Date: Feb 02, 2023
To: "Adolfo Paz-Silva" adolfo.paz@usc.es
From: "Biological Control" support@elsevier.com
Subject: Decision on submission to Biological Control

ELSEVIER

Manuscript Number: BCON-D-22-00729R1

GELATIN TREATS CONTAINING FILAMENTOUS FUNGI TO PROMOTE SUSTAINABLE CONTROL OF HELMINTHS AMONG PETS AND ZOO ANIMALS

Dear Dr Paz-Silva,

Thank you for submitting your manuscript to Biological Control.

I am pleased to inform you that your manuscript has been accepted for publication.

My comments, and any reviewer comments, are below.
Your accepted manuscript will now be transferred to our production department. We will create a proof which you will be asked to check, and you will also be asked to complete a number of online forms required for publication. If we need additional information from you during the production process, we will contact you directly.

We appreciate you submitting your manuscript to Biological Control and hope you will consider us again for future submissions.

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Kind regards,
Raquel Campos-Herrera
Associate Editor

Biological Control

Editor and Reviewer comments:

Biological Control

GELATIN TREATS CONTAINING FILAMENTOUS FUNGI TO PROMOTE SUSTAINABLE CONTROL OF HELMINTHS AMONG PETS AND ZOO ANIMALS

--Manuscript Draft--

Manuscript Number:	BCON-D-22-00729R1
Article Type:	Research Paper
Keywords:	edible; prevention; nematodes; Mucor circinelloides; Duddingtonia flagrans
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Abstract:	The appropriateness of edible formulations of the parasiticide fungi Mucor circinelloides (ovicide) and Duddingtonia flagrans (larvicide) for the control of helminths affecting animals has been tested in two assays. Firstly, gelatin-based treats were manufactured with a blend of 3 – 6·107 chlamydospores each fungus and provided to three groups of six puppies each passing eggs of Toxocara canis, thrice a week for eight weeks. Feces collected at the end of this period were analyzed during four weeks to estimate the egg viability, the counts of infective eggs and the soil contamination index. The usefulness of the edible formulation comprised checking for the easiness of preparation and administration, the level of acceptance by the targeted animals and the expiration interval. A similar assay was conducted on a group of six captive baboons, but some refused to ingest the gelatins and others did it with difficulty. The second assay consisted of drying the gelatin treats and administering (thrice a week for eight weeks) to other two groups of six pups each infected by T. canis (different from those in assay I), and to six baboons captive in a zoo and passing eggs of Trichuris sp. (this probe on baboons was performed in triplicate at a four-month interval). Feces collected after eight weeks were examined for four weeks as mentioned previously. Viability of eggs of T. canis was significantly halved with the two edible formulations, and 44% reached the infective stage. In the feces of baboons taking dry gelatins, 50% eggs of Trichuris sp. remained viable, and 14% attained infectivity. Soil contamination by these nematodes was 20 and 5%, respectively. It was concluded that gelatin treats containing chlamydospores of a blend of M. circinelloides and D. flagrans offer a highly effective and practical formulation to develop strategies for the biological control of helminths affecting pets. In addition to retaining the high helminthicidal activity, the dehydration of the gelatins provides a very safe edible formulation, easy to store at room temperature and to give to companion animals and captive wild species, thus its administration is strongly advised to reduce the risk of infection by soil-transmitted helminths.
Suggested Reviewers:	Jackson V Araújo, PhD Senior Lecturer, Federal University of Vicosa Department of Animal Science jvictor@ufv.br His great and valuable contribution to the development of biological control of parasites
	Pedro Mendoza, PhD Head of Department, National Research Institute for Forestry Agriculture and Livestock

	Research Center for Veterinary Parasitology pedromdgives@yahoo.com High experience on isolation of parasiticide fungi
Response to Reviewers:	

Dear Editor,

I have the pleasure to submit a corrected version of the paper formerly entitled "**GELATIN TREATS CONTAINING FILAMENTOUS FUNGI TO PROMOTE SUSTAINABLE CONTROL OF HELMINTHS AMONG ANIMALS**" for consideration for publication in the *Biological Journal.*

The current manuscript has been corrected according to the reviewers comments and suggestions, which implies also a modification of the title to **GELATIN TREATS CONTAINING FILAMENTOUS FUNGI TO PROMOTE SUSTAINABLE CONTROL OF HELMINTHS AMONG PETS AND ZOO ANIMALS**. We have made a serious and important effort to take into consideration all the changes advised, but some of them remain due to several comments are not easy to understand by us. All this has been conveniently explained, but we are open to further modifications in order to gain understanding.

We thank sincerely all the proposal the reviewers did because the manuscript has been significantly enhanced because of the explanations added to the present version. We apologize if some points remain unclear, and, we are available to revise those questions pending of clarification, with the aim to offer a fully comprehensive paper to the audience.

Expecting to hear from you as soon as possible, best regards!

Adolfo Paz Silva, DVM, PhD, DipEVPC, EBVS®European Veterinary Specialist in Parasitology

Zoonoses and Public Health - Senior Lecturer

Faculty of Veterinary - University of Santiago de Compostela

SPAIN

Answer to the Reviewer 1:

Reviewer #1: I could see that a valuable work has been performed on the manuscript. I am glad that the authors accepted (almost) all suggestions. All small mistakes in the paper were corrected and the structure of the paper was improved.

We appreciate sincerely your kind comments, suggestions and indications, and are fully convinced that they contribute to improve significantly the quality and understanding of the manuscript.

However, I would like to propose some suggestions and small corrections to new mistakes occurred during this improvement process:

1) Please include the names of helminths in the graphical abstract as was done with the names of fungi.

Ans.: Thanks for the suggestion, you are right, and this information is interesting to understand the activity of the fungi.

2) On p. 3 line 10: replace "(Baron et al., 2019; Poveda et al., 2020, 2021)" by: "(Baron et al., 2019; Poveda et al., 2020; Poveda, 2021)" as the latter is the lone author of the article.

Ans.: This change has been done in the current manuscript.

3) On p. 3 lines 11-14: put the dates of the taxonomic publications of the fungi in brackets, as in the rest of the references, such as:

Pochonia chlamydosporia (Goddard) Zare & W. Gams (2001)

Mucor circinelloides Tiegh (1875)

Duddingtonia flagrans (Dudd.) R.C. Cooke (1969)
Monacrosporium thaumasium (Drechsler) de Hoog & Oorschot (1985)

Ans.: This indication has been applied in the amended version of the manuscript.

4) Include Figure 1 in the appropriate section (2.4. Study design).
Ans.: A mention to Fig. 1 has been added to Section 2.4.1.

5) On p. 7 line 19: include closing parenthesis: "baboons)".

Ans.: This indication has been applied in the amended version of the manuscript.

6) In section "2.6. Evaluation of the usefulness of edible products containing chlamydospores of parasiticidal fungi", I suggest referring to the levels (0, 1, 2 and 3) to be taken into account in the evaluation (as shown in Table 1).

Ans.: According to your comment, this section has been improved with the levels considered in the evaluation:

"The criteria considered for the evaluation of the appropriateness of edible formulations with chlamydospores of *M. circinelloides* and *D. flagrans* for the control of helminths among animals consisted of easiness of manufacturing and administration (0: No problems; 1: Easy; 2: Medium difficulty; 3: Difficult), acceptance level by the target animals (0: Immediate (< 5 s); 1: 5 - 20 s; 2: 21 – 60 s; 3: Rejected) and the expiration interval (0: < 7 days; 1: < 15 days; 2: < 30 days; 3: > 30 days)."

7) On page 12, line 16: replace "TP" with "PRG".

Ans.: Thanks for your correction, which has been applied to the manuscript.

8) On p. 13, line 11: correct "66% and 96 - 49%" by "48% and 95 - 49%".

Ans.: We are sorry for this mistake, which has been amended.

9) On p. 13, line 13: correct "42 ± 6% were obtained in CRG, by 41 ± 12% in CRDG" to "50% were obtained in PRG, by 48% in PRDG".

Ans.: We are sorry for this mistake, which has been amended.

10) In Table 4: Identify CP1 and CP2 as they appear on p. 13 line 15.

Ans.: This lack of information has been solved in the Table 4.

11) On p. 17 line 4: correct "(Goosens et al., 2005)" to "(Goossens et al., 2005)".

Ans.: The reference has been amended.

12) In the Results, reference is made to tables but not to figures (Fig. 3a and 3b and Fig. 4). Include references where appropriate.

Ans.: We are sorry, but these figures had been referenced in the previous manuscript, and in the actual version they can be found in page 12, lines 13 and 16 (Fig. 2a); page 13, line 8 (Fig. 2b); page 14, line 4 (Fig. 3).

13) On p. 24 Line 23: correct "Ortiz Pérez, D.O., Sánchez Muñoz, B., ..." by Ortiz-Pérez, D.O., Sánchez Muñoz, B., ..." (add hyphen).

Ans.: Thanks for your kind suggestion, but this change has not been included due to there was a mistake when citing this author, then it should be Ortiz Pérez et al. Accordingly, this error has been amended in the manuscript.

14) On p. 26 Line 15: insert reference:
Tiegh P. E. L. Van (1875). Mucor circinelloides. Annls Sci. Nat., Bot., ser. 6 1: 94.

Ans.: We apologize for forgetting to include this reference. This error has been fixed.

15) Finally, I consider that Figure 2 is not necessary as the appearance of the treats (gelatinous and dehydrated) is already shown in the graphical abstract.

Ans.: We agree with you, and this figure has been deleted in the present version of the manuscript.

Thanks for your time, effort, and kindness in reviewing our manuscript, which has been significantly improved.

Answer to the Reviewer 2:

Reviewer #2: This study assessed the use of the combination of two helminthophagous fungi Duddingtonia flagrans and Mucor circinelloides for controlling helminths in two animal species puppies and baboons. In general this is a very interesting and original study that shows important results in reducing the egg viability the counts of infective eggs and the soil contamination index attributed to consumption of gelatin (either in wet or dry material) containing spores of both fungi. I find this manuscript as an important contribution to the use of two natural antagonists of parasitic nematodes in these two animal species. However, many deficiencies were identified that make unsuitable this manuscript to publish; unless authors are able to make major changes to their manuscript to achieve a polished final version.

Ans.: We thank you for your time, effort and kindness in reviewing our manuscript, and we will try to incorporate all the modifications you have pointed out, so that it complies with the necessary requirements for publication.

My comments and suggestions to this manuscript are the following ones:

Title:
The title is too wide and vague in relation to the term "Animals". This term does not say too much. It does not reflex the real content of this research. I think it should mention that the study was performed using puppies and baboons. Or perhaps "in companion pets and Zoo animals", and in material and methods clarify that these two animal species were used as models of study. The title has to be more descriptive according to the essence of the study.

*Ans.: According to your suggestion, the previous title "***GELATIN TREATS CONTAINING FILAMENTOUS FUNGI TO PROMOTE SUSTAINABLE CONTROL OF HELMINTHS AMONG ANIMALS***" has been changed to "***GELATIN TREATS CONTAINING FILAMENTOUS FUNGI TO PROMOTE SUSTAINABLE CONTROL OF HELMINTHS AMONG PETS AND ZOO ANIMALS***", due to pets appears to include companions also.*

Highlights

Last issue: Gelatins and dry gelatins are useful in halving the risk of helminthoses by 50%

I think the term "halving" means reducing to half, itself; then, "by 50%" seems to be a pleonasm.
Ans.: You are right, and "by 50%" has been deleted.

In Page 8, issue 2.2.2. Assay I: Testing edible gelatin foods

Did you use a positive control group with an anthelmintic drug of common use and a negative control? It so, it would be great!

Ans.: We appreciate your indications, but it is not possible to apply them in the zoological park where the current investigation has been carried out, due to the management conditions of the animals in each case.

…six 3 mo old, replace it with …six 3 month old,

Ans.: Thanks for your suggestion, which has been considered throughout the corrected version of the manuscript.

Same page, lines 9 to 12

This paragraph is a bit unclear. Could you be more descriptive? at what treatments correspond everyone of the three groups? For instance, group 1 = non-treated; group 2 = treated group and group 3 = ?

Please, describe your experimental groups in the easiest and clearest way you can.

Ans.: We consider that you are refereeing to Section 2.4.1. because the control groups are defined there. We are sorry, but we are not able to understand your question. The three groups of puppies were managed equally: control samples were those taken prior to the administration of chlamydospores formulated as gelatins or dry gelatins, and treated samples one week after the animals took the edible formulations of chlamydospores.

In page 8, line 18, Issue 2.4.3 Assay II

Why did you use two groups in this assay and three groups in the previous one?
Ans.: Because of breeding of dogs did not occur throughout the year, and the kennel takes a break.

Same page, line 19

Mixing puppies and baboons in one experiment cause a bit confusion, please, try to be more simple to achieve a much easier and understandable redaction.
It would be easier to separate the experiment by animal species and to describe how many animals were used in total and divided in 3 or 2 groups of "n" animals each, and the three groups were treated as follows: ...etc.

Ans.: Thanks for your interesting suggestion, but the main objective in the current investigation consisted of evaluating the usefulness of two new formulations containing chlamydospores of two helminthicide fungi. Your indication is very helpful, but it seems more appropriate to reach information on the animal species, and our purpose is to stress the usefulness of the edible formulations.

Page 11, Issue 3.2.1. Appropriateness of edible formulations

Where in Materials and Method section did you describe the method to assess the level of difficulty to prepare the edible formulations and the difficulty to be administered or accepted by animals?

A well descriptive section about the procedure to assess the properties of edible formulations in Material & Method section it would be great!

what criterion did you use to differentiate the difficulty to prepare the gelatins?

I think this evaluation was a bit too subjective. What is "easy" what is "hard"? You have to describe these criteria in a sub-section in Materials and Methods. An additional reference for this assessment (if available) it would be great.

Ans.: Thanks for your interesting proposal. Unfortunately, we were not able to find some references to assess the properties of edible formulations. We have tried to add more explanation to this item, and this section has been changed as follows:

"The criteria considered for the evaluation of the appropriateness of edible formulations with chlamydospores of *M. circinelloides* and *D. flagrans* for the control of helminths among animals consisted of:

- Easiness of manufacturing:

  0: No problems - Reading and interpreting the protocol is very easy and, together with the elaboration, takes < 5 min.

  1: Easy - Reading and interpreting the protocol is easy and, together with the elaboration, requires 5 - 10 min.

  2: Medium difficulty - Reading and interpreting the protocol is somewhat difficult, and together with the elaboration takes 10 - 20 min.

  3: Difficult - Reading and interpreting the protocol is difficult, and together with the elaboration it requires > 20 minutes.

- Easiness of administration:

  0: No problems - Treats are placed in the feeder along with cereal or feed.

  1: Easy - The treats are chopped up and placed in the feeder.

2: Medium difficulty - It is necessary to tempt the animals with cereals or feed to get them to approach the feeders, and at this time treats are provided.

3: Difficult - It is needed to physically separate or even immobilize the animals.

- Acceptance level by the target animals:

0: Immediate (< 5 s).

1: High (5 - 20 s).

2: Moderate (21 – 60 s).

3: Rejected.

- Expiration interval:

0: < 7 days.

1: < 15 days.

2: < 30 days.

3: > 30 days."

Page 14 line 21

Regarding the cite: Luangsa-ard.
Is this a proper cite?
If so, should be Luangsa-ard et al., 2022.
If you mean that these were the authors describing for the first time Purpureocillium lilacinum then delete it from the list of references or correct it!

Same page, line 24 about F.B. Rocha, P. Chaverri & Jaklitsch 2015
Same situation! Please, correct it!

Page 15, line 1
Schroers, Samuels, Seifert & W. Gams 1999
is this a cite? if so, please correct it as Schroers et al., 1999;
or is only the authors than described this species for the first time? like F.B. Rocha. P. Chaverri & Kaklistsch 2015 for Trichoderma then correct it please.

Ans.: These references were included at the request of another reviewer, to indicate the first description of each species of fungus. We apologize that the format was not appropriate. In this new version, the years have been added between brackets, according to the Editor indication.

Page 17 line 2 about Moudgil et al., 2018
This cite is not included into the list of references
I found Moudgil and Singla, 2018.
This cite will have to be corrected

Ans.: Thanks for your comment. The reference has been included in the new version of the manuscript.

Important comment!

I think making a pool of feces by homogenizing the three samples diminish the robustness of your results. Why you did not analyze the three samples in an individual way. This would allow to get information about three individual animals and it would get robust results to ensure a greater statistic validity.

Ans.: This is a very interesting proposal, but the current procedure was followed considering that the eggs may not be uniformly distributed in the feces, so the results could depend on the portion taken for examination. The three samples corresponding to each animal were analyzed individually first to ensure the presence of helminth eggs, then they were mixed to obtain a homogeneous sample to examine, and in this way, to evaluate the effect of the fungi on the eggs. Your suggestion would be more appropriate if the eggs were distributed homogeneously.

In summary,
This is an important study that deserves to be published.
Although, quite a lot improvement has to be done before being accepted.

My decision is: Major Revision

Thanks for your time, effort, and kindness in reviewing our manuscript, which has been significantly improved. We hope this new version fulfils the requisites for its publication, though suggestions and comments are welcome for the purpose to enhance both quality and understanding.

GELATIN TREATS CONTAINING FILAMENTOUS FUNGI TO PROMOTE SUSTAINABLE CONTROL OF HELMINTHS AMONG ~~ANIMALS~~PETS AND ZOO ANIMALS

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Field Code Changed

Abstract

The appropriateness of edible formulations of the parasiticide fungi *Mucor circinelloides* (ovicide) and *Duddingtonia flagrans* (larvicide) for the control of helminths affecting animals has been tested in two assays. Firstly, gelatin-based treats were manufactured with a blend of $3 – 6 \cdot 10^7$ chlamydospores each fungus and provided to three groups of six puppies each passing eggs of *Toxocara canis*, thrice a week for eight weeks. Feces collected at the end of this period were analyzed during four weeks to estimate the egg viability, the counts of infective eggs and the soil contamination index. The usefulness of the edible formulation comprised checking for the easiness of preparation and administration, the level of acceptance by the targeted animals and the expiration interval. A similar assay was conducted on a group of six captive baboons, but some refused to ingest the gelatins and others did it with difficulty. The second

assay consisted of drying the gelatin treats and administering (thrice a week for eight weeks) to other two groups of six pups each infected by *T. canis* (different from those in assay I), and to six baboons captive in a zoo and passing eggs of *Trichuris* sp. (this probe on baboons was performed in triplicate at a four-month interval). Feces collected after eight weeks were examined for four weeks as mentioned previously.

Viability of eggs of *T. canis* was significantly halved with the two edible formulations, and 44% reached the infective stage. In the feces of baboons taking dry gelatins, 50% eggs of *Trichuris* sp. remained viable, and 14% attained infectivity. Soil contamination by these nematodes was 20 and 5%, respectively. It was concluded that gelatin treats containing chlamydospores of a blend of *M. circinelloides* and *D. flagrans* offer a highly effective and practical formulation to develop strategies for the biological control of helminths affecting pets. In addition to retaining the high helminthicidal activity, the dehydration of the gelatins provides a very safe edible formulation, easy to store at room temperature and to give to companion animals and captive wild species, thus its administration is strongly advised to reduce the risk of infection by soil-transmitted helminths.

Keywords: edible, prevention, nematodes, *Mucor circinelloides*, *Duddingtonia flagrans*

1. Introduction

An important number of parasites develop in the soil until attaining the stages that can infect animal species and even humans. Nearly all grazing animals are exposed to helminths, gastrointestinal nematodes and trematodes mainly (Charlier et al., 2016), while more than 50% pets are infected (D'ambroso et al., 2022; Safarov et al., 2022),

thus a great economic effort is made to minimize the damage through the administration of effective treatments. It has been estimated that more than 400 million € are spent yearly on anthelmintics in the European Union (Morgan et al., 2013), but this strategy reveals to be insufficient to prevent new frequent infections, so that regular deworming is necessary, which can result in increased environmental contamination by chemical residues or the appearance of strains resistant to anthelmintics (anthelmintic resistance) (Vercruysse et al., 2018).

Among the alternatives investigated to helminth control practices based on anthelmintics only, application of saprophytic filamentous fungi (SFF) as biological control agents against plant-parasitic nematodes and insect pests has been successfully carried out for decades (Baron et al., 2019; Poveda et al., 2020; Poveda, 2021). Some fungal species, in particular *Pochonia chlamydosporia* (Goddard) Zare & W. Gams (2001) and *Mucor circinelloides* Tiegh (1875), are antagonists of eggs of trematodes or gastrointestinal nematodes, whereas *Duddingtonia flagrans* (Dudd.) R.C. Cooke (1969) or *Monacrosporium thaumasium* (Drechsler) de Hoog & Oorschot (1985) develop traps in their mycelium to catch larvae of strongyles parasitizing animals (Canhão-Dias et al., 2020; Araújo et al., 2021). Moreover, it has been demonstrated the lack of adverse effects of *D. flagrans* on soil nematodes (Saumell et al., 2016), together with the innocuousness of daily administration of nutritional pellets containing chlamydospores of *M. circinelloides* and *D. flagrans* ~~during two years~~ to heifers for two years (Voinot et al., 2021b).

Nevertheless, the use of SFF has slowed down in domestic, sylvatic, or captive wild species. In addition to regulatory issues, an important concern relies on proper ways to ensure that spores are correctly given to the animals. Most recent efforts have been focused on formulations that improve their oral administration to livestock, and a

significant part of the trials consisted of providing them in grain seeds (Chandrawathani et al., 2002; Paraud et al., 2005), cereal flour (Healey et al., 2018; Braga et al., 2020; de Oliveira et al., 2021), and in a lesser extent, nutritional pellets (Aguilar-Marcelino et al., 2017; Hernández et al., 2018c; Voinot et al., 2020), energy blocks (Sagüés et al., 2011) or milled cereal soaked with aqueous solutions (Palomero et al., 2020; Voinot et al., 2021a). So far, only two commercial formulations containing chlamydospores of *D. flagrans* are available, Bioworma® and Bioverm® (Healey et al., 2018; Fausto et al., 2021).

According to the management of the animals, notable differences exist especially regarding their nutrition. Whereas livestock species are provided regularly (daily) with feed supplementation, this is not the case for others as horses or certain wild individuals captive in zoological parks. For the purpose to improve the potential for biological control of animal parasites through orally delivery of SFF, edible gelatin-based treats were elaborated with chlamydospores of a blend of *M. circinelloides* and *D. flagrans*, then tested on puppies, and baboons captive in a zoological garden. A second trial consisted of investigating the helpfulness of freeze-drying the gelatin foods.

2. Material and methods

2.1. Parasiticide fungi

Different strains of soil saprophytic fungi (SSF) were isolated from feces of livestock and captive animals, and soil samples also, then deposited at the Spanish Type Culture Collection (CECT, Valencia, Spain) (Arias et al., 2013; Hernández et al., 2017). In the present study, the ovicidal species *M. circinelloides* (CECT 20824) and the larvicidal *D. flagrans* (CECT 20823) were co-cultured in a submerged medium (COPFr) at RT with

the aim to get elevated numbers of chlamydospores (Hernández et al., 2018a). The medium was composed by water, wheat and mineral salts (Arias et al., 2013).

2.2. Formulation of parasiticidal fungi as edible gelatin

2.2.1. Gelatin

Two types of gelatin-based foods were prepared in the Lab, one was obtained by mixing gelatin (Juncá Gelatines S.L., Spain), honey and liquid medium containing $\approx 3 - 5 \cdot 10^6$ chlamydospores of both fungi / mL, *M. circinelloides* and *D. flagrans*. The concentration was estimated by means of a Neubauer chamber (Ojeda-Robertos et al., 2008), using a total of 10 25-µL aliquots / L medium.

The blend was completely homogenized, heated under microwave for a brief period, placed into silicone molds and finally quenched at 4-6 ºC to enhance gelling. This formulation enabled that each food provided a dosage of $3 - 6 \cdot 10^7$ chlamydospores of each parasiticide fungus. (Additional information can not be provided due to it is pending of registration).

To assess if the numbers of fungal chlamydospores did vary significantly through the gelification process, each month for a six-month period a quantity of 30 gelatins were each soaked into 10 mL water at 37ºC and stirred until completely melted. A total of five 25 µL of each blend was examined under an optical microscope at 20X by using a Neubauer chamber and counts indicated as numbers of chlamydospores / mL.

The reduction of the numbers of chlamydospores during the gelification was estimated according to the formula:

$$\text{Reduction gelification (\%)} = [1 - (\text{Counts}_{\mathbf{GELATIN}} / \text{Counts}_{\mathbf{SUBMERGED\ CULTURE}})] \times 100$$

2.2.2. Dry gelatins

In order to increase the gelatin shelf life, part of the pieces was frozen at -35ºC and then water was removed by freeze-drying. The final products were packed into reusable plastic bags and stored at RT until administrated.

Examination of the dried gelatins was conducted to ascertain if the procedure might decrease the numbers of chlamydospores. Every month for six months, 30 gelatins were freeze-dried, then rehydrated by submerging each one into 10 mL water at 37ºC and stirred until complete melted. Finally, five 25 µL-aliquots of each solution were taken, and spores counted in a Neubauer chamber under an optical microscope at 20X and expressed as numbers per mL.

The reduction of the numbers of chlamydospores during the drying process was estimated as follows:

$$\text{Reduction drying (\%)} = [1 - (\text{Counts}_{\mathbf{DRY\ GELATIN}} / \text{Counts}_{\mathbf{GELATIN}})] \times 100$$

Finally, for the purpose to determine if storage might influence the numbers of chlamydospores, a total of 160 gelatins were freeze-dried, then 20 analyzed monthly for eight months as pointed above, and results shown as numbers of chlamydospores / mL.

2.3. Animals

Evaluation of the two edible foods prepared with a blend of chlamydospores of *M. circinelloides* and *D. flagrans* was carried out in 3-month old Griffon bleu de Gascogne puppies (‘‘Soñar’’ commercial breeding kennel, 42º57’32.689’’ N, 7º40’40.78’’ W; Lugo, NW Spain), and adult baboons (*Papio hamadryas*) captive in the “Marcelle

Zoological Park" (43°4′14.71″ N, 7°37′53.50″ W; Outeiro de Rei, NW Spain) (Palomero et al., 2020). First, infection by helminths was analyzed by means of the McMaster coprological technique with saturated saline solution (ρ = 1.2 g / dL), which makes possible the identification of protozoan cysts / oocysts and eggs of cestodes and nematodes (Hernández et al., 2018a; Voinot et al., 2021a). Briefly, three grams of each sample were weighed and placed in one bottle, then emulsified in 42 mL of water and shaken vigorously until completely broken down. This emulsion was filtered through a 150 µm pore diameter sieve and passed to two 15 mL glass tubes. After centrifuging at 1500 rpm for 10 min, the supernatant was discarded and the sediment homogenized in saturated saline solution, then aliquots taken carefully from the superior part of the tubes to fill a McMaster chamber, and finally observed under a light microscope at 10X (Viña et al., 2020). This procedure was repeated two times for each sample, and results expressed as eggs per gram of feces (EPG).

2.4. Design of study

2.4.1. Control groups

Because of the impossibility of keeping captive animals in control and treated groups at the same time, at the starting of the study three fecal samples were collected per each individual directly from the soil~~,~~ and homogenized by mixing vigorously (Fig. 1). Feces of puppies were labelled as CP (control puppies), and those belonging to baboons as CB (control baboons). Three grams of each mixture were placed into eight plastic containers provided with four holes in the side walls for allowing proper aeration, then covered with a plastic lid and finally brought to a meadow for a 4-week period (Hernández et al., 2018b). The contents of two containers per animal were examined

weekly by means of a modified McMaster technique (Hernández et al., 2018b; Voinot et al., 2021a). This modification consisted of that after centrifuging at 1500 rpm for 10 min, the sediment was homogenized in saturated saline solution, then aliquots of 100 µL were taken carefully from the top of the tubes, placed between glass slides and coverslips, and examined under a light microscope at 10X and 20X until a minimum of 100 eggs was analyzed (Viña et al., 2020). This procedure was repeated two times for each sample. It is important to note that identical numbers of *control* and *treated* groups were observed in each assay.

2.4.2. Assay I: Testing edible gelatin foods

Three groups of six 3-month old Griffon bleu de Gascogne puppies were given one gelatin with fungal spores thrice a week for eight weeks (PRG, puppies receiving gelatins). After this period, three fecal samples were collected per each animal directly from the soil, homogenized by mixing vigorously and proceeded as mentioned above (2.4.1.).

The same designing was performed on baboons, but two of them refused to ingest these treats, and irregular and infrequent intake was recorded among the others. Accordingly, the assay was abandoned.

2.4.3. Assay II: Testing edible dried gelatin foods

Two groups of six Griffon bleu de Gascogne puppies (3-month old) (different to those in Assay I), and one group of six adult baboons were used in the second assay for testing edible dried gelatins ~~(Fig. 2)~~. As mentioned in 2.4.1., control fecal samples were

prepared at the starting of the assay. Later, all animals were given one dry gelatin treat, thrice a week for an interval of eight weeks, and at the end of this period three fecal samples were collected (per each animal) directly from the soil and homogenized by mixing vigorously. Next step consisted of placing three grams of each mixture into eight plastic containers and labelling them as PRDG (puppies receiving dried gelatins) or BRDG (baboons receiving dried gelatins), then continuing as stated prior (2.4.1.). Given the impossibility of having a greater number of baboons, this assay was performed two more times, at intervals of 4 months.

The experimental design was approved by the Ethical committee of the University of Santiago de Compostela (Spain; protocol number CTM2015-65954b) and complied with the Directive 2010 / 63 / EU.

2.5. Evaluation of the parasiticide effect

Eggs of helminths observed by means of the modified McMaster test were classified as enduringly unviable when presenting loss of shell continuity, contraction or rupture, cytoplasmic vacuolization, and abnormal morphology of inner larvae (Cazapal-Monteiro et al., 2015; Hernández et al., 2018b). Likewise, the viable eggs were classified into infective when presenting a L2 larva inside for the ascarids, and a L1 for the trichurids.

The reduction of the viability (VR) and the Soil Contamination Index (SCI) were calculated according to Viña et al. (2020):

$$VR\ (\%) = [(100 - \%\ \text{Viable EPG}_{\text{Week}X})/\%\ \text{Viable EPG}_{\text{Week}0}] \times 100$$

$$SCI\ (\%) = (\%\ \text{Viable Eggs} \times \%\ \text{Eggs with L2}) / 100$$

2.6. Evaluation of the usefulness of edible products containing chlamydospores of parasiticidal fungi

The criteria considered for the evaluation of the appropriateness of edible formulations with chlamydospores of *M. circinelloides* and *D. flagrans* for the control of helminths among animals consisted of:

- E easiness of ~~preparation and administration~~manufacturing:
  - 0: No problems - Reading and interpreting the protocol is very easy and, together with the elaboration, takes < 5 min.
  - 1: Easy - Reading and interpreting the protocol is easy and, together with the elaboration, requires 5 - 10 min.
  - 2: Medium difficulty - Reading and interpreting the protocol is somewhat difficult, and together with the elaboration takes 10 - 20 min.
  - 3: Difficult - Reading and interpreting the protocol is difficult, and together with the elaboration it requires > 20 minutes.
- Easiness of a~~and a~~dministration:
  - ~~(~~0: No problems - Treats are placed in the feeder along with cereal or feed.
  - 1: Easy - The treats are chopped up and placed in the feeder.
  - 2: Medium difficulty - It is necessary to tempt the animals with cereals or feed to get them to approach the feeders, and at this time treats are provided.
  - 3: Difficult - It is needed to physically separate or even immobilize the animals.
- ~~A; 1: Easy; 2: Medium difficulty; 3: Difficult),~~ acceptance level by the target animals:
  - ~~(~~0: Immediate (< 5 s).

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- ~~1~~: High (5 - 20 s).
- ~~2~~: Moderate (21 – 60 s).
- ~~3~~: Rejected.

- ~~) and the~~ Eexpiration interval:
  - ~~(~~0: < 7 days.
  - ~~1~~: < 15 days.
  - ~~2~~: < 30 days.
  - ~~3~~: > 30 days~~)~~.

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2.7. Statistical analysis

Distribution of data collected in the current research was analyzed first for their normal distribution, by means of the Kolmogorov-Smirnov and the Levene's tests. Most data were not normally distributed, hence the Mann-Whitney U and Kruskal-Wallis non-parametric probes were run.

All tests were carried out by using the statistical package SPSS, version 20 (IBM SPSS Inc., Chicago, IL, USA), at a significance level of $P < 0.05$.

Due to differences between the replicates of each assay were not observed, these data were pooled and analyzed as a single set.

3. Results

3.1. Identification of parasites in coprological analyses

Eggs of the gastrointestinal nematodes *T. canis* and of *Trichuris* sp. were identified in the feces of puppies and baboons, respectively.

3.2. Analysis of edible formulations with chlamydospores of *M. circinelloides* and *D. flagrans*

3.2.1. Appropriateness of edible formulations

Preparation of gelatins with chlamydospores of parasiticidal fungi resulted very simple (Table 1), the administration was very easy and these foods well accepted (puppies took them in less than five seconds) for treats less than 15 days old. After 21 days, many of the gelatins had lost their consistency, making it difficult to administer. In contrast, baboons did not accept the gelatins as willingly, as some refused, and others did so with difficulty and after a period (more than 10 minutes).

Dry gelatins were very easy to prepare also (Table 1) and preserved at RT for more than 8 months, without any sign of alteration. Administration was very simple and well accepted by pups (took them in less than five seconds), but occasionally it was needed to break them into short pieces and mix with fruit, cereal grains or seeds for baboons.

3.2.2. Counts of chlamydospores in edible foods

After the elaboration of gelatins, non-significant variations were recorded in the numbers of chlamydospores of *M. circinelloides* or *D. flagrans* with respect to the initial quantities in the submerged culture (Table 2), and an average reduction of 1.78 ± 0.83% (Z= -1.239, *P*= 0.215) and 1.89 ± 0.73% (Z= -1.214, *P*= 0.225) was respectively obtained.

Changes in the counts of chlamydospores of both parasiticide fungi after the gelatins were dried are summarized in Table 2. A significant reduction in the numbers was

achieved, with average values of 8.87 ± 2.81% (Z= -5.608, *P*= 0.001) for *M. circinelloides* and 9.99 ± 2.69% (Z= -5.387, *P*= 0.001) for *D. flagrans*.

Table 3 contains data on the numbers of chlamydospores during an 8-month storage period. Non-significant variations were observed with the time elapsed since drying (*P* > 0.05).

3.3. Assay I: gelatin-based foods given to puppies

As drawn in Table 4, viability of eggs of *T. canis* was 96 ± 2% at the beginning of the study, and four weeks later was reduced to 90 ± 4% in the control samples (CP), by 48 ± 9% in the PRG (puppies receiving gelatins), which represents a VR of 7% and 50%, respectively. Statistical differences between the two groups were demonstrated (Z= -6.363, *P*= 0.001).

Infective eggs of *T. canis* (containing a L2) appeared from the 1st week (wk) (Fig. ~~3a~~2a) in CP, and values around 80% were obtained at the end of the study. In PRG, eggs with L2 were first observed since the 3rd wk, with the highest percentages (44%) by the end of the assay (Fig. ~~3b~~2a) (Z= -3.447, *P*= 0.001).

The values of the Soil Contamination Index (SCI) increased along the study until 70% (4th wk) in CP, by 20% in ~~TP~~ PRG (Table 4).

3.4. Assay II: Evaluation of dehydrated gelatin-based pieces

3.4.1. In puppies

The viability of eggs of *T. canis* in the control puppies (CP) decreased slightly (2 – 7%) for one month, with values near to 90% (Table 4). In the puppies receiving dehydrated gelatins containing chlamydospores of *M. circinelloides* and *D. flagrans* (PRDG), a significant reduction between the 2nd wk (VR= 13%) and the 4th wk (VR= 48%) was recorded (Z= -5.666, *P*= 0.001).

The presence of infective eggs (with L2) in the controls (CP) was recorded since the 2nd wk, the values increased fast and the highest percentages were obtained at the end of the study (81%) (Fig. ~~3b~~2b). In the PRDG, eggs with a L2 were observed after three weeks, and found in 44% at the end of the assay (Z= -2.595, *P*= 0.009).

Concerning the SCI, values around 73% and 20% were obtained at the end of the study in CP and PRDG, respectively (Table 4).

Comparison between the parasiticide effect reached by providing puppies gelatins or dry gelatins is summarized in Table 4. Viable eggs of *T. canis* oscillated between 96 – ~~66~~48% and ~~96~~ 95 – 49% in puppies receiving gelatins or dried gelatins, respectively, with reduction values about 50% (Z= -0.092, *P*= 0.927). Regarding the infective eggs, percentages of ~~42 ± 6~~50% were obtained in CRG, by ~~41 ± 12~~48% in CRDG, with a SCI of 20% in both groups (Z= -0.455, *P*= 0.649). Differences between results obtained in the respective control groups (CP1 and CP2) were not significant (Z= -0.083, *P*= 0.934) and (Z= -0.753, *P*= 0.451).

3.4.2. In baboons

Viability of eggs of *Trichuris* sp. in the control feces of baboons (CB) maintained at 89 – 97% (Table 4), while in BRDG reduced to 44% after four weeks of exposure to the

parasiticide fungi. After one month, the values of VR were 8% in CB and 54% in BRDG (Z= -6.667, *P*= 0.001).

Infective eggs (containing a L1 inside) were first observed since the 3rd wk in the controls (CB), and percentages close to 30% attained at the 4th wk (Figs. 43). In the feces exposed to the chlamydospores (BRDG), 14% of the eggs had a L1 at the end of the study (Z= -3.777, *P*= 0.001).

After four weeks, the SCI values for the control baboons (CB) were 26%, and 5% in those receiving dry gelatins (BRDG) (Table 4).

Discussion

The main advantages of using certain saprophytic filamentous fungi for the control of helminths affecting animal species are centered on the possibility of reducing the risk of infection to prevent damage to hosts and minimize the need for frequent application of chemical dewormers (Ortiz-Pérez et al., 2017; Mendoza et al., 2018). In the current investigation, a new way of administration of SFF consisting of gelatin-based treats enriched with chlamydospores of *M. circinelloides* and *D. flagrans* given to pups infected by *T. canis* and baboons by *Trichuris* sp., has been tried. These foods were easy to prepare and puppies took them quickly (in less than five minutes). Four weeks later, viability of eggs of the gastrointestinal nematode *T. canis* in fecal samples was halved, in agreement with previous investigations involving puppies given nutritional pellets pre-blended with the fungal chlamydospores (Hernández et al., 2018a). Some *in vitro* studies pointed out that filamentous fungi such as *P. chlamydosporia* or *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (2011) have a notable antagonistic activity on eggs of *T. canis* (Carvalho et al., 2010;

Maciel et al., 2012; Araujo et al., 2013). By spraying spores of *Trichoderma atrobrunneum* F.B. Rocha, P. Chaverri & Jaklitsch (2015) or *Clonostachys rosea* Schroers, Samuels, Seifert & W. Gams (1999) directly on feces of pigs, Viña et al. (2020) reported an ovicidal effect on 50% and 66% (respectively) on the eggs of the ascarid *Ascaris suum*, similar to that attained on eggs of other ascarid, *Toxascaris leonina*, in feces of captive lynxes exposed to *M. circinelloides* (Hernández et al., 2018b). By opposite, some baboons refused to take the gelatins and others did so with difficulty. Another issue was the need for keeping gelatins refrigerated to maintain their consistency, but melting after 15-20 days complicated their handling and administration beyond this interval. In an attempt to prolong their shelf-life, the gelatin-based treats were dehydrated and tested on other groups of puppies infected by *T. canis*, as well as on baboons passing eggs of *Trichuris* sp. Similar results to those described in the first assay (with gelatin treats) were attained among the puppies, in agreement also with a previous study involving pups receiving nutritional pellets sprayed the same blend of parasiticide chlamydospores (Hernández et al., 2018a). Viability of eggs of *Trichuris* sp. in baboon feces was halved, but dried gelatins were occasionally chopped and mixed with fruit, cereals, or seeds to facilitate intake. Decreasing water activity to almost zero avoids the disintegration of treats, which can be easily handled and transported, besides being preserved at room temperature for months. When stored taking care to avoid humidity and direct exposure to sunlight, the external appearance is not modified and fungal growth does not occur, which is relevant for animal owners or keepers.

Much of the research conducted so far involving the use of certain strains of SFF against different helminths capable of infecting animals has been focused on livestock, mainly ruminants (Vilela et al., 2016; Canhão-Dias et al., 2020; Rodrigues et al., 2020; Araújo et al., 2021). For this purpose, suitable formulations have been targeted that

allow oral administration of fungal spores/mycelium as cereal meal, nutritional pellets or energy blocks (Sagüés et al, 2011; Healey et al., 2018; Voinot et al., 2021a). However, it should be noted that certain animal species (as pets) do not receive these formulations due to the absence of commercially available products (so far), or because they are not part of their nutrition. In many areas, horses are kept under grazing regimes and rarely (if ever) receive supplementation (Hernández et al., 2018c). In the case of captive animal species in zoos, certain aspects related to economic profit can complicate the situation. There is a high number of species with particular nutritional requirements, resulting in the quantities of feedstuff regularly produced being far lower than those for livestock. All these concerns seem to severely interfere with the possibilities of considering strategies based on biological agents for the control of helminths, so new ones should be designed to help enhance the implementation of this sustainable solution.

Regarding the preparation of the two edible formulations, a crucial issue relies on that the concentration of chlamydospores might be reduced. As expected, minor and non-significant variations were obtained in the preparation of the gelatin treatments. However, the fungal chlamydospores counts were found to decrease significantly by about 10% after freeze-drying the gelatins, though it is noticeable to point that a similar parasiticidal activity was found among puppies receiving the gelatin-treats or those given the dried gelatins. Moreover, in the present investigation, slight and non-significant variations were observed during an 8-month storage period, which underlines the beneficial effect that can be achieved by drying the gelatins.

Unlike helminths that affect plants, which are always found in the soil, in those that infect animals or even people only some stages will be found in the environment and others inside the hosts. For decades, deworming of animals has often been considered

the only measure for helminth control, and overusing or underdosing identified as dosage errors which can lead to efficacy percentages lower than expected, and even to the development of nematode resistance to anthelmintics (Vercruysse et al., 2011; Moudgil ~~et al~~& Singla~~.~~, 2018; Lahat et al., 2021). This emphasizes the need for additional preventive strategies to improve and extend the success of treatment, and to evade the development of resistance to anthelmintics (Goossens et al., 2005). By considering that animals become infected by their exposure to infective stages developing in the soil (environment), the ability to permanently damage the eggs of certain helminths, or to stop or delay egg-development reveals essential to prevent it. In the present research, administration of gelatins or dried gelatins with chlamydospores of *M. circinelloides* and *D. flagrans* to puppies resulted in percentages of infective eggs of *T. canis* half of those developing in the absence of chlamydospores; data collected in baboons given dried gelatins showed that eggs of *Trichuris* sp. with a first-stage larva inside (the infective stage) dropped to one third. It appears very conceivable that success of any program for the control of these parasites depends on decreasing the risk of animals can infect again, which is directly related to the level of soil contamination by infective stages (Maesano et al., 2014; Palomero et al., 2020). In the present research, estimation of the Soil Contamination Index (based on the percentages of viable eggs which developed to the infective stage) showed values in control feces (without exposure to the fungi) of puppies were three-fold higher than in the treated samples (collected after two months taking chlamydospores), and five-fold higher in baboons, confirming that the risk of infection can be decreased threefold in the presence of SFF, as demonstrated by Viña et al. (2020).

Conclusions

Elaboration of edible gelatins with chlamydospores of *M. circinelloides* and *D. flagrans* affords a very interesting and practical formulation for the administration of these saprophytic filamentous fungi to pets, though it is necessary to keep them under refrigeration to maintain their consistency. Dehydration of these gelatins does not affect their helminthicidal activity, provides very safe foods easy to preserve at room temperature, and to administer to pets and wild captive animals as treats, with the aim to reach a notable beneficial effect in reducing their risk of infection by soil-transmitted helminths. These formulations appear highly interesting and attractive to develop successful strategies for the control of helminths among pets (private houses, dog kennels or shelters), and animals captive in zoological parks.

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Competing interests

All authors declare the absence of any financial or personal interests that could inappropriately influence the present investigation. The final article has been approved by all authors.

Author contributions

Cristiana Filipa Cazapal-Monteiro: Data curation, Investigation, Writing – Original draft; Antonio Miguel Palomero: Conceptualization, Methodology, Validation; Cándido Viña: Data curation, Investigation, Resources; Rami Salmo: Data curation, Investigation; José Ángel Hernández: Validation, Visualization; Rita Sánchez-Andrade: Conceptualization, Writing - review & editing, Funding acquisition; Adolfo Paz-Silva: Project administration, Conceptualization, Writing - review & editing, Funding acquisition; María Sol Arias: Formal Analysis, Conceptualization, Methodology, Supervision, Validation.

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FIGURE CAPTIONS

Figure 1 Design of the study for testing the appropriateness of edible formulations of *M. circinelloides* (ovicide) and *D. flagrans* (larvicide) for the control of helminths affecting pups and captive baboons.

~~Figure 2 Gelatin-treats elaborated with chlamydospores of *M. circinelloides* and *D. flagrans* were cryodesiccated prior to their administration to puppies and captive baboons.~~

Figure ~~3~~ 2 Infective eggs of *T. canis* in feces of puppies. a) Effect of the administration of gelatin-treats containing chlamydospores of *M. circinelloides* and *D. flagrans*. b) Effect of the administration of dry gelatin-treats containing chlamydospores of *M. circinelloides* and *D. flagrans*. PRG: feces taken after an interval of eight weeks providing the gelatins to the puppies; CP: fecal samples collected prior to the administration of the fungi (untreated controls); PRDG: feces taken after an interval of eight weeks giving dry gelatins to the puppies. Results are means ± 2SD. (*): significant differences between the two groups.

Figure ~~4~~ 3 Infective eggs of *Trichuris* sp. in feces of captive baboons. BRDG: feces taken after an interval of eight weeks giving dry gelatins with chlamydospores of *M. circinelloides* and *D. flagrans* to captive baboons; CB: fecal samples collected prior to the administration of the fungi (untreated controls). Results are means ± 2SD. (*): significant differences between the two groups.

TABLE CAPTIONS

Table 1 Appropriateness of edible formulations for the administration of chlamydospores of the parasiticide fungi *Mucor circinelloides* and *Duddingtonia flagrans*.

Table 2 Counts of chlamydospores of *M. circinelloides* and *D. flagrans* in gelatins and dried gelatins.

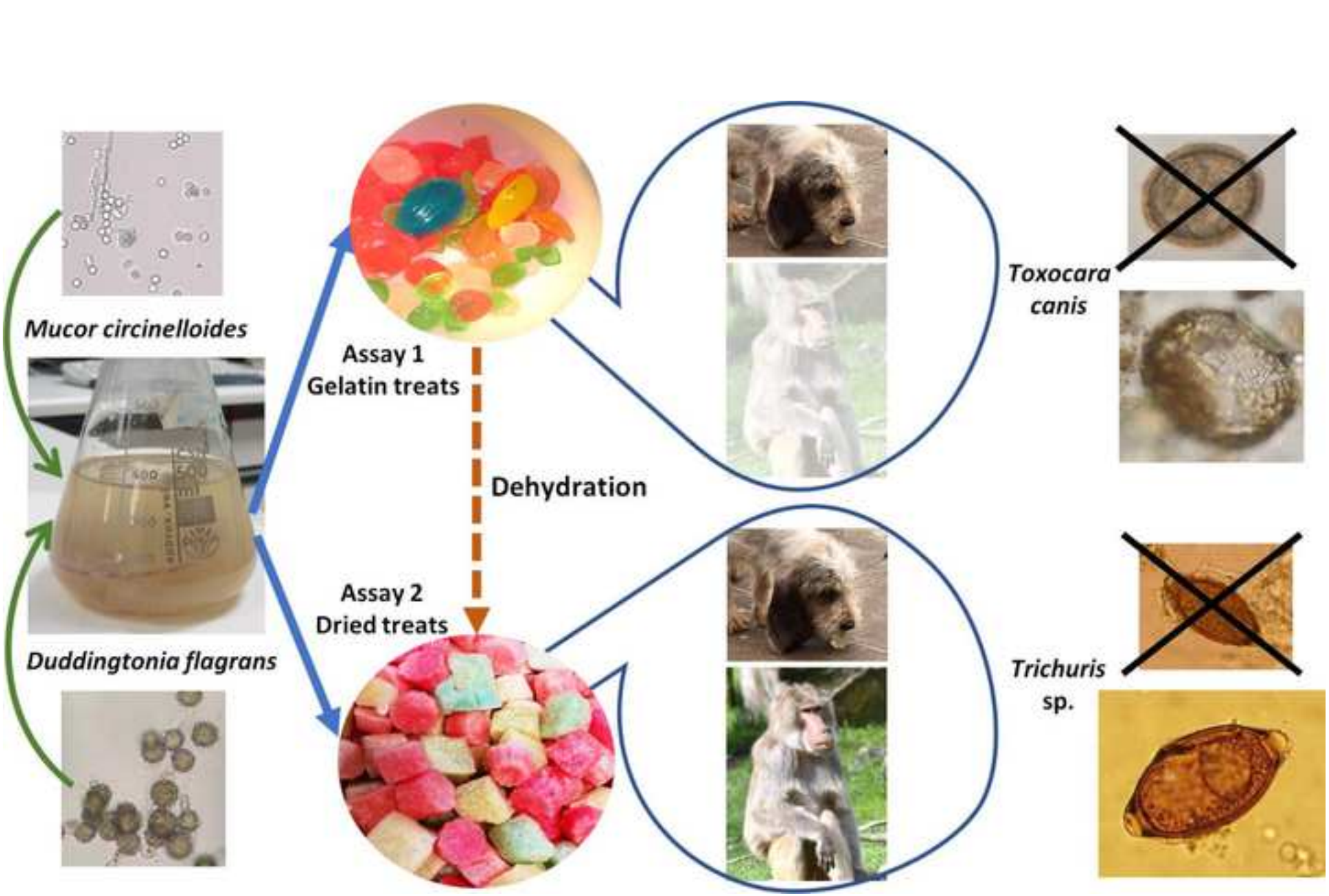
Table 3 Variations on counts of chlamydospores of *M. circinelloides* and *D. flagrans* in dried gelatins during an 8-month storage period.

Table 4 Analysis of viability of eggs of *T. canis* and *Trichuris* sp. in feces of puppies and captive baboons. STH: soil-transmitted helminths; VE: viable eggs; VR: viability reduction; PRG: feces taken after an interval of eight weeks providing the gelatins to the puppies; CP: fecal samples collected prior to the administration of the fungi (untreated controls); PRDG: feces taken after an interval of eight weeks giving dry gelatins to the puppies; BRDG: feces taken after an interval of eight weeks giving dry gelatins with chlamydospores of *M. circinelloides* and *D. flagrans* to captive baboons; CB: fecal samples collected prior to the administration of the fungi (untreated controls).

Highlights

- Helminths can affect almost all grazing animals, and up to 80% pets
- Ingestion of certain fungi helps in reducing the risk of infection by helminths
- Gelatins with fungi are easily prepared and dehydration guarantees long durability
- Gelatins and dry gelatins are useful in halving the risk of helminthoses ~~by 50%~~

Graphical Abstract



GELATIN TREATS CONTAINING FILAMENTOUS FUNGI TO PROMOTE SUSTAINABLE CONTROL OF HELMINTHS AMONG PETS AND ZOO ANIMALS

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Abstract

The appropriateness of edible formulations of the parasiticide fungi *Mucor circinelloides* (ovicide) and *Duddingtonia flagrans* (larvicide) for the control of helminths affecting animals has been tested in two assays. Firstly, gelatin-based treats were manufactured with a blend of $3 – 6 \cdot 10^7$ chlamydospores each fungus and provided to three groups of six puppies each passing eggs of *Toxocara canis*, thrice a week for eight weeks. Feces collected at the end of this period were analyzed during four weeks to estimate the egg viability, the counts of infective eggs and the soil contamination index. The usefulness of the edible formulation comprised checking for the easiness of preparation and administration, the level of acceptance by the targeted animals and the expiration interval. A similar assay was conducted on a group of six captive baboons, but some refused to ingest the gelatins and others did it with difficulty. The second

assay consisted of drying the gelatin treats and administering (thrice a week for eight weeks) to other two groups of six pups each infected by *T. canis* (different from those in assay I), and to six baboons captive in a zoo and passing eggs of *Trichuris* sp. (this probe on baboons was performed in triplicate at a four-month interval). Feces collected after eight weeks were examined for four weeks as mentioned previously.

Viability of eggs of *T. canis* was significantly halved with the two edible formulations, and 44% reached the infective stage. In the feces of baboons taking dry gelatins, 50% eggs of *Trichuris* sp. remained viable, and 14% attained infectivity. Soil contamination by these nematodes was 20 and 5%, respectively. It was concluded that gelatin treats containing chlamydospores of a blend of *M. circinelloides* and *D. flagrans* offer a highly effective and practical formulation to develop strategies for the biological control of helminths affecting pets. In addition to retaining the high helminthicidal activity, the dehydration of the gelatins provides a very safe edible formulation, easy to store at room temperature and to give to companion animals and captive wild species, thus its administration is strongly advised to reduce the risk of infection by soil-transmitted helminths.

Keywords: edible, prevention, nematodes, *Mucor circinelloides*, *Duddingtonia flagrans*

1. Introduction

An important number of parasites develop in the soil until attaining the stages that can infect animal species and even humans. Nearly all grazing animals are exposed to helminths, gastrointestinal nematodes and trematodes mainly (Charlier et al., 2016), while more than 50% pets are infected (D'ambroso et al., 2022; Safarov et al., 2022),

thus a great economic effort is made to minimize the damage through the administration of effective treatments. It has been estimated that more than 400 million € are spent yearly on anthelmintics in the European Union (Morgan et al., 2013), but this strategy reveals to be insufficient to prevent new frequent infections, so that regular deworming is necessary, which can result in increased environmental contamination by chemical residues or the appearance of strains resistant to anthelmintics (anthelmintic resistance) (Vercruysse et al., 2018).

Among the alternatives investigated to helminth control practices based on anthelmintics only, application of saprophytic filamentous fungi (SFF) as biological control agents against plant-parasitic nematodes and insect pests has been successfully carried out for decades (Baron et al., 2019; Poveda et al., 2020; Poveda, 2021). Some fungal species, in particular *Pochonia chlamydosporia* (Goddard) Zare & W. Gams (2001) and *Mucor circinelloides* Tiegh (1875), are antagonists of eggs of trematodes or gastrointestinal nematodes, whereas *Duddingtonia flagrans* (Dudd.) R.C. Cooke (1969) or *Monacrosporium thaumasium* (Drechsler) de Hoog & Oorschot (1985) develop traps in their mycelium to catch larvae of strongyles parasitizing animals (Canhão-Dias et al., 2020; Araújo et al., 2021). Moreover, it has been demonstrated the lack of adverse effects of *D. flagrans* on soil nematodes (Saumell et al., 2016), together with the innocuousness of daily administration of nutritional pellets containing chlamydospores of *M. circinelloides* and *D. flagrans* to heifers for two years (Voinot et al., 2021b).

Nevertheless, the use of SFF has slowed down in domestic, sylvatic, or captive wild species. In addition to regulatory issues, an important concern relies on proper ways to ensure that spores are correctly given to the animals. Most recent efforts have been focused on formulations that improve their oral administration to livestock, and a significant part of the trials consisted of providing them in grain seeds (Chandrawathani

et al., 2002; Paraud et al., 2005), cereal flour (Healey et al., 2018; Braga et al., 2020; de Oliveira et al., 2021), and in a lesser extent, nutritional pellets (Aguilar-Marcelino et al., 2017; Hernández et al., 2018c; Voinot et al., 2020), energy blocks (Sagüés et al., 2011) or milled cereal soaked with aqueous solutions (Palomero et al., 2020; Voinot et al., 2021a). So far, only two commercial formulations containing chlamydospores of *D. flagrans* are available, Bioworma® and Bioverm® (Healey et al., 2018; Fausto et al., 2021).

According to the management of the animals, notable differences exist especially regarding their nutrition. Whereas livestock species are provided regularly (daily) with feed supplementation, this is not the case for others as horses or certain wild individuals captive in zoological parks. For the purpose to improve the potential for biological control of animal parasites through orally delivery of SFF, edible gelatin-based treats were elaborated with chlamydospores of a blend of *M. circinelloides* and *D. flagrans*, then tested on puppies, and baboons captive in a zoological garden. A second trial consisted of investigating the helpfulness of freeze-drying the gelatin foods.

2. Material and methods

2.1. Parasiticide fungi

Different strains of soil saprophytic fungi (SSF) were isolated from feces of livestock and captive animals, and soil samples also, then deposited at the Spanish Type Culture Collection (CECT, Valencia, Spain) (Arias et al., 2013; Hernández et al., 2017). In the present study, the ovicidal species *M. circinelloides* (CECT 20824) and the larvicidal *D. flagrans* (CECT 20823) were co-cultured in a submerged medium (COPFr) at RT with

the aim to get elevated numbers of chlamydospores (Hernández et al., 2018a). The medium was composed by water, wheat and mineral salts (Arias et al., 2013).

2.2. Formulation of parasiticidal fungi as edible gelatin

2.2.1. Gelatin

Two types of gelatin-based foods were prepared in the Lab, one was obtained by mixing gelatin (Juncá Gelatines S.L., Spain), honey and liquid medium containing ≈ $3 – 5 \cdot 10^6$ chlamydospores of both fungi / mL, *M. circinelloides* and *D. flagrans*. The concentration was estimated by means of a Neubauer chamber (Ojeda-Robertos et al., 2008), using a total of 10 25-µL aliquots / L medium.

The blend was completely homogenized, heated under microwave for a brief period, placed into silicone molds and finally quenched at 4-6 ºC to enhance gelling. This formulation enabled that each food provided a dosage of $3 – 6 \cdot 10^7$ chlamydospores of each parasiticide fungus. (Additional information can not be provided due to it is pending of registration).

To assess if the numbers of fungal chlamydospores did vary significantly through the gelification process, each month for a six-month period a quantity of 30 gelatins were each soaked into 10 mL water at 37ºC and stirred until completely melted. A total of five 25 µL of each blend was examined under an optical microscope at 20X by using a Neubauer chamber and counts indicated as numbers of chlamydospores / mL.

The reduction of the numbers of chlamydospores during the gelification was estimated according to the formula:

$$\text{Reduction gelification (\%)} = [1 - (\text{Counts}_{\mathbf{GELATIN}} / \text{Counts}_{\mathbf{SUBMERGED\ CULTURE}})] \times 100$$

2.2.2. Dry gelatins

In order to increase the gelatin shelf life, part of the pieces was frozen at -35ºC and then water was removed by freeze-drying. The final products were packed into reusable plastic bags and stored at RT until administrated.

Examination of the dried gelatins was conducted to ascertain if the procedure might decrease the numbers of chlamydospores. Every month for six months, 30 gelatins were freeze-dried, then rehydrated by submerging each one into 10 mL water at 37ºC and stirred until complete melted. Finally, five 25 µL-aliquots of each solution were taken, and spores counted in a Neubauer chamber under an optical microscope at 20X and expressed as numbers per mL.

The reduction of the numbers of chlamydospores during the drying process was estimated as follows:

$$\text{Reduction drying (\%)} = [1 - (\text{Counts}_{\mathbf{DRY\ GELATIN}} / \text{Counts}_{\mathbf{GELATIN}})] \times 100$$

Finally, for the purpose to determine if storage might influence the numbers of chlamydospores, a total of 160 gelatins were freeze-dried, then 20 analyzed monthly for eight months as pointed above, and results shown as numbers of chlamydospores / mL.

2.3. Animals

Evaluation of the two edible foods prepared with a blend of chlamydospores of *M. circinelloides* and *D. flagrans* was carried out in 3-month old Griffon bleu de Gascogne puppies (‘‘Soñar’’ commercial breeding kennel, 42º57’32.689’’ N, 7º40’40.78’’ W; Lugo, NW Spain), and adult baboons (*Papio hamadryas*) captive in the “Marcelle

Zoological Park" (43°4′14.71″ N, 7°37′53.50″ W; Outeiro de Rei, NW Spain) (Palomero et al., 2020). First, infection by helminths was analyzed by means of the McMaster coprological technique with saturated saline solution (ρ = 1.2 g / dL), which makes possible the identification of protozoan cysts / oocysts and eggs of cestodes and nematodes (Hernández et al., 2018a; Voinot et al., 2021a). Briefly, three grams of each sample were weighed and placed in one bottle, then emulsified in 42 mL of water and shaken vigorously until completely broken down. This emulsion was filtered through a 150 µm pore diameter sieve and passed to two 15 mL glass tubes. After centrifuging at 1500 rpm for 10 min, the supernatant was discarded and the sediment homogenized in saturated saline solution, then aliquots taken carefully from the superior part of the tubes to fill a McMaster chamber, and finally observed under a light microscope at 10X (Viña et al., 2020). This procedure was repeated two times for each sample, and results expressed as eggs per gram of feces (EPG).

2.4. Design of study

2.4.1. Control groups

Because of the impossibility of keeping captive animals in control and treated groups at the same time, at the starting of the study three fecal samples were collected per each individual directly from the soil and homogenized by mixing vigorously (Fig. 1). Feces of puppies were labelled as CP (control puppies), and those belonging to baboons as CB (control baboons). Three grams of each mixture were placed into eight plastic containers provided with four holes in the side walls for allowing proper aeration, then covered with a plastic lid and finally brought to a meadow for a 4-week period (Hernández et al., 2018b). The contents of two containers per animal were examined

weekly by means of a modified McMaster technique (Hernández et al., 2018b; Voinot et al., 2021a). This modification consisted of that after centrifuging at 1500 rpm for 10 min, the sediment was homogenized in saturated saline solution, then aliquots of 100 µL were taken carefully from the top of the tubes, placed between glass slides and coverslips, and examined under a light microscope at 10X and 20X until a minimum of 100 eggs was analyzed (Viña et al., 2020). This procedure was repeated two times for each sample. It is important to note that identical numbers of *control* and *treated* groups were observed in each assay.

2.4.2. Assay I: Testing edible gelatin foods

Three groups of six 3-month old Griffon bleu de Gascogne puppies were given one gelatin with fungal spores thrice a week for eight weeks (PRG, puppies receiving gelatins). After this period, three fecal samples were collected per each animal directly from the soil, homogenized by mixing vigorously and proceeded as mentioned above (2.4.1.).

The same designing was performed on baboons, but two of them refused to ingest these treats, and irregular and infrequent intake was recorded among the others. Accordingly, the assay was abandoned.

2.4.3. Assay II: Testing edible dried gelatin foods

Two groups of six Griffon bleu de Gascogne puppies (3-month old) (different to those in Assay I), and one group of six adult baboons were used in the second assay for testing edible dried gelatins. As mentioned in 2.4.1., control fecal samples were

prepared at the starting of the assay. Later, all animals were given one dry gelatin treat, thrice a week for an interval of eight weeks, and at the end of this period three fecal samples were collected (per each animal) directly from the soil and homogenized by mixing vigorously. Next step consisted of placing three grams of each mixture into eight plastic containers and labelling them as PRDG (puppies receiving dried gelatins) or BRDG (baboons receiving dried gelatins), then continuing as stated prior (2.4.1.). Given the impossibility of having a greater number of baboons, this assay was performed two more times, at intervals of 4 months.

The experimental design was approved by the Ethical committee of the University of Santiago de Compostela (Spain; protocol number CTM2015-65954b) and complied with the Directive 2010 / 63 / EU.

2.5. Evaluation of the parasiticide effect

Eggs of helminths observed by means of the modified McMaster test were classified as enduringly unviable when presenting loss of shell continuity, contraction or rupture, cytoplasmic vacuolization, and abnormal morphology of inner larvae (Cazapal-Monteiro et al., 2015; Hernández et al., 2018b). Likewise, the viable eggs were classified into infective when presenting a L2 larva inside for the ascarids, and a L1 for the trichurids.

The reduction of the viability (VR) and the Soil Contamination Index (SCI) were calculated according to Viña et al. (2020):

$$VR\ (\%) = [(100 - \%\ \text{Viable EPG}_{\text{Week}X})/\%\ \text{Viable EPG}_{\text{Week}0}] \times 100$$

$$SCI\ (\%) = (\%\ \text{Viable Eggs} \times \%\ \text{Eggs with L2}) / 100$$

2.6. Evaluation of the usefulness of edible products containing chlamydospores of parasiticidal fungi

The criteria considered for the evaluation of the appropriateness of edible formulations with chlamydospores of *M. circinelloides* and *D. flagrans* for the control of helminths among animals consisted of:

- Easiness of manufacturing:

  0: No problems - Reading and interpreting the protocol is very easy and, together with the elaboration, takes < 5 min.

  1: Easy - Reading and interpreting the protocol is easy and, together with the elaboration, requires 5 - 10 min.

  2: Medium difficulty - Reading and interpreting the protocol is somewhat difficult, and together with the elaboration takes 10 - 20 min.

  3: Difficult - Reading and interpreting the protocol is difficult, and together with the elaboration it requires > 20 minutes.

- Easiness of administration:

  0: No problems - Treats are placed in the feeder along with cereal or feed.

  1: Easy - The treats are chopped up and placed in the feeder.

  2: Medium difficulty - It is necessary to tempt the animals with cereals or feed to get them to approach the feeders, and at this time treats are provided.

  3: Difficult - It is needed to physically separate or even immobilize the animals.

- Acceptance level by the target animals:

  0: Immediate (< 5 s).

  1: High (5 - 20 s).

2: Moderate (21 – 60 s).

3: Rejected.

- Expiration interval:

0: < 7 days.

1: < 15 days.

2: < 30 days.

3: > 30 days.

2.7. Statistical analysis

Distribution of data collected in the current research was analyzed first for their normal distribution, by means of the Kolmogorov-Smirnov and the Levene's tests. Most data were not normally distributed, hence the Mann-Whitney U and Kruskal-Wallis non-parametric probes were run.

All tests were carried out by using the statistical package SPSS, version 20 (IBM SPSS Inc., Chicago, IL, USA), at a significance level of $P < 0.05$.

Due to differences between the replicates of each assay were not observed, these data were pooled and analyzed as a single set.

3. Results

3.1. Identification of parasites in coprological analyses

Eggs of the gastrointestinal nematodes *T. canis* and of *Trichuris* sp. were identified in the feces of puppies and baboons, respectively.

3.2. Analysis of edible formulations with chlamydospores of *M. circinelloides* and *D. flagrans*

3.2.1. Appropriateness of edible formulations

Preparation of gelatins with chlamydospores of parasiticidal fungi resulted very simple (Table 1), the administration was very easy and these foods well accepted (puppies took them in less than five seconds) for treats less than 15 days old. After 21 days, many of the gelatins had lost their consistency, making it difficult to administer. In contrast, baboons did not accept the gelatins as willingly, as some refused, and others did so with difficulty and after a period (more than 10 minutes).

Dry gelatins were very easy to prepare also (Table 1) and preserved at RT for more than 8 months, without any sign of alteration. Administration was very simple and well accepted by pups (took them in less than five seconds), but occasionally it was needed to break them into short pieces and mix with fruit, cereal grains or seeds for baboons.

3.2.2. Counts of chlamydospores in edible foods

After the elaboration of gelatins, non-significant variations were recorded in the numbers of chlamydospores of *M. circinelloides* or *D. flagrans* with respect to the initial quantities in the submerged culture (Table 2), and an average reduction of 1.78 ± 0.83% (Z= -1.239, *P*= 0.215) and 1.89 ± 0.73% (Z= -1.214, *P*= 0.225) was respectively obtained.

Changes in the counts of chlamydospores of both parasiticide fungi after the gelatins were dried are summarized in Table 2. A significant reduction in the numbers was

achieved, with average values of 8.87 ± 2.81% (Z= -5.608, *P*= 0.001) for *M. circinelloides* and 9.99 ± 2.69% (Z= -5.387, *P*= 0.001) for *D. flagrans*.

Table 3 contains data on the numbers of chlamydospores during an 8-month storage period. Non-significant variations were observed with the time elapsed since drying (*P* > 0.05).

3.3. Assay I: gelatin-based foods given to puppies

As drawn in Table 4, viability of eggs of *T. canis* was 96 ± 2% at the beginning of the study, and four weeks later was reduced to 90 ± 4% in the control samples (CP), by 48 ± 9% in the PRG (puppies receiving gelatins), which represents a VR of 7% and 50%, respectively. Statistical differences between the two groups were demonstrated (Z= -6.363, *P*= 0.001).

Infective eggs of *T. canis* (containing a L2) appeared from the 1st week (wk) (Fig. 2a) in CP, and values around 80% were obtained at the end of the study. In PRG, eggs with L2 were first observed since the 3rd wk, with the highest percentages (44%) by the end of the assay (Fig. 2a) (Z= -3.447, *P*= 0.001).

The values of the Soil Contamination Index (SCI) increased along the study until 70% (4th wk) in CP, by 20% in PRG (Table 4).

3.4. Assay II: Evaluation of dehydrated gelatin-based pieces

3.4.1. In puppies

The viability of eggs of *T. canis* in the control puppies (CP) decreased slightly (2 – 7%) for one month, with values near to 90% (Table 4). In the puppies receiving dehydrated gelatins containing chlamydospores of *M. circinelloides* and *D. flagrans* (PRDG), a significant reduction between the 2nd wk (VR= 13%) and the 4th wk (VR= 48%) was recorded (Z= -5.666, *P*= 0.001).

The presence of infective eggs (with L2) in the controls (CP) was recorded since the 2nd wk, the values increased fast and the highest percentages were obtained at the end of the study (81%) (Fig. 2b). In the PRDG, eggs with a L2 were observed after three weeks, and found in 44% at the end of the assay (Z= -2.595, *P*= 0.009).

Concerning the SCI, values around 73% and 20% were obtained at the end of the study in CP and PRDG, respectively (Table 4).

Comparison between the parasiticide effect reached by providing puppies gelatins or dry gelatins is summarized in Table 4. Viable eggs of *T. canis* oscillated between 96 – 48% and 95 – 49% in puppies receiving gelatins or dried gelatins, respectively, with reduction values about 50% (Z= -0.092, *P*= 0.927). Regarding the infective eggs, percentages of 50% were obtained in CRG, by 48% in CRDG, with a SCI of 20% in both groups (Z= -0.455, *P*= 0.649). Differences between results obtained in the respective control groups (CP1 and CP2) were not significant (Z= -0.083, *P*= 0.934) and (Z= -0.753, *P*= 0.451).

3.4.2. In baboons

Viability of eggs of *Trichuris* sp. in the control feces of baboons (CB) maintained at 89 – 97% (Table 4), while in BRDG reduced to 44% after four weeks of exposure to the

parasiticide fungi. After one month, the values of VR were 8% in CB and 54% in BRDG (Z= -6.667, *P*= 0.001).

Infective eggs (containing a L1 inside) were first observed since the 3rd wk in the controls (CB), and percentages close to 30% attained at the 4th wk (Fig. 3). In the feces exposed to the chlamydospores (BRDG), 14% of the eggs had a L1 at the end of the study (Z= -3.777, *P*= 0.001).

After four weeks, the SCI values for the control baboons (CB) were 26%, and 5% in those receiving dry gelatins (BRDG) (Table 4).

Discussion

The main advantages of using certain saprophytic filamentous fungi for the control of helminths affecting animal species are centered on the possibility of reducing the risk of infection to prevent damage to hosts and minimize the need for frequent application of chemical dewormers (Ortiz Pérez et al., 2017; Mendoza et al., 2018). In the current investigation, a new way of administration of SFF consisting of gelatin-based treats enriched with chlamydospores of *M. circinelloides* and *D. flagrans* given to pups infected by *T. canis* and baboons by *Trichuris* sp., has been tried. These foods were easy to prepare and puppies took them quickly (in less than five minutes). Four weeks later, viability of eggs of the gastrointestinal nematode *T. canis* in fecal samples was halved, in agreement with previous investigations involving puppies given nutritional pellets pre-blended with the fungal chlamydospores (Hernández et al., 2018a). Some *in vitro* studies pointed out that filamentous fungi such as *P. chlamydosporia* or *Purpureocillium lilacinum* (Thom) Luangsa-ard, Houbraken, Hywel-Jones & Samson (2011) have a notable antagonistic activity on eggs of *T. canis* (Carvalho et al., 2010;

Maciel et al., 2012; Araujo et al., 2013). By spraying spores of *Trichoderma atrobrunneum* F.B. Rocha, P. Chaverri & Jaklitsch (2015) or *Clonostachys rosea* Schroers, Samuels, Seifert & W. Gams (1999) directly on feces of pigs, Viña et al. (2020) reported an ovicidal effect on 50% and 66% (respectively) on the eggs of the ascarid *Ascaris suum*, similar to that attained on eggs of other ascarid, *Toxascaris leonina*, in feces of captive lynxes exposed to *M. circinelloides* (Hernández et al., 2018b). By opposite, some baboons refused to take the gelatins and others did so with difficulty. Another issue was the need for keeping gelatins refrigerated to maintain their consistency, but melting after 15-20 days complicated their handling and administration beyond this interval. In an attempt to prolong their shelf-life, the gelatin-based treats were dehydrated and tested on other groups of puppies infected by *T. canis*, as well as on baboons passing eggs of *Trichuris* sp. Similar results to those described in the first assay (with gelatin treats) were attained among the puppies, in agreement also with a previous study involving pups receiving nutritional pellets sprayed the same blend of parasiticide chlamydospores (Hernández et al., 2018a). Viability of eggs of *Trichuris* sp. in baboon feces was halved, but dried gelatins were occasionally chopped and mixed with fruit, cereals, or seeds to facilitate intake. Decreasing water activity to almost zero avoids the disintegration of treats, which can be easily handled and transported, besides being preserved at room temperature for months. When stored taking care to avoid humidity and direct exposure to sunlight, the external appearance is not modified and fungal growth does not occur, which is relevant for animal owners or keepers.

Much of the research conducted so far involving the use of certain strains of SFF against different helminths capable of infecting animals has been focused on livestock, mainly ruminants (Vilela et al., 2016; Canhão-Dias et al., 2020; Rodrigues et al., 2020; Araújo et al., 2021). For this purpose, suitable formulations have been targeted that

allow oral administration of fungal spores/mycelium as cereal meal, nutritional pellets or energy blocks (Sagüés et al, 2011; Healey et al., 2018; Voinot et al., 2021a). However, it should be noted that certain animal species (as pets) do not receive these formulations due to the absence of commercially available products (so far), or because they are not part of their nutrition. In many areas, horses are kept under grazing regimes and rarely (if ever) receive supplementation (Hernández et al., 2018c). In the case of captive animal species in zoos, certain aspects related to economic profit can complicate the situation. There is a high number of species with particular nutritional requirements, resulting in the quantities of feedstuff regularly produced being far lower than those for livestock. All these concerns seem to severely interfere with the possibilities of considering strategies based on biological agents for the control of helminths, so new ones should be designed to help enhance the implementation of this sustainable solution.

Regarding the preparation of the two edible formulations, a crucial issue relies on that the concentration of chlamydospores might be reduced. As expected, minor and non-significant variations were obtained in the preparation of the gelatin treatments. However, the fungal chlamydospores counts were found to decrease significantly by about 10% after freeze-drying the gelatins, though it is noticeable to point that a similar parasiticidal activity was found among puppies receiving the gelatin-treats or those given the dried gelatins. Moreover, in the present investigation, slight and non-significant variations were observed during an 8-month storage period, which underlines the beneficial effect that can be achieved by drying the gelatins.

Unlike helminths that affect plants, which are always found in the soil, in those that infect animals or even people only some stages will be found in the environment and others inside the hosts. For decades, deworming of animals has often been considered

the only measure for helminth control, and overusing or underdosing identified as dosage errors which can lead to efficacy percentages lower than expected, and even to the development of nematode resistance to anthelmintics (Vercruysse et al., 2011; Moudgil & Singla, 2018; Lahat et al., 2021). This emphasizes the need for additional preventive strategies to improve and extend the success of treatment, and to evade the development of resistance to anthelmintics (Goossens et al., 2005). By considering that animals become infected by their exposure to infective stages developing in the soil (environment), the ability to permanently damage the eggs of certain helminths, or to stop or delay egg-development reveals essential to prevent it. In the present research, administration of gelatins or dried gelatins with chlamydospores of *M. circinelloides* and *D. flagrans* to puppies resulted in percentages of infective eggs of *T. canis* half of those developing in the absence of chlamydospores; data collected in baboons given dried gelatins showed that eggs of *Trichuris* sp. with a first-stage larva inside (the infective stage) dropped to one third. It appears very conceivable that success of any program for the control of these parasites depends on decreasing the risk of animals can infect again, which is directly related to the level of soil contamination by infective stages (Maesano et al., 2014; Palomero et al., 2020). In the present research, estimation of the Soil Contamination Index (based on the percentages of viable eggs which developed to the infective stage) showed values in control feces (without exposure to the fungi) of puppies were three-fold higher than in the treated samples (collected after two months taking chlamydospores), and five-fold higher in baboons, confirming that the risk of infection can be decreased threefold in the presence of SFF, as demonstrated by Viña et al. (2020).

Conclusions

Elaboration of edible gelatins with chlamydospores of *M. circinelloides* and *D. flagrans* affords a very interesting and practical formulation for the administration of these saprophytic filamentous fungi to pets, though it is necessary to keep them under refrigeration to maintain their consistency. Dehydration of these gelatins does not affect their helminthicidal activity, provides very safe foods easy to preserve at room temperature, and to administer to pets and wild captive animals as treats, with the aim to reach a notable beneficial effect in reducing their risk of infection by soil-transmitted helminths. These formulations appear highly interesting and attractive to develop successful strategies for the control of helminths among pets (private houses, dog kennels or shelters), and animals captive in zoological parks.

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Competing interests

All authors declare the absence of any financial or personal interests that could inappropriately influence the present investigation. The final article has been approved by all authors.

Author contributions

Cristiana Filipa Cazapal-Monteiro: Data curation, Investigation, Writing – Original draft; Antonio Miguel Palomero: Conceptualization, Methodology, Validation; Cándido Viña: Data curation, Investigation, Resources; Rami Salmo: Data curation, Investigation; José Ángel Hernández: Validation, Visualization; Rita Sánchez-Andrade: Conceptualization, Writing - review & editing, Funding acquisition; Adolfo Paz-Silva: Project administration, Conceptualization, Writing - review & editing, Funding acquisition; María Sol Arias: Formal Analysis, Conceptualization, Methodology, Supervision, Validation.

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FIGURE CAPTIONS

Figure 1 Design of the study for testing the appropriateness of edible formulations of *M. circinelloides* (ovicide) and *D. flagrans* (larvicide) for the control of helminths affecting pups and captive baboons.

Figure 2 Infective eggs of *T. canis* in feces of puppies. a) Effect of the administration of gelatin-treats containing chlamydospores of *M. circinelloides* and *D. flagrans*. b) Effect of the administration of dry gelatin-treats containing chlamydospores of *M. circinelloides* and *D. flagrans*. PRG: feces taken after an interval of eight weeks providing the gelatins to the puppies; CP: fecal samples collected prior to the administration of the fungi (untreated controls); PRDG: feces taken after an interval of eight weeks giving dry gelatins to the puppies. Results are means ± 2SD. (*): significant differences between the two groups.

Figure 3 Infective eggs of *Trichuris* sp. in feces of captive baboons. BRDG: feces taken after an interval of eight weeks giving dry gelatins with chlamydospores of *M. circinelloides* and *D. flagrans* to captive baboons; CB: fecal samples collected prior to the administration of the fungi (untreated controls). Results are means ± 2SD. (*): significant differences between the two groups.

TABLE CAPTIONS

Table 1 Appropriateness of edible formulations for the administration of chlamydospores of the parasiticide fungi *Mucor circinelloides* and *Duddingtonia flagrans*.

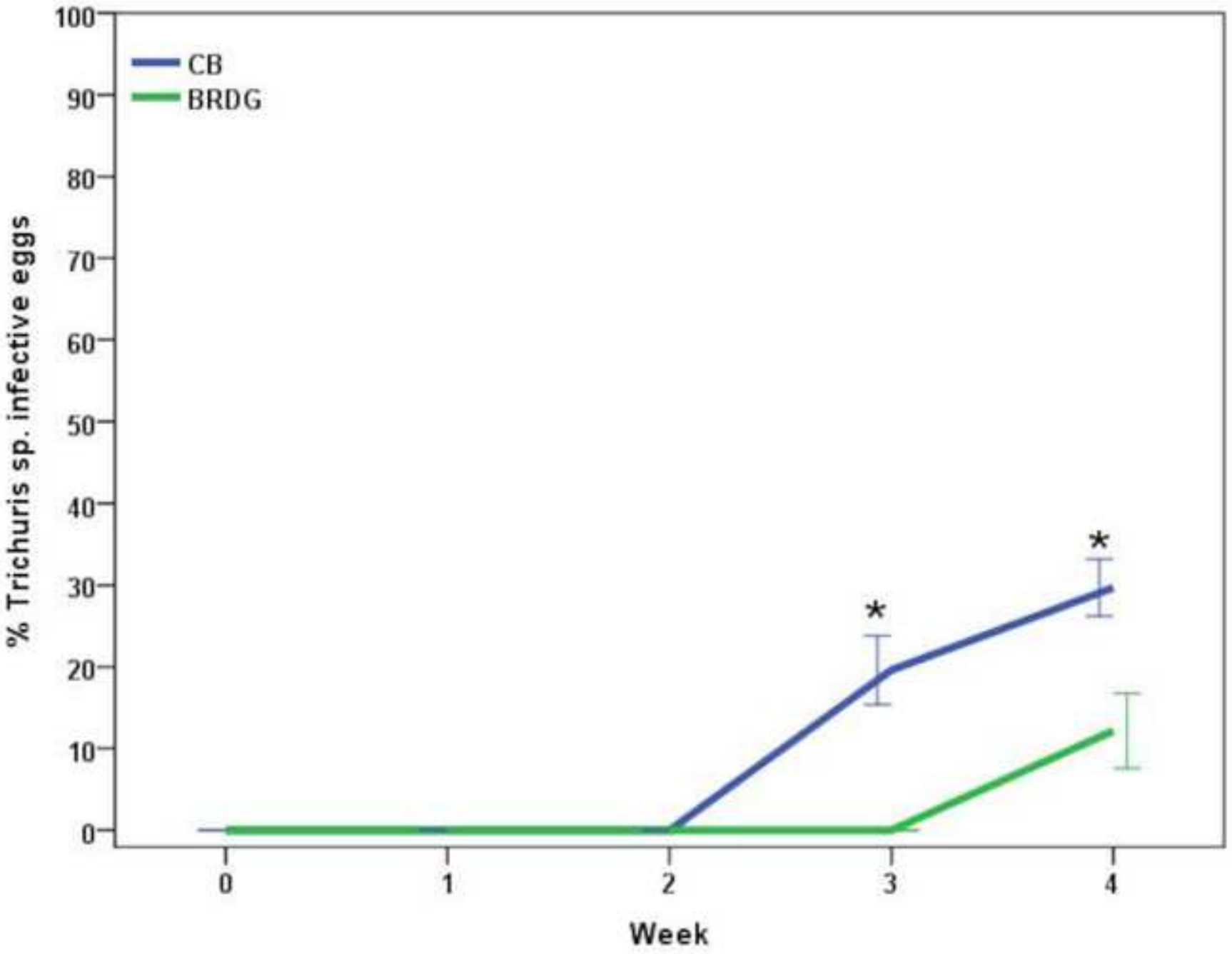
Table 2 Counts of chlamydospores of *M. circinelloides* and *D. flagrans* in gelatins and dried gelatins.

Table 3 Variations on counts of chlamydospores of *M. circinelloides* and *D. flagrans* in dried gelatins during an 8-month storage period.

Table 4 Analysis of viability of eggs of *T. canis* and *Trichuris* sp. in feces of puppies and captive baboons. STH: soil-transmitted helminths; VE: viable eggs; VR: viability reduction; PRG: feces taken after an interval of eight weeks providing the gelatins to the puppies; CP: fecal samples collected prior to the administration of the fungi (untreated controls); PRDG: feces taken after an interval of eight weeks giving dry gelatins to the puppies; BRDG: feces taken after an interval of eight weeks giving dry gelatins with chlamydospores of *M. circinelloides* and *D. flagrans* to captive baboons; CB: fecal samples collected prior to the administration of the fungi (untreated controls).

Figure

Click here to access/download;Figure;Fig3.jpg



Graphical Abstract

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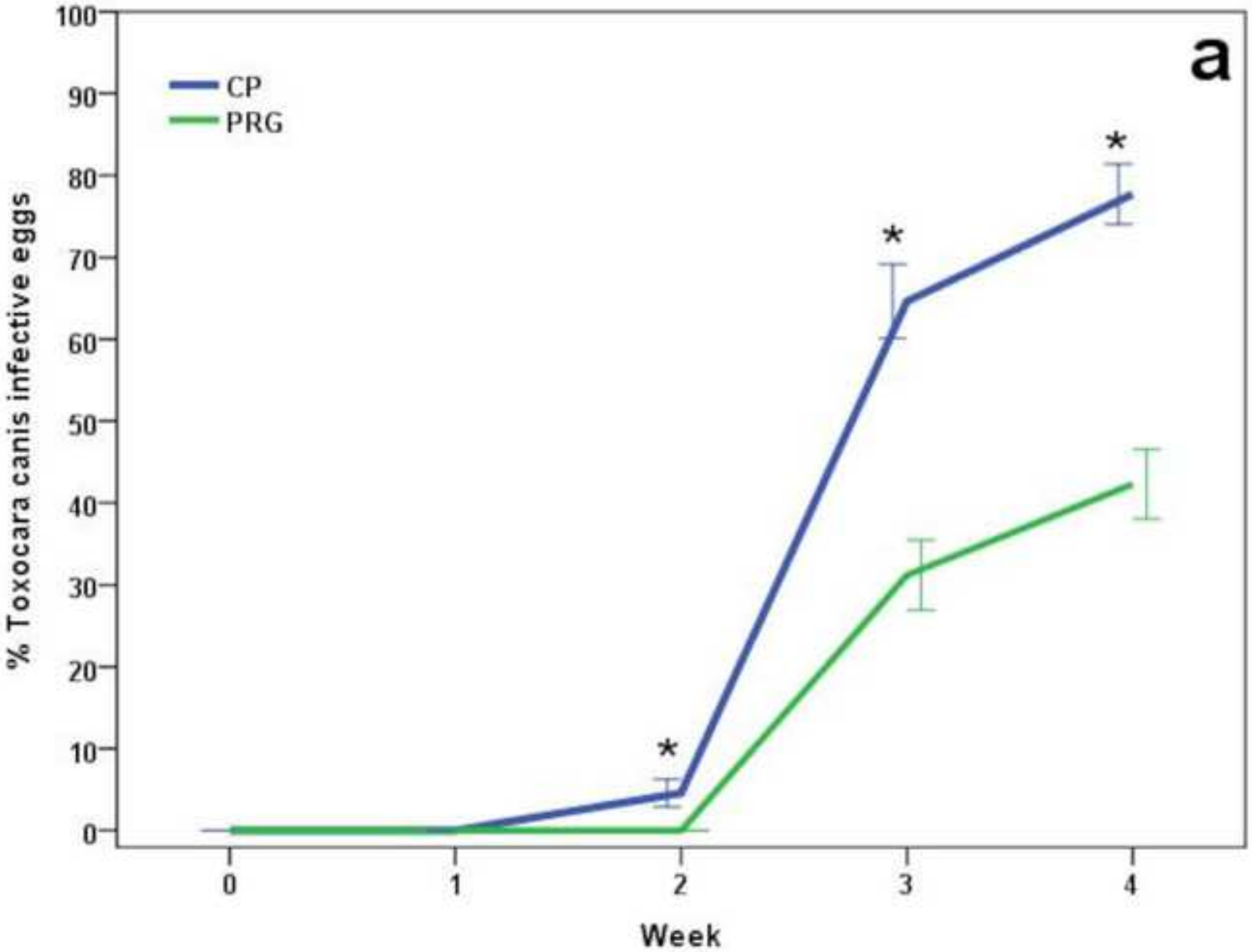
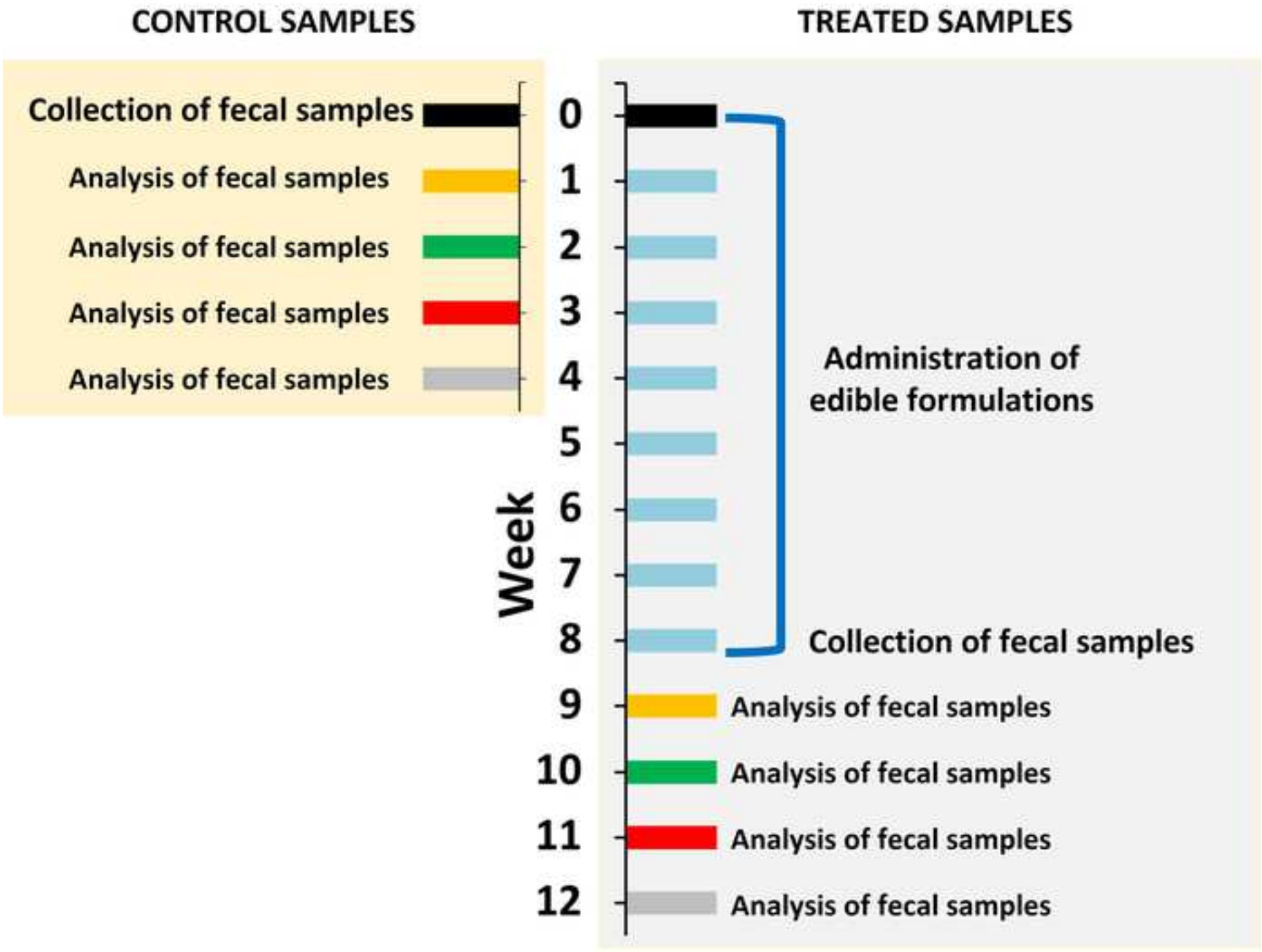


Figure



Figure

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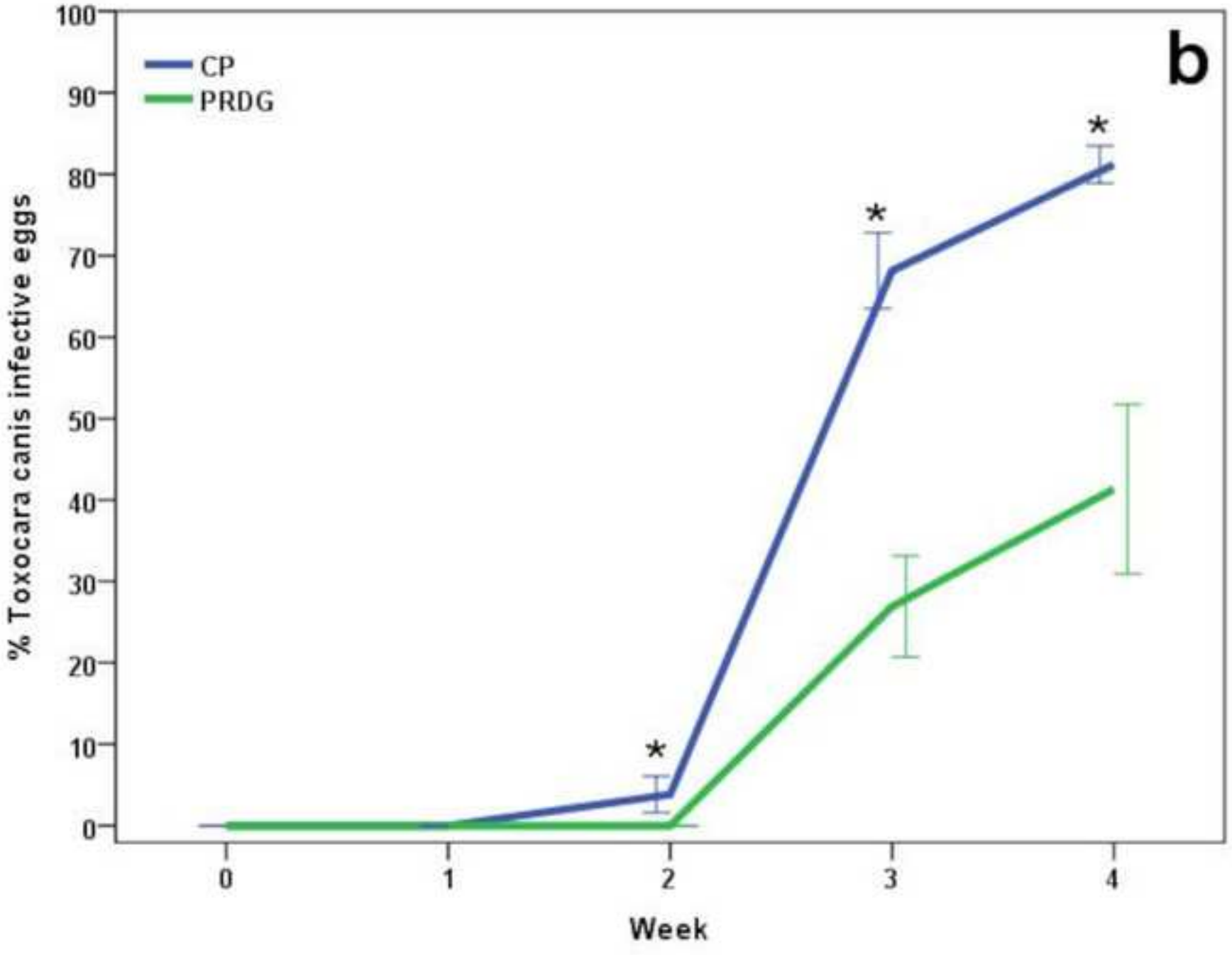

	Qualification	Interpretation	Gelatin	Dry gelatin
Manufacturing	0	No problems	X	X
	1	Easy		
	2	Medium difficulty		
	3	Difficult		
Administration	0	No problems	X	X
	1	Easy		
	2	Medium difficulty		
	3	Difficult		
Acceptance level	0	Immediate (< 5 s)	X	X
	1	High (5 - 20 s)		
	2	Moderate (21 – 60 s)		
	3	Rejected		
Expiration interval	0	< 7 days		
	1	< 15 days	X	
	2	< 30 days		
	3	> 30 days		X

Month		*Mucor circinelloides* (chlamydospores / mL)				*Duddingtonia flagrans* (chlamydospores / mL)			
		Average (CI 95%)	SD	% Reduction gelification	% Reduction drying	Average (CI 95%)	SD	% Reduction gelification	% Reduction drying
1	Submerged culture	$4.2 \cdot 10^6$ $((2.9 – 5.6) \cdot 10^6)$	$1.5 \cdot 10^5$	1.7 ± 1.3 (0 – 13)		$4.1 \cdot 10^6$ $((2.8 – 5.4) \cdot 10^6)$	$1.5 \cdot 10^5$	1.7 ± 0.7 (1.5 – 2)	
	Gelatin	$4.2 \cdot 10^6$ $((3.3 – 5) \cdot 10^6)$	$9.1 \cdot 10^4$	U= -0.503 *P*= 0.615	8.5 ± 2.6 (7.5 – 9.5)	$4 \cdot 10^6$ $((2.5 – 5.5) \cdot 10^6)$	$1.7 \cdot 10^5$	U= -0.510 *P*= 0.610	9.1 ± 2.7 (8.1 – 10.1)
	Dry gelatin	$3.7 \cdot 10^6$ $((0.9 – 6.5) \cdot 10^6)$	$3.2 \cdot 10^5$		U= -2.122 *P*= 0.034	$3.6 \cdot 10^6$ $((3.2 – 4) \cdot 10^6)$	$0.4 \cdot 10^5$		U= -1.804 *P*= 0.071
2	Liquid culture	$4 \cdot 10^6$ $((2.9 – 5.6) \cdot 10^6)$	$5 \cdot 10^5$	1.4 ± 0.9 (0 – 9)		$4.8 \cdot 10^6$ $((3.2 – 6.5) \cdot 10^6)$	$1.8 \cdot 10^5$	1.9 ± 0.9 (1.6 – 2.7)	
	Gelatin	$3.9 \cdot 10^6$ $((3.6 – 4.2) \cdot 10^6)$	$0.3 \cdot 10^5$	U= -0.636 *P*= 0.525	9.1 ± 3.3 (7.8 – 10.3)	$4.7 \cdot 10^6$ $((3.3 – 6.1) \cdot 10^6)$	$1.6 \cdot 10^5$	U= -0.554 *P*= 0.579	11.1 ± 2.9 (10 – 12.2)
	Dry gelatin	$3.5 \cdot 10^6$ $((2.8 – 4.2) \cdot 10^6)$	$0.8 \cdot 10^5$		U= -2.499 *P*= 0.012	$4.3 \cdot 10^6$ $((1.9 – 6.6) \cdot 10^6)$	$2.6 \cdot 10^5$		U= -2.669 *P*= 0.008
3	Liquid culture	$4.4 \cdot 10^6$ $((3.2 – 5.6) \cdot 10^6)$	$1.4 \cdot 10^5$	1.1 ± 0.5 (0 – 5.5)		$4.7 \cdot 10^6$ $((3.9 – 5.5) \cdot 10^6)$	$0.9 \cdot 10^5$	1.8 ± 0.6 (1.6 – 2)	
	Gelatin	$4.4 \cdot 10^6$ $((3.4 – 5.4) \cdot 10^6)$	$1.1 \cdot 10^5$	U= -0.717 *P*= 0.473	8.1 ± 2.6 (7.1 – 9.1)	$4.6 \cdot 10^6$ $((3.7 – 5.5) \cdot 10^6)$	$1 \cdot 10^5$	U= -0.547 *P*= 0.584	9.4 ± 2.7 (8.4 – 10.4)
	Dry gelatin	$4.1 \cdot 10^6$ $((1.7 – 6.4) \cdot 10^6)$	$2.6 \cdot 10^5$		U= -2.469 *P*= 0.014	$4.3 \cdot 10^6$ $((2.1 – 6.4) \cdot 10^6)$	$2.4 \cdot 10^5$		U= -1.982 *P*= 0.048
4	Liquid culture	$4.4 \cdot 10^6$ $((0.15 – 1) \cdot 10^7)$	$6.6 \cdot 10^5$	1.8 ± 1.3 (0 – 14)		$4.4 \cdot 10^6$ $((1.6 – 7.3) \cdot 10^6)$	$3.2 \cdot 10^5$	1.9 ± 0.7 (1.7 – 2.2)	
	Gelatin	$4.3 \cdot 10^6$ $((1 – 9.6) \cdot 10^6)$	$5.9 \cdot 10^5$	U= -0.747 *P*= 0.455	9.5 ± 1.9 (8.7 – 10.2)	$4.4 \cdot 10^6$ $((1.1 – 7.6) \cdot 10^6)$	$3.6 \cdot 10^5$	U= -0.591 *P*= 0.554	9.7 ± 2.5 (8.7 – 10.6)
	Dry gelatin	$3.9 \cdot 10^6$ $((0.97 – 8.5) \cdot 10^6)$	$5.1 \cdot 10^5$		Z= -2.610 *P*= 0.009	$3.9 \cdot 10^6$ $((1.8 – 7.7) \cdot 10^6)$	$4.2 \cdot 10^5$		U= -2.189 *P*= 0.029

5	Liquid culture	$4.3 \cdot 10^6$ $((0.41 – 1.3) \cdot 10^7)$	$9.4 \cdot 10^5$	0.95 ± 0.07 (0.3 – 1.6)		$4.3 \cdot 10^6$ $((0.07 – 8.6) \cdot 10^6)$	$4.8 \cdot 10^5$	1.8 ± 0.8 (1.5 – 2.1)	
	Gelatin	$4.3 \cdot 10^6$ $((0.41 – 1.3) \cdot 10^7)$	$9.3 \cdot 10^5$	U= -0.569 *P*= 0.569	8.9 ± 2.8 (7.8 – 9.9)	$4.3 \cdot 10^6$ $((0.1 – 8.4) \cdot 10^6)$	$4.6 \cdot 10^5$	U= -0.673 *P*= 0.501	10.2 ± 2.2 (9.4 – 11.1)
	Dry gelatin	$3.8 \cdot 10^6$ $(0.5 – 1.3 \cdot 10^7)$	$1 \cdot 10^6$		U= -2.395 *P*= 0.017	$3.8 \cdot 10^6$ $(1.2 – 8.8 \cdot 10^6)$	$5.5 \cdot 10^5$		U= -2.625 *P*= 0.009
6	Liquid culture	$5.4 \cdot 10^6$ $((1.7 – 9.1) \cdot 10^6)$	$4.1 \cdot 10^5$	0.9 ± 0.4 (0 – 4.7)		$3.8 \cdot 10^6$ $((3 – 4.6) \cdot 10^6)$	$0.9 \cdot 10^5$	2.1 ± 0.6 (1.8 – 2.3)	
	Gelatin	$5.4 \cdot 10^6$ $((1.5 – 9.1) \cdot 10^6)$	$4.3 \cdot 10^5$	U= -0.673 *P*= 0.501	9.2 ± 3.4 (8 – 10.5)	$3.7 \cdot 10^6$ $((2.9 – 4.5) \cdot 10^6)$	$0.1 \cdot 10^5$	U= -0.532 *P*= 0.595	10.5 ± 2.7 (9.5 – 11.5)
	Dry gelatin	$4.9 \cdot 10^6$ $((0.05 – 1) \cdot 10^7)$	$6.1 \cdot 10^5$		U= -2.418 *P*= 0.016	$3.2 \cdot 10^6$ $((2.6 – 3.8) \cdot 10^7)$	$0.7 \cdot 10^5$		U= -2.203 *P*= 0.028

	Mucor circinelloides (chlamydospores / mL)		*Duddingtonia flagrans* (chlamydospores / mL)	
Month	Average CI 95%	SD	Average CI 95%	SD
1	$4.01 \cdot 10^6$ $(3.7 - 4.32) \cdot 10^6$	$6.62 \cdot 10^5$	$3.82 \cdot 10^6$ $(3.41 – 4.24) \cdot 10^6$	$8.87 \cdot 10^5$
2	$3.83 \cdot 10^6$ $(3.65 - 4.01) \cdot 10^6$	$3.85 \cdot 10^5$	$3.9 \cdot 10^6$ $(3.56 – 4.24) \cdot 10^6$	$7.32 \cdot 10^5$
3	$4.29 \cdot 10^6$ $(4.08 - 4.50) \cdot 10^6$	$4.44 \cdot 10^5$	$3.56 \cdot 10^6$ $(3.31 – 3.81) \cdot 10^6$	$5.36 \cdot 10^5$
4	$3.99 \cdot 10^6$ $(3.84 - 4.14) \cdot 10^6$	$3.18 \cdot 10^5$	$3.64 \cdot 10^6$ $(3.43 – 3.85) \cdot 10^6$	$4.57 \cdot 10^5$
5	$3.99 \cdot 10^6$ $(3.76 - 4.23) \cdot 10^6$	$5.08 \cdot 10^5$	$3.88 \cdot 10^6$ $(3.71 – 4.04) \cdot 10^6$	$3.46 \cdot 10^5$
6	$4.1 \cdot 10^6$ $(3.86 - 4.35) \cdot 10^6$	$5.24 \cdot 10^5$	$3.62 \cdot 10^6$ $(3.39 – 3.85) \cdot 10^6$	$4.9 \cdot 10^5$
7	$4.13 \cdot 10^6$ $(3.94 - 4.33) \cdot 10^6$	$4.1 \cdot 10^5$	$4.06 \cdot 10^6$ $(3.88 – 4.24) \cdot 10^6$	$3.84 \cdot 10^5$
8	$4.18 \cdot 10^6$ $(3.97 - 4.39) \cdot 10^6$	$5.19 \cdot 10^5$	$3.85 \cdot 10^6$ $(3.51 – 4.2) \cdot 10^6$	$8.5 \cdot 10^5$
Kruskal-Wallis test	$\chi^2 = 12.607$ $P = 0.082$		$\chi^2 = 12.756$ $P = 0.078$	

Formulation	Animal species	Groups	STH	Week	Nr Eggs analyzed		% VE (x ± SD)		% VR		% SCI	
					CP1	PRG	CP1	PRG	CP1	PRG	CP1	PRG
Gelatin	Puppies *Canis familiaris* Linnaeus, 1758	3	*Toxocara canis*	0	2043	2066	97 ± 3	96 ± 2	-	-	-	-
				1	1946	1958	95 ± 2	92 ± 3	2	4	-	-
				2	1919	1923	93 ± 3	81 ± 5	4	15	4	-
				3	1932	1874	91 ± 3	66 ± 9	6	31	59	21
				4	1882	1854	90 ± 4	48 ± 9	7	50	70	20
				Week	CP2	PRDG	CP2	PRDG	CP1	PRDG	CP2	PRDG
	Puppies *Canis familiaris* Linnaeus, 1758	2	*Toxocara canis*	0	1322	1320	97 ± 2	95 ± 2	-	-	-	-
				1	1354	1268	95 ± 3	91 ± 2	2	5	-	-
				2	1276	1265	93 ± 5	83 ± 4	4	13	4	-
				3	1292	1259	92 ± 2	67 ± 8	4	30	63	18
				4	1270	1254	90 ± 5	49 ± 8	7	48	73	20
Dry gelatin				Week	CB	BRDG	CB	BRDG	CB	BRDG	CB	BRDG
	Baboons *Papio hamadryas* Linnaeus, 1758	3	*Trichuris* sp.	0	2008	2079	97± 2	96 ± 3	-	-	-	-
				1	1970	1931	95 ± 3	89 ± 4	2	7	-	-
				2	1930	1935	92 ± 4	83 ± 3	5	13	-	-
				3	1894	1866	90 ± 3	69 ± 5	6	27	18	-
				4	1864	1851	89 ± 5	44 ± 6	8	54	26	5

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: