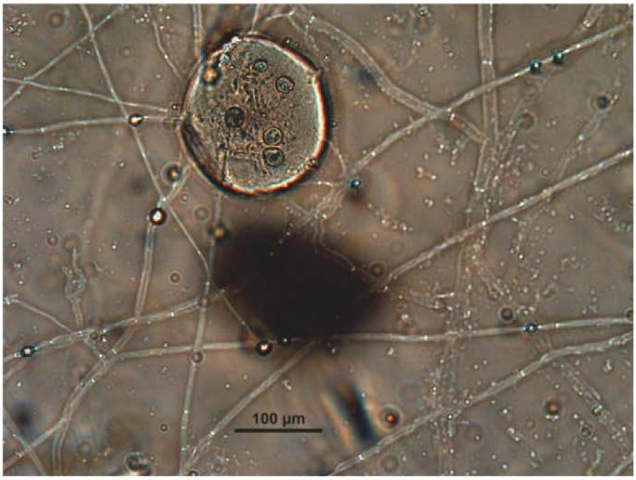

Figure 5. Chlamydospores of *Mucor* inside of an egg of *P. equorum*.

As summarized in Table 1, the number of fungal species with ovicidal activity in each of the fecal samples ranged between 1 and 2, whereas the most abundant predaceous fungi were, in four samples, *Trichoderma* and *Verticillium*, in three samples, *Fusarium*, *Mucor*, and *Penicillium*, *Gliocladium* in two samples, and *Lecanicilium* only in one.

No morphological differences regarding the effect the soil fungi developed have been recorded.

Table 1. Isolation of predaceous fungi with ovicidal activity against eggs of the helminths *Calicophoron daubneyi* and *Parascaris equorum*, in feces of wild captive animals ("Marcelle Natureza" zoological park, NW Spain). WA: water agar. MEA: malt extract agar. CMA: corn meal agar. PGA: potato glucose agar. AT: wheat extract agar with chloramphenicol.

Captive Animals			Fungal Isolation			
Common Name	**Scientific Name**	**Parasites Diagnosed**	**Culture**	**Subculture 1**	**Subculture 2**	**Genera Identified**
Coati	*Nasua nasua*	Nematodes: Strongyles	WA	MEA		*Trichoderma*
				CMA		*Trichoderma* *Verticillium*
Raccoon	*Procyon lottor*	Nematodes: Strongyles	WA	MEA		*Mucor*
				CMA		*Mucor*
Eurasian lynx	*Lynx lynx*	Nematodes: Strongyles	WA	MEA	PGA	*Fusarium*
				CMA		*Gliocladium*
Brown bear	*Ursus arctos*	Nematodes: Strongyles	WA	MEA		*Trichoderma*
				CMA		*Trichoderma*
Goat	*Capra hircus* spp.	Coccidia	WA	MEA	PGA	*Verticillium*
		Nematodes: Strongyles		CMA		*Verticillium*
Mouflon	*Ovis musimon*	Coccidia	WA	MEA		*Fusarium* *Penicillium*
		Nematodes: Strongyles		CMA		*Fusarium*
Gazelle	*Gazella cuvieri*	Coccidia	WA	MEA		*Mucor*
		Nematodes: Strongyles		CMA		*Mucor* *Penicillium*
Axis	*Axis axis*	Nematodes: Strongyles	WA/AT	MEA		*Verticillium* *Lecanicillium*
				CMA		*Verticillium* *Lecanicillium*
Bison	*Bison bison*	Coccidia	WA	MEA		*Trichoderma*
		Nematodes: Strongyles		CMA		*Trichoderma*
Dromedary	*Camelus dromedarius*	Nematodes: Strongyles	WA	MEA	PGA	*Trichoderma*
				CMA		*Trichoderma* *Verticillium*
Guanaco	*Lama guanicoe*	Coccidia	WA	MEA	PGA	*Gliocladium*
		Nematodes: Strongyles		CMA		*Gliocladium*
Falabella	*Equus caballus*	Coccidia	WA	MEA		*Fusarium*
				CMA		*Fusarium* *Penicillium*
Wallaby	*Macropus rufogriseus*	-	WA	MEA		*Mucor*
				CMA		(Sordariaceae)

4. Discussion

The presence of soil fungi antagonists of egg parasites in fecal samples of wild captive animals was investigated. Formerly, only eggs of strongyles were detected in their feces. After culturing these fecal samples, seven isolates with ovicidal activity were obtained. Two of them, identified to genus level as *Gliocladium* and *Penicillium*, were able to adhere to eggshell only and therefore classified as type 1 ovicidal fungi. These are specimens found frequently in soil samples [35,36], and there is no available information concerning their effect on the eggs of helminths infecting animals. Some investigations reported their usefulness as a biocontrol agent against plant pathogens as *Rhizoctonia solani*, *Phytium ultimum*, and *Meloydogine incognita* [37–39].

Five fungal specimens isolated from the feces of the captive animals were identified to the genus level as *Fusarium*, *Lecanicillium*, *Mucor*, *Trichoderma*, and *Verticillium*. When eggs of the gastric fluke *Calicophoron daubneyi* and the roundworm *Parascaris equorum* were exposed to these fungi, it was observed that hyphae attached to the eggshells and penetrated and destroyed the inner embryo, so they were classified as type 3 ovicidal fungi. If the mechanical pressure was the main factor enabling the penetration of the hypha through the eggshell, then the fungi should be fixed to the egg surface in such a way that it would be able to develop a high pressure on the structure of eggshell, particularly on the mechanically resistant chitin–protein complex of the chitinous layer of *Ascaris lumbricoides* eggs. The penetration organ physically damages the eggshell, though specific enzymes may be involved. The damaged ascarosid layer no longer performs its role of the osmotic barrier. The embryo, if it has not yet been infected by the fungus, can be killed by the injurious substances from the outer environment, which can freely diffuse into the egg [34].

Prior investigations indicated the antagonistic activity of several fungal species belonging to the genera *Fusarium* and *Trichoderma* on eggs of the roundworm *Toxocara canis* [40–42]. The ovicidal activity of *Pochonia chlamydosporia* (formerly *Verticillium chlamydosporium*) has been widely reported on eggs of trematodes (*Echinostoma paraensei*, *Fasciola hepatica*) and ascarids (*T. canis*, *P. equorum*) [19,20,43].

Transmission of many parasites affecting animals occurs in the soil, where the infectious agents develop part of their life-cycle. This enhances the importance to control the numbers of infective stages of parasites, especially when animals are maintained always in the same paddocks, where the continuous shedding of eggs favors the accumulation of infective stages [5].

Different actions have been suggested to minimize the risk of infection in grazing animals, such as the rotation of pastures, the alternation of animal species pasturing in the same paddock, the manual collection of manure or drainage [44,45]. Nevertheless, these procedures can not be applied in different regimes, as occurs in zoological parks, and the number of dewormings per year is frequently increased to eliminate parasitic infections in the animals. As a consequence, selection of parasite strains resistant to different chemical compounds [46].

Nematodes in the soil are exposed to many organisms such as bacteria, viruses, fungi, predatory nematodes, and mites, and some have the ability to parasite and destroy them [47]. These beneficial organisms are called biological agents, which should be highly antagonistic to parasites, selective in their activity (acting on parasites but not on crop plants or higher animals), to growth on artificial media at suitable pH and temperature ranges, and easy to formulate in a working way [48]. In the current research, five of the isolated fungi developed in plates containing a medium composed of corn meal agar and a type 3 effect on eggs of *C. daubneyi* and *P. equorum* was recorded. No pathogen effects have been reported after the administration of *M. circinelloides*, *D. flagrans*, or *Pochonia chlamydosporia* to sheep, cattle, and domestic and wild horses [2,5,7,49,50]. In recent years, the formulation of fungi in pelleted feed by adding mycelium or spores of *D. flagrans* and *M. circinelloides* provided successful results in terms of preventing infection by helminths in horses, enhancing thus their distribution as biocontrol agents [2,51].

Parasitic stages present in soil can also affect humans. Soil-transmitted helminth infections caused by ascarids, such as *Toxocara canis*, *Ascaris suum*, *Toxascaris leonina*, and *Baylisascaris procyonis*, and trichurids are transmitted to humans through the accidental ingestion of eggs containing a second stage larva inside [21,42]. Larvae of ancylostomids can penetrate through the skin and especially affect people enjoying recreational locations with, for example, sandy areas or recreational surfaces in parks [52,53]. Parasiticide fungi could be distributed by spraying them into aqueous solutions, providing a useful tool for lessening the risk of infection to children playing in those places or adults taking a sunbath.

Our results demonstrated the existence of saprophytic fungi with ovicidal activity in the feces of captive animals from the "Marcelle Natureza" zoological park (Lugo, NW Spain), and a similar variety of predaceous fungi was observed in all samples examined. Most of the former surveys conducted on fecal samples collected from domestic livestock have been focused on the finding predaceous fungi with larvicidal activity only [4,13,14,54].

In the present investigation, the presence of fungal specimens in feces indicates that they can survive the gastrointestinal tract, and the observation of an ovicidal type 3 effect in five of the isolates demonstrates that fungi retained their biological activity on the eggs of helminths, confirming thus their potential as biological control agents against helminths transmitted through eggs. Their impact on the eggs of strongyle nematodes remains unknown because, according to the weather and/or season, the development of a mobile phase (larva) inside might be slower than the time required for the fungi to develop their ovicidal activity. Further studies are in progress to elucidate this issue.

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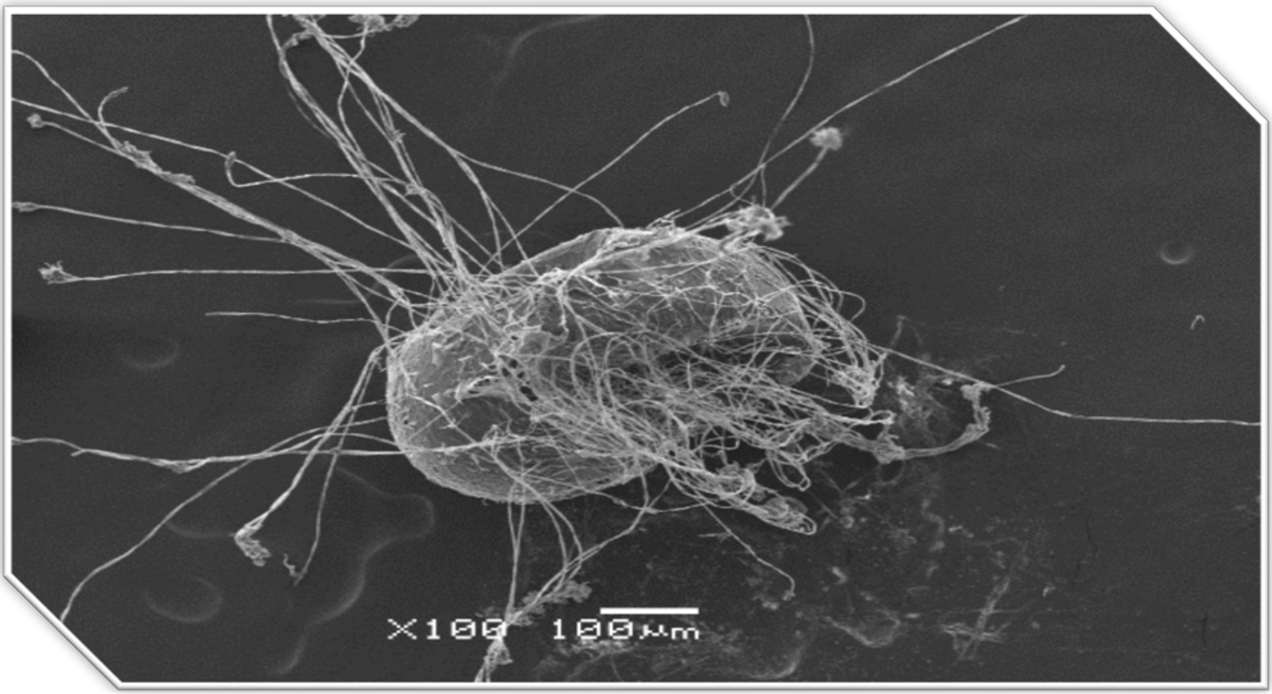
6. PAPER Nº 2:

A COMBINED EFFORT TO AVOID STRONGYLE INFECTION IN HORSES IN AN OCEANIC CLIMATE REGION: ROTATION GRAZING AND PARASITICIDAL FUNGI



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Parasites & Vectors

RESEARCH Open Access

A combined effort to avoid strongyle infection in horses in an oceanic climate region: rotational grazing and parasiticidal fungi

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Abstract

Background: An approach to preventing strongyle infection in horses was tested, comprising rotational pasturing and the administration of spores of two parasiticidal fungi, *Mucor circinelloides* and *Duddingtonia flagrans*.

Methods: Twenty-two adult Spanish Sport Horses were dewormed with ivermectin (1 mg *pour-on*/kg body weight) and then randomly divided into three groups. G-1 was maintained with continuous grazing, and G-2 and G-3 were kept on a four-paddock rotation system. Commercial pelleted feed (2.5 kg/horse) was supplied to G-1 and G-2 twice a week; horses in G-3 received pellets containing 2×10^6 spores/kg of each fungus. Fecal samples were analyzed by the flotation method to estimate the reduction in the fecal egg counts (FECR), the percentage of horses shedding eggs (PHR), and the egg reappearance period (ERP).

Results: Third-stage larvae were identified in fecal pats as *Cyathostomum* (*sensu lato*) types A, C and D, *Gyalocephalus capitatus*, *Triodontophorus serratus*, *Poteriosthomum* spp., *Strongylus vulgaris* and *S. edentatus*. Two weeks after treatment, the FECR values were 100% in G-1, 96% in G-2 and 99% in G-3; the PHR values were 100% in G-1, 75% in G-2 and 88% in G-3. A strongyle ERP of 6 weeks was observed in G-1, ERP of 10 weeks was observed in G-2, and ERP of 16 weeks was observed in G-3. The counts of eggs per gram of feces (EPG) were > 300 EPG in G-1 and G-2 but remained below 250 EPG in G-3 throughout the observation period of 12 months.

Conclusions: These results suggest that horse strongyle infection could be decreased by combining rotational pasturing with feeding pellets containing the spores of parasiticidal fungi.

Keywords: Horses, Strongyles, Rotational pasturing, Pelleted feed, Integrated control, *Duddingtonia flagrans*, *Mucor circinelloides*

Background

Horses maintained on pasture receive important benefits that include not only nutritional benefits but also the opportunity to exercise and to socialize [1]. Many helminths develop an external phase of their life-cycle in the soil, including trematodes, cestodes and nematodes [2]; thus horses on pasture are at risk of infection by consuming the infective stages of these helminths with herbage [3]. Among the gastrointestinal nematodes, strongyles are commonly reported in grazing horses. Eggs passed in feces develop a larva 1 (L1) stage inside, which hatches and molts into L2 and then L3, the infective stage [4].

High numbers of viable L3s have been recorded as surviving for long periods in pastures in oceanic climate areas, and even in regions where cold climate conditions occur during certain months of the year [5, 6]. An oceanic climate, also termed a maritime or marine west coast climate, is characterized by temperatures within a

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narrow range and reliable rainfall throughout the year, with warm summers and mild, cool winters. This climate can be found in regions along the west coasts at the middle latitudes of all continents, and in Chile, New Zealand and Tasmania and is responsible for grass that grows almost continuously, except in winter [7].

With the aim of removing parasitic infective stages from the environment where horses feed, grassland rotation is recommended [8]. Grassland rotation is a practice wherein animals are moved among different pastures, with each pasture having two phases, grazing and resting. Some strategies of biological control against strongyles include the utilization of soil saprophytic fungi such as *Duddingtonia flagrans* or *Monacrosporium thaumassium* [9], which are able to create traps in their mycelium where larvae are captured, immobilized and finally digested [10]. Therefore, administration of those spores has been advised as an appropriate procedure to prevent horse infection by strongyles [11, 12]. As occurs with other ovicidal fungi, *Mucor circinelloides* can attach to the egg-shell of the parasite, penetrate inside and destroy the inner content [13, 14]. An antagonistic effect of *M. circinelloides* has been shown on the eggs of helminths such as *Fasciola hepatica* and *Ascaris suum* that are shed in feces of cattle and pigs, respectively [15]. Recently, the manufacturing of pelleted feed with spores of *M. circinelloides* and *D. flagrans* has been suggested as an easy way to ensure the presence of fungal stages in the feces, where strongyle eggs hatch and develop to larvae [14, 16]. Herein, the possibility of enhancing the beneficial effect of rotational grazing by providing pelleted feed manufactured with the spores of *M. circinelloides* and *D. flagrans* has been evaluated.

Methods

Soil saprophytic fungi

Spores of *Mucor circinelloides* (ovicidal activity) and *D. flagrans* (larvicidal activity) were cultured in the submerged medium COPFr (patent Nr PCT/ES2014/070110) for 1.5–2 months at room temperature, until a concentration $\geq 1 \times 10^8$ spores/l medium was reached [17]. These fungal species were isolated in the Laboratory of the COPAR Research Group (University of Santiago de Compostela, Spain) [18].

The spores were added during the industrial manufacturing (mixing phase) of commercial pelleted feed (Pro-Horse Club®, Nanta, Spain), at a concentration of 2×10^6 spores/kg concentrate of each fungus [16, 19].

Horse management

This study was carried out in a stud farm for Spanish Sport Horses (SSHs) located in Xul (Friol, Lugo, North-West (NW) Spain, 42°58'49"N, 7°48'45"W), which contains 22 adult SSHs. Six of the horses are kept stabled and are exercised twice a day by jumping, then graze for 1–2 h in the same paddock (approximately 4.5 ha) every day (continuous grazing) before returning to their stalls.

The other 16 SSHs are maintained outdoors and are dedicated to recreation (riding along the forest). There are eight fenced wooded meadows, each approximately 2.5 ha, that are available on the stud farm, and two groups of pastures are considered to enable horse management; eight of the SSHs feed on four meadows (named A-D) throughout the year, and the other eight horses pasture on the other four meadows (called 1–4). In this way, a four-paddock rotation consisting of six weeks grazing and 18 weeks rest for each pasture is conducted observed. Every 1.5 months, horses are moved to the next pasture; thus, the rotation was completed after six months. Paddocks are randomly decided each year, and all horses graze all the pastures after two years.

Waterers are placed in each meadow to provide water *ad libitum*, and feeders are supplied where pelleted feed is provided twice a week; wheat straw and barley are provided when grass is scarce (from December to February). No actions have been previously performed on the pastures to reduce the presence of parasites.

Climatic parameters

Data regarding the temperature (maximum, minimum and average), relative humidity (%), frost days, rainfall (l/m^2) and water balance (l/m^2) were collected monthly from an automatic weather station (Corno do Boi, Friol, Lugo, NW Spain, 43°02'24"N, 7°53'24"W) located 5 km away from the farm and at the same altitude.

Experimental design

The experiment was carried out between September 2014 and October 2015. Based on previous assays involving selective therapy [20], a threshold of 300 strongyle eggs per gram of feces (EPG) was established as the criterion for horse deworming at the beginning of the study. The horses received ivermectin *pour-on* [1 mg of Noromectin 0.5%/kg body weight (bw), (Norbrook Laboratories, Newry, UK)] [7, 20] and were classified into three groups:

(i) G-1: six SSHs maintained under a continuous grazing regimen and each receiving a total of 2.5 kg of pelleted feed twice weekly (on Monday and Thursday);
(ii) G-2: eight SSHs maintained under a rotational grazing regimen and each provided 2.5 kg of pellets twice weekly;
(iii) G-3: eight SSHs kept under a rotational grazing regimen and each provided 2.5 kg of pellets containing spores of *M. circinelloides* and *D. flagrans* twice weekly.

Horses remained in the same group throughout the entire trial.

Coprological probes

Over the course of one year, feces were collected individually from the rectums of the horses. Five grams of each sample were analyzed by using the flotation test and saturated NaCl solution (ρ = 1.20 g/ml; sensitivity = 30 eggs/gram, EPG) [20].

The strongyle species infecting the horses was identified through the analysis of fecal cultures. Prior to deworming, ten grams of feces were taken from each horse and pooled according to their group. Then, the pats were incubated for 19 days at 22–25 °C. After being collected by using the Baermann technique, third-stage larvae (L3) were identified under an optical microscope (Leica DM2500; Leica Microsistemas, Barcelona, Spain) in accordance with morphological keys [21].

The presence of spores of *M. circinelloides* and *D. flagrans* in the feces of the horses in G-3 was also investigated. During the first five days of feeding the pellets with fungal spores, five grams of feces were individually taken from each horse in this group, emulsified in 40 ml water and passed through a 150 μm sieve. Two 15 ml/tubes were filled with the filtered solution and centrifuged at 1500× *rpm* for 5 min. After discarding the supernatant, the sediment was re-suspended in 2 ml water. Aliquots of 50 μl were placed between a glass slide and a coverslip, and observed under an optical microscope for spore identification (10–20×).

Evaluation of the efficacy

The efficacy of the strategy against the strongyles was assessed by calculating the values of the FECR (fecal egg-count reduction) and the PHR (reduction of the number of horses positive to the flotation test) [22]:

$$\text{FECR}\ (\%) = \left[1-\left(\text{FEC}_{\text{post-treatment}}/\text{FEC}_{\text{pre-treatment}}\right)\right] \times 100$$

$$\text{PHR}\ (\%) = [1-(\text{No.of positive horses}_{\text{post-treatment}}/\text{No.of positive horses}_{\text{pre-treatment}})] \times 100$$

The egg reappearance period (ERP) was estimated by considering the week after treatment when the FECR values dropped below 90% [22].

Adverse effects

The analysis of possible side effects in the G-3 horses after the ingestion of pellets with spores was performed by checking the activity of the digestive, reproductive and respiratory systems, and the skin integrity, in search of any disorder [16]. The possibility of pellets having strange odors or consistencies, or fungal growth, was checked.

Statistical analysis

The FECR and PHR values are expressed as percentages and 95% confidence intervals. The egg-output kinetics are represented as the mean ± 2SD. Because the Kolmogorov-Smirnov test showed that the values of strongyle egg-output were not normally distributed (statistic = 0.088, P = 0.001), these data were analyzed by means of the non-parametric Kruskal-Wallis and Mann-Whitney U two-sided tests (α = 0.05) [24]. Significant differences were considered when $P < 0.05$, and the time points with significant differences between groups are indicated in the figures. All tests were done using SPSS for Windows (v. 20.0; SPSS Inc., Chicago, IL, USA).

Results

Climatic variations

As summarized in Fig. 1, temperatures oscillated between 34 °C (June) and -2 °C (February), and frost days were recorded from December to March. Rainfall peaked in January, and the lowest values were achieved in June; relative humidity higher than 81% was observed throughout the study. The water balance, or the difference between the accumulated rainfall and evapotranspiration, was negative from May to July.

Strongyle species identified

Eggs of strongyles were observed in the feces of all horses. In the fecal pats, the larvae were identified as *Cyathostomum* (*sensu lato*) type A (63%), type C (14%) and type D (15%); *Gyalocephalus capitatus* (1%), *Triodontophorus serratus* (2%), *Poteriosthomum* spp. (2%), *Strongylus vulgaris* (2%) and *S. edentatus* (1%).

Fungal spores in the feces

Spores of *M. circinelloides* and *D. flagrans* were first detected in all the G-3 horses two days after pellets were provided (Fig. 2).

Efficacy of deworming

At the beginning of the study, values of strongyle egg-output higher than 300 EPG were observed in all the horses. As summarized in Table 1, the fecal counts of strongyle eggs were reduced in the three groups within two weeks after the administration of the macrocyclic lactone; the FECR values were 100% in G-1 horses, 96% in G-2 horses and 99% in G-3 horses. A PHR value of 100% was achieved in G-1, 75% in G-2 and 88% in G-3.

The ERP was 6 weeks (1.5 months) in G-1 horses, 10 weeks (2.5 months) in G-2 horses and 16 weeks (4 months) in G-3 horses.

Dynamics of strongyles egg-output

The kinetics of strongyles eggs in feces are drawn in Fig. 3. In the horses kept under continuous grazing and given

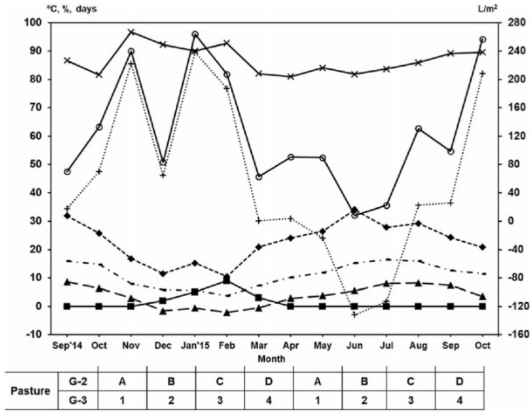


Fig. 1 Variations of climatic parameters in an oceanic climate area (NW Spain). *Key*: ♦, maximum temperature (°C); ＿, average temperature (°C); ▲, minimum temperature (°C); ×, relative humidity (%); ■, number of frost days; ○, rainfall (l/m^2); +, water balance (l/m^2)

commercial pellets (G-1), the average EPG counts increased gradually from the 1st month after treatment (m. a. t.) to the end of the study, and average numbers from 306 (3rd m. a. t.) to 630 (12th m. a. t.) were achieved. FECR values between 80 and 0% were recorded until the end of the trial. One month after the deworming treatment, half of the horses passed eggs in their feces (PHR = 50%), and 15 days later (6 weeks after the deworming) all the equines were positive for the presence of strongyle eggs (Table 1).

The EPG numbers rose progressively by 1 m. a. t. in the G-2 horses (maintained under rotational grazing and provided commercial pellets), and values of approximately 300 EPG were observed at the 5th m. a. t. (Fig. 3). The egg-output levels increased above 500 EPG by 8 m. a. t., and the highest numbers were obtained at the end of the field trial (> 600 EPG). The average FECR values oscillated between 77 and 0%. Half of the horses were positive to the flotation test 6 weeks after treatment (PHR = 50%), and all were positive by 3 m. a. t. (14 weeks) (Table 1).

In the horses under rotational pasturing and supplemented with pellets containing fungal spores (G-3), the strongyle EPG counts rose steadily between the 1st and the 6th m. a. t., when an average value of 201 was observed. From this month to the 12th m. a. t., the egg-output numbers ranged between 196–249 EPG. By the 3rd m. a. t. (12 weeks) strongyle eggs were found in half of the SSHs, and all were positive for the presence of strongyle eggs at the 6th m. a. t. (26 weeks) (Table 1). The FECR percentages ranged between 83–54% throughout the study. PHR values greater than or equal to 50% were recorded until the 3rd m. a. t. (14 weeks). Only one horse in this group exceeded the threshold of 300 EPG, at the 9th m. a. t.

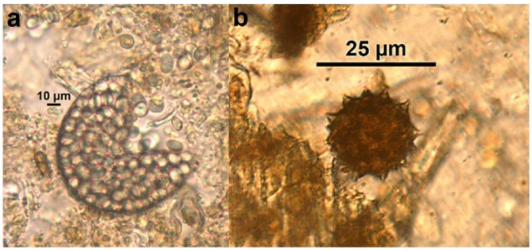


Fig. 2 Spores of *Mucor circinelloides* (**a**) and *Duddingtonia flagrans* (**b**) in feces of Spanish Sport Horses under a four-paddock rotation system, two days after provided pellets containing 2 × 10^6 spores/kg of each fungus

Table 1 Values of fecal egg count reduction (FECR) and coprological positive horses reduction (PHR) in Spanish Sport Horses under different pasturing regimes in an oceanic climate area (NW Spain)

WAT	Continuous grazing		Rotational grazing			Rotational grazing + parasiticide fungi		
	G-1 (*n* = 6)[a]		G-2 (*n* = 8)[b]			G-3 (*n* = 8)[c]		
	FECR (95% CI)	PHR (95% CI)	Pasture	FECR (95% CI)	PHR (95% CI)	Pasture	FECR (95% CI)	PHR (95% CI)
2	100	100	A	96 (94–98)	75 (50–100)	1	99 (98–100)	88 (65–100)
3	99 (97–99)	83 (46–100)		96 (94–98)	75 (50–100)		98 (97–99)	88 (65–100)
4	91 (87–93)	50 (10–90)		93 (91–96)	63 (29–96)		98 (96–99)	75 (45–100)
5	90 (87–93)	50 (10–90)		92 (90–95)	63 (29–96)		96 (95–98)	75 (45–100)
6	80 (76–84)	0		92 (89–95)	50 (15–85)		96 (94–98)	75 (45–100)
7	72 (68–77)	0	B	92 (89–95)	50 (15–85)	2	95 (94–97)	75 (45–100)
8	54 (49–59)	0		90 (87–93)	38 (4–71)		95 (93–97)	63 (29–96)
9	52 (47–57)	0		91 (88–93)	38 (4–71)		94 (93–96)	63 (29–96)
10	51 (46–56)	0		77 (72–81)	38 (4–71)		93 (9195)	63 (29–96)
12	17 (13–20)	0		69 (65–74)	38 (4–71)		93 (9195)	50 (15–85)
14	15 (12–19)	0	C	58 (53–63)	0	3	91 (8893)	50 (15–85)
16	14 (11–18)	0		39 (34–44)	0		83 (79–86)	38 (4–71)
20	3 (1–4)	0	D	24 (20–28)	0	4	74 (70–77)	38 (4–71)
24	0	0		22 (18–26)	0		63 (59–67)	25 (0–55)
26	0	0	A	0	0	1	60 (56–65)	0
30	0	0		0	0		60 (56–65)	0
32	0	0	B	0	0	2	64 (60–68)	0
36	0	0		0	0		61 (57–66)	0
38	0	0	C	0	0	3	54 (50–59)	0
42	0	0		0	0		60 (56–65)	0
44	0	0	D	0	0	4	59 (55–63)	0
48	0	0		0	0		54 (50–58)	0

Abbreviations: *WAT* weeks after treatment, *CI* confidence interval, *FECR* fecal egg count reduction, *PHR* positive horses reduction

[a]G-1: horses dewormed [1 mg Ivermectin/kg bw *pour on*, Noromectin 0.5%, (Norbrook Laboratories, Newry, UK)], maintained under continuous grazing and given pellets without fungal spores

[b]G-2: SSHs dewormed (1 mg Ivermectin/kg bw *pour on*), kept under rotational grazing and provided pellets without fungal spores

[c]G-3: SSHs dewormed (1 mg Ivermectin/kg bw *pour on*), kept under rotational grazing and given pellets containing 2×10^6 spores of *Mucor circinelloides* + 2×10^6 spores *Duddingtonia flagrans*/kg

The data obtained in the first six months show that the average values of strongyle egg-output were 1.5-fold higher in G-1 than in G-2 and 3-fold in G-1 higher than in G-3. From the 7th to the 12th m. a. t., the average levels of EPG in G-1 and G-2 were very similar and were 2.6-fold higher than in G-3 (χ^2 = 71.263, df = 2, P = 0.001).

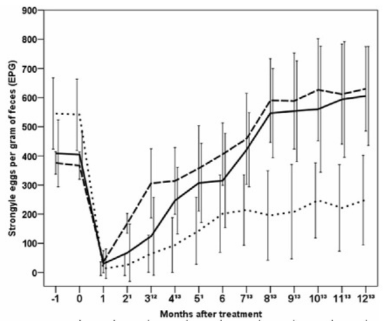
Significant differences were observed in the values of strongyle egg-output according to the group of horses (χ^2 = 59.138, df = 2, P = 0.001). By means of the Mann-Whitney U test, significant differences were identified between G-2 and G-3 (U = -5.988, P = 0.001) and between G-1 and G-2 (U = -5.650, P = 0.001) (Fig. 3).

Adverse effects

None of the horses in G-3 rejected the pellets enriched with fungal spores. Normal appetite, digestive activity, reproductive behavior and breathing were recorded in all the horses. No signs of skin damage or disorder were observed during the assay. No alterations concerning pellet smell or thickness were observed, nor did hyphae grow on the pellet surface.

Discussion

Grazing regimes offer horses nutrition and the opportunity to exercise and to interact with other individuals [23], but infection by gastrointestinal nematodes such as strongyles can be enhanced when infective stages (L3 larvae) are ingested with grass, and therefore, deworming is frequently needed. In the current study, the topical administration of ivermectin to horses under continuous pasturing provided successful results, based on the values achieved 14 days after treatment for the reduction in the fecal egg counts (FECR: 100%) and in the numbers of horses passing eggs of strongyles in feces (PHR: 100%),



Pasture	G-2	A	B	C	D	A	B	C	D
	G-3	1	2	3	4	1	2	3	4

Fig. 3 Kinetics of strongyle egg-output in Spanish Sport Horses under different pasturing regimes in an oceanic climate area (NW Spain). *Key*: (— —) G-1: continuous grazing and given pellets without fungal spores; (—) G-2: four-paddock rotation system and pellets without fungal spores; (••••••) G-3: four-paddock rotation system and pellets containing 2×10^6 spores of *Mucor circinelloides* + 2×10^6 spores *Duddingtonia flagrans*/kg. Points mean the average value, and error bars mean 2SD. Superscripts indicate time points differences between the strongyle egg-output values from G-1 and G-2 (**1**), G-2 and G-3 (**2**) and G-1 and G-3 (**3**)

which agreed with the results of prior studies in the same area [7, 20]. The coprological assessment of anthelmintic efficacy comprises the estimation of FECR and thus concerns the effect on adult worms, but ivermectin presents a high activity against adult worms and larvae in the gut lumen, and low activity against encysted (hypobiotic) larvae [24]. Although the deworming was successful, the strongyle egg reappearance period was 6 weeks after treatment, probably due to the mobilization of encysted L3 larvae from the mucosa wall to the intestinal lumen to develop into adult stages [25]. Bearing in mind that in the current study, horses always fed on a pasture previously grazed by parasitized equines, another explanation involves the ingestion of L3s, which can attain the adult stage in 5–6 weeks, from the contaminated pasture [25]. The observation of EPG values higher than 300 (the threshold set at the beginning of the current study) from the 3rd month after treatment implies that deworming would be necessary again.

Several attempts have been made to avoid having horses that feed on grasslands become highly contaminated by L3 larvae, such as the regular collection of feces or pasture rotation [26]. In the present investigation, one group of horses maintained under grassland rotation involving a grazing period of 1.5 months and a rest phase of 4.5 months, was treated with ivermectin *pour-on*. Successful results were also observed according to the values of FECR (96%), although two out of eight horses continued to pass eggs in their feces (PHR: 75%). The strongyle egg reappearance period (ERP) was longer than in horses under continuous grazing (10 *vs* 6 weeks), as was the period in which all the equines became positive to the flotation test (14 *vs* 6 weeks). In addition to hypobiotic L3 larvae mobilizing to the gut lumen and developing to adult worms, the maintenance of horses in the same meadow for 1.5 months and then moving them to another paddock seemed to reduce their exposure to L3 strongyle larvae, and average egg-output values that were 49% lower than those in horses under continuous grazing during the first six months after treatment were obtained.

Rotational grazing is helpful to keep horses out of areas highly contaminated by L3 strongyles [27], and the numbers of infective L3s decrease with the length of the paddock resting period, so an infected pasture could return to a low level of risk of infection after a rest period of 3–6 months [28]. In the present investigation, horses re-entered the same pasture after a rest period of 4.5 months, and during this period (7–12 m. a. t.) the counts of the eggs of strongyles were 7% lower than in continuous grazing horses. These data appear to show that rotation decreases the level of pasture contamination, and consequently, infection is delayed or slowed down. However, the equines were re-infected, and at the end of the trial (12 m. a. t.), similar numbers of eggs of strongyles were recorded in the feces of either continuously grazing or rotationally grazing horses. Moreover, the observation of fecal egg-output values higher than 300 EPG starting at five months after treatment indicates that deworming is needed again after three pasture rotations. One possible solution to limit the numbers of strongyles in the pastures could rely on shortening the grazing period to less than 1.5 months [29] by increasing the number of rotated pastures. Because this is seldom possible, helpful measures appear necessary to reduce the viability of larvae of strongyles in the grass, or to avoid having the larvae develop from eggs inside the feces and move to the herbage.

The hatching of strongyle eggs and their further development from L1 to L3 larvae requires levels of soil moisture higher than 15–20% and takes 3 days at 25–35 °C, 15–24 days at 10 °C, or several weeks at temperatures < 10 °C [30]. Strongyle eggs can remain viable below 6 °C, although hatching does not occur [31]. In the area of study, average temperatures higher than 10 °C were recorded during all the months except between December and March (winter), when frost days were detected. The observation of high percentages of relative humidity (> 81%), together with notable rainfall values, except during the summer, and a negative water balance, only at the end of spring and at the beginning of summer, indicates that the soil moisture supports the growth of grasses nearly all year and offers a suitable environment for the development and survival of free-living stages of strongyles almost year-round. In addition to this, the eggs and larvae of strongyles are able to survive inside fecal pats and soil, even during cold periods [5]; under low levels of humidity and rainfall, L3s tend to remain in the feces, avoiding migration onto contiguous grass [5, 6], and fecal pats appear to become their main reservoirs [32]. Therefore, promising results could be attained by taking action against the strongyles in the feces.

Some saprophytic fungi are characterized by their antagonistic activity against different parasitic stages in the feces of infected animals. *Pochonia chlamydosporia* and *Mucor circinelloides* can destroy more than 50% of viable eggs of helminths such as *Calicophoron daubneyi*, *Toxocara canis* or *Trichuris* spp. [13, 18]. *Duddingtonia flagrans* is able to trap and then eliminate 94% of cyathostomin L3 when spores are directly sprayed on the feces of infected horses [33], and 27–99% when chlamydospores are given orally to horses from a similar climatic place (Curitiba, Brazil) [12]. Because the spores of these fungi can survive the industrial manufacturing of pelleted feed and the gastrointestinal tract, once administered to horses [16], in the present investigation, a third group of horses that was under rotational grazing was treated with ivermectin and provided twice a week with pellets industrially enriched with a blend of spores of *M. circinelloides* and *D. flagrans*. Deworming was effective (FECR: 99%), but one out of eight horses was positive to the flotation test 14 days after treatment (PHR: 88%). In comparison with horses under the same regime but feeding on pellets without spores, the ERP was extended by a further 1.5 months, and the period required for all the horses to shed strongyle eggs was extended by a further three months. Only one of the horses receiving pellets with spores exceeded the threshold of 300 EPG, and no adverse effects were recorded. Recently, an ERP of seven months has been reported among horses under continuous grazing and receiving daily pellets with spores of *M. circinelloides* and *D. flagrans*, and all the horses excreted eggs of strongyles by 15 months after their deworming [16].

When the pasture rotation ended (6 first months), a 50% reduction in the EPG values was observed in the horses given pellets with fungal spores compared with those given pellets without spores. After the horses re-entered the meadows they had previously grazed and completed the pasture rotation, the reduction in the EPG values was 59%. Since horses under rotational grazing were randomly distributed into two groups by the owner, as occurred with the designation of the pastures, it is suggested that the twice a week administration of pelleted feed with spores contributes to a lower risk of infection by strongyles. Previous investigations performed on continuously grazing horses showed that the numbers of fecal strongyle eggs decreased by 35–73% after providing them with a weekly ration of pellets with the mycelia of *D. flagrans* for six months [11], and the daily administration of pelleted feed with chlamydospores of *D. flagrans* was correlated with significant EPG reductions after 6 months (78%) and 12 months (67%) [24]. The results from the present research appear to indicate that fungi in the feces negatively influence the development of eggs and/or larvae in the feces, decreasing the level of contamination. Despite the high efficacy of *D. flagrans* in trapping and destroying strongyle larvae [11, 33], no data are available concerning the effect of *M. circinelloides* on strongyle eggs, although its ovicidal activity against the eggs of trematodes and ascarids has been demonstrated [15].

Conclusions

The effective control of horse strongyles needs to include strategies to reduce the numbers of infective larvae in pastures, which will make it possible to decrease the frequency of anthelmintic treatments. In oceanic climate regions, rotational grazing contributes to delaying horse infection by strongyles, but the long-term persistence of the viable stages in the soil/herbage requires additional measures to avoid their migration from the feces to the grass, unless short grazing periods (< 6 weeks) or a high number of grazed pastures can be used. Supplementation twice weekly with pellets industrially manufactured with the spores of *M. circinelloides* and *D. flagrans* ensures the presence of these two parasiticidal fungi in the feces, which can reduce the development of strongyles in the environment and thus the risk of horse infection. The current results were obtained during a one-year period, and a more extensive follow-up study is in progress to gain information to confirm these initial findings.

Abbreviations

FECR: Fecal egg-count reduction; PHR: Reduction of the numbers of horses shedding eggs; ERP: Egg reappearance period; EPG: Eggs per gram of feces; SSH: Spanish Sport Horses; L1: First-stage larva; L2: Second-stage larva; L3: Third-stage larva; m. a. t: Month after treatment

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Authors' contributions
JAH, CFC, FLA and JMS took the samples and analyzed them. RS, AP and MSA designed the study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval
Not applicable.

Consent for publication
Not applicable.

Competing interests
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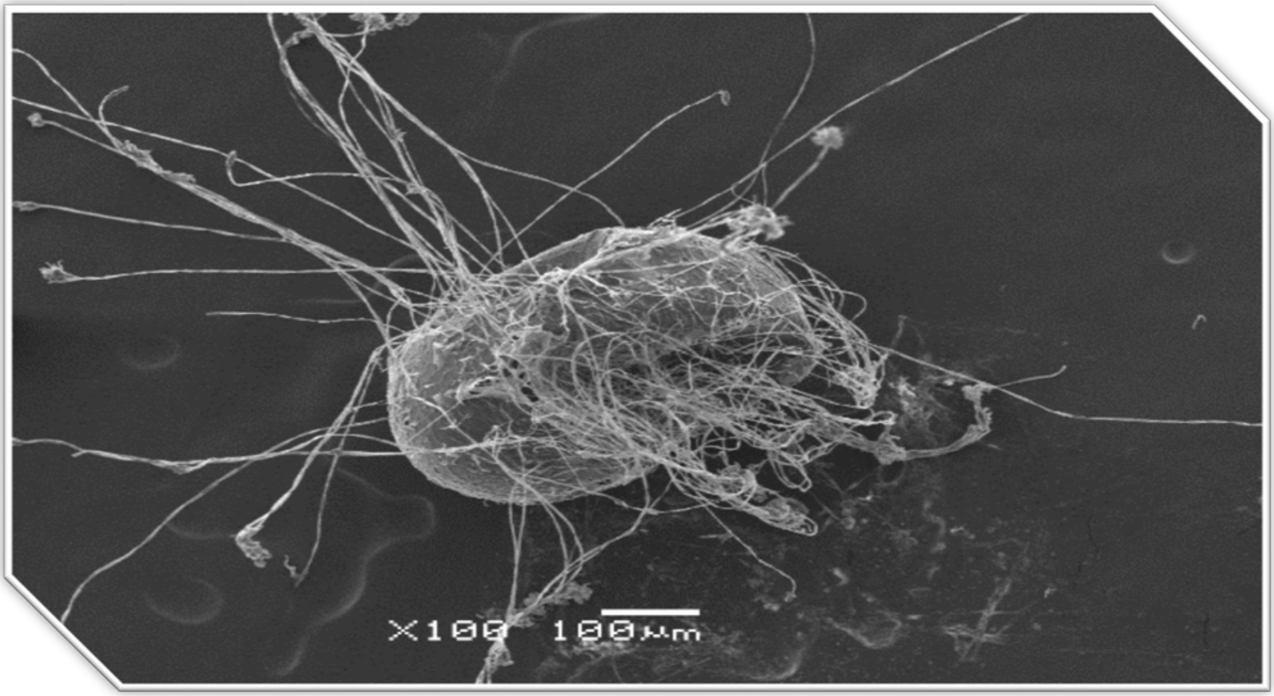
7. PAPER Nº 3:

BIOLOGICAL CONTROL OF SOIL TRANSMITTED HELMINTHS (STHs) IN A ZOOLOGICAL PARK BY USING SAPROPHYTIC FUNGI



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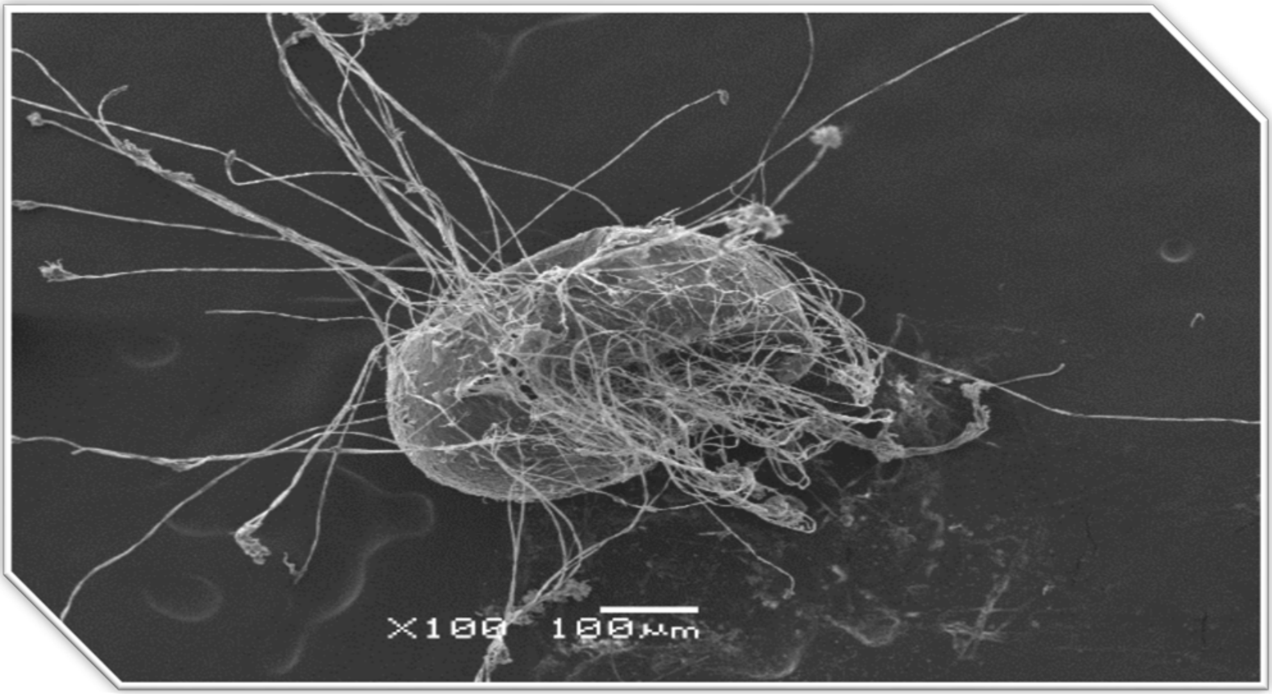

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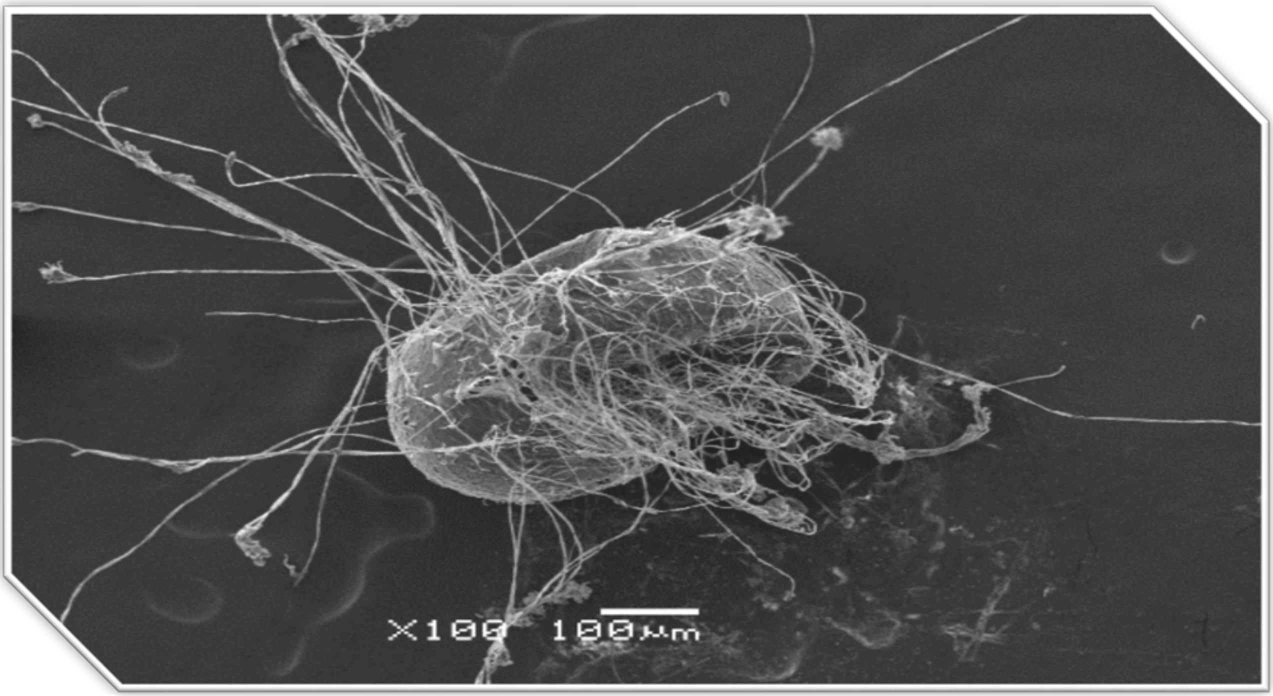


Chapter

Advantageous Fungi against Parasites Transmitted through Soil

José Ángel Hernández Malagón, Cristiana Filipa Cazapal-Monteiro, Rodrigo Bonilla Quintero, Antonio Miguel Palomero Salinero, María Isabel Silva Torres, Mathilde Voinot Messnier, María Vilá Pena, Ángel Romasanta Blanco, José Pedreira García, Adolfo Paz Silva, Rita Sánchez-Andrade Fernández and María Sol Arias Vázquez

Abstract

Although many fungal specimens are responsible for human and/or animal infection, other species are advantageous for preventing the infection by soil-transmitted zoonotic parasites. Infection occurs by the accidental ingestion of parasitic stages (cysts, oocysts, eggs, and larvae), their active penetration through the skin or through direct contact. Numerous species of helminths develop an external phase in the soil where the infective stages are attained, thus mammals become infected when grazing, drinking, or accidentally. Ectoparasites as ticks perform also in the soil the phase from egg to larva. Different soil saprophytic fungi that turn into predatory agents when parasitic stages are near have been isolated and described. These species are capable of destroying the pathogens or irreversibly decreasing their viability, providing thus a very interesting and sustainable tool to reduce environmental contamination by pathogenic agents. In the last year, a profound knowledge on the most appropriate fungal species, together with the proper way to disseminate them, has been acquired.

Keywords: *Mucor circinelloides*, *Duddingtonia flagrans*, parasiticide, soil, STHs, zoonoses

1. Introduction

1.1 Organisms in soil

The definition of soil according to the sciences of the earth and life points to the external part of the earth's crust, which is biologically active and tends to develop on the surface of the rocks emerged by the influence of weather and living beings. It is also frequent that this concept includes a complex set of physical, chemical, and biological elements that make up the natural substrate in which life develops on the surface of the continents. The soil is the habitat of a specific biota of microorganisms (bacteria and fungi), plants, and small animals that constitute the edaphon.

In recent decades, there has been an increasing concern for soil biodiversity, on the basis that the interactions between microorganisms, animals, and plants provide an undoubted benefit to the well-being of mammalian species, including man [1]. This biodiversity conditions both the possibilities of feeding these species, oxygenation, as well as the control of the risk of certain diseases. For these reasons, it is not difficult to understand that soil biodiversity is directly affected by global changes caused by man, especially those related to land use, urbanization, agriculture, deforestation, and desertification, which leads to the logical conclusion that the careful and sustainable use of soils would guarantee their benefits.

Different studies have indicated that exposure to soil microorganisms decreases the prevalence of allergic diseases [2]; taking into account the predictions that around the year 2050 two-thirds of the world population will reside in cities, the stimulation of the immune system by soil organisms will be reduced, and therefore allergy cases will increase.

Other researches highlight the increase in the appearance of bacterial species resistant to most known antibiotics, and the same happens with some parasites, such as helminths. The use of remedies found in the soil, such as certain types of fungi, has not yet come to be considered as a solution to the aforementioned problems. It is interesting to know that some bacteria capable of synthesizing effective antibiotics against *Mycobacterium tuberculosis* have been isolated in the soil [3]. It should also be noted the production of molecules with parasiticide action from fungi [4]. Special mention should be made of the use of some fungal species in the control of certain endoparasites that, once in the soil, complete a series of phases until they reach the infective stage [5]. In recent years, very important achievements have been made in the large-scale production of saprophytic fungal spores that are found in the soil, such as *Mucor circinelloides* or *Duddingtonia flagrans*, filamentous species that are in contact with eggs or larvae of some parasites, respectively. They have the capacity to destroy them or limit their viability [6, 7]. In this way, it is possible to reduce the risk of infection in people, and also in animals that are in pasture.

1.2 Pathogenic organisms transmitted through the soil

Pathogenic organisms belong mainly to five main groups, viruses, bacteria, fungi, and parasites (protozoa, helminths, and ectoparasites) [8]. From an academic and disease control approach, the importance of soil lies in the fact that a significant number of pathogens are found in this habitat, and sometimes they are accidentally ingested by animals and people, causing important disorders. There are some organisms that do not require ingestion, being able to spread their pathogenicity through bites or penetrating the skin.

Table 1 summarizes different examples of pathogens present in the soil. It is important to note that most soil organisms do not constitute a health risk, and pathogenic species represent only a minority. Nor should we forget that some species are opportunistic (*Pseudomonas* and *Enterobacter*) and can cause alterations in mammals, although in the soil they are actually antagonists of root pathogens of some plant species, or can act as growth promoters of some plants and even as decomposers of organic matter [9]. Other pathogens need to develop part of their cycle in the soil, to complete their evolution until the infective phase. These are organisms that can survive in the soil for long periods of time, and include spores, eggs, or even larvae. These are obligate pathogens that temporarily reside in the soil, and that are transmitted to mammals by direct contact, by vectors, or through accidental ingestion [10]. For these reasons, it is necessary to know the ecology of

Bacteria	*Bacillus anthracis*	*Agrobacterium tumefaciens*
	Listeria monocytogenes	*Escherichia coli*
	Salmonella spp.	*Clostridium* spp.
Fungi	*Aspergillus* spp.	*Histoplasma capsulatum*
	Coccidioides immitis	
Protozoa	*Naegleria fowleri*	*Toxoplasma gondii*
Helminths	*Ascaris* spp.	*Taenia* spp.
	Ancylostoma spp.	*Strongylus* spp.
Ectoparasites	*Pulex irritans*	*Ixodes* spp.

Table 1.
Numerous pathogens can be found in the soil.

the interactions between the soil and the various organisms to determine why some are prevalent and persist under certain conditions.

The concept of *Soil Borne Human Diseases* offers a very accurate introduction about the role that soil can play in the transmission of certain diseases [11]. However, it is obvious that this idea is a bit limited, since not only the human species will experience the risk of contracting diseases from this habitat. From an etiological point of view, *pathogenic organisms* are defined as those whose habitat is the soil, and *pathogens transmitted by the soil* as organisms that can survive for long periods of time in the soil, and need to do so to infect the host and continue their biological cycle, but they are not part of the soil [12]. Some of the most frequent endoparasites affecting people and animals, such as roundworms, cestodes, or strongyles, belong to this group, and they are characterized by undergoing a series of changes in the soil to the infecting stage. Part of the biological cycle of some ectoparasites such as fleas or ticks occurs in the soil also. This underlines the importance of soil as an adequate medium to certain parasites can survive and develop to infective stages, pending of proper hosts ingest them (flatworms, roundworms, whipworms), contact with soil (hookworms) or walk near (ectoparasites). Regardless of their origin (animal/human), control of parasites affecting mammals requires some action on the stages in the group, since parasiticide therapy acts on the parasites living and affecting them only; thus, the risk of reinfection is elevated, even though successful treatments are applied.

1.3 Mammal parasites developing in the soil

Soil provides a suitable habitat to different organisms as plants can grow and develop, serving as food for the survival of many living creatures (insects and micromammals). This environment enables mammals as herbivores to graze and carnivores to find their feeding.

Most known parasites associated to soil are defined as *soil-transmitted helminths* (STHs), which involve well-known species belonging to flatworms, tapeworms, or roundworms. Helminths can develop a direct cycle in the soil, but an intermediate host is required for some species, and paratenic hosts participate in the transmission of several infections. On the basis of the zoonotic role of different parasites developing in the soil, it is necessary to know the external phase of their life cycle.

Transmission of STHs involves that eggs are passed in the feces of infected individuals. Once in the soil, flatworms (trematodes and cestodes) need to complete several stages inside an intermediate host, to attain the infective stage. *Fasciola hepatica* and *Schistosoma mansoni* (flatworms) are related to humid environments where a number of aquatic or amphibious snails take part. After some stages are completed and exit-off the snails, the infective stages known as metacercariae mature in herbage or water, and infection occurs by the ingestion of herbage or water contaminated [13].

Roundworms (*Ascarids*) represent the most spread nematodes around the world. Although these are host species-specific pathogens, humans can be involved as paratenic hosts for many of them such as roundworms infecting domestic animals (*Toxocara canis*, *Ascaris suum*) or wild species (*Baylisascaris procyonis* and *Toxascaris leonina*). Infection occurs by the accidental ingestion of larvated eggs (containing a second-stage larva inside) (**Figure 1**).

Whipworms (*Trichuris* spp.) have a similar cycle to roundworms. Transmission occurs by the oral ingestion of eggs holding a first larva.

In the case of *Ancylostoma*, nematodes (hookworms), embryonated eggs are passed in the feces and once in the soil, the first-stage larva (L3) emerges and molts to a second-stage larva and then to a third-stage larva, the infective stage. Infection can occur either by oral ingestion of L3 or through the skin [14].

It is well recognized that ticks need to suck blood from mammals for surviving, but sometimes it is forgotten that these ectoparasites develop part of their life cycle in the soil also. Gravid adult females drop off the final host to the ground to lay eggs. Under appropriate conditions, the egg hatches into a larva, which waits for an appropriate mammal to bite for feeding and then transform into nymph.

Appropriate conditions (moisture and temperature) must concur in the soil to improve the development of parasites to their infective stages. Nevertheless, evolution of parasites can be delayed until unfavorable circumstances appear, especially low temperatures. Some of them such as roundworms and whipworms are able to survive viable for long periods, even under temperatures below zero [15]. This

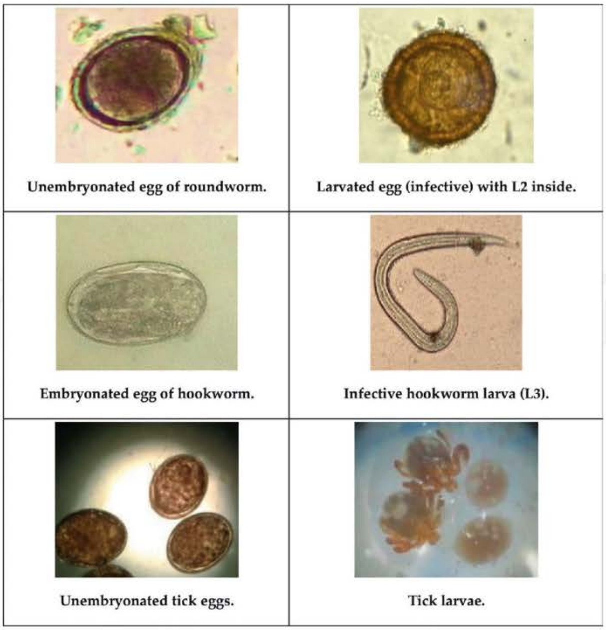


Figure 1.
Numerous parasitic stages can be found in the soil (COPAR archive).

resistance is conferred by their eggshells, composed of at least four layers, uterine (mucopolysaccharides), vitelin, chithinous, and lipidic (inner). Eggs of ticks can also survive in the environment unless the solar light falls directly on them.

Larval stages (first, second, or third) from nematodes exhibit a certain degree of resistance, and it has been reported they can subsist under snowy areas [16]. Dry soils in spite of very humid areas are preferred by immature hookworms [5], like sandy places. This explains the cutaneous infection of people enjoying outdoor activities on beaches, parks, etc. from touristic areas.

1.4 Importance of infection by parasites from the soil

Human STHs are frequent in Asia, Africa, and South America, being absent in Western Europe and developed countries. Nevertheless, these diseases have reemerged due to immigration, travel, and business. Also in recent years, populations of ticks are increasing in urban areas, as well as orchards, parks, and gardens [17].

There are four main STHs affecting humans, *Toxocara canis* (roundworm), *Ancylostoma duodenale* and *Necator americanus* (hookworm), and *Trichuris trichiura* (whipworm). Between 1.5 and 2 billion people, it is believed that they are probably infected worldwide [18]. The presence of these parasites is associated to low standards of hygiene, poverty, and malnutrition because infection takes place by the accidental ingestion of eggs or through cutaneous contact with larvae of hookworms. It is necessary the exposure to feces of pets, mainly dogs. As advised by the WHO (World Health Organization), periodic administration of albendazole and mebendazole is helpful to reduce the incidence of these parasitoses. Deworming is the most applied measure against STHs, and extension of treatment (increment of frequency) looks like a valuable solution, although there is a potential emergence of drug resistance as observed in veterinary medicine [19]. By considering that infections originate from fecal contamination of the environment, mammals can become reinfected frequently after parasiticide treatment is administered. Consequently, actions on the environment are required to reduce the exposure to infective stages, mainly consisting of the use of latrines, together with hygienic behaviors.

Dogs are the definite hosts for *T. canis* and *N. americanus*, thus another question to address concerns the possibility of humans and animals sharing infections by parasites, the so-called parasitic zoonoses. As explained previously, transmission occurs in the same way, but the presence of infected animals becomes essential for human infection. In this case, control appears more difficult, due to the impossibility to ensure that pets receive an appropriate deworming therapy. The problem aggravates when considering that wild/uncontrolled animals can live near persons, because there is no way to perform control of their parasites by the administration of antiparasitic drugs. In some countries, it is not rare to observe feces of stray dogs or cats, foxes or raccoons, in private gardens, public parks, or even beaches. As stated above, humans might become infected by roundworms or hookworms, and despite infection, it is not completed, serious damage could be provoked attributable to the erratic migration of immature stages across the organism [20]. At this point, it seems necessary to remember that second-stage larvae of *Toxocara canis* (dog), *T. cati* (cat), *Ascaris suum* (pig), or *Baylisascaris procyonis* (raccoon) can cause a visceral larva migrans syndrome after these larvae are released at the gut level. Infection by *T. canis* can be responsible for an ocular larva migrans [21], while *B. procyonis* is associated to a devastating neurological syndrome, with children being the riskiest group due to their tendency to play with ground, or take and leave sand in the mouth [22]. The possibility of human infection through the exposure to eggs of roundworms on the coat of dogs has also been considered [23], which remarks the importance of these parasites are easily transmitted to their owners.

2. Beneficial soil fungi

2.1 Antagonists of helminths

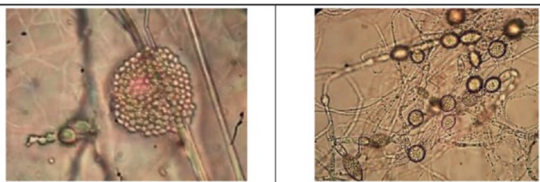
By considering that a great number of pathogens develop in the soil, one interesting question refers to why mammals did not infect more frequently, or why low to moderate infections are usually detected. Infection depends on the density of pathogens and risky situations such as accidental ingestion or active passage through the skin (helminths) or walking by places with vegetation (ectoparasites). Then, it could be expected that exposure to natural environments might represent a great hazard, thus enjoying natural habitats should be avoided (or even forbidden).

As mentioned previously, a great number of fungal species can be found in the soil, together with many other organisms such as viruses, bacteria, earthworms, insects, etc. Some of these species are saprophytic and feed on organic matter, but in the presence of parasitic stages such as eggs or larvae, they shift to predatory agents. Hyphae develop and the mycelium grows toward the parasites in an attempt to take certain nutrients, nitrogen and carbon mainly [24]. Other fungal species feed on different species of fungi, as succeeds with some mites.

It has been demonstrated that certain soil saprophytic fungi such as *Duddingtonia flagrans* are able to adapt to the numbers of larvae of nematodes developing from eggs shed in feces of infected grazing horses [25]. In the absence of nutrients, fungi can remain as resting stages (spores). It should be emphasized that different organisms interact simultaneously on the ground, thus soil fungi do not persist for long periods (4 months) and need to be replaced by new structures such as spores, mycelium, etc. [26]. This must be taken into account when soil fungi are going to be used under biological control strategies. Other interesting finding consists of the absence of activity on nonparasitic organisms (**Figure 2**).

Based on experiments with plants, traditionally the fungal antagonists of parasites comprise nematode-trapping species (larvicidal), predacious agents, endoparasitic fungi, and egg parasitic fungi (ovicidal) [27]. In the last decades, this classification applies also for defining the activity of soil fungi against parasites affecting mammals (**Table 2**).

In natural conditions, when the environment does not result altered by humans, soil albeits not only fungi but other microorganisms as viruses, bacteria, earthworms, insects... A number of filamentous fungi feed on organic detritus, certain



Zygospore of *M. circinelloides*. | Hyphae and chlamydospores of *D. flagrans*.

Figure 2.
Filamentous fungi develop hyphal nets in the soil, and reproduce by spores (COPAR archive).

Effect	Species		Action against
Ovicidal	*Pochonia chlamydosporia*	*Mucor circinelloides*	Flatworms Roundworms (ascarids) Whipworms
	Purpureocillium lilacinus	*Verticillium chlamydosporium*	
	Trichoderma spp.	*Gliocladium* spp.	
Larvicidal	*Duddingtonia flagrans*	*Monacrosporium* spp.	Hookworms Roundworms (strongyles)
	Arthrobotrys spp.		

Table 2.
Filamentous soil fungi antagonists of parasites in the soil.

coprophagous beetles participate in enriching the ground by decomposing organic matter as manure, some mites feed on fungi, and several fungi do it also. This means that an equilibrium situation takes place, where organisms are controlled mutually, and explains also why low risk of infection is usually observed. When agricultural procedures affecting the surface of the ground are performed, this habitat is transformed, and beneficial organisms drop or disappear. As a consequence, the density of pathogens increases, accordingly the risk of exposure among mammals increases and they can become infected.

Several investigations pointed the efficacy provided by some fungi to limit the viability of eggs of roundworms [28]. As drawn in **Figures 3** and **4**, the addition of spores of *M. circinelloides* to the feces of dogs infected by *T. canis* decreased their viability by half after a period of 30 days [29]. When the spores were sprayed onto feces of raccoons parasitized by *B. procyonis*, egg viability reduced by two-thirds also, in agreement with previous experiments [30].

A notable efficacy has been reported against larvae of hookworms by using trapping-nematode fungi such as *D. flagrans*. A 57–73.2% reduction of the numbers of the third-stage larvae of *Ancylostoma* spp. has been obtained, and the counts of larvae decreased by 24.5–63% when exposed to chlamydospores of *D. flagrans* [31, 32].

By taking into account that the aforementioned parasites are STHs, the use of ovicidal and larvicidal fungi could be strongly helpful to limit the development of parasites to infective stages in the soil. One interesting question refers to the proper way to spread the fungi to ensure their contact with the parasites. Because the eggs of parasites are shed by feces, the most useful procedure looks to try that fungi

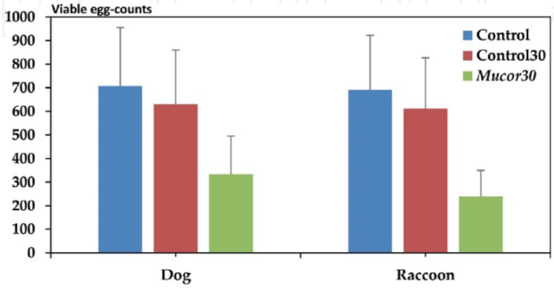


Figure 3.
Viability of eggs of Toxocara canis (left) and Baylisascaris procyonis (right) after 30 days of exposure to spores of Mucor circinelloides (Mucor30) or distilled water (Control 30). Points mean average values and bars the SD [29].

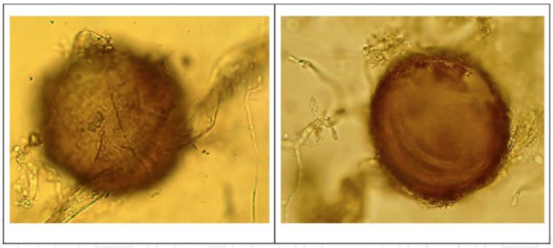


Figure 4.
The soil filamentous fungus M. circinelloides is able to attach to the eggshells of roundworms such as T. canis, colonize, penetrate, and absorb the inner content (COPAR archive).

are in the feces at the same time, and for this purpose, oral administration could be appropriate. Several investigations demonstrated that the spores of *Pochonia chlamydosporia*, *Mucor circinelloides*, and *Duddingtonia flagrans* can survive the passage through the gastrointestinal tract of different animal species, and retained their antagonistic activity [6, 33, 34]. Later, several assays were performed by adding spores or mycelium of *Pochonia chlamydosporia* or *Duddingtonia flagrans* during the handmade elaboration of nutritional pellets [35–37]. More recently, the capability of fungal spores to resist the industrial fabrication of pelleted feed has been demonstrated [38, 39]. The usefulness of pellets containing spores of *M. circinelloides* and *D. flagrans* has been tested on grazing horses, and highly successful results were obtained. Through this strategy, it was possible to reduce the frequency of deworming from 4 years to 1–1.5 years [7, 40]. This approach has also been assayed on wild captive equids maintained in a zoological park, and as a result the administration of anthelmintics was significantly lessened [41], supporting the results previously collected by administering the spores as a premixed feed [6].

2.2 Entomopathological agents

It has been explained that ectoparasites develop part of their cycle in the soil. After mating on the host, gravid female ticks engorge completely and drop to the ground, where thousands of eggs are laid mainly in places protected from sun and desiccation, with vegetation. Later than a variable period, depending on temperature and humidity, eggs hatch and larvae exit off, addressing to plants, pending of a host to attach and suck blood for molting into nymphae. *Beauveria bassiana* and especially *Metarhizium anisopliae* are the most investigated entomopathogenic fungi capable of infecting and damaging ticks [42, 43]. Trials consisted of the topical administration of oil solutions, targeted against immature or adult stages [44]. The aim is to reduce the indiscriminate use of chemical acaricides, for avoiding contamination of food and environment, as well as the appearance of chemical resistance among tick populations [45].

There is little information available concerning the possible effect of fungi on tick eggs in the soil. **Figure 5** summarizes the results collected after the exposure of eggs of *Rhipicephalus boophilus* to spores of *M. circinelloides*. The fungal activity was estimated by measuring the percentage of egg viability, and the hatching percentage, i.e., the percentage of larvae hatched after 15 days. Fungal growth started on the eggshells 4 days after exposure, and by 6 days, hyphae penetrated inside.

Viability of ticks' eggs decreased to 80% in the controls-untreated eggs, and to 38% in those exposed to the filamentous fungus. The hatching percentage was 45% in the controls, by 15% in the *Mucor*-treated eggs.

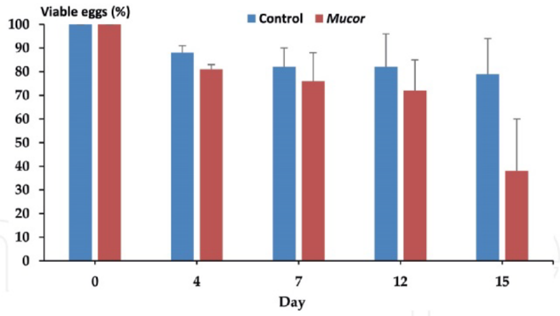


Figure 5.
Viability of eggs of the tick R. boophilus exposed to spores of M. circinelloides (Mucor) or distilled water (Control). Points mean average values and bars the SD (COPAR archive).

Four phases have been described during the activity that the ovicidal fungus *Verticillium chlamydosporium* perform on eggs of helminths, i.e., contact, attachment, penetration, and deliberation [46]. The fungus *M. circinelloides* develops a similar activity on both the eggs of helminths and ticks (**Figure 6**). When the spores contact with the parasites, hyphae grow toward the eggshell and colonize it. Those hyphae facing the eggshell in perpendicular are able to penetrate inside. This is possible due to the involvement of the *appressorium*, a pressing organ consisting of a flattened and thickened hypha, which is provided of a *haustorium*, a specialized branch which penetrates the tissues of the host and absorbs nutrients and water [47]. This mechanism enables the fungus to take all the inner content of the egg, without losing anything. Once completed, hyphae exit off and colonize other egg (deliberation).

In view of the mentioned results, certain soil fungi seem very promising agents for limiting the viability and evolution of tick eggs in the soil, contributing to decrease the risk of infestation. One possible approach could rely on preparing aqueous solutions containing the fungal spores, and spreading by using airless

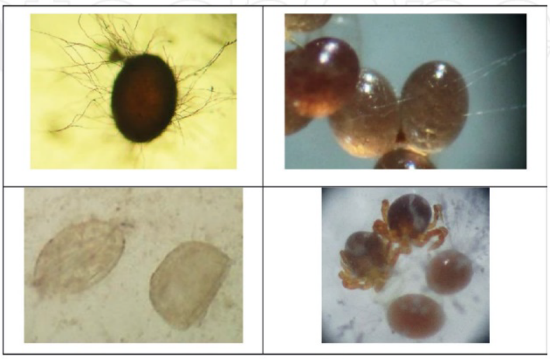


Figure 6.
Hyphae of M. circinelloides grow and attach to the eggshells of ticks, penetrate and destroy them (COPAR archive).

sprayers. This would provide a solution to limit the risk of infestation in outdoor areas as waysides or the edge of grass along the roadsides, gardens, or even farms. Reduction in the presence of ticks in the soil also provides a sustainable and preventive tool to avoid damage to humans and animals.

2.3 Biofuel production

Some strains of several soil fungal species have been isolated according to their ability to convert fungal oils into esters, providing thus a sustainable way to obtain biofuel [48, 49]. The interest of microbial oils has increased as they are now used as commercial sources of several nutritionally important polyunsaturated fatty acids [50].

2.4 Health and soil fungal employment

Despite fungi being mostly considered responsible for fungal diseases that can range from nonsevere to mortal illnesses, fungal infections have become a serious health problem in immunocompromised patients largely.

Opposite to *Duddingtonia flagrans* and *Monacrosporium thaumasium*, the infection by *Mucor circinelloides* has been associated to clinical cases of mucormycosis, a sporadic and life-threatening infection caused by Mucorales. These are fungi distributed far and wide in the environment, in particular on woody surfaces and soils, where it can be easily isolated [27].

Several reports indicated nosocomial infection by *M. circinelloides* among immunocompromised people with skin wounds, or suffering diabetes mellitus [51].

Among animals, infection by *M. circinelloides* has been diagnosed in one Vietnamese potbellied pig presenting clinical signs of pneumonia, but information regarding the habitat or the level of inbreeding has not been provided [52].

Until now, long-term assays comprising the frequent administration (daily or twice a week) of a blend of spores of *M. circinelloides* and *D. flagrans* have been developed in pasturing horses. One group of seven horses received daily pellets containing the fungal spores during 64 weeks, and no adverse effects regarding respiratory, digestive, reproductive, or cutaneous damage were recorded [7]. Other group of eight horses was given pellets twice a week with the spores for a 1-year period, and after testing the activity of the respiratory, digestive, and reproductive systems, no alterations were recorded [41]. No signs of damage on skin integrity were observed.

Until now, there have not been reported any problem with people producing and managing spores/mycelium for longer than 10 years.

2.5 Conclusions

Inasmuch as STHs are transmitted through soil, it seems essential to develop measures on the environment to avoid reinfection, and the abusive administration of parasiticides. Some STHs originate from animals (domestic and wild), and helpful actions to reduce the risk of transmission are also required. Besides public education and hygienic behaviors, other activities should be applied to limit the presence and survival of infective stages of parasites. There have been described several species of soil fungi antagonists of eggs or larvae of helminths and ticks. Although several cases of disease have been linked to soil fungi, the absence of disease among people managing them or among animals receiving fungal structures seems to reinforce their safety, unless the patients are immunocompromised. The use of soil fungi against infections transmitted across ground gives a sustainable

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measure to prevent damage to persons and animals, and might allow us to limit the administration of antiparasitic drugs to imperative situations only.

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Conflict of interest

All authors declare the absence of any financial or personal interests that could inappropriately influence or bias the current work. The final chapter has been approved by all the authors.

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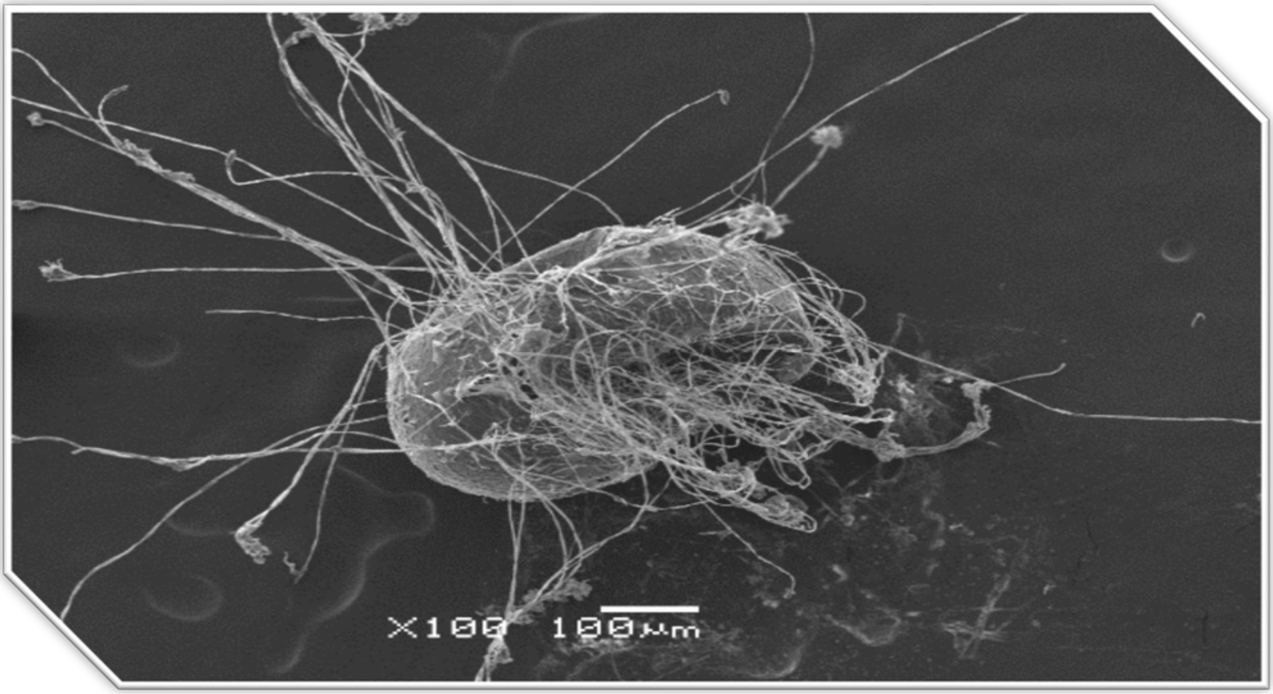
10. DISCUSSION



10. DISCUSSION

The decreased efficiency of some antiparasitic drugs, identified decades ago, sometimes worsened by the existence of strain resistance to certain active ingredients, indicated the need to design useful procedures to lower the risk of parasitic infection, prevent their development in animals and human beings, and the use of drug therapies in necessary situations only, thus preserving the efficiency of antiparasitic drugs. In the wild, some pathogenic forms are naturally regulated by certain organisms such as viruses, bacteria, invertebrates, nematodes and even saprophytic fungi, reaching a balanced situation that may be broken if the upper layers of the soil are destroyed (Arias et al. 2011b). Some fungal species feed on decomposing organic matter (saprophytes) and parasitic cysts, eggs or larvae. In our case study, fungi were isolated from stools collected straight from vegetated areas in a zoological park where 13 wild different animal species lived; when these fungi were put in contact with *Calicophoron daubneyi* and *Parascaris equorum* eggs, *Gliocladium* (*Purpureocillium*) and *Penicillium* were only capable of adhering to the shell and therefore, were classed as having type 1 ovicidal activity. The other five different fungal species, belonging to the genus *Fusarium*, *Lecanicillium*, *Mucor*, *Trichoderma* y *Verticillium*, developed hyphae capable of adhering to the shell of *C. daubneyi* and *P. equorum* eggs, breaking into it and destroying the inner embryo; as a result, their ovicidal activity was classed as type 3, as described by (Lýsek and Krajcí 1987).

The different fungal forms identified and isolated from the faeces of animals kept in captivity are frequently described in the soil (Siddiqui and Mahmood 1996; Vargas Gil et al. 2009), but there is no sufficient information in respect of their effect on parasites found on that very same soil and that could potentially affect animals and human beings.

Some studies report that *Gliocladium* and *Penicillium* could be used in the control of plant pathogens such as *Rhizoctonia solani*, *Phytium ultimum* and *Meloydogine incognita* (McQuilken et al. 2001; Siddiqui and Futai 2009). Evidence shows that there is an obvious antagonic activity of the fungal species *Fusarium* and *Trichoderma* against *Toxocara canis* eggs (Ciarmela, M. L. et al. 2010), and of Pochonia *chlamydosporia* against trematodes eggs (*Echinostoma paraensei*, *Fasciola hepatica*) and ascarids (*T. canis, P. equorum*) (Lelis et al. 2014; Hiura et al. 2015).

The transmission of many parasites may take place at ground level, partly because the exogenous phase of their life cycle occurs on the soil. Hence the importance of having the number of parasitic infective phases under control, specially when animals are always kept and maintained in the same plots of land, where a continuous elimination of parasites eggs occurs (Arias et al. 2013a). Different actions have been proposed in order to minimise the risk of livestock infection, such as the periodic manual removal and collection of faeces, pastures rotation or the shifting of livestock species (Colvin et al. 2008). Nevertheless, these measures may not be applied on certain regimes, such as for instance, zoological parks. As a consequence, there is an increase in parasitic infections that results in a more frequent application of conventional treatments and a possible stimulation of a selection of multi-drug resistant parasite strain (Geurden et al. 2014).

Those parasites in the soil are exposed to many organisms such as bacteria, viruses, fungi, predatory nematodes or mites; some of them are capable of parasitising and destroying those parasites (Fritzen et al. 2010). These beneficial organisms, named *biological agents*, must be highly antagonistic and selective (they must be have activity against parasites but not against any other organisms, plants or animals).

They should also have the ability to grow in culture means under adequate pH and temperature conditions (Geurden et al. 2014). In our case study, five of those isolated fungi grew in corn meal agar plates and they also reported a type 3 ovicidal activity against *C. daubneyi* and *P. equorum* eggs (Lýsek and Krajcí 1987). It should be noted that no pathogenic effects were reported in sheep, bovines or wild or domestic equines after the administration of M. *circinelloides*, *D. flagrans* or *Pochonia chlamydosporia* spores, nor in human beings who worked in closed contact with these species (Ojeda-Robertos et al. 2008a; Silva et al. 2014).

Some parasites stages present in the soil may affect human beings. For instance, ascarids such as *Toxocara canis*, *Ascaris suum*, *Toxascaris leonina* or *Baylisascaris procyonis* and tricurids, are transmitted to humans via an accidental ingestion of eggs containing infective larvae (De Souza Maia Filho et al. 2013); ancylostomatidaea larvae may break the skin and affect people whilst enjoying leisure time in sandy areas or parks (Criado et al. 2012).

The presence of fungi in faeces indicates that they can survive passage through the gastrointestinal tract; furthermore, the evidence of a type 3 ovicidal effect in three of five isolated fungi also demonstrates that their biological effect against helminths is preserved, thus confirming their potential against other soil transmitted pathogens. This fact can be seen as an interesting contribution, since most of the fungi identified in livestock faeces are species with a larvicide only activity (Mendoza-De Gives et al. 2006; Kelly et al. 2009). The possible fungal ovicidal effect against strongylid nematodes eggs is still unknown since different seasons or climate differences may affect the development of mobile stages (larvae) which could end up being shorter than the period of time needed for fungi to develop their antagonist activity.

Almost 100% of the equine global population are infected by strongylid nematodes. These nematodes eggs hatching process and the subsequent development from L1 to L3 (infective stage) require soil moisture levels of over 15-20%, and this transformation would complete in 3 days at 25-35°C, in 15-24 days at 10°C, o in several weeks at temperatures under 10°C (Mfitilodze and Hutchinson 1987). The eggs of these gastrointestinal nematodes may remain viable under

6°C, although hatching shall not take place (Smith 2015)). In some areas in the province of Lugo (Spain), mean temperatures throughout the year are typically over 10°C, except between december and march, when risk of frost is detected. The observation of high percentages of relative humidity (81%), along with notable precipitation (except during the summer time) and a negative hydria balance towards the end of spring and beginning of summer, indicate that the soil humidity allows the growing of pastures almost throughout the whole year and offers an adequate environment for the development and survival of free-living stages of strongylids almost all year round. Even though these nematodes eggs and larvae are capable of surviving inside faeces and soil (even during cold time) with high levels of humidity and rain, L3 tend to remain inside faecal matter, not migrating towards contiguous grass (Kuzmina et al. 2006). As a result, prevention of the strongylids infection could be based upon acting at faecal level, via oral administration, for example, of fungal elements (spores, mycelium), that is, as *food additives*, in such way that pathogens and antagonists would be in direct contact putting a halt to the development of parasites into infective stages.

Numerous studies have resorted to solid cultures based on bacteriological agar (Braga et al. 2010a) to obtain high concentrations of fungal spores (Saumell et al. 2016) that are subsequently administered by means of water based solutions, handmade pelleted feeds or mineral blocks (Mendoza-De Gives et al. 2006; Aguilar et al. 2008; Saumell et al. 2016). All of these are valid options, but they entail extra tasks for farmers or animal carers, which could potentially have an impact on the final use.

The discovery of the correct development and growth of *Mucor circinelloides* and *Duddingtonia flagrans* in liquid means (Arias et al. 2013b) (COPFr) made it possible to disperse the fungi as food additives and administer them either in a premix before the livestock daily feed (Arias *et al*., 2013) or in industrial pelleted feed with added parasitice fungal spores (Hernández et al. 2016).

In this present study, a group of strongylids infected horses maintained in a rotational pasture system receive an effective pour on ivermectin based treatment (*Faecal Egg Count Reduction*, FECR: 99%). They were also fed an industrial pelleted feed with added parasiticidal fungal spores from M. *circinelloides* and *D. flagrans*, reporting an extended *Egg reappearance period* (ERP) of 1,5 months in comparison to another group of horses which received identical treatment but were fed an industrial pelleted feed without any added parasiticidal fungal spores. The period of time that lapsed before all horses started to eliminate strongylid eggs in their faeces got extended for a further three months. Reportedly, only one horse fed with pelleted feed with spores eliminated over 300 EPG, and no adverse effects were detected. These results support previous findings in a similar assay studying horses maintained in a continuous grazing system, which reported an ERP of 7 months and all the equines excreted stronglylids eggs 15 month after deworming treatments (Hernández et al. 2016).

With the intention of maintaining horses free from strongylid L3 highly contaminated pastures, rotational grazing is advised to lower L3 soil levels, noting that an infected area could return to safe levels after a period of 3-6 months (Kumar et al. 2013).The fact that the total egg counts in horses fed with regular feed pellets were 7% lower when reintroduced to the same paddock after a 4,5 rest period, seems to confirm that rotational grazing lower pastures contamination levels and as a result, the infection is either slowed down or delayed. Nevertheless, those animals managed in continuous or rotational pastures which were fed with pelleted feed without spores, ended up getting re-infected and at the end of the study (twelve months post-treatment) and similar egg elimination levels were registered. Five months post treatment, over 300 EPG were registered, indicating the need for a new deworming treatment after three rotations. A possible solution to limit the number of strongylids in the paddocks, could be to shorten grazing times to periods under 1,5 months (Colvin et al. 2008) and increase the number of paddocks; however, these measures are difficult to implement due to animal handling issues and the availability of plots of land.

At the end of the rotational grazing period (6 first months), elimination levels in horses fed with pelleted feed with fungal spores decreased by 50%, in comparison to those that received food without spores. Once the animals returned to previously grazed paddocks and the rotation was complete, a reduction in EPG levels of 59% was observed. Previous studies about horses maintained in continuous grazing showed reduction in their levels of strongylid eggs eliminated in faeces of some 35-73%, although in these cases they were fed a weekly pelleted feed ration with mycelium of *D. flagrans* for six months (Hernández et al. 2016); the administration of daily rations of pelleted feed with chlamydospores of *D. flagrans* was related to significant total egg count reductions of 78% after 6 months and 67% after 12 months (Lester et al. 2013).

Amongst those S*oil-Transmitted Helminth* (STHs), *Toxocara canis*, *Trichuris vulpis* y *Ancylostoma caninum* can affect domestic, wild carnivores and human beings. Infection occurs mainly due to exposure to parasite stages (ingestion of eggs or larvae, or percutaneous entrance of L3 of *A. caninum*) that became infective after a variable period of time in the soil. Human beings may accidentally get infected with theses helminths, reinforcing the zoonotic risk associated with animal stools.

Various measures have been recommended to achieve a reduction in parasites environmental concentrations, consisting in improving education of pet owners, hygiene, manual stools collection, and the avoidance of animal defecation in public areas or the restriction of pets in public places (Traversa 2012). Some others, such as to cover sandy spaces in parks with canvas sheets to stop pets from defecating in these areas, are only viable in small places, as it occurs with the regular collection of faeces (Vanhee et al. 2015). The pouring of chemicals or hot water directly over the stools with the intention of destroying STHs eggs such as *Baylisascaris procyonis*, which affects racoons, coatis and even people (Blizzard 2010), was also proposed; but these strategies are difficult to implement and may harm the environment.

Transmission of STHs frequently occurs between pregnant mothers and their offspring infected adult females eliminate STHs eggs in the environment where the offspring get infected.

Furthermore, a vertical transmission (lactogenic or transplacental) of the ascarid nematode *T. canis* takes place between bitches and puppies; it is also possible to get transmitted via ingestion of larvae in paratenic hosts (Lee et al. 2010; Schnieder et al. 2011). Yet another different transmission may exist between mothers and puppies, involving somatic larvae that remain silent for many years in different tissues, and are mobilised during pregnancy infecting following litters born already infected by *T. canis* or *A. caninum* (Overgaauw and van Knapen 2013). In this situation, frequent deworming is considered to be the most reasonable option in breeding kennels or kennels in general (Traversa et al. 2014). With the intention of preventing the transmission of helminths from mothers to offspring, bitches can undergo treatment protocols consisting of daily administrations of fenbendazole between day 40 of pregnancy and 14 days postpartum, or macrocyclic lactones between day 40 and 55 of pregnancy (ESCCAP 2017). It has been suggested that the total production of eggs of *T. canis* could be reduced by 29% if 90% of the animals receive therapy four times a year (Nijsse et al. 2015).

In order to limit the survival of STHs in dogs' faeces, the following assay was presented: spores of two different filamentous fungi with antagonistic effect over helminths eggs (*Mucor circinelloides*) and larvae (*Duddingtonia flagrans*) were mixed with dry food before feeding the puppies (premix). The usefulness of this formulation was proved since fungal spores were found in faecal samples one day into the treatment, showing their ability to survive the passage through the gastrointestinal tract (Carvalho et al. 2009). Analysis of the faeces 28 days later, revealed a significant reduction (of at least by 50%) of the viability of *T. canis*, *T. leonina* or *T. vulpis* eggs, thus confirming the fungal ovicidal effect. Previous studies indicated successful results when using spores of *M. circinelloides*, *Trichoderma* spp. or *Verticillium* spp. directly over faeces of dogs and pigs infected with *T. canis* y *Ascaris suum*, respectively (Arias et al. 2013b; Cortiñas et al. 2015). Silva et al. 2010 indicated a reduction of 29,5-94,8% in the viability of *T. vulpis* in Petri dishes. In addition to this, in our current study, the number of infective *A. caninum* L3 was reduced by two thirds in the faeces of those puppies which received fungal spores, highlighting the great nematophagous activity of *D. flagrans* against *A. caninum* larvae (Maciel et al. 2010).

STHs eggs excreted in shady areas with vegetation and humidity, require a variable period of time to develop into infective stages, that is, eggs containing L2 (*T. canis*, *T. leonina*, *T. vulpis*) or L3 (*A. caninum*). It is crucial when to consider the fungi as parasites antagonists, since these organisms must act against parasites before they reach their infective stage. It has been established a period of time of over three weeks for *T. canis*, *T. leonina* and *T. vulpis*, and two or more weeks for L3 of *A. caninum* (Kramer De Mello et al. 2014). In this study, the antagonistic fungal effect was assessed 28 days after the administration of spores to puppies, reporting a significant reduction in the viability of ascarids, trichurids and ancylostomids eggs and no adverse side effects. As a result, we can safely conclude that the addition of spores in the premix may be considered to be a safe, innovative, sustainable and successful means to reduce the risk of soil contamination by STHs such as *T. canis*, *T. leonina*, *T. vulpis* and *A. caninum*.

The inclusion of parasiticidal fungal spores in the premix or through pelleted feed could be classed as *food additives*. The European Union establishes that all products destined to animal feed shall improve the quality of their rations and the products of animal origin, as well as the animals' condition and their health. From this definition we may conclude that the *additive* acts upon the animal that ingest it, and in the case of fungi as biological agents, this is not much so, since the aim is to use the animal as a ***vehicle***, to facilitate the contact between pathogens and antagonists in the faecal matter. The aim is to interrupt the development of parasites in the faeces, and not to act upon the host.

Food additives used in animal nutrition, based on their properties, may be categorised as technological (preservatives, stabilisers, antioxidants…), organoleptic (food colouring, flavouring), nutritional (vitamins, trace elements, amino acids…) and zoo technical (digestives, stabilisers of gastrointestinal flora, substances which improve the environment …). In this classification we can include the use of fungal spores as *food additives*, because there is a clear intention to improve the environment by reducing the risk of parasitic infections.

The spread of fungi in water based solutions can be seen as a different means of dissemination, using sprays to facilitate pulverisation over surfaces, pastures, stables, etc. In this case, the preparations containing spores or mycelium would be classed as ***biocides,*** defined as active *ingredients, compounds (which contain one or more active ingredients) or microorganisms intended to destroy, render harmless, counteract, neutralise o exert a controlling effect on any harmful or nocive organism by chemical or biological means.* Their classification is currently broken down in 23 product types depending on their use, although they may be classified in four large main groups.

The EU regulatory framework for biocides has been defined by the Directive 98/8/EC, of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market, transposed to our Spanish legal system through the Royal Decree 1054/2002 of 11 October 2002, amended by the Order PRE/1982/2007 of 29 June 2007, regulating the assessment process for registration, authorisation and marketing. For obvious reasons, the biocide shall not pose a risk to human beings, animals or the environment.

This use as a biocide could result of huge usefulness to control *Soil-Transmitted Helminths (*STHs) in wild species kept in captivity in zoological parks, since as it was previously mention in relation to pets, these helminths are still considered to be an important issue with complex solution for the presence of viable stages in the soil favour the existence of persistence infections (Arias et al. 2013a; Mazurkiewicz-Zapałowicz et al. 2014). Amongst the STHs, ascarids and trichurids infective stages (eggs containing a L2 or L1, respectively) are capable of surviving for months or even years until they are ingested (Dabrowska et al. 2014; Traversa et al. 2014).

In our study, we proved a decreased viability of *Toxascaris leonina* y *Trichuris* sp. eggs excreted by lynxes and dromedaries kept in captivity in a zoological park when their stools were sprayed with a water based solution containing spores of three filamentous saprophytic fungi (*Mucor circinelloides*, *Verticillium* sp. and *Trichoderma atrobrunneum*). Previous studies carried out in the same park indicated that the viability of *Baylisascaris procyonis* eggs, an ascarid parasite which typically affects racoons, coatis… or even humans, significantly decreased after spraying spores of some filamentous saprophytic fungi (*M. circinelloides*, *Puerperocillium* (*Paecilomyces) lilacinus* and *Verticillium* sp.) on the faeces of captive racoons (Cazapal-Monteiro et al., 2015). Similar findings were reported when spraying spores of *M. circinelloides* over pigs stools infected with *Ascaris suum* eggs (Cortiñas et al. 2015).

Ascarids and trichurids eggs are released unembryonated in the faeces, and the infective stage is not reached until after an important period of time in the soil (Okulewicz et al. 2012). Taking into account the latency in the embryogenesis process as a useful tool to reduce the viability of trichurids eggs and the presence of infective stages in the soil, the potential ovistatic effect of *M. circinelloides* and V*erticillium* sp. on *T. leonina* eggs was studied.

The existence of a larger percentage of non-developed viable eggs (in zygote stage) in presence of fungal spores seems to indicate a delayed evolution into infective stages, as suggested in previous studies in relation to *T. canis* and *A. suum* eggs (Kuźna-Grygiel et al. 2001). In our present study, similar results were obtained when we pulverised spores of *M. circinelloides* and *T. atrobrunneum*.

It should be remarked that applying regular deworming treatments is the only effective measure against animal parasites in zoological parks, and preventive measures are seldom in place. Some recommendations to limit the risk of infection by STHs are the manual recollection and appropriate disposal of pet stools, as well as the application of regular deworming treatments (Arias et al. 2013b; Traversa et al. 2014). Even though stools are removed on a daily basis from the different paddocks in the zoological park, before visitors start to arrive in the morning and sometimes even once or twice during visiting hours, the existence of a persistent infection means that parasites eggs in the soil develop into infective stages. As it has been previously mentioned, the direct flaming of faeces and ground with torches has been proposed as a measure to prevent infections caused by *B. procyonis* (Page et al. 2011); however, this seems to be not only a difficult measure to apply when dealing with captive animals, but also highly dangerous. Rotational grazing is also advisable to limit livestock parasites infections (Relf et al. 2013), but again, it is certainly not an easy solution for zoological parks (Hernández et al. 2017).

In our case study, the introduction of filamentous fungal spores into faeces containing *T. leonina* or *Trichuris* sp. eggs resulted in a reduction of their viability by ≥50%, and the egg development was delayed by over a third. These very interesting results appear to confirm that some filamentous saprophytic fungi, typically found in the soil, may become parasites eggs predators, even under wet conditions (Arroyo et al. 2017).

It's been demonstrated that *Verticillium* sp. and *M. circinelloides*, in contact with the shell of some parasites eggs, are able to develop hyphae capable of penetrating into the egg and destroying the embryo (Lýsek and Stěrba 1991; Arias et al. 2013b). On the contrary, no sing of shells destruction or mycelium penetration has been reported in *Ascaris suum* eggs when they were exposed to *Paecilomyces frequentans*, nor in *Toxocara canis* eggs in contact with *P. fumosoroseus* or *T. viride*; as a consequence it has been considered the possible release of metabolites as responsible for the morphological damage in the eggs and the delay in the embryos development (Mazurkiewicz-Zapałowicz et al. 2014). An outstanding finding in our investigation was the observation of a large percentage of *T. leonina* eggs with broken shells, as well as a high number of L2 outside of the eggs, which were therefore rendered unviable and would survive little time on the environment.

A possible explanation to these findings could be that, in presence of fungi in the soil, parasites egg shells weaken and rupture, making it possible for L2 to be released, unviable an incapable of surviving in the medium. Although Silva et al. 2010 indicated that

Pochonia chlamydosporia hyphae were capable of penetrating through *Trichuris trichiura* egg shells, in the course of our investigation, the main structural alterations observed in *Trichuris* sp. eggs were the result of the effect of *M. circinelloides* and *T. atrobrunneum* hyphae by eliminating polar plug, and the subsequent penetration into the eggs.

Fungi are frequently used as biological agents in the control of plant diseases and plagues: however, the number of studies involving the use of fungi in the control of animal parasites is not so large, especially in the faecal natural environments. Quite limited is also the information on biological control of parasites in captive animals. The administration of industrial pellet food containing spores of *D. flagrans* to equines maintained in captivity in zoological parks proved a significant reduction in strongylidae infections (Arias et al. 2013b). Different studies involving livestock animals also reported successful results when orally administering spores of *P. chlamydosporia*, *M. circinelloides*, *D. flagrans* or *Monacrosporium thaumasium* in water based solutions mixed with industrial feed or even industrial pelleted feed (Braga et al. 2010b; Araujo et al. 2012; Arroyo et al. 2016). Special importance should be placed on the fact that no adverse side effects were reported in relation to respiratory, gastrointestinal, reproductive systems or at skin level, in wild or domestic animals kept in captivity that were administered spores of *M. circinelloides* y *D. flagrans* (Arias et al. 2013b; Hernández et al. 2016). The biological control of STHs seems very useful in zoological parks due to the possibility of halving the presence of infective stages of *T. leonina* y *Trichuris* sp., as well as the delay of their development by over a third, in the case of captive lynxes and dromedaries.

An interesting question would be to determine which is the appropriate manner to guarantee a uniform spread of fungi in the faeces.

Due to the fact that fungal spores were obtained, in our case study, in submerged cultures, the direct spraying of spores over faeces happened to be very useful, especially when animals are kept in small or medium sized plots of land.

Some ectoparasites develop part of their life cycle in the soil.

After mating in the host, female ticks feed and fall to the ground where they release thousands of eggs, particularly in vegetated areas, protected from direct sun and desiccation. After a variable period of time, depending on temperature and humidity conditions, the eggs hatch releasing the larvae, which move towards different plants awaiting for new hosts to pass nearby, adhere to them, feed on their blood, transform into nymphs and eventually, becoming adult ticks.

There is little information regarding the effect of parasiticidal fungi on soil ticks eggs. *Beauveria bassiana*, and especially *Metarhizium anisopliae*, are two studied entomopathogenic species with the ability to infect and damage ticks ,as it has been reported in numerous assays which consisted in the topical administration of oil based solutions directed at adult ticks or immature stages with the intention of reducing the indiscriminate use of chemical acaricides, thus avoiding contamination of food and environment as well as the existence of chemical resistances (Camargo et al. 2012, 2016; Aw and Hue 2017; Prado-Rebolledo et al. 2017).

The reported decreased viability of *Rhipicephalus boophilus* eggs by 38% and decreased hatching levels by 15%, highlights the usefulness of *M. circinelloides* spores to perform control of those endo and ectoparasites which present life cycle stages in the soil.

In view of the obtained results, it is possible to state that water based solutions containing fungal spores could be sprayed in order to limit the risk of infection and infestation levels in open spaces such as footpaths, gardens or even farms.

The innocuity of parasiticidal fungi is a controversial aspect. Even though most fungi are considered pathogenic and responsible for several diseases, ranging from moderate to severe or fatal, mycoses are an important element to take into account when dealing with immunocompromised individuals.

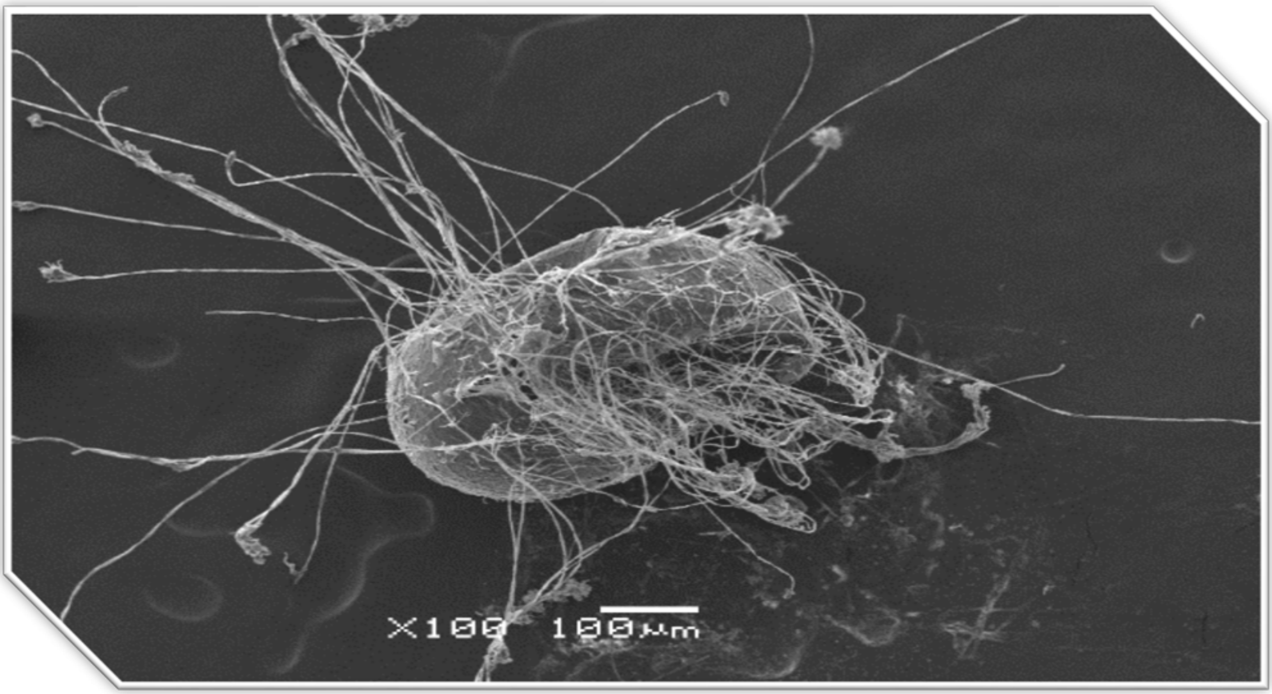
Infection by *Mucor circinelloides* has been linked to **mucormycosis** clinical cases, an sporadic infection but potentially fatal, caused by fungi belonging to the order Mucorales, and in particular, caused by *Rhizopus* spp. (Sephton-Clark et al. 2018).

These cosmopolitan fungi are particularly found in surfaces and wooden soils, where they can be easily isolated. Numerous reports indicated the existence of nosocomial infections by *M. circinelloides* in immunocompromised people, patients with skin wounds or suffering from diabetes mellitus (Garcia-Hermoso et al. 2018). In animals, an infection by *M. circinelloides* was diagnosed in a Vietnamese pig presenting pneumonia clinical signs, but no further information was offered regarded to its habitat (Evans et al. 2018). On the contrary, there are long term studies based on the frequent administration (daily or twice a week) of a mixture of spores of *M. circinelloides* and *D. flagrans* in grazing horses which did not report the existence of adverse side effects related to damage to the respiratory, gastrointestinal, reproductive system or at skin level (Hernández et al. 2016).

Up to this day, no issues have been reported in people who participated in the handling, manufacture or administration of spores/mycelium for over ten years.

The future looks bright, since *M. circinelloides* offers other advantages such as the ability to transform fungal oils into esters, which is a sustainable way of obtaining bio fuels (Carvalho et al. 2015). Over the last years, there has been an increased interest in microbial oils, for they are currently being used as commercial sources of various polyunsaturated fatty acids with nutritional importance (Tang et al. 2015).

11. CONCLUSIONS



11. CONCLUSIONS

Results obtained in the current study led us to CONCLUDE THAT:

1.- Samples of feces of animal species offer a useful source of fungi with parasiticide activity, innoucuous to humans, plants and animals, and capable to survive the digestive tract.

2.- Formulation of spores of *Mucor circinelloides* as a food additive in pelleted feed provides a very beneficial and practical tool to ensure the distribution of this parasiticide fungus.

3.- The possibility of culturing the filamentous fungus *M. circinelloides* offers a helpful way to the distribution of spores as a biocide directly on the ground, which results very interesting to avoid infections by soil-transmitted parasites.

4.- The fungus *M. circinelloides* can be used in biological control strategies to prevent infestation by ticks, due to their ability to penetrate the eggshell and destroy the inner structures.

5.- Two different formulations seem interesting from a business perspective, water solution as biocide to spread on the soil, and pelleted feed as food additive to nourish animals.

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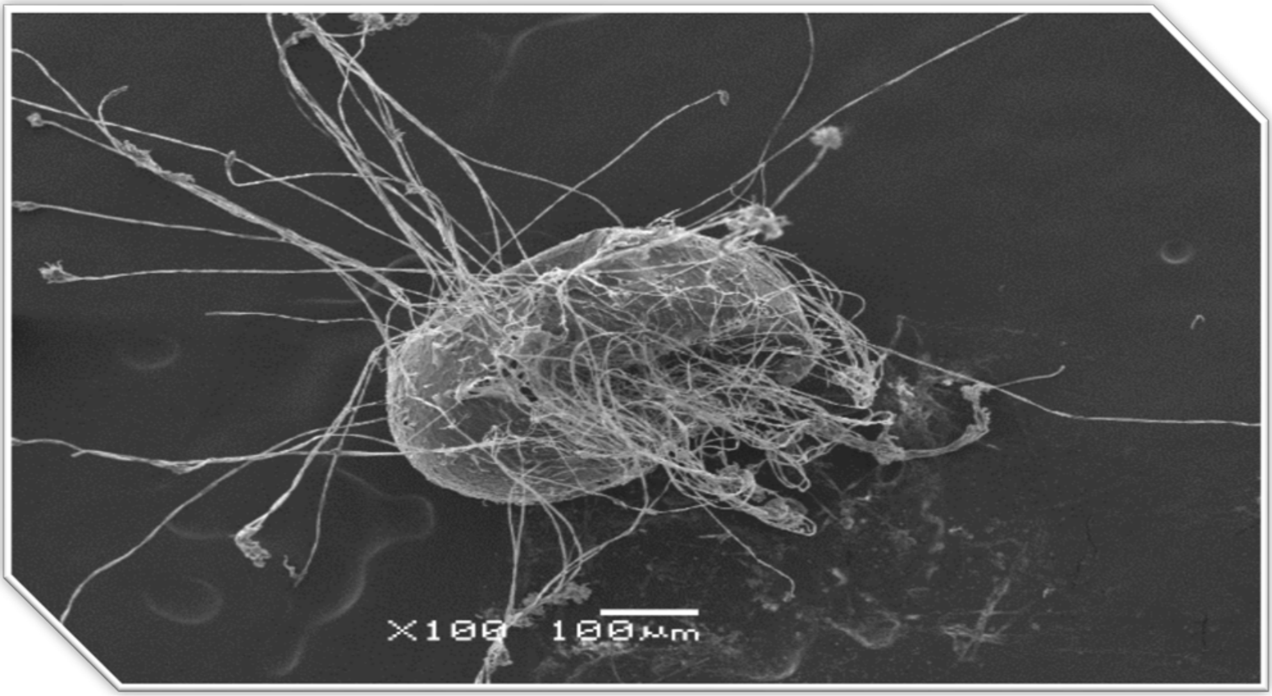

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