UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

INTEGRATED CONTROL OF AVIAN GASTROINTESTINAL PARASITES: OPTIMIZING THEIR COPROLOGICAL DIAGNOSIS AND BIOCONTROL USING PREDATORY FUNGI

JOÃO MIGUEL PESTANA LOZANO

Orientador(es): Professor Doutor Luís Manuel Madeira de Carvalho

Professora Doutora Maria Manuela Castilho Monteiro de Oliveira

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Sanidade Animal

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Título da Tese ou Dissertação: Integrated control of avian gastrointestinal parasites: optimizing their coprological diagnosis and biocontrol using predatory fungi

Ano de conclusão (indicar o da data da realização das provas públicas): 2025

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“Life is made of giving and receiving”

Traditional Portuguese saying

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CONTROLO INTEGRADO DOS PARASITAS GASTROINTESTINAIS DAS AVES: OTIMIZAÇÃO DO SEU DIAGNÓSTICO COPROLÓGICO E BIOCONTROLO USANDO FUNGOS PREDADORES

Resumo

Nos últimos 30 anos registaram-se melhorias no controlo do parasitismo gastrointestinal (GI) dos animais, incluindo o desenvolvimento de métodos de diagnóstico coprológico mais sensíveis e precisos, como o Mini-FLOTAC (MF), e a utilização de fungos predadores como uma solução sustentável de controlo parasitário. Este projeto procurou otimizar o método MF para o diagnóstico do parasitismo GI avícola, e isolar fungos predadores e avaliar as suas potencialidades para uso no controlo parasitário nas aves. Procedeu-se primeiro ao diagnóstico do parasitismo GI numa exploração avícola e em três coleções de aves exóticas, em Portugal continental, com recurso ao método MF. Identificaram-se infeções por *Eimeria* spp. em galinhas poedeiras e pavões, *Capillaria* spp., *Trichostrongylus tenuis* e *Strongyloides pavonis* em pavões, bem como *Libyostrongylus douglassii* em avestruzes e emas. Foram isolados sete fungos filamentosos das fezes dos galiformes, posteriormente identificados como *Mucor circinelloides* (n=6) e *M. lusitanicus* (n=1). Todos os fungos revelaram aptidão para destruir oocistos de *Eimeria* spp. *in vitro*, sendo que o isolado *M. circinelloides* FR1 alcançou a maior eficácia coccidicida, 22%, após 14 dias de exposição. Avaliou-se também a suscetibilidade *in vitro* destes fungos face a sete antiparasitários (albendazol, fenbendazol, levamisol, ivermectina, lasalocida, amprólio e toltrazuril), e nenhum revelou ser suscetível aos antiparasitários testados, independentemente das suas concentrações. A caracterização do perfil de virulência de *M. circinelloides* FR1 revelou apenas a presença de genes codificadores de recetores de ferro (FOB1 and FOB2), permease de ferro (FTR1), fatores de ribosilação do ADP (ARF2, ARF6) e GTPase (CDC42), sendo que o isolado apenas testou positivo para a produção de lecitinase. Realizaram-se administrações orais dos esporos deste isolado fúngico em galinhas poedeiras e pavões, e que revelaram que este fungo não afetou a aparência e consistência das fezes das aves (70-100% de fezes normais), nem as abundâncias relativas e diversidades-alfa de bactérias e fungos nativos do seu trato digestivo. Por fim, verificou-se que a administração de esporos deste fungo a pavões reduziu significativamente as cargas fecais de *Eimeria* spp. até 92%, após 2 meses de ensaio. Os resultados deste projeto revelam a utilidade do MF no diagnóstico coprológico do parasitismo GI em aves, e representam o primeiro relato sobre o isolamento de fungos predadores a partir de fezes de aves. Por fim, demonstrou-se a segurança e eficácia de *M. circinelloides* FR1 no controlo integrado do parasitismo GI em aves domésticas e exóticas.

Palavras-chave: Aves, Parasitas Gastrointestinais, Mini-FLOTAC, Fungos Predadores.

INTEGRATED CONTROL OF AVIAN GASTROINTESTINAL PARASITES: OPTIMIZING THEIR COPROLOGICAL DIAGNOSIS AND BIOCONTROL USING PREDATORY FUNGI

Abstract

In the last 30 years, several improvements were achieved in the control of animals' gastrointestinal (GI) parasitism, including the development of more sensitive and precise coprological diagnosis techniques, like Mini-FLOTAC (MF), as well as the use of predatory fungi as a sustainable solution for parasite control. This project aimed to optimize the MF method for the diagnosis of avian GI parasitism, and to isolate predatory fungi and assess their potentialities in avian parasite control. First, the diagnosis of GI parasitism was performed in a poultry farm and three exotic bird collections, in Portugal mainland, using the MF technique. *Eimeria* spp. infections were identified in laying hens and peacocks, *Capillaria* spp., *Trichostrongylus tenuis* and *Strongyloides pavonis* in peacocks, as well as *Libyostrongylus douglassii* in ostriches and emus. Seven filamentous fungi were isolated from Galliformes feces, furtherly identified as *Mucor circinelloides* (n=6) and *M. lusitanicus* (n=1). All fungi were capable of destroying *Eimeria* spp. oocysts *in vitro*, with *M. circinelloides* isolate FR1 achieving the highest coccidicidal efficacy, 22%, after 14 days of exposure. All fungi were checked for potential *in vitro* susceptibilities to seven antiparasitic drugs (albendazole, fenbendazole, levamisole, ivermectin, lasalocid, amprolium and toltrazuril), and all were not susceptible to the tested antiparasitic drugs, independently of their concentrations. The characterization of the virulence profile of *M. circinelloides* FR1 revealed only the presence of genes coding for iron receptors (FOB1 and FOB2), iron permease (FTR1), ADP-ribosylation factors (ARF2, ARF6) and GTPase (CDC42), having the isolate only tested positive for lecithinase production. Oral administrations of spores of this fungal isolate were performed in laying hens and peacocks, which revealed that this fungus did not affect birds' feces appearance and consistency (70-100% of normal feces), neither the relative abundances and alfa-diversities of their native gut bacteria and fungi. Finally, it was verified that the administration of this fungus spores to peacocks reduced significantly their *Eimeria* spp. fecal shedding up to 92%, after two months of trial. Results from this project point out the usefulness of MF in the coprological diagnosis of GI parasitism in birds, and provide the first report regarding the isolation of predatory fungi from bird feces. Finally, the safety and efficacy of using *M. circinelloides* FR1 in the integrated control of domestic and exotic birds GI parasitism was demonstrated.

Keywords: Birds, Gastrointestinal Parasites, Mini-FLOTAC, Predatory Fungi.

CONTROLO INTEGRADO DOS PARASITAS GASTROINTESTINAIS DAS AVES: OTIMIZAÇÃO DO SEU DIAGNÓSTICO COPROLÓGICO E BIOCONTROLO USANDO FUNGOS PREDADORES

Resumo alargado

As aves domésticas e exóticas mantidas em explorações avícolas em modo de produção extensivo ou biológico, e em coleções ornitológicas públicas ou privadas, e instituições zoológicas, encontram-se expostas frequentemente ao mesmo ambiente durante um longo período, e são sujeitas a irregulares tratamentos com antiparasitários, promovendo uma maior exposição das aves a diversos agentes patogénicos, nomeadamente parasitas gastrointestinais (GI) como coccídias, capilarídeos, ascarídeos e estrongilídeos. Nos últimos 30 anos registaram-se importantes avanços nas metodologias de controlo do parasitismo GI dos animais, com o diagnóstico das infeções por parasitas GI com recurso a métodos mais sensíveis e precisos, como o Mini-FLOTAC (MF), e a utilização de fungos predadores com ação antiparasitária, a representarem duas das abordagens de controlo integrado parasitário mais estudadas em equídeos, ruminantes e animais de companhia, e sendo recentemente propostas para utilização em aves. O presente projeto de doutoramento visou a otimização do método MF para o diagnóstico do parasitismo GI avícola, e o isolamento de fungos predadores e avaliação da eficácia e segurança do seu uso no controlo parasitário nas aves.

A primeira fase deste projeto consistiu na implementação do método MF no diagnóstico de infeções por parasitas GI em diversas coleções de aves. Entre Julho 2020 – Abril 2021, foram colhidas 142 amostras fecais de galinhas poedeiras, pavões e ratites, pertencentes a quatro coleções de aves localizadas nos distritos de Lisboa e Santarém (Portugal Continental), tendo sido processadas com o método MF e culturas fecais para identificação de parasitas GI e determinação das respetivas cargas fecais (oocistos ou ovos por grama de fezes, OoPG ou OPG, respetivamente) e prevalências. Em paralelo, o método de McMaster foi utilizado também para determinação das cargas parasitárias fecais, e comparação com os valores obtidos com o método MF. Por fim, procedeu-se ao cálculo da sensibilidade e especificidade relativas, considerando o método de McMaster como de referência. A implementação do MF permitiu detetar a maior carga fecal média de coccídias do género *Eimeria* nos pavões da segunda coleção de aves (502 OoPG), seguidos dos pavões da primeira coleção (107 OoPG), galinhas poedeiras (24 OoPG), e pavões da terceira coleção (9 OoPG). Os diagnósticos com MF realizados nos pavões revelaram também infeções por *Capillaria* spp., *Trichostrongylus tenuis* e *Strongyloides pavonis*, enquanto as avestruzes e as emas testaram positivo para o nematode *Libyostrongylus douglassii*. O método MF alcançou sensibilidades e especificidades relativas superiores a 70% na deteção de infeções por *Eimeria* spp. em Galliformes, por helmintes nos pavões, e por *L. douglassii* nas ratites. Por

fim, o protocolo do MF para animais exóticos detetou as maiores cargas fecais de ovos de *L. douglassii* nas avestruzes, comparativamente com o método de McMaster e com os protocolos do MF para pequenos e grandes animais. Os resultados da primeira fase deste projeto permitiram concluir que o protocolo do MF para animais exóticos é a melhor alternativa ao convencional método de McMaster para o diagnóstico de infeções por parasitas GI em aves domésticas e exóticas.

Na segunda fase deste projeto pretendeu-se averiguar a utilidade das fezes das aves Galliformes analisadas na fase anterior, e também de fezes de frangos campestres positivas para coccídias, para isolamento de fungos com potencial ação antiparasitária. Um total de 58 amostras fecais foram cultivadas em Agar-Água e Agar-Trigo, para isolamento e purificação de colónias de fungos filamentosos. Todos os isolados foram identificados morfologicamente ao nível do género, com base na textura e cor das colónias, e morfologia e medições dos esporângios, hifas e conídios. De seguida, procedeu-se à extração do DNA genómico de cada isolado, com a região ITS1-5.8S-ITS2 do rDNA a ser posteriormente amplificada e sequenciada. Por fim, foi testada *in vitro* a potencial atividade lítica desenvolvida por cada isolado fúngico face a oocistos de *Eimeria* spp., em Agar-Água e em culturas fecais. Obtiveram-se sete isolados fúngicos, tendo sido todos identificados morfologicamente como pertencentes ao género *Mucor* e, após extração do DNA e sequenciação da região ITS1-5.8S-ITS2 do rDNA, como *Mucor circinelloides* (isolados FR1, FR2, FR2, SJ, SJ2 e QP2) e *Mucor lusitanicus* (isolado QP1). Todos os isolados exibiram atividade lítica contra os oocistos de coccídias, com os isolados FR3, QP2 e SJ1 a apresentarem eficácias coccidiostáticas (inibição da esporulação dos oocistos) superiores a 70%, e os isolados FR1, QP2 e QP1 a revelarem eficácias coccidicidas (destruição dos oocistos) de 22%, 14% e 8%, respetivamente, após 14 dias de incubação. Os resultados desta fase permitiram descrever pela primeira vez o isolamento de fungos predadores a partir de fezes de aves e confirmação das suas atividades antiparasitárias face a coccídias.

A terceira fase do projeto consistiu em avaliar *in vitro* a suscetibilidade dos sete fungos predadores isolados na fase anterior face aos principais antiparasitários utilizados em clínica de aves, como os anti-helmínticos albendazol, fenbendazol, levamisol e ivermectina, os coccidiostáticos lasalocida e amprólio, e o coccidicida toltrazuril (concentrações entre 0,0078 – 4 µg/mL), utilizando para o efeito microplacas de 96 poços preenchidas com meio de cultura RPMI 1640, e placas de Agar-Sabouraud (AS). Os resultados obtidos permitiram concluir que a exposição de todos os isolados de *Mucor* face aos sete antiparasitários não inibiu a sua germinação, tendo sido detetado o crescimento de cada isolado fúngico em meio RPMI 1640, após 48 h de exposição, e também em meio AS após exposição à maior concentração de cada fármaco. Os resultados obtidos nesta fase do projeto sugerem a compatibilidade da

utilização conjunta destes isolados de *Mucor* e de antiparasitários no controlo integrado dos parasitas GI das aves.

A quarta fase do projeto foi delineada com o objetivo de caracterizar o perfil de virulência do fungo predador *M. circinelloides* (FMV-FR1), que revelou a maior ação coccidicida durante a segunda fase do projeto, e análise do potencial impacto dos seus esporos na comunidade microbiana comensal do trato GI de aves. O DNA genómico deste fungo foi extraído e sujeito a Whole-Genome Sequencing (WGS) para pesquisa de genes codificadores para fatores de virulência. Procurou-se testar também este fungo para a expressão de seis fatores de virulência, nomeadamente proteinase, lecitinase, DNase, gelatinase, hemolisina e produção de biofilme, em meios de cultura e condições de incubação próprios. Por fim, desenvolveu-se um ensaio *in vivo* baseado na administração *per os* de esporos deste fungo a galinhas poedeiras e pavões, três vezes por semana, durante três meses, com as fezes colhidas ao longo do ensaio a serem utilizadas para extração de DNA genómico e sequenciação das regiões 16S e 25S-28S do rRNA. Todas as amostras fecais foram caracterizadas de acordo com a sua aparência e consistência (normais *vs* diarreicas e/ou hemorrágicas), servindo também como indicador de homeostasia intestinal. Verificou-se nesta fase do projeto que o genoma do fungo *M. circinelloides* FR1 apresenta genes codificadores para permease de ferro (FTR1), recetores de ferro (FOB1 e FOB2), fatores de ribosilação do ADP (ARFs) (ARF2 e ARF6), e GTPase (CDC42), bem como apenas testou positivo para a atividade de lecitinase. O ensaio *in vivo* revelou que o microbioma fecal das galinhas poedeiras era dominado por bactérias dos filos Firmicutes e Proteobacteria, e o dos pavões por Firmicutes and Bacteroidetes, enquanto o micobioma fecal de ambas as espécies de aves era maioritariamente composto por fungos dos filos Ascomycota e Basidiomycota. Para além disso, este ensaio revelou que as administrações de esporos de *M. circinelloides* FR1 não interferiram com a diversidade-alfa de bactérias e fungos nas galinhas poedeiras e pavões, com os respetivos valores a não diferirem significativamente entre os três momentos de colheitas fecais ($p=0,62$ e $p=0,15$, respetivamente), bem como não modificaram a qualidade das fezes das aves, com 70-100% das amostras a apresentarem aparência e consistência normais ao longo do ensaio. Os resultados globais desta fase do projeto sugerem a ausência de virulência do isolado *M. circinelloides* FR1, embora sejam necessários mais estudos para concluir acerca da segurança da administração de esporos deste fungo predador nas aves.

O ensaio *in vivo* desenvolvido na quarta fase deste projeto incluiu também análises parasitológicas realizadas nas amostras fecais colhidas na coleção de pavões, para determinação da eficácia do fungo *M. circinelloides* FR1 na redução das infeções por coccídias nestas aves, consistindo assim na quinta fase do projeto. Para o efeito, as amostras fecais colhidas a cada duas semanas foram processadas com o método MF para identificação

e quantificação da carga parasitária GI, sendo que a eficácia do tratamento foi calculada para cada instante de colheita, usando como controlo a carga parasitária inicial. No início do ensaio, os pavões apresentavam um nível severo de infeção por coccídias do género *Eimeria* (20106 ± 8034 OoPG), tendo sido verificado que a administração de esporos do isolado *M. circinelloides* FR1 resultou numa tendência decrescente nas cargas fecais de coccídias, com uma eficácia de redução significativa até 92%, após 60 dias de ensaio (p=0,012).

Os resultados globais deste projeto de doutoramento fornecem um importante contributo para o controlo integrado do parasitismo GI em aves, revelando a utilidade do método MF para um rápido e sensível diagnóstico de infeções parasitárias em clínicas, explorações avícolas e instituições zoológicas, bem como propondo a utilização do fungo predador nativo *M. circinelloides* FR1 para um controlo parasitário mais sustentável e seguro nas aves.

Palavras-chave: Aves, Parasitas Gastrointestinais, Mini-FLOTAC, Fungos Predadores.

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List of abbreviations

- CFU – Colony Forming Unit
- CLSI – Clinical & Laboratory Standards Institute
- DMSO – Dimethyl Sulfoxide medium
- EPG – Eggs per Gram of Feces
- EUCAST – European Committee on Antimicrobial Susceptibility Testing
- FOCR – Fecal Oocyst Count Reduction
- FOSR – Fecal Oocyst Sporulation Reduction
- FOVR – Fecal Oocyst Viability Reduction
- GI – Gastrointestinal
- IH – Intermediate Host
- L1 – First-stage Larvae
- L3 – Third-stage Larvae
- MF – Mini-FLOTAC
- OD – Optical Density
- OPG – Oocysts per Gram of Feces
- PBS – Phosphate Buffered Saline solution
- RPMI 1640 – Roswell Park Memorial Institute 1640 medium
- rRBCs – Rabbit Red Blood Cells
- SA – Sabouraud Agar medium
- WA – Water Agar medium
- WFA – Wheat-Flour Agar medium
- WGS – Whole-Genome Sequencing

Introductory note

The current thesis was specially elaborated to obtain the degree of Doctor in Veterinary Sciences, specialization in Animal Health, from the Faculty of Veterinary Medicine, University of Lisbon (Portugal). The document was written and structured based on the guidelines established by the University of Lisbon and its Faculty of Veterinary Medicine. Its main text is divided in seven chapters, with chapters I-VI being linked to six published articles (Figure 1).

Chapter I provides an introduction to free-range and organic poultry production, the importance of zoological parks for the conservation of bird species, and the challenges these systems pose for birds' health, with emphasis on gastrointestinal (GI) parasitic infections. Then, it displays a complete literature review regarding the aetiology, life cycle, pathogenicity, and common prevention and treatment procedures for infections caused by the main GI parasites in Galliformes and ratites, which were the two bird groups assessed in this study. Moreover, an introduction to the integrated control of GI parasitic infections in birds is provided, with highlight for new coprological diagnosis techniques and the use of predatory fungi for parasites' sustainable control. This chapter ends with a published literature review regarding previous studies assessing the potentialities of using predatory fungi for the biocontrol of avian GI parasites.

Chapters II-VI correspond to each task of the current Doctoral project, which resulted in five published original research articles, and thus all text provided in each of these chapters was retrieved directly from the respective articles. They include results from: a) the implementation of the Mini-FLOTAC method in the diagnosis of GI parasitic infections in domestic and exotic birds, and the comparison of its analytic results with the McMaster method (Chapter II); b) the isolation of native predatory fungi from bird feces and assessment of their *in vitro* lytic activity towards coccidia oocysts (Chapter III); c) the assessment of predatory fungi susceptibilities to common avian antiparasitic drugs (Chapter IV); d) the analysis of the virulence profile of predatory fungi and their interactions with the avian gut commensal microbiome and mycobiome (Chapter V); e) the evaluation of the efficacy of predatory fungi in reducing *in vivo* the coccidia parasitism in peacocks (Chapter VI).

Finally, chapter VII includes an integrated discussion of all results recorded in each task of this thesis, as well as the general conclusions matching each initially established objective. Moreover, information regarding further research is provided at the end of this chapter.

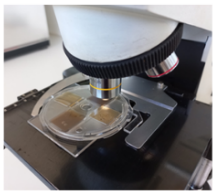
Chapter I

Introduction and literature review:

- Coccidia and helminths of poultry, peacocks and ratites
- Integrated control of avian gastrointestinal parasites
  - Coprological diagnosis
  - Biocontrol of avian gastrointestinal parasites using predatory fungi: current status, challenges, and opportunities **(Article 1)**

Chapter II

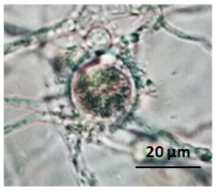
Improving the coprological diagnosis of gastrointestinal parasites in birds:



Implementation of Mini-FLOTAC in routine diagnosis of coccidia and helminth infections in domestic and exotic birds **(Article 2)**

Chapter III

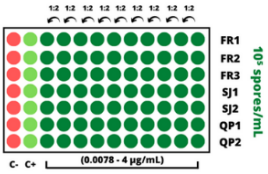
Searching for new native predatory fungi in birds' feces:



Isolation of saprophytic filamentous fungi from avian fecal samples and assessment of its predatory activity on coccidian oocysts **(Article 3)**

Chapter IV

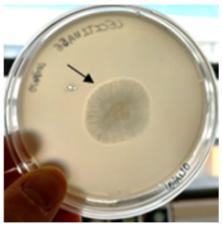
Compatibility of the joint administration of predatory fungi and antiparasitic drugs:



First insights on the susceptibility of native coccidicidal fungi *Mucor circinelloides* and *Mucor lusitanicus* to different avian antiparasitic drugs **(Article 4)**

Chapter V

Safety of *M. circinelloides* FR1 and interactions with avian commensal gut microorganisms:



Analysing the safety of the parasiticide fungus *Mucor circinelloides*: first insights on its virulence profile and interactions with the avian gut microbial community **(Article 5)**

Chapter VI

***In vivo* performance of *M. circinelloides* FR1 in reducing coccidia parasitism in peacocks:**

Assessing the efficacy of the ovicidal fungus *Mucor circinelloides* in reducing coccidia parasitism in peacocks **(Article 6)**

Chapter VII

General discussion, conclusions, and future perspectives

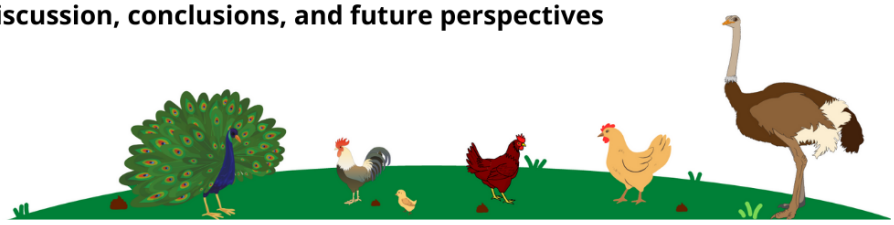


Figure 1. Schematization of thesis chapters (figure created using Canva®; www.canva.com).

General objectives

The current doctoral project aimed to optimize two of the most important avian gastrointestinal (GI) parasite integrated control approaches, namely parasite coprological diagnosis and biological control using predatory fungi. As such, the project had the following objectives:

i) To improve the detection of GI parasitic infections in domestic and exotic birds, using several coprological techniques;

ii) To assess the possibility of using avian feces to isolate native predatory fungi;

iii) To analyse the susceptibility of the isolated predatory fungi species to antiparasitic drugs;

iv) To characterize the virulence profile of the isolated predatory fungus having the highest potential for biocontrol and its effect in the avian gut microbiome and mycobiome;

v) To evaluate the effect of using parasiticide fungi in controlling the coccidia parasitism in peacocks.

CHAPTER I – Introduction and literature review

Linked publication

Lozano J, Almeida C, Oliveira M, Paz-Silva A, Madeira de Carvalho L. 2022. Biocontrol of avian gastrointestinal parasites using predatory fungi: current status, challenges, and opportunities. Parasitologia. 2:37-44. doi:10.3390/parasitologia2010004.

Author contributions: João Lozano – conceptualization, methodology, investigation, resources, data curation, writing (original draft), funding acquisition; Cristina Almeida – conceptualization, investigation, writing (original draft); Manuela Oliveira – validation, writing (review and editing), supervision; Adolfo Paz-Silva – validation, writing (review and editing), supervision; Luís Madeira de Carvalho - methodology, validation, resources, writing (review and editing), supervision, project administration, funding acquisition.

1. General introduction

Consumers are generally increasing their demand towards poultry meat and eggs produced in sustainable farming systems (Pettersson et al. 2016), stimulating farmers to find an equilibrium between animal welfare, food safety, environment preservation, social equity, and farm's economic viability (Font-i-Furnols 2023). Despite the worldwide dominance of intensive poultry systems, which are of most importance to satisfy the nutritional needs of an exponentially growing human population, small and mid-scale poultry production continues to play a crucial role in the economy and subsistence of rural areas in developing countries (FAO 2023), having become a recent noteworthy trend in developed countries, both in rural and peri-urban areas, for production or ornamental purposes, or to be kept as pets (Elkhoraibi et al. 2014).

Estimations in the European Union (EU) point to 2-5% of the total broiler heads to correspond to slow-growing breeds, used in both alternative systems, with France representing the largest organic chicken meat producer, accounting for 35% of all EU organic poultry heads. Also, in EU, free-range and organic laying hens represent 16% and 5% of total hen heads, thus highlighting the economic importance of "type 1" and "type 2" egg production (respectively) in the European poultry sector (Augère-Granier 2019).

Free-range poultry production is mainly characterized by the use of slow-growing breeds which have access to the outdoor environment, consume commercial feed and complement it with pasture, native earthworms and insects, and are subjected to irregular or absent chemical pathogen control, whereas in certified organic poultry production, which shares some similarities with the free-range system, some particular restrictions are yet imposed by applied legislation, namely European (Regulation (EU) 2018/848) and North-American (USDA National Organic Program), such as birds' mandatory access to outdoor areas and consumption of certified organic pasture and/or feed, recommendation of autochthonous breeds production, and prohibition of preventive treatments using antimicrobial and antiparasitic drugs (Fanatico et al. 2009; Elkhoraibi et al. 2014; Palacios and Sarmiento 2018) (Figure 2).

Moreover, zoological institutions, which often harbour domestic and exotic bird flocks, have been developing important roles for our society and animal wildlife, namely the establishment of animal conservation, research, and visitors' education programs (Miranda et al. 2023). *In situ* and *ex situ* animal conservation are of extreme importance, especially for Vulnerable, Endangered, or Critically Endangered animal species or breeds. According to the last report from The International Union for Conservation of Nature (IUCN Red List 2022), 13% of bird species are Threatened and 6% are Near Threatened at the European level, thus

highlighting the importance of their conservation. Moreover, among avian species, chickens have currently the highest number of breeds at risk of extinction (29%), and the proportion of avian species with unknown status of conservation is even higher (64%) than the observed for mammal species (55%) (FAO 2022).

The intrinsic characteristics of general free-range or organic poultry farms, and public or private ornithological collections, namely the exposition of birds for long periods of time to the same outdoor environments, contact with free-ranging wild avifauna, irregularity or absence of antiparasitic drug treatments and of indoor and outdoor sanitizing, lead to a high exposure to several pathogens, namely GI parasites like coccidia, capillarids, ascarids and strongylids, which have different life cycles, pathogenic potentials, and control procedures (Papini et al. 2012; Carrera-Játiva et al. 2018; Lolli et al. 2019; Lozano et al. 2019).

The next sub-chapter provides information regarding coccidia and nematodes of clinical and economic importance in Galliformes and ratites, which were the groups of parasites and birds studied in the current Doctoral project.

Figure 2. Photos from two of the free-range Galliformes' flocks included in this study, namely chickens (A) and peacocks (B) (originals).

2. Coccidia and GI nematodes of Galliformes and ratites

2.1. *Eimeria* spp.

Coccidia are protozoan organisms belonging to the phylum Apicomplexa, and are the most ubiquitous GI parasites in domestic, exotic, and captive wild birds. *Eimeria* is definitely the most prevalent genus in this group, resulting in pathogenic and economic impacts in the poultry industry (Fatoba and Adeleke 2018; Blake et al. 2020; Mesa-Pineda et al. 2021), and in birds kept at zoological institutions (Panayotova-Pencheva 2013; Hofstatter and Guaraldo 2015; Carrera-Játiva et al. 2018).

Eimeria spp. infections in free-range Galliformes, like chickens, laying hens, turkeys, and peacocks, can reach prevalences up to 100%, and shedding levels even higher than 10,000 oocysts per gram of feces (OPG), within the same bird flock, depending on several factors, namely the age of the flock and presence of juveniles, implemented antiparasitic drug treatments and sanitizing programs, waste management, nutrition, and climate (Prakashbabu et al. 2017; Carrisosa et al. 2021; Lozano et al. 2021a; Zhang et al. 2022) .

Moreover, according to a previous study in Romania, coccidia infections can be responsible for economic losses of nearly 3,200 € per flock, due to mortality (35%) and poor feed conversion (65%) (Györke et al. 2016), and for annual losses of nearly 12 billion € in the poultry industry worldwide (Blake et al. 2020). Although few studies have reported coccidia infections in ratites, there are previous reports describing enteric coccidiosis caused by *Eimeria* spp. in kiwi juveniles (*Apteryx* spp.) (Doneley 2006; Morgan et al. 2013; Coker et al. 2023), thus revealing the sanitary impact that this coccidian genus can have in ratites kept at zoos and rescue centres.

One of the biological characteristics of the genus *Eimeria* is its host-specificity, and thus the capacity of only parasitizing a single host species or a group of related hosts (Chapman 2014). One-hundred years ago, Johnson (1923) demonstrated for the first time this characteristic, as he failed to experimentally infect several bird species with sporulated *Eimeria* sp. oocysts from chickens. An example of this characteristic is the order Galliformes, in which seven *Eimeria* species have been described in chickens and laying hens' flocks, namely *Eimeria acervulina*, *Eimeria mitis*, *Eimeria praecox*, *Eimeria tenella*, *Eimeria necatrix, Eimeria brunetti* and *Eimeria maxima* (Lozano et al. 2019; Mesa-Pineda et al. 2021), while peacocks can be infected by nine *Eimeria* species, like *Eimeria arabica*, *Eimeria kharjensis*, *Eimeria mandali*, *Eimeria mayurai*, *Eimeria mutica*, *Eimeria pavonina*, *Eimeria pavonis*, *Eimeria patnaiki* and *Eimeria riyadhae* (Titilincu et al. 2009; Jaiswal et al. 2013; Zhang et al. 2022). Moreover, and despite few studies have addressed the pathogenicity of *Eimeria* spp. infections in ratites, a previous report described the identification of *Eimeria kiwii*, *Eimeria mantellii*,

Eimeria apteryxii, *Eimeria paraurii* and *Eimeria paopaoii* in kiwi juveniles (Coker et al. 2023), as well as *Eimeria* spp. infections in rheas (Reissig et al. 2001) and emus (Gallo et al. 2020).

2.1.1. Life cycle and pathogenicity

Despite the wide diversity of *Eimeria* species infecting domestic and exotic birds, they all share common characteristics regarding their life cycle, which has two main phases: exogenous and endogenous. The first phase, also known as “sporogony”, is initiated with the excretion of immature oocysts with feces to the environment, which under adequate temperature (20-30°C) and relative humidity (16-75%), start to sporulate within 1-2 days, depending on the species. The sporulation process ends with the formation of four sporocysts, each containing two sporozoites, conferring the infectant potential to the oocyst (Figure 3). Then, birds become infected after ingestion of soil, feces or pasture containing sporulated oocysts. The general enzymatic microenvironment of the GI tract and mechanical action of the gizzard alter the permeability of the oocyst shell, leading to its rupture and consequent releasing of sporozoites into the intestinal lumen. Then, the sporozoites invade the enterocytes and differentiate into trophozoites, which develop the parasitophorous vacuole, enlarge, and differentiate into first generation schizonts. These structures are responsible for producing merozoites, which are then released in the intestinal lumen and invade other epithelial cells, leading to more generations of schizonts. After two or more generations resulting from sexual reproduction, the schizonts can either produce merozoites, or enter the sexual reproduction phase, “gametogony”, by differentiating into macrogametocytes and microgametocytes, which produce macrogametes and microgametes, respectively. Fertilization leads to a diploid zygote, which differentiates into an immature oocyst and is finally expelled with feces (Waldenstedt et al. 2001; McDougald and Fitz-Coy 2008; Mesa-Pineda et al. 2021).

Coccidia of this genus have diverse pathogenic potential, and infection can be developed within the small or large intestines, depending on the tropism of each *Eimeria* species. For example, in chickens and laying hens, which are the bird hosts in which *Eimeria* spp. infections are better described, the most pathogenic coccidia are *E. brunetti*, *E. maxima*, *E. necatrix* and *E. tenella*, with the first three establishing infections in all compartments of the small intestine, while the former being the only responsible for infecting caecal enterocytes in this bird species (McDougald and Fitz-Coy 2008; Lozano et al. 2019). Also, regarding peacocks, in which *Eimeria* spp. infections are not so well studied, it has already been reported that the preferable site of infection for *E. mutica* and *E. kharjensis* within the GI tract is the ileum (Alyousif and Al-Shawa 1998). Moreover, a previous study described extra-intestinal

coccidiosis in kiwis, namely renal, hepatic, splenic and pulmonary disease (Morgan et al. 2013).

Coccidiosis can be symptomatic, with clinical signs ranging between diarrheic feces, sometimes with blood, prostration, and even mortality in more acute cases, while sub-clinical Coccidiosis include reductions on feed and water consumption, and consequently weight loss (McDougald and Fitz-Coy 2008; Lozano et al. 2019).

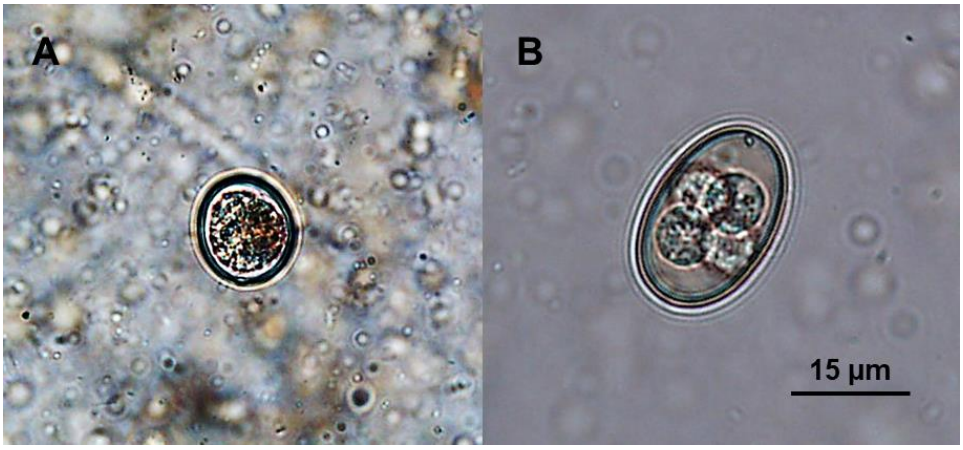


Figure 3. Photos from immature (A) and infective (B) *Eimeria* spp. oocysts, with the later containing four sporocysts, each with two sporozoites; scale at 15 µm (originals).

2.1.2. Prevention and treatment

The control of avian coccidiosis in poultry farms and ornithological collections is mainly performed using coccidiostatic and coccidicidal drugs, and in less extension through vaccination, disinfectants, and improved nutrition and handling practices (Mesa-Pineda et al. 2021).

There are two types of antiparasitic drugs for coccidia control, based on their mode of action and output: coccidiostatics and coccidicidals. The first have the function of interrupting the life cycle of the parasite, by interfering with its replication and growth, although their effect can be reversible once the drug is removed from the bird's feed, while the second can either destroy or cause irreversible damage on the parasite. Moreover, these drugs can be classified according with their chemical origin into i) synthetic drugs, which are produced by chemical synthesis and inhibit different biochemical pathways of the parasite; ii) ionophore drugs, which are by-products of *Streptomyces* spp. and *Actinomadura* spp. fermentation that destroy coccidia by interfering with mono or divalent cation channels present on their cell's membrane and thus affecting its osmotic balance; and iii) mixed drugs, which combine synthetic and ionophore compounds (Peek and Landman 2011). Examples of coccidiostatic molecules are

amprolium, lasalocid, nicarbazin and quinolones, whereas toltrazuril and diclazuril have coccidicidal activity (Peek and Landman 2011; Attree et al. 2021; Mesa-Pineda et al. 2021). Despite their proven efficacy, drugs misuse (e.g., over or infra-dosage, incorrect frequency, and absence of molecule rotation) in poultry production has historically been the main cause for the rising problematic of antiparasitic drug resistance, which for some drugs like buquinolate, lasalocid and salinomycin was recorded as quick as one year after their market introduction (Noack et al. 2019).

Vaccination with live non-attenuated or live attenuated vaccines has been also suggested as complementary solution to coccidiostatic and coccidicidal drugs. The first vaccine type consists in isolating oocysts of virulent strains from bird feces, followed by their reintroducing in larger amounts in the flocks, without attenuating their pathogenicity, while in the second type only the first oocysts excreted by animals on feces are used, which are the offspring of the *Eimeria* spp. precocious line and have a shorter life cycle, thus causing less damage in the intestinal epithelium and reducing the production of further immature oocysts. Currently, only live attenuated vaccines are authorized in Europe, namely Coccivac®, Immucox®, Paracox® and Livacox®, which are considered as the safest coccidiosis vaccines, whereas non-attenuated vaccines, like Advent® Inovocox®, Immucox®, and Coccivac®, are still used in North America, Asia and Africa. Despite having more immunogenic potential, a major drawback of using non-attenuated vaccines is the potential capacity of causing clinical coccidiosis if not properly managed (Mesa-Pineda et al. 2021; Zaheer et al. 2022).

Other preventive approaches for coccidiosis control have been showing interesting results, namely improved sanitizing between flocks, use of fluid or vapour forms of ammonium hydroxide, or a combination of formol and sodium dodecylbenzene sulphonate, or calcium hydroxide and ammonium sulphate, as well as the administration of natural products like prebiotics and probiotics, plant extracts and essential oils (Attree et al. 2021; Jamil et al. 2022).

2.2. Nematodes

Domestic and exotic birds are also prone to nematode infections, especially on free-range systems, in which birds have access to outdoor soil and pasture, where parasites' eggs and/or infective larvae are accumulated (Thapa et al. 2015; Lolli et al. 2019; Groves 2021). Several GI nematode species have been identified in domestic and exotic Galliformes worldwide, namely ascarids (e.g., *Ascaridia galli*), heterakids (*Heterakis* spp.), capillarids (*Capillaria* spp.), strongylids (e.g., *Trichostrongylus tenuis*) and *Strongyloides* spp., with the overall nematode infections reaching prevalences as high as 70% in free-range or organic laying hen flocks. *Ascaridia galli* is the most commonly detected nematode in this bird species

(Thapa et al. 2015; Lolli et al. 2019; Carrisosa et al. 2021; Groves 2021), while *Capillaria* spp., *Trichostrongylus tenuis* and *Strongyloides pavonis* can reach prevalences between 18-51% in peacocks (Titilincu et al. 2009; Rosa de Almeida 2022). Moreover, *Codiostomum struthionis* and *Libyostrongylus* spp. were also identified in captive ratites across the globe, especially in ostriches, emus, and rheas (Ponce Gordo et al. 2002; Nemejc and Lukesova 2012; Ederli and Rodrigues de Oliveira 2015).

2.2.1. Life cycles and pathogenicity

2.2.1.1. Ascarids and Heterakids

Ascaridia galli is the largest roundworm affecting Galliformes, and its life cycle starts with the excretion of immature eggs (Figure 4) to the environment, together with feces, after which 1-3 weeks are needed for them to become infective, due to the development of an infective larva in their interior, which depends on oxygen, temperature, and relative humidity conditions (Tarbiat et al. 2015). In free-range bird collections, annelids can occasionally serve as paratenic hosts, by accumulating infective eggs, which are then consumed by birds, despite not being the most common way of transmission. The life cycle resumes once birds ingest the infective eggs through contaminated soil, feces, water and/or feed, and thus beginning the endogenous phase. The eggs are carried by peristaltic movements through the GI tract, and larvae hatch mainly in the anterior section of jejunum's lumen after 24 hours (Ferdushy et al. 2012), and become attached to the intestinal epithelium, which is known as the histotrophic stage, that can last between 3-54 days until the final maturation to adult stage, after two consecutive molts (Yazwinski and Tucker 2008; Zajac and Conboy 2012a; Shohana et al. 2023).

The pathogenicity of this nematode is often associated with the thickening of the intestinal epithelium and appearance of hemorrhagic spots along with edema, as well as ulcerative proventriculitis (Brar et al. 2016; Shohana et al. 2023), with the accumulation of adult forms being capable of blocking the GI lumen in more severe infections (Yazwinski and Tucker 2008). This ascarid is also responsible for raising the susceptibility of birds to other microbial pathogens, as concurrent infections by *Escherichia coli* and *Pasteurella multocida* were already reported (Dahl et al. 2002; Permin et al. 2006). Clinical signs of ascaridiosis often include diarrhoea and anaemia, together with loss of appetite and weight, which in more acute cases can lead to death (Yazwinski and Tucker 2008; Höglund et al. 2023).

Heterakids' infections have also been reported in Galliformes, mainly by *Heterakis gallinarum* (the most frequently described in this bird order), along with *Heterakis isolonche* and *Heterakis dispar*. Their life cycle is direct, with immature eggs being excreted with feces

to the environment, and reaching the infective stage within two weeks. Once ingested by the host, larvae hatch within the small intestine lumen and migrate to caeca in approximately one day, where they reach the adult stage. Occasionally, *Heterakis* spp. eggs can be ingested by annelids, where larvae hatch and reside for months (Yazwinski and Tucker 2008; Groves 2021).

Infections by *Heterakis* spp. present different levels of pathogenicity, with *H. dispar* being considered as relatively non-pathogenic, whereas interactions of *H. isolonche* and *H. gallinarum* with the caecal epithelium lead to inflammation, thickening, nodules formation, diarrhoea, and weight loss. However, the main problem associated with infections by heterakids, especially *H. gallinarum*, is the capacity of its eggs to vehiculate the flagellated protozoa *Histomonas meleagridis*, which is responsible for causing histomonosis (the well-known “Black-Head” disease), which is characterized by the degradation of the caecal and hepatic tissues, causing necrosis, and consequently yellowish droppings, and the darkening of the birds’ wattle and comb skins (Yazwinski and Tucker 2008; Zajac and Conboy 2012a; Daş et al. 2021; Beer et al. 2022).

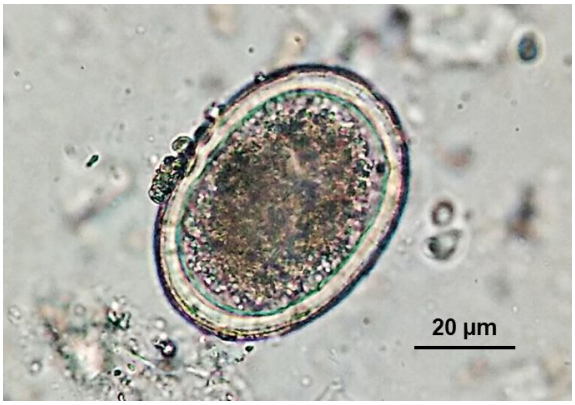


Figure 4. Photo from an *Ascaridia galli* egg identified in a laying hen fecal sample; scale at 20 µm (original).

2.2.1.2. *Capillaria* spp.

A total of six *Capillaria* species are known to infect domestic and exotic Galliformes, namely *Capillaria annulata* and *Capillaria contorta* (tropism for oesophagus and crop), *Capillaria caudinflata*, *Capillaria bursata* and *Capillaria obsignata* (small intestine), and *Capillaria anatis* (caeca). Non-embryonated eggs are expelled with feces to the environment (Figure 5) and reach the first larval stage (L1) within 9-14 days. The life cycle of *C. contorta*, *C. obsignata* and *C. anatis* is direct and thus eggs containing L1 larva are infective for birds, which hatch inside the respective GI tract sections and reach the adult stage after four

consecutive molts, whereas *C. annulata*, C. *caudinflata* and *C. bursata* have indirect life cycles, since non-embryonated eggs are first ingested by annelids (IH), in which reach the infective stage after 14-21 days. Their final consumption by birds closes the life cycle of the parasite (Yazwinski and Tucker 2008; Zajac and Conboy 2012a).

Capillarids can be responsible for severe infections in Galliformes, with *C. contorta* and *C. annulata* causing catarrhal inflammation and thickening of the oesophagus and crop epithelia, whereas *C. caudinflata*, *C. bursata*, *C. obsignata* and *C. anatis* establish hemorrhagic enteritis in small and large intestines, which leads to diarrhoea with blood, and anaemia in more acute cases (Yazwinski and Tucker 2008; Groves 2021).

Figure 5. Photo from a *Capillaria* sp. egg identified in a peacock fecal sample; scale at 20 µm (original).

2.2.1.3. Strongylids and *Strongyloides* spp.

Strongylids are also identified in the GI tract of domestic and exotic birds, with *T. tenuis* being common in poultry and exotic Galliformes, and ratites (Yazwinski and Tucker 2008; Nemejc and Lukesova 2012; Tomza-Marciniak et al. 2014; Rosa de Almeida 2022). Birds from the former order are also prone to infections by *Codiostomum struthionis*, as well as by *Libyostrongylus douglassii*, *Libyostrongylus dentatus* and *Libyostrongylus magnus*, having the former only been identified in Africa (Ponce Gordo et al. 2002; McKenna 2005; Nemejc and Lukesova 2012). They all have a typical direct life cycle, where non-embryonated eggs are expelled with feces (Figure 6), and larvae emerge within two days in the environment, becoming infective (L3) after two weeks. Birds are infected by picking up third stage larvae, which then migrate to different GI sections, where they molt twice and reach the adult stage. The preferential site for *T. tenuis* and *C. struthionis* is the birds' caeca, whereas *Libyostrongylus* spp. develop tropism to the proventricular epithelium (Yazwinski and Tucker 2008; Ederli and Oliveira 2009, 2014).

Infections by strongylids in Galliformes and ratites often lead to the inflammation and thickening of the GI epithelia and congestion of blood vessels, evolving to mucoid diarrhoea, weight loss and anaemia, and even death in more severe infections, especially in chicks. In wild Galliformes, it has been demonstrated that infections by the nematode *T. tenuis* are responsible for the periodical declining of the Scottish Red Grouse population (*Lagopus lagopus scotica*), due to heavy mortality of the juveniles in spring and autumn (Friend and Franson 1999; Ferreira 2015), whereas emus can present mucoid diarrhoea, with blood (Yazwinski and Tucker 2008). Moreover, infections by the nematode *L. douglassii* are of major relevance in ostriches, causing the “Rotten Stomach” disease in chicks and less frequently in adult birds. This parasite can either reside deeply in the proventriculus glands (late larval stages), causing proventriculitis and excessive mucus secretion, leading to glands impaction, or remain attached to its surface, where they feed on blood and cause inflammation (McKenna 2005; Nemejc and Lukesova 2012).

Finally, infections by roundworms of the genus *Strongyloides* have already been reported in poultry and peacocks, namely by *Strongyloides avium* and *Strongyloides pavonis*, respectively (Yazwinski and Tucker 2008; Titilincu et al. 2009; Rosa de Almeida 2022). They have a particular life cycle, different from most birds’ nematodes. Eggs are expelled with feces to the environment and larvae hatch in less than one day, after which develop directly to free-living adult males and females, or to infective larvae (heterogonic or homogonic pathways, respectively). Third stage larvae (L3), which origin from free-living specimens, are the infective stages that can be transmitted to birds via feces, soil, feed, or pasture, as well as by invasion of the skin, and then present a visceral migration and accumulate in the small and large intestines, where they develop and differentiate into parthenogenetic females, which is the parasitic stage of the life cycle (Yazwinski and Tucker 2008; Rosa de Almeida 2022).

Infections by *Strongyloides* spp. in birds can lead to caecal wall thickening and tissue necrosis, and eventually to thin fecal discharges containing blood, as well as larval migration to the lungs, by hemato-lymphatic route, and potentially responsible for pneumonia, with juvenile birds being more likely to become infected by this nematode (Yazwinski and Tucker 2008; Rosa de Almeida 2022).

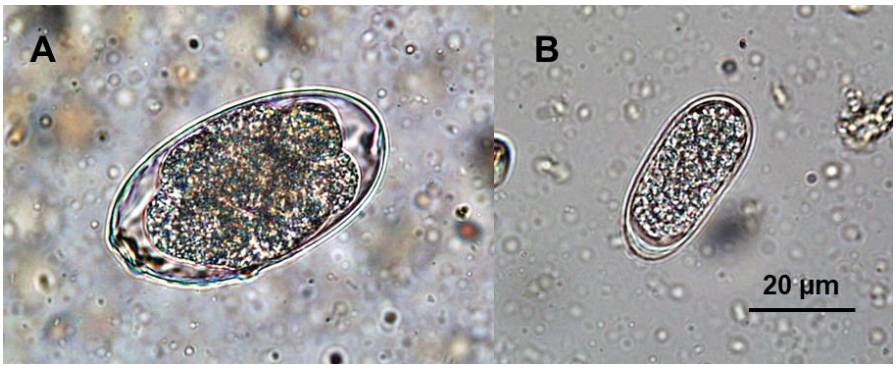


Figure 6. Photos from *Trichostrongylus tenuis* (A) and *Strongyloides pavonis* (B) eggs, identified in peacock fecal samples; scale at 20 µm (originals).

2.2.2. Prevention and treatment

Anthelminthic use for the preventive control of nematode infections in organic poultry is not permitted by general organic farming legislation worldwide, and free-range poultry and exotic bird flocks are either treated intermittently during their production lifetime or are not subjected to any parasite chemical control (Elkhoraibi et al. 2014; Thapa et al. 2015).

Common dewormers used for treating avian nematode infections include: i) benzimidazoles (e.g., fenbendazole, albendazole, flubendazole), which act on the parasite by binding to its tubulin molecules and inhibiting the formation of microtubules, thus arresting cell division, as well by inhibiting fumarate reductase and consequently blocking the mitochondrial activity and associated energy production; ii) macrocyclic lactones (e.g., ivermectin), which bind to glutamate-receptors and trigger chloride influx, hyperpolarizing the nematodes' neurons and preventing the propagation of action potentials, and thus leading to the parasite's paralysis and death; iii) imidazothiazole-derivatives (e.g., levamisole), which act as nicotinic agonists, by binding to acetylcholine receptors and blocking the neural transmission, thus disturbing the nematode's neuromuscular system and causing its contraction and paralysis (Coles and Lynn 2014).

Moreover, other control solutions have been proposed by several authors, such as the correct cleaning and disinfection of the housings, regular changing of floor and nest covering material, removal of fecal material from enclosures, and good drainage on the outdoor soil (Nemejc and Lukesova 2012; Liebhart et al. 2017; Groves 2021).

3. Integrated control of GI parasitic infections in birds

As mentioned previously, the control of the main avian parasitic diseases at clinical, farm or zoological park level, is mainly achieved using antiparasitic drugs, which are frequently administrated to birds as the only preventive and/or treatment strategy, and without any previous laboratorial diagnosis (Peek and Landman 2011; Erez et al. 2023).

However, this kind of approach is of limited utility, since antiparasitic drugs target mainly the endogenous stages of the parasites' life cycles and thus do not act on oocysts, eggs and larvae accumulated in feces, soil and/or pasture, and it also raises serious health, economic and ecological concerns for birds, farms and zoological parks, consumers, and the environment. Drug misuse in bird collections often results in low treatment efficacy and development of drug resistance, as well as accumulation of pharmacological residues in bird's carcass, soil, and even groundwaters (Abbas et al. 2011; Mund et al. 2017; Mooney et al. 2021; Martins et al. 2022).

The historical unsustainable use of antiparasitic drugs, and its associated consequences, stimulated the search and implementation of novel complementary solutions, an approach known as "Integrated Control". This concept has original roots in agriculture, linked to the control of plant pathogens, and gradually evolved to other sectors, namely Veterinary Medicine (Braga and Araújo 2014; Herrera-Estrella et al. 2016).

This concept has several definitions (Stenberg et al. 2021), and regarding the control of animal parasitosis it can be generally defined as the combination of antiparasitic drugs with other approaches, such as vaccination, routine diagnosis of infections, animal handling, feed and pasture management, sanitization (cleaning and disinfection), or natural solutions like herbal extracts and essential oils, probiotics and prebiotics, algae, and fungi, aiming to prevent and control animal diseases caused by ecto- and endoparasites (Madeira de Carvalho et al. 2012; Ellse and Wall 2014; Andriantsoanirina et al. 2022; Ahmed et al. 2023) (Figure 7).

Routine diagnosis of infections using novel quantitative-qualitative approaches, like the coprological method Mini-FLOTAC, and feeding predatory fungi to animals, have been two of the most studied sustainable solutions for the control of GI parasitic infections in several domestic and exotic animal species, such as horses, ruminants, pets, being more recently proposed for application in birds (Cringoli et al. 2017; Araújo et al. 2021; Salmo et al. 2024), and therefore being introduced in the following sections.

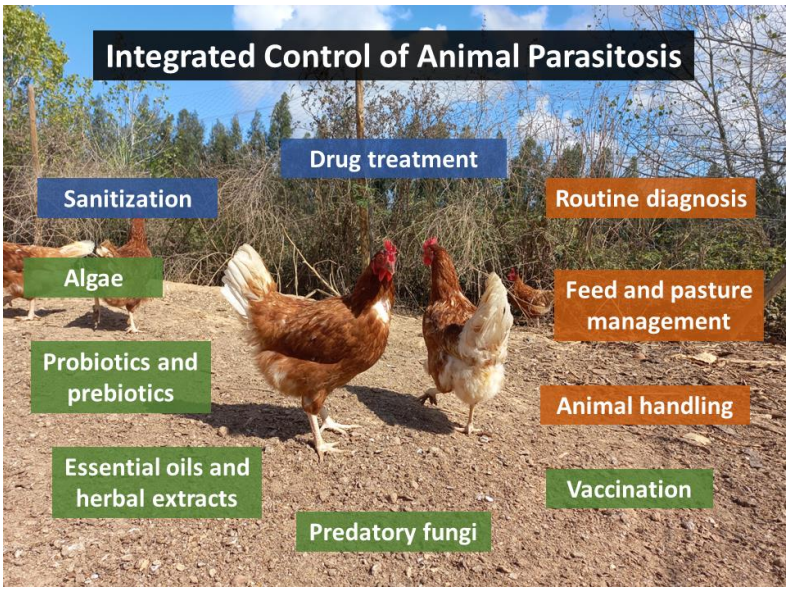


Figure 7. Scheme of the main approaches used for parasite integrated control, namely chemical, biological, and technical solutions (blue, green, and orange boxes, respectively) (original).

3.1. Coprological diagnosis

Understanding the real parasitism status of the bird is of major importance prior to any medical intervention. Feces are the only kind of bird samples that can be collected without the need for animals' immobilization and offer the opportunity to study the avian GI parasitic community, estimate the severity of infections, and consequently perform a more accurate parasite control and efficient use of material resources (Seivwright et al. 2004; Englar 2023).

There are several diagnosis techniques based on using fecal samples (coprological diagnosis), namely quantitative, qualitative, and quantitative-qualitative. Quantitative methods are used to estimate the concentration of coccidia oocysts and helminth eggs in the excreta (oocysts or eggs per gram of feces, OPG or EPG, respectively), with the McMaster method being the most commonly used in clinics, diagnostics labs, and research institutions, worldwide, whereas qualitative methods like Willis-Flotation, Natural or Centrifugal Sedimentation, and Fecal Cultures (for coccidia and helminths), and Modified Ziehl-Neelsen (for *Cryptosporidium* spp. and *Giardia* spp.), allow to check the presence of parasitic elements in feces, and identify them at genus or species level (Smith 2008; Shapiro 2010; Zajac and Conboy 2012b).

Moreover, FLOTAC and Mini-FLOTAC are examples of quantitative-qualitative or dual-purpose methods, since their implementation offers the possibility of estimating the oocysts, eggs, or larvae concentration on feces, and simultaneously visualize the membranes and

some organelles of parasitic elements at total microscope magnifications up to 400x, thus allowing to estimate the coccidia or helminth shedding, and simultaneously identify parasites at genus or even species level. These techniques are known for having higher diagnostic sensitivity and precision, and for being more user-friendly and of practical usage, in comparison with the McMaster method (Cringoli et al. 2010, 2017). The FLOTAC method involves the centrifugation of a fecal dilution in tap water and mixing the sediment with a chosen saturated flotation solution (e.g., sucrose, sodium chloride, zinc sulphate, with different specific gravities), followed by its transfer to the FLOTAC reading chamber, which is again centrifugated to force the parasitic elements to float and attach to the counting grids (Cringoli et al. 2010). Moreover, the Mini-FLOTAC method shares the main principles of the FLOTAC method, although it relies on using only two devices, such as the Fill-FLOTAC, which is the device that allows to weight and mix the feces with the chosen flotation solution, to filtrate the fecal suspension, and to directly transfer it to the second device, which is the reading chamber, without any previous centrifugation (Cringoli et al. 2017). Each solution provides different diagnosis results, since sucrose and sodium chloride solutions are mainly used to identify coccidia oocysts, and nematode and cestode eggs, whereas zinc sulphate allows also to identify *Giardia* spp. cysts, and trematode eggs (Maesano et al. 2014; Alvarado-Villalobos et al. 2017; Capasso et al. 2019) (Figure 8).

All these characteristics support the idea of Mini-FLOTAC as being more suitable for a practical and rapid diagnosis of GI parasitic infections in animals, and previous (but still scarce) research has demonstrated the usefulness of this technique for the diagnosis of *Eimeria* spp. and ascarids infections in poultry, achieving sensitivities of up to 100% and precisions of 80-92% (Bortoluzzi et al. 2018; Daş et al. 2020; Lozano et al. 2021b).

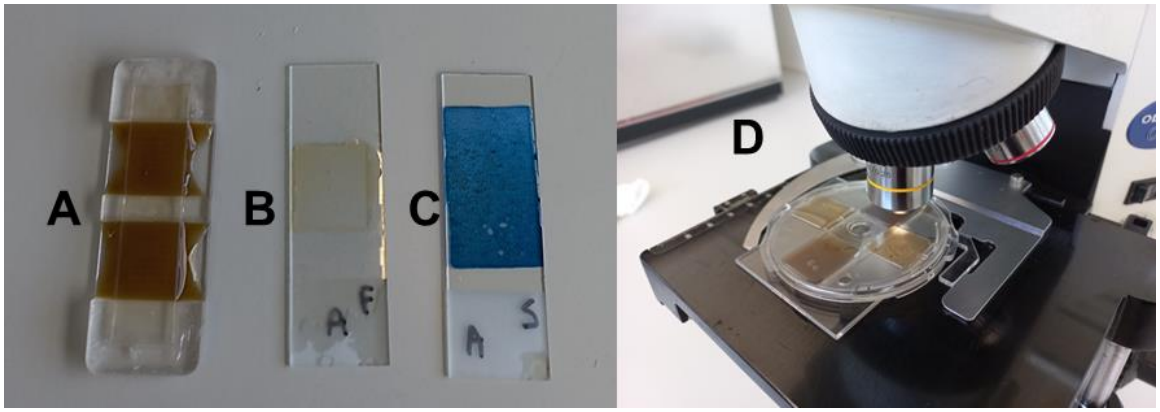


Figure 8. Photos of the end-products of the previously mentioned coprological methods, namely the McMaster reading chamber (A), Willis-Flotation (B) and Natural Sedimentation slides (C), and the Mini-FLOTAC reading chamber (D) (originals).

3.2. Introduction to predatory fungi and their applications in avian parasite control

The study of predatory fungi and their applications in Veterinary Medicine and Animal Production have been constantly attracting the interest of the scientific community, being considered as an accurate, innovative, and sustainable ally in the integrated control of animals' GI parasitic infections (Araújo et al. 2021; Palomero et al. 2021; Mendoza de Gives et al. 2022).

Predatory fungi are saprophytic and filamentous fungi which develop different mechanical and enzymatic mechanisms to capture and destroy the exogenous forms of animals' GI parasites, namely parasites' eggs (ovicidal fungi) or infective larvae (larvicidal fungi), and thus breaking their life cycles on the environment (Madeira de Carvalho et al. 2012; Braga and Araújo 2014). Fungal spores are often administrated to animals via feed (10^4-10^6 spores/kg feed), and less frequently via drinking-water, and even sprayed on pasture or feces (Madeira de Carvalho et al. 2011; Cortiñas et al. 2015; Voinot et al. 2021; Paz-Silva et al. 2023). The ability of chlamydospores to survive to the GI passage has already been demonstrated in ruminants, horses, dogs, and chickens (Madeira de Carvalho et al. 2012; Silva et al. 2017; Braga et al. 2020; Rodrigues et al. 2021), after which they are excreted with feces to the environment, and finally germinate and destroy parasitic elements in both fecal and peri-fecal microenvironments (Figure 9).

Most research regarding the biocontrol of GI parasites using predatory fungi has been performed in ruminants (Aguilar-Marcelino et al. 2017; Healey et al. 2018; Branco de Oliveira et al. 2021; Voinot et al. 2021), horses (Madeira de Carvalho et al. 2011; Arias et al. 2013a; Hernández et al. 2016), dogs (Araujo et al. 2012; Viña et al. 2022; Paz-Silva et al. 2023), and captive wild animals (Cazapal-Monteiro et al. 2015; Palomero et al. 2021; Paz-Silva et al. 2023). Research performed in birds is still scarce, and to the author's knowledge there were no scientific publications regarding the application of predatory fungi in the control of avian coccidia infections, prior to the development of the current doctoral project. The next sub-section provides a description of the current status and future perspectives regarding the use of predatory fungi in avian parasite control.

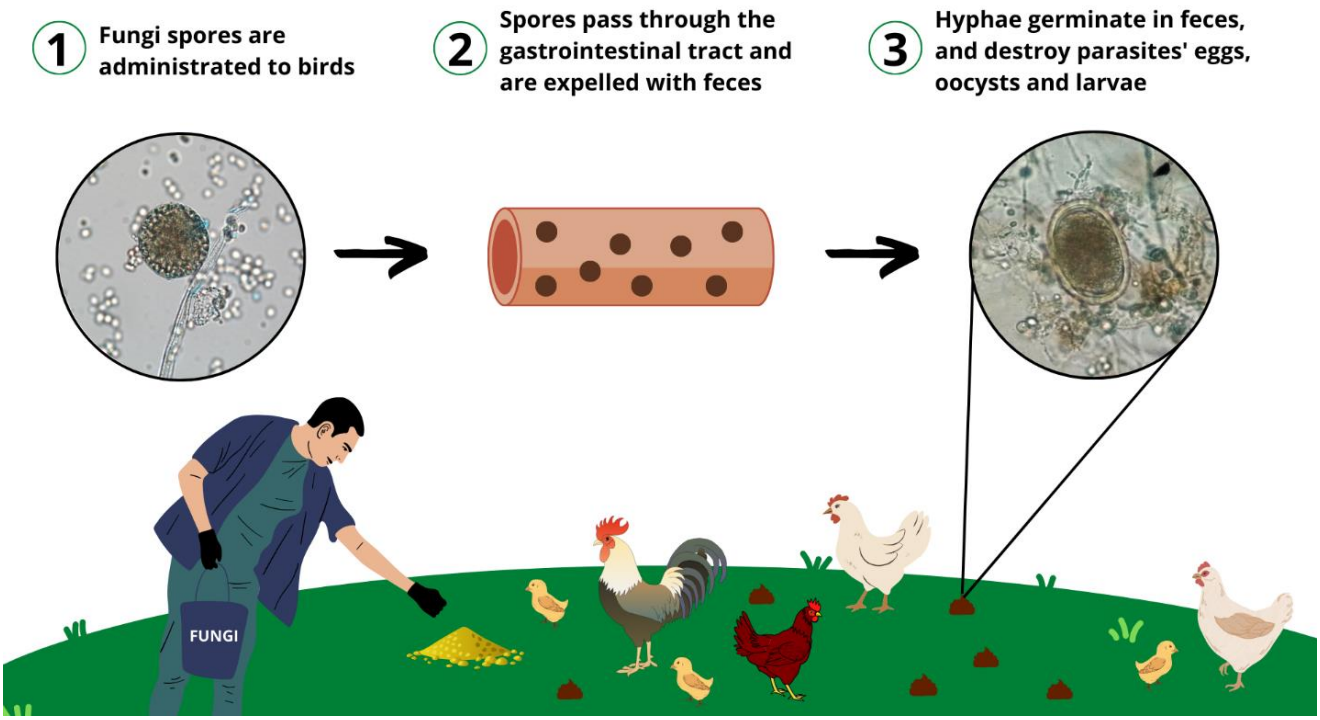


Figure 9. Illustration on the main principles regarding the administration of predatory fungi to birds, and their mode of action towards parasitic elements (figure created using Canva® - www.canva.com; photos regarding fungal structures and the destruction of the *Ascaridia galli* egg are original).

3.2.1. Article 1 – “Biocontrol of avian gastrointestinal parasites using predatory fungi: current status, challenges, and opportunities”

Abstract

This review describes the current research status regarding the implementation of predatory fungi in the biological control approach of bird gastrointestinal (GI) parasitosis. The main GI parasites of Galliformes (e.g., broilers, layers, peacocks, pheasants) and ratites (e.g., ostriches, emus, rheas) are addressed, as well as their impact on farms, zoos, and private collections. The main characteristics regarding biocontrol with predatory fungi are briefly described, such as their mode of action and efficacy against GI parasites of different animal hosts. The state of the art regarding the use of predatory fungi in birds is reviewed here by describing all associated articles already published in the main databases, techniques, and their main findings. Ovicidal fungi such as *Pochonia chlamydosporia*, *Metarhizium* spp. and *Acremonium* spp., and larvicidal fungi, namely *Duddingtonia flagrans*, *Arthrobotrys* spp. and *Monacrosporium thaumasium*, have shown promising predacious activity against ascarid eggs and nematode larvae from chickens and ostriches, both *in vitro* and *in vivo*, also revealing tolerance to the GI passage in chickens and maintenance of predacious capacity. Further studies are needed to understand the fungi–parasite–host gut microbiota interactions and target other avian GI parasitic species, such as nematodes, coccidia, cestodes, and trematodes.

Keywords: Birds, Intestinal Parasites, Biological Control, Predatory Fungi.

3.2.1.1. Gastrointestinal parasites of Galliformes and ratites

Domestic and exotic birds are commonly exposed to a wide variety of generalist or host-specific gastrointestinal (GI) parasites, with different life cycles and levels of pathogenicity (Yazwinski and Tucker 2008; Thapa et al. 2015; Fatoba and Adeleke 2018; Lozano et al. 2019; Attree et al. 2021; Lozano et al. 2021c; Mesa-Pineda et al. 2021; Nath et al. 2021).

In Galliformes kept on free-range farms, zoos, and public gardens (e.g., broilers, layers, peacocks, pheasants), coccidia infections caused by *Eimeria* spp. and *Isospora* spp. can reach prevalence and shedding values up to 80% and 15,000 oocysts per gram of feces (OPG), respectively (Titilincu et al. 2009; Papini et al. 2012; Jaiswal et al. 2013; Prakashbabu et al. 2017; Lolli et al. 2019; Carrisosa et al. 2021; Lozano et al. 2021c) and are currently responsible for average losses of approximately 12 billion annually worldwide in the poultry industry (Blake et al. 2020; Attree et al. 2021). Nematode infections are also a serious problem in Galliformes, being ascarids (e.g., *Ascaridia galli*), heterakids (e.g., *Heterakis gallinarum* and *H. isolonche*), capillarids (e.g., *Capillaria* spp.), strongyles (e.g., *Trichostrongylus tenuis*), and *Strongyloides* spp., the most frequent and pathogenic species (Titilincu et al. 2009; Papini et al. 2012; Jaiswal et al. 2013; Ilić et al. 2018; Lolli et al. 2019; Lozano et al. 2021c; Nath et al. 2021; Valadão et al. 2021).

Larger birds like ratites (e.g., ostriches, emus and rheas), which are commonly kept in zoos worldwide for ornamental exhibition and occasionally in farms for production purposes, are also susceptible to GI parasitic infections, and nematodes belonging to the genera *Libyostrongylus* and *Codiostomum* are of most clinical importance, especially *Libyostrongylus douglassii*, which is responsible for the “rotten stomach” disease (Jansson and Christensson 2000; Ponce Gordo et al. 2002; McKenna 2005; Ederli and Rodrigues de Oliveira 2015; Kummrow 2015; Lozano et al. 2021c).

The control of these agents based solely on the administration of antiparasitic compounds (e.g., anticoccidials and anthelminthics) is of limited utility, since they do not act on the environmental forms of the parasites. In addition, common drug misuse in livestock farms often leads to efficacies lower than expected, appearance of drug resistance, and potential contamination of the environment with drug residues (Köhler 2001; Beynon 2012; Noack et al. 2019; Selzer and Epe 2021).

New complementary strategies are being proposed for integrated GI parasite control in domestic and wild animals kept in captivity, namely the use of predatory fungi as an accurate, innovative, natural, and sustainable tool (Canhão-Dias et al. 2020; Araújo et al. 2021).

3.2.1.2. Biocontrol of GI parasites using predatory fungi

Over the past 20 years, there has been an increasing interest in research regarding the use of predatory fungi (also referred as “nematophagous fungi”, or more recently “helminthophagous fungi”) for the biocontrol of animal gastrointestinal parasites, in complement with drug treatments.

These are saprophytic filamentous fungi belonging mainly to the phyla Ascomycota and Mucoromycota, often found in agricultural soil and organic decaying matter, which play a role in the recycling of carbon, nitrogen, and other elements originating from nematode degradation (Braga and Araújo 2014). Besides their common saprophytic characteristics, these fungi also have the ability to predate intestinal parasites of animals, especially the eggs and larvae, which serve as an additional source of nutrients for fungal growth. Their tolerance to the animal’s gastrointestinal transit has already been demonstrated, being expelled with feces to the soil, where they start predating parasitic forms, especially in micro-fecal and peri-fecal environments (Madeira de Carvalho et al. 2012).

There are three main groups of predatory fungi, defined according to their mode of action: larvicidal, ovicidal, and endoparasitic, the first two being the most commonly used in biocontrol trials. For larvicidal fungi such as *Duddingtonia flagrans*, *Arthrobotrys* spp., and *Monacrosporium thaumasium*, the main feature is the production of a wide diversity of traps (e.g., constricting rings, non-constricting rings, adhesive nodules, and ramifications), whose formation is stimulated by the presence of helminth larvae. For ovicidal fungi, namely *Mucor circinelloides*, *Pochonia chlamydosporia*, *Verticillium* spp., *Purpureocillium lilacinum* (formerly known as *Paecilomyces lilacinus*) and *Trichoderma* spp., the main characteristic consists of their ability to predate helminth eggs, and it is the presence of parasite eggs that triggers fungal hyphae migration towards their cuticula, in which mechanic and enzymatic activity are developed (Braga and Araújo 2014).

Both larvicidal and ovicidal fungi have been used in several *in vitro* and *in vivo* experiments, being unanimously considered an accurate and sustainable tool for the control of GI parasites, resulting in a reduction in the number of eggs per gram of feces (EPG) of 60–97% in field trials with grazing animals (Madeira de Carvalho et al. 2011; Healey et al. 2018; Canhão-Dias et al. 2020; Branco de Oliveira et al. 2021; Palomero et al. 2021; Voinot et al. 2021). The lack of adverse effects of *D. flagrans* on soil nematodes (Saumell et al. 2016), as well as the innocuousness of *M. circinelloides* and *D. flagrans* on several animal species (Hernández et al. 2016; Voinot et al. 2021) should also be underlined.

These fungi have already been isolated in America (Soto-Barrientos et al. 2011; Falbo et al. 2013; Ojeda-Robertos et al. 2019; Arroyo-Balán et al. 2021; Ocampo-Gutiérrez et al.

2021), Europe (Hernández et al. 2017), Asia (Liu et al. 2015; Xue et al. 2018), Oceania (Larsen et al. 1994; Faedo et al. 1997), and even in Antarctica (Gray and Smith 1984), and two commercial formulations of *D. flagrans* are already commercially available in Australia and New Zealand (BioWorma® - NCIMB 30336, BioWorma, Sydney, Australia) and in Brazil (Bioverm® - AC001, GhenVet Saúde Animal, Paulínia, Brazil).

3.2.1.3. Testing the use of predatory fungi against avian GI parasites: state of the art

Despite the increasing number of studies in this topic, most of them are focused on the biocontrol of intestinal parasites affecting ruminants and horses, and there is a lack of research regarding the use of predatory fungi in other animals, such as birds.

A literature search was performed in November 2021, in PubMed, Scopus, Web of Science and Google Scholar databases, using the search string "(predatory fungi OR predacious fungi OR duddingtonia OR arthrobotrys OR monacrosporium OR mucor OR pochonia OR verticillium OR paecilomyces OR trichoderma) AND (coccidia OR helminth OR nematode)". Title and abstract analysis were performed, only research articles in English and published from 1990 until 2021 were included, and other types of publications (e.g., reviews, letters, and editorials) were excluded. It was found that only 5 publications were related to *in vitro* and *in vivo* experiments using predatory fungi against avian GI parasites (4 original research articles and 1 research note), carried out in Brazil and Denmark (Table 1).

Table 1. *In vitro* and *in vivo* research performed with predatory fungi against avian GI parasites.

Type of Assay	Fungal Species (Biotype)	Target Organism	Study Objectives	Reference
In vitro	*D. flagrans* (AC001 ; CG722) *A. cladodes* (CG719)	*L. douglassii*	Test larvicidal activity against L3 larvae	(Braga et al. 2013)
	P. chlamydosporia (Biotype 10) *Me. brunneum* (KVL04-57; KVL16-26) *Me. carneum* (KVL16-33) *Acremonium* sp. (KVL16-34)	*A. galli* *H. gallinarum*	Test ovicidal activity in different soil types; isolate native ovicidal fungi	(Thapa et al. 2017)
In vivo	*D. flagrans* (AC001; CG722) *M. thaumasium* (NF34A)	*Panagrellus* spp.	Test GI passage in chickens and evaluate the maintenance of germination and larvicidal capacities	(Silva et al. 2017)
	P. chlamydosporia (VC4)	*A. galli* *H. gallinarum*	Test GI passage in chickens and evaluate the maintenance of germination and ovicidal capacities	(Valadão et al. 2020)
	P. chlamydosporia (Biotype 10)	*A. galli* *H. gallinarum*	Test ovicidal activity in different soil types; evaluate the interaction soil-fungi in birds worm population and burdens, and egg counting	(Thapa et al. 2018)

The first *in vitro* experiment with predatory fungi against avian intestinal parasites was reported 9 years ago by (Braga et al. 2013). The study aimed to test the larvicidal activity of two isolates of *D. flagrans* (AC001 and CG722) and one isolate of *Arthrobotrys cladodes* (CG719) on infective larvae (L3) of *L. douglassii*. The assays were performed in plates with Water-Agar medium (WA, 2%) and the number of non-preyed L3 was counted daily, for seven days of incubation, in all treated and control groups. Percentage reductions of L3 were found to be significant between test and control plates, totalizing efficacies of 85.2% (isolate AC001), 81.2% (CG722), and 89.2% (CG719). Isolates did not differ in the daily mean of non-preyed L3, but all of them differed significantly from control plates, and therefore these isolates offer potential to be used in the biocontrol of GI nematodes of ratites.

Another *in vitro* study was conducted in Denmark by (Thapa et al. 2017), which aimed to test the performance of *P. chlamydosporia* (Biotype 10) and *Metarhizium brunneum* (KVL04-57) against non-embryonated ascarid eggs (*A. galli* and *Heterakis* spp.) in sterilized and non-sterilized soils. Egg recovery was examined before and after incubation at 22°C for 30 days. In sterilized soil, results were significantly influenced by the interaction between fungal treatment and incubation time, with egg count differing between treatments and controls after

30 days of incubation, and *P. chlamydosporia* and *Me. brunneum* showing reduction efficacies of 46% and 30%, respectively. However, in non-sterilized soil, the outcomes were slightly different, with both fungal and control plates showing significant egg recovery reductions (68–77%). In this case, only *Me. brunneum* treatment resulted in slight but significant reductions in comparison with controls and *P. chlamydosporia* plates. These results suggest that resource competition between predatory fungi and native soil microbiota may interfere negatively with the performance of fungal isolates, as well as rejects the hypothesis of potential environmental impact on soil microbiota caused by the administration of these fungi.

In this study, the authors also aimed to evaluate the survival of ascarid eggs in different soil types, both in sterilized and non-sterilized soil, after 30 days of incubation at 22°C. For sterilized soils, only incubation time and soil type had a significant interaction on egg recovery. For non-sterilized soils, the egg counts were significantly reduced in all soil types, ranging from 38% to 99%. Non-sterilized soils exhibiting the highest ovicidal activities were also used to isolate, identify, and test the antagonistic effect of native fungi against ascarid eggs. Fungal isolates belonged to the genera *Metarhizium* and *Acremonium*; however, none of the three isolates revealed predatory efficacies higher than 34% after 28 days of exposure. These results also suggest that soil has inherent biotic egg-degrading properties, namely due to its native microbiota.

Predatory fungi have also been tested *in vivo* in chickens and hens, with the first published report dating back to 2017. The study developed by (Silva et al. 2017) aimed to test the maintenance of germination and larvicidal capacities of *D. flagrans* (AC001; CG722) and *M. thaumasium* (NF34A) after passing through the GI tract of chickens. For this purpose, four experimental groups with two chickens were considered: three groups were provided with autoclaved concentrate feed mixed with 1 mL of an aqueous solution containing 6.4 x 10^4 spores of each isolate (test groups), and 1 group received feed mixed with distilled water (control group), on a daily basis. Fecal samples were collected 6, 12, 24, 48, and 74 hours post-administration, and placed in Petri dishes with WA medium. Suspensions containing larvae of the free-living nematode *Panagrellus* spp. were also added to each plate, followed by incubation at 25°C for 12 days, to test mycelial growth and average number of recovered larvae in each period of administration. Fungal structures from all isolates were observed at 6, 12, and 24 h post-administration, confirming the ability of spores to resist the GI passage in chickens. In addition, the highest percentage of reduction in the number of recovered larvae was identified at 6 h post-administration, averaging reduction rates of approximately 35% to 71%, with only isolate AC001 showing a significant reduction in comparison with the control plates. Despite larvicidal activity being tested against free-living nematodes, results from this

study can be extrapolated to parasitic nematodes affecting bird species, due to a similar mode of action.

A study conducted by Valadão et al. (2020) also aimed to test the maintenance of germination and ovicidal capacities of *P. chlamydosporia* (VC4) after GI transit in chickens, with an experimental design similar to the previously mentioned study. A group of 22 chickens was divided into two experimental groups: both groups received a supplementation of shredded corn for 7 days, after which only the test group started to receive the supplement inoculated with *P. chlamydosporia*. Samples were collected in each group after 0, 6, 8, 10, 12, 18, and 24 h post-administration, and placed in plates with WA medium, followed by incubation at 25°C for 30 days, to check for the growth of *P. chlamydosporia*. The authors reported the identification of VC4 isolate only in samples from the test group, and 6 h post administration. VC4 isolates obtained after 30 days of incubation were used for further *in vitro* tests in WA medium, aiming to check the maintenance of ovicidal activity against *A. galli* and *H. gallinarum* eggs. A significant reduction in egg viability was observed after 74 h of incubation and the highest rates were recorded after 144 h, totalizing approximately 60% and 40% for *A. galli* and *H. gallinarum*, respectively.

Finally, a study performed by Thapa et al. (2018) aimed to evaluate the performance of *P. chlamydosporia* (Biotype 10) in reducing worm burden and ascarid egg count in hens, by jointly giving the fungus with sterilized and non-sterilized soil. These soils were previously used in *in vitro* trials aiming to evaluate the egg recovery in sterilized and non-sterilized substrates inoculated with *P. chlamydosporia*. For the *in vivo* trial, birds were fed with the same soils together with the morning meal, comprising four experimental groups: sterilized control soil (SC), sterilized soil with fungus (SF), non-sterilized control soil (NC), and non-sterilized soil with fungus (NF). The study aimed to analyse worm recovery, fecal eggs counts, and *A. galli* Igγ levels after fungal administration. A significant interaction between soil sterility and fungal treatment on ascarid worm burden was observed, which decreased significantly only in hens fed with sterilized soil inoculated with *P. chlamydosporia*, in comparison with the other three treatments. However, this scenario was completely different from that observed for egg counting, in which the overall EPG in the SF group was significantly higher than in groups SC and NC, but not versus the NF group. In addition, hens from the SF group had significant higher proportions of the three largest worm length categories (1.5-3.0 cm, 3.0-5.0 cm, 5.0-8.0 cm), in comparison with the other groups. This was an interesting result since the SF group had the lowest mean worm burden of *Ascaridia galli* and the highest abundance of mature worms, which allowed to conclude that reduced exposure modified *A. galli* populations. As stated by the authors, if all ascarid forms are not eradicated from the farm's soil or litter, the remaining eggs might therefore lead to long-term serious infection outbreaks in flocks. These

results emphasize the need to optimize parasite control programs in farms, targeting the reduction of environmental contamination with eggs and thus avoiding episodes of re-infection.

3.2.1.4. Further Research

Although only five research articles related with the use of predatory fungi against GI parasites of birds have been published to date, overall results reveal their potential effectiveness against nematode eggs and larvae and suggest their possible use in parasite control programs for domestic and exotic birds.

Despite their promising utility, some questions remain to be addressed. One of them refers to the impact of fungal administration on bird intestinal microbiota and if it can have a potential probiotic effect, besides their activity on fecal and soil environment. Interactions between the intestinal microbiota diversity and the chicken's productivity has been demonstrated by several authors, although depending on the type of sample used for 16S rDNA sequencing (e.g., small intestine, large intestine, feces), with generally a higher bacterial diversity being found in the intestine of chickens with greater feed conversion ratio (Carrasco et al. 2019). A growing number of studies aiming to characterize the relationships between parasites and the gut microbiota in several animal hosts has also been observed. For example, (Huang et al. 2018) demonstrated that, in chickens, coccidiosis modulated the avian gut microbiota towards a lower bacterial diversity and relative abundances of *Lactobacillus* and *Faecalibacterium*, in contrast to higher abundances of *Clostridium*, *Lysinibacillus* and *Escherichia* after fecal analysis. Therefore, it would be interesting to analyse the influence of predacious fungi administration on host intestinal microbiota, and to investigate if they can have a potential dual action on parasitism by regulating the gut microbiota and predating environmental forms.

More *in vitro* studies are needed to test these fungi against other bird GI parasites. Promising results already obtained against ascarid eggs and nematode larvae also reveal that it would be interesting to check the efficacy of ovicidal fungi against coccidia oocysts, cestode, and trematode eggs, as well as larvicidal fungi against L3 larvae from other nematode species. In addition, more *in vivo* studies using fungal formulations need to be performed in several species of domestic and exotic birds, kept in farms, zoos, or private collections, and evaluate the long-term kinetics of egg/oocyst shedding in the environment.

Since these fungi are often found in agricultural soils and animal feces, there is a great opportunity for scientific centres working on this topic to isolate native fungal species with predatory capacity and establish mycological collections, and routinely test them against GI

parasites, namely from birds, both *in vitro* and *in vivo*, setting up the basis for developing more biocontrol products with market application.

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CHAPTER II – Improving the coprological diagnosis of gastrointestinal parasites in birds

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Linked publication:

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Article 2 – "Implementation of Mini-FLOTAC in routine diagnosis of coccidia and helminth infections in domestic and exotic birds"

Abstract

Mini-FLOTAC (MF) has recently been proposed for the fecal quantification of gastrointestinal (GI) parasites in birds due to its higher sensitivity and precision in comparison with the McMaster method. The current research aimed to test the use of MF in routine diagnosis of coccidia and helminth infections in several domestic and exotic bird collections in Portugal. Between July 2020 and April 2021, a total of 142 fecal samples from organic layers, peacocks and ratites were collected in four Portuguese bird collections and processed using MF and fecal cultures to identify and calculate GI parasite shedding and prevalence. The McMaster method was also used to compare the shedding levels obtained for both quantitative techniques. MF's relative sensitivity and specificity were also assessed, using McMaster as the reference technique. The implementation of MF resulted in an average *Eimeria* spp. shedding higher in peacocks from bird collection 2 (502 OPG), followed by peacocks from collection 1 (107 OPG) and organic layers (24 OPG) and peacocks from collection 3 (9 OPG). Peacocks were also positive for *Capillaria* spp., *Trichostrongylus tenuis* and *Strongyloides pavonis*, whereas ostriches and emus were infected by *Libyostrongylus douglassii*. The MF protocol for exotic animals and the McMaster method did not differ significantly for each parasitic agent and bird species, and MF achieved relative sensitivities and specificities higher than 70% for Galliform *Eimeria* spp., peacock helminths and ratites' *L. douglassii* infections. Higher *L. douglassii* EPG values were identified using the MF protocol for exotic species (2 g of feces/38 mL of sucrose solution), followed by McMaster 2/28, MF 5/45 and MF 2/18. The use of MF allowed for obtaining different intestinal parasitic populations in several bird species and locations, and MF 2/38 is globally proposed as the most suitable protocol for bird fecal samples as an alternative to the McMaster method in the diagnosis of avian intestinal parasitic infections.

Keywords: Poultry, Exotic Birds, Gastrointestinal Parasites, Mini-FLOTAC, Portugal.

1. Introduction

Free-range poultry and captive wild birds are commonly housed in limited areas with high animal stocking and access to the environment and co-habit with other bird species and wild avifauna, consequently being exposed to a wide variety of generalist or host-specific gastrointestinal (GI) parasites, namely coccidia and helminths, which are still responsible for severe health and economic concerns in poultry farms and zoological collections worldwide (Papini et al. 2012; Ederli and Rodrigues de Oliveira 2015; Lozano et al. 2019; Blake et al. 2020; Carrera-Játiva et al. 2020; Carrisosa et al. 2021).

Coccidia infections in Galliformes from free-range farms, zoos and public gardens can reach prevalence and shedding values of up to 80% and 15,000 oocysts per gram of feces (OPG), respectively (Titilincu et al. 2009; Papini et al. 2012; Jaiswal et al. 2013; Prakashbabu et al. 2017; Lolli et al. 2019; Carrisosa et al. 2021). Avian helminth infections are also a reality in traditional free-range farms, private exotic collections and public gardens, with ascarids (e.g., *Ascaridia galli*), heterakids (e.g., *Heterakis gallinarum* and *H. isolonche*), *Trichostrongylus tenuis*, *Strongyloides* spp. and *Capillaria* spp. being the most prevalent and pathogenic nematodes in domestic and exotic Galliformes such as poultry and peacocks (Titilincu et al. 2009; Papini et al. 2012; Jaiswal et al. 2013; Ilić et al. 2018; Lolli et al. 2019).

Birds of the order Struthioniformes, such as ostriches, emus and rheas, are also frequently housed in zoos and occasionally in farms across the globe, and GI parasitism by helminths is of the most clinical importance in these birds. Infections caused by *Libyostrongylus douglassii* are noteworthy, which is the most common and pathogenic nematode in ostriches and other ratites, being responsible for rotten stomach disease. There are also other species of *Libyostrongylus*, such as *L. magnus* and *L. dentatus*, the latter having only been recorded in North America thus far. *Codiostomum struthionis* is also commonly found in ratites, inhabiting the distal cecum and upper rectum of adult birds and occasionally being responsible for hemorrhagic processes and oedema in the cecum's mucosa (Jansson and Christensson 2000; Ponce Gordo et al. 2002; McKenna 2005; Ederli and Rodrigues de Oliveira 2015; Kummrow 2015).

Over the past seven years, Mini-FLOTAC (MF) has been used in routine parasitological diagnosis in several animal species, and most of the studies concluded that this technique is a good alternative to the traditional McMaster technique, allowing simultaneous identification of helminth eggs and coccidia oocysts with relatively higher sensitivity, accuracy and precision. Established in the Unit of Parasitology and Parasitic Diseases of the Department of Veterinary Medicine and Animal Production (University of Naples Federico II, Naples, Italy), the manufacturer proposes three MF protocols for animals, namely for small animals (e.g., dogs

and cats), herbivores (e.g., ruminants and horses) and exotic species (e.g., birds and reptiles), which involve different fecal dilutions and detection limits (Cringoli et al. 2017; Capasso et al. 2019; Maurelli et al. 2020).

Despite the constant annual increment in studies involving the use of MF in several animal species, its implementation in epidemiological studies in birds has been extremely scarce thus far, and there is a lack of consensus regarding the optimal protocol for the diagnosis of coccidia and helminth infections in these hosts. However, recent studies with MF in birds have demonstrated its potential in the diagnosis of common avian coccidia and nematodes, with some achieving sensitivities of up to 100% (Bortoluzzi et al. 2018; Coker et al. 2020; Daş et al. 2020; Lozano et al. 2021b).

The current research aimed to implement and optimize MF in the routine diagnosis of GI parasitic infections in several domestic and exotic bird species from different collections across Portugal and compare the resulting shedding levels with the traditional McMaster method.

2. Materials and methods

2.1. Bird collections and fecal samplings

Between July 2020 and April 2021, a total of 142 fecal samples from organic layers (*Gallus gallus domesticus*) (n = 46), peacocks (*Pavo cristatus*) (n = 68), ostriches (*Struthio camelus*) (n = 9) and emus (*Dromaius novaehollandiae*) (n = 19) (Figure 10) were collected from a poultry farm and several exotic bird collections in Portugal. The sampling sites were located in the Lisbon and Santarem districts, comprising bird collection 1 (Lisbon, 38°42′50.241″ N 9°8′2.182″ W), bird collection 2 (Lisbon, 38°45′30.44″ N 9°9′23.83″ W), bird collection 3 (Abrantes, 39°26′52.595″ N 8°10′24.949″ W) and a poultry farm (Lourinhã, 39°13′54.373″ N 9°17′2.235″ W), whose species and housing conditions are summarized in Table 2.

Figure 10. Domestic and exotic birds selected for this study: (A) organic layers, (B) peacocks, (C) ostriches and (D) emus (originals).

Table 2. Sampling periods, bird species, quantity of samples and outdoor areas.

Period	Collection	Species	No. of birds	Age	Samples	Outdoor area
October-December 2020	Bird collection 1 (Lisbon)	Peacocks	20	3 months-9 years	29	9600 m^2
April 2021	Bird collection 2 (Lisbon)	Peacocks	40	3 months-19 years	25	6000 m^2
September 2020-February 2021	Bird collection 3 (Abrantes)	Ostriches	2	4 years	9	50,000 m^2
		Emus	6	7-14 years	19	
		Peacocks	3	3-6 years	14	6000 m^2
July-November 2020	Poultry farm (Lourinhã)	Organic layers	200	16 months	46	1700 m^2

Fresh fecal samples were randomly collected after excretion to the environment, deposited in individual plastic bags and then immediately transported in a cooling bag to the Laboratory of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine, University of Lisbon, where they were stored in a refrigerator (4-5 °C) for 1 week, processed and analyzed.

This research followed the daily activity of the selected farm and bird collections in strict collaboration with the owners and assistant veterinarians, and no interferences were made in the regular health management of all collections.

2.2. Coprological techniques

All samples were processed and analyzed with the Mini-FLOTAC technique, aiming to calculate gastrointestinal parasites' shedding (eggs and oocysts per gram of feces – EPG and OPG, respectively), whereas fecal cultures were used for taxonomical identification of coccidia and helminths with environmental larval development. The McMaster method was also used in each sample to compare average EPG and OPG data with MF (Zajac and Conboy 2012b; Cringoli et al. 2017; Lozano et al. 2021b).

2.2.1. Mini-FLOTAC

The Mini-FLOTAC protocol followed the guidelines proposed by the manufacturer for exotic species (MF 2/38): 2 g of feces was added to the corresponding Fill-FLOTAC device and mixed with 38 mL of saturated sucrose solution (specific gravity 1.2); then, the fecal suspension was transferred to the (previously assembled) reading chamber and left for 10 min to rest on the lab bench before rotating the top piece of the reading chamber (Figure 11). Coccidia oocysts and helminth eggs were identified and counted in a light microscope (100x), using detection limits of 10 OPG and 10 EPG, respectively (Cringoli et al. 2017; Lozano et al. 2021b).

Figure 11. Mini-FLOTAC and Fill-FLOTAC devices used for quantitative coprology (original).

The three Mini-FLOTAC protocols proposed by the manufacturer for small animals (2 g/18 mL of saturated sucrose solution, specific gravity 1.2), exotic species (2 g/38 mL) and herbivores (5 g/45 mL) were also tested in 8 ostrich fecal samples positive for the nematode *L. douglassii*, with shedding values higher than 1000 EPG, using detection limits of 5, 10 and 5 EPG, respectively, aiming to compare the resulting EPG levels between the MF and McMaster protocols (Cringoli et al. 2017).

The relative sensitivity of Mini-FLOTAC was calculated as the percentage of true positive reads (TP) in the sum of false negative (FN) and TP reads, while Mini-FLOTAC's relative specificity was calculated as the percentage of true negative reads (TN) in the sum of false positive (FP) and TN reads (Daş et al. 2020; Lozano et al. 2021b). These parameters were calculated for Galliform coccidia, peacock helminths and ratites' *L. douglassii*, assuming the McMaster method as the reference technique due to its historic and frequent use for quantitative copromicroscopy in most parasitology laboratories (Gordon and Whitlock 1939; Cringoli et al. 2010; Bortoluzzi et al. 2018).

2.2.2. McMaster

For the McMaster method, 2 g of each fecal sample was mixed with 28 mL of saturated sucrose solution (specific gravity 1.2), and the filtered suspension was transferred to a McMaster slide. Parasitic forms were identified and counted under a light microscope (100x), using a detection limit of 50 OPG and 50 EPG (Zajac and Conboy 2012b).

2.2.3. Fecal cultures

Fecal cultures for oocyst sporulation were performed only with samples positive for coccidia, using 5-10 g of each fecal sample, which were placed on Petri dishes with potassium dichromate (2%) and incubated for 1 week at 26 °C. Sporulated oocysts were identified based on their size and the number of sporocysts inside to the genus level. Fecal cultures for helminths were only conducted with samples positive for nematodes with environmental larval development using 5-10 g of each fecal sample, which were placed inside plastic cups and incubated for 2 weeks at 26 °C. Infective larvae (L3) obtained from ostrich samples were analyzed in terms of their morphology and measures and compared with current reports in the literature for different species of *Libyostrongylus* and *Codiostomum* (Ederli et al. 2008; Zajac and Conboy 2012b; Ederli and Rodrigues de Oliveira 2014; López-Osorio et al. 2020).

2.3. Statistical analysis

The software Microsoft® Excel®, for Microsoft 365 MSO (Microsoft Corporation, Redmond, WA, USA, 2021), was used for data storage and table and chart editing, and the software GraphPad InStat®, version 3.0 for Windows (GraphPad Software, San Diego, CA, USA, 2021), was used for statistical analysis.

Data normality was assessed with the Kolmogorov-Smirnov test, and for every group of animals and quantitative technique, EPG and OPG data failed the normality test ($p<0.0001$). These results determined the use of the following non-parametric tests: Kruskal-Wallis test for *Eimeria* OPG comparison between each sampling site; Mann-Whitney test for helminth EPG comparison between peacocks from collections 1 and 2 and between ostriches and emus from collection 3; Wilcoxon matched pairs test for Mini-FLOTAC and McMaster comparison in Galliform *Eimeria* spp., peacock helminths and ratites' *L. douglassii*. For the Mini-FLOTAC optimization trial in ostrich samples, EPG data passed the Kolmogorov-Smirnov test ($p>0.10$), and the results obtained for each protocol were compared using the Tukey-Kramer multiple comparisons test. A significance level of $p<0.05$ was used for all statistical tests.

3. Results

3.1. Epidemiological results with Mini-FLOTAC in domestic and exotic birds

The current research with Mini-FLOTAC revealed an overall *Eimeria* spp. prevalence higher in peacocks from bird collection 3 (43%), followed by organic layers (41%) and peacocks from bird collections 1 and 2 (29% and 25%, respectively). The overall coccidia prevalence in Galliformes was 35%. However, the average *Eimeria* spp. shedding was higher in peacocks from bird collection 2 (502 OPG) in comparison with peacocks from collection 1 (107 OPG) and organic layers (24 OPG) and peacocks from collection 3 (9 OPG), which were statistically significant differences ($p<0.0001$). Additionally, the average *Eimeria* OPG in all Galliformes reached 160 OPG (Table 3).

Table 3. Epidemiological results obtained with Mini-FLOTAC for each bird species.

Collection	Bird species	GI parasites	OPG | EPG (Min-Max)	Prevalence (%)
Bird collection 1	Peacocks	*Eimeria* spp.	107 (0-750)	29
		Helminths	145 (0-2000)	14 (*Capillaria* spp.) 14 (*S. pavonis*)
Bird collection 2	Peacocks	*Eimeria* spp.	502 (0-1800)	25
		Helminths	66 (0-1000)	8 (*T. tenuis*) 4 (*S. pavonis*)
Bird collection 3	Ostriches	*L. douglassii*	2731 (500-5700)	100
	Emus		60 (0-420)	32
	Peacocks	*Eimeria* spp.	9 (0-30)	43
Poultry farm	Organic layers	*Eimeria* spp.	24 (0-300)	41
Average *Eimeria* spp., Galliformes			160 (0-1800)	35
Average Helminths, Peacocks			70 (0-2000)	15
Average Helminths, ratites			1396 (0-5700)	66

Helminth species were identified in all bird collections except for the poultry farm, in which *Eimeria* spp. oocysts were the only intestinal parasitic forms found. Collections 1 and 2 exhibited different helminthic populations: birds from collection 1 were positive for *Capillaria* spp. (14%) and *S. pavonis* (14%), and birds from collection 2 were positive for *T. tenuis* (8%) and *S. pavonis* (4%). Helminth EPG was higher in peacocks from collection 1 (145 EPG) in contrast to collection 2 (66 EPG), despite the fact that their differences were not significant ($p=0.58$), and peacocks from collection 3 were not found infected by any helminth.

Ratites from collection 3 were infected by *L. douglassii*, with frequencies of 100% and 32% and average shedding levels of 2731 and 60 EPG for ostriches and emus, respectively, with the EPG results differing significantly between ratite species ($p<0.0001$) (Figure 12).

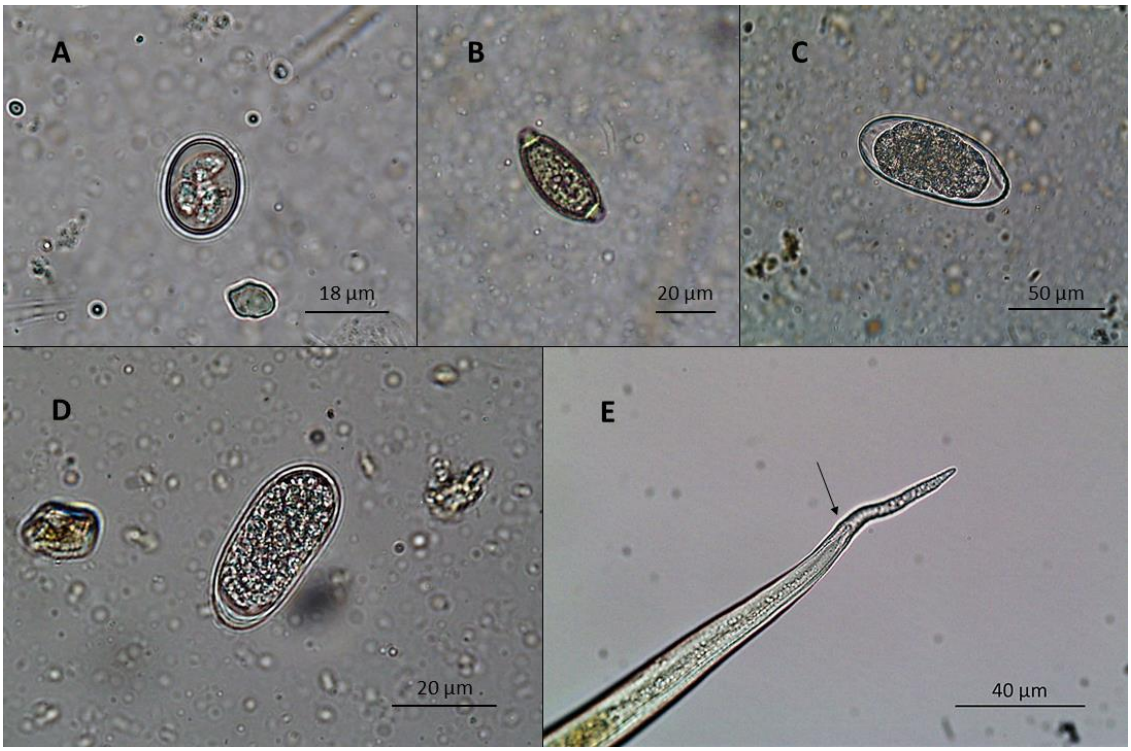

Figure 12. (A) *Eimeria* sp. oocyst; (B) *Capillaria* sp. egg; (C) *Trichostrongylus tenuis* egg; (D) *Strongyloides pavonis* egg; (E) tail and sheath end of *Libyostrongylus douglassii* L3 identified in emus, highlighting the typical tail-end knob format (black arrow) (originals).

3.2. McMaster and Mini-FLOTAC comparison

The McMaster and Mini-FLOTAC techniques resulted in similar average shedding values for *Eimeria* spp. in Galliformes (188 and 160 OPG, respectively) and *L. douglassii* in ratites (1647 and 1396 EPG, respectively), and their differences were not significant ($p=0.17$ and $p=0.67$ for Galliformes and ratites, respectively). Helminths' average shedding values in the aggregate community of peacocks from collections 1 and 2 reached 16 and 105 EPG for McMaster and MF, respectively, and the techniques also did not differ significantly ($p=0.08$) (Table 4).

Table 4. Mini-FLOTAC and McMaster shedding data obtained for each bird group.

Bird groups	GI parasites	Mini-FLOTAC EPG | OPG (Min–Max)	McMaster EPG | OPG (Min–Max)
Galliformes	*Eimeria* spp.	160 (0–1800)	188 (0–5100)
Peacocks (Collections 1 and 2)	Helminths	105 (0–2000)	16 (0–400)
Ratites	*L. douglassii*	1396 (0–5700)	1647 (0–10,000)

The relative sensitivity of the MF technique for Galliform coccidia, peacock helminths and *L. douglassii* in ratites reached 86%, 86% and 100%, respectively, and the relative specificity of MF was 70%, 100% and 87%, respectively.

The MF optimization trial in ostrich fecal samples positive for *L. douglassii* resulted in higher EPG levels for the MF 2/38 protocol (2028 ± 636 EPG), followed by McMaster (1788 ± 635 EPG), MF 5/45 (1553 ± 543 EPG) and MF 2/18 (1410 ± 550 EPG) (Figure 13). Differences were statistically significant between the pairs MF 2/18 – MF 2/38 ($p<0.001$), MF 2/18 – McMaster ($p<0.05$) and MF 5/45 – MF 2/38 ($p<0.01$). For the pairs MF 5/45 – MF 2/18, MF 2/38 – McMaster and MF 5/45 – McMaster, no significant differences were observed ($p>0.05$ for each pair).

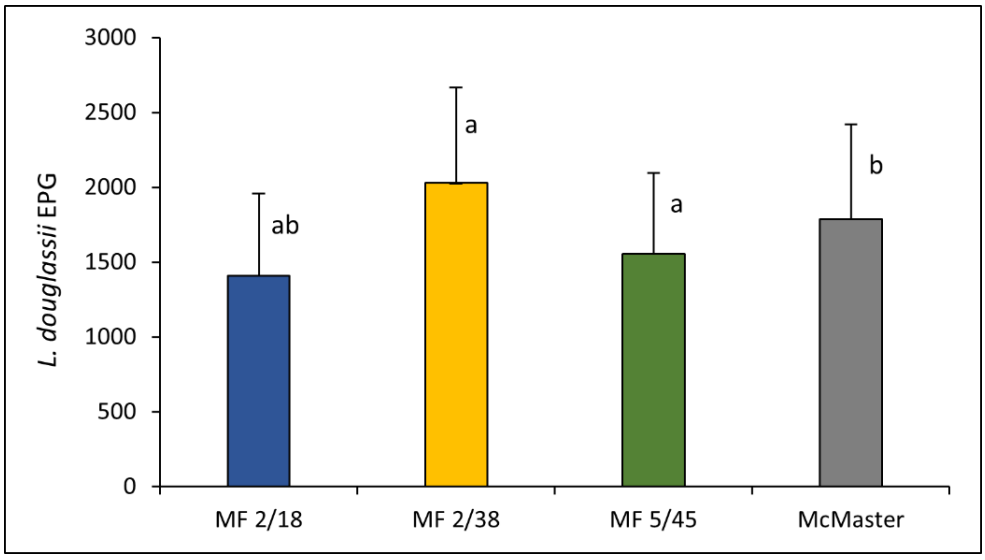

Figure 13. *Libyostrongylus douglassii* average EPG values and standard deviations in Mini-FLOTAC for small animals (MF 2/18), exotic animals (MF 2/38) and herbivores (MF 5/45) and McMaster. a: significant differences between MF protocols; b: significant difference between MF 2/18 and McMaster.

4. Discussion

The current research focused on the innovative implementation of Mini-FLOTAC in the routine diagnosis of gastrointestinal parasitic infections in several species of domestic and exotic birds in different housing conditions and kept for different purposes. The use of this technique for epidemiological purposes revealed different parasitic scenarios in each sampling site regarding the intestinal parasites identified and their respective shedding levels and frequencies.

Coccidia belonging to the genus *Eimeria* were the most prevalent intestinal parasites in organic layers and peacocks from all locations, which confirms this group of parasites as ubiquitous in several species of domestic and exotic Galliformes (Zajac and Conboy 2012a; Jaiswal et al. 2013; López-Osorio et al. 2020; Carrisosa et al. 2021).

Organic layers from the poultry farm had a moderate *Eimeria* spp. prevalence (41%), similar to previous research in free-range/organic broiler and layer flocks, which highlights the importance of regular monitoring of birds' health status, namely through periodic blood and fecal samplings for parasitological analysis (Prakashbabu et al. 2017; Lolli et al. 2019; Carrisosa et al. 2021). These birds were not subjected to any antiparasitic drug program since organic animal production is extremely regulated in the European Union, and it is forbidden to use antiparasitic drugs for prophylactic purposes, which therefore poses a higher risk of developing parasitic diseases in organic flocks. However, the overall *Eimeria* spp. shedding in these birds was considerably low (24 OPG), which can be explained by the advanced age of the flock (16 months) and the low stocking density, reflecting a potential equilibrium between the parasite and the host immune system, which is common in older domestic birds (Shamim et al. 2015; Kaboudi et al. 2016; Prakashbabu et al. 2017; Carrisosa et al. 2021).

The period of the year in which sampling took place may have also influenced the resulting *Eimeria* spp. shedding in organic layers, as the Mediterranean summer season is frequently long, dry and hot, commonly ending in late October, which offers adverse environmental conditions for oocysts' survival and sporulation in the soil, thus limiting their dissemination between birds, as concluded by other authors in different countries and climatic conditions (Shamim et al. 2015; Kaboudi et al. 2016; Prakashbabu et al. 2017; Lolli et al. 2019). This research did not identify helminths in samples from organic layers, unlike other publications regarding parasitological assays in birds kept in organic, free-range or backyard conditions (Thapa et al. 2015; Carrisosa et al. 2021; Saraiva et al. 2021). Possible explanations may be the low stocking density and advanced age of the flock, which limit the dissemination of eggs among birds – particularly relevant in parasites with direct life cycles (e.g., ascarids) (Yazwinski and Tucker 2008; Zajac and Conboy 2012a). The adverse impact of direct sunlight

and high temperatures frequently recorded during summertime in the Mediterranean region may have also affected the survival of helminth eggs in the environment (Thapa et al. 2015).

Regarding the three peacock communities included in this research, Lisbon bird collections 1 and 2 had birds infected by coccidia and helminths, while samples from collection 3 were only positive for *Eimeria* spp. Bird collection 2 showed the highest recorded coccidia shedding level in peacocks (502 OPG) in comparison with bird collections 1 and 3. The high coccidia shedding level in peacocks from collection 2 might have been influenced by the season in which sampling took place, since springtime is the breeding period for peacocks, leading to an increase in the number of chicks which are more prone to be infected by coccidia than older birds, as well as being a season in the Mediterranean region normally characterized by moderate temperatures and relative humidity and sporadic episodes of intense rainfall, which are optimal conditions for oocyst sporulation in the soil and horizontal transmission among birds (Titilincu et al. 2009; Shamim et al. 2015; Kaboudi et al. 2016; Prakashbabu et al. 2017; Lolli et al. 2019).

Differences were also observed in the helminth populations of peacocks from collections 1 and 2. *Capillaria* eggs were only identified in collection 1, *T. tenuis* infections were only detected in peacocks from collection 2 and *S. pavonis* eggs were identified in samples from both collections. These results allow for confirming the susceptibility of peacocks to helminths (Titilincu et al. 2009; Jaiswal et al. 2013), both by ingestion of infective forms directly from feces or soil, or through their intermediate hosts, and by being exposed to wild free-ranging avifauna, which often leads to episodes of cross-transmission (Carrera-Játiva et al. 2020). All intestinal parasites identified in these communities are the first of their kind to be reported in ornamental peacocks from Portuguese public gardens.

Ostriches and emus from bird collection 3 were infected by *L. douglassii*, which is the most pathogenic helminth in ratites, and this is in accordance with other findings in ostriches in South America (Ederli et al. 2008; Ederli and Rodrigues de Oliveira 2015; Mariño-González et al. 2017), Asia (Eslami et al. 2007), Oceania (Barton and Seward 1993; Button et al. 1993; More 1996; McKenna 2005), Africa (Mukaratirwa et al. 2004) and Europe (Jansson and Christensson 2000; Ponce Gordo et al. 2002).

Despite commonly being considered specific to ostriches, *L. douglassii* was first identified 21 years ago in emus kept in Sweden (Jansson and Christensson 2000), which, at the time, suggested the potential cross-transmission of this helminth between different ratite species. Since then, no other research has identified this nematode across the ratites group, but it has been suggested that infections would likely be infrequent in these bird species (McKenna 2005). The current study detected infections by *L. douglassii* in emus, reaching a

prevalence of 32%, and confirmed the cross-transmission of the host-species barrier. However, the shedding levels were very low (60 EPG) when compared to ostriches (2731 EPG), and their differences were statistically significant, which allows us to conclude that ostriches are indeed more prone to be infected by this helminth than emus are. It must be noted that in bird collection 3, these two ratite species were normally separated in distinct parks, and infections were only recorded in emus when they were mixed with ostriches in the same area, which explains the low average shedding level of *L. douglassii* in emus and the host-specificity of this nematode, while revealing that cross-transmission for this helminth might indeed occur in the ratites group (Jansson and Christensson 2000). These results in emus also have implications in zoo animal management, since it is advisable to not mix different species of ratites in the same areas to avoid serious outbreaks of Libyostrongylosis in these birds.

The overall shedding and prevalence of *L. douglassii* in this study were higher than noted in previous research regarding this helminth in ratites, which is an interesting result since the majority of samples were collected during the winter season; low temperatures could therefore potentially limit helminth infections in these birds. The results from this study suggest the survival and maintenance of the infectious capacity of *L. douglassii* L3 larvae on soil during wintertime, as revealed by previous research conducted in Scandinavia (Jansson et al. 2002).

The implementation of the Mini-FLOTAC exotic animal protocol in bird fecal samples allowed the identification of the most common species of gastrointestinal parasites in the selected domestic and exotic birds, and both the relative sensitivities and specificities of this technique for all groups of intestinal parasites reached values higher than 70%. The use of MF in the detection of *Eimeria* spp. infections in Galliformes reached a relative sensitivity of 86%, similarly to previous research regarding the comparison of the MF and McMaster techniques in the detection of free-range poultry *Eimeria* spp. infections (Lozano et al. 2021b).

Samples from ratites, which had an average shedding higher than 1000 EPG, reached a relative sensitivity of 100%, meaning that for this level of shedding, there was no difference between both techniques and each positive sample averaged a true positive read with MF. These results are in accordance with previous research using the MF and McMaster methods for the detection of ascarid eggs in chicken feces, whose authors concluded that MF tended to be more sensitive than McMaster only at lower EPG levels, while the difference between them was not significant for shedding levels higher than 50 EPG (Daş et al. 2020).

Comparison of McMaster and MF regarding Galliform *Eimeria* spp., peacock helminths and ratites' *L. douglassii* shedding allowed for concluding that these techniques reached similar OPG and EPG results, which did not differ significantly regardless of the parasitic agent.

These results are similar to previous research with MF and McMaster in the detection of avian *Eimeria* spp., in which the authors also did not identify significant differences between these two techniques, using the same protocol (Cringoli et al. 2017; Lozano et al. 2021b).

The MF optimization trial using ostrich samples positive for *L. douglassii* allowed us to observe that the protocol established by the manufacturer for exotic animals resulted in higher shedding values in comparison with the other MF protocols (small animals and herbivores) and the McMaster method. The mean EPG values obtained with the MF exotic animal protocol differed significantly from the protocols for small animals and herbivores, but it did not differ from the McMaster method. One of the reasons for obtaining higher and statistically significant EPG results with the exotic animal protocol in comparison with the other MF protocols may have been due to the conjugating effect of a clearer reading and a higher multiplication factor as a result of the dilution used (1:20) (Cringoli et al. 2017; Bortoluzzi et al. 2018). On the other hand, since the dilutions were quite similar, the lack of significance between the McMaster technique and the MF 2/38 protocol may be explained by the difference in the multiplication factor between these techniques. Even though the McMaster technique revealed a poorer resolution and ranked second in terms of mean EPG, its multiplication factor of 50 EPG was enough to counter an eventual significant difference with MF 2/38.

The fact that both MF 2/38 and MF 5/45 did not differ significantly when compared to the McMaster method reflects the identical results these three techniques can achieve. Furthermore, since MF 2/38 achieved the highest EPG levels when compared to the other MF protocols and the McMaster protocol and differed significantly from the MF 5/45 protocol, it allows us to conclude that MF 2/38 can indeed be globally considered the best alternative to the McMaster method.

5. Conclusions

The current research accurately implemented Mini-FLOTAC in the routine diagnosis of gastrointestinal parasites in several domestic and exotic bird species and allowed us to identify different parasitic scenarios in the selected bird communities in Portugal, being the first European report in terms of using this technique in different avian species kept in captivity for different purposes, namely for egg production or ornamental exhibition.

The types of bird species, age amplitude of flocks, access to the environment, exposure to wild avifauna and season were potential key factors responsible for the wide diversity of intestinal parasitic species identified in this research. Galliformes were mainly infected by coccidia belonging to the genus *Eimeria* and helminths such as *Capillaria* spp., *T. tenuis* and *S. pavonis*, with differences between organic layers and peacocks, and this is the first national

report of gastrointestinal parasitism in peacocks from public gardens. Moreover, this study identified *L. douglassii* infections in ratites, which is the most pathogenic helminth in this group of birds, and the cross-infection and breaking of the host-species barrier for this helminth was confirmed in emus, being the first report in more than 20 years.

Comparison of the MF and McMaster techniques in Galliformes, peacocks and ratites allowed us to conclude that the MF exotic animal protocol is the best alternative to the McMaster method in birds, and therefore, the current study proposes this MF protocol for routine diagnosis of avian gastrointestinal parasitosis.

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CHAPTER III – Searching for new native predatory fungi in birds' feces

Linked publication:

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Article 3 – "Isolation of saprophytic filamentous fungi from avian fecal samples and assessment of its predatory activity on coccidian oocysts"

Abstract

Fungal strains used in the biocontrol of animal gastrointestinal parasites have been mainly isolated from pasture soil, decaying organic matter, and feces from herbivores and carnivores. However, their isolation from birds and assessment of predatory activity against avian GI parasites has been scarce thus far. This research aimed to isolate filamentous fungi from avian fecal samples and evaluate their predatory activity against coccidia. A pool of 58 fecal samples from chickens, laying hens, and peacocks, previously collected between July 2020-April 2021, were used for isolation of filamentous fungi and assessment of their *in vitro* predatory activity against coccidian oocysts, using Water-Agar medium and coprocultures. The Willis-flotation technique was also performed to obtain concentrated suspensions of oocysts. A total of seven *Mucor* isolates was obtained, being the only fungal taxa identified, and all presented lytic activity against coccidia. Isolates FR3, QP2 and SJ1 had significant coccidiostatic efficacies (inhibition of sporulation) higher than 70%, while isolates FR1, QP2 and QP1 had coccidicidal efficacies (destruction of the oocysts) of 22%, 14% and 8%, respectively, after 14 days of incubation, being a gradual and time-dependent process. To our knowledge, this is the first report regarding the isolation of native predatory fungi from avian feces and demonstration of their lytic activity against coccidia.

1. Introduction

Avian coccidiosis is one of the most important parasitic diseases affecting domestic and exotic birds worldwide, being responsible for severe health and economic concerns in poultry farms, ornithological parks, and private bird collections (Fatoba and Adeleke 2018; Lozano et al. 2019; Blake et al. 2020; Lozano et al. 2021c; Mesa-Pineda et al. 2021).

The control of this parasitic disease is mainly achieved through chemotherapy (e.g., anticoccidials) and vaccines. However, due to increasing concerns regarding antiparasitic drug resistance, extensive research has been conducted aiming at developing new alternative or complementary strategies to control coccidiosis in bird collections, including feed improvement, house cleaning and disinfection, as well as natural solutions like herbal extracts, essential oils, probiotics and prebiotics, and algae (Quiroz-Castañeda and Dantán-González 2015; Levine et al. 2018; Mesa-Pineda et al. 2021; Mohsin et al. 2021; Zaheer et al. 2022). More recently, Portuguese, Spanish, Brazilian and Danish researchers have been proposing the use of predatory fungi (also known as “nematophagous fungi” or “helmintophagous fungi”) with larvicidal and ovicidal characteristics as a complement to antiparasitic drugs for the control of gastrointestinal parasitic infections in domestic and exotic birds (Lozano et al. 2022).

The biocontrol of animal gastrointestinal parasitic infections using predatory fungi has already proved to be an accurate and sustainable complement to antiparasitic drugs, achieving efficacies of up to 97% in reducing the parasite egg shedding (number of eggs per gram of feces, EPG) in horses and ruminants (Madeira de Carvalho et al. 2011; Arias et al. 2013a; Aguilar-Marcelino et al. 2017; Ortiz Pérez et al. 2017; Healey et al. 2018; Canhão-Dias et al. 2020; Araújo et al. 2021; Branco de Oliveira et al. 2021; Palomero et al. 2021; Rodrigues et al. 2021; Voinot et al. 2021; Mendoza de Gives et al. 2022; Sobral et al. 2022). However, only a few *in vitro* and *in vivo* studies have assessed the performance of predatory fungi against parasites affecting other animal hosts, namely birds, dogs, raccoons and wapitis (Frassy et al. 2010; Araujo et al. 2012; Soares et al. 2014; Cazapal-Monteiro et al. 2015; Palomero et al. 2021; Lozano et al. 2022; Viña et al. 2022).

Predatory fungi are also known for their ubiquity, having been mostly isolated from agricultural soil, decaying organic matter, and animal feces (Braga and Araújo 2014). Studies performed in America, Europe, Asia, Oceania and Antarctica have reported the isolation of filamentous fungi with ability to predate intestinal parasitic forms, from feces belonging to a wide diversity of animal species, including: sheep, goats and bovines (Larsen et al. 1994; Soto-Barrientos et al. 2011; Liu et al. 2015; Hernández et al. 2017; Arroyo-Balán et al. 2021); water buffalo (Ojeda-Robertos et al. 2019); donkeys (Arroyo-Balán et al. 2021); coati, raccoon, Eurasian lynx, Brown bear, mouflon, gazelle, bison, dromedary, guanaco and wallaby

(Hernández et al. 2017); and horses (Soto-Barrientos et al. 2011; Hernández et al. 2017). The most commonly isolated taxa of predatory fungi with larvicidal properties are *Duddingtonia flagrans* (Dudd.) R.C. Cooke (1969), *Arthrobotrys* spp., and *Monacrosporium* spp., while *Pochonia chlamydosporia* (Goddard) Zare & W. Gams (2001), *Mucor circinelloides* Tiegh (1875), *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (2011), *Verticillium* spp., and *Trichoderma* spp. have shown to present ovicidal properties (Madeira de Carvalho et al. 2012; Braga and Araújo 2014; Araújo et al. 2021). Nevertheless, studies on the isolation of these fungi from avian fecal samples have not yet been reported in the scientific literature.

The current research aimed to isolate native filamentous fungi from fecal samples of domestic and exotic birds and assess its *in vitro* predatory activity on coccidian oocysts.

2. Methods

2.1. Fecal samplings and coprological analysis

A total of 89 fecal samples from free-range chickens and laying hens (*Gallus gallus domesticus*; n=46) and peacocks (*Pavo cristatus*; n=43), were previously assessed using several coprological techniques, such as McMaster, Mini-FLOTAC and Coprocultures, in the scope of a recent study performed at the Laboratory of Parasitology and Parasitic Diseases (LPPD) of Faculty of Veterinary Medicine, University of Lisbon (Lozano et al. 2021c), which reported that 58 samples (65%) were positive for at least one gastrointestinal parasite taxon, namely coccidia of the genus *Eimeria*, and nematodes like *Capillaria* sp., *Trichostrongylus tenuis* and *Strongyloides pavonis*. Samples belonged to healthy animals, which did not show any clinical signs of gastrointestinal disorders, namely diarrhoea and/or feces with blood.

These samples were collected between July 2020 and April 2021, in a poultry farm (PF) and two exotic bird collections located in Lisbon (SJ) and Santarem (QP) districts (Portugal). The poultry farm is located in Northwestern Lisbon (39°13’54.373’’ N 9°17’2.235’’ W) and harbours two separate populations of 200 free-range chickens and 200 laying hens, while the exotic bird collections SJ and QP have 20 and 3 peacocks, being located in central Lisbon (38°42’50.241’’ N 9°8’2.182’’ W) and Abrantes (39°26’52.595’’ N 8°10’24.949’’ W), respectively.

Fecal samples were immediately collected after excretion, packed in plastic bags, and transported to LPPD, being stored in a refrigerator (4°C) for a maximum length of 1 week, until further processing.

2.2. Isolation and morphological identification of filamentous fungi

A total of 58 avian fecal samples positive for gastrointestinal parasites were used for isolation and identification of filamentous fungi, at both the LPPD and the Laboratory of Mycology of the Faculty of Veterinary Medicine – University of Lisbon (Portugal). The idea of using only these samples was based on the premise that this kind of fungi have as main ability the predation and destruction of parasite eggs, oocysts and larvae, and thus this procedure would stimulate the growth of potential predaceous fungi and restrict the development of other fungal groups.

For this purpose, approximately 1 g of each fecal sample was placed on the surface of Water-Agar medium (WA, 2%), and then incubated at 26°C for 3 weeks. Once filamentous fungi growth was recorded, individual colonies were subjected to 3-4 passages, using Wheat-Flour Agar (WFA, 2%) and incubation cycles of 26°C for 1 week, until achieving pure cultures. Two replicates were used for each fecal sample (Hernández et al. 2017).

All isolates were subjected to morphological identification at the genus level, based on (Cooke and Godfrey 1964; Hernández et al. 2017; Arroyo-Balán et al. 2021; Ocampo-Gutiérrez et al. 2021). Measurements (length and width) and morphology description were carried out for a total of 10 sporangia and hyphae (200x and 400x total magnification), and conidia (1000x total magnification, in immersion oil), using a lactophenol cotton blue stain and a light microscope. Also, macroscopical characterization of the colonies was performed for each isolate, regarding its texture and colour.

Suspensions of spores were established for each isolate using distilled-water, and their final concentration was calculated using the Neubauer chamber. All fungal suspensions were standardized to 10^6 spores/mL.

Fungal isolates were preserved in Petri dishes and glass flasks with WFA at room temperature, and 850 µL of each fungal aqueous suspension were stored at -20°C in cryotubes with 15% (v/v) sterile glycerol (Vellanki et al. 2018).

2.3. Molecular characterization of fungal isolates

2.3.1. DNA extraction

DNA extraction from all fungal isolates was performed using the E.Z.N.A.® Fungal DNA Mini kit (Omega Bio-Tek, Norcross, GA). A calibrated 1 µL swab was used to collect fresh mycelia from each fungal isolate. This procedure was repeated 5 times, and the total mycelia volume was placed in the respective 2 mL microcentrifuge tubes, to which 600 µL of lysis buffer FG1 were also added. The mixture was vortexed to disperse all clumps and incubated at 65°C

for 10 min. Then, 140 µL of FG2 buffer (glacial acetic acid) were added, and the suspension was vortexed. Tubes were incubated on ice for 5 min, and then centrifuged at 10,000 x g for 10 min. Supernatants were transferred to new microcentrifuge tubes, to which were also added 0.7 volumes of isopropanol. After vortex, suspensions were centrifuged at 10,000 x g for 2 min. The supernatants were discharged, and 300 µL of sterile distilled water were added to each DNA pellet, and then vortexed. A total of 4 µL of RNase A was added to each tube, followed by 150 µL of FG3 buffer (guanidine hydrochloride) and 300 µL of 100% ethanol, always using the vortex to mix the suspensions. Further steps were performed using HiBind® DNA Mini Columns to eliminate polysaccharides, phenolic compounds, and enzyme inhibitors from fungal lysates, due to its reversible nucleic acid-binding. Pure DNA was eluted in 200 µL of sterile distilled water, and its purity and concentration were checked using NanoDrop™ (Thermo Fisher Scientific Inc., Waltham, USA). Tubes were finally stored at -20 °C.

2.3.2. Amplification of ribosomal DNA

Amplification of rDNA was performed for each isolate targeting the ITS1-5.8S-ITS2 region, using 10-66 ng of genomic DNA and primers ITS1 (TCC GTA GGT GAA CCT GCG G) and ITS4 (TCC TCC GCT TAT TGA TAT GC). These procedures followed the guidelines described by Arroyo-Balán et al. (2021) and Lau et al. (2007), and the PCR reaction was performed in a 25 µL volume, composed by: 0.4 µL ITS1 (0.8 µM), 0.4 µL ITS4 (0.8 µM), 10 µL DNA template, 10 µL NZYTaq II Green Master Mix (NZYTech, Lisbon, Portugal) and 4.2 µL molecular biology water. A negative control was also used, by replacing DNA for water.

Thermocycling conditions were the following: an initial denaturation step at 95°C for 10 min, followed by 60 cycles composed by a denaturation step at 94°C for 15 s, an annealing step at 55°C for 30 s, and an extension step at 72°C for 30 s. Finally, an extension step was performed at 72°C for 5 min. The amplicons were analysed by agarose-gel (1.5%) electrophoresis, stained with 2.5 µL of GreenSafe Premium (NZYTech), and including the NZYDNA Ladder VI (50-1500 bp; NZYTech). The gel was run at 85 V for 40 min and visualized using the equipment ChemiDoc and the Image Lab™ Software (Bio-Rad Laboratories, Inc., California, USA).

Each PCR product was purified using magnetic beads (MCLAB, California, USA) for DNA precipitation, followed by a pellet wash with 85% ethanol and subsequent elution in MiliQ water. The obtained supernatants were used for further sequencing. Purified PCR products were sequenced using the ITS1 primer and BigDye™ Terminator version 3.1 Cycle Sequencing Kit, and also with the equipment DNA Analyzer 3730 XL (Thermo Fisher Scientific Inc.).

Sequences were assessed using Chromas Software, version 2.6.6 (Technelysium Pty, Ltd., South Brisbane, Australia), and their quality was checked based on well-defined peaks of the nucleotides in the chromatograms. Sequences were then blasted using the Blastn Suite (BLAST®) of the National Centre for Biotechnology Information (NCBI), to perform a preliminary analysis of sequences significant alignments and select fungal taxa for comparative purposes in the phylogenetic analysis. For each fungal isolate, a Top 3 of sequence similarity was established based on the results from Blastn Suite.

2.3.3. Phylogenetic analysis

Sequences were manually edited using MEGA software, version 11.0.11 (Tamura et al. 2021), to check for the quality of sequences and undetected nucleotides, remove primers, as well as to perform their alignment using the MUSCLE algorithm. Based on the BLAST® search, ITS1-5.8S-ITS2 region sequences from isolates *Mucor circinelloides* CBS 195.68 (accession number: NR_126116.1), *Mucor lusitanicus* CBS 108.17 (accession number: NR_126127.1), *Mucor racemosus* f. *racemosus* CBS 260.68 (accession number: NR_126135.1) and *Mucor fragilis* (accession number: FJ904925.1) were also included. The IQ-TREE web server (Trifinopoulos et al. 2016) was used to generate a maximum likelihood phylogenetic tree from 1000 replications. The ModelFinder option of IQ-TREE was set for auto-determination of the best model (Kalyaanamoorthy et al. 2017). The ultrafast bootstrapping tool (1000 bootstrapped alignments) was chosen to obtain node support statistics, with its branches being only supported by bootstrap values above 50 (Hoang et al. 2018). The phylogenetic tree was visualized and edited using MEGA software, and *M. racemosus* was chosen to root the tree, since in the initial maximum likelihood phylogenetic tree, this reference strain was clearly more phylogenetically distant from the other fungal isolates and reference strains, and both rooting the tree on this strain and on Midpoint resulted in identical phylogenetic trees.

The ITS1-5.8S-ITS2 nucleotide sequences of all seven fungal isolates were deposited in the GenBank database under the following accession numbers: ON150886 (*M. circinelloides*, FR1), ON150887 (*M. circinelloides*, FR2), ON150888 (*M. circinelloides*, FR3), ON150889 (*M. lusitanicus*, QP1), ON150890 (*M. circinelloides*, QP2), ON150891 (*M. circinelloides*, SJ1) and ON150892 (*M. circinelloides*, SJ2).

2.4. *In vitro* biological control trials against avian coccidia

Two types of biocontrol trials were conducted aiming to test the predatory activity of all fungal isolates against avian coccidia: a qualitative assay in Petri dishes containing WA medium, and a quantitative-qualitative coproculture assay (Hernández et al. 2017).

To obtain concentrated suspensions of oocysts, fecal samples from chickens, laying hens and peacocks, positive for *Eimeria* spp., were processed using the Willis-flotation technique. Briefly, two grams of feces were mixed with 28 mL of saturated sucrose solution (specific gravity 1.2); the fecal suspension was filtrated and poured to 10 mL test tubes, until the formation of a convex meniscus, on which a coverslip was placed; test tubes were left on the lab bench for 10 min, and the coverslip was then washed with distilled water to new test tubes, which were centrifugated at 2000 rpm for 10 min; the supernatant was partially removed, leaving just 1 mL in each tube; the sediment and supernatant were mixed using a Pasteur pipet, and 100 µL of the oocysts suspension were visualized using a light microscope, at 400x total magnification. Two reads were performed and the total oocyst count was multiplied by 10 to calculate the coccidia concentration (i.e., oocysts/mL).

In the first assay, a total volume of 500 µL of each fungal isolate (10^6 spores/mL) were inoculated on the surface of WA medium, to which 1 mL of oocyst suspension was also added, with a mean concentration of 140 oocysts/mL. Two replicates were used for each isolate, and a positive control was also used to assess the survival of the oocysts without fungal inoculate and test contamination by other fungal species. Plates were sealed with Parafilm and incubated at room temperature for 30 days. Then, plates were observed for identification of predatory activity, which was characterized as follows: hyphae attachment to the oocysts capsule but without morphological damage (activity type 1); the oocysts capsule and inner structures exhibiting morphological changes, but without fungal penetration (type 2); hyphae penetrate into the oocyst cytoplasm, grow inside, and destroy it (type 3) (Lýsek et al. 1982; Cazapal-Monteiro et al. 2015; Hernández et al. 2017).

The second assay aimed to evaluate the fungal isolates efficacy on degrading the oocysts, following exposure to the fecal microenvironment. Four grams of peacock fecal samples from the exotic collection SJ, positive for *Eimeria* spp. (n=20), were gently mixed and placed in eight plastic cups. A total of 4 mL of fungal suspensions (10^6 spores/mL) were added to the respective test cups (one per fungal isolate, n=7), while 4 mL of distilled water were poured onto the control cup (n=1). Then, cups were covered with perforated aluminium foil and left incubating for two weeks, at 26°C. After one and two weeks of incubation, two flotations were performed in each test and control cups, using 2 g of feces randomly picked from distinct parts of the sample and mixing it with 28 mL of saturated sucrose solution (specific gravity 1.2),

aiming to calculate the proportion of sporulated/unsporulated and viable/unviable oocysts (after one week) and the proportion of viable/unviable oocysts (in each week). Two reads were performed in each cup and timeframe, by counting a total of 100 oocysts per read.

For each fungal isolate and timeframe (7 and 14 days), the fecal oocyst viability reduction (FOVR) (1) and fecal oocyst sporulation reduction (FOSR) (2) were calculated as follows (Viña et al. 2020; Palomero et al. 2021; Voinot et al. 2021):

$$\text{FOVR (\%)} = [1 - (\text{VIABILITY test} / \text{VIABILITY control})] \times 100 \quad (1)$$

$$\text{FOSR (\%)} = [1 - (\text{SPORULATION test} / \text{SPORULATION control})] \times 100 \quad (2)$$

The characterization of the oocysts' appearance was adapted from the procedures established for ascarid eggs by (Cazapal-Monteiro et al. 2015), with oocysts being considered as unviable if at least one of the following characteristics was observed: inner structures poorly marked, oocysts abnormal shape, cytoplasm containing vacuoles, and/or capsule disruption.

Also, since most *Eimeria* species affecting Galliformes sporulate in less than 2 days, at temperatures ranging between 20 and 30°C (McDougald and Fitz-Coy 2008; Mesa-Pineda et al. 2021), the FOSR assessment was performed according to the following criteria: at the end of the first week of incubation, the identification of non-sporulated oocysts in the test cups was attributed to a coccidiostatic activity developed by the exposure to the respective fungal isolates.

2.5. Statistical analysis

The software Microsoft® Excel® for Microsoft 365 MSO (Microsoft Corporation, Redmond, WA, USA), was used for data storage, and table and chart editing.

The software IBM® SPSS® Statistics version 27 for Windows (IBM Corporation, Armonk, NY, USA) was used for the initial descriptive statistics (mean and standard errors). Also, this software was used to build 2 x 2 tables using data from the *in vitro* trial (viability and sporulation), aiming at performing a Chi-Square test, to compare the results obtained between the oocysts exposed to each fungal isolate (test cups) and to water (control cup). Moreover, this test was used to assess the time-dependency of the ovicidal activity developed by each fungal isolate on oocysts. A significance level of $p<0.05$ was used for all tests.

3. Results

3.1. Fungi isolation and identification

From the pool of 58 feces belonging to chickens, laying eggs, and peacocks, it was possible to obtain seven isolates of filamentous fungi: three from chickens (FR1, FR2 and FR3) and four from peacocks (SJ1 and SJ2 – exotic bird collection SJ; QP1 and QP2 – exotic bird collection QP).

Macroscopic and microscopic fungal characterization revealed similar results for most isolates: ovoidal and hyaline conidia, without septa; yellowish and non-branched sporangia, supported by a columella; hyphae without septa; grey-white and fluffy colonies. However, the isolate QP1 had conidia with an oblong shape, and hyphae thinner than the other isolates (Figure 14; Table 5). Thus, morphological assessment allowed to presumptively identify all isolates as *Mucor* sp. No other fungal taxa were identified.

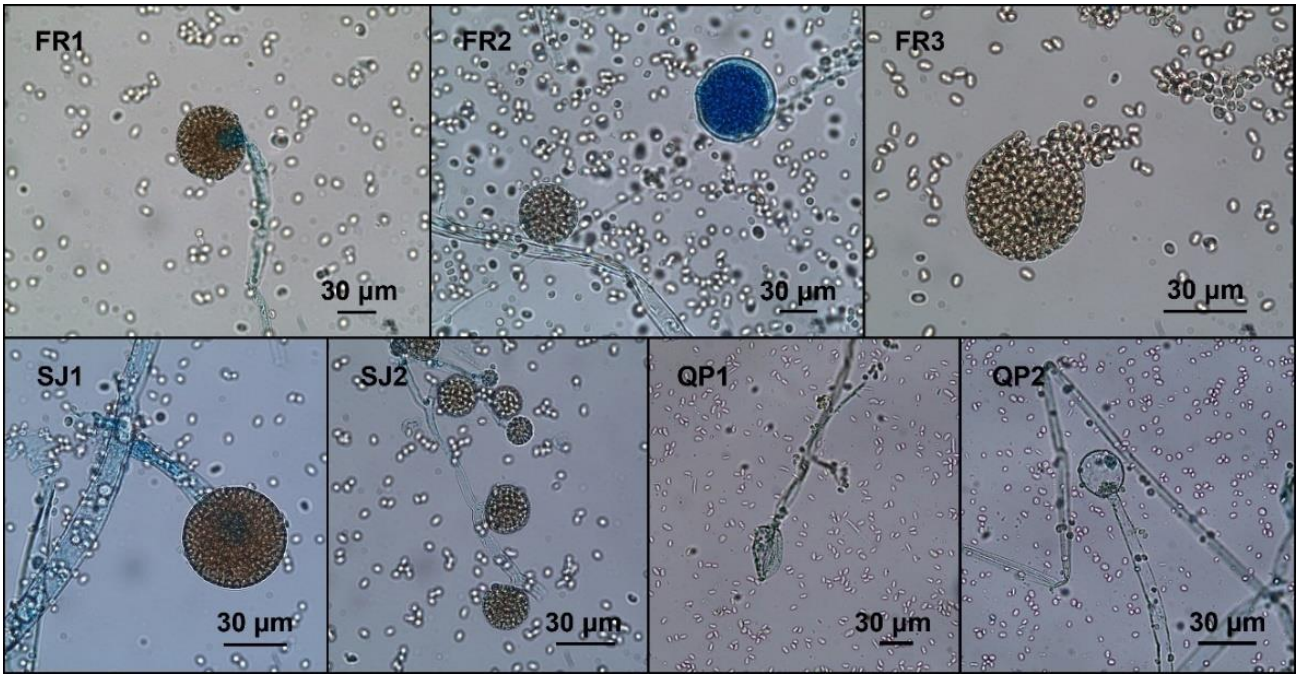

Figure 14. Conidia, sporangia, and hyphae of *Mucor* isolates (FR1 – *Mucor circinelloides*; FR2 – *Mucor circinelloides*; FR3 – *Mucor circinelloides*; SJ1 – *Mucor circinelloides*; SJ2 – *Mucor circinelloides*; QP1 – *Mucor lusitanicus*; QP2 – *Mucor circinelloides*; originals).

Table 5. Mean (± standard error) measures for conidia and sporangia length and width, and hyphae length.

Isolates	Conidia		Sporangia		Hyphae Length
	Length (µm)	Width (µm)	Length (µm)	Width (µm)	(µm)
FR1	4.9 ± 0.3	3.7 ± 0.3	48 ± 3.4	47 ± 3.4	11 ± 1.3
FR2	5.5 ± 0.3	4 ± 0.3	45 ± 3.8	41 ± 3.8	11 ± 0.7
FR3	6.6 ± 0.3	4.4 ± 0.2	47 ± 3.7	46 ± 3.2	13 ± 1.1
SJ1	4.5 ± 0.3	3.4 ± 0.4	45 ± 4.2	42 ± 3.1	10 ± 1.1
SJ2	4.3 ± 0.2	3.1 ± 0.2	27 ± 2.0	26 ± 1.8	12 ± 1.5
QP1	4.6 ± 0.2	2.9 ± 0.2	30 ± 3.2	27.5 ± 3.1	6.8 ± 1.4
QP2	5.4 ± 0.5	3.8 ± 0.3	26.5 ± 2.3	24.5 ± 2.3	6.3 ± 1.5

The upload of ITS1-5.8S-ITS2 sequences to Blastn Suite allowed to establish a Top 3 of similarity for each fungal isolate: FR1 had over 99.5% similarity with two *Mucor circinelloides* strains (GenBank accession numbers OW988287 and OW985400); FR2 had over 99% similarity with three *M. circinelloides* strains (FN598920, HQ914900, and KJ584557); FR3 had 99.8% similarity with *two M. circinelloides* strains (MK396486 and KT336541); SJ1 had 100% similarity with three *M. circinelloides* strains (KX620480, OW987678, and OW987665); SJ2 had over 99.5% similarity with three *M. circinelloides* strains (MT991775, NR_126116, and FJ713065); QP1 had 99.8% similarity with *Mucor* sp. (MK164174), *Mucor lusitanicus* (OP163597) and *Mucor racemosus* (MN726736); and QP2 had 100% similarity with two *M. circinelloides* strains (MT603934 and OW988287) (Table 6).

Phylogenetic analysis of the ITS1-5.8S-ITS2 sequences of all isolates, using the strains *Mucor circinelloides* CBS 195.68, *Mucor lusitanicus* CBS 108.17, *Mucor racemosus* f. *racemosus* CBS 260.68 and *Mucor fragilis*, obtained also from BLAST analysis, allowed to identify isolates FR1, FR2, FR3, SJ1, SJ2 and QP2 as *Mucor circinelloides* rDNA sequences, whereas the isolate QP1 was identified as *Mucor lusitanicus* (Figure 15).

Table 6. Similarity and query coverage percentages of each isolate sequence, in comparison with reported strains (Blastn).

Isolates Reported strains	Similarity (%)	Query coverage (%)	GenBank accession nr.
Isolate FR1			
Mucor circinelloides IBT2M2	99.65	93	OW988287
M. circinelloides	99.49	95	OW985400
Mucor sp. 033b	98.48	93	MW789352
Isolate FR2			
M. circinelloides IBT2H2	99.31	93	FN598920
M. circinelloides OUCMBI101096	99.15	96	HQ914900
M. circinelloides Sz8H	99.15	96	KJ584557
Isolate FR3			
M. circinelloides MDM14	99.83	96	MK396486
M. circinelloides M37	99.83	96	KT336541
Mucor sp. BAB-4784	99.83	96	KR154996
Isolate SJ1			
M. circinelloides AW1085	100	95	KX620480
M. circinelloides	100	95	OW987678
M. circinelloides	100	95	OW987665
Isolate SJ2			
M. circinelloides JEHAN37	99.83	97	MT991775
M. circinelloides CBS195.68	99.83	97	NR_126116
M. circinelloides E2A	99.67	97	FJ713065
Isolate QP1			
Mucor lusitanicus WZ-900	99.83	94	OP163597
Mucor racemosus GZ20190123	99.83	94	MN726736
Mucor sp. REB-039A	99.83	94	MK164174
Isolate QP2			
M. circinelloides CMRC545	100	96	MT603934
M. circinelloides	100	96	OW988287
Mucor sp. F8-2018	100	96	MW789352

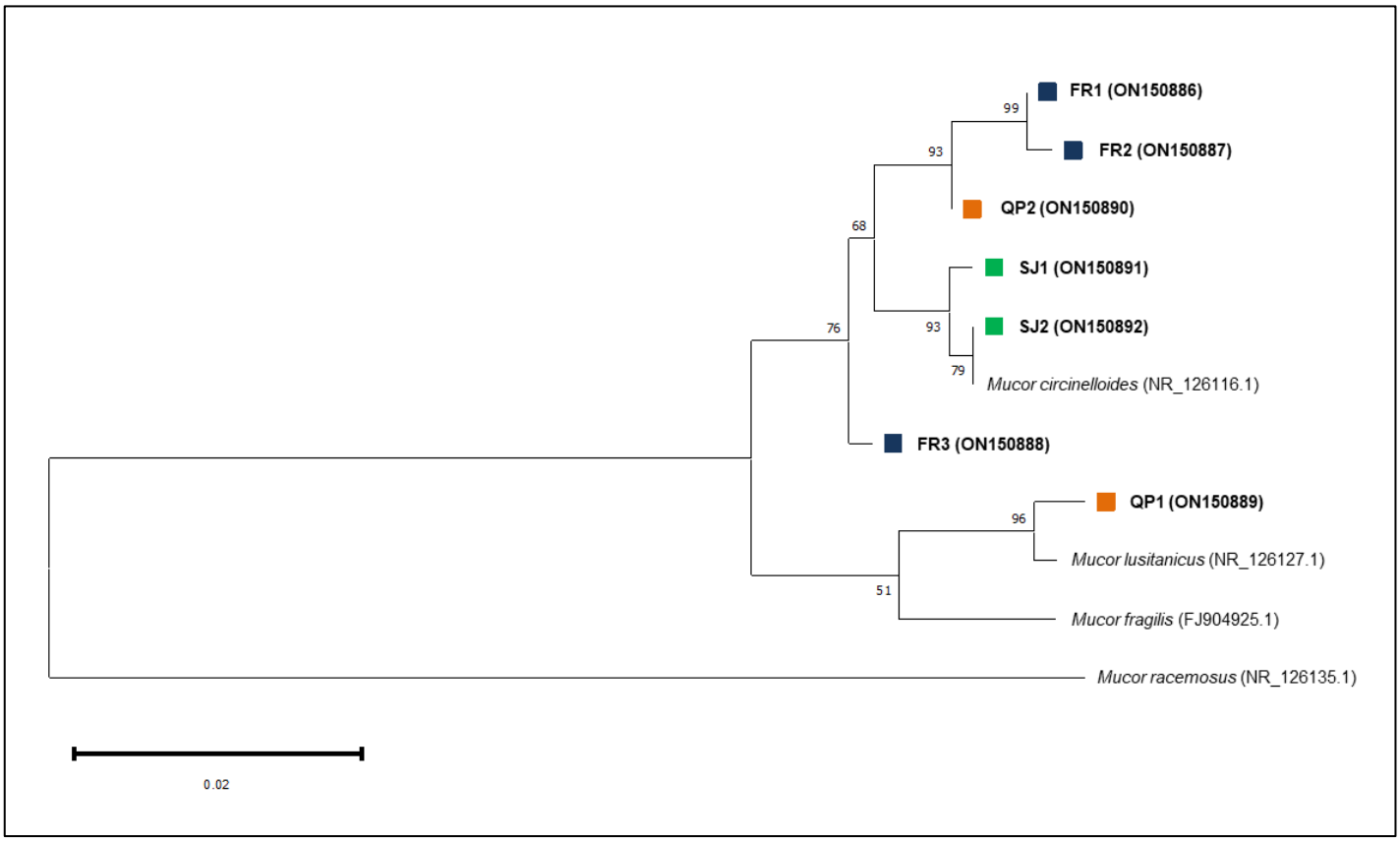

Figure 15. Maximum likelihood phylogenetic tree based on ITS1-5.8S-ITS2 region, including isolates' and GenBank reference strains' sequences. The likelihood's bootstrap is shown in each branch, and only values above 50 were considered. All seven *Mucor* isolates are displayed in bold, followed by their respective GenBank accession numbers. Coloured squares represent the bird collections from which fungi were obtained: blue – chickens; green and orange – peacocks from exotic collections SJ and QP, respectively.

3.2. *In vitro* biocontrol trials

In vitro trials revealed that all isolates developed predacious activity against *Eimeria* oocysts, both in WA medium (Figure 16) and in coprocultures (Figure 17). It was possible to observe that the presence of oocysts triggered the development of fungi hyphae and their adhesion to the oocysts' capsules (activity type 1). Also, during 30 days of fungal exposure, oocysts started to change their morphology, showing their inner structures poorly marked, and developing vacuoles (type 2), until spores started proliferating within the oocyst cell and finally leading to its disruption (type 3).

Fungal isolates showed different lytic performances when exposed to the fecal microenvironment (plastic cups trial), in terms of reducing coccidia sporulation and damaging the oocysts structure: isolates FR3, QP2, SJ1, SJ2 and FR2 had significant reduction efficacies of 85% ($p<0.001$), 85% ($p<0.001$), 73% ($p=0.001$), 69% ($p=0.001$) and 65% ($p=0.003$), respectively, on limiting the sporulation of oocysts, while isolates FR1, QP2 and QP1 had significant reduction efficacies of 22% ($p<0.001$), 14% ($p<0.001$) and 8% ($p=0.01$), respectively, on destroying the oocysts, but only after 14 days of exposure. Also, the ovicidal efficacy was time-dependent, since the oocyst viability differed significantly between the first and second weeks of the trial, after exposure to the isolates FR1 ($p<0.001$) and QP1 ($p=0.001$). The oocysts viability remained stable in the control cup during the assay, with percentages of viability equal to 97% and 94%, after 1 and 2 weeks of incubation, respectively (Table 7), with no statistical differences being recorded between both weeks ($p=0.302$).

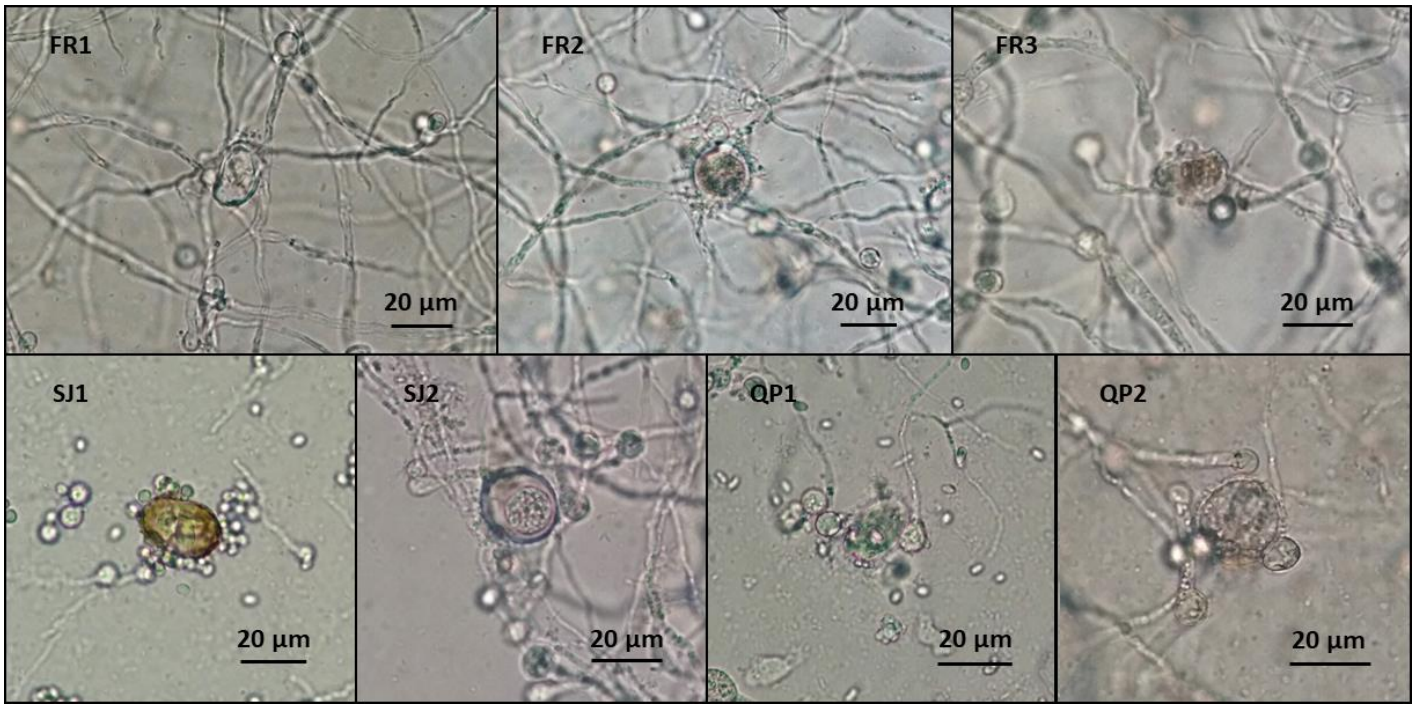

Figure 16. Predation developed by *Mucor* isolates against coccidian oocysts, in WA medium (FR1 – *Mucor circinelloides*; FR2 – *Mucor circinelloides*; FR3 – *Mucor circinelloides*; SJ1 – *Mucor circinelloides*; SJ2 – *Mucor circinelloides*; QP1 – *Mucor lusitanicus*; QP2 – *Mucor circinelloides*; originals).

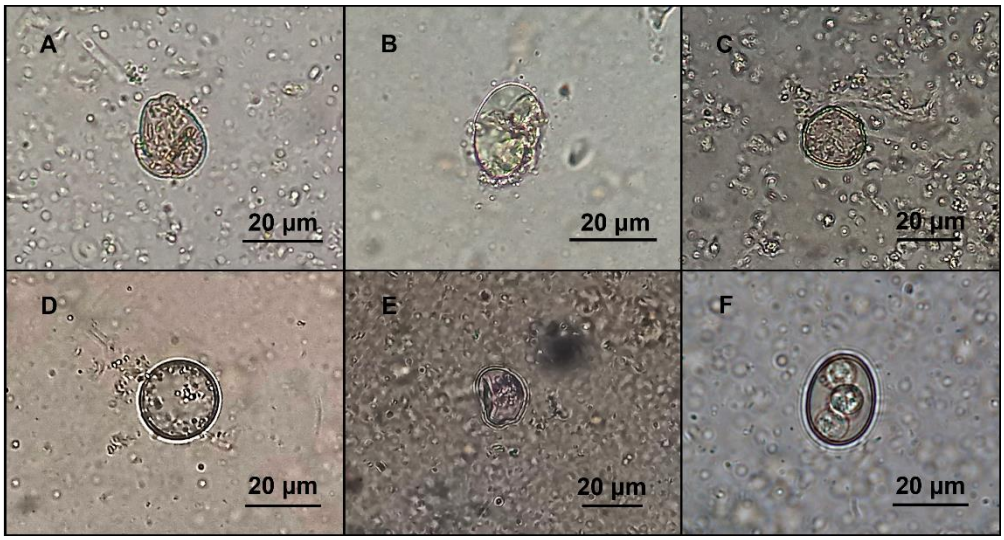

Figure 17. *Eimeria* sp. oocysts showing different morphological changes, following coprocultures with fungi (A, C, E – oocyst capsule deformation; B, D – oocyst disruption and loss of cytoplasmic content; F – sporulated oocyst; originals).

Table 7. Quantification of the predacious activity developed by each fungal isolate against coccidia. Sporulated and viable oocyst percentage reads (R1 and R2) and its mean and standard errors (SE) are provided, as well as the average reduction in oocyst sporulation (FOSR) and viability (FOVR) in comparison with control, after 7 and 14 days of incubation; significant results ($p<0.05$) are highlighted in bold and marked with an asterisk.

Isolates	FOSR					FOVR									
	7 days					7 days					14 days				
	R1 (%)	R2 (%)	Mean ± SE (%)	Reduction (%)	p	R1 (%)	R2 (%)	Mean ± SE (%)	Reduction (%)	p	R1 (%)	R2 (%)	Mean ± SE (%)	Reduction (%)	p
FR1	8	10	9 ± 1	31	0.20	92	95	93.5 ± 1.5	3	0.17	67	81	74 ± 7	**22***	<0.001
FR2	5	4	4.5 ± 0.5	**65***	0.003	94	96	95 ± 1	2	0.46	92	93	92.5 ± 0.5	2	0.47
FR3	1	3	2 ± 1	**85***	<0.001	91	95	93 ± 2	4	0.12	97	90	93.5 ± 3.5	1	0.73
SJ1	4	3	3.5 ± 0.5	**73***	0.001	98	98	98	0	-	90	92	91 ± 1	3	0.21
SJ2	3	5	4 ± 1	**69***	0.001	94	94	94	3	0.24	93	90	91.5 ± 1.5	3	0.27
QP1	7	11	9 ± 2	31	0.20	98	95	96.5 ± 1.5	0	-	87	87	87	**8***	0.01
QP2	2	2	2	**85***	<0.001	94	92	93 ± 1	4	0.12	82	81	81.5 ± 0.5	**14***	<0.001
Control	15	11	13 ± 2	-	-	97	96	96.5 ± 0.5	-	-	93	96	94.5 ± 1.5	-	-

4. Discussion

Predatory fungi are a group of saprophytic filamentous fungi known for their ability to predate and destroy larvae, eggs and oocysts from parasites affecting animals and plants. Besides these functional characteristics, they are also known for other attributes, namely the possibility to be isolated from a wide diversity of environmental samples, including agricultural soil, decaying organic matter and animal feces. Their isolation from fecal matter, which frequently also harbours environmental forms of intestinal parasites, proves that fungi and parasites naturally establish relationships in the fecal and soil microenvironment, with the formers being a nutritional source for predatory fungi (Madeira de Carvalho et al. 2012; Braga and Araújo 2014; Araújo et al. 2021). Also, the isolation of this type of fungi from feces belonging to healthy animals demonstrates the equilibrium in which these microorganisms are within the intestinal environment, and thus their innocuity to immunocompetent animals (Hernández et al. 2016; Vilela et al. 2016, 2018; Araújo et al. 2021; Palomero et al. 2021; Voinot et al. 2021; Viña et al. 2022).

To our knowledge, this study allowed to isolate for the first time filamentous fungi with predatory capacities from bird fecal samples, suggesting that birds are also “natural shedders” of this kind of fungi, as previously reported by several authors for mammal species, namely ruminants, horses and carnivores kept in farms and zoological parks (Larsen et al. 1994; Soto-Barrientos et al. 2011; Liu et al. 2015; Hernández et al. 2017; Ojeda-Robertos et al. 2019; Arroyo-Balán et al. 2021).

For this research, the use of avian fecal samples positive for intestinal parasites, together with their initial inoculation on a poor medium like Water-Agar, allowed to restrict the groups of fungi able to develop in this medium and stimulate the growth of only potential predatory fungi. Also, besides WA medium, the isolation steps also featured Wheat-flour Agar for rapid hyphae growth, purification, and storage (Arias et al. 2013b). The use of these two media, with no antibiotic supplementation, allowed to accurately isolate and store predacious fungi, in a quicker and economical approach in comparison with other more nutritive mediums like Sabouraud Agar, Corn Meal Agar, Potato Dextrose Agar or Malt Extract Agar, which are often supplemented with Chloramphenicol for these procedures.

Filamentous fungi isolates were obtained in all selected locations and from the two bird model species used, despite no isolates being obtained from laying hens’ samples. Morphological analysis allowed to conclude that all isolates belong to the genus *Mucor*, with qualitative and quantitative results tracked for conidia, sporangia and hyphae being in accordance with published literature regarding this genus (Kidd et al. 2016; Vellanki et al. 2018). Also, molecular assessment based on rDNA ITS1-5.8S-ITS2 sequences led to the

identification of two fungal species, *Mucor circinelloides* (FR1, FR2, FR3, SJ1, SJ2 and QP2) and *Mucor lusitanicus* (QP1), and thus proving that these target sequences are indeed suitable to be used in the molecular identification of predatory fungi, as demonstrated in other studies (Kelly et al. 2008; Xue et al. 2018; Arroyo-Balán et al. 2021; Ocampo-Gutiérrez et al. 2021; Pérez-Anzúrez et al. 2022).

All fungal isolates developed lytic activity against coccidian oocysts in WA medium and within the fecal microenvironment, allowing to identify all stages of predatory activity. Regarding the first assay, predating efficacies differed between strains, with FR1 and QP2 having been the most accurate on destroying *Eimeria* spp. oocysts (efficacies of 22% and 14%, respectively), while strains FR3, QP2 and SJ1 presented significant coccidiostatic efficacies, higher than 70%. These results are in accordance with previous studies performed by Portuguese and Spanish researchers (Cazapal-Monteiro et al. 2015; Hernández et al. 2017), which demonstrated *in vitro* predatory activity developed by *M. circinelloides* against eggs and oocysts from intestinal parasites affecting different animal hosts. Moreover, it constitutes the first original research article reporting the coccidicidal and coccidiostatic activity of *Mucor* spp. against avian coccidia. Also, the current research reveals for the first time the predatory skills developed by *M. lusitanicus* against parasitic forms, which had a significant impact on the oocysts' viability after 14 days of incubation (8%), despite presenting an efficacy lower than the ones obtained for the other strains. The ability of *Mucor* spp. to predate avian intestinal parasitic forms is one of the research lines of Spanish and Portuguese authors belonging to the COPAR research group (Faculty of Veterinary – University of Santiago de Compostela) and the LPPD (Faculty of Veterinary Medicine – University of Lisbon), respectively.

The detection of significant results for fungal coccidicidal activity only after 14 days of incubation, and the significant differences between data from 7 and 14 days, for isolates FR1 and QP1, confirms that the predatory activity developed by this kind of fungi is a gradual and time-dependent process. It is the presence of the parasite that triggers the development of fungi hyphae towards it and their adhesion to its capsule (Madeira de Carvalho et al. 2012; Braga and Araújo 2014; Cazapal-Monteiro et al. 2015; Hernández et al. 2017; Araújo et al. 2021). Fungal hyphae migration towards parasite eggs and oocysts may be considered as one of the most critical stages of the predatory process. Hyphae must grow and reach the parasite, which is a process that can be delayed by native biotic and abiotic factors within the fecal microenvironment, and thus affecting the performance and speed of fungal action. The avian fecal microbiota, which is composed by a wide diversity of native microorganisms, namely bacteria of the Phyla Firmicutes and Proteobacteria (Grond et al. 2018), fungi of the Phyla Ascomycota and Basidiomycota (Robinson et al. 2022), and other microorganisms, may have

negatively influenced the performance of each fungal strain, due to resource competition and degradation of the fungal strains. It has been suggested that predatory fungi survival within the soil and fecal microenvironment is affected by biotic factors such as the presence of microorganisms with fungistatic characteristics (Saumell et al. 2016). Furthermore, a study performed in Denmark (Thapa et al. 2017) demonstrated that the in-soil predatory performance of *Pochonia chlamydosporia* and *Metarhizium brunneum*, two ovicidal fungi species, against avian ascarid eggs (*Ascaridia galli* and *Heterakis gallinarum*), is affected by the soil's microbiota. All these factors need to be considered when planning a biocontrol assay, namely the optimal dosage of spores to counter these limiting factors.

Finally, since all *Mucor* strains were isolated from avian fresh fecal samples, and showed interesting lytic activity on coccidian oocysts, it can be suggested that these strains resisted to the gastrointestinal passage in chickens and peacocks, and maintained both their germination and predatory capacities, as previously demonstrated in birds for the ovicidal fungus *P. chlamydosporia* (Valadão et al. 2020) and larvicidal fungi *D. flagrans* and *Monacrosporium thaumasium* (Silva et al. 2017). The fact that all *Mucor* isolates were obtained from feces belonging to healthy birds, allows also to suggest their innocuity to immunocompetent birds, as demonstrated by other researchers for horses (Hernández et al. 2016), sheep (Voinot et al. 2021), dogs (Viña et al. 2022) and wapitis (Palomero et al. 2021).

To our knowledge, this study was the first performed worldwide aiming to isolate and identify native predatory fungi from bird feces and test their *in vitro* efficacy against avian *Eimeria* spp. oocysts. Results suggest that *Mucor circinelloides* strains FR1 and QP2 are the most promising to be used in future *in vitro* and *in vivo* biocontrol trials.

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CHAPTER IV – Compatibility of the joint administration of predatory fungi and antiparasitic drugs

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Linked publication:

Lozano J, Cunha E, Madeira de Carvalho L, Paz-Silva A, Oliveira M. 2024. First insights on the susceptibility of native coccidicidal fungi *Mucor circinelloides* and *Mucor lusitanicus* to different avian antiparasitic drugs. BMC Vet Res. 20:63. doi:10.1186/s12917-024-03909-z.

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Article 4 – "First insights on the susceptibility of native coccidicidal fungi *Mucor circinelloides* and *Mucor lusitanicus* to different avian antiparasitic drugs"

Abstract

The combined application of predatory fungi and antiparasitic drugs is a sustainable approach for the integrated control of animal gastrointestinal (GI) parasites. However, literature addressing the possible interference of antiparasitic drugs on the performance of these fungi is still scarce. This research aimed to assess the *in vitro* susceptibility of six native coccidicidal fungi isolates of the species *Mucor circinelloides* and one *Mucor lusitanicus* isolate to several antiparasitic drugs commonly used to treat GI parasites' infections in birds, namely anthelminthics such as Albendazole, Fenbendazole, Levamisole and Ivermectin, and anticoccidials such as Lasalocid, Amprolium and Toltrazuril (drug concentrations of 0.0078 – 4 µg/mL), using 96-well microplates filled with RPMI 1640 medium, and also on Sabouraud Agar (SA). This research revealed that the exposition of all *Mucor* isolates to the tested anthelminthic and anticoccidial drug concentrations did not inhibit their growth. Fungal growth was recorded in RPMI medium, after 48 h of drug exposure, as well as on SA medium after exposure to the maximum drug concentration. Preliminary findings from this research suggest the potential compatibility of these *Mucor* isolates with antiparasitic drugs for the integrated control of avian intestinal parasites. However, further *in vitro* and *in vivo* studies are needed to confirm this hypothesis.

Keywords: Gastrointestinal Parasites, Predatory Fungi, *Mucor* spp., Antiparasitic Drugs, Susceptibility.

1. Introduction

Domestic, exotic, and wild birds kept in captivity often contact with the same outdoor area for long periods of time, and thus are highly prone to re-infections caused by the infective forms of several gastrointestinal (GI) parasites, namely coccidia and helminths, which are responsible for clinical or sub-clinical diseases, and major economic concerns in poultry farms, zoological parks, and private bird collections (Ilić et al. 2018; Lolli et al. 2019; Blake et al. 2020; Lozano et al. 2021c).

Their prevention and treatment are still frequently performed exclusively with antiparasitic drugs, namely coccidiostatics (e.g, Amprolium and Lasalocid), coccidicidals (e.g., Toltrazuril) and anthelminthics (e.g., Benzimidazoles and Macrocyclic Lactones), whose incorrect use often leads to antiparasitic drug resistance, and accumulation of drug residues in bird carcasses, soil, and ground-waters (Abbas et al. 2011; Mund et al. 2017; Mooney et al. 2021; Martins et al. 2022).

Since the early 1990's, researchers from around the world have been proposing the integration of predatory fungi in animal health programs in farms, zoos, and private animal collections, serving as a complement to antiparasitic drugs for the control of GI parasitic infections in domestic, companion, exotic and captive wild animals (Madeira de Carvalho et al. 2012; Braga and Araújo 2014; Lozano et al. 2022; Mendoza de Gives et al. 2022).

The main attribute of these fungi lays on their ability to destroy parasites' infective stages (oocysts, eggs, or larvae), and thus breaking their life cycles in the environment. The most frequently reported predatory fungal taxa are: *Duddingtonia flagrans*, *Arthrobotrys* spp. and *Monacrosporium* spp., which are larvicidal fungi and thus predate and destroy nematodes' infective larvae (L3); *Pochonia chlamydosporia*, *Mucor circinelloides* and *Verticillium* spp., which have shown to present ovicidal properties, destroying both nematodes' eggs and coccidia oocysts (Madeira de Carvalho et al. 2012; Braga and Araújo 2014; Cazapal-Monteiro et al. 2015; Hernández et al. 2017; Lozano et al. 2023; Paz-Silva et al. 2023).

Considering that fungi of the order Mucorales are commonly associated to opportunistic infections (Hassan and Voigt 2019; Tahiri et al. 2023), ensuring their safety for animals is a mandatory step while designing a parasite biocontrol program, namely through anatomopathological, cytotoxicity, hematological and fecal analysis. In fact, all previous studies revealed that parasitized animals receiving *M. circinelloides* spores maintained or even improved the hematological parameters and feces consistency and appearance, and also without damaging the internal tissues (Hernández et al. 2016; Palomero et al. 2021; Viña et al. 2022; Voinot et al. 2022). Predatory fungi are administrated to animals always in controlled

programs, with constant monitorization of any side effects (Araújo et al. 2021; Voinot et al. 2022; Paz-Silva et al. 2023).

Moreover, ensuring their environmental innocuity is essential, namely to free-living nematodes which have an important role in soil and plant roots' oxygenation. For this purpose, a previous study developed by Saumell et al. (2016) demonstrated that the presence of *D. flagrans* spores in ovine feces does not have any effect on its natural colonization by free-living nematodes and other native predatory fungi, and thus not posing any environmental concern.

Combining antiparasitic drug treatments with predatory fungi administrations is of major importance, to target parasites' endogenous and exogenous stages (Madeira de Carvalho et al. 2012; Araújo et al. 2021). However, information regarding the possible negative effect of antiparasitic drugs in the survival of predatory fungi spores is still scarce, being a critical step in the design of an integrated parasite control program. Previous *in vitro* and *in vivo* research performed with the larvicidal fungi *D. flagrans* and *Arthrobotrys* spp., and the ovicidal fungi *Paecilomyces* spp. and *Verticillium chlamydosporium* (furtherly reclassified as *P. chlamydosporia*), have revealed these fungal taxa as being susceptible to variable concentrations of Ivermectin and several Benzimidazoles (Sanyal et al. 2004; Singh et al. 2010; Ferreira et al. 2016; Vieira et al. 2017). However, there is little information on the possible susceptibility of other predatory fungi taxa to different anticoccidial and anthelmintic drugs.

The current research aimed to assess for the first time the potential susceptibility of seven native ovicidal fungi of the genus *Mucor* to different antiparasitic drugs commonly used in Avian Medicine.

2. Methods

A total of seven *Mucor* isolates of the species *M. circinelloides* (FMV-FR1, FMV-FR2, FMV-FR3, FMV-SJ1, FMV-SJ2, FMV-QP2) and *Mucor lusitanicus* (FMV-QP1), belonging to the native predatory fungi collection of the Parasitology and Parasitic Diseases Lab, Faculty of Veterinary Medicine – University of Lisbon, and with proven parasiticide activity towards avian coccidia (Lozano et al. 2023), were used in this research. All fungal isolates were previously obtained from chicken and peacock fecal samples, and subjected to morphological and molecular identification through amplification of rDNA's ITS1-5.8S-ITS2 region and further sequencing using the ITS1 primer (Lozano et al. 2023). Moreover, isolates were maintained in Wheat-Flour Agar (WFA, 2%), at room temperature, as previous research revealed this medium to be a good alternative to other more nutritive mediums like Corn Meal, Potato Dextrose or Malt Extract agar, for rapid hyphae growth and storage of purified ovicidal fungi cultures (Hernández et al. 2017; Lozano et al. 2023).

Fresh mycelium was collected from each fungal isolate, using a calibrated 1 µL swab, and diluted in distilled water, with spores' final concentration being calculated using the Neubauer chamber. Fungal concentrations were standardized to 10^6 spores/mL.

All fungal isolates were checked against several antiparasitic drugs commonly used to treat coccidia and helminth infections in birds, namely Ivermectin (Purity ≥ 90%, Molecular Weight (MW) = 875.1 g/mol, Solubility in DMSO = 50 mg/mL; Merck Life Science, S.L., Lisbon, Portugal), Lasalocid (Purity ≥ 97%, MW = 612.77 g/mol, Solubility = 100 mg/mL; Ehrenstorfer GmbH, Augsburg, Germany), Albendazole (Purity ≥ 98%, MW = 265.33 g/mol, Solubility = 17 mg/mL; Merck Life Science, S.L., Lisbon, Portugal), Amprolium (Purity ≥ 98%, MW = 315.24 g/mol, Solubility = 2 mg/mL; Merck Life Science, S.L., Lisbon, Portugal), Toltrazuril (Purity ≥ 98%, MW = 425.38 g/mol, Solubility = 25 mg/mL; Merck Life Science, S.L., Lisbon, Portugal), Fenbendazole (Purity ≥ 98%, MW = 299.35 g/mol, Solubility = 30 mg/mL; Merck Life Science, S.L., Lisbon, Portugal) and Levamisole (Purity ≥ 98%, MW = 240.75 g/mol, Solubility = 10 mg/mL; Merck Life Science, S.L., Lisbon, Portugal).

Techniques used in this assay were based on the international standards proposed by CLSI for assessing filamentous fungi susceptibility to antimicrobial drugs (CLSI Reference Method M38 2021), as well as in previous research with larvicidal and ovicidal fungi (Vieira et al. 2017).

Stock solutions of each antiparasitic drug were prepared according to the manufacturers' instructions and each drug's solubility, having all been dissolved in Dimethyl Sulfoxide medium (DMSO) (Avantor, Inc., Radnor, Pennsylvania, USA) to a concentration of 1 mg/mL (5 mg of each drug diluted in 5 mL of DMSO). Working solutions of 100 µg/mL were prepared for each drug using also DMSO (100 µL of stock solution diluted in 900 µL of DMSO), followed by dilution in Roswell Park Memorial Institute medium (RPMI 1640) (Biowest, Missouri, USA) to a concentration of 8 µg/mL (80 µL of working solution diluted in 920 µL of RPMI).

Serial dilutions (1:2) were performed in 96-well microplates, using drug concentrations ranging between 0.0078 – 4 µg/mL, with wells containing a final fungal concentration of 10^5 spores/mL (100 µL of each fungus and 100 µL of each drug concentration). Positive and negative controls (100 µL of each fungus and 100 µL of RPMI medium, and only 200 µL of RPMI, respectively) were also used to test fungal growth in RPMI medium and contamination, respectively. Plates were incubated at 26°C for 48h, using a compressor-cooled incubator. Three independent assays were performed, using two replicates for each fungal isolate and drug. After incubation, each well's bottom was checked for mycelia growth, by directly visualization (naked eye), with two possible outcomes: the lack of mycelia in the bottom of the

test wells means a fungistatic effect of the respective drug concentration, whereas the growth of fungal mycelia means that the isolate is not susceptive.

The total suspension (200 µL) in wells containing the highest drug concentration (4 µg/mL) was finally transferred to Sabouraud Agar (SA), having these plates been also incubated at 26°C for 48h, to check for the maintenance of fungal growth after exposure to the respective antiparasitic drug, and with also two possible outcomes: the lack of fungal growth on this medium means a fungicide effect promoted by the corresponding drug, whereas colony growth means that the isolate was not susceptive to the corresponding drug. Both approaches were used for all fungal isolates, even if fungal growth was recorded in all test wells, to counter any dubious mycelia growth result, and therefore using the assay on SA medium as the final proof for any fungal susceptibility to antiparasitic drugs (Figure 18).

The chosen drug concentration range of 0.0078 – 4 µg/mL was based on: i) Vieira et al. (2017), who used drug concentrations of 0.0078 – 4 µg/mL for albendazole, thiabendazole and ivermectin, 0.003 – 1.875µg/mL for levamisole, and 0.004 – 2.5 µg/mL for closantel, and reported Minimum Inhibitory Concentrations (MIC's) as low as 0.031 – 4 µg/mL for *A. oligospora*, *D. flagrans* and *P. lilacinus* (furtherly reclassified as *Purpureocillium lilacinum*); ii) Sanyal et al. (2004) and Singh et al. (2010) studies, who reported fungal growth inhibitions at Albendazole and Triclabendazole concentrations of 1 – 4.5 µg/mL, after spores being fed to Ruminants; iii) therapeutic dosages of ivermectin as low as 0.8 – 1 µg/mL (drinking-water) in some exotic birds species, namely canaries (Tully 2007). This information was used as a starting point for establishing the drug range in the current research, and find which drugs are compatible with the used native fungal isolates.

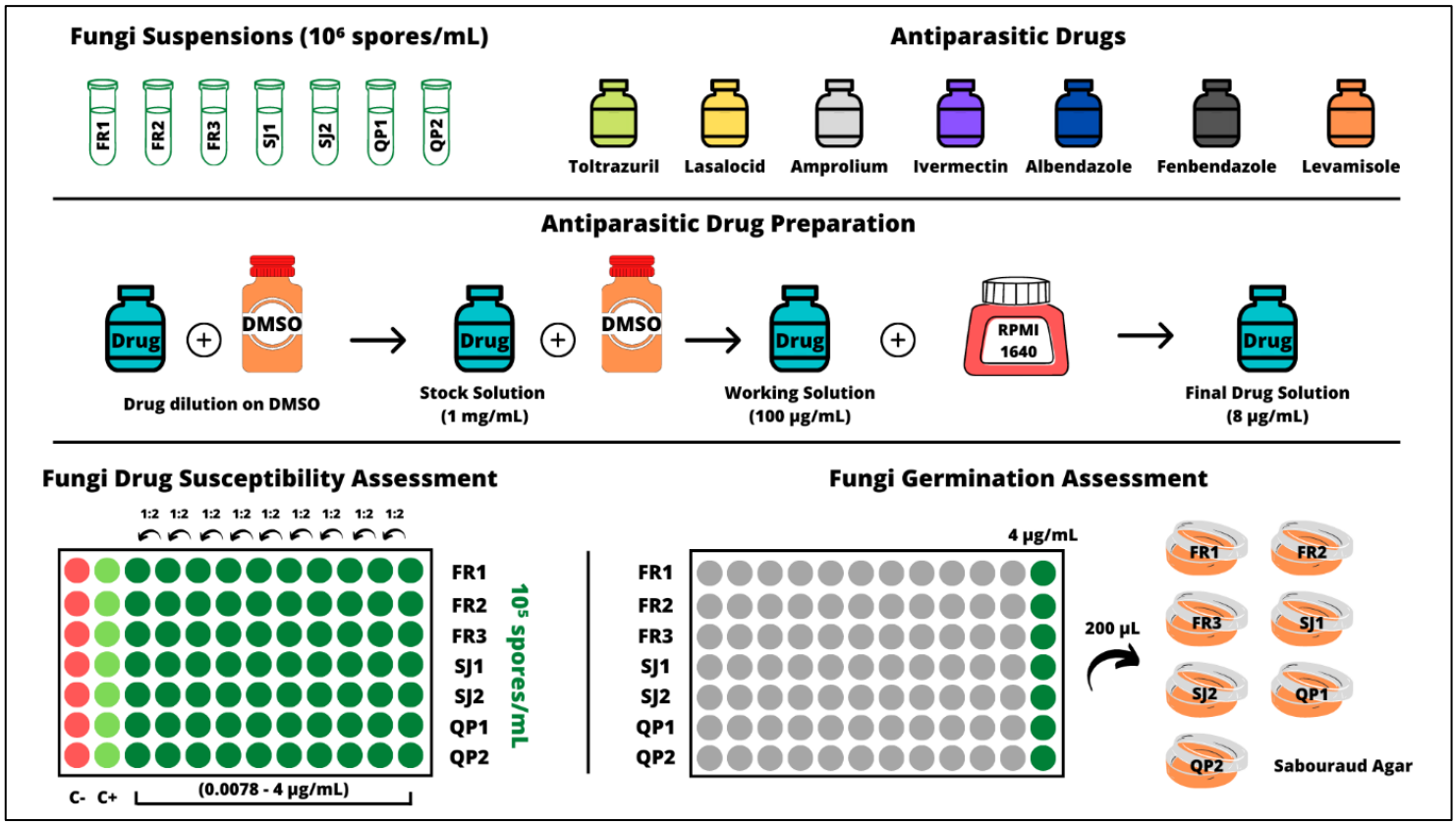

Figure 18. Workflow followed in the current study (figure created using Canva®; www.canva.com). FR1 – *Mucor circinelloides* isolate FMV-FR1; FR2 – *Mucor circinelloides* isolate FMV-FR2; FR3 – *Mucor circinelloides* isolate FMV-FR3; SJ1 – *Mucor circinelloides* isolate FMV-SJ1; SJ2 – *Mucor circinelloides* isolate FMV-SJ2; QP1 – *Mucor lusitanicus* isolate FMV-QP1; QP2 – *Mucor circinelloides* isolate FMV-QP2.

3. Results

Antiparasitic drug susceptibility experiments revealed that all seven *Mucor* isolates were not susceptible to the antiparasitic drugs tested and for all their assessed concentrations. Fungal growth was observed after two days of incubation, as demonstrated by the detection of mycelium growth in the bottom of test and positive control wells. These results reveal that spores' survival and mycelium growth were not affected by the exposure to antiparasitic drugs. Also, no fungal growth was recorded in the negative control, and thus confirming no contamination during the assay (Figures 19 and 20).

Moreover, all fungal isolates maintained their germination capacity even after exposition to the maximum drug concentration of 4 µg/mL, for all anticoccidials and anthelmintics, as demonstrated by the macroscopical visualization of colonies growth on SA medium (Figures 21 and 22).

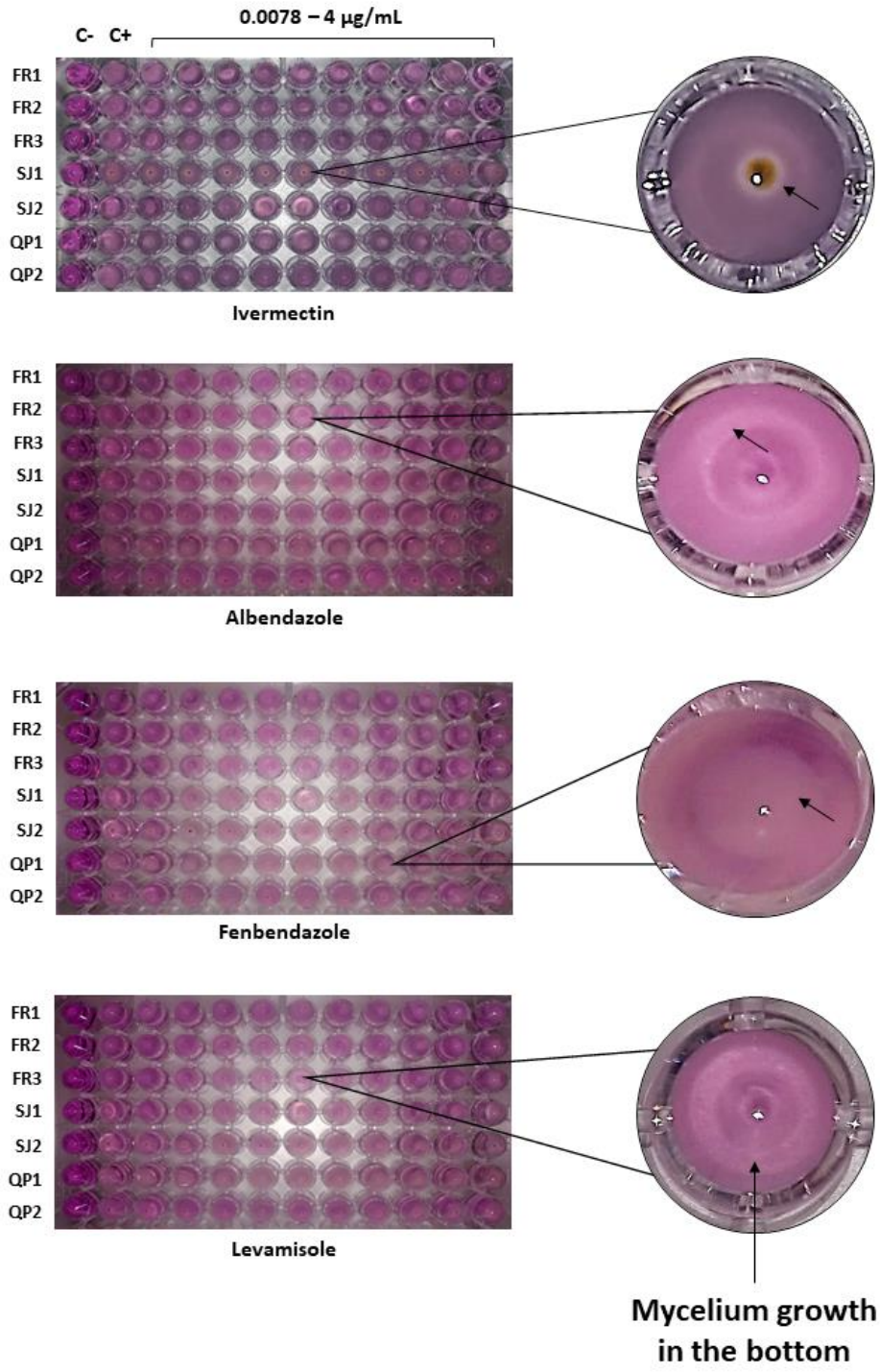

Figure 19. Fungal growth recorded for each *Mucor* isolate after exposition to each anthelminthic drug concentration. FR1 – *Mucor circinelloides* isolate FMV-FR1; FR2 – *Mucor circinelloides* isolate FMV-FR2; FR3 – *Mucor circinelloides* isolate FMV-FR3; SJ1 – *Mucor circinelloides* isolate FMV-SJ1; SJ2 – *Mucor circinelloides* isolate FMV-SJ2; QP1 – *Mucor lusitanicus* isolate FMV-QP1; QP2 – *Mucor circinelloides* isolate FMV-QP2; C+: positive control (medium and fungi), C-: negative control (only medium); magnifications for each drug plate illustrate examples of growth detected in those wells (black arrows).

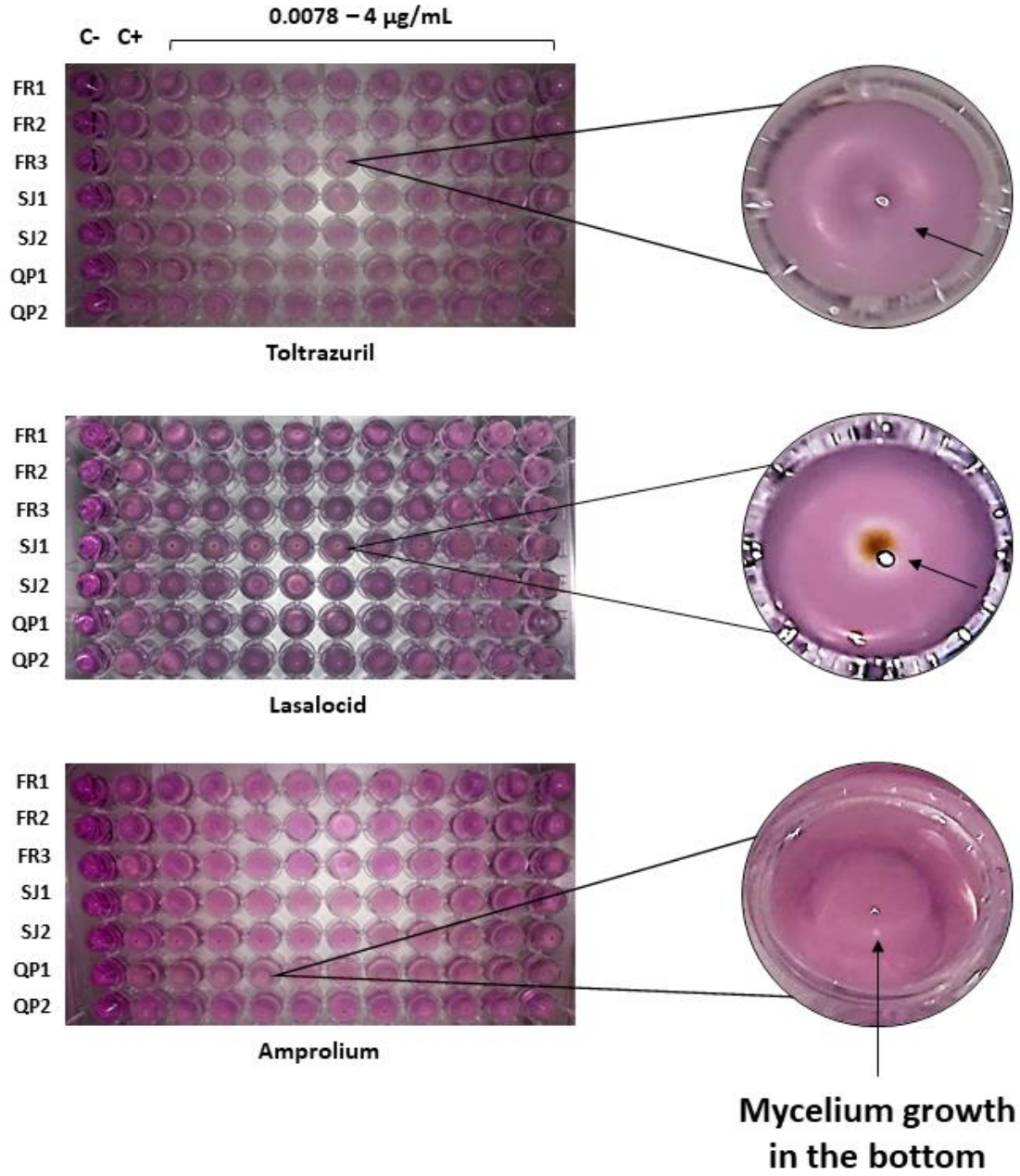

Figure 20. Fungal growth recorded for each *Mucor* isolate after exposition to each anticoccidial drug concentration. FR1 – *Mucor circinelloides* isolate FMV-FR1; FR2 – *Mucor circinelloides* isolate FMV-FR2; FR3 – *Mucor circinelloides* isolate FMV-FR3; SJ1 – *Mucor circinelloides* isolate FMV-SJ1; SJ2 – *Mucor circinelloides* isolate FMV-SJ2; QP1 – *Mucor lusitanicus* isolate FMV-QP1; QP2 – *Mucor circinelloides* isolate FMV-QP2; C+: positive control (medium and fungi), C-: negative control (only medium); magnifications for each drug plate illustrate examples of growth detected in those wells (black arrows).

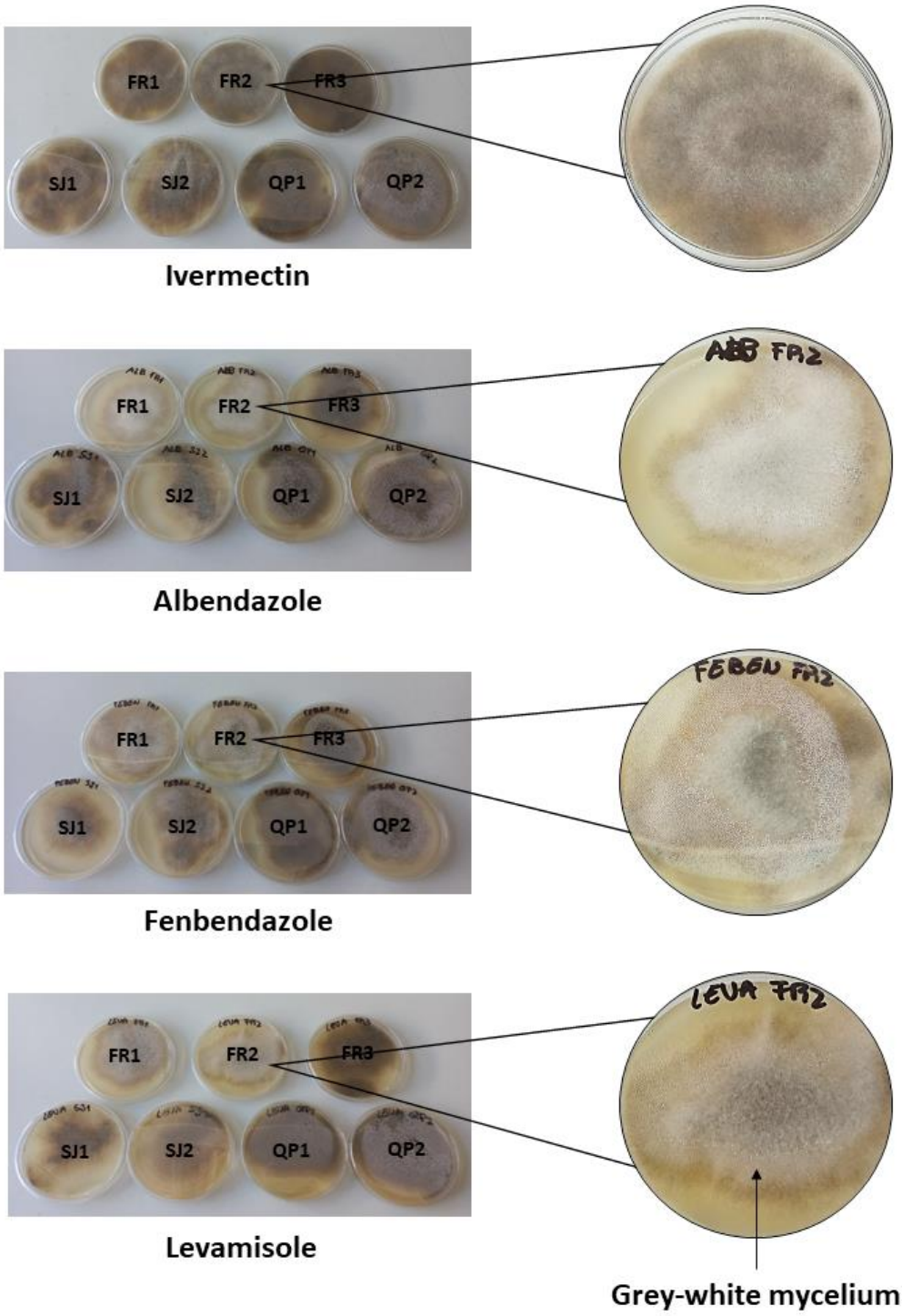

Figure 21. Fungi germination on Sabouraud Agar, after exposition to the maximum anthelminthic concentration of 4 µg/mL. FR1 – *Mucor circinelloides* isolate FMV-FR1; FR2 – *Mucor circinelloides* isolate FMV-FR2; FR3 – *Mucor circinelloides* isolate FMV-FR3; SJ1 – *Mucor circinelloides* isolate FMV-SJ1; SJ2 – *Mucor circinelloides* isolate FMV-SJ2; QP1 – *Mucor lusitanicus* isolate FMV-QP1; QP2 – *Mucor circinelloides* isolate FMV-QP2; magnifications provide an insight on the growth of typical *Mucor* spp. colonies on Sabouraud agar (black arrow).

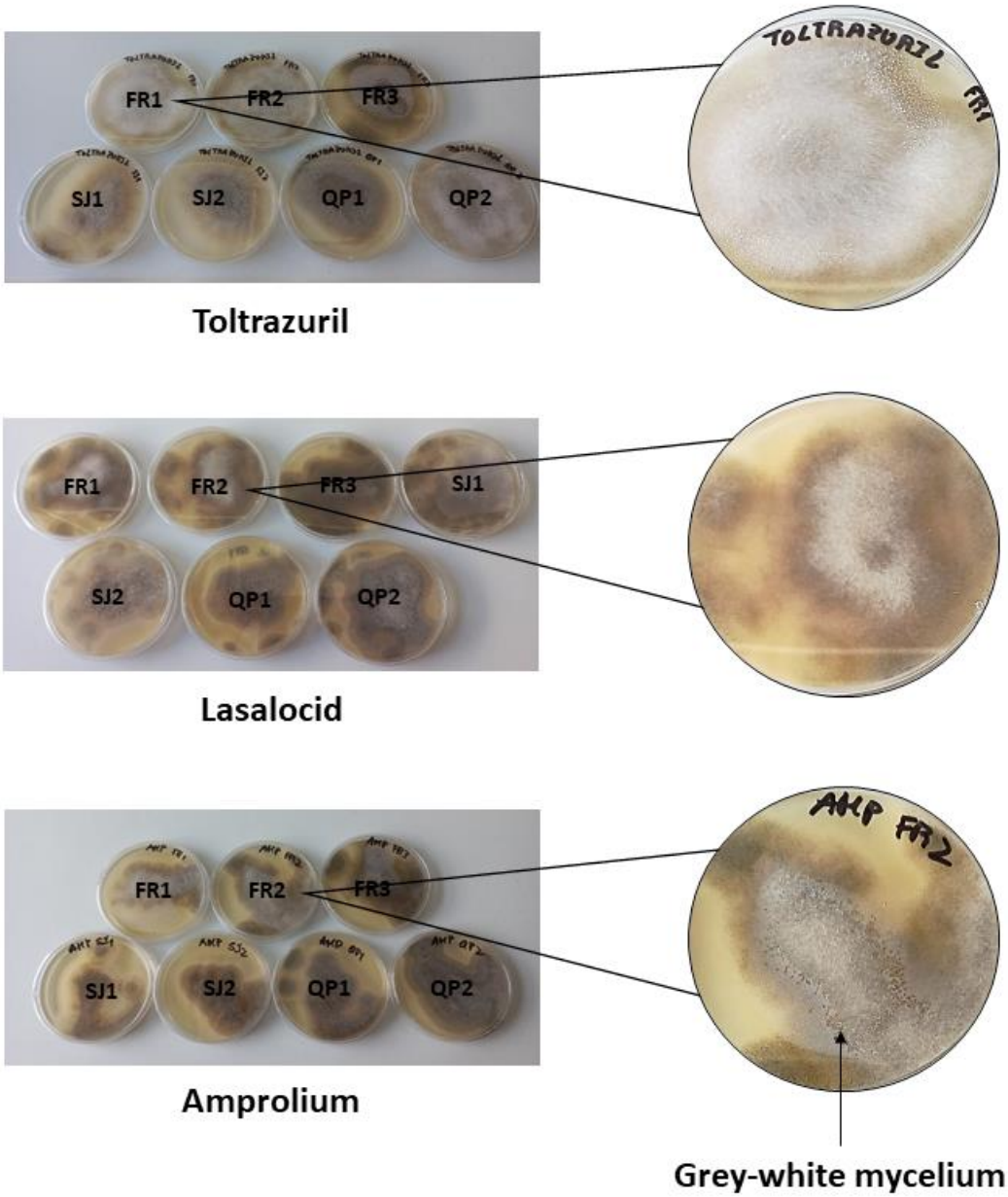

Figure 22. Fungi germination on Sabouraud Agar, after exposition to the maximum anticoccidial concentration of 4 µg/mL. FR1 – *Mucor circinelloides* isolate FMV-FR1; FR2 – *Mucor circinelloides* isolate FMV-FR2; FR3 – *Mucor circinelloides* isolate FMV-FR3; SJ1 – *Mucor circinelloides* isolate FMV-SJ1; SJ2 – *Mucor circinelloides* isolate FMV-SJ2; QP1 – *Mucor lusitanicus* isolate FMV-QP1; QP2 – *Mucor circinelloides* isolate FMV-QP2; magnifications provide an insight on the growth of typical *Mucor* spp. colonies on Sabouraud agar (black arrow).

4. Discussion

Assessing predatory fungi susceptibility to antiparasitic drugs is an important step in the optimization of parasite biocontrol programs, and despite fungi are not exposed to the initial drug concentration administrated to animals, in the intestinal microenvironment, any fungal incompatibility to a given drug might interfere with its germination capacity, and further efficacy on destroying parasitic forms (Sanyal et al. 2004; Singh et al. 2010; Ferreira et al. 2016; Vieira et al. 2017).

Results obtained in this research revealed that all studied *Mucor* isolates were not susceptible to anticoccidial and anthelminthic drugs, independently of the drug concentration, maintaining their germination capacity after drug exposure. These results are in contrast with previous research performed *in vitro* with larvicidal and ovicidal fungi (Ferreira et al. 2016; Vieira et al. 2017). *In vitro* susceptibility of predatory fungal species to antiparasitic drugs was first described by Ferreira et al. (2016), who reported that Ivermectin and Albendazole concentrations as low as 0.08 mg/mL had an inhibitory effect on *Paecilomyces* spp. growth of approximately 11-63% and 60-79%, respectively. Also, another study performed by Vieira et al. (2017), revealed that Albendazole, Thiabendazole, Ivermectin, Levamisole and Closantel had an inhibitory effect on the growth of *D. flagrans*, *Arthrobotrys oligospora*, and *P. lilacinus*. Thus, it can be concluded that for a given predatory fungus strain, a previous *in vitro* assessment of antiparasitic drug susceptibility might be of most importance to establish the optimal combination of drug treatment and fungal administration.

Also, it is of major importance that studies on this topic standardize the protocol used for assessing fungal susceptibility to different antiparasitic drugs, by following the international guidelines established by the Clinical & Laboratory Standards Institute (CLSI), for filamentous fungi (CLSI Reference Method M38 2021), as performed in our study, or by the European Committee on Antimicrobial Susceptibility Testing (EUCAST), which is also another worldwide recognized organization in the field of medical testing.

A possible explanation for the lack of susceptibility of all *Mucor* isolates to the tested antiparasitic drugs, namely to Benzimidazoles, might rely on the incapacity of these drugs to bind to its β-tubulins. These proteins are essential for microtubules' stabilization during the interphase stage of the fungal cells' cycle, and it has been demonstrated that Benzimidazoles' binding to these proteins arrests fungal cell division (Sanyal et al. 2004; Zhou et al. 2016; Oliveira et al. 2020). A systematic identification of tubulin genes from 59 representative fungi reported that *M. circinelloides* has four β-tubulin genes (Zhao et al. 2014), and therefore any mutation on these genes might interfere with the drugs' binding sites, as previously demonstrated for different fungal taxa (Minagawa et al. 2021). However, further studies are

needed to confirm this hypothesis, namely through Whole-Genome Sequencing (WGS) of the tested *Mucor* isolates.

Our study followed the standard M38 established by CLSI, which is one of the leading worldwide organizations to provide guidelines and standards in medical laboratory testing. The used standard only mentions a qualitative analysis for assessing the susceptibility of fungi to drugs, which was enough in the current study to check if fungi isolates were capable of growing during and after drug exposition. Moreover, the current study also included some relevant modifications of the CLSI standard M38, which only established the application of a negative control well (with only RPMI medium), considering it as sufficient for this kind of antimicrobial susceptibility assays, and just for testing contamination. Our team complemented the assays by including a positive control (fungi and RPMI), to perform a more robust analysis, and check if fungal isolates were capable of growing in RPMI when not exposed to antiparasitic drugs, as described also by Vieira et al. (2017). Besides, two trials were performed (in microplates and SA medium), to confirm the lack of susceptibility of all *Mucor* isolates to the different antiparasitic drugs, by checking if fungi were capable of growing on SA medium following exposition to the maximum drug concentration (4 µg/mL). Although a qualitative analysis is enough to assess if a certain fungal isolate is susceptible to a given antimicrobial drug, it would be also interesting if further studies in this topic include a quantitative analysis, namely the quantification of Colony Forming Units (CFUs) and each well's absorbance, as well as measuring colony radial growth in different timepoints following drug exposition. Moreover, and despite predatory fungal spores and drugs are often administrated separately to animals in different timepoints, with an interval of 14-21 days post-drug treatment (Palomero et al. 2021; Rodrigues et al. 2021; Voinot et al. 2021), and thus fungi are not exposed to the initial drug dosage, but instead to lower concentrations due to its metabolization in the gastrointestinal tract, it would be interesting in further studies to assess if these native *Mucor* isolates maintain their lack of susceptibility to several therapeutic dosages used for the tested antiparasitic drugs, and thus conclude if a drug wash-out period is necessary before administrating fungal spores.

5. Conclusion

To our best knowledge, this is the first report to reveal the compatibility of different isolates of the genus *Mucor* to the most common avian antiparasitic drugs, and thus suggesting these parasiticide fungi as potential candidates to be combined with the most common anticoccidials and anthelmintics in integrated parasite biocontrol programs in domestic and exotic bird collections. However, further *in vitro* and *in vivo* studies are needed and in current progress to confirm these hypotheses.

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CHAPTER V – Safety of *M. circinelloides* FR1 and interactions with avian commensal gut microorganisms

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Linked publication:

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Author contributions: João Lozano – conceptualization, resources, investigation, data curation, formal analysis, writing (original draft); Eva Cunha – resources, investigation; Cristina Almeida – resources, investigation; Mónica Nunes – conceptualization, resources, investigation; Ricardo Dias – conceptualization, resources, investigation; Eduardo Vicente – resources, investigation; Daniela Sebastião – resources, investigation, supervision; Sérgio Henriques – resources, investigation, supervision; Luís Madeira de Carvalho – resources, investigation, writing (review and editing), supervision; Adolfo Paz-Silva – resources, investigation, writing (review and editing), supervision; Manuela Oliveira – resources, investigation, writing (review and editing), supervision.

Article 5 – "Analysing the safety of the parasiticide fungus *Mucor circinelloides*: first insights on its virulence profile and interactions with the avian gut microbial community"

Abstract

Parasiticide fungi are considered an accurate, sustainable, and safe solution for the biocontrol of animal gastrointestinal (GI) parasites. This research provides an initial characterization of the virulence of the native parasiticide fungus *Mucor circinelloides* (FMV-FR1) and an assessment of its impact on birds' gut microbes. The genome of this fungus was sequenced to identify the genes coding for virulence factors. Also, this fungus was checked for the phenotypic expression of proteinase, lecithinase, DNase, gelatinase, hemolysin, and biofilm production. Finally, an *in vivo* trial was developed based on feeding *M. circinelloides* spores to laying hens and peacocks three times a week. Bird feces were collected for 3 months, with total genomic DNA being extracted and subjected to long-read 16S and 25S-28S sequencing. Genes coding for an iron permease (FTR1), iron receptors (FOB1 and FOB2), ADP-ribosylation factors (ARFs) (ARF2 and ARF6), and a GTPase (CDC42) were identified in this *M. circinelloides* genome. Also, this fungus was positive only for lecithinase activity. The field trial revealed a fecal microbiome dominated by Firmicutes and Proteobacteria in laying hens, and Firmicutes and Bacteroidetes in peacocks, whereas the fecal mycobiome of both bird species was mainly composed of Ascomycetes and Basidiomycetes fungi. Bacterial and fungal alpha-diversities did not differ between sampling time points after *M. circinelloides* administrations ($p=0.62$ and $p=0.15$, respectively). Although findings from this research suggest the lack of virulence of this *M. circinelloides* parasiticide isolate, more complementary *in vitro* and *in vivo* research is needed to conclude about the safety of its administration to birds, aiming at controlling their GI parasites.

Keywords: Avian Parasitology, Parasiticide Fungi, Virulence Factors, Microbiome, Mycobiome.

1. Introduction

Parasiticide fungi are a functional group of microorganisms known for their ability to destroy the exogenous forms of gastrointestinal (GI) parasites, namely coccidia oocysts, and also helminth eggs and larvae (Madeira de Carvalho et al. 2012). Fungi like *Duddingtonia flagrans*, *Arthrobotrys oligospora* and *Monacrosporium thaumasium*, develop trapping structures to immobilize and destroy nematodes' infective larvae (larvicidal fungi), whereas *Mucor circinelloides* and *Pochonia chlamydosporia,* destroy coccidia oocysts and helminth eggs (ovicidal fungi) (Braga and Araújo 2014; Cazapal-Monteiro et al. 2015; Lozano et al. 2023). The majority of research on this topic has been performed in ruminants and horses (Madeira de Carvalho et al. 2011; Healey et al. 2018; Canhão-Dias et al. 2020; Voinot et al. 2021) and more recently extended to the control of parasites affecting other animal hosts, namely dogs, birds, and captive wild animals (Palomero et al. 2021; Lozano et al. 2023; Paz-Silva et al. 2023).

Considering that some fungi of the order Mucorales, such as *M. circinelloides*, are commonly linked to opportunistic infections, which may lead to mucormycosis in immunocompromised humans and animals (Seyedmousavi et al. 2018), studying their safety is a mandatory step to implement this parasite biological control approach at field-level.

Birds' native gut microbiota (i.e., bacterial community) is mainly composed by bacteria of the phyla Firmicutes, Proteobacteria and Bacteroidetes, with its diversity and relative abundances being influenced by several endogenous and exogenous factors, such as diet, age, sex, health status and environmental microorganisms (Grond et al. 2018). Although most studies are still focused on animal gastrointestinal (GI) bacteria, new information is being recorded for animals' enteric fungal communities (i.e., mycobiota), revealing that the Ascomycota and Basidiomycota phyla are the most dominant in the GI tract of poultry (Cafarchia et al. 2019; Robinson et al. 2022). Since parasiticide fungi spores are often administrated orally to animals and then pass through the GI tract and are finally expelled with feces to the environment, where they develop larvicidal or ovicidal activities toward parasitic forms (Madeira de Carvalho et al. 2012; Braga and Araújo 2014), ensuring that fungal formulations do not disturb the native gut micro- and mycobiota is also crucial for maintaining intestinal homeostasis, which is, to our knowledge, a topic not yet studied.

The current research aimed to perform an initial characterization of the virulence profile of the native parasiticide fungus *Mucor circinelloides* (FMV-FR1) and assess its potential influence on birds' GI native bacterial and fungal communities in the scope of a larger project aiming at controlling peacocks' coccidia.

2. Materials and methods

2.1. Fungal isolate

This study focused on a *M. circinelloides* (FMV-FR1) isolate belonging to the Laboratory of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine, University of Lisbon (LPPD-FMV), with previously demonstrated parasiticide activity toward avian coccidia (Lozano et al. 2023). This isolate was stored in wheat-flour agar medium (WFA, 2%) at room temperature and in a wheat broth (10 grams of wheat grains per 1 L of distilled water). This broth was previously autoclaved and transferred to sterilized plastic bottles, after which it was inoculated with WFA cubes of 2.25 x 2.25 x 2.25 cm, containing mycelia from *M. circinelloides*, and then left at room temperature for 1 month, with a slope of 45° (Arias et al. 2013b).

Fungal mycelium from the first formulation was used in the first phase of this study (virulence profile assessment), whereas the wheat broth enriched with fungal spores was used in the second phase (*in vivo* trials).

2.2. Phase 1 – assessing *M. circinelloides* virulence profile

2.2.1. Whole-genome sequencing (WGS)

Extraction of *M. circinelloides* DNA was made by phenol/chloroform followed by precipitation with sodium acetate and ethanol, and finally resuspended in Tris-EDTA buffer (Aamir et al. 2015). The obtained DNA was purified using AMPure XP beads (Beckman Coulter, High Wycombe, UK), and its quality and concentration were assessed by NanoDrop One and Qubit 4 Fluorometer (Thermo Fisher Scientific Inc., Waltham, USA).

The isolate's genomic DNA was subjected to WGS using Oxford Nanopore PromethION (P24), with R10.4.1 flow cell (FLO-PRO114M) and Ligation Sequencing Kit V14 (SQK-LSK114) following the manufacturer's instructions (Oxford Nanopore Technologies, Oxford, UK). After 17h, sequencing yielded 1,950,000 long read sequences with a size N50 of 18Kb. A total of 21.27 Gb of data were produced (500 × genome coverage). Raw reads were classified by MinKNOW (Oxford Nanopore Technologies, Oxford, UK) based on the average read quality score > 7 and then assembled using the pipeline Canu (version 2.2), with the parameters "genomeSize=39mb" and "-nanopore". The output FASTA file from Canu was polished using the tool Medaka (version 1.6.0), with the parameter "-m r103_hac_g507".

The pipeline Funannotate (https://github.com/nextgenusfs/funannotate) was used to explain the assembled genome with the following commands: funannotate clean; funannotate sort; funannotate mask; funannotate predict with the parameter *"Mucor racemosus"*;

funannotate iprscan, using the pipeline InterProScan (version 5.61-93.0); and funannotate annotate. Gene IDs recorded correspond to hypothetical genes given by the Funannotate pipeline.

Protein sequences associated with fungal pathogenicity were retrieved from the Database of Fungal Virulence Factors (DFVF, http://sysbio.unl.edu/DFVF/Download/AllGenes.txt) (Lu et al. 2012) and Pathogen-Host Interactions Database (PHI-BASE, https://www.phi-base.org) (Urban et al. 2022). Protein sequences generated by the Funannotate pipeline were used to perform a BlastP against DFVF and PHI-BASE databases, using a local instance of SequenceServer 2.0.0, BLASTP 2.12.0+, and with the following parameters: e-value 10^{-6}, matrix BLOSUM62, gap-open 11, gap-extend 1, filter F (Priyam et al. 2019). Cut-offs equal to 10^{-6} for e-value and >80% for genes identity were chosen based on the Funannotate pipeline, Chaudhuri and Ramachandran (2014), and on the standardized procedures of the Biosystems and Integrative Sciences Institute of the Faculty of Sciences, University of Lisbon.

2.2.2. Phenotypic expression of virulence factors

This step aimed to assess the phenotypic expression of common six microbial virulence factors by the *M. circinelloides* isolate (FMV-FR1), namely the enzymes proteinase, lecithinase, DNase, gelatinase, and hemolysin, and also biofilm production. All media, incubation conditions, and expected outcomes for each virulence factor are summarized in Table 8. The analysis of the phenotypic expression of all virulence factors was performed by direct visualization of each plate and following all procedures from previous research by Cunha et al. (2023) and Raposo et al. (2023) and their control plates.

Table 8. List of virulence factors assessed in *Mucor circinelloides* (FMV-FR1), culture media and incubation conditions used for this purpose, and expected phenotypic expression.

Virulence factors	**Media**	**Incubation conditions**	**Outcomes**
Proteinase	Skim milk medium: skim milk powder and bacteriological agar (VWR, Leuven, Belgium)	26°C for 72h	Positive result – appearance of a clear zone surrounding the colonies
Lecithinase	Tryptic Soy agar supplemented with 10% egg yolk emulsion (VWR, Leuven, Belgium)		Positive result – appearance of a white precipitate around the colonies
DNAse	DNase medium (Thermo Fisher Scientific – Remel, Lenexa, USA) supplemented with 0.01% toluidine blue (Merck, Darmstadt, Germany)		Positive result – appearance of pink halos around the colonies
Gelatinase	Nutrient Gelatine agar (Oxoid, Hampshire, United Kingdom)		Positive result – cumulative effect of gelatine liquefaction after incubation and maintenance of its liquid consistency after cooling at 4°C for 30 minutes
Hemolysin	Columbia agar supplemented with 5% sheep blood (bioMérieux, Marcy-l'Etoile, France)		α-Haemolysis (partial activity) – appearance of green halos around the colonies β-Haemolysis (full activity) – clearing halos Negative result – absence of halos
Biofilm production	Red Congo agar: Brain-Hearth Infusion Broth (VWR, Leuven, Belgium), bacteriological agar (VWR, Leuven, Belgium), and Red Congo reagent (Sigma-Aldrich, Steinheim, Germany)		Positive result – black coloured medium around the colonies: - Strong producer: after 24h - Medium producer: after 48h - Weak producer: after 72h

Moreover, a second experiment was performed aiming at quantifying the rate of red blood cell destruction by measuring the absorbance of hemoglobin (Hb) release after exposure to fungal spores. Two blood samples were collected from two different male lionhead rabbits younger than 1-year old on the day before the experiment and during routine clinic consultations at “Exoclinic” (Lisbon, Portugal). Blood was taken from the lateral saphenous vein into EDTA tubes and stored in a refrigerator at 4°C. Then, a protocol adapted from Mendonça et al. (2021) was applied. For each blood sample, a total of 1 mL of blood was centrifuged at 4,000 rpm, for 5 minutes, and the resulting pellet was washed two times in 1X phosphate-buffered saline (PBS) solution (150 mM NaCl, 5 mM Na_2HPO_4, 1.7 mM KH_2PO_4; pH 7.4) by centrifugation at 4,000 rpm, for 5 minutes, until obtaining a clear supernatant. Then, the supernatant was discharged, and the pellet containing rRBCs was resuspended in PBS solution to a final concentration of 0.5% (vol/vol). *M. circinelloides* suspension with an average concentration of 6.04 x 10^6 spores/mL was also prepared in PBS solution by using a Neubauer chamber to count fungal conidia. Serial dilutions (1:2) were performed in microplates, with test wells containing 100 µL of each fungus concentration and 100 µL of rRBCs’ suspension, corresponding to a final fungal concentration ranging between 10^4 and 10^6 spores/mL. Positive and negative controls were also performed (100 µL of Triton (1%) and 100 µL of rRBCs’ suspension, or only 200 µL of PBS, respectively). Microplates were incubated at 37°C for 1h with 100 rpm stirring and then centrifuged at 4,000 rpm at 4°C for 5 minutes. Supernatants were then transferred to a new microplate, and hemolysis was determined by Hb release, measured by absorbance at 450 nm using a FLUOstar OPTIMA microplate reader (BMG LABTECH, Offenburg, Germany) and the software BMG LABTECH OPTIMA (version 2.20 R14). In this experiment, each blood sample evaluation was performed in three repetitions, each with two replicates.

2.3. Phase 2 – effect of *M. circinelloides* spores in avian native gut bacteria and fungi

2.3.1. Domestic and exotic bird collections

Between July and December 2022, two *in vivo* trials were performed in two different avian collections located in Lisbon district, Portugal: a laying hens' flock (*Gallus gallus domesticus*) from a livestock farm (39°13'54.373" N 9°17'2.235" W) and a peacock flock (*Pavo cristatus*) from São Jorge Castle (38°42'50.241" N 9°8'2.182" W).

The trial with laying hens was performed between July and September 2022. The flock was composed of 100 birds, 1-year old, kept under free-range conditions, with an outdoor area of 625 m^2 and a housing of 45 m^2 with feeders, water drinkers, perches, and nests. The trial with peacocks was performed between October and December 2022, with the flock composed of 58 birds (44 adults and 14 juveniles), free ranging in an outdoor area of 4,700 m^2.

According to the routine practices performed in each bird collection, laying hens and peacocks were fed with avian commercial feed once or twice per day, respectively. Also, both bird flocks were not subjected to any antimicrobial drug treatment at least 6 months prior to and during the trials.

2.3.2. Fungal formulations

M. circinelloides spores' suspensions in wheat broth were added to feeders at farm level prior to feeding birds. This approach was chosen due to the poor climatization of the farm's storage room, which would contribute to the rapid growth of fungi on feed and eventually to its rejection by the animals.

The procedure was adapted from the protocol described by Palomero et al. (2021). Two hand sprayers were filled with the *M. circinelloides* suspension (10^6 spores/mL), which was then sprayed on feed after being placed in feeders. Laying hens received a dose of 6.8 x 10^7 spores/kg of feed in each administration timepoint.

For peacocks, the administration procedure was slightly different. Bird feed doses enriched with *M. circinelloides* spores were previously prepared in the LPPD-FMV, adapting the procedures reported by Voinot et al. (2021). Each formulation was composed of 600 grams of bird feed mixed with 60 mL of fungal suspension, which was dried at 27°C for 30 minutes using an incubator. Individual doses were prepared in sealed plastic bags, and peacocks received 1.01 x 10^8 spores/kg of feed at each administration timepoint.

2.3.3. Experimental design

In both bird collections, a total of 33 fungus administrations were performed *per os* three times a week. Then, the number of samples collected on each bird flock was determined based on the availability of fresh feces at the control time point in laying hens, 20 samples, in which the first trial was performed. Thus, the same quantity of samples was collected in further sampling timepoints, each lasting 2 weeks, for 3 months, and in both bird flocks, with the only exceptions being observed for the control and t30-day time points in peacocks, in which it was only possible to collect 17 and 16 fresh fecal samples, respectively. Since it was not possible to establish two separate groups (test and control) in each bird flock, the initial samples served as control (t0 days), whereas samples from t15 to t90 days corresponded to the test phase, as also described by Paz-Silva et al. (2023) (Figure 23). Feces were packed in individual plastic bags and immediately transported in a cooling bag to the LPPD-FMV.

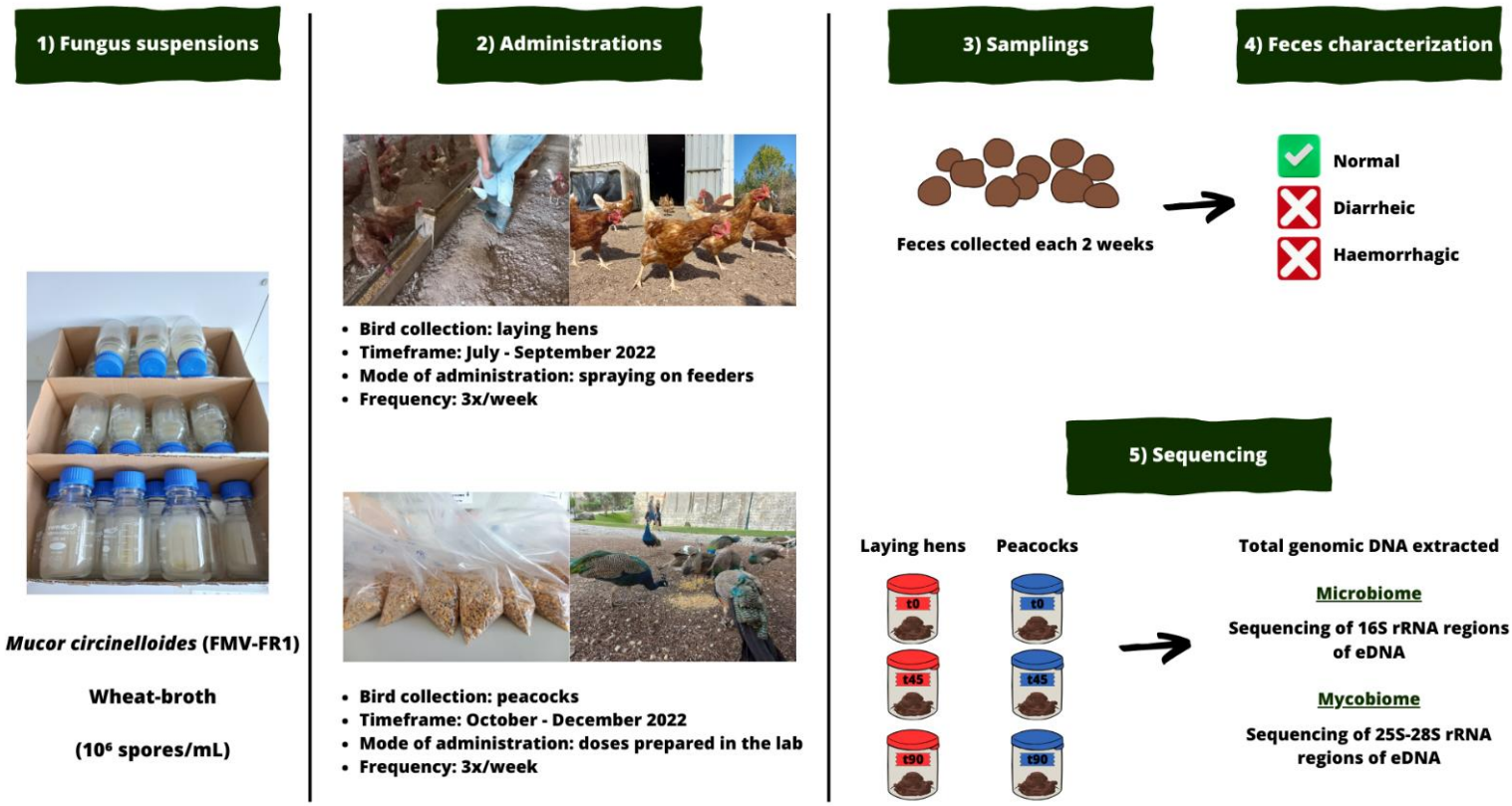

Figure 23. Experimental design established for assessing the effect of *Mucor circinelloides* (FMV-FR1) spores on birds' feces appearance and consistency and on their fecal microbiome and mycobiome (figure created using Canva® - www.canva.com; photos from fungal suspensions, bird flocks, and mode of administration are original).

In each sampling time point and in both bird flocks, fecal samples' appearance (normal vs hemorrhagic) and consistency (normal vs diarrheic) were analyzed (Figure 24), with the number of samples in each category being quantified and compared with those from the control time point. Then, all feces from the control, t45 and t90days time points were gently mixed and homogenized, forming three aggregated sub-samples per bird flock, which were then placed in sterile plastic flasks and stored in a freezer (-20°C) until further processing. Since *in vivo* trials were performed in bird groups, the procedure of aggregating feces in each sampling time point aimed to reduce the variability between individuals and thus establish sub-samples that would be more representative of the overall flocks' resident bacterial and fungal communities.

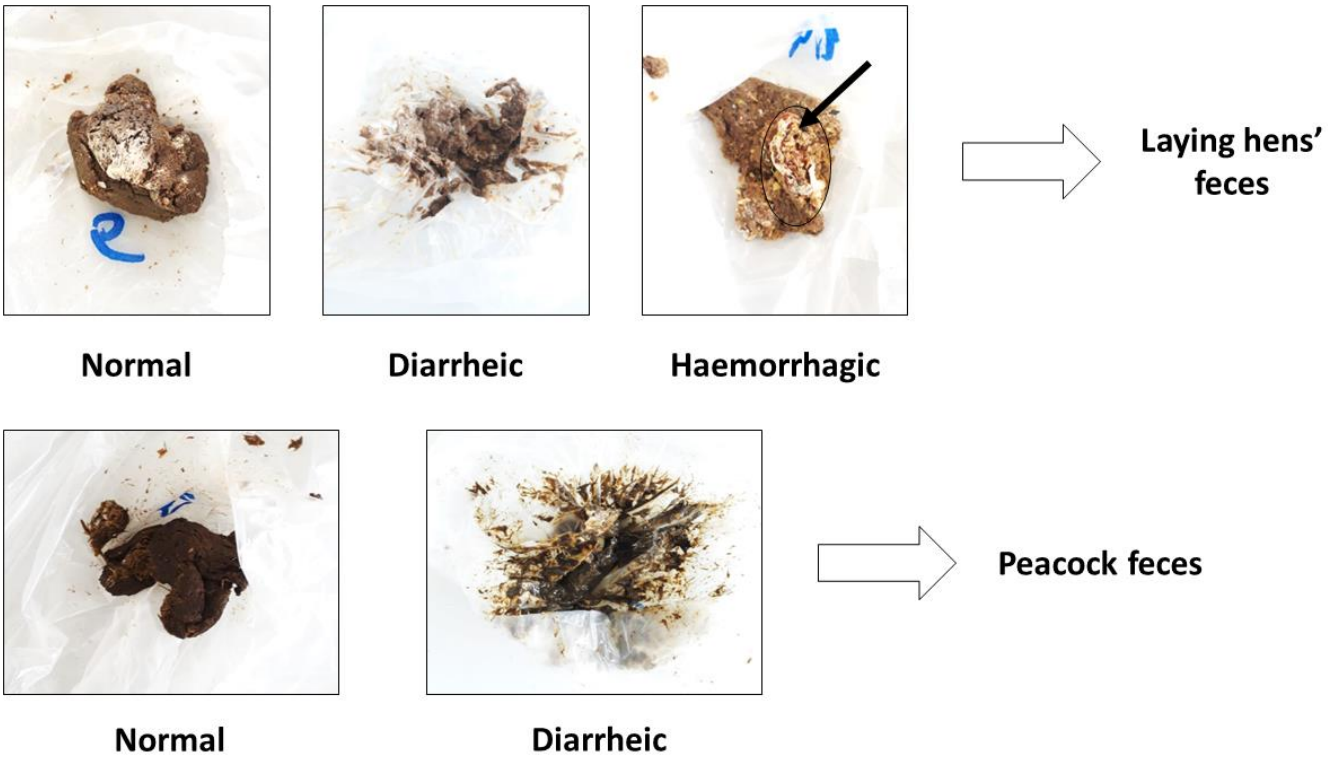

Figure 24. Qualitative scale used for fecal samples' appearance and consistency characterization (normal vs diarrheic or hemorrhagic); black circle and arrow highlight the blood clot identified in a laying hen's fecal sample (original photos).

2.3.4. Microbiome and mycobiome sequencing

Total genomic DNA was extracted from all aggregated sub-samples using the DNeasy PowerMax Soil Kit (QIAGEN, Venlo, Netherlands), following the manufacturer's instructions.

Each sample was subjected to microbiome and mycobiome characterization (bacterial and fungal sequences, respectively) by sequencing the 16S and 25S-28S rRNA regions of eDNA. Samples were analyzed by a customized analytical pipeline developed by BioISIGenomics for long-read targeted nanopore sequencing to obtain high-accuracy taxonomical classification. The current approach has been validated through ZymoBIOMICS Microbial Community Standard, and sequencing runs were carried out on the GridION X5 sequencing platform. Sequencing data was obtained from 16S and 25S-28S rRNA amplicons, low-quality reads were removed, and the remaining reads were size selected (keeping reads between 1,200 and 1700 bp) using prinseq-lite (Schmieder and Edwards 2011). Taxonomic classification was performed using a lowest common ancestor approach, with indexing based on k-mers mapping to the lowest common ancestor of all genomes known to contain a given k-mer (Wood et al. 2019). Following classification, data were rarefied and subjected to phyla and genera relative abundances analysis (percentage of each phylum or genus reads in the total of raw reads) as well as alpha-diversity group analysis based on calculating the Shannon diversity (Shannon 1948) and Pielou evenness (Pielou 1966) indexes using the Qiime2 software (version 2019.4.0) (Bolyen et al. 2019). Three technical replicates were performed for each aggregated sub-sample (nine technical replicates for each bird flock).

2.4. Statistical analysis

Data regarding the quantitative hemolysis assay were subjected to descriptive analysis (mean and standard error values) using the software IBM SPSS Statistics, version 27 (IBM Corporation, Armonk, NY, EUA). Also, a normality analysis was performed using the Shapiro-Wilk test ($n<50$), and it was concluded that the OD results obtained for wells containing rRBCs and each fungal concentration and the positive and negative controls were normally distributed (10^4 spores/mL: $p=0.20$; 10^5 spores/mL: $p=0.13$; 10^6 spores/mL: $p=0.10$; positive control: $p=0.19$; and negative control: $p=0.31$). Thus, the one-way ANOVA with *post hoc* LSD test was used to compare the results between these five groups of OD data. Moreover, fecal appearance and consistency were compared between each trial's time point in both bird flocks using the Fisher's Exact test.

Microbiome and mycobiome data were treated using the software R, version 4.1.2 (The R Foundation, https://www.r-project.org/foundation/). Alpha-diversity group analysis was

subjected to the Kruskal-Wallis test to compare bacterial and fungal fecal diversities between each sampling time point. A significance level of $p<0.05$ was used for every statistical test.

3. Results

3.1. *Mucor circinelloides* (FMV-FR1) virulence factors

The complete analysis of *M. circinelloides* (FMV-FR1) genome revealed six predicted genes coding for virulence factors, above the 80% identity cut-off, namely the gene FTR1, which encodes for an iron permease (Protein ID: I1BRD6; *Rhizopus arrhizus*, 83.98% identity; PHI database), the genes FOB1 (Protein ID: I1BW02; *Rhizopus arrhizus*, 83.07% identity; PHI database) and FOB2 (Protein ID: I1CCV9; *Rhizopus arrhizus*, 86.67% identity; PHI database), which encode for iron receptors, the genes ARF2 (Protein ID: Q5AND9; *Candida albicans*, 90.06% identity; PHI database) and ARF6 (Protein ID: G4N9S6; *Magnaporthe oryzae*, 80.45% identity; PHI database), which encode for ADP-ribosylation factors, as well as the gene CDC42 (Protein ID: Q2PBY8_CLAPU; *Claviceps purpurea*, 86.84% identity; DFVF database), which encodes for a Rho-like GTPase.

All other common Mucorales virulence factors, such as iron ferroxidase (FET3 gene), spore coat proteins (COTH2 and COTH3 genes), protein kinase A (PKAR gene), endonucleases (DCL1 gene), 14-α sterol demethylases (CYP51 gene), transcription factors (ATF1 gene), heteromeric G-protein beta subunit (GPB1 gene), siderophores (ARN1, SREA, BIR1 and AFT1 genes) and chitinase (GH18), were detected under the threshold of 80% identity (Table S1).

Regarding the phenotypic expression of virulence factors, based on the culture media available for this assay, this *M. circinelloides* isolate was found to be positive only for lecithinase activity. Negative results were obtained for the other virulence factors tested, with fungal growth not leading to any media changes (Figure 25).

Also, the quantitative hemolysis assay resulted in OD reads of 1.22 ± 0.11, 1.17 ± 0.09, 1.12 ± 0.10 after rabbit red blood cells (rRBCs) being exposed to the fungal concentrations of 10^4, 10^5, and 10^6 spores/mL, respectively, whereas the positive and negative controls had absorbances of 5.55 ± 0.96 and 1.05 ± 0.10, respectively. No differences were identified between the absorbances obtained for microplate wells containing rRBCs and each fungal concentration ($p=0.95$ between 10^6 and 10^5 spores/mL; $p=0.88$ between 10^6 and 10^4 spores/mL; and $p=0.93$ between 10^5 and 10^4 spores/mL), as well as between these wells and negative control ($p=0.91$, 0.86, and 0.79, for comparisons between the negative control and the wells containing rRBCs and 10^6, 10^5, and 10^4 spores/mL, respectively). Moreover, the

mean absorbance in the positive control was significantly higher than in wells containing rRBCs and each fungal concentration and the negative control ($p<0.01$ for all comparisons) (Figure 26). Thus, aggregated results from these qualitative and quantitative assays confirm that *M. circinelloides* tested negative for hemolysin production.

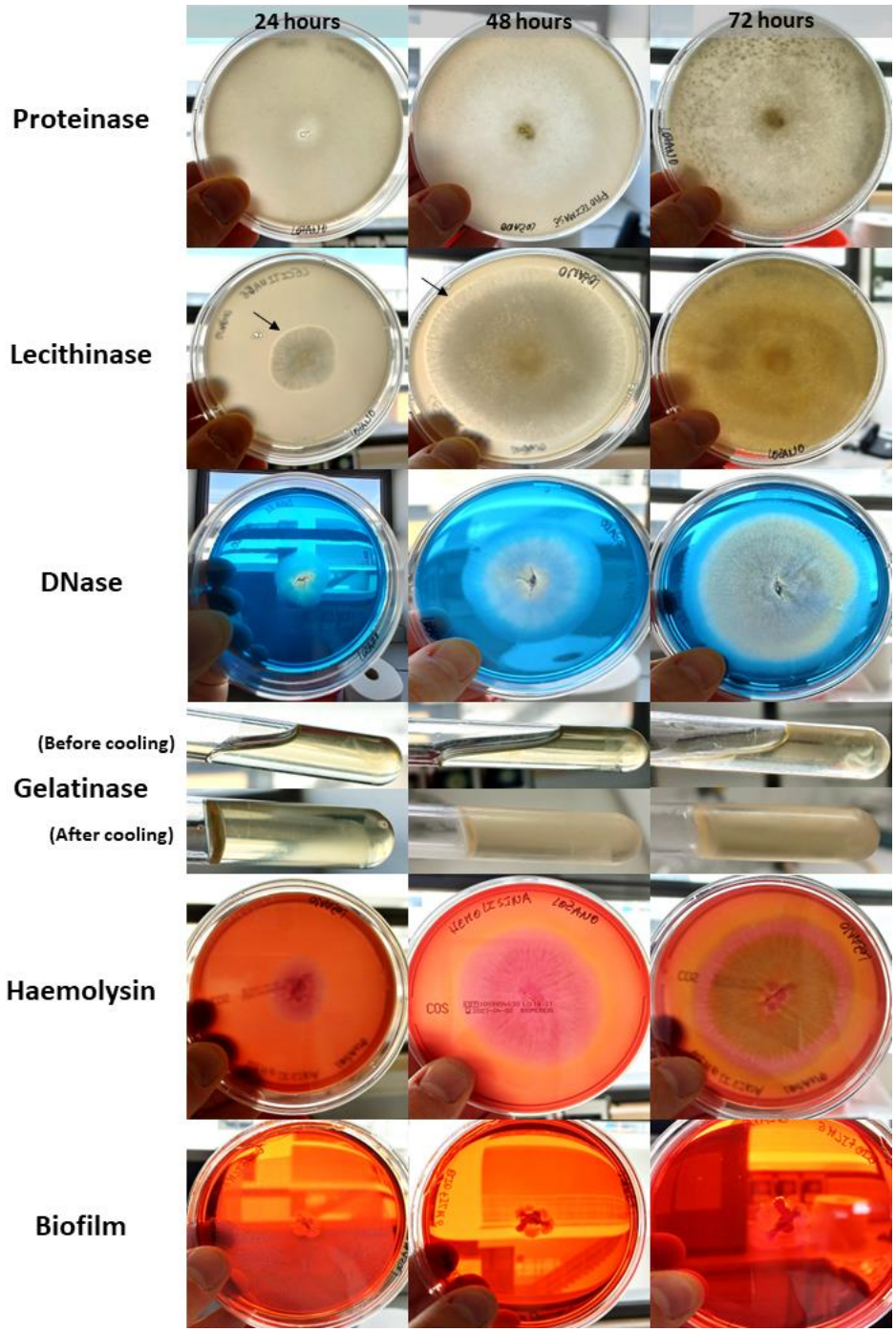

Figure 25. Phenotypic expression of six virulence factors by *Mucor circinelloides* (FMV-FR1). Positive results for lecithinase activity are revealed by a clearing halo surrounding the fungal colony, observed after 24 and 48 hours of incubation (black arrows). This isolate tested negative for all other virulence factors.

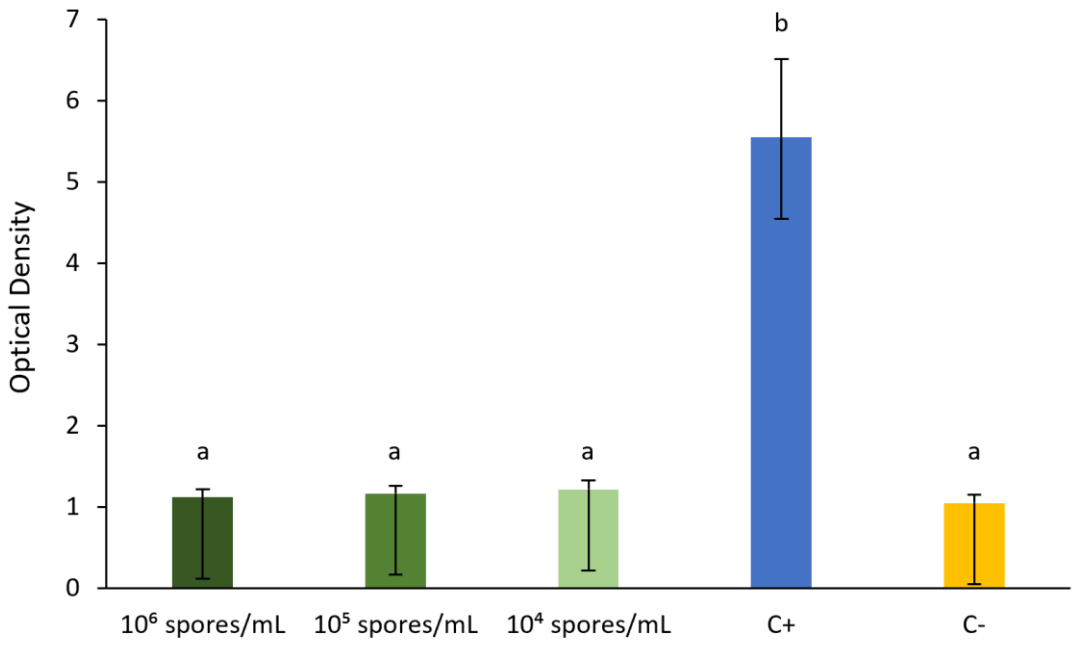

Figure 26. Optical density (OD) results (mean ± standard error) obtained for test wells containing rRBCs and each fungal concentration (green bars), and for positive (with rRBCs + Triton 1%) and negative control wells (with PBS) (C+ and C-, respectively). OD results were measured by absorbance at 450 nm. Bars sharing the same superscript letter correspond to non-significant differences (p>0.05).

3.2. Impact of *M. circinelloides* in the avian intestinal micro- and mycobiome and homeostasis

Fecal microbiome (i.e., bacteria) sequencing revealed that bacteria of the phyla Firmicutes (74% ± 0.05% - 89% ± 0.34%) and Proteobacteria (4% ± 0.1% - 16% ± 0.1%) were the most abundant in the GI tract of laying hens, with the genus *Lactobacillus* being the most represented in the entire study (22% ± 0.1% - 48% ± 0.04%) (Figure 27). Guts from peacocks were mainly colonized by Firmicutes (62% ± 0.1% – 66% ± 0.1%) and Bacteroidetes (30% ± 0.1% – 31% ± 0.1%), and *Prevotella* spp., *Lachnoclostridium* spp., and *Blautia* spp. were the most abundant genera (Figure 28). Overall, bacterial phyla and genera relative abundances remained similar during both *in vivo* trials after birds were fed with *M. circinelloides* spores, and the aggregated alpha-diversity for laying hens and peacocks did not differ between each sampling time point (p=0.62).

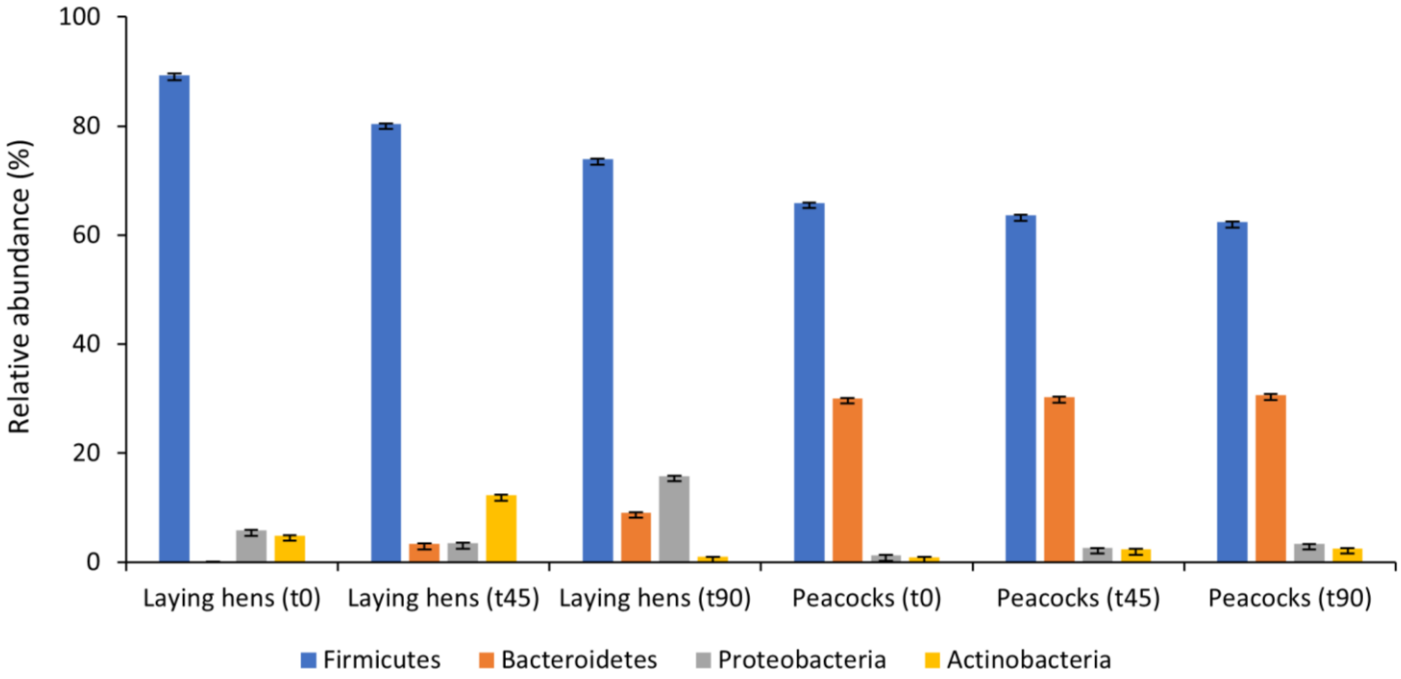

Figure 27. Dynamics of native GI bacteria phyla during the *in vivo* trials in laying hens and peacocks following *Mucor circinelloides* spores' administrations.

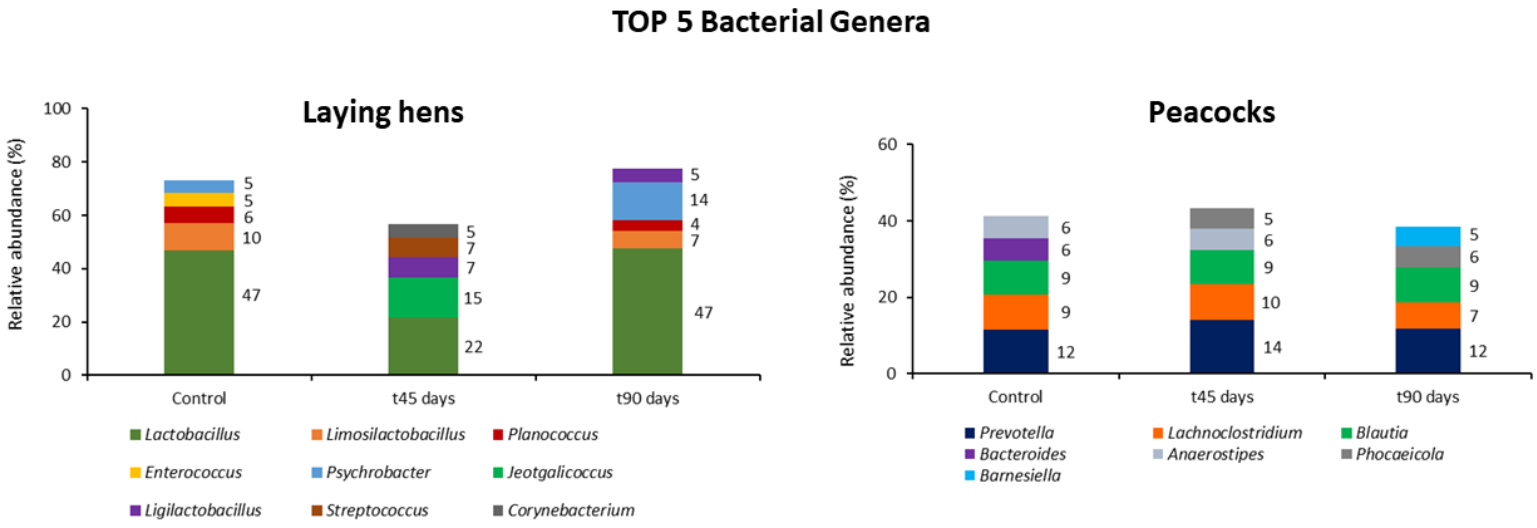

Figure 28. Top five bacterial genera in each fecal sampling time point, measured by relative abundance, following the administration of *Mucor circinelloides* spores to laying hens and peacocks.

The gut mycobiomes of laying hens and peacocks were found to be dominated by fungi of the phyla Ascomycota and Basidiomycota (55% ± 0.1% - 88% ± 0.1%), with the exception of peacocks after 90 days of fungal administrations, in which Mucoromycota was the second most abundant phylum (22% ± 0.1%) (Figure 29). Also, gut fungal genera composition differed between laying hens and peacocks, with the feces of the first being mainly composed of fungi of the genus *Kazachstania* at the beginning and end of the *in vivo* trial (22% ± 0.1% and 17% ± 0.04%), whereas *Pezicula*, *Coprinellus,* and *Antarctomyces* were the most abundant genera in the GI tract of peacocks at t0, t45, and t90 days (19.5% ± 0.2%, 21.96% ± 0.003%, and 55.7% ± 0.1%, respectively) (Figure 30). Moreover, no *M. circinelloides* sequences were detected in feces from both bird collections in every trial's time point. As observed for the microbiome, the fungal diversity was not affected by the administration of *M. circinelloides* spores, as the aggregated alpha-diversity did not differ between each sampling time point (p=0.15).

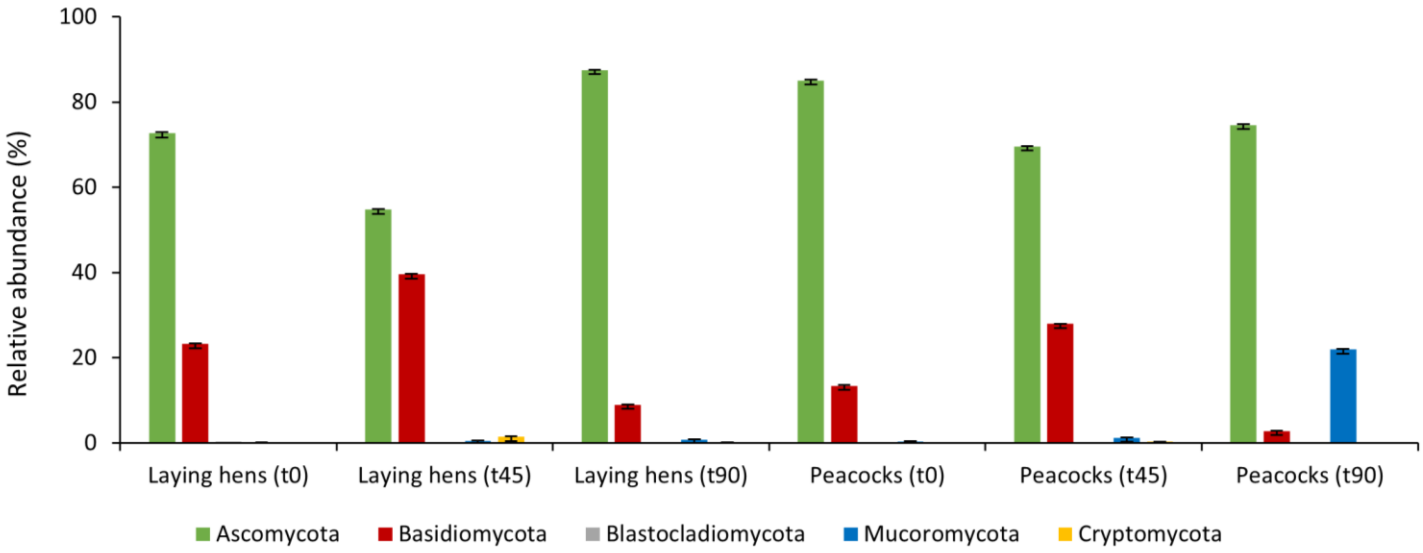

Figure 29. Dynamics of native GI fungi phyla during the *in vivo* trials in laying hens and peacocks following *Mucor circinelloides* spores' administrations.

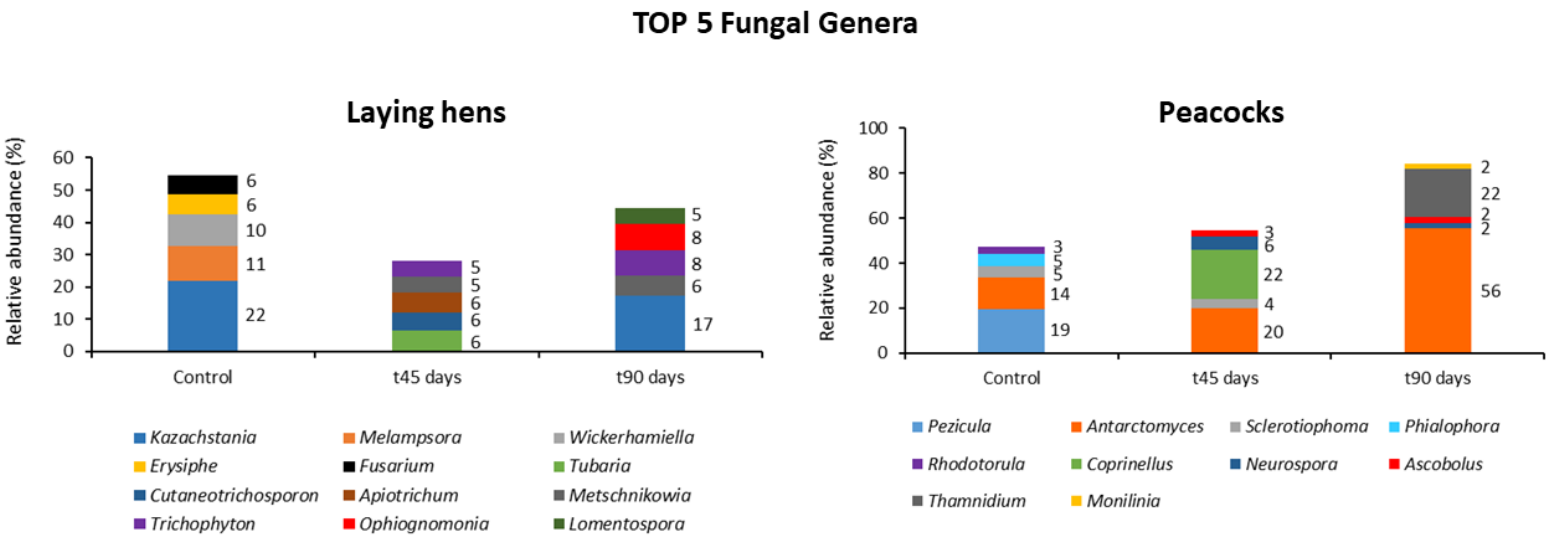

Figure 30. Top five fungal genera in each fecal sampling time point, measured by relative abundance, following the administration of *Mucor circinelloides* spores to laying hens and peacocks.

Moreover, fecal appearance analysis revealed that laying hens' intestinal homeostasis seemed to remain constant during the *in vivo* trial, with 70%-100% of the samples showing normal appearance. In fact, a significant increase in the proportion of normal feces after 45 days of fungal feeding was recorded (100%, p=0.02) in comparison with those from the control time point. Also, 88%-100% of peacock fecal samples were classified as normal regarding their appearance and consistency, never differing significantly from those of the control time point (Table 9). Finally, birds exhibited normal behaviour, and no changes were observed on skin and feathers throughout the trials.

Table 9. Intestinal homeostasis evaluation performed during the assays in laying hens and peacocks, measured by the prevalence of normal fecal samples in comparison with diarrheic or hemorrhagic feces.

Time point	Laying Hens					Peacocks				
	N	D	H	% N (N/T)	p-value	N	D	H	% N (N/T)	p-value
Control	14	3	3	70	NA[b]	15	2	0	88	NA
T15 days	18	0	2	90	0.235	19	1	0	95	0.58
T30 days	16	3	1	80	0.716	15	1	0	94	0.52
T45 days	20	0	0	100[c]	0.02	20	0	0	100	0.20
T60 days	17	2	1	85	0.45	20	0	0	100	0.20
T75 days	18	0	2	90	0.235	20	0	0	100	0.20
T90 days	19	0	1	95	0.09	20	0	0	100	0.20

[a]N, normal feces; D, diarrheic feces; H, hemorrhagic feces; and T, total (N+D+H).

[b]NA – Not applicable.

[c]Significant differences in comparison with the control time point ($p<0.05$).

4. Discussion

Research with ovicidal and larvicidal fungi has revealed promising results for their use in the biological control of avian GI parasites, namely coccidia, ascarids, and strongyles (Braga et al. 2013; Thapa et al. 2018; Lozano et al. 2023). Despite their demonstrated efficacy in destroying parasitic forms, ensuring the safety of these fungi for animals and technicians is a crucial step when choosing the most suitable fungal strains for parasite management in farms, zoos, and public/private animal collections.

The whole-genome sequencing (WGS) of this *M. circinelloides* isolate allowed to identify six predicted genes coding for virulence factors, namely an iron permease (FTR1), iron receptors (FOB1 and FOB2), ARFs (ARF2 and ARF6), as well as a Rho-like GTPase (CDC42). As iron is essential for fungal survival, the permease FTR1 and iron receptors FOB1 and FOB2

play an important role in free iron uptake and, thus, in the virulence of Mucorales fungi, especially when the proteins lactoferrin and transferrin fail to chelate the free iron available in host tissue fluids as a consequence of altered metabolism, lysis of red blood cells, or even trauma (Bullen et al. 2006; Lax et al. 2020). Also, studies have been demonstrating that ARFs play an essential role in *M. circinelloides* dimorphism and virulence, such as by regulating the biogenesis of vesicles involved in hyphal apical growth (Patiño-Medina et al. 2018; Lax et al. 2020). Moreover, the GTPase CDC42 is essential for hyphae morphogenesis in filamentous fungi due to its role in the generation of cell polarity (Virag et al. 2007), and its coding gene has been also identified in the genome *P. chlamydosporia*, another ovicidal fungus species. In this particular case, the detection of the CDC42 gene can also be seen as an important attribute of this *M. circinelloides* isolate since hyphae germination and migration towards parasite eggs and oocysts is the first step of the parasiticide activity developed by this kind of fungi (Braga and Araújo 2014; Lozano et al. 2023).

Also, regarding the phenotypic expression of virulence factors, the current study showed that the FMV-FR1 *M. circinelloides* isolate only tested positive for lecithinase activity. This enzyme hydrolyses the phospholipid lecithin, which is a structural component of the animal cell membrane, and thus might play a role in fungal pathogenicity and eventually lead to host cell lysis (Ghannoum 2000). Negative results for hemolysin and biofilm assays are in contrast with previous research performed in other medically important fungi, namely *Aspergillus*, *Candida*, *Fusarium*, *Cryptococcus*, and *Coccidioides*, which reported these fungal taxa as being hemolysin producers and having biofilm forming abilities in abiotic and biotic substrates (Fanning and Mitchell 2012; Nayak et al. 2013).

In the second phase of the present study, the analysis of birds' gut microbial communities was performed on fresh fecal samples from both avian hosts, collected immediately after excretion. This is an easy, non-invasive, and accurate approach for estimating the overall bacterial and fungal communities in animals GI' tract by sequencing their fecal microbiome and mycobiome (Grond et al. 2018). Fecal microbiome sequencing results from laying hens revealed that their guts were overall colonized by bacteria of the phyla Firmicutes, followed by Proteobacteria, and the genus *Lactobacillus* had the highest relative abundance, whereas the GI microbiota of peacocks was mostly dominated by Firmicutes and Bacteroidetes. These results are in accordance with Grond et al. (2018) and Carrasco et al. (2019), who reported that in physiological conditions, the native GI microbiota of most bird species, namely Galliformes, is mainly dominated by Firmicutes, and described the crucial role of these bacterial communities in the biosynthesis of short-chain fatty acids (byproducts of fermentation), which are important energy and carbon sources for bird's nutrition. The abundance of some bacterial taxa belonging to this phylum, namely *Lactobacillus* spp., has

been positively associated with higher productivity in poultry (Carrasco et al. 2019). Also, *Lactobacillus* spp. and *Bacillus subtilis* are examples of bacteria with probiotic properties, contributing for the regulation of birds' gut microbiota towards higher proportions of beneficial bacteria and thus improving the animals' intestinal homeostasis and nutrient uptake (Reis et al. 2017; Carrasco et al. 2019). Animals' GI microbiota is influenced by several biotic and abiotic factors, and the identification of the Bacteroidetes phylum as the second most abundant in peacocks during the entire trial and with similar relative abundances in every sampling time point might be related to the feed composition, which contained corn grains and sunflower seeds, among other constituents. The presence of these components in feed may have stimulated the growth of bacteria from this phylum since they play an important role in degrading complex plant polysaccharides, such as cellulose (Grond et al. 2018).

The phyla Ascomycota and Basidiomycota were the most abundant in the GI tract of laying hens and peacocks throughout the assays, with the exception of the last fecal sampling time point in peacocks, in which fungi of the phylum Mucoromycota were the second most abundant after Ascomycota. Although most studies regarding the characterization of fungal intestinal communities have been performed in humans and rodents, recent studies revealed that the GI mycobiota of chickens is mainly colonized by Ascomycota and, in less extension, by Basidiomycota fungi, irrespective of the GI tract's section (Robinson et al. 2022), which is in accordance with the current study results. Also, yeasts of the genus *Kazachstania* were identified as the most abundant at two sampling time points (beginning and end of the trial) in laying hens, being a genus highly represented in the GI tract of other monogastric species, such as pigs, and with a reported positive role in promoting intestinal epithelial glycolysis (Hu et al. 2023).

The increase in the relative abundance of the Mucoromycota phylum in peacock feces, at the end of the *in vivo* trial, is another interesting result observed in this study. The last weeks of this assay were marked by flood episodes in Lisbon downtown, as a result of intense rainfall, which led the birds' owners to house them together. Mucorales fungi are known for their ubiquity and a rapidly growing mycelium (Hoffmann et al. 2013), and thus birds' stocking conditions observed for that period might have increased fungal spores' transmission through fecal-oral route, and consequently being responsible for a higher fecal excretion of Mucorales spores at the end of the trial.

Also, mycobiome sequencing failed to specifically detect *M. circinelloides* sequences in feces from laying hens and peacocks in each sampling time point, suggesting that spores from this fungus were not capable of colonizing the GI tract of birds. Although further anatomopathological analysis is needed to confirm this hypothesis in birds, the incapability of

M. circinelloides to colonize the GI tract of other animal species has already been demonstrated in a previous study in ruminants (Voinot et al. 2022).

The overall results obtained in each bird flock suggest that feeding *M. circinelloides* spores to laying hens and peacocks did not alter their GI microbiome and mycobiome, with *Lactobacillus* spp. and *Prevotella* spp. being the top bacterial genera in all sampling time points, in laying hens and peacocks, while the fungal genus *Kazachstania* was the most abundant in laying hens' feces at the beginning and end of the trial. Some fluctuations were still observed in microbial phyla and genera throughout the study, which could have been influenced by several biotic and abiotic factors. For example, age is a biological factor that has been shown to influence birds' gut microbial composition, with previous studies in chickens revealing that the cecum of newly hatched chicks is mainly dominated by Clostridiaceae, whereas *Lactobacillus* represents 25% of all cecal bacterial genera at three days of age and then its abundance decreases up to 100 times when broilers reach 42 days (Gong et al. 2008; Kers et al. 2020; Bindari and Gerber 2022). However, there are still few publications addressing the interactions between age evolution in animal groups and their gut microbial composition and diversity, especially for mycobiome, with a previous study reporting the transition from a *Trichosporon* spp. dominance in the cecum of broilers aging 14 days to *Microascus* spp. at 28 days of age, despite no difference being recorded in the fungal alfa-diversity (Robinson et al. 2020). Moreover, some shifts observed in fecal microbiome and mycobiome analysis may have been caused by the intrinsic instability of the fecal bacterial and fungal communities, which rapidly change their relative abundances depending on storage conditions (Bindari and Gerber 2022). These limitations of the current study suggest that further research should include a previous separation of birds into two distinct groups, with the control group receiving feed not supplemented with fungal spores, as well as more fecal samplings to cover a broader timeframe of analysis.

Despite the identification of several predicted genes in the *M. circinelloides* (FMV-FR1) genome coding for virulence factors and a positive lecithinase activity, overall results from this virulence profile analysis, combined with the lack of interference of *M. circinelloides* spores in birds' gut microbial diversities and the absence of alterations in birds' fecal appearance and consistency, allow to have an initial insight on the lack of pathogenicity of this *M. circinelloides* isolate to birds. The absence of differences in the bacterial and fungal alfa-diversities, after birds are fed with *M. circinelloides* spores, is another interesting result from the current study, as microbial equilibrium is essential for intestinal homeostasis (Bindari and Gerber 2022; Chen et al. 2022).

Parasiticide fungi have always been tested on animals in controlled health programs, with constant monitoring of any eventual side effect developed by the animals. Previous *in vivo* studies in which parasitized animals received *M. circinelloides* (CECT 20824) spores found that their hematological parameters (e.g., red blood cells, hemoglobin, hematocrit, white blood cells, and lymphocytes) remained constant or even improved after fungus administrations (Hernández et al. 2016; Voinot et al. 2021). Also, the anatomopathological analysis of different dairy cow tissues revealed no signs of damage caused by *M. circinelloides* (CECT 20824) and *D. flagrans* (CECT 20823) spores (Voinot et al. 2022).

To the best of our knowledge, this study represents the first report regarding the analysis of the virulence profile of a *M. circinelloides* parasiticide isolate and the assessment of its potential impact on animals' GI core bacterial and fungal communities. Despite overall results suggesting that this *M. circinelloides* isolate does not offer any health risk for birds, in the scope of a parasite biological control program, more *in vitro* and *in vivo* studies are needed to confirm this hypothesis, namely assessing the phenotypic expression of more potential virulence factors, collecting blood samples for hematological analysis, performing anatomopathological analysis in birds receiving spores of *M. circinelloides* FMV-FR1, and finally integrating the microbial results with parasitological data by comparing the dynamics of gut microbiome and mycobiome following the reduction of the GI parasitic population caused by the administration of fungi with parasiticidal properties.

Data availability: Whole-genome sequencing data recorded for *Mucor circinelloides* isolate FMV-FR1 has been uploaded to the NCBI database with the BioProject accession code "PRJNA1065632" (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1065632) and genome submission code "SUB14153967".

Supplemental material:

Table S1 (Spectrum04078-23-s0001.xlsx). List of hypothetical genes coding for virulence factors, after WGS of the tested *M. circinelloides* isolate. (https://journals.asm.org/doi/10.1128/spectrum.04078-23#supplementary-materials).

Ethics approval: This study was approved by the Ethical Committee for Research and Teaching of the Faculty of Veterinary Medicine – University of Lisbon (CEIE 019/2022), and also authorized by the owners of each bird collection. Administration of fungal formulations were performed *per os*, through feed, and fecal samples were collected after excretion, with no direct manipulation of the animals. Moreover, the study followed the normal daily routine of each bird collection. Finally, rabbit blood samples used in the quantitative hemolysis assay were taken from healthy animals and during routine consultations for recommended blood analysis.

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CHAPTER VI – *In vivo* performance of *M. circinelloides* FR1 in reducing coccidia parasitism in peacocks

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Linked publication:

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Article 6 – "Assessing the efficacy of the ovicidal fungus *Mucor circinelloides* in reducing coccidia parasitism in peacocks"

Abstract

The biological control of gastrointestinal (GI) parasites using predatory fungi has been recently proposed as an accurate and sustainable approach in birds. The current study aimed to assess for the first time the efficacy of using the native ovicidal fungus *Mucor circinelloides* (FMV-FR1) in reducing coccidia parasitism in peacocks. For this purpose, an *in vivo* trial was designed in the resident peacock collection (n=58 birds) of the São Jorge Castle, at Lisbon, Portugal. These animals presented an initial severe infection by coccidia of the genus *Eimeria* (20106 ± 8034 oocysts per gram of feces, OPG), and thus received commercial feed enriched with a *M. circinelloides* suspension (1.01 x 10^8 spores/kg feed), thrice-weekly. Fresh feces were collected every 15 days to calculate the coccidia shedding, using the Mini-FLOTAC technique. The same bird flock served simultaneously as control (t0 days) and test groups (t15 – t90 days). The average *Eimeria* sp. shedding in peacocks decreased up to 92% following fungal administrations, with significant reduction efficacies of 78% (p=0.004) and 92% (p=0.012) after 45 and 60 days, respectively. Results from this study suggest that the administration of *M. circinelloides* spores to birds is an accurate solution to reduce their coccidia parasitism.

Keywords: Peacocks, Coccidia, Predatory Fungi, *Mucor circinelloides*, Mini-FLOTAC, Portugal.

1. Introduction

Galliformes kept under free-range conditions in farms, zoos and private collections are highly prone to gastrointestinal (GI) parasitism caused by coccidia of the genera *Eimeria* and *Isospora*, and by nematodes such as *Capillaria* spp., *Ascaridia galli*, *Heterakis* spp., *Trichostrongylus tenuis* and *Strongyloides* spp. (Ilić et al. 2018; Lolli et al. 2019; Lozano et al. 2019; Carrera-Játiva et al. 2020; Andreopoulou et al. 2022; Zhang et al. 2022) .

Coccidia, in particular, are responsible for severe health concerns in domestic and exotic birds, being of clinical (characterized by swelling of the intestinal wall and hemorrhages, leading to diarrhea and/or hemorrhagic feces, and even to death) or subclinical importance (associated with limited enteritis and loss of fluids, leading to poor absorption of nutrients), with poultry coccidia being the most studied (Lozano et al. 2019; Mesa-Pineda et al. 2021), resulting in average economic losses for the poultry industry of nearly 12 € billion annually worldwide (McDougald and Fitz-Coy 2008; Blake et al. 2020). Moreover, a total of nine *Eimeria* species have already been described in peacocks, namely *Eimeria arabica*, *E. kharjensis*, *E. mandali*, *E. mayurai*, *E. mutica*, *E. pavonina*, *E. pavonis*, *E. patnaiki* and *E. riyadhae* (Titilincu et al. 2009; Jaiswal et al. 2013; Zhang et al. 2022), and despite not being so well studied, it is known that *E. mutica* and *E. kharjensis* develop their pathogenic activity on peacock's ileum (Alyousif and Al-Shawa 1998).

Prevention and treatment of avian GI parasitic infections is still frequently performed exclusively with antiparasitic drugs, whose incorrect use often leads to low treatment efficacies, drug resistance, accumulation of drug residues on carcasses and contamination of soil and ground-waters (Abbas et al. 2011; Mund et al. 2017; Mooney et al. 2021; Selzer and Epe 2021; Martins et al. 2022) .

Over the last 30 years, efforts have been made by the scientific community to develop new complementary approaches for the integrated control of GI parasitic infections in animals, namely the use of predatory fungi, also referred in the literature as "nematophagous", "helmintophagous" or "parasiticide fungi" (Braga and Araújo 2014). These saprophytic filamentous fungi are mostly found in agricultural soil and animal feces (Falbo et al. 2013; Liu et al. 2015; Hernández et al. 2017; Ojeda-Robertos et al. 2019; Arroyo-Balán et al. 2021; Lozano et al. 2023) , and their main functional characteristic relies on the ability to capture and destroy environmental forms of GI parasites (larvae, eggs, and oocysts), by means of mechanical and enzymatic activities, and thus breaking the parasites' life cycles on their exogenous stages. The most known predatory fungal taxa are *Duddingtonia flagrans* (Dudd.) R.C. Cooke (1969), *Arthrobotrys oligospora* Fresen., (1850), and *Monacrosporium thaumasium* (Drechsler) de Hoog & Oorschot (1985), which are able to destroy nematodes'

infective larvae (L3) (larvicidal fungi), whereas *Pochonia chlamydosporia* (Goddard) Zare & W. Gams (2001) and *Mucor circinelloides* Tiegh (1875) present ovicidal activity towards helminth eggs and coccidian oocysts (ovicidal/coccidicidal fungi) (Madeira de Carvalho et al. 2012; Braga and Araújo 2014; Viña et al. 2022).

Despite the majority of studies have addressed the use of these fungi to control ruminants (Healey et al. 2018; Branco de Oliveira et al. 2021; Voinot et al. 2021) and horses (Madeira de Carvalho et al. 2011; Arias et al. 2013a; Canhão-Dias et al. 2020) GI parasites, research on this topic has been recently extended to other animal species, namely dogs, birds, and captive wild animals (Araujo et al. 2012; Cazapal-Monteiro et al. 2015; Palomero et al. 2021; Lozano et al. 2023; Paz-Silva et al. 2023; Salmo et al. 2024) . Regarding bird parasite control, it has been revealed that *P. chlamydosporia*, *D. flagrans*, *Arthrobotrys* spp. and *M. thaumasium* are promising candidates for the biological control of helminth infections, namely in chickens, laying hens and ostriches, and also confirmed chlamydospores' tolerance to the avian GI biochemical environment (Braga et al. 2013; Silva et al. 2017; Thapa et al. 2018; Valadão et al. 2020; Lozano et al. 2022). Recently, Lozano et al. (2023) described for the first time the isolation of seven native *Mucor* spp. isolates (*M. circinelloides* and *M. lusitanicus*) from chicken and peacock fecal samples, and confirmed their coccidiostatic and coccidicidal activity towards *Eimeria* sp. oocysts, with the isolate *M. circinelloides* FR1 achieving the highest efficacy on destroying coccidia oocysts. However, field trials using predatory fungi to control GI parasitic infections in birds are still lacking.

The current research aimed to evaluate *in vivo* the efficacy of *M. circinelloides* (FMV-FR1) in reducing coccidia infections in an ornamental peacock collection.

2. Methods

2.1. Peacock collection

This trial was performed on the resident peacock collection (*Pavo cristatus*) of São Jorge Castle, a national monument located in Lisbon downtown, Portugal (38°42'50.241" N 9°8'2.182" W). The flock was composed by 58 birds (44 adults and 14 chicks), kept freely in an outdoor area of 4700 m^2. According to previous research performed at the LPPD-CIISA-FMV, infections by *Eimeria* sp., *Capillaria* sp. and *Strongyloides pavonis* were identified in this peacock collection (Lozano et al. 2021c).

These birds were normally fed twice per day with a formulation composed by corn, wheat, barley, sorghum, soyabean meal, sunflower seeds, calcium carbonate, monocalcium phosphate, soyabean oil, sodium chloride, molasses, and lard (nutritional composition: 15.8%

Crude Protein, 4.25% Crude Fibre, 5% Ash, 3.45% Crude Fat, 0.8% Calcium, 0.43% Phosphorus, 0.09% Sodium, 0.75% Lysine and 0.32% Methionine), given *ad libitum* at two different locations.

In this bird collection, the assistant veterinarians annually perform a single oral administration of Toltrazuril (25 mg/kg) to the juveniles (March-April), and the entire group receives febantel (15 mg/kg), pyrantel pamoate (5 mg/kg) and praziquantel (5 mg/kg), as a pre-mixture. However, no antiparasitic drug treatment was performed at least 6 months prior and during the trial.

2.2. Preparation of the fungal suspension

A native *Mucor circinelloides* (FMV-FR1) ovicidal isolate, belonging to the predatory fungi collection of the Laboratory of Parasitology and Parasitic Diseases of the Faculty of Veterinary Medicine – University of Lisbon (LPPD-CIISA-FMV), whose parasiticide activity towards avian coccidia was previously confirmed (Lozano et al. 2023), was used in the current study. This isolate was stored in Wheat-Flour Agar (2%) medium, at room temperature, in a dark and dry environment, and cultured according to Arias et al. (2013b). Briefly, a wheat broth was prepared, using 10 grams of wheat grains per 1 L of distilled water. After autoclaving, 50 mL of broth were transferred to 20 plastic bottles, previously washed, and sterilized with UV-light. Wheat Agar cubes of 2.25 x 2.25 x 2.25 cm, containing mycelia from *M. circinelloides*, were cut and added to the broth in each bottle, which were then left at room temperature with a slope of 45° for one month. Finally, fungal suspensions of 10^6 spores' fold/mL were established, using a Neubauer chamber to count *Mucor* conidia and chlamydospores, and kept in a dry environment until its further use.

2.3. Fungal administrations

For this assay, bird feed doses containing *M. circinelloides* spores were previously prepared in the laboratory, following the protocol proposed by Voinot et al. (2021). For each dose, 600 g of peacock feed were mixed with 60 mL of the fungal suspension with 10^6 spores/mL. The mixture was dried at 27 °C, for 30 min, using an incubator, and then packaged individually in sealed plastic bags, with peacocks receiving 1.01 x 10^8 spores/kg of feed (Figure 31).

The assay lasted between October – December 2022 and was based on the procedures described by Palomero et al. (2021) and Voinot et al. (2021). During this timeframe, a total of 33 fungal oral administrations were performed, thrice weekly. Since it was not

possible to set two separate groups (test and control), the same flock served simultaneously as control (t0 days) and test group (t15-t90 days).

Weeks (W)	Fungal administrations (FA)	Fecal samplings (FS)
Control	FA 1-3	FS 1
W 1		-
W 2	FA 4-6	FS 2
W 3	FA 7-9	-
W 4	FA 10-12	FS 3
W 5	FA 13-15	-
W 6	FA 16-18	FS 4
W 7	FA 19-21	-
W 8	FA 22-24	FS 5
W 9	FA 25-27	-
W 10	FA 28-30	FS 6
W 11	FA 31-33	-
W 12		FS 7

Figure 31. Experimental design of the *in vivo* trial performed in the selected peacock collection (figure created using Canva®; www.canva.com).

2.4. Parasitological analysis

A total of 20 fresh fecal samples were collected from the environment, immediately after excretion, every 15 days, with exception for t0 days (n=17 samples) and t30 days (n=16 samples), and then individually packed in plastic bags and stored in a refrigerator (4 °C) at the LPPD-CIISA-FMV, for maximum one week.

All samples were processed using the coprological technique Mini-FLOTAC, to identify coccidia oocysts and helminth eggs, and calculate their fecal shedding (oocysts or eggs per gram of feces, OPG or EPG). For this purpose, the Mini-FLOTAC protocol followed the guidelines proposed by the manufacturer and referred in the literature for exotic animals (Cringoli et al. 2017; Lozano et al. 2021b,c). Briefly, 2 g of feces were mixed with 38 mL of saturated sucrose solution (specific gravity 1.2), using the Fill-FLOTAC device; the resulting fecal suspension was transferred to the counting chamber, and left resting on the lab bench for 10 min, to allow parasitic forms to float and attach to the counting grids; then, the top disk was rotated clock-wise, and all coccidia oocysts and helminth eggs were identified and counted in an optical microscope (100x), using an analytic sensitivity of 10 OPG/EPG.

At the beginning of the trial, peacocks revealed very low burdens of *Capillaria* sp. (1.2 ± 0.81 EPG), which was the only helminth identified, and therefore only coccidia shedding was considered for further analysis.

The average coccidia OPG was calculated for each sampling timepoint, and treatment efficacy was determined based on the coccidia fecal oocyst count reduction (FOCR), using the following formula (Madeira de Carvalho et al. 2011; Arias et al. 2013a; Palomero et al. 2021):

$$FOCR\ (\%) = [1 - (OPG_{test\ day}/OPG_{day\ 0}]$$

2.5. Side effects

Peacocks were regularly examined for any side effects resulting from fungal administrations, namely feed rejection, modifications on normal bird behavior, feathers appearance, skin lesions, and diarrhea or blood on droppings.

2.6. Climate conditions

Weather data recorded in the Lisbon Municipality, for the 12 weeks of the trial, namely average temperature (°C) and rainfall (mm), were retrieved from the regional platform "CLIMA.AML: Rede de Monitorização e Alerta Meteorológico Metropolitano" (URL: https://www.clima.aml.pt).

2.7. Statistical analysis

Calculations for mean values and standard errors were performed using the software IBM® SPSS® Statistics, version 27 for Windows (IBM Corporation, Armonk, NY, USA), which was also used for all further statistical analysis. It was observed that the coccidia OPG data failed the Shapiro-Wilk normality test ($n < 50$ samples for every timepoint; $p<0.001$). Thus, the coccidia OPG recorded in each test time point was compared with control using the Mann-Whitney test. Also, a correlation analysis was performed to assess a possible statistical association between coccidia OPG and the weather variables “average temperature” and “rainfall”, using the Spearman’s Test. A significance level of $p<0.05$ was used for all statistical tests.

3. Results

Initial coprological results revealed that this peacock collection was severely infected with *Eimeria* sp. at the beginning of the trial (20107 ± 8034 oocysts per gram of feces, OPG). Following fungal administrations, it was possible to observe an overall decrease in oocysts’ shedding, with OPG reduction efficacies ranging between 59% to 92%, and with the decreased values at timepoints t45 and t60 being significant ($p=0.004$ and $p=0.012$, respectively) (Figure 32). Furthermore, it was also observed that the average OPG did not reach the initial level during the entire assay.

Weather data tracked for the Lisbon municipality, in which the peacock collection was located, revealed an average temperature and rainfall of 16.4°C (11.5-21.3°C, min-max.) and 5.96 mm (0-26.4 mm, min-max), respectively, during the study period (Figure 33). Also, a rainfall increase was observed in weeks 9 and 10, characterized by flood episodes in Lisbon downtown. Both temperature and rainfall did not significantly correlate with the coccidia shedding values ($p=0.12$ and $p=0.48$ for the correlations “*Eimeria* OPG – temperature”, and “*Eimeria* OPG – rainfall”, respectively).

Finally, no side effects were recorded after feeding peacocks with *M. circinelloides* spores, namely no changes on normal bird behavior, feed consumption, and feather appearance.

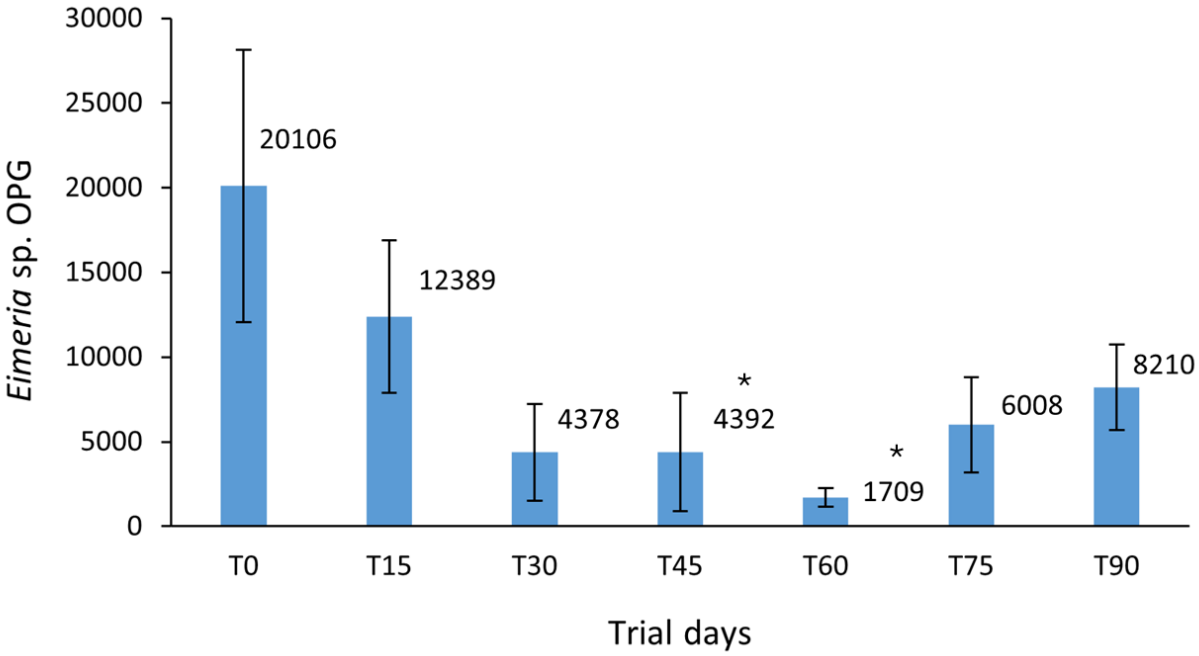

Figure 32. *Eimeria* sp. oocyst shedding dynamics during the trial in peacocks. Blue bars represent mean shedding values (± standard errors). Asterisks on days 45 and 60 mean significant differences in comparison with the control time point (p=0.004 and p=0.012, respectively).

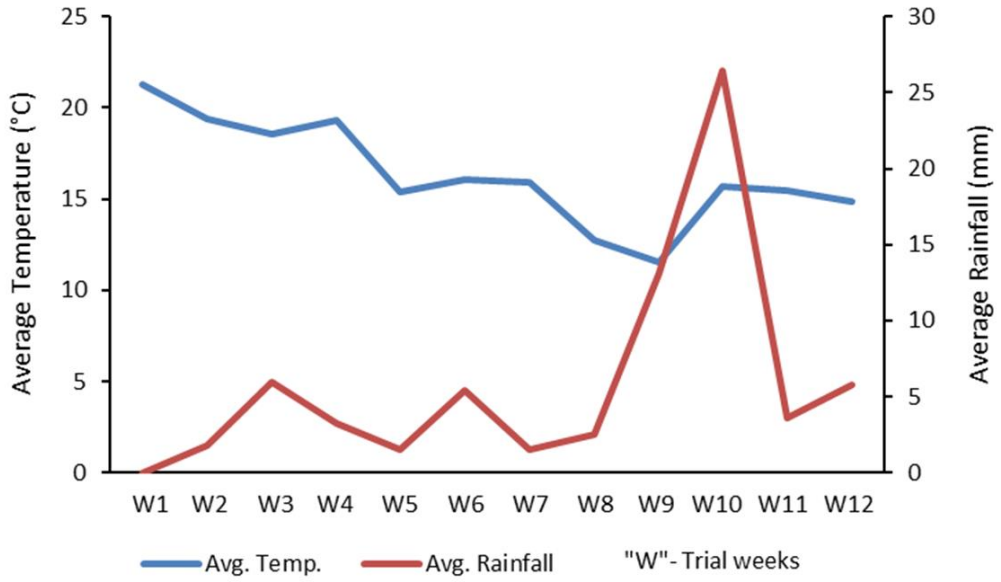

Figure 33. Average daily temperature (blue line, °C) and rainfall (red line, mm) recorded for the Lisbon Municipality, during the 12 weeks of the in vivo trial in peacocks (data retrieved from CLIMA.AML, https://www.clima.aml.pt).

4. Discussion

Predatory fungi are a promising solution for the integrated parasite management of animals kept in captivity in farms, zoos, and private collections, serving as a complement to the conventional antiparasitic drug treatments (Madeira de Carvalho et al. 2012; Araújo et al. 2021; Paz-Silva et al. 2023). However, studies on this topic traditionally focus on horses and ruminants, and to our knowledge no large-scale field trials were previously performed aiming to assess the efficacy of predatory fungi in the reduction of avian GI parasitism.

Parasitological results from this study allowed to conclude that the parasiticide activity developed by *M. circinelloides* against *Eimeria* spp. oocysts reduced the environmental contamination by this parasite, with significant OPG reduction efficacies up to 92%, after 60 days of fungal feeding. These results demonstrated that the predacious activity developed by this type of fungi is a gradual process, as observed in other *in vivo* trials performed with *M. circinelloides* in dogs (Viña et al. 2022; Paz-Silva et al. 2023), sheep (Voinot et al. 2021), baboons (Paz-Silva et al. 2023) and wapitis (Palomero et al. 2021), which reported significant reductions in helminth egg shedding after a minimum of one month of routine fungal administrations.

The presence of parasitic forms (eggs, oocysts, and larvae) triggers predatory fungi hyphae germination and colonization of the fecal and surrounding soil's microenvironments, with the final attachment to the oocyst/egg capsule prompting the first stage of ovicidal/coccidicidal activity (Madeira de Carvalho et al. 2012; Braga and Araújo 2014; Cazapal-Monteiro et al. 2015). This can be considered one of the most critical stages of the whole ovicidal process, together with the penetration of the parasite's capsule, since fungi need to compete against other fecal and soil commensal microorganisms, attach on the parasite capsule and penetrate it through mechanical (appressorium and haustorium) and enzymatic activity (e.g., proteases, chitinases, collagenases and lipases) (Madeira de Carvalho et al. 2012; Yang et al. 2013; Braga et al. 2015; Herrera-Estrella et al. 2016; Freitas Soares et al. 2023) .

This trial was conducted mostly during the Autumn season, in which moderate average temperatures and rainfall were recorded in the Lisbon Municipality. Despite these weather variables being favorable for coccidia oocysts to reach the infective stage, which can even be accomplished at temperatures lower than 20 °C, and especially during Spring and Autumn seasons (Ahad et al. 2015; Shamim et al. 2015), no significant correlations were obtained between weather and coccidia shedding. Thus, the possibility that meteorological conditions could contribute to differences observed in the parasitism results was discarded. Moreover, data collected regarding peacock samples suggest that this *M. circinelloides* isolate tolerated

and maintained its predatory activity at overall temperatures and rainfall between 11.5-21.3 °C and 0-26.4 mm, respectively, being in accordance with previous *in vitro* and *in vivo* research using the strain *M. circinelloides* CECT 20824 for the biological control of strongyles, ascarids and trematodes (Cazapal-Monteiro et al. 2015; Cortiñas et al. 2015; Palomero et al. 2021). It has also been demonstrated that *D. flagrans*, *P. chlamydosporia* and *Monacrosporium sinense* Xing Z. Liu & K.Q. Zhang (1994) maintain their germination capacity on different culture media even at temperatures lower than 20 °C (Wang et al. 2019; Oliveira et al. 2022).

Despite the general OPG decreasing trend observed during the trial, following fungal spores administrations, a slight increase was recorded on the last two weeks, which coincided with the intense rainfall period recorded for the Lisbon district, as previously mentioned. Between t67-90 days of trial, and due to climate conditions, peacocks were not let free ranging in the monument's outdoor area, and thus were sheltered in close contact with each other, leading to a higher exposition to feces contaminated with coccidia oocysts, and consequently stimulating re-infections. Avian *Eimeria* spp. have a very short life cycle, with a prepatent period of minimum four days (McDougald and Fitz-Coy 2008; Lozano et al. 2019), and thus increasing bird stocking density can trigger re-infections and increase coccidia shedding (Attree et al. 2021). These results also point out to the need for a constant monitorization of parasite shedding and clinical signs, during a parasite biocontrol program using predatory fungi, since treatments with antiparasitic drugs might be necessary to complement the action of these fungi.

This was also the first biological control trial to use the coprological technique Mini-FLOTAC for assessing the dynamics of coccidia shedding following the administration of predatory fungi to animals. Since 2014, there has been an increment in studies reporting the use of Mini-FLOTAC in routine diagnosis of GI parasitic infections in several animal species, being unanimously considered a good alternative to the traditional McMaster technique (Cringoli et al. 2017; Maurelli et al. 2020) . Recent studies with Mini-FLOTAC in birds have revealed its usefulness for the diagnosis of coccidia and helminth infections, achieving sensitivities of up to 100% (Bortoluzzi et al. 2018; Coker et al. 2020; Daş et al. 2020; Lozano et al. 2021b,c). Since typical *in vivo* studies with predatory fungi always aim to compare control and test groups and obtain the respective oocyst/egg shedding or L3 reduction efficacies, the use of more sensitive coprological techniques like MF is of major importance, allowing to detect the real differences between groups and determine more statistically robust treatment efficacies.

Finally, one of the major concerns regarding the use of predatory fungi to control GI parasites is whether this kind of fungi can be harmful to birds, and during this trial no side

effects were recorded following the administration of *M. circinelloides* spores to peacocks. These outcomes are in accordance with extensive research performed with this fungal species in horses, ruminants, and dogs (Hernández et al. 2016; Voinot et al. 2021; Viña et al. 2022; Voinot et al. 2022), in which authors confirmed the lack of pathogenicity of *M. circinelloides* spores to these animal species.

Results from this research offer the opportunity to develop further studies in the topic of parasite biocontrol. It would be interesting if further *in vivo* trials could include: (i) a wider period of research (e.g., 6-12 months of spores' administrations and fecal collections), to evaluate the long-term efficacy of *M. circinelloides* in maintaining the coccidia fecal shedding at basal levels in birds, as extensively demonstrated for strongyles affecting domestic and exotic herbivores (Madeira de Carvalho et al. 2011; Vilela et al. 2016; Healey et al. 2018; Palomero et al. 2021); (ii) the separation of birds in test and control groups, and if possible according to their age (e.g., juveniles and adults), since younger birds are typically more prone to be infected by coccidia than adults (Shamim et al. 2015; Kaboudi et al. 2016), and thus parasiticide fungi efficacy might differ between age groups; (iii) the collection of blood samples for haematological analysis to conclude more about the safety of this fungus, as previously performed in horses and ruminants fed with *M. circinelloides* and *D. flagrans* spores (Vilela et al. 2012; Voinot et al. 2021; Voinot et al. 2022); (iv) a previous characterization of the different *Eimeria* species residing in the GI tract of the respective birds, to check if the parasiticide efficacy of *M. circinelloides* would differ between *Eimeria* species.

To our best knowledge, this is the first report of an *in vivo* trial performed in exotic birds kept under real ornamental conditions, using a native ovicidal fungus previously isolated from birds, and aiming to evaluate its efficacy in reducing coccidia parasitism. This study revealed that feeding *M. circinelloides* to peacocks did not result in any side effects for birds, while achieving significant reduction efficacies of 78-92% in their coccidia parasitism. Overall results allow to propose *M. circinelloides* as a good fungal candidate for an accurate, safe, and sustainable parasite control program in birds.

Ethics approval: This study was approved by the Ethical Committee for Research and Teaching of the Faculty of Veterinary Medicine – University of Lisbon (CEIE 019/2022) and also received the written consent of the bird collection owner. Fungal administrations and fecal samplings were performed without any direct manipulation of the animals, and the study followed the normal daily procedures of the peacock collection. All methods were carried out in accordance with relevant guidelines and regulations, as well as authors complied with the ARRIVE guidelines.

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CHAPTER VII – General discussion, conclusions, and future perspectives

1. General discussion

The control of animal GI parasitic infections based on the exclusive use of antiparasitic drugs, without any previous laboratorial diagnosis and integration with other complementary solutions like vaccination, pasture rotation or administration of probiotics, herbal extracts and predatory fungi, is still a common, but unsustainable, practice in domestic and exotic animal collections (Peek and Landman 2011; Hernández et al. 2018; Erez et al. 2023), contributing to the increasing problematic of drug resistance and bioaccumulation of pharmacological residues in the animals, soil and groundwaters (Abbas et al. 2011; Mooney et al. 2021; Martins et al. 2022). Moreover, the design of treatment protocols for exotic animals is often challenging for veterinarians, since it often consists of adapting the procedures used in domestic animals, which can result in low treatment efficacies and even have a toxic effect in animal hosts (Panayotova-Pencheva 2016).

With the aim to counter all these drawbacks associated with the exclusive and irrational use of antiparasitic drugs in domestic and exotic animals, the scientific community working in the fields of Veterinary Parasitology and Biological Control developed noteworthy efforts since the end of the 1990's decade, namely the search for novel parasite diagnosis and control solutions such as the establishment of the FLOTAC and Mini-FLOTAC methods (Cringoli et al. 2010; Cringoli et al. 2017), and the use of filamentous fungi with parasiticide activity as a green solution for reducing environmental contamination with eggs, oocysts or infective larvae, and thus prevent re-infections (Madeira de Carvalho et al. 2012; Arias et al. 2013c; Braga and Araújo 2014).

In the first phase of this Doctoral project, the implementation of the Mini-FLOTAC method allowed to identify different GI parasitic populations and fecal shedding in the four selected domestic and exotic bird collections. The identification of *Eimeria* spp. infections in laying hens and peacocks, and nematode infections in exotic birds, namely by *Capillaria* sp., *T. tenuis* and *S. pavonis* in peacocks, and by *L. douglassii* in ostriches and emus, corroborated previous available literature in Galliformes (Pérez Cordón et al. 2008; Carrera-Játiva et al. 2018; Ilić et al. 2018; Lozano et al. 2021a; Rosa de Almeida 2022; Zhang et al. 2022), and ratites' parasites (Jansson and Christensson 2000; Ponce Gordo et al. 2002; Ederli and Rodrigues de Oliveira 2015; Mariño-González et al. 2017), kept for production or ornamental purposes.

The detection of some of these parasites, namely of *Eimeria* spp. oocysts and *Capillaria* spp. eggs in Galliformes feces, are noteworthy results since these parasites can lead to moderate or severe diarrhoea episodes, sometimes with blood coagula, and may eventually lead to death, whereas subclinical diseases are often linked to decreased nutrient absorption

and consequently weight loss, anorexia, and prostration, and thus resulting in overall health and economic concerns for poultry farms and zoological institutions (Yazwinski and Tucker 2008; Lozano et al. 2019; Groves 2021; Mesa-Pineda et al. 2021). Besides, the higher coccidia shedding in peacock feces collected during Springtime, which is the breeding period of this bird species, highlights the importance of performing coprological analysis during this critical timeframe of birds life cycle, as chicks and juveniles are more prone to be infected by coccidia, and thus serve as natural disseminators of coccidia oocysts within the flock (Titilincu et al. 2009; Shamim et al. 2015; Kaboudi et al. 2016; Prakashbabu et al. 2017; Lolli et al. 2019). Moreover, the detection of *L. douglassii* eggs in ostriches and emus' feces is another relevant result, not only because it is by far the most pathogenic nematode in ostriches (McKenna 2005), but also confirms its cross-transmission among other ratite species like emus, which was suggested for the first time more than 20 years ago (Jansson and Christensson 2000). These results are also of practical application for zoo birds' management, since separating ratites' species in different areas may be a recommended approach to avoid the cross transmission of this pathogenic nematode.

Also, results from this study's phase allowed to identify different results for coccidia and helminth shedding obtained with the Mini-FLOTAC and McMaster methods. In the initial comparisons at field-level, parasites' shedding data did not differ between techniques in all bird species which is in accordance with previous research using both coprological techniques in the diagnosis of coccidia and nematode infections in poultry (Cringoli et al. 2017; Daş et al. 2020; Lozano et al. 2021b). Regarding the comparison of techniques' analytical results performed in the second part of this study's phase, it was possible to identify that the Mini-FLOTAC's protocol for exotic animals detected a higher and significant *L. douglassii* fecal shedding, in comparison with the other Mini-FLOTAC dilutions, while not differing from the McMaster method. Overall results suggested that the Mini-FLOTAC's protocol for exotic animals is the most suitable alternative to the McMaster method for the diagnosis of coccidia and helminth infections in birds, as reported in previous studies (Cringoli et al. 2017; Lozano et al. 2021b,c; Rosa de Almeida 2022), having this protocol been selected to be used in all further coprological diagnosis during the Doctoral program's timeframe.

The project's second phase focused on the isolation of parasiticide fungi from bird feces, with all isolates being identified morphologically as belonging to the genus *Mucor* and, after DNA extraction and sequencing of the rDNA ITS1-5.8S-ITS2 region, as *Mucor circinelloides* (FR1, FR2, FR2, SJ, SJ2 and QP2) and *Mucor lusitanicus* (QP1). These results represent the first isolation of this kind of fungi from bird feces, and suggest that the selected free-range chicken and peacock flocks, which were comprised by healthy birds, were naturally exposed to these fungal isolates in the respective outdoor areas, being in equilibrium within

the avian intestinal environment (Hernández et al. 2016; Vilela et al. 2016, 2018; Araújo et al. 2021; Palomero et al. 2021; Voinot et al. 2021; Viña et al. 2022). Also, results reveal that bird feces can be a suitable substrate for the isolation of filamentous fungi with predatory capacity, as demonstrated in previous studies performed in ruminants and horses kept in livestock farms, and carnivores from zoological institutions (Soto-Barrientos et al. 2011; Hernández et al. 2017; Ojeda-Robertos et al. 2019; Arroyo-Balán et al. 2021). Moreover, the isolation of these *Mucor* species from bird fresh fecal samples points out for fungal spores' tolerance to the different biochemical microenvironments of the avian GI tract, as previously demonstrated for the ovicidal fungus *P. chlamydosporia* (Valadão et al. 2020) and for the larvicidal fungi *D. flagrans* and *M. thaumasium* (Silva et al. 2017).

In this study's phase, it was possible to observe that all fungal isolates were capable of destroying *Eimeria* spp. oocysts, not only in WA medium but also when exposed to the fecal microenvironment, being in accordance with previous studies which also assessed the parasiticide activity of another *M. circinelloides* strain (CECT 20824) towards coccidia oocysts extracted from wild boar (Cruz 2015) and poultry feces (Lozano 2019), towards *Baylisascaris procyonis* eggs from raccoon feces (Cazapal-Monteiro et al. 2015), and towards *Calicophoron daubneyi* and *Parascaris equorum* eggs from bovine and equine feces, respectively (Hernández et al. 2017). Furthermore, *Mucor* isolates FR1 and QP2 had the best performance in destroying coccidia oocysts, achieving efficacies of 22% and 14%, respectively, while isolates FR3 and QP2 had the highest coccidiostatic effect, 85% for both. Also, *Mucor* coccidicidal efficacy revealed to be time-dependent, as significant results for FR1 and QP1 isolates were only obtained after 14 days of spores inoculation in bird feces. These results confirm that the ovicidal or coccidicidal activity developed by predatory fungi is a gradual process, characterized by hyphae migration towards parasite eggs or oocysts, adhesion to their shell, penetration, and full consumption of its organelles (Madeira de Carvalho et al. 2012; Braga and Araújo 2014; Cazapal-Monteiro et al. 2015). Since *M. circinelloides* isolate FR1 had the most promising results regarding its parasiticide activity towards coccidia oocysts, the project's last two phases included its characterization through several safety and efficacy tests, whose results are furtherly discussed.

The project's third phase was characterized by an *in vitro* assessment of the susceptibilities of all seven native *Mucor* isolates to several antiparasitic drugs commonly used in Avian Medicine. The design of this study's phase was based on the fact that: i) parasite biocontrol programs with predatory fungi often consist of deworming animals at the beginning of the assay and separating them in test and control groups, with fungal spores administrations being then performed until the end of the assay (Palomero et al. 2021; Rodrigues et al. 2021; Voinot et al. 2021); and ii) previous *in vitro* and *in vivo* studies performed with the larvicidal

fungi *D. flagrans* and *A. oligospora*, and ovicidal fungi *Paecilomyces* spp. have already reported their susceptibility to several anthelmintic drugs' concentrations, such as albendazole, thiabendazole, ivermectin, levamisole and closantel, with inhibitory effects on fungal germination being observed for ivermectin and albendazole concentrations as low as 0.08 mg/mL (Sanyal et al. 2004; Ferreira et al. 2016; Vieira et al. 2017; Zegbi et al. 2024).

Data recorded for all *Mucor* isolates were in contrast with the findings from previously mentioned studies, since none of them was susceptible to the tested antiparasitic drugs, independently of their concentration, which was observed by the maintenance of fungal germination capacity during the exposition to drugs in liquid medium, followed by inoculation in Sabouraud Agar. These results may be explained by an incapacity of antiparasitic drugs to bind to these *Mucor* isolates' β-tubulins, which are proteins essential for microtubules stabilization during fungal cell division, and thus the main target of most anti-mycotic Benzimidazole molecules (Sanyal et al. 2004; Zhou et al. 2016; Oliveira et al. 2020).

All procedures performed during this phase followed the standard M38 established by CLSI (CLSI Reference Method M38 2021), which is an internationally recognized organization in medical laboratory testing. Performing research based on standardized guidelines if of most importance for its reproducibility and to validate results, and this case some relevant improvements were even added to the methodologies, namely: i) to include a positive control (fungi and RPMI) in the microplate assay, to check if fungal isolates were capable of growing in liquid RPMI medium without the exposition to antiparasitic drugs, as described also by (Vieira et al. 2017); and ii) to include a complementary inoculation in Sabouraud Agar to check if fungi maintained their germination capacity in solid medium, after being exposed to the maximum antiparasitic drug concentration. Further studies in this topic could also include a quantitative analysis of fungal susceptibility to antiparasitic drugs, by measuring wells' absorbances in the microplate assay, as well as tracking colony radial growth in solid medium.

Results obtained in this study's phase are of practical importance by suggesting the compatibility of using *M. circinelloides* and *M. lusitanicus* in parallel with the most common avian antiparasitic drugs, in parasite biocontrol programs in domestic and exotic birds.

The study's fourth phase aimed to provide insights on the safe application of the native parasiticide fungi isolate *M. circinelloides* FR1, through the molecular and phenotypical characterization of its virulence profile, and the *in vivo* evaluation of the potential effect of its spores in laying hens and peacock GI commensal bacteria and fungi communities, being two crucial steps in the design of safe parasite biocontrol programs using this kind of fungi.

The analysis of *M. circinelloides* FR1 genome predicted the existence of six genes coding for virulence factors above the pre-defined threshold of 80% identity, which were

associated with different *Mucor* biochemical processes, namely an iron permease (FTR1) and iron receptors (FOB1 and FOB2), which play important roles in filamentous fungi iron uptake (Bullen et al. 2006; Lax et al. 2020), as well as ADP-ribosylation factors (ARFs) (ARF2, ARF6) and a Rho-like GTPase (CDC42), linked to hyphae morphogenesis and apical growth (Virag et al. 2007; Patiño-Medina et al. 2018; Lax et al. 2020). Moreover, phenotypic assays showed that this *Mucor* isolate was only positive for lecithinase activity, which is an enzyme that catalyses the hydrolysis of the phospholipid lecithin, a structural component of the animal cell membrane, and thus playing a potential role in host cell lysis (Ghannoum 2000).

Fecal microbiome sequencing results recorded during the *in vivo* trial in birds allowed to conclude that their feeding with *M. circinelloides* spores did not interfere with the overall bacterial and fungal communities residing in their GI tracts, with laying hens' feces being dominated by bacteria of the phylum Firmicutes, in which *Lactobacillus* was the most abundant genus, followed by the phylum Proteobacteria. Regarding peacocks' feces, they were mainly positive for bacteria of the phyla Firmicutes and Bacteroidetes, being all these results in accordance with previous research in Galliformes (Grond et al. 2018; Carrasco et al. 2019). Moreover, the fungal commensal community of both laying hens and peacocks' GI tracts was dominated by the phyla Ascomycota and Basidiomycota throughout the assays, as previously reported in chickens (Robinson et al. 2022).

Despite the overall results obtained in laying hens and peacocks suggesting that the administration of *M. circinelloides* spores did not interfere with overall maintenance of the bacteria and fungi fecal compositions, with *Lactobacillus* spp. and *Prevotella* spp. being the top bacterial genera in laying hens and peacocks, respectively, while the fungal genus *Kazachstania* was overall the most abundant in laying hens' feces, some fluctuations were still observed in microbial phyla and genera throughout the study, to which several biotic and abiotic factors may have contributed. Age has already been reported as an influencing factor in what concerns birds' GI microbial composition, with previous studies in chickens revealing that *Lactobacillus* spp. represents 25% of all caecal bacteria in chicks, with its abundance decreasing up to 100 times after six weeks (Gong et al. 2008; Kers et al. 2020; Bindari and Gerber 2022). Moreover, a previous study on birds' GI mycobiome reported the dominance of *Trichosporon* spp. in 14-days old broilers' caeca, which was overpassed by fungi of the genus *Microascus* at four weeks of age (Robinson et al. 2020). Besides, bacteria and fungi fecal communities have an intrinsic instability, with their taxa relative abundances depending on the samples' storage conditions (Bindari and Gerber 2022).

Feed is another abiotic factor which may have contributed to the detection of particular bacteria taxa during the trials. For example, the identification of the Bacteroidetes phylum as

one of the most abundant in peacocks' feces can be explained by the feed composition, in which the presence of corn grains and sunflower seeds may have stimulated the growth of bacteria from this phylum, who are known to be able to degrade complex plant polysaccharides (Grond et al. 2018).

During both trials, it was also possible to observe that the administration of *M. circinelloides* FR1 spores did not interfere with commensal gut bacteria and fungi alfa-diversities, which is essential for the maintenance of the intestinal homeostasis (Bindari and Gerber 2022; Chen et al. 2022), being these findings also complemented by the absence of significant variations in birds feces' appearance and consistency.

Finally, another interesting result obtained during these two trials was the absence of *M. circinelloides* sequences in feces from laying hens and peacocks, at the three sampling timepoints, which suggests that spores of this parasiticide fungus were not capable of colonizing the GI tract of both bird species, as previously reported for this fungal taxon in ruminants (Voinot et al. 2022). However, further anatomopathological analysis are needed to confirm this results in birds.

The *in vivo* trial performed in the study's fourth phase also included several parasitological analyses of the selected peacock collection to check the efficacy of *M. circinelloides* FR1 in reducing coccidia parasitism in these birds, being the fifth objective of the current Doctoral project. Parasitological results recorded during the *in vivo* trial allowed to conclude that *M. circinelloides* FR1 was capable of reducing the coccidia parasitism in peacocks, with significant reduction efficacies of 78-92% after 45-60 days of fungal feeding, respectively, and thus being time-dependent, as also observed in the *in vitro* trials performed at the current study's second phase. These results corroborated the existent literature in this topic, since other *in vivo* trials previously performed in several mammal species, namely dogs (Viña et al. 2022; Paz-Silva et al. 2023), ruminants (Voinot et al. 2021), non-human primates (Paz-Silva et al. 2023) and wapitis (Palomero et al. 2021), also reported that feeding *M. circinelloides* CECT 20824 spores to these animals only achieved significant helminth reduction efficacies after a minimum period of one month of fungal administrations.

Also, the identification of significant coccidia reductions during the Autumn season represents another interesting finding, by suggesting that *M. circinelloides* FR1 spores maintained their predatory activity at overall temperatures and rainfall between 11.5-21.3 °C and 0-26.4 mm, respectively, as previously observed for the *M. circinelloides* strain CECT 20824 (Cazapal-Monteiro et al. 2015; Cortiñas et al. 2015; Palomero et al. 2021) and other predatory fungi taxa like *D. flagrans*, *P. chlamydosporia* and *M. sinense*, which are all known

to maintain their germination capacity at temperatures lower than 20 °C (Wang et al. 2019; Oliveira et al. 2022).

Besides weather conditions, other factors may influence the parasite shedding dynamics in the scope of biocontrol trials using predatory fungi, such as changes in animal stocking. The end of the *in vivo* trial was characterized by two flood episodes recorded in the Lisbon district, which led to the need for temporary sheltering of peacocks. This modification in animal stocking potentially led to a higher exposition to feces contaminated with coccidia oocysts and stimulated re-infections (Attree et al. 2021), which thus may have contributed to the increase in coccidia shedding recorded during that timeframe.

Combined results of the final two phases allowed to conclude that feeding *M. circinelloides* FR1 to laying hens and peacocks did not interfere with their gut bacterial and fungal communities' overall compositions and alfa-diversities, while achieving significant reductions in coccidia parasitism in peacocks, without any side effect.

2. Conclusions

The current Doctoral project aimed to optimize two solutions for the integrated control of avian GI parasites, namely the implementation of Mini-FLOTAC in routine diagnosis of GI parasitism in domestic and exotic birds, and the isolation and characterization of native predatory fungi from bird feces and evaluation of their parasiticide potential towards coccidia oocysts.

Results of this project allow to conclude that:

i) The implementation of the Mini-FLOTAC method allowed to identify different parasitic populations in several Portuguese domestic and exotic bird collections, with highlight to *Eimeria* spp. oocysts, and *Capillaria* spp., *T. tenuis* and *S. pavonis* eggs in Galliformes feces, and *L. douglassii* infections in ratites. Moreover, this study also found that the Mini-FLOTAC protocol for exotic animals achieved identical results in comparison with the conventional McMaster method, and thus being proposed for a complete quantitative-qualitative diagnosis of avian GI parasitic infections in farms and zoological institutions;

ii) Bird feces are suitable biological substrates for the isolation of native predatory fungi. Besides, all predatory fungal isolates obtained from chickens and peacocks' feces were identified as belonging to the genus *Mucor*, with six isolates corresponding to *M. circinelloides* and one to *M. lusitanicus*. These isolates were capable of limiting coccidia sporulation and

even destroying the oocysts, with the most promising coccidicidal results being observed for the *M. circinelloides* isolate FR1;

iii) All native *Mucor* isolates tested did not reveal any *in vitro* susceptibility to the most common avian antiparasitic drugs, and thus suggesting for the first time that parasiticide fungi of the genus *Mucor* can be integrated in conventional birds' deworming programs, with their germination capacity not being affected by antiparasitic drugs' molecules;

iv) Although the genome of the native *M. circinelloides* FR1 isolate presented six predicted genes coding for several virulence factors, namely iron receptors, an iron permease, ADP-ribosylation factors and a GTPase, with the isolate also testing positive for the phenotypic expression of lecithinase, the integration of these results with the lack of its interference in the avian GI bacterial and fungal communities and diversity, as well as the absence of impact in birds' feces consistency and appearance, suggests that this parasiticide fungus does not offer any health risk to Galliformes;

v) The native *M. circinelloides* FR1 isolate was capable of significantly reducing the coccidia parasitism in a peacock collection, with a maximum efficacy of 92% after two months of exposure, having been to the author's best knowledge the first parasite biocontrol trial to include the administration of predatory fungi spores to birds kept under real ornamental conditions.

Considering the rising problematic of antiparasitic drug resistance in Avian Medicine, which offers several challenges for the prevention and treatment of parasitic infections in domestic, exotic, and wild captive birds, overall results from the current project provide several noteworthy contributes to the integrated control of avian GI parasites, by revealing that Mini-FLOTAC is a suitable method to be used in clinics, farms, and zoological institutions for an accurate, quick, non-expensive and "in-house" diagnosis of avian GI parasitism, as well by proposing the administration of *M. circinelloides* FR1 spores to birds as a more sustainable procedure for controlling birds' GI parasitism.

3. Future perspectives

Overall results obtained in every phase of the current Doctoral project offer the opportunity to perform further research in the field of integrated control of avian GI parasites.

Future research could target the isolation of native larvicidal fungi from bird feces and farm soil, and perform their *in vitro* testing against nematode infective larvae, such as from *T. tenuis* and *L. douglassii*. This would allow also to establish mixed fungal cultures (i.e., ovicidal and larvicidal fungi) for further *in vivo* trials. Also, it would be of most value to evaluate the parasiticide activity of the seven native *Mucor* isolates towards ascarids/heterakids and capillarids' eggs, since these are also parasites of clinical importance in avian medicine.

Regarding the analysis of predatory fungi susceptibility to antiparasitic drugs, further studies should also include the measuring of colony radial growth in different timepoints to further characterize the effect of each antiparasitic drug and respective concentrations in the germination rate of each fungal isolate.

More *in vivo* parasite biocontrol trials are currently being planned, since the author and his team aim to evaluate the efficacy of *M. circinelloides* FR1 and other native predatory fungi in reducing coccidia and helminth parasitism in other bird collections, and conclude more about its safety for these hosts. Future *in vivo* trials should cover a broader timeframe of analysis (i.e., 6 months – 1 year), to monitor the dynamics of oocyst/egg fecal shedding and check any potential interference of climate conditions in fungi parasiticide activity. Also, it would be interesting to collect blood samples for haematological analysis, and eventually perform anatomopathological analysis in birds receiving predatory spores, to conclude more about the safety of this parasite control approach for birds. All these tasks will allow to set up to basis to develop a biocontrol product with potential market application.

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