UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

NEW ADMINISTRATION FORMULA OF PARASITICIDE FUNGI SPORES TO PREVENT INFECTION BY GASTROINTESTINAL NEMATODES IN PASTURING HORSES

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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“Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth.”

Jules Verne in *Journey to the Centre of the Earth*

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RESUMO

NOVA FÓRMULA DE ADMINISTRAÇÃO DE FUNGOS PARASITICIDAS PARA PREVENIR INFEÇÃO POR NEMÁTODES GASTROINTESTINAIS EM CAVALOS DE PASTOREIO (LUGO, ESPANHA)

A resistência a anti-helmínticos em cavalos tem vindo a aumentar recentemente e a procura por métodos de controlo alternativos levou ao desenvolvimento de abordagens complementares como o controlo biológico. Esta abordagem usa fungos parasiticidas, como *Duddingtonia flagrans* e *Mucor circinelloides*, no controlo da população parasitária e estudos recentes têm-se focado no desenvolvimento de novos métodos de administração. Seguindo esta tendência, uma fórmula nova e alternativa foi desenvolvida utilizando um produto liofilizado que contém esporos de *D. flagrans* e *M. circinelloides* para o controlo de nematodes gastrointestinais em cavalos.

Após fabrico do produto e verificação da morfologia normal dos esporos, estes foram testados para crescimento *in vitro*. Um total de 20 placas de Petri foram semeadas com uma mistura de 0.1 g de produto e 0.5 ml de água em meio sólido. As placas foram mantidas a 25ºC em escuridão total e todas demonstraram desenvolvimento de novos esporos passados 10 dias.

Após a verificação *in vitro*, o produto foi administrado *per os* a cavalos para observar o seu efeito nas contagens fecais de ovos (CFO) por grama (OPG). Assim, um grupo de 5 cavalos em pastoreio foi escolhido para receber 10 g de produto cada (com esporos de *M. circinelloides* e um total de cerca 10^5 clamidosporos de *D. flagrans* por cavalo) 3 vezes por semana, de setembro a março. Outro grupo de 7 cavalos numa pastagem adjacente foi utilizado como controlo. Após tratamento com unção contínua de Ivermectina em setembro de 2017, uma amostra fecal de cada cavalo foi colhida mensalmente e o CFO foi avaliado utilizando a técnica de McMaster modificado. Apenas ovos de nemátodes gastrointestinais, nomeadamente estrongilídeos, foram observados com esta técnica. A média de OPG de cada grupo foi comparada para cada mês e no total do estudo para observar o efeito de redução do tratamento fúngico. Diferenças estatisticamente significativas entre os dois grupos foram observadas em fevereiro, redução de 72%, março, redução de 64%, e no total, 66% de redução. Cavalos no grupo de teste só passaram o limiar de 300 OPG dois meses depois dos cavalos do grupo controlo. Em novembro e janeiro foram realizadas culturas fecais em todas as amostras, demonstrando apenas a existência de larvas de ciatostomíneos.

Este estudo permitiu com sucesso o desenvolvimento de uma nova fórmula para administração oral de fungos parasiticidas para cavalos com base num produto liofilizado, aumentando as futuras possibilidades de desenvolvimento e aplicações de produtos. Novas e aperfeiçoadas formas de controlo biológico devem ser desenvolvidas e implementadas para aumentar o controlo de parasitas e diminuir os casos de resistência a anti-helmínticos.

Palavras-chave: Controlo parasitário, controlo biológico, fungos parasiticidas, *Duddingtonia flagrans*, *Mucor circinelloides*, produto liofilizado, estrongilídeos, cavalos.

ABSTRACT

NEW ADMINISTRATION FORMULA OF PARASITICIDE FUNGI SPORES TO PREVENT INFECTION BY GASTROINTESTINAL NEMATODES IN PASTURING HORSES (LUGO, SPAIN)

Anthelmintic resistance in horses has increased in recent years and the continuous search for alternative control methods has led to the development of complementary approaches such as biological control. This approach can make use of parasiticide fungi, such as *Duddingtonia flagrans* and *Mucor circinelloides*, in parasite population control and recent research has been focused on the development of new administration methods. Following this line of research, a new and alternative formula has been developed by using a lyophilized product that contained both *D. flagrans* and *M. circinelloides* spores for the control of gastrointestinal nematodes in horses.

After the product manufacture and the normal spore morphology were assessed, these were tested for *in vitro* growth. A total of 20 Petri dishes were assembled with a mix of 0.1 g of product and 0.5 ml of water in solid media. The assembled plaques were kept at 25ºC in total darkness and all showed the development of new fungi spores after 10 days.

Following the *in vitro* assessment, the product was administered *per os* to horses in order to observe their effect in the faecal egg count (FEC) of eggs per gram (EPG). Thus, one group of 5 horses in a pasture was chosen to receive 10 g of product (with *M. circinelloides* spores and a total of around 10^5 *D. flagrans* chlamydospores per horse), 3 days a week starting in September, and another group of 7 horses in an adjacent pasture remained as control. Following treatment with Ivermectin pour-on in September 2017, a faecal sample was collected from each horse on a monthly basis and FEC was assessed using a Modified McMaster technique. Only gastrointestinal nematode eggs, namely strongyle eggs, were observed with this technique. The EPG average from each group was compared for each individual month and overall to see the reduction effect achieved with the fungi treatment. Statistically significant differences were found between the two groups in February (72% reduction), March (64% reduction), and overall, 66% reduction. The horses in the test group only reached a cut-off value of 300 EPG two months after the horses in the control group. In November and January, faecal culture method was applied to all faecal samples, showing only the presence of cyathostomin larvae.

This study allowed the successful development of a new formula for the administration of parasiticide fungi to horses, based on lyophilized product, which increases the possibilities for future product development and application. New and improved ways of biological control should be developed and implemented to increase parasite control and reduce anthelmintic resistance cases.

Keywords: Parasite control, biological control, parasiticide fungi, *Duddingtonia flagrans*, *Mucor circinelloides*, lyophilized product, strongyles, horses.

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LIST OF ABBREVIATIONS AND SYMBOLS

AAC – Water, Agar, Chloramphenicol

ATC – Wheat, Agar, Chloramphenicol

CFO – Contagens fecais de ovos

COPAR – Research group for the control of parasites affecting animals and humans: detection, prevention and treatment

CPG – Chlamydospores per gram

ELISA - Enzyme-Linked Immunosorbent Assay

EPG – Eggs per gram

ERP – Egg reappearance period

FEC – Faecal egg count

FECRT – Faecal egg count reduction test

FVM-USC – Faculty of Veterinary Medicine, University of Santiago de Compostela

i.e. – *id est* (that is)

L1 – First stage larvae

L2 – Second stage larvae

L3 – Third stage larvae

L4 – Fourth stage larvae

L5 – Fifth stage larvae

LL3 – Late third stage larvae

NaCl – Sodium chloride

OPG – Ovos por grama

rpm – Rotations per minute

SD – Standard deviation

CHAPTER 1 – INTRODUCTION AND RESEARCH AIMS

Alternative and complementary approaches in parasite control have been developed and researched due to the increasing anthelmintic resistance and rise of new environmental concerns in the last decades. Of those approaches, biological control using parasiticide fungi has seen a big development for the past 30 years, with special regards to the discovery of new species and distribution methods.

Biological control is a pest control method through the use of biological antagonists. When applied to horse parasite control, it makes use of parasiticide fungi that affect either larvae or eggs in their free-range forms (Larsen, 2000). It is already used for the control of Nematodes and Trematodes that affect domestic animals, namely cattle, horses and small ruminants.

There is not a single species of fungi or distribution method ideal for all the situations and environments. Every scenario should be assessed individually to choose the adequate way to apply the most appropriate parasiticide fungi.

The major goal of this dissertation was to develop a new viable and experimental formula for oral administration of parasiticide fungi for the control of gastrointestinal nematodes in horses. This can be divided into 3 specific aims:

1. To develop a lyophilized product with viable *Duddingtonia flagrans* chlamydospores and *Mucor circinelloides* spores.
2. To assess fungi *in vitro* growth after product manufacture.
3. To assess fungi predatory activity against gastrointestinal nematodes after being fed to pasturing horses.

1.1 INTERNSHIP WITH COPAR RESEARCH GROUP.

Between September 13th and December 15th, the trainee did an internship at the Parasitic Diseases Laboratory of the Faculty of Veterinary Medicine, University of Santiago de Compostela (Campus of Lugo) (FVM-USC) (Spain). With a total of 500 hours, this internship was supervised by Prof. Doctor Adolfo Paz-Silva, coordinator of COPAR Research Group, a group from the Animal Pathology Department, FMV-USC, and embodied in the research project "*Formulating spores of parasiticide fungi in edible gelatines for the prevention of soil-transmitted helminthezoonoses*".

The activity of the COPAR Research Group, which stands for "Control of parasites affecting animals and humans: detection, prevention and treatment", focuses on the biological control of parasitic zoonoses, diseases shared by animals and humans, through the use of soil innocuous fungi.
While in the internship, the trainee engaged in all the activities in which COPAR takes part and collaborates. Starting with the diagnostic of parasitic diseases, collaborated collecting blood and faecal samples from dogs and horses that took part in research projects for further analyses. Blood samples were mostly used for the research of *Babesia* spp. and *Theileria* spp., using a blood smear technique, or antibodies agaisnt *Dicrocelium* sp. and *Fasciola* sp. by means of serological techniques as *ELISA*. Faecal samples sent to the laboratory were analysed using faecal egg count (FEC) techniques, such as modified McMaster technique (used as both a quantitative and qualitative method) and sedimentation, faecal culture and modified Baerman technique. These tests were used to identify a large array of parasites in very different hosts, such as baboons, pigs, lynxes, bison, and many more. Following the diagnosis, he helped in the treatment of different parasitic diseases, resorting to pharmacological treatment and other approaches such as biological control.

Besides all the work that dealt directly with parasites, the trainee was inducted to help with the biological control research held at COPAR, specifically dealing with parasiticide fungi. With the intention of developing a research on the subject of parasiticide fungi (mainly with *Duddingtonia flagrans* and *Mucor circinelloides*), he got a specific theoretical training on how they develop, act and how they should be handled at a laboratory level. During the remaining of the internship, he was responsible for the continuous development of these fungi in the laboratory which has several steps: isolation of pure fungi culture from already prepared solid cultures; preparation of solid and submerged culture mediums; assemblage of solid and submerged fungi cultures using the previous media and isolated fungi culture; counting of fungal spores per ml in submerged media (between coverslip and glass slide for *D. flagrans* and in a Neubauer chamber for *M. circinelloides*) for further usage in product manufacture;

assessment of fungi activity against parasite eggs and larval stages, either in faecal cultures or in solid media with previously added larvae and eggs.

Furthermore, there were visits to some institutions that currently work with COPAR such as the Zoological Park Marcelle Natureza (Outeiro de Rei, Lugo) (developing research among captive wild animals), Galician Horse Breed Associations (Santiago de Compostela) (Puraga, Centro de Referencia e Mostra do Cabalo de Pura Raza Galega and Granja Gayoso Castro) and “Scooby” (Medina del Campo, Valladolid), an animal sanctuary and shelter. These visits allowed to have a glance at which species COPAR works with and how the theoretical approaches are applied in real life situations.

1.2 INTERNSHIP IN LABORATORY OF PARASITOLOGY AND PARASITIC DISEASES OF FMV-ULISBOA

The trainee continued his work in the Laboratory of Parasitology and Parasitic Diseases of FMV-ULisboa where it learned about larvae identification and observed both larvae parasiticide fungi present in faecal samples. This knowledge was then applied in the present work (described further below). Besides this specific training for the present work, the trainee enroled in the daily activities in the laboratory from December to January and March to May, helping with diagnostic tests such as the ones already mentioned in the previous chapter and other students’ works with the fungi.

CHAPTER 2 – BIBLIOGRAPHIC REVIEW

2.1 IMPORTANT GASTROINTESTINAL NEMATODES OF HORSES

A parasite is an organism that lives in or on a larger organism and can only survive and prosper due to the expense of this host. Since the early Egyptian medical records that there are reports of what are, almost certainly, parasitic infections in humans (Cox, 2002). The interaction between parasite and host can be somewhat insignificant, without any visible changes in the host biology and quality of life but can also reach the extreme of host death. The parasite genus, density and lesion it inflicts, in interaction with the nourishment and development state of the host, will ultimately decide what kind of interaction ends up occurring. Helminths or worms are a group of animals that includes parasites, affecting not only animals but also humans and plants, and free-range non-parasite organisms essential for the biology of an ecosystem (Bowman, 2014). Helminths are divided into two major phyla, Nematoda and Platyhelminthes, and a minor phylum, Acanthocephala, being the former two the most relevant in equine medicine (Sallé & Cabaret, 2015; Taylor, Coop, & Wall, 2016).

Nematodes are helminths belonging to phylum Nematoda, commonly called roundworms due to their cross-section appearance. The study of the transmission and development of parasitic nematodes began in the middle of the 19th century with *Trichinella spiralis*. The class Secernentea is the one that includes most of the important parasitic nematodes in veterinary medicine. Nematodes are relatively similar between each other, with worm-like appearance and covered by a cuticle, and at least 16,000 species have been described (Anderson, 2000). In the Nematoda, there is sexual dimorphism and it allows, most of the time, to distinguish between genera using characteristics from either male or female reproductive tracts (Taylor et al., 2016).

2.1.1 FAMILY STRONGYLIDAE

The family Strongylidae belongs to the superfamily Strongyloidea, which includes several other families. Strongylidae contains the most commonly found parasites in horses using FEC techniques (American Association of Equine Practitioners [AAEP], 2013). Most of the adult nematodes in this family are found in the mucosal surface of the intestine while the larvae can have different development routes inside the host depending on the species (Taylor et al., 2016). There are two important subfamilies concerning infection in horses, the Cyathostominae and Strongylinae, usually called equine or horse strongyles (Anderson, 2000; Sallé & Cabaret, 2015).

In the 1960s and 1970s, the Strongylinae (large strongyles) had the most important parasitic species regarding equine medicine, namely *Strongylus vulgaris*, and the Cyathostominae, small strongyles or cyathostomins, were not considered as important. This situation as reversed due to the big control over *Strongylus vulgaris* and the appearance of widely spread anthelmintic resistance in cyathostomins (AAEP, 2013; Love, Murphy, & Mellor, 1999). The exogenous free-living stages, including larvae and eggs of the Strongylidae family, are extremely resilient in the environment, withstanding a wide range of temperatures, from freezing to above 40ºC, surviving up to months within the faecal matter (Nielsen, Kaplan, Thamsborg, Monrad, & Olsen, 2007).

2.1.1.1 CYATHOSTOMINAE

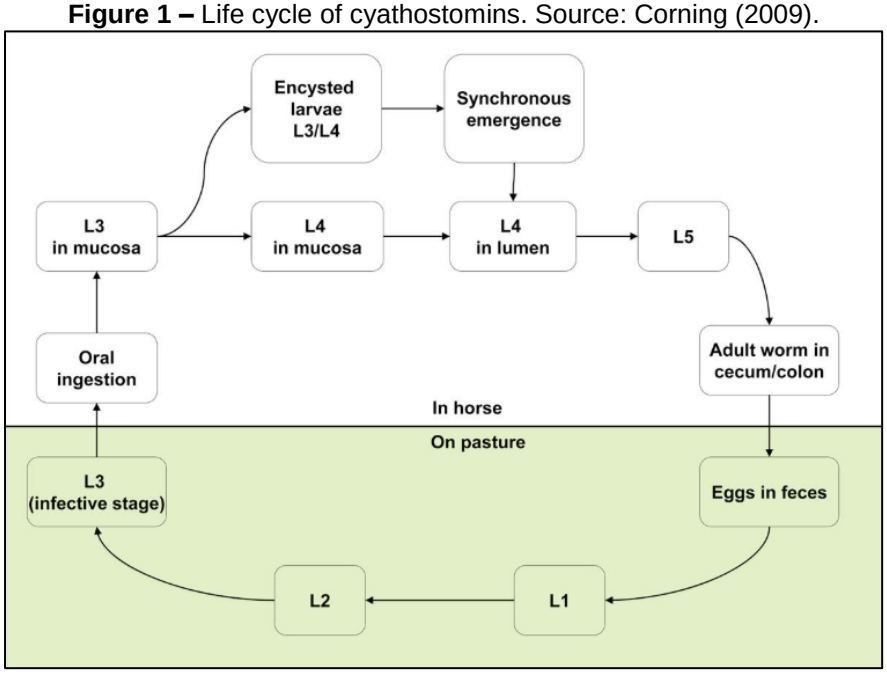
Cyathostomins group contains around 50 species spreading over several genera, four of them considered the most important, *i.e. Cyathostomum, Cylicocyclus, Cylicodonthophorus,* and *Cylicostephanus* (Taylor et al., 2016). From these genera, the species *Cylicostephanus longibursatus*, *Cylicocyclus nassatus*, and *Cyathostomum catinatum* represent 70 and 80% of the total cyathostomins population in horses (Kaplan, 2002). The subclass Cyathostominae is considered nowadays the most important and frequently reported strongyle group in horses. This is due to their increased importance in cases of anthelmintic resistance, the susceptibility of large strongyles to macrocyclic lactones which led to their eradication in well-controlled farms. Other nematode parasites (such as *Parascaris equorum*) affect almost exclusively foals, and the remaining (such as pinworms), are not as pathogenic as cyathostomins (Corning, 2009; Kaplan, 2002; Reinemeyer & Nielsen, 2013; Sallé & Cabaret, 2015)

2.1.1.1.1 LIFE CYCLE

All cyathostomins have an almost identical direct life cycle (Figure 1). Eggs are shed to the environment with the faeces, embryonate and the first larval stages (L1) hatch in as little as 4 days. Under optimal temperature (25-33ªC) and humidity (57-63%), L1 develops to the second stage larvae (L2) and, ultimately, into third stage larvae (L3), the infective stage (Nielsen et al., 2007; Taylor et al., 2016). The L3 larvae start to migrate from the faeces to the surrounding pasture and are ingested by horses while grazing. After ingestion, the L3 migrate to the large intestine where they invade the mucosa and become “encysted”, entering a hypobiotic state (Corning, 2009). This encysted L3 can remain within the intestinal wall for up to 2 years and no chemical treatment is 100% effective against them (Monahan, Chapman, Taylor, French, & Klei, 1996; Reinemeyer & Nielsen, 2013). Eventually, these L3s develop into late L3s (LL3) and then to fourth stage larvae (L4). The later are released from the cyst, enter the lumen of the large intestine and become into fifth stage larvae (L5), which finally mature to adult worms.

These adults are usually found in different places in the large intestine and cecum, depending on the species, and females start to shed eggs as soon as 5 weeks after infection (Reinemeyer & Nielsen, 2013).

Figure 1 – Life cycle of cyathostomins. Source: Corning (2009).



2.1.1.1.2 PATHOGENICITY AND CLINICAL SIGNS

A large cyathostomin burden is not an indication that an animal is going to develop clinical signs of disease, and there are reports that showed horses harbouring thousands of cyathostomes without any detectable illness (Love et al., 1999). The more severe cases of disease caused by cyathostomin infections occur in late winter/early spring and with a big burden of encysted L4, where there is a massive and simultaneous emergence of these larvae from the cysts to the intestine. This excystment can lead to severe cases of larval cyathostominosis, which is a clinical syndrome with performance decline, weight loss, diarrhoea, anaemia, dehydration, ventral oedema and death, in up to 50% of the cases, due to inflammatory enteropathy (Bowman, 2014; Love et al., 1999). Encystment of L3s can produce similar clinical signs despite not being as severe as the excystment, and adult cyathostomins are relatively harmless. Although much more common in foals up to 3 years of age, infection with clinical signs can also happen in adults (Reinemeyer & Nielsen, 2013; Taylor et al., 2016).

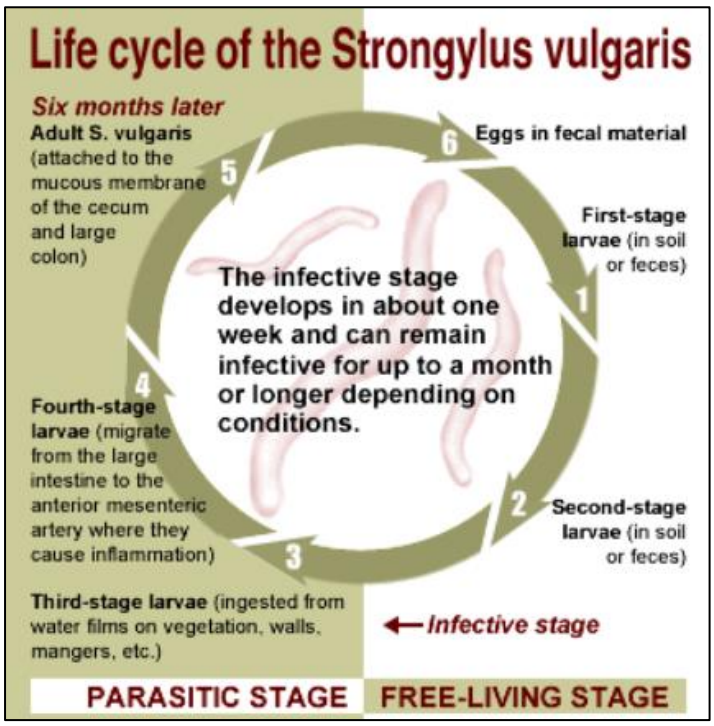
2.1.1.2 STRONGYLINAE

The members of subclass Strongylinae are considered the most pathogenic nematodes that infect horses. The genus *Strongylus* has three different species that are usually considered the most important, *Strongylus edentatus, S. equinus* and *S. vulgaris,* being the last one the most pathogenic (Reinemeyer & Nielsen, 2013). Although they are recently considered less important than cyathostomins due to their lower prevalence, they are still a high priority in horse parasite control (AAEP, 2013; Sallé & Cabaret, 2015). The previously referred three species of the genus *Strongylus* have considerable differences when it comes to their life cycle, larvae migration in the host and their pathogenicity (Bowman, 2014; Reinemeyer & Nielsen, 2013; Taylor et al., 2016). Due to this difference, *S. vulgaris* will serve as a model species since it is considered the most pathogenic.

2.1.1.2.2 *Strongylus vulgaris* LIFE CYCLE

S. vulgaris follows the same kind of development in its free-range lifeforms as cyathostomins, in which it develops into an L1, L2 and L3, the infective stage, in 4 to 20 days, depending on the environment temperature and humidity (Anderson, 2000; McCraw & Slocombe, 1976). The horses ingest the L3 when they graze on the pasture, migrating to the lumen of the small intestine before reaching the cecum and ventral colon (Bowman, 2014). Around 2 days after being swallowed, the larvae penetrate the mucosa and the submucosa where they moult to L4 in the following 2 to 3 days (Figure 2). The L4 migrate to the arterioles of the submucosa, reach the cecal and ventral colic arteries by day 8 and the cranial mesenteric and ileo-ceco-colic arteries by days 11 to 14 (McCraw & Slocombe, 1976). There are reports of larvae that continue migrating and can even attain the aorta near the left ventricle (Cranley & McCullagh, 1981). The larvae stay in the arteries for around 4 months where they moult to L5. These L5 are led back through the bloodstream, over the intima layer of arteria, to the large intestine, where they end up encased in small nodules in the submucosa of the caecum and ventral colon (Reinemeyer & Nielsen, 2013). Subsequently, the nodules rupture and release the L5 into the intestinal lumen, in which they mature to adults after 6 to 8 weeks (McCraw & Slocombe, 1976). The adults start to shed eggs in faeces around 6 months after infection (Round, 1969).

Figure 2 – Life cycle of *S. vulgaris*. Source: http://articles.extension.org/pages/10280/strongyles-in-horses



2.1.1.2.3 PATHOGENICITY AND CLINICAL SIGNS IN HORSES INFECTED BY *Strongylus vulgaris*

Most of the acute clinical signs and health problems caused by *S. vulgaris* infections are caused by the larval stages, as occurs with cyathostomins (McCraw & Slocombe, 1976). Larval migration to the mesenteric and ileo-ceco-colic arteries is the cause of the thrombo-embolic colic syndrome due to the release of previously formed thrombi. These blood clots are a consequence of larval migration damaging the endothelium, leading to inflammation and thickening of the arterial wall (Taylor et al., 2016). The clinical signs associated with these larval migrations are hyperthermia, loss of appetite, painful colic when the thrombus become embolized, abdominal distress, and, occasionally death (McCraw & Slocombe, 1976). An erratic migration of larvae will cause local inflammation as reported by Cranley & McCullagh (1981), where larvae that migrate to the heart can lead to ischaemic myocardial fibrosis. Adults are usually not pathogenic but when in high numbers, they may cause a high amount of local inflammation and ulceration due to the morphology of their buccal capsules and feeding habits, leading to anaemia, emaciation, poor coat and poor performance (Bowman, 2014; McCraw & Slocombe, 1976). Rupture of nodules can cause haemorrhages, even if not as problematic and frequent as with cyathostomins excystment, and occasionally lead to fatal cases (Bowman, 2014).

2.1.1.3 DIAGNOSIS OF STRONGYLE INFECTION

Although the diagnosis can be performed by taking in account the clinical signs and the presence of strongyle eggs in qualitative and quantitative faecal analysis techniques, there is the need for specific techniques to differentiate between large strongyles and small strongyles due to the similarities between the eggs. The strongyle eggs dimensions are about 50 µm x 100 µm, oval-shaped, with a smooth surface and identifiable cells (Anderson, 2000; Reinemeyer & Nielsen, 2013). One of these techniques and the most used is the faecal culture where the eggs can develop to L3 which will be further identified according to morphological keys (Madeira de Carvalho, Fazendeiro, & Afonso-Roque, 2008; Reinemeyer & Nielsen, 2013). Both the faecal culture technique and larval identification applied during this research will be described further below in the Material and Methods chapter. Since these techniques require time and experience for a regular and reliable diagnosis, some molecular techniques have been developed to differentiate between adults species by analysing faecal samples (Traversa et al., 2007), others can be applied to detect prepatent infections using the horse serum (Andersen et al., 2013; Nielsen, Vidyashankar, et al., 2014). The diagnosis of encysted cyathostomin larvae continues to be a challenge because there is not a single direct test that can detect and quantify the number of encysted larvae, although there have been recent developments in molecular diagnosis using *ELISA* (Mitchell et al., 2016). There is also the possibility in necropsies to retrieve and identify adults and larvae and to check for lesions correspondent to the ones caused by the migrating larval stages (Bowman, 2014).

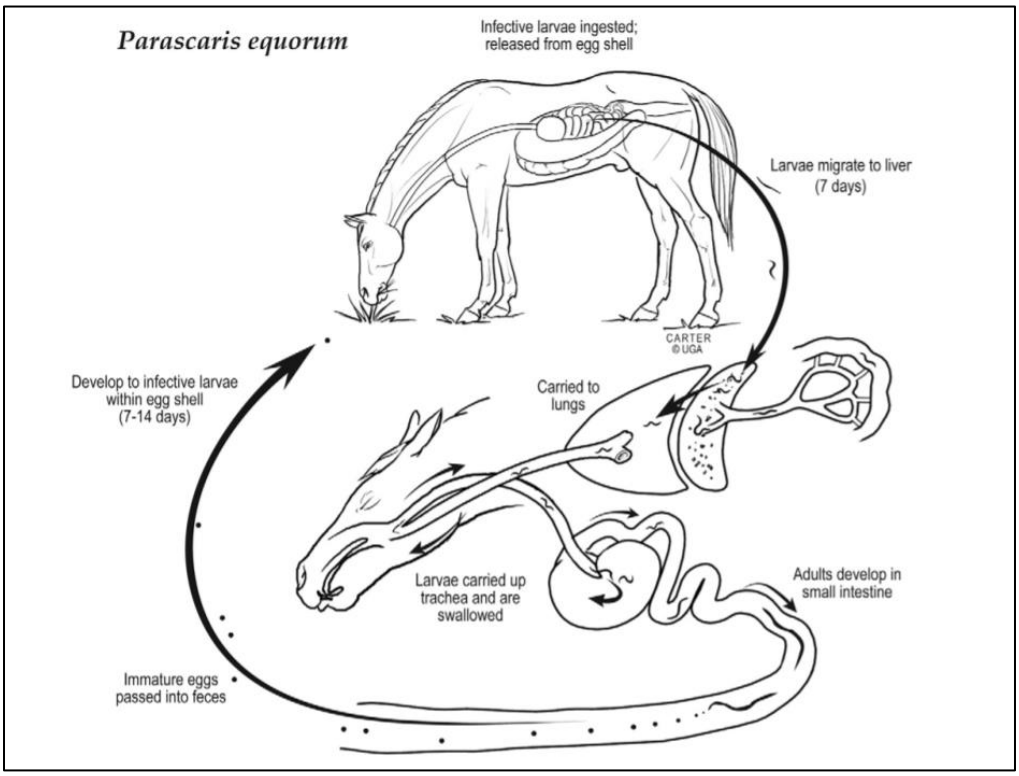
2.1.2 ASCARIDAE – *Parascaris* SPP.

The family Ascaridae, belonging to the phylum Nematoda, has some of the biggest adult worms affecting animals, namely genus *Parascaris*, with adults reaching up to 50 cm in length (Bowman, 2014; Hernández Malagón, 2014). *Parascaris equorum* and *P. univalens* are the species infecting horses and, while they are anatomically identical, the former is the most studied and believed to be the cause of most horse infections (Bowman, 2014; Clayton, 1986; Nielsen, Wang, et al., 2014). Recently, it has been reported that the prevalence of *P. univalens* probably has been underestimated over the years and that this parasite might actually be the most prevalent in horses (Nielsen, Wang, et al., 2014)

2.1.2.1 LIFE CYCLE

Like most ascaridoids in domestic animals, *P. equorum* and *P. univalens* have a direct life cycle and, since so little is known about the later, it is being considered the same (Reinemeyer & Nielsen, 2013). Eggs are expelled in the faecal matter and contain one single cell that further develops into an embryo, morula, L1 and L2, the infective stage (Figure 3). This development may range from 10 days to 6 weeks, depending on environmental conditions such as humidity and temperature (25ºC to 35ºC). Horses, mainly foals, get infected when they ingest the eggs containing the L2 which later hatches when it reaches the small intestine (Arroyo Balán, 2017; Bowman, 2014). After hatching, the L2 become into L3, penetrate the gut and reach the liver in 24 hours, in which they remain for around a week. Following this week, the larvae migrate to the lungs using the caudal vena cava, heart and pulmonary artery until they finally reach the alveoli. The larvae stay in the lungs until about four weeks after infection, ascending afterwards in the mucus to the tracheoesophageal area (Reinemeyer & Nielsen, 2013; Taylor et al., 2016). After ascending, the larvae are swallowed and reach the intestinal tract once again, where they mature and reach the adult stage, usually remaining in the duodenum and proximal jejunum unless in heavy infections, where they can be found all through the small intestine (Clayton, 1986). Adults of *P. equorum* usually start to shed eggs approximately 75-80 days after infection and there are reports that show the presence of adults in horses intestine as early as two weeks after infection (Clayton, 1986; Reinemeyer & Nielsen, 2013).

Figure 3 – Life cycle of *P. equorum*. Source: Boyle & Houston (2006).



2.1.2.2 PATHOGENICITY AND CLINICAL SIGNS

Clinical signs of infection are mainly seen in foals due to the development of immunity as horses age, although they can also be observed in adults (Clayton, 1986; Lyons, Tolliver, & Collins, 2006). The first signs of infection are associated with larvae migration and consist mainly of coughing and nasal discharge, while in a necropsy we can see white, fibrous scars ("milk spots") under the liver capsule, usually accompanied by oedema and haemorrhage in the lungs (Clayton, 1986; Reinemeyer & Nielsen, 2013). Following the larval development into adults, the clinical signs can develop into diarrhoea, lethargy, weight loss, rough hair coat, and gastrointestinal impaction with colic (Bowman, 2014; Reinemeyer & Nielsen, 2013). As Nielsen (2016) reports, an infection with a large number of *P. equorum* adults should be taken into consideration when foals show signs of small intestinal impaction following deworming.

2.1.2.3 DIAGNOSIS OF INFECTION BY *Parascaris SPP.*

These nematodes are usually diagnosed in foals younger than 2 years of age, but can also be found in adults (Clayton, 1986, Francisco, I. et al., 2009). Diagnosis is based on the presence of *P. equorum* eggs in faeces, the clinical signs and, in a necropsy, the presence of adults and lesions in the organs in which the larvae migrate (Bowman, 2014). The eggs of *P. equorum* (80-100 µm) are almost spherical, dark-brown coloured and have a rough thick external wall, allowing them to be extremely resilient, resisting chemical and physical agents, and able to remain viable for several years (Bowman, 2014; Hernández Malagón, 2014). Even though there is a cellular response with eosinophilia associated with larvae migration, being a typical occurrence in many parasitic infections should not be considered specific for parascariosis (Clayton, 1986). The only way to completely distinguish between *P. equorum* and *P. univalens* is through genomic sequencing and analysis (Nielsen, Wang, et al., 2014).

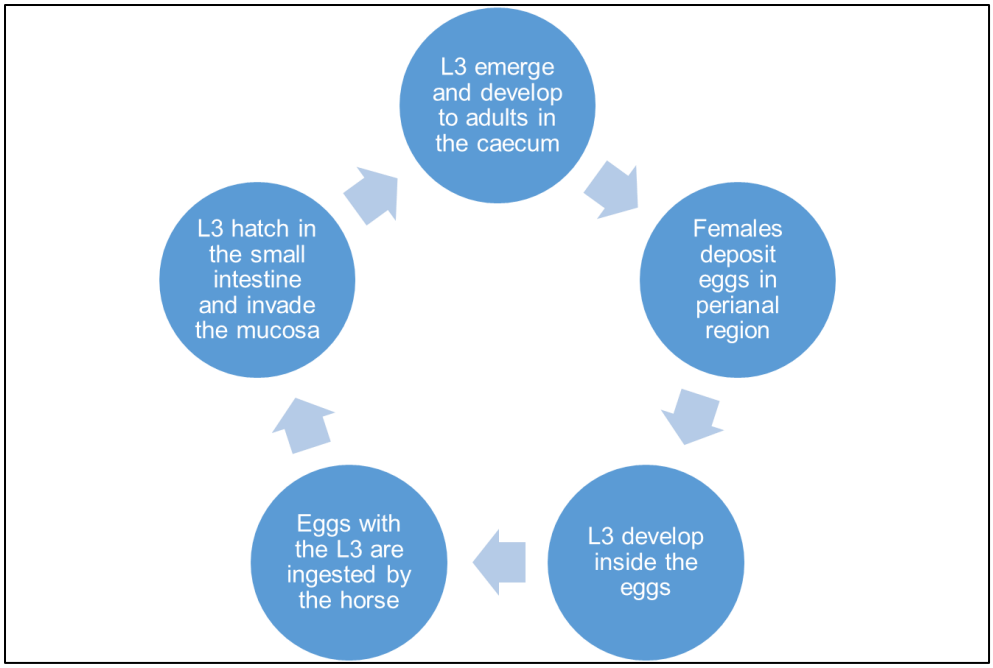
2.1.3 OXYURIDAE – *Oxyuris equi*

The members of the Oxyuridae family are also referred has "pinworms", due to their pin-shaped tail, and it gets its name after *Oxyuris equi*, the common and large pinworm of horses (Beveridge & Emery, 2014; Bowman, 2014). Although previously relatively nonpathogenic, *O. equi* has shown some changes on its biology and anthelmintic resistance which has led to an increased challenge in its control (Wolf, Hermosilla, & Taubert, 2014).

2.1.3.1 LIFE CYCLE

The life cycle is direct, and horses get infected by ingesting the eggs present in the environment containing an L3 (Figure 4). Although the L3 hatch in the small intestine, they migrate to the anterior large bowel and invade the crypts of Lieberkühn (Wolf et al., 2014). After developing for 3-11 days to L4, the larvae emerge and start feeding on the mucosa going through L5 until they mature to adult, 150 days later (Reinemeyer & Nielsen, 2014). Adults are usually found in the dorsal colon but females, when ready for oviposition, migrate to the anus and deposit up to 60,000 eggs in yellow gelatine like masses that can adhere to almost any surface and contaminate the environment (Bowman, 2014). After 3-5 days in the environment, the infective stage is reached (L3) (Reinemeyer & Nielsen, 2013; Wolf et al., 2014).

Figure 4 – Life cycle of *O. equi* (original).



2.1.3.2 PATHOGENICITY AND CLINICAL SIGNS

The most evident and common clinical sign of infection by *O. equi* is pruritus caused by the adhesive egg masses deposited by the adult females that can lead to horses constantly rubbing their tails on different surfaces (leading to egg dispersion), eventually ending with their tail heads tangled, hair scarce, or even wounded. These symptoms are rather distinct and lead to a condition known as “rat-tailed” (Bowman, 2014; Reinemeyer & Nielsen, 2014). Although relatively nonpathogenic in moderate infections, in high-density infections with L4 can lead to signs of abdominal discomfort due to inflammation of the ventral colon and caecum caused by their mouth structure and feeding activity (Reinemeyer & Nielsen, 2014).

2.1.3.3 DIAGNOSIS OF *Oxyuris equi* INFECTION

Diagnosis is mainly based on the clinical signs, especially the “rat-tailed” condition. Usual coprological techniques bare no results in the diagnosis of the disease due to female adults depositing the eggs directly on the perianal area (Bowman, 2014). A technique named “Scotch Tape Technique” was developed for Oxyuridae diagnosis, in which a piece of transparent scotch tape is applied (with the sticky side down) to the perianal area of the horses and then transferred to a microscope slide, with one or two droplets of water, for further observation under a microscope (Reinemeyer & Nielsen, 2013). This “Scotch Tape Technique” allows the observation of any *O. equi* egg present. These eggs are 42x 90 µm in size, yellow to brownish in colour, slightly flattened on one side and have an operculum-like plug in one of its ends (Bowman, 2014; Foreyt, 2002).

2.2 PARASITE CONTROL PRACTICES

Parasite control is achieved by blocking any single event from a parasite life cycle, preventing its development to the infective stage. Most of the efforts taken into parasite control involve the use of anthelmintics (chemical treatments), which disrupt life cycle events within the hosts. There is a variety of alternative strategies that act not only in the host but also the parasite itself, the environment or a combination of these and should be applied in parasite control programs, complementing the treatment with anthelminthics (Bowman, 2014; Madeira de Carvalho et al., 2011; Reinemeyer & Nielsen, 2013). Modern parasite control practices have also moved from an eradication goal to maintenance of sustainable levels of parasitism (Reinemeyer & Nielsen, 2013).

2.2.1 ANTHELMINTIC APPROACHES

As reviewed by Lyons, Tolliver, & Drudge (1999), there are ancient treatments that were meant to target parasitic diseases, more specifically, helminths in horses. Such treatments would include the use of other animals’ and humans’ faeces, black soap, hens’ eggs, and others. It is only in the early 1900s, with the introduction of carbon disulfide, and in 1940s, with the launch of phenothiazine, that control and treatment of parasites starts to diverge from mere empirical knowledge (Lyons et al., 1999). In spite of an increase in the number of reported anthelmintic resistances, chemical compounds are still needed to treat horses and to support other strategies focused on parasite control (Nielsen, 2016; Nielsen et al., 2018; Reinemeyer & Nielsen, 2013).

There are currently 5 classes of anthelmintic commercially available and regulated for horses: Benzimidazoles (fenbendazole and oxibendazole), Tetrahydropyrimidines (pyrantel salts), Macrocyclic Lactones (ivermectin and moxidectin), Heterocyclic Compounds (piperazine), and Isoquinoline-Pyrazines (praziquantel) (AAEP, 2013; Bowman, 2014; Matthews, 2014). While there were several parasite control programs used over the years, most of the anthelmintic treatment regimens used the interval-dose program described in the 1960s by Drudge & Lyons (1966) as a base concept for their development (Reinemeyer & Nielsen, 2013).

The interval-dose programs follow a time fixed treatment of all horses on a farm, all year long, with a regular interval of 2, 3 or 4 months and without any diagnostic or efficacy evaluation (Reinemeyer & Nielsen, 2013). This was first designed to deal with the threat of *S. vulgaris* but continues to be applied in farms all over the world (Drudge & Lyons, 1966; Nielsen et al., 2018; Relf, Morgan, Hodgkinson, & Matthews, 2012; Sallé & Cabaret, 2015). This systematic approach and similar methods are considered by most equine parasitologists to be the main source of anthelmintic resistance nowadays. This statement is also applied to the rotational program, in which, in theory, rotation of anthelmintics with different mechanism of action would reduce the number of resistance cases that could appear in a near future (Kaplan & Nielsen, 2010).

The most commonly accepted as the correct chemical approach by parasitologists is called the selective or targeted treatment (Reinemeyer & Nielsen, 2013). This approach requires the diagnosis of parasitism and FEC, quantification of the numbers of eggs per gram (EPG) being excreted in faeces, on all horses from a farm. Following this step, the horses that have surpassed a certain FEC threshold (ranging 200-500 EPG) are submitted to treatment with an anthelmintic that specifically targets the observed parasite (Francisco, R. et al., 2012; Krecek, Guthrie, van Nieuwenhuizen, & Booth, 1994). This approach would also be able to reduce anthelmintic administration by up to 82% (Lester et al., 2013) Despite being highly acceptable, this approach can lead to the reappearance of previously controlled parasites such as *S. vulgaris* (Nielsen, Vidyashankar, Olsen, Monrad, & Thamsborg, 2012).

2.2.2 ALTERNATIVE APPROACHES

Even though the pharmacological approach is the most common and observed, there has been a search for new methods that can aid in the control of parasites and support those referred before, keeping parasite burdens within acceptable levels and reducing resistance cases. Adequate hygiene and management of the pasture, genetic selection, nutrition, plant extracts, vaccines and biological control through parasiticide fungi, are the most commonly mentioned methods of alternative approaches that act on the parasites but also in the environment and the host (Arroyo Balán, 2017; Shalaby, 2013; Taylor et al., 2016).

Pasture is essential for the development of certain parasites as described in previous chapters. Thus, adequate control of the environment can help to prevent most of the infections from happening and spreading (AAEP, 2013). There are numerous recommendations for pasture management and hygiene that can aid with parasite control. Easy actions such as regular removal of faeces can result very useful regarding parasite control, limiting transmission and movement of larvae from the faeces to the surrounding pasture, thus avoiding horse infection. Other actions such as mowing and rotating pastures, mixing or alternating grazing species, and quarantine practices are all part of a good and sustainable management of a farm or grassland and are also very effective in minimizing parasite transmission (Barger, 1997; Reinemeyer & Nielsen, 2013; Taylor et al., 2016).

Genetic selection has been applied over the years to improve production parameters of domestic animals but can also be used to increase resistance to parasite infections. Although not as a short-term approach (can take up to 8-10 years in sheep), the results can be quite satisfactory, not only in terms of parasite control and overall parasite burden but also in production parameters such as body growth and body condition score as a result (Behnke et al., 2003; Bisset, Morris, McEwan, & Vlassof, 2001; Taylor et al., 2016).

Nutritional status correlates directly with the possibility of horses becoming infected and a bad nutrition condition diminishes their response to infection. It is crucial to make sure that horses are in a healthy condition so that pathophysiological status such as reduced appetite and changes in nutrient metabolism caused by parasites are easier to manage and control (Smith, Panickar, Urban, & Dawson, 2018). The amount of protein intake and the resistance to gastrointestinal nematodes have been correlated, so higher protein intake is associated to the capacity of the host to overcome and control parasite infections (Coop & Kyriazakis, 2001). Besides this, copper oxide supplements can be given in order to reduce parasite burden, as it directly affects them, and base levels of copper in the host are needed for an adequate immune response (Knox, 2002; Shalaby, 2013).

Plant extracts can be administered to animals and act as anthelmintics, even though this has mostly been tested *in vitro* and still needs more validation with *in vivo* studies (Githiori, Athanasiadou, & Thamsborg, 2006). From the diversity of plants with parasiticide activity, only the common garlic (*Allium sativum*) can inhibit ascarid egg eclosion and development (Hernández Malagón, 2014).

So far there is only one vaccine developed to control equine protozoal myeloencephalitis caused by *Sarcocystis neurona* (Marsh et al., 2004). There have been advances in the overall development of vaccines in recent years which lead to more commercially available vaccines against cattle lungworm (*Dictyocaulus viviparus*) and poultry protozoa (*Eimeria* spp.) (Taylor et al., 2016).

2.3 BIOLOGICAL CONTROL

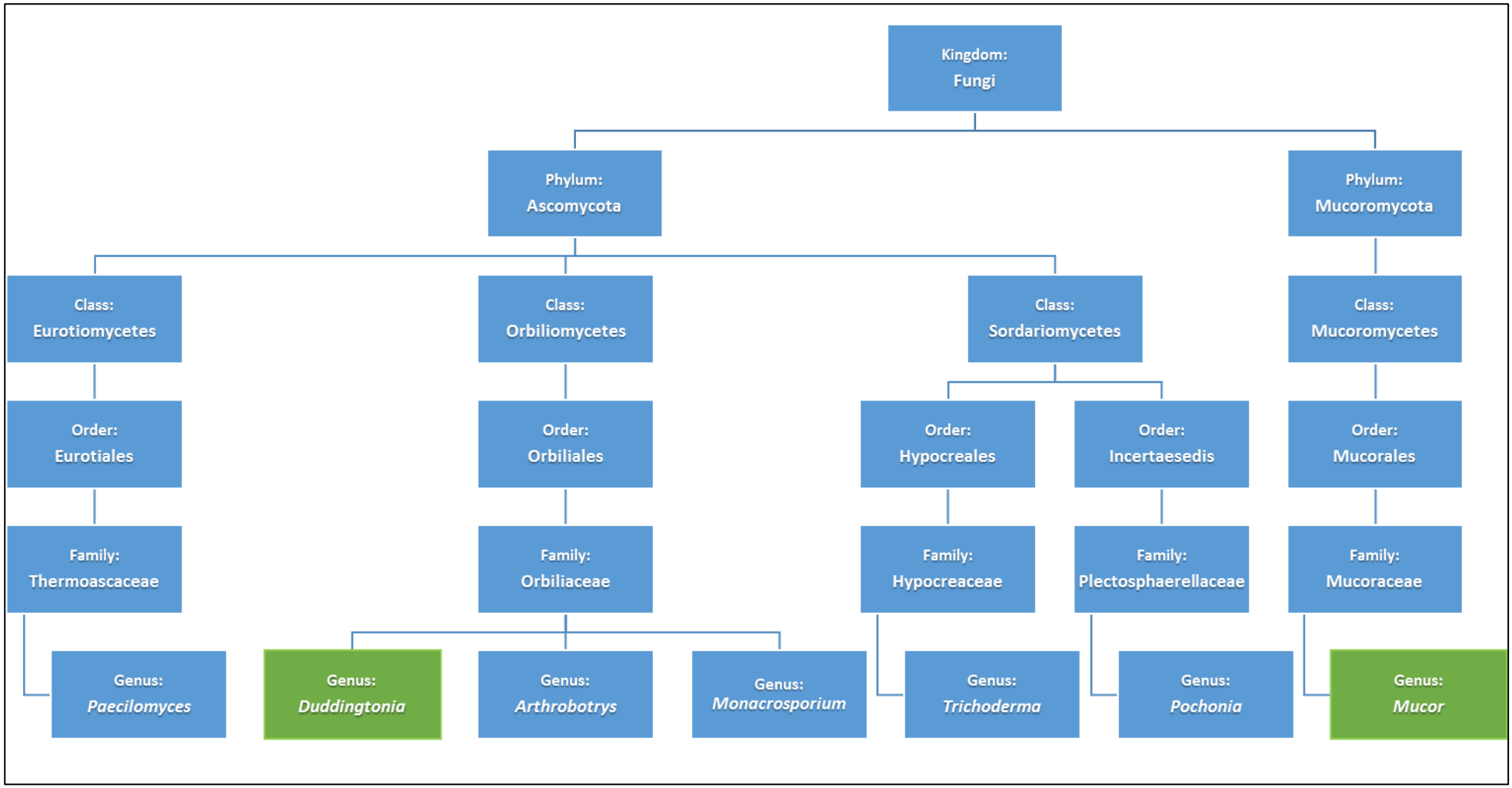
With the rise in the concern regarding environmental sustainability and the increase of anthelmintic resistance in horse nematodes, research for new or improved methods of parasite control has seen a great development over the last 30 years (Suárez, 2017). As described for the approaches developed in the previous chapter, biological control has been increasingly developed and used as a complementary component to the use of anthelmintics in parasite control, with a special regard to Brasil and Spain where a lot of research has been performed in recent years (Braga et al., 2009; Cazapal-Monteiro, 2015; Cruz, 2015). Biological control can be defined as pest control using biotic agents, which in the control of horses nematode usually involves the use of parasiticide fungi, and is designed to keep pest population at a non-harmful level (Larsen, 2000; Madeira de Carvalho et al., 2011).

2.3.1 PARASITICIDE FUNGI

Fungi used as biological control agents are eukaryotes and can be found in all kind of environments. They usually possess structures called hyphae, with a cell wall composed of chitin but without chlorophyll or cellulose, forming a mycelium (a mass of hyphae), with both sexual and asexual reproductive behaviours producing conidia or spores. In adverse environmental conditions, the hyphae can produce chlamydospores, which are spores with a very thick wall, capable of withstanding harsh conditions and developing new hyphae once under favorable conditions (Barron, 1977). Even though most of the parasiticide fungi are saprophytes, they can use other organisms, such as free-range stages of parasitic nematodes, as a nutrient resource, if the opportunity and need arise (Alexopoulos & Mims, 1979; Dijksterhuis, Veenhuis, Harder, & Nordbring-Hertz, 1994).

Since late 19th century that is known the activity of certain fungi, namely *Arthrobotrys oligospora*, against plant nematodes (Zopf, 1888; cited by (Cruz, 2015)), and over 200 species have already been recorded (Nordbring-Hertz, Jansson, & Tunlid, 2011). Parasiticide fungi can be divided into three different categories according to their parasitic abilities: the nematode-trapping or larvicide fungi (such as *Duddingtonia*, *Arthrobotrys* and *Monacrosporium*), ovicide fungi (namely *Mucor*, *Pochonia, Paecilomyces* and *Trichoderma*), and endoparasitic fungi (for example *Drechmeria* and *Harposporium*) (Braga & Araújo, 2014; Nordbring-Hertz et al., 2011). Endoparasitic fungi are usually not considered when making a biological control program due to their inability to grow in the soil, dependence on the presence of water and nematodes for their development both *in vitro* and *in vivo* (Braga & Araújo, 2014). In Figure 5 are represented the most studied parasiticide fungi.

Figure 5 - Schematic representation of parasiticide fungi taxonomic classification (original) (Raghukumar, 2017; Spatafora et al., 2016).



2.3.1.1 SOIL LARVICIDE FUNGI

Nematode-trapping or predator fungi are the most studied and known specimens used in biological control programs, with *Duddingtonia flagrans, Arthrobotrys* spp*.* and *Monacrosporium* spp. being the prime representatives (Braga & Araújo, 2014). These fungi are characterized by the development of a large network of hyphae to capture nematode larvae in the soil, where they can be frequently isolated, for ensuring their nourishing, especially carbon and nitrogen (Arias, Cazapal-Monteiro, Suárez, et al. (2013). Morphogenesis (and hyphae growth) is influenced by several environmental aspects such as fungi feeding possibilities, temperature, soil and faecal larval density, where the contact between fungi and larvae or their compounds is the most effective inducer of trap formation (Arias et al., 2013, Dijksterhuis et al., 1994; Madeira de Carvalho et al., 2011; Su et al., 2017).

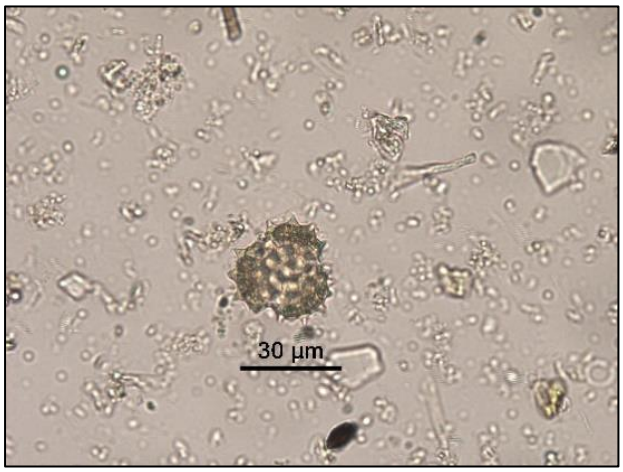
The larvicide fungi can have different trap mechanisms that derivate from their hyphae growth and one species can use one or more of these to capture the larvae: adhesive branches, adhesive two-dimensional and tri-dimensional networks, adhesive knobs, constrictive rings and non-constrictive rings (Arroyo Balán, 2017; de Ulzurrun & Hsueh, 2018). Several phases occur until nematode destruction, beginning with the attraction of nematodes due to components released by the mycelium. Following this, there is a phase of adhesion where the parasites get attached to the trap structures on contact or, in the case of constrictive rings, the traps themselves encase the nematodes. Once parasites get fully immobilized, the hyphae produce enzymes and invasive tube-like hyphae that penetrate the parasite cuticle. Once inside the parasite, the fungi start digesting and end up completely destroying it (Nordbring-Hertz et al., 2011).

2.3.1.1.1 *Duddingtonia flagrans*

Isolated by Duddington for the first time in 1949, it is only since the beginning of the 1990s *Duddingtonia flagrans* has become one of the most studied parasiticide fungi. This was mostly due to its ability to survive passage through the animals intestinal tract (Larsen, 2000; Madeira de Carvalho et al., 2011). Chlamydospores are thick-walled resting spores (Figure 6), produced when the fungus grows older, from a hyphal cell, and can last more than 20 months in a dried air environment (Barron, 1979). A recent study by Arroyo Balán et al. (2014) showed their ability to withstand the treatment used in factories for the production of nutritional pellets, while others showed the capacity of surviving lyophilization processes (Santurio et al., 2009). The production of spores in submerged cultures increases the possiblities of spreading them by using different ways of administration (Arias, Cazapal-Monteiro, Suárez, et al., 2013), and thus *D.flagrans* becomes into a suitable candidate for future integration in animal feeding habits. Besides the survival capacity due to the production of chlamydospores, *D. flagrans* is

able to quickly produce a large number of conidia and therefore is able to spread in the faecal matter in a short timespan (Grønvold et al., 1993).

Figure 6 - *D. flagrans* chlamydospore present in a horse faecal sample (original)



Trap formation in *D. flagrans* mycelium begins with the first germinative hyphae, where lateral branches start to develop and loop. Hyphae continue to grow and curve until they anastomose with the original one or another, repeating until they form non-constricting rings, constricting rings and tri-dimensional networks that can capture nematode larvae (Barron, 1979; Grønvold et al., 1996). These rings are covered with an adhesive substance that entraps the larvae not relying solely on the constriction mechanism. Following the entrapment, chitinases and proteases are produced to help with the solubilization of cuticle and digestion of the nematodes (Braga et al., 2015; Suárez, 2017). There are already reports that show the possibility of usage not only for nematode control but also in vector control, such as *Aedes aegypti* (Braga et al., 2016).

Concerns regarding the effect on non-parasite nematodes and the soil were tested by Saumell et al. (2016) showing no significant effect on free-living nematode populations and lasting no longer than 2 months in the environment. There has been no record of adverse effects regarding *D. flagrans* oral administration in horses (Hernández et al., 2016; Suárez, 2017).

2.3.1.2 SOIL OVICIDAL FUNGI

Ovicidal fungi show activity mostly against eggs, although some of them can also damage adults and larvae. The most researched telluric fungi with ovicide effect are *Pochonia chlamydosporia*, *Paelomyces lilacinus*, *Dactyella ovoparasitica*, *Trichoderma* spp. and, one of the focused in this essay, *Mucor circinelloides* (Braga & Araújo, 2014; Hernández Malagón, 2014). Most nematodes eggs rapidly develop into larvae, which reduces the activity of these fungi on them. In contrast to other nematodes such as strongyles, larvae belonging to the

Ascaridae or Oxyuridae do not develop free-range larvae in the environment, so these fungi have enough time to act and destroy eggs (Cortiñas et al., 2015).

There are four different phases that these fungi develop over a parasite egg. After the hyphae contact with the eggshell, they adhere and penetrate inside, then the inner embryo is destroyed and hyphae exit off in search of new eggs (deliberation) (Arroyo Balán, 2017; Hernández Malagón, 2014). Contact phase begins when the fungi present in the soil or faeces get in physical contact with parasite eggs as a result of hyphae net development from spores or mycelium (Irving & Kerry, 1986). Following contact, the hyphae produce an *appressorium*, which is a swollen portion of these hyphae in contact with the egg that adheres to the eggshell (Barron, 1977). Only hyphae that adhere nearly perpendicularly to the egg are able to penetrate it, although the mechanism is still not completely identified, not knowing what is the relation between physical pressure and certain released enzymes (Cazapal-Monteiro, 2015). Once it penetrates, the fungus continues to grow inside the eggs, keeping the shell intact but destroying and nurturing on all the egg contents, including the embryos. Once there are no more nutrients, the fungus feeds on the eggshell and releases the recently formed hyphae, ready to infect other eggs (Lýsek & Stěrba, 1991).

Even though there are studies using ovicide fungi, most of these are *in vitro* and performed in Petri dishes, only a few evaluate the direct effect in the helminth eggs present in the faeces (Arroyo Balán, 2017; Cortiñas et al., 2015).

2.3.1.2.1 *Mucor circinelloides*

Mucor circinelloides was previously studied as a way to produce biodiesel and animal feed and only recently it is viewed as a possible fungus for biological control due to its ovicidal characteristics (Arias, Cazapal-Monteiro, Suárez, et al., 2013; Vicente et al., 2009). Belonging to the phylum Mucoromycota (previously Zygomycota) it develops through both sexual and asexual life cycles, with the formation of hyphae and sporangia in the former, and zygospores with meiospores in the later, both structures represented in Figure 7 (Li et al., 2011; Raghukumar, 2017). A recent study by Cortiñas et al. (2015) has shown that, just like *D. flagrans*, *M. circinelloides* has the ability to survive passage through animals intestinal tract.

Figure 7 - *M. circinelloides* zygospores (1.) and hyphae (2.) (original)

This species is able of developing on eggs of trematodes (*Calicophoron daubneyi* and *Fasciola hepatica*) and nematodes (*Toxocara canis, Baylisascaris procyonis, Ascaris suum*, *Parascaris equorum* or *Trichuris* spp.). It has the capacity of not only to delay development and diminish their viability but also to destroy the embryos (Arroyo Balán, 2017; Cazapal-Monteiro et al., 2015; Cortiñas et al., 2015; Hernández Malagón, 2014).

There are no reports of disease caused by the ingestion of this fungus in animals (Arroyo Balán, 2017; Suárez, 2017). Some human cases regarding mucormycosis have been described among hospitalized human patients with immunodeficiency (Ribes, Vanover-Sams, & Baker, 2000).

2.3.2. PARASITICIDE FUNGI DISTRIBUTION

Over the years there have been studies to find the best methods to disseminate the spores in the environment in order to act on free-range parasite stages (Table 1). Most of the studies have been developed *in vitro*, but other found ways to apply these fungi in the pasture or directly to the animals (Arroyo Balán, 2017). There is probably no perfect way to distribute these fungi so every situation should be assessed individually, taking into consideration factors such as the environment, the purpose of the farm/pasture, the animals and their parasites.

Table 1 - Fungi distribution methods and some related researches.

Fungi distribution method	Research
Directly in the soil	Arias, Cazapal-Monteiro, Suárez, et al. (2013)
Fungi soaked cereals	Waller, Knox, & Faedo (2001)
Feed premixes	Cazapal-Monteiro et al. (2014)
Mineral/energy blocks	Sagüés et al. (2011)
Slow-release bolus	Waller, Faedo, & Ellis (2001)
Pellets	Hernández et al. (2016)
Gelatines	Vilá Pena (2017)

2.3.2.1 DIRECTLY ON THE SOIL

The development of (submerged) liquid media for the growth of *D. flagrans* and *M. circinelloides*, enables new ways of dispersing spores through the pasture by using sprayers and other devices (Arias et al., 2012; Arias, Cazapal-Monteiro, Suárez, et al., 2013). This method allowed to reduce FEC in the tested areas to around 50 to 100 strongyle EPG, while horses in control areas shed 250 to 300 strongyle EPG (Arias et al., 2012). The procedure could be easily applied in areas such as parks, leisure places, zoological enclosures or small farms (Arroyo Balán, 2017; Suárez, 2017). Despite the high efficacy when applied directly to the soil, this method is not as useful in large extensions of land or in high animal density areas (Paz-Silva et al., 2011).

2.3.2.2 ORAL ADMINISTRATION

Direct administration to animals enchances the direct contact between fungi and parasites in faeces, where eggs and larvae develop, allowing the fungi to grow and act as soon as they leave the intestinal tract (Arroyo Balán, 2017; Larsen et al., 1995). Fungi can be given directly to the animals by feeding fungi soaked cereals, animal feed premixes, mineral/energetic blocks, slow-release bolus, sodium alginate or nutritional pellets, or gelatines.

Cereals provide an ideal substrate for fungi spores growth and large-scale production. Studies by Waller, Knox, et al. (2001) showed the ability of *D. flagrans* spores embedded in cereals to survive the passage through the intestinal tract in sheep and reduce the number of larvae present in faecal plot. These authors suggested that this would be a good method to implement biological control in intensive farming systems. Other proposal by Sagüés, Purslow, et al.

(2011) consisted of feeding animals with these fungi during critical time points, such as calving, due to to the rise in faecal parasite egg excretion.

Fungi can be directly incorporated into feed concentrate usually mixing it with liquid media containing spores or with spores collected from the surface of agar solid cultures (Arroyo Balán, 2017). Studies by Arias, Cazapal-Monteiro, Valderrábano, et al. (2013) and Cazapal-Monteiro et al. (2014) showed that after providing spores of *D. flagrans* and *M. circinelloides* to wild animals captive in a zoological park, previously treated according to selective therapy indications, the values of FEC remained under 300 EPG for over a year.

Mineral and energy blocks are a way for animals to intake a supplement of certain nutrients in deficiency, especially in areas with limited land and feed resources (Knox, 1996). This appears to be an effective way to control parasites in sheep, with advantages of price, working as a nutritional supplement and a vehicle for fungi, and remaining viable for up to 2 years at room temperature of 24ºC without losing activity. Despite being effective, there is the disadvantage of being reliant on individual animal consumption of the blocks which is almost never consistent (Sagüés, Fusé, et al., 2011; Waller, Knox, et al., 2001).

Slow-release boluses have been used in ruminants for the last 40 years to provide several different compounds, such as anthelmintics like Ivermectin (Barth, Heinze-Mutz, Roncalli, Schlüter, & Gross, 1993). Waller, Faedo, et al. (2001) tested the same mechanism of slow-release boluses as a way of constantly providing *D. flagrans* chlamydospores to sheep instead of feeding them. This procedure continues to release spores up to 3 weeks after administration, but the author refers that it should be over 4 weeks to be a viable method.

Pellets are given to most livestock, due to the capacity of ensuring a complete and balanced nutrition. Several formulations have been tried to incorporate fungi in hand made sodium alginate pellets (Araújo, Stephano, & Sampaio, 2000; Braga et al., 2009; Tavela et al., 2013; Vilela et al., 2016) and industrial manufactured pellets (Arroyo Balán, 2017; Arroyo Balán et al., 2014; Hernández et al., 2016; Suárez, 2017) with a promissing high success rate in the control of parasites affecting sheep and horses. Parasiticide fungi have been incorporated in sodium alginate at the stage of myceliium, but the studies with industrial pellets involved the addition of spores and chlamydospores due to their higher resistance. This is important for pellet production because temperatures higher than 70ºC can be reached (Arroyo Balán et al., 2014; Vilela et al., 2016). It has been demonstrated that the addition of fungal spores to industrial pellets does not modify their shelf life (Arroyo Balán, 2017).

Gelatine capsules have been used as a physical vehicle of fungi administration with the incorporation of lyophilized chlamydospores (Buske, 2010; Santurio et al., 2009) or spores directly collected and diluted from the surface of agar solid cultures (Faedo, Larsen, & Waller, 1997). A very recent investigation by Vilá Pena (2017) demonstrated the effectiveness of orally administered fresh edible gelatines containing a blend of spores of *D. flagrans* and *M. circinelloides* against cyathostomins when given to grazing horses.

CHAPTER 3 - MATERIAL AND METHODS

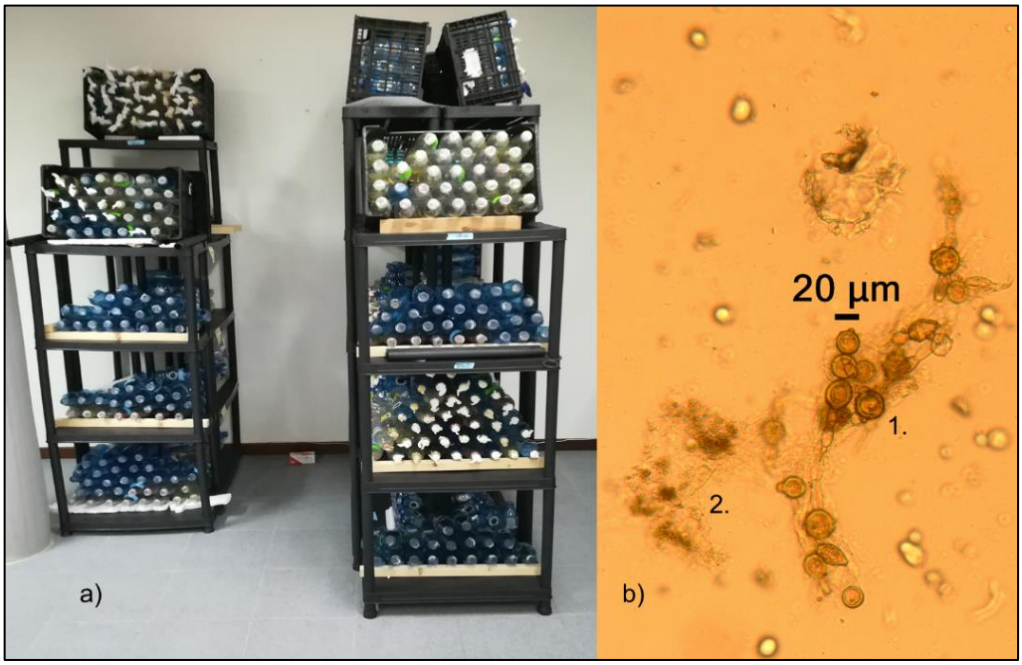
3.1 DEVELOPING LYOPHILIZED ORAL PRODUCT WITH VIABLE SPORES OF *Duddingtonia flagrans* AND *Mucor circinelloides*

Following the recent research trends on how to develop new formulations containing parasiticide fungi, a novel idea emerged on using lyophilized product already containing spores. Theoretically, this would allow an easier storage and a more stable product like those already used in many pharmaceutical compounds (Bosmans, 1974). The experimental design has been approved by the Ethical committee of the University of Santiago de Compostela, complies with the Directive 2010/63/EU and partly supported by the Research Project protocol number CTM2015-65954-R (Spanish Ministry of Economy and Competitiveness; FEDER).

3.1.1 PRODUCT MANUFACTURE

Product was manufactured at the Laboratory of the COPAR Research Group (Faculty of Veterinary, Lugo, Spain). First, a submerged medium (COPFr – patent PCT/ES2014/070110), was prepared, by mixing (per litre of distilled water) 500 ml of commercial chicken broth, 0.423 mg FhrAPS protein (recombinant protein of *Fasciola hepatica* tegument) and 12.5 g of wheat (*Triticum aestivum*). Spores of *Duddingtonia flagrans* (CECT 20823) and *Mucor circinelloides* (CECT 20824) (Arias, Cazapal-Monteiro, Suárez, et al., 2013; Vilá Pena, 2017) were finally added. A minimal concentration of 3300 *D. flagrans* spores/ml (100 spores/30 µl) is required, while that of *M. circinelloides* is not relevant due to the elevated growth rate observed in the culture (Figure 8).

Figure 8 - a) Production of spores of *D. flagrans* and *M. circinelloides* in submerged media (original). b) *D. flagrans* chlamydospores (1.) and *M. circinelloides* spores (2.) in liquid medium (original).



The concentration is calculated by counting the number of chlamydospores in a 30 µl aliquot of the submerged medium between coverslip and glass slide in an optical microscope (Leica DM2500, Spain) and using the following formula:

$$Number\ of\ chlamydospores\ per\ mililiter = \frac{Number\ of\ chlamydospores \times Water\ volume}{Aliquot\ volume}$$

Eight grams of powder gelatine are added per 100 ml of submerged medium and left to hydrate for 10 minutes at room temperature. Meanwhile, 2.5-3 g of previously autoclaved (20 minutes at 121ºC) horse feed pellets are poured into plastic moulds. After the 10 minutes have elapsed, the solution is heated in a microwave (1050 W) for 30-45 seconds (if the total volume was 100-300 ml), or 1.5-2 minutes (if the total volume was 450-600ml) and homogenized with a spatula. The temperature of the solution must reach 50-75ºC.

The solution is then poured into the silicone moulds, kept at room temperature (so it starts to gel) and then transferred to a refrigerator (4-6ºC) until fully gelled. Products were retrieved from the moulds using a metal spatula and stored in plastic/glass boxes at -35ºC.
When frozen, the products were transferred to a lyophilization machine (“Alpha 1-2 LD plus”, Christ, B. Braun Biotech®, Germany) for about 3 days until the process is over. The lyophilization process has two major steps: protein solution freezing and then drying the frozen solution under vacuum. Drying is divided into two substeps of removing the frozen water and removing non-frozen bound water (Wang, 2000).

Lyophilized products were then mashed in a blender until they reach dimensions ranging from dust particles to 2 cm by 2 cm blocks, so they are easier to store and administer to the horses. Two and a half kg of product were stored in a plastic box and kept at room temperature for further use (Figure 9).

Figure 9 - Product manufacture steps (original): a) Pouring gelatine mixture into silicone moulds. b) Lyophilization machine. c) Product mashing in a blender.

3.1.2 SPORE VIABILITY ASSESSMENT

With the aim to verify that chlamydospores remained unaltered and to assess the amount within the product, 20 random samples of 0.03 g of product each, were thoroughly mixed with 0.4 ml distilled water in eppendorf tubes (Figure 10). After mixing, the presence of *M. circinelloides* was verified and the amount of *D. flagrans* chlamydospores per gram (CPG) of lyophilized product calculated by counting the number of chlamydospores in a 30 µl aliquot of the mixture, between coverslip and glass slide in an optical microscope and using the following formula:

$$CPG = \frac{Number\ of\ chlamydospores \times Water\ volume}{Aliquot\ volume \times Compound\ weight}$$

Figure 10 - Lyophilized product mixed with distilled water in eppendorf tubes (original).

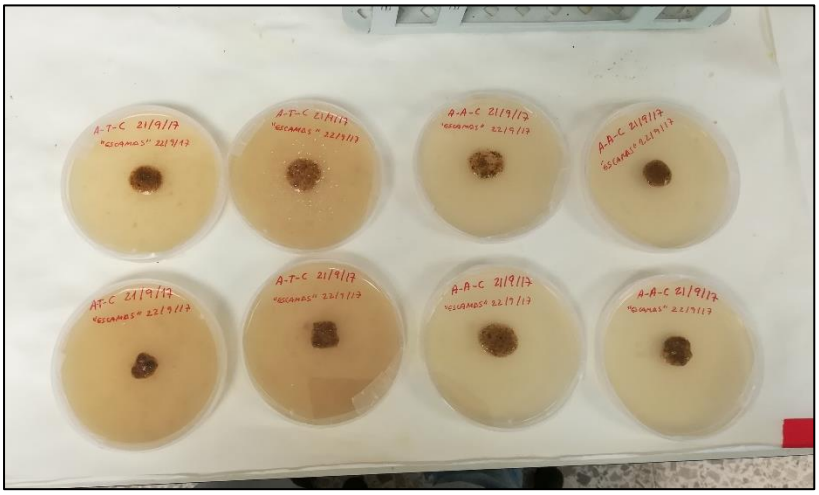
Three months later, to verify the absence of degradation, changes, abnormal odour, as well as that chlamydospores numbers and viability remained unchanged, a visual exam followed by the previous counting protocol were applied to the product kept within the plastic box.

3.2 ASSESSING FUNGI *IN VITRO* GROWTH AFTER PRODUCT MANUFACTURE

Even with the presence of seemingly viable spores within the product, there was no certainty that these would be able to continue their life cycle according to normal morphogenesis. Then, Petri dishes with Wheat-Agar-Chloramphenicol (ATC) and Water-Agar-Chloramphenicol (AAC) media were placed with product to verify if there was any development of new spores and the lack of abnormalities.

Previously, 8 Petri dishes with ATC solid medium and 12 Petri dishes with AAC solid medium were prepared (Figure 11). ATC medium is composed of 20 g of agar, 20 g of corn wheat flour, 1 L of distilled water, and, for every 1 L of medium, 500 mg of Chloramphenicol while AAC medium is composed of 20 g of agar, 1 L of distilled water, and, for every 1 L of medium, 500 mg of Chloramphenicol. These mediums were previously autoclaved (121ºC, 20 minutes) and, when the temperature dropped to 37ºC, poured into sterile Petri dishes while inside a laminar flow cabinet. They were kept under UV light for 12 hours before being sealed with Parafilm M® and left at room temperature until used (Arroyo Balán, 2017; Hernández Malagón, 2014). Following medium preparation, 0.1 g of lyophilized product were mixed with 0.5 ml of distilled water in eppendorf tubes and then poured at the centre of the medium surface using a discardable plastic Pasteur pipette and resealed with Parafilm M®.

Figure 11 - Petri dishes assembled with solid medium and product for fungi growth (original).



After assembled, Petri dishes were kept at 25ºC ± 2ºC in total darkness. 10 days after being assembled, each plate was analysed for the presence of new spores using an optic microscope.

3.3 ASSESSING FUNGI PREDATORY ACTIVITY AFTER PASTURING HORSES BEING FED

Following product manufacture, fungi predatory activity against gastrointestinal nematodes was assessed following product oral administration to horses. A total of 12 Galician Pure Breed horses, 4-8 years old, with no clinical signs were selected at the “Gayoso Castro” farm (property of Provincial Deputation of Lugo, Spain) (Figure 12), and divided into 2 groups. The first group (Group T), was constituted by 5 horses (Figure 13) and received product containing fungal spores, while the second group (Group C) was composed by 7 horses serving as controls, without receiving product. Both groups were kept in two adjacent pastures each with 2 hectares with constant access to grazing. These pastures have already been occupied by horses for the last ten years.

Figure 12 - Air view of the two pastures from Provincial Deputation of Lugo used in this study (Map data ©2017 Google).

Figure 13 – Galician Pure Breed horses from group T (original)

From 26th September 2017 to 22nd March 2018, each horse from group T was hand-fed with 10 g of product (Figure 14), 3 days a week (Monday, Wednesday and Friday). With the results from the chapter 5.2.2 already gathered, 10 g of product would be enough to give 10^5 chlamydospores to each horse, considering some waste on their behalf. This would mean a weekly average around 3-4 x 10^5 *D. flagrans* chlamydospores per horse.

Figure 14 - Preparation of individual product doses (original).

3.3.1 ASSESSING FUNGI EFFECT ON HORSES' FEC

The effect of fungi on gastrointestinal nematodes was established by comparing the EPG values in faeces of the two groups by means of FEC techniques and reduction formulas.
On September 26, the day in which the administration of fungi started, faecal samples from all horses in the two different groups were collected. Samples were taken directly from the rectum or from fresh faeces on the ground (only when sure of the source animal identification) using disposable insemination gloves and kept at 3-6 ºC until analysed once in the laboratory.

The samples were analysed using a modified McMaster technique to assess the genus/species (if possible) and the proportion they existed in both groups. Horses that reached a cut-off value of 300 gastrointestinal nematodes' EPG were treated with Ivermectin pour-on (5 mg of ivermectin (Paramectin Pour-on®) per 5 kg of body weight) (Francisco, I.et al., 2011; Reinemeyer & Nielsen, 2013). The decision to use this was made due to the wild nature of the horses, making harder to use other types of Ivermectin administrations such as oral.

After 13 days (9th of October 2017), the same procedure of collecting and analysing was used to evaluate treatment efficacy (AAEP, 2013). Any horse above the same cut-off was treated again with a dosage of 10 mg of Ivermectin per 5 kg of body weight and all the horses in both groups got reevaluated after 14 days (23rd of October 2017). From the 13th of November 2017 until 22nd March 2018, faecal samples from each horse were collected monthly as previously described and evaluated using the modified McMaster technique. It is important to mention that, on November 15th, the two groups swapped pasture with each other (the decision was taken by the farm direction).
The following formula was applied to each month and overall (from November to March) giving us an idea of the EPG reduction effect when comparing between groups (Cruz, 2015).

$$\% \, Reduction \; = \left[1 - \left(\frac{T \; group \; EPG \; average}{C \; group \; EPG \; average}\right)\right] \times 100$$

Faecal egg count reduction test (FECRT) is used to determine the efficacy of anthelmintics. This test should only be used in a group context, not at individual level, and makes use of the following formula, applied whenever faeces were collected and processed with modified McMaster technique. (AAEP, 2013):

$$FECRT \; (\%) = \frac{EPG \; (Pre \; treatment) - EPG \; (Post \; treatment)}{EPG \; (Pre \; treatment)} \times 100$$

When using Ivermectin, FECRT above 98% means that the strongyle population is susceptible to the treatment, 95-98% suspect to resist and below 95% is resistant to the anthelmintic. Values below 95% should first be an indication to repeat treatment ir order to discard false cases of resistance (AAEP, 2013).

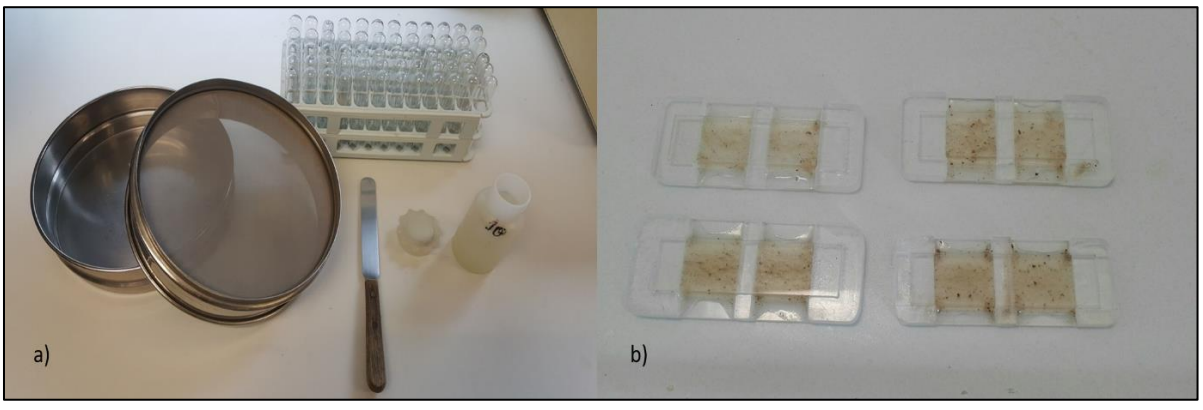
Egg-Reappearance Period (ERP) is the period between the last treatment with anthelmintics and the reappearance of relevant strongyle egg shedding (AAEP, 2013; Lyons et al., 2011). It makes use of the previous formula results so that the weeks between treatment and FECRT below 90% correspond to the ERP. For strongyles treated with Ivermectin, ERP is considered normal when it is around 6-8 weeks.

3.3.1.1 MODIFIED MCMASTER TECHNIQUE

Modified McMaster technique is a quantitative technique for FEC useful to assess the EPG numbers in a faecal sample. Despite being a quantitative technique, it can also be used as qualitative and allow for parasite identification.

The used technique follows the procedure found in the Manual of Veterinary Parasitological Laboratory Techniques (Ministry of Agriculture, Fisheries and Food [MAAF], 1986), with some modifications (Figure 15). Three grams of a faecal sample are introduced in a plastic flask with 50 ml capacity, and mixed 42 ml of water to achieve an almost homogenous solution. The solution is then filtered using a mesh with 150 µm pore diameter. After being filtered, the solution is poured into two 12 ml centrifuge tubes and centrifuged at 2000 rotations per minute (rpm) for 10 minutes. All supernatant is discarded using a vacuum pump and approximately 8 ml of saturated sodium chloride (NaCl) solution (ρ= 1.2 g/ml) is added. Using a discardable plastic Pasteur pipette, the content of the centrifuge tubes is mixed and retrieved to fill the two chambers in a McMaster chamber.

Figure 15 – Modified McMaster technique (original): a) Material needed for preparation. b) McMaster slides ready for microscopical examination.



The McMaster chamber is examined under a microscope using 100x magnification, focusing the existing grid and counting the number of parasite eggs. The EPG is then calculated using the following formula (50 EPG sensitivity):

$$EPG = \frac{Number\ of\ eggs\ in\ McMaster\ chamber\ \times 45\ ml}{0.30\ ml\ \times 3\ g}$$

3.3.2 ASSESSING LARVAE POPULATION

Faecal samples collected in November 2017 and January 2018 were analysed by means of the faecal culture method, for the identification of L3 genus and the species when possible, and the larvae population ratio present in the faeces.

3.3.2.1 FAECAL CULTURE METHOD

Faecal culture is used to identify and quantify the number of L3 larvae present in faeces. For this purpose, 10 g from the previously collected faecal samples of all horses were put in plastic boxes (Figure 16) provided with holes to allow oxygenation. Every 2-3 days they were humidified with water using a discardable Pasteur pipette. Boxes were kept for 30 days at room temperature in order to allow the fungi to grow and larvae to develop (Paz Silva, personal communication, November 13, 2017).

Figure 16 - Plastic boxes with faecal samples for faecal culture method (original).

Once the 30 days have elapsed, the larvae are collected using a modified Baermann technique (Vilá Pena, 2017). First, a 12 ml centrifuge tube is placed at the end of a rubber tube attached to a funnel. Following the assembly of the apparatus, all the faecal content from each box is collected, put on a filter paper with 200 µm pore diameter and put on the top of the funnel. The apparatus is then filled with tepid water until it covers the faeces, allowing the larvae to migrate

from the faeces to the water and then to the end of the centrifugal tube (Figure 17). After 12 to 24 hours, each tube is collected and centrifuged at around 1000 rpm for 5 minutes. Supernatant is removed using a vacuum pump until around 2 ml to 5 ml are left in the tube, depending on the amount of deposit left. The content of each tube is mixed and a 100 µl aliquot is retrieved using a micropipette. This aliquot is then analysed between coverslip and glass slide in an optical microscope for the presence and identification of larvae according to the keys suggested by Madeira de Carvalho et al. (2008). All the content of each tube was analysed according to this method.

Figure 17 - Assembled Baermann apparatus for the retrieval of larvae (original).

3.4 DATA STORAGE AND STATISTICAL ANALYSES

Data obtained in the current study were stored in a Microsoft Excel 2016® datasheet and analysed using The R Project for Statistical Computing version 3.4.1. The Shapiro-Wilk test was used to test the normality of the samples for both CPG and EPG.

CPG in the first and second assessments was transformed using a Log_{10} base. The Welch Two Sample T-Test was applied to see if there were differences between CPG counts in the two assessments.

EPG for each horse group were analysed as a total (from November to March) and in each individual month. The Welch Two Sample T-Test was applied if the results were normal and a Mann-Whitney U-test when not, signalling differences between groups T and C.

Chi-square tests were applied for larvae population present in November and January with the same aim described above.

All tests were conducted at a significance level of 5% ($p < 0.05$).

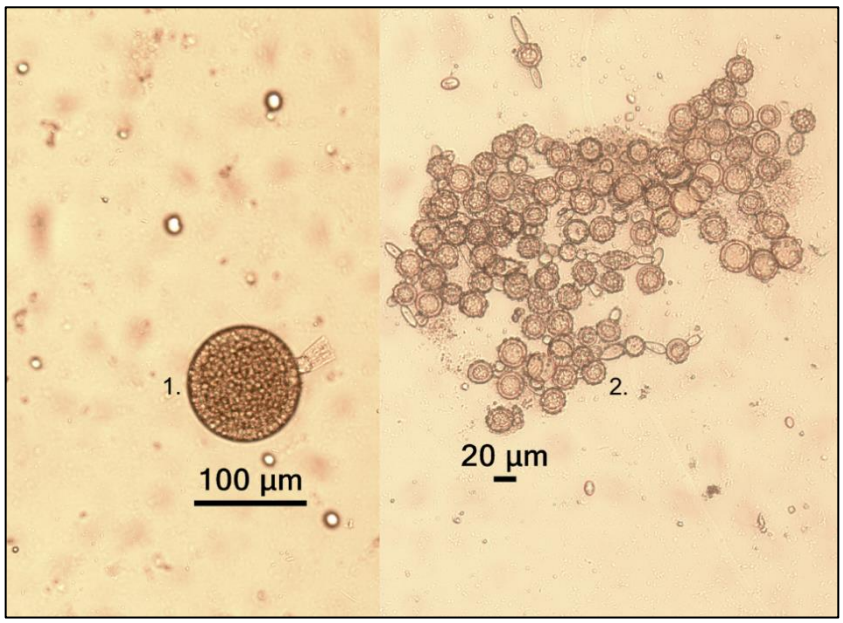
CHAPTER 4 – RESULTS

4.1 PRODUCT MANUFACTURE AND SPORE VIABILITY

Lyophilized product weight ranged from 4 to 7 g, depending on the mould format and number of nutritional pellets used.

In the first assessment of spore viability after production, all 20 samples of product contained seemingly unaltered and viable spores of *D. flagrans* and *M. circinelloides*. One of the samples showed an undamaged *M. circinelloides* zygospore, as shown in Figure 18. *D. flagrans* chlamydospores were counted and averaged 1.5 x 10^4 CPG (*SD* = 14448). Thus, 10 g of product administered *per os* to each horse should guarantee a minimum ingestion of 10^5 chlamydospores.

Figure 18 - *M. circinelloides zygospores* (1.) and *D. flagrans* chlamydospores (2.) present in product (original).



Three months after storing the product in a plastic box, no degradation, alteration, abnormal odour, colour or macroscopic fungi growth were observed. Spores of *D. flagrans* and *M. circinelloides* did not show any change regarding their morphology. Ten assessments of chlamydospores concentration showed an average of 2.2 x 10^4 CPG (*SD* = 5302), showing a narrower range of results than the first assessment. The results for both the first and second assessment of chlamydospores concentration are scrutinised in Appendix A.

Log_{10} results were found normal under Shapiro-Wilk test ($w = 0.97$, $p = 0.5744$) and statistically different between both assessments with Welch Two Sample T-Test ($t(26) = -3.79$, $p < 0.001$).

4.2 FUNGAL GROWTH *IN VITRO*

After 10 days, all 20 Petri dishes assembled with lyophilized product showed growth of new *D. flagrans* hyphae and chlamydospores, as well as *M. circinelloides* hyphae and zygospores, outside the initial central area. It is important to note that growth was substantially more exuberant in ATC medium than AAC medium (Figure 19 and Figure 20).

Figure 19 - Macroscopic aspect of fungi growth in Petri dishes (original). a) Example of growth in ATC medium. b) Example of growth in AAC medium.

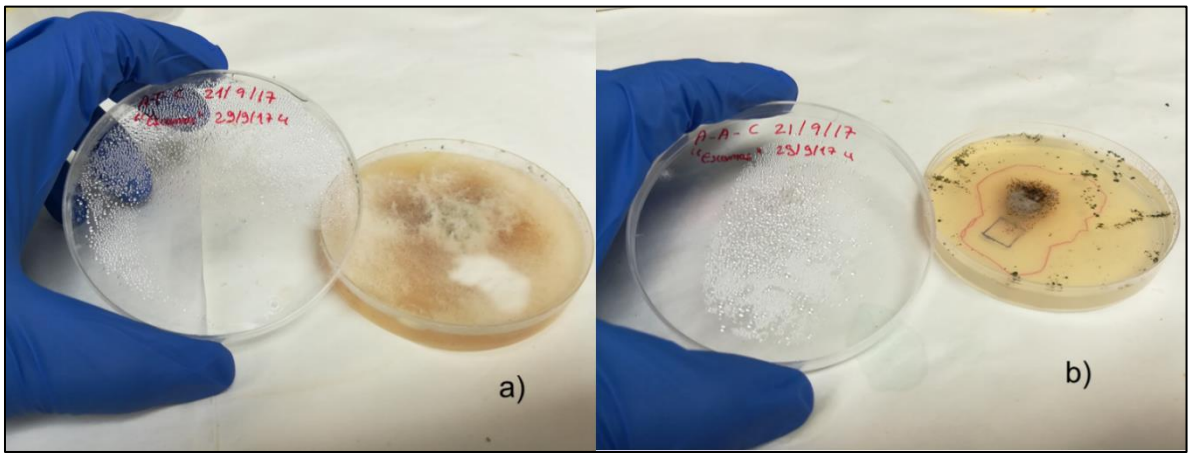
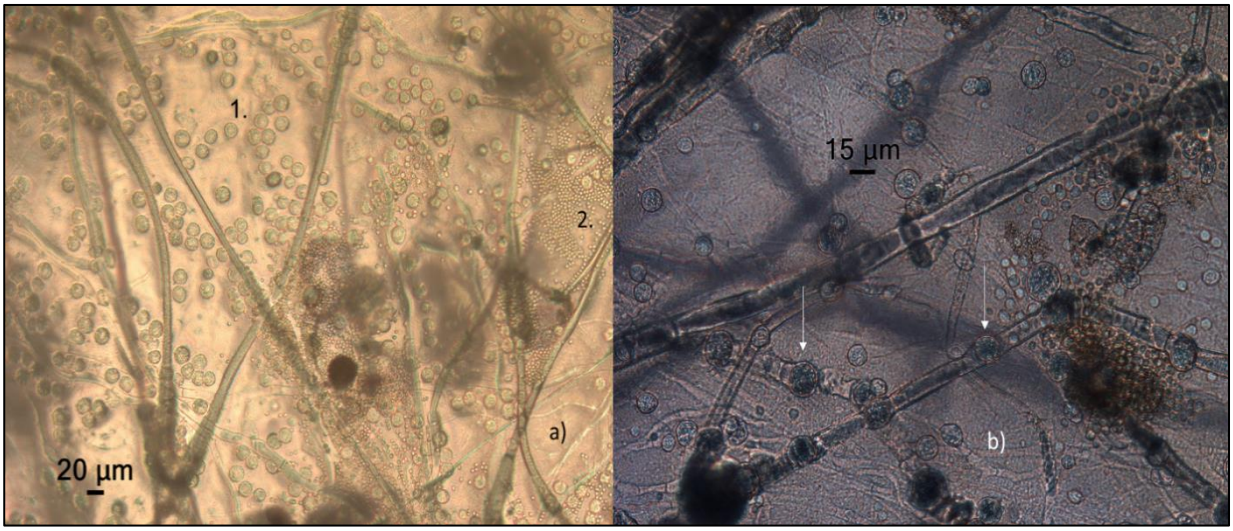


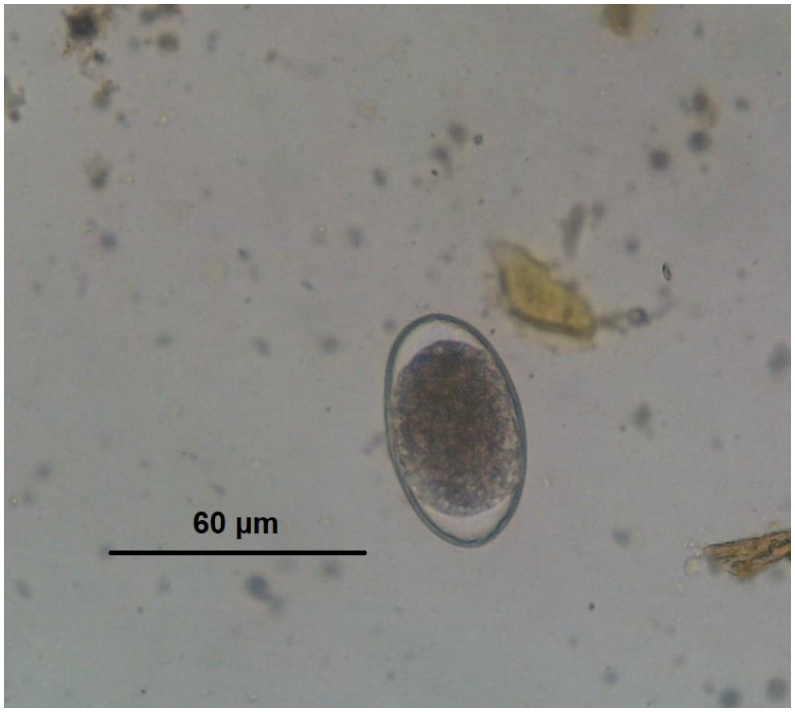
Figure 20 - Microscopic aspect of fungal growth in Petri dishes (original). a) *D. flagrans* conidia (1.) and *M. circinelloides* spores (2.) present in ATC medium. b) White arrows indicate the formation of new *D. flagrans* chlamydospores from the present hyphae present in AAC medium.



4.3 FUNGI INFLUENCE IN HORSES' FEC

In the first FEC performed on 26th September 2017 all horses in both groups T and C surpassed the 300 EPG cut-off for gastrointestinal nematodes, namely strongyle eggs (Figure 21). No other parasite eggs or larvae were observed throughout this study. All horses were then treated with Ivermectin pour-on due to these results.

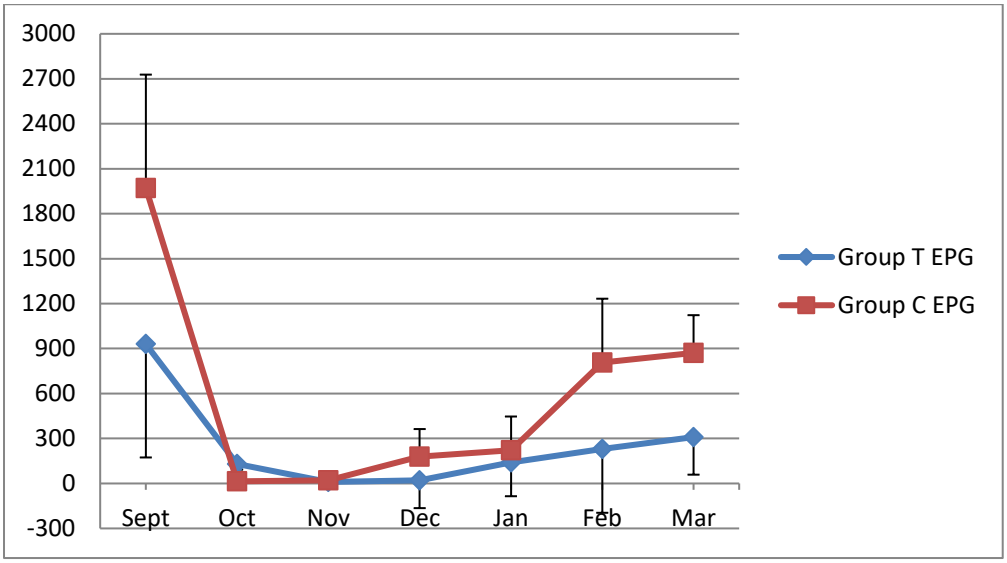
Figure 21 – Horse strongyle egg observed with modified McMaster Technique (original).



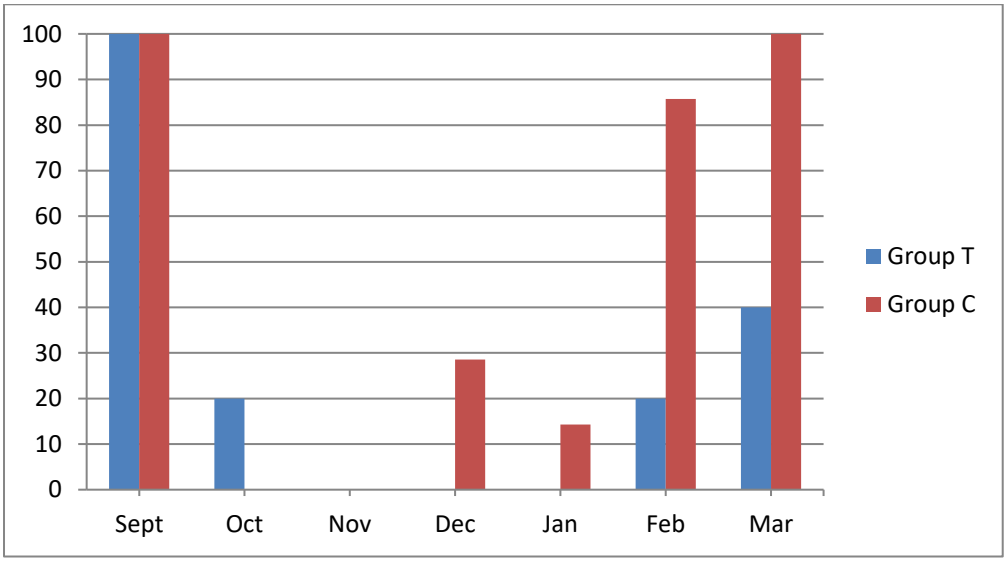
Thirteen days after treatment with Ivermectin, the second FEC showed that only one horse in group T (horse 7487) had 650 EPG and one horse in group C (horse 7020) had 100 EPG. All other horses had no observable eggs in the faecal samples. Due to these results, horse 7487 in group T was treated again with Ivermectin pour-on.
On 23rd October all horses were assessed again for FEC and only the horse 7020 in group C showed the excretion of parasite eggs in faeces. This assessment was performed 4 weeks after the treatment with Ivermectin for all horses, 2 weeks for horse 7487 in group T.

The FECs of all horses in group T was below the 300 EPG cut-off until February 2018, lasting two more months without the need for treatment than the horses in group C. In the control group, in December two horses had already surpassed the 300 EPG cut-off. In February only one horse in group T exceeded the cut-off, in contrast to what happened in group C where all, but one had already gone over it. The EPG average of both groups throughout the study are represented in Graphic 1 and percentage of horses exceeding the cut-off are shown in Graphic 2. Individual FEC results are scrutinised in Appendix B.

Graphic 1 - Average EPG for groups T and C from September 2017 to March 2018.



Graphic 2 – Percentage of horses exceeding 300 EPG cut-off in group T and group C.



Statistically significant differences between group T and C FEC were found overall, from November to March (w = 0.79, p < 0.001 and U = 600.5, p = 0.01391), February (w = 0.93, p = 0.3763 and $t(9)$ = 3.13, p = 0.0121) and March (w = 0.94, p = 0.5422 and $t(10)$ = 4.32, p = 0.001573).

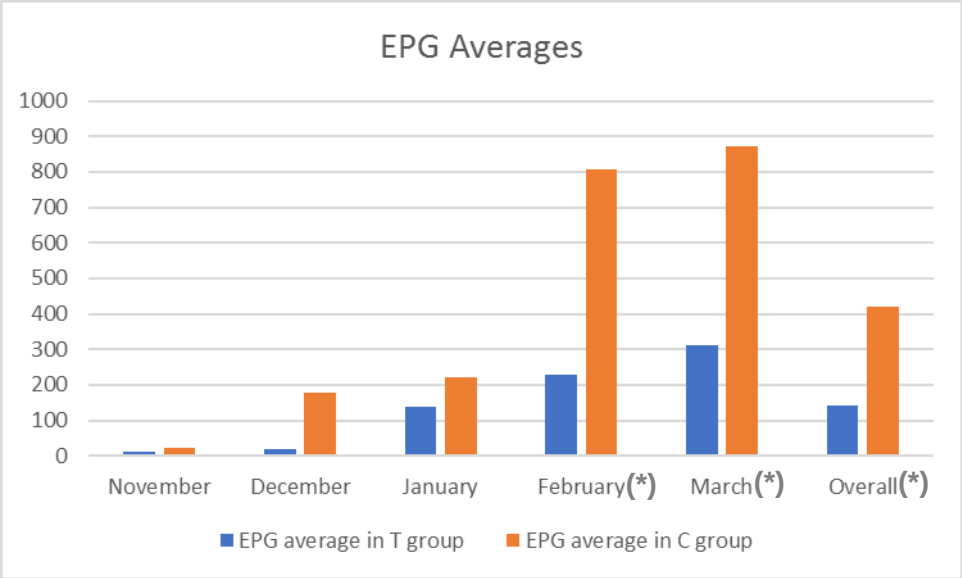
No statistically significant differences between groups were found in November (w = 0.61, p < 0.001 and U = 21.5, p = 0.4879), December (w = 0.73, p = 0.001704 and U = 29.5, p = 0.0528) and January (w = 0.68, p < 0.001 and U = 21, p = 0.6187).

To apply the reduction formula described in the Material and Methods section, from November to March, an arithmetic mean of all horses' EPG from each group was obtained. These results are displayed in Table 2 and Graphic 3.

Table 2 - Average EPG for groups T and C from November 2017 to March 2018, overall and respective % Reduction. (*) highlights where EPG was statistically different between groups.

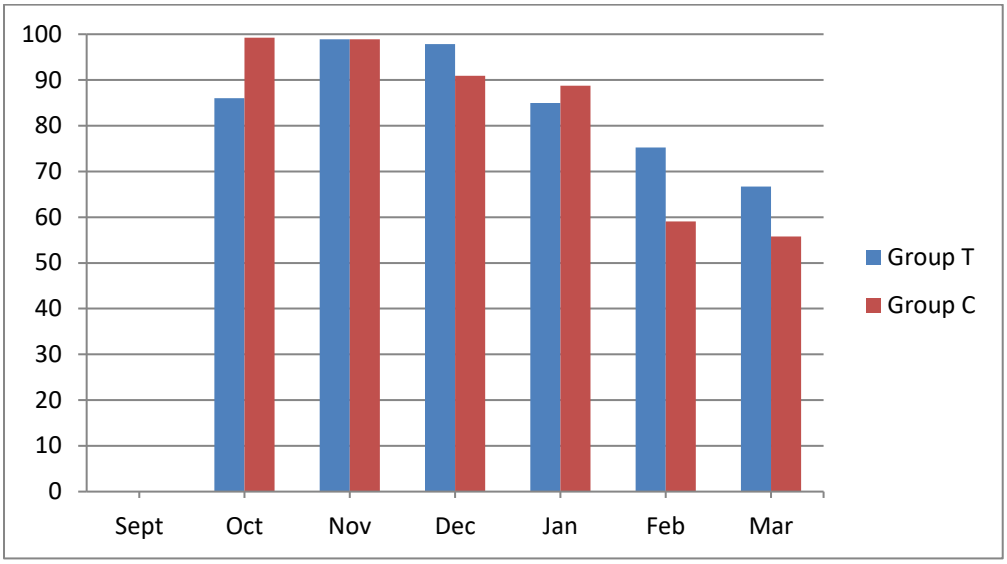
	Average EPG in group T	Average EPG in group C	% Reduction
November	10	21	53
December	20	179	89
January	140	221	37
February (*)	230	807	72
March (*)	310	871	64
Overall (*)	142	420	66

Graphic 3 - Average EPG for groups T and C from November 2017 to March 2018 and overall. (*) highlights where EPG was statistically different between groups.



FECRT (Graphic 4) was 99% for the first two months (around 7 weeks) after treatment with anthelmintics in both groups except in October for group T due to the already mentioned case of horse 7487.

Graphic 4 – FECRT results for group T and C.



As observed in Graphic 4, an ERP of 16 weeks was found in both groups.

It is important to note that *D. flagrans* chlamydospores and *M. circinelloides* spores were found in group T faecal samples and no horse rejected the product. No clinical signs associated with parasite activity or adverse reaction to product was recorded for the duration of this study.

4.4 LARVAE POPULATION

Only larvae belonging to the subfamily Cyathostominae were found in the faecal cultures from November and January.

In November, 73.9% of the collected larvae in the group T were identified as *Cyathostomum* spp. type A (Figure 22), 14.5% *Cyathostomum* spp. type C (Figure 23) and 11.6% *Cyathostomum* spp. type D (Figure 24). In group C 74,2% of the larvae were *Cyathostomum* spp. type A, 14.8% *Cyathostomum* spp. type C and 11% *Cyathostomum* spp. type D. Chi-square test, x^2 (2, N = 539) = 0.058, p = 0.9715, showed no stastically significant difference between both groups.

In January, no *Cyathostomum* spp. type C was found and *Cyathostomum* spp. type D was found only in group C. Group T only had *Cyathostomum* spp. type A (100%) larvae, while group C had 94.8% of the larvae identified as *Cyathostomum* spp. type A and 5.2% were *Cyathostomum* spp. type D. Chi-square test, x^2 (1, N = 726) = 8.88, p = 0.002876, showed a statistically significant difference between both groups.

Figure 22 – *Cyathostomum* spp. type A, with 8 intestinal cells (first 2 cells as a pair side by side, followed by 6 cells in a single row) (original).

Figure 23 - *Cyathostomum* spp. type C, with 8 intestinal cells (first 4 cells as pairs side by side, followed by 4 cells in a single row) (original).

Figure 24 - *Cyathostomum* spp. type D, with 8 intestinal cells (8 cells in a single row) (original).

CHAPTER 5 - DISCUSSION

The present study aimed to develop a new viable formula for oral administration of parasiticide fungi for the control of gastrointestinal nematodes in horses. The proposed formula of lyophilized product could be a viable alternative in pasturing horses or other type of horse management like stabulation, working as a treat or a reward for behaviour, without the need for constant administration.

5.1 PRODUCT MANUFACTURE AND SPORE VIABILITY

A correct storage and conservation method of parasiticide fungi is essential to maintain their activity after prolonged storage periods (Mota, Campos, & Araújo, 2003) and lyophilization is one the best techniques to keep fungi viable, stable and unspoiled while stored (Bosmans, 1974).

Santurio et al. (2009) reported the first use of lyophilization in *Duddingtonia flagrans* chlamydospores, where their resistance to the process was demonstrated, even though a 10-fold reduction after treatment occurs. Bosmans (1974) had already reported a successful lyophilization in *Arthrobotrys* genus. *D. flagrans* was previously classified as *Arthrobotrys flagrans* (Barron, 1979) but Bosmans (1974) does not specify the species. *Mucor circinelloides* is also able to resist lyophilization process (Von Arx & Schipper, 1978; Bosmans, 1974). Beside these studies, there is no information about the ability of a blend of spores of *D. flagrans* and *M. circinelloides* to survive lyophilization while already inserted in a gelatinous substance. Lyophilizing products containing the fungal spores allow their direct administration to animals, avoiding the troubles of appropriate dosage and preservation of freshly prepared gelatine capsules as indicated by Santurio et al. (2009).

Lyophilized product remained unchanged for more than a three months period and equally treated products with fungi remain unspoiled up to six months (Bunse & Steigleder, 1991) or even 10 years (Bosmans, 1974). This seems to point that fungi do not develop while the product was stored in the plastic box. The presence of visible hyphal growth would hinder compliance from farmers when it comes to feeding the animals with these products.

The differences in the two assessments could be explained by the different sample size between them (20 in the first and 10 in the second) and their small number (De Winter, 2013). Is it required to recall that it was used a minimum concentration of chlamydospores from the liquid medium. With this in mind, different product samples could have had more spores than others before lyophilization. We can also take into account the size of the product that is assessed. A 0.03 g sample can be very small and different people did the assessments, possibly leading to distinct ways of randomly selecting them.

There is no commercial product, with parasiticide fungi, that is developed and sold in Europe with the aim of being used for the control of human or animal parasites (Arroyo Balán, 2017). The lyophilization procedure would allow manufacturers, mainly industrial factories, to develop a stable product that can compete with already existing ones. It should be emphasized that industrial animal nutritional pellets industrially manufactured, should present a shelf life of three months (Arroyo Balán, 2017). Easier storage is also an advantage for farmers as lyophilized products wouldn't need any kind of refrigeration, unlike fresh gelatines vehicles (Vilá Pena, 2017), needing only to be stored in a slightly dry environment like a sealed plastic box.

5.2 *IN VITRO* GROWTH

It has been stated that nutrient-rich media such as ATC stimulate the growth of hyphae, while nutrient-poor media like AAC do it for conidia, spores and chlamydospores (Kamp & Bidochka, 2002). This would explain the macroscopic difference between fungi growth in ATC and AAC media, where the former has a more exuberant macroscopic development compared to the later. Although *D. flagrans* and *M. circinelloides* showed no sign of antagonism, there are other blends of soil fungi showing it, preventing each other to develop in an optimal way (Arias, Cazapal-Monteiro, Suárez, et al., 2013).

After heat treatment in the manufacturing of nutritional pellets, the spores of *D. flagrans* and *M. circinelloides* developed new hyphae 5 and 4 days, respectively, and new spores by 17 and 8 days, respectively, after being assembled in a solid medium containing agar and wheat (Arroyo Balán, 2017). Opposite to this, without heat treatment, Arias, Cazapal-Monteiro, Suárez, et al. (2013) showed the growth of new *D. flagrans* and *M. circinelloides* as soon as 7 days after inoculation in the agar-wheat solid medium. Grønvold et al. (1996) showed the presence of new *D. flagrans* chlamydospores after 5 days in Corn-Meal-Agar medium. The results of the present study seem to be in agreement with the last two studies, showing the presence of new spores from both species in all plaques, after 10 days. Fungi could be relatively less affected by lyophilization and develop faster than the ones in which the heat treatment applied in pellet fabrication was used, but this should be further tested under similar conditions. This could be an advantage, due to a faster fungi development over the eggs and larvae present in the faeces.

5.3 EFFECT ON HORSE FEC

Only strongyle eggs were found in all horses from September to March. These results agree with the ones in this area of Spain for pasturing horses (Francisco, I. et al., 2009; Vilá Pena, 2017). Since only strongyle eggs were observed, treatment with Ivermectin was correctly applied, as recommended for this parasitological status (Bowman, 2014). On 9th October, following the treatment with Ivermectin, all animals had an FEC below the 300 EPG cut-off except one. This could have been due to the difficult to estimate the appropriate weight of the horses (indigenous) and, as a consequence, their right dosage. The second treatment (10 mg of Ivermectin per 5 kg of body weight) led to a total reduction of EPG, supporting the previous statement. FECRT results of 99% are in conformity with what is expected to happen when strongyle population is still susceptible to treatment with Ivermectin (AAEP, 2013). These results are in line with Francisco, I. et al. (2011), Hernández et al. (2016) and Vilá Pena (2017) regarding the successfulness of Ivermectin pour-on agaisnt strongyle infections.

The ERP of about 16 weeks found in both groups is higher than the ones usually reported for Ivermectin (AAEP, 2013; Lyons et al., 2011). Not only this would mean that treatment was effective agaisnt adults but also encysted L3 due to ERP being higher than prepatent period of strongyles. This would lead to believe that egg-shedding after treatment would be due to the development of new larvae infecting horses after grazing. It is important to remember that a switch between padocks was made in November, so horses from group T switched to group C padock and vice versa. Although *D. flagrans* was not able to withstand in the environment for more than 2 months after being released in the faeces (Saumell et al., 2016), the fungi would still be able to act for these 2 months after the switch, reducing the number of larvae present and able infect horses in group C. The FECs in February and March were already made without the influence of fungi in the environment for group C, contrary to what happened to group T, leading to an higher increase in EPG in the former.

In Oceanic climate areas, such as those used in this research, there is a rise in FEC from most parasites in spring and autumn (Nielsen et al., 2007). An increase in FECs for all horses in February and March is consistent with the life cycle and pathology previously described for strongyle parasites because their ability to release more eggs when environmental conditions become more adequate for larvae development (Love et al., 1999; Taylor et al., 2016). Despite surpassing FEC cut-off, the horses were not treated again with anthelmintics to observe the effect of the fungi once EPG starts to increase.

Using the lyophilized product, FEC ranged from an average 807 EPG in group C to 230 in group T, which means a 72% reduction in February, and from 871 EPG in group C to 310

EPG in group T, a 64% reduction in March. Statistically significant differences in the FECs were observed during these two months, just like with the overall FECs, with a reduction of 66% between both groups. Horses from group T had FECs below the threshold of 300 EPG for three months longer than in group C, which would mean a decrease of two or three treatments with anthelmintics over the course of a year. As pointed before, a simple usage of targeted treatments with FEC techniques would reduce the anthelmintic usage by up to 82% (Lester et al., 2013), so a combined use of this treatment with parasiticide fungi could be a major benefit in parasite control. Due to the only difference between the two groups of horses consisted of receiving lyophilized product with parasiticide fungi, it seems correct to assume that they keep their activity despite the lyophilization treatment, as demonstrated in *in vitro* assays. Accordingly, this represents a viable and alternative formula of administration to those that already exist.

A reduction of 69% of *Haemonchus contortus* larvae in water agar media by using lyophilized *D. flagrans* chlamydospores, when compared to the control group, has been reported. This study showed that their ability to resist passage through the sheep gastrointestinal tract while keeping their parasitic activity (Santurio et al., 2009). Later, Santurio et al. (2011) reported that a group of sheep receiving lyophilized chlamydospores (daily average of 10^6 per animal) was able to keep FECs of Trichostrongylidae below 500 EPG for 12 months and had statistically significant differences between control and test groups, before the former being treated with anthelmintics. The lyophilized spores of *D. flagrans* showed no decrease in its parasiticide activity at different temperature ranges following passage through sheep intestinal tract (Buske, 2010).

Other fungi used in biological control such as *Arthrobotryis oligospora* (Nalepina, Matskevich, Kozhukhar & Teplyakova, 1990), *Arthrobotriys musiformis* (Garcia, 2007), *Arthrobotryis* robusta and *Monacrosporium thaumassium* (Mota et al., 2003) were already stored using a lyophilization process and tested with success. This shows the possibility of applying this treatment with other parasiticide fungi better suited for different situations and environments.

Despite the rising number of studies with parasiticide fungi, there are only a few studying the action of larvicide and ovicide fungi on parasites when mixed and used together. The combined use of both types of parasiticide fungi could help to enlarge the spectrum of action and even support the final effect, where ovicide fungi would damage parasite eggs and destroy the embryos while larvicide fungi would act over the larvae that were still able to develop (Arroyo Balán, 2017). Some of these studies use the same fungi strains as the ones from the present work but with different administration formulas, concentration and frequency.

Vilá Pena (2017) worked with the same *D. flagrans* and *M. circinelloides* strains but inserted in fresh edible gelatines vehicles, without any additional treatment after gelation. From November to May, spores were given two days a week (Tuesday and Friday) to the same horses used in the present work, with the control group also being the same. Each horse received an average of 4 x 10^5 chlamydospores each administration, meaning 8 x 10^5 per week. Comparing to the present work it was used a doubled amount of chlamydospores. The EPG counts were statistically different between the groups from December to May, more specifically March, April and May, similar to the ones observed in the present study. Reduction in EPG for the group receiving chlamydospores reached 65% when compared to the control group, compared to the 66% (72% when significant) in the present study. FECRT in the test group decreased from 96% in December to 62% January and 21% in February, a more significant decrease than in the present study where it went from 98% to 85% and then 75%. These results are very similar despite a different anthelmintic administration and a lower frequency of administration but higher numbers of chlamydospores in the referred study. Besides, as soon as there is a steady increase in temperature, it is noted a higher predatory activity, as remarked by Madeira de Carvalho et al. (2008), which can also explain an increase of fungi development and consequently a higher parasiticide activity in February and March.
It should be reminded that the fresh gelatines used by Vilá Pena (2017) needed refrigeration after being produced and would only last about 3 weeks, in contrast with the minimum 3 months of lyophilized product conservation at room temperature. Despite the higher temperature when comparing refrigeration and room temperature, it has been shown this has no effect on fungi parasiticide activity (Fitz-Aranda et al., 2015). Regardless of the higher cost and time involved in the production, lyophilized products have the big advantage of an easier storage and longer shelf-life.

Another study with the same fungi strains was performed by Hernández et al. (2016) but the spores were added to nutritional pellets during the industrial manufacturing. Horses in the test group were fed on a daily basis with 2.5 kg of pellets containing 2 x 10^6 *D. flagrans* chlamydospores and 2 x 10^6 *M. circinelloides* spores per kg. A total of 5 x 10^6 chlamydospores were given each day, 35 x 10^6 per week, almost over 100 times the amount used in the present work. With this, the referred study was able to keep strongyle egg output below 300 EPG for over 64 weeks after an anthelmintic treatment in the test group and some statistically significant differences were also found between the test and the control groups. This administration formula was more effective in keeping EPG values below the established cut-off than the one in the present work, but the differences appear to be related to a more frequent and larger administration dose of fungi. Although *D. flagrans* is capable of reducing cyathostomin egg output with a lower concentration of chlamydospores as well as with a higher concentration, the higher frequency and the 100-fold amount of chlamydospores given to horses can explain

the difference between the present and preceding study of Hernández et al. (2016) (Paz-Silva et al., 2011).

A similar study in horses, reported by Braga et al. (2009), giving *D. flagrans* mycelium in sodium alginate pellets, two times a week, achieved a 46.2% average reduction in cyathostomin EPG after three months. In a field trial conducted in Portugal consisting of daily feeding horses with 5 x 10^5 chlamydospores/kg of body weight mixed in the feed premix, no statistical differences in the EPGs between treated and not treated groups was obtained, but a reduction of over 60% in the group averages was observed (Madeira de Carvalho et al., 2011).
Both these studies used a different strain of *D. flagrans* than the one used in the present study, AC001 in Braga et al. (2009) and Troll A in Madeira de Carvalho et al. (2011), which could explain some of the differences in the results. Even though the frequency in which chlamydospores were given to the horses by Madeira de Carvalho et al. (2011) is similar to the one reported by Hernández et al. (2016), the total number of spores fed was increased by 20-fold the amount per week. Taking into account that administration formula and fungus strain were also different, the results were significantly different giving the idea of a more effective formula or a strain with more parasiticide activity.

Other studies have been researching the use of *D. flagrans* and *M. circinelloides* in the control of horse parasites with relative success, just like it happens with livestock parasites.
The formula used in this present study allowed us to achieve comparable results to other studies already performed, even with a lower concentration of fungi spores and lower frequency of administration, showing a valuable promise for future product development. This formula can be applied when horses are not fed daily with pellets, as a treat, for sport and teaching horses.

5.4 LARVAE POPULATION

The larvae population in November was similar in both groups and only Cyathostominae specimens were found. These results agree with previous studies reporting that 95-99% of the strongyle eggs in horses belong to cyathostomins (AAEP, 2013; Reinemeyer, Smith, Gabel, & Herd, 1984). *Cyathostomum* spp. type A was the most common, followed by *Cyathostomum* spp. type C and *Cyathostomum* spp. type D.

Vilá Pena (2017) found similar results with the same horse groups, with the exception that *Gyalocephalus capitatus* larvae were also identified. Madeira de Carvalho (2001) found in horses that 75% of the *Cyathostomum* L3 belonged to *Cyathostomum* spp. type A, 12% to *Cyathostomum* spp. type C and 10% *Cyathostomum* spp. type D, very close to the ones reported in the present study. The presence of fungi in group T appeared to have no difference in larvae type ratio between groups.

In January a different scenario happened with the absence of *Cyathostomum* spp. type C in both groups and no *Cyathostomum* spp. type D in group T. *Cyathostomum* spp. type C larvae belong to *Cylicostephanus longibursatus*, *Cylicostephanus calicatus* and *Cylicostephanus hybridus* (Madeira de Carvalho et al., 2008).

The absence of *Cyathostomum* spp. type D in group T is significant and explains the observable differences between the two groups in January. Thus, new studies should be performed to test if *D. flagrans* and *M. circinelloides* affect different *Cyathostomum* spp. types of larvae and eggs in distinct ways.

CHAPTER 6 - CONCLUSION

This study allowed the successful development of a new viable formula for oral administration of parasiticide fungi spores to prevent the infection by gastrointestinal nematodes in pasturing horses, increasing the range of possibilities for future product development and application. Thus the aims for this study have been achieved:

1. Development of lyophilized product with viable *D. flagrans* chlamydospores and *M. circinelloides* spores.
2. Regular *in vitro* growth of the fungi after product manufacture.
3. Fungi kept their predatory activity against gastrointestinal nematodes as significant differences were found between both horse groups EPG.

Biological control continues to be one of the best complementary methods to be used in parasite control in order to reduce the frequency of parasiticide treatments and thus anthelmintic resistance. By developing new formulas for the distribution of parasiticide fungi it will allow manufacturers and farmers the choice to apply it in different situations, making easier a better and more rational use of anthelmintic treatments.

Research on parasiticide fungi for biological control of parasites will continue to be developed as more formulae for their administration, new schedules of administration and new fungi strains are discovered. In the future, research will provide the industrial development of products for an easy access as well as more choices, so that every situation can have a wide range of products to choose from.

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APPENDIX A – Chlamydospores concentration assessment.

First Concentration Assessments (CPG)	Second Concentration Assessments (CPG)
10560	23620
7920	17000
58080	18600
11000	15500
8360	27900
8600	22300
11666	18440
5720	32750
9240	21000
11440	24800
9300	
8888	
3960	
7920	
7333	
24200	
15555	
20000	
51480	
9240	

APPENDIX B

		FEC (EPG) 26/09/2017	FEC (EPG) 09/10/2017	FEC (EPG) 23/10/2017	FEC (EPG) 13/11/2017	FEC (EPG) 14/12/2017	FEC (EPG) 23/01/2018	FEC (EPG) 08/08/2018	FEC (EPG) 14/03/2018
T group	**5055**	800	0	0	0	0	50	0	150
	7481	550	0	0	0	50	150	150	300
	7487	300	650	0	50	50	150	550	650
	7488	2300	0	0	0	0	150	250	200
	9001	700	0	0	0	0	200	200	250
C group	**Ojitos**	1200	0	0	0	100	200	900	850
	7020	2850	100	100	50	500	700	650	750
	7482	1300	0	0	0	200	100	850	950
	7483	1250	0	0	0	50	250	500	800
	7484	1800	0	0	50	0	200	1000	1250
	7486	2650	0	0	50	350	50	1550	1050
	9000	2750	0	0	0	50	50	200	450