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Abstract: Thirty-two Friesian cattle under a leaders/followers four-day rotation and passing eggs of trematodes and gastrointestinal nematodes (GIN) were studied in two trials for the integrated control of these helminths over two years. In the first trial, the effect of rotational pasturing was assessed on a group of leaders (milking cows, G-L1) and followers (dried-off cows and heifers, G-F1) supplemented daily with commercial nutritional pellets. In the second trial, leaders (G-L2) and followers (G-F2) were maintained under a rotational pasturing regime; the cows received daily commercial pelleted feed and heifers pellets manufactured with a blend of parasiticide fungi (3x105 chlamydospores of both Mucor circinelloides and Duddingtonia flagrans/kg pellet). Deworming via closantel and albendazole was performed in cows in each trial at the beginning of their drying periods, and fourteen days later, the fecal egg-count reductions (FECR) of Calicophoron daubneyi and GIN were from 94-100% (average 98%), while the percentages of reduction of cattle shedding eggs (CPCR) were from 50-100% (average 77% and 82%, respectively). The heifers were dewormed one time only, at the beginning of each trial, and the values of FECR and CPCR were 100% against C. daubneyi and 96% and 83%, respectively, against GIN. Over a period of 24 months, significantly higher numbers of helminth egg-output were observed in G-L1, with the lowest numbers in G-F2. C. daubneyi egg output was reduced by 5% (G-L1) and 42% (G-F1) at the end of trial 1 and by 83% (G-L2) and 100% (G-F2) at the end of trial 2; the numbers of GIN egg-output decreased by 13% (G-L1) and 18% (G-F1) at the end of trial 1, and by 72% (G-L2) and 85% (G-F2) at the end of trial 2. No adverse effects were detected in cattle taking pellets enriched with fungal spores (G-F2). It is concluded that long-term ingestion of spores of M. circinelloides and D. flagrans provides a valuable tool to improve the effect of rotational grazing and to lessen the risk of infection by C. daubneyi and GIN in

dairy cattle, and accordingly, the performance of integrated control programs.

Highlights

- Deworming only is insufficient to control helminths affecting grazing cattle
- Control of helminths in grazing cows needs to integrate deworming plus prevention
- Long pasture resting periods are needed to prevent helminth infection in grazing ruminants
- Daily ingestion of fungal chlamydospores improves the usefulness of rotational grazing

INTEGRATING THE CONTROL OF HELMINTHS I~~O~~N DAIRY CATTLE: DEWORMING, ROTATIONAL GRAZING AND NUTRITIONAL PELLETS WITH PARASITICIDE FUNGI

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Abstract

Thirty-two Friesian cattle under a leaders/followers four-day rotation and passing eggs of trematodes and gastrointestinal nematodes (GIN) were studied in two trials for the integrated control of these helminths over two years. In the first trial, the effect of rotational pasturing was assessed on a group of leaders (milking cows, G-L1) and followers (dried-off cows and heifers, G-F1) supplemented daily with commercial nutritional pellets. In the second trial, leaders (G-L2) and followers (G-F2) were maintained under a rotational pasturing regime; the cows received daily commercial pelleted feed and heifers pellets manufactured with a blend of parasiticide fungi ($3x10^5$ chlamydospores of both *Mucor circinelloides* and *Duddingtonia flagrans*/kg pellet). Deworming via closantel and albendazole was performed in cows in each trial at the beginning of their drying periods, and fourteen days later, the fecal egg-count reductions (FECR) of *Calicophoron daubneyi* and GIN were from 94-100% (average 98%), while the percentages of reduction of cattle shedding eggs (CPCR) were from 50-100% (average 77% and 82%, respectively). The heifers were dewormed one time only, at the beginning of each trial, and the values of FECR and CPCR were 100% against *C. daubneyi* and 96% and 83%, respectively, against GIN. Over a period of 24 months, significantly higher numbers of helminth egg-output were observed in G-L1, with the lowest numbers in G-F2. *C. daubneyi* egg output was reduced by 5% (G-L1) and 42% (G-F1) at the end of trial 1 and by 83% (G-L2) and 100% (G-F2) at the end of trial 2; the numbers of GIN egg-output decreased by 13% (G-L1) and 18% (G-F1) at the end of trial 1, and by 72% (G-L2) and 85% (G-F2) at the end of trial 2. No adverse effects were detected in cattle taking pellets enriched with fungal spores (G-F2). It is concluded that long-term ingestion of spores of *M. circinelloides* and *D. flagrans* provides a valuable tool to improve the effect of rotational grazing and to lessen the risk of infection by *C. daubneyi* and GIN in dairy cattle, and accordingly, the performance of integrated control programs.

The control of helminths among dairy cattle under pasturing regimes presents serious difficulties due to the presence of infective stages in the soil ensures their infection. Thirty-two Friesian cattle managed under a leaders/followers four-day rotation and passing eggs of trematodes and gastrointestinal nematodes (GIN) were involved in two assays for the integrated control of these helminths. In the first assay, leaders (milking cows, G-L1) and followers (heifers, G-F1) were supplemented daily with commercial nutritional pellets; in the assay, the leaders (milking cows, G-L2) received daily commercial pelleted feed also whereas the followers (heifers, G-F2) fed on pellets manufactured with a blend of parasiticide fungi ($3x10^5$ spores of *Mucor circinelloides* and $3x10^5$ of *Duddingtonia flagrans* / Kg pellet). Deworming consisting of closantel + albendazole was administered to all heifers (G-F1 and G-F2) at the beginning of each assay, and cows (G-L1 and G-L2) during the dry periods. The fecal egg count reductions (FECR) of *Calicophoron daubneyi* and GIN were 94-100% (average 98%), while the percentages of reduction of cattle shedding helminth eggs were 50-100% (average 77% and 82%, respectively). During a period of 24 months, significantly higher numbers of helminths egg-output were observed in G-L1, and the lowest in G-F2. At the end of the trial, the values of *C. daubneyi* egg-output in assay 1 reduced by 5% (G-L1) and 42% (G-F1), whereas in the assay 2 diminution was of 83% (G-L2) and 100% (G-F2); the numbers of GIN egg-output in assay 1 decreased by 13% (G-L1) and 18% (G-F1), and in assay 2 by 72% (G-L2) and 88% (G-F2). Heifers in G-F2 never refused to take the pellets enriched with fungal spores, and no adverse effects were detected. It is concluded that long-term ingestion of spores of *M. circinelloides* and *D. flagrans* provides a valuable tool to improve the effect of rotational grazing and lessen the risk of infection by *C. daubneyi* and GIN in grazing dairy cattle. Manufacturing of nutritional pellets with spores of parasiticide fungi enhances their distribution without adding additional tasks to livestock keepers, and accordingly, the performance of integrated control programs.

Key words: biological control, ruminants, *Mucor circinelloides*, *Duddingtonia flagrans*, chlamydospores, sustainability

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Introduction

The numbers of grazing cattle have increased markedly, partly because this is a cost-effective regime, and partly because of the preoccupation on animal welfare in food production (Thornton, 2010). Trematodes belonging to Fasciolidae and Paramphistomidae and can frequently affect ruminants on humid habitats, where appropriate conditions are present for the development of the external phase in their life cycle (Szmidt-Adjidé et al., 2000). Gastrointestinal nematodes (GIN) are helminths that often infect grazing ruminants through the ingestion of third larvae (the infective stages) (Smith & Sherman, 2009; Craig, 2009).

Control of helminths in dairy cattle becomes especially difficult because deworming is only allowed during the dry period, which occurs consecutively on farms throughout the year. Therefore, treated and untreated cows can share the same pastures, and untreated ruminants continue to shed eggs in the feces, increasing the risk of all cows ingesting infective stages of helminths (e.g., eggs, larvae) while feeding on grass.

With the aim to diminish the presence of infective stages in the soil, rotational grazing regimes characterized by ruminants feeding on grass for a time interval (grazing period), followed by other rest periods, are advised (Undersander et al., 2002). In the last years, biological strategies for the control of helminths based on the use of certain soil saprophytic fungi have been reported. Some species (*Duddingtonia flagrans*, *Monacrosporium thaumassium*) are able to trap the infective stages of GIN (Araújo et al., 2004), and others can produce antagonistic effects on helminth eggs as trematodes, cestodes and nematodes (*Pochonia chlamydosporia*, *Mucor circinelloides*, *Purpureocillium lilacinum*) (Cazapal-Monteiro et al., 2015; Cortiñas et al., 2015); they can also act against the miracidia of *C. daubneyi* (Arroyo et al., 2017). Former studies have stated the usefulness of providing cattle chlamydospores in water solutions (Assis et al., 2013; Silva et al., 2014; Saumell et al., 2015), mass mycelia of *D. flagrans* or *M. thaumassium* in alginate pellets (Dias et al., 2018), or as a blend of spores of *D. flagrans* and *M. circinelloides* in industrially manufactured pellets (Hernández et al., 2016). Recently, a commercial formulation containing chlamydospores of *D. flagrans* was registered (Healey et al., 2018). The efficacy and safety of combining rotation of grasslands with providing pellets enriched with chlamydospores of *M. circinelloides* and *D. flagrans* were successfully tested on horses (Hernández et al., 2018a).

In the current investigation, the helpfulness of the integrated control of helminths among dairy cattle based on anthelmintic treatment, pasture rotation and feeding on pellets manufactured with chlamydospores of *M. circinelloides* and *D. flagrans* was analyzed.

The numbers of pasturing cattle have increased markedly, partly due to this is a cost-effective regime, and partly to the preoccupation on animal welfare in food production (Thornton, 2010). Helminths as trematodes belonging to Fasciolidae and Paramphistomidae can frequently affect ruminants grazing on humid habitats, where concur appropriate conditions for the development of the external phase in their life-cycle, characterized by an embryo called miracidium origins inside the eggs, exits off and swims to infect the intermediate hosts, amphibious snails of the genera *Lymnaea*, *Planorbis*, *Indorbis* or *Fossaria* (Szmidt-Adjidé et al., 2000). After completing different phases, the cercariae released from the snails become into metacercariae, the infective stages. Gastrointestinal nematodes (GIN) are helminths often infecting pasturing ruminants; eggs passed in the feces of infected hosts develop a first-stage larva (L1) inside, exits off and feeds on organic matter, then moult into a second larva, which also feeds and transform into a third larva (the infective stage); this process takes 0.5-2 months (Smith & Sherman, 2009; Craig, 2009).

Control of helminths in dairy cattle becomes especially difficult due to deworming is only allowed during the dry period, which occurs consecutively on farms throughout the year. Therefore, treated and untreated cows can share the pastures, and untreated ruminants remain shedding eggs in the feces, increasing the risk of all cows ingest infective stages of helminths (eggs, larvae, cysts…) while feeding on grass.

Rotational pasturing regimes characterized by ruminants feeding on grass for a time interval (grazing period), followed by other rest period, are advised for diminishing the presence of infective stages in the soil (Undersander et al., 2002). In the last years, there have been reported soil saprophytic fungi with larvicidal activity (*Duddingtonia flagrans*, *Monacrosporium thaumassium*) (Araújo et al., 2004), or ovicidal effect (*Pochonia chlamydosporia*, *Mucor circinelloides*, *Purpureocillium lilacinum*) (Cazapal-Monteiro et al., 2015; Cortiñas et al., 2015). Few investigations stated their usefulness on cattle, focused on strongyles or trematodes separately (Assis et al., 2013; Silva et al., 2014; Saumell et al., 2015). Former studies showed successful results by means of mass mycelia of *D. flagrans* or *M. thaumassium* in alginate pellets (Dias et al., 2018), or as a blend of spores of *D. flagrans* and *M. circinelloides* in industrially manufactured pellets (Hernández et al., 2016). Recently, a commercial formulation containing chlamydospores of *D. flagrans* has been registered (Healey et al., 2018). The efficacy and safety of combining rotation of grasslands with providing pellets enriched with spores of *M. circinelloides* and *D. flagrans* were successfully tested on horses (Hernández et al., 2018a).

In the current investigation, the usefulness of the integrated control of helminths among dairy cattle based on anthelmintic treatment, pasture rotation and feeding on pellets manufactured with spores of *M. circinelloides* and *D. flagrans* has been analyzed.

Material and methods

Area of study

The present research was carried out in Galegos (Riotorto, Lugo, NW Spain, 43º 15' 43.9" N - 7º 12' 36.0" W), an agricultural and mountainous area (250-734 m altitude) under an oceanic climate, which covers ca. 16 km^2 crossed by profuse streams watering the grasslands and is mostly dedicated to dairy farming and forestry production (pine and eucalyptus).

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Fungal chlamydospores

A submerged culture (COPFr) was used to obtain chlamydospores of *M. circinelloides* and *D. flagrans* simultaneously (Arias et al., 2013a). The concentration was adjusted to $1x10^8$ chlamydospores of each fungus/L before manufacturing the pelleted feed.

A submerged culture (COPFr) was used to obtain simultaneously chlamydospores of *Mucor circinelloides* and *Duddingtonia flagrans* (Arias et al., 2013a). The concentration was adjusted to $1x10^8$ spores of each fungus / L before manufacturing the pelleted feed.

Addition of fungal chlamydospores to nutritional pellets

Pelleted feed (NANFOR, Begonte, Lugo, Spain) containing corn, dehulled soya flour, barley, sunflower seed flour, soya hulls, rape seed flour, calcium carbonate, palm kernels as expeller, sugarcane molasses and sodium chloride was used in the current study. The analytical composition includes crude protein (20.5%), crude fat (2.3%), crude fiber (9.3%), ash content (7.9%), calcium (1.6%), phosphorus (0.55%), sodium (0.32%), magnesium (0.54%), vitamin A (20000 UI/kg), vitamin D3 (2750 UI/kg) and vitamin E (45 UI/kg).

According to previous investigations (Hernández et al., 2016), a blend of $3x10^5$ chlamydospores of *M. circinelloides* and $3x10^5$ chlamydospores of *D. flagrans* was added per kg of feedstuff during the mixing phase of the elaboration of the pellets, after which the feedstuff was conditioned by an injecting steam (75 ºC for 90 s) and passed through the pelletizer (Arroyo et al., 2016; Hernández et al., 2018a). The final product was cooled, dried, packed into 40-kg bags, and stored for a minimum of three months at room temperature, avoiding direct sunlight.

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According to previous investigations (Hernández et al., 2016), a blend of $3x10^5$ spores of *M. circinelloides* + $3x10^5$ spores of *D. flagrans* was added per Kg of feedstuff during the mixing phase of the elaboration of the pellets, then conditioned by injecting steam (75ºC for 90 seconds) and lastly passed through the pelletizer (Arroyo et al., 2016; Hernández et al., 2018a). The final product was cooled, dried and packed into 40 Kg bags, and stored for a minimum of three months at room temperature, avoiding direct sunlight.

Cattle management

Friesian cattle reared in the current farm are stabled overnight and graze on perennial ryegrass and white clover during the daytime, under a leaders/followers rotational scheme consisting of the most-needing cattle (leaders: milking cows) taking the best feed, followed four days later by the individuals with lower nutritional requirements (followers: dried cows and heifers) (Fig. 1). With the aim to ensure that pasture plants can recover and regrow, the leaders do not come back to the same paddock after two months (pasture resting period). Hay is given when green forage is insufficient, from December to February. All cattle receive 2.5 kg commercial pelleted feed daily, distributed in the morning and in the night, and corn ensilage.

Every month, four cows are dried off, dewormed and managed as followers (with the heifers) until they calve (after approximately two months). Calving occurs year round, and calves are removed from the dam soon after birth and reared outdoors until they are weaned as heifer replacements.

Cattle grazed on perennial ryegrass and white clover during the daytime by following a four-day rotation scheme (Fig. 1) and were stabled overnight. Hay was given when green forage was insufficient, from December to February. All cattle received daily 2.5 Kg commercial pelleted feed distributed in the morning and in the night, and corn ensilage.Calving happens all year round and calves are removed from the dam soon after birth and reared outdoors until weaning as heifer replacements. Every month, four cows are dried and dewormed (Table 1), and managed with the heifers until they calve.Calving happens all year round and calves are removed from the dam soon after birth and reared outdoors until weaning as heifer replacements.

Experimental scheme

Over two years, Friesian cattle were maintained into two trials (Table 1).

~~During two years, Friesian cattle (20 cows and 12 heifers) were maintained under a leaders/followers system consisting of milking ruminants (Leaders) pastured ahead of the dried-off cows and heifers (Followers) (Fig. 1).~~

G-L1 (leaders): ten milking cows (4-6 yr) were grazed on a paddock during four days, after which they were moved to other pasture. Commercial pellets were given daily. Every month, two cows were dried off, dewormed (see below) and maintained with the followers (G-F1) until calving (approximately two months later).

G-F1 (followers): eight cattle (heifers and dried off cows) grazed a paddock four days after the leaders grazed. a) Six heifers (12-14 mo) received, at the beginning of the trial only, closantel (10 mg/kg bodyweight (bw) ENDOEX, SP Veterinaria, Spain) and albendazole (7.5 mg/kg bw ALBENDEX, SP Veterinaria, Spain); b) cows were treated at the start of their respective drying periods with closantel and albendazole as explained above. Supplementation consisting of commercial pellets was provided daily.

Trial 2: analyzing the effect of rotational grazing and the intake of pellets with fungal chlamydospores.

G-L2 (leaders): ten milking cows (4-6 yr) fed on a paddock for four days, after which they were brought to another pasture. Every month, two cows were dried off, dewormed and maintained in the group of followers (G-

F2) until calving (approximately two months). Commercial pellets were given daily.

G-F2 (followers): eight cattle (heifers and dried off cows) grazed on a paddock four days after the leaders. a) Six heifers (12-14 mo) were treated one time only with closantel and albendazole as previously described at the beginning of the trial only; supplementation was given daily, consisting of commercial pellets manufactured with $3x10^5$ chlamydospores of *M. circinelloides* and $3x10^5$ *D. flagrans* per kg of pelleted feed. b) Cows were dewormed at the start of the drying period with closantel and albendazole as explained above. Commercial pellets (without fungal chlamydospores) were provided daily.

A total of thirty different areas pastured by cattle for more than 7 years, with flooded zones, were randomly divided into two lots of 15 each and were used for every trial. As explained before, a grazing interval of four days and a resting period of two months were observed. Accordingly, the pastures were completely rotated after two months in each trial (Fig. 1). It is important to note that every group of ruminants fed on the same 15 grasslands throughout the study, which were never shared by cattle belonging to the different trials.

Assay 1: assessing the effect of rotational pasturing.

G-L1 (Leaders): ten milking cows (4-6 yr) which had been dewormed with closantel (10 mg / Kg bw ENDOEX, SP Veterinaria, Spain) + albendazole (7.5 mg / Kg bw ALBENDEX, SP Veterinaria, Spain) at least two months before the experiments started.

G-F1 (Followers): six heifers (12-14 mo) treated with closantel + albendazole.

Two cows from the G-L1 were dried-off monthly, treated at the beginning of the drying period and maintained in the G-F1 group until calving (during two months approximately), when they returned to the G-L1.

Assay 2: analyzing the effect of the intake of pellets with fungal spores + rotational grazing.

G-L2 (Leaders): ten milking cows (4-6 yr), dewormed with closantel + albendazole at least two months before.

G-F2 (Followers): six heifers (12-14 mo) treated with closantel + albendazole and supplemented with spore-inoculated commercial pellets at a dosage (per Kg) of $3x10^5$ spores of *M. circinelloides* and $3x10^5$ *D. flagrans*.

Every month, two cows from the G-L2 were dried-off, dewormed (as described above) and maintained in the group of G-F2 until calving (for about two months), when they were moved to the G-L2 again.

A total of thirty different areas pastured by cattle since more than 7 years, with flooded zones, were randomly divided into two lots of 15 each which were used for every assay. The scheme consisted of a grazing period of four days and a resting period of two months. Accordingly, the pastures were complete rotated after two months in each assay (Fig. 1). It is important to note that every group of ruminants fed on the same 15 grasslands throughout the study, which were never shared by cattle belonging to the different assays.

Coprological probes

Fecal samples were collected monthly from the rectum of each ruminant and divided into two subsamples of five grams; one was analyzed through a flotation probe with saturated NaCl solution (specific gravity= 1.20) (Sensitivity= 10 eggs per gram feces, EPG) and the other by using the sedimentation test (Se= 10 EPG) (Francisco et al., 2009). The results are expressed as EPG.

For the purpose of identifying the gastrointestinal nematodes infecting cattle, feces were individually collected at the beginning of the trial; pools containing five grams from every ruminant were prepared for each group and incubated over 17 days at 22-25°C according to Hernández et al. (2016). The Baermann technique was applied to collect the third-stage larvae (L3), and identification was performed under an optical microscope employing morphological keys (van Wyk & Mayhew, 2013).

~~Every month, fecal samples were collected individually from the rectum of each ruminant and two subsamples of five grams were prepared; one was analyzed through the flotation probe with saturated NaCl solution (ρ= 1.20 g / dL) (Sensitivity= 30 eggs per gram feces, EPG) and the other by using the sedimentation test (Se= 50 EPG) (Sanchís et al., 2013; Hernández et al., 2016). Results were expressed as EPG.~~

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Evaluation of the efficacy

With the objective of assessing the efficacy of deworming, fecal samples were individually collected from the rectum of each ruminant at days 0 and 14 after treatment and analyzed by means of the flotation and sedimentation probes. The reductions in fecal egg counts (FECR) and in the numbers of cattle positive by coprological tests (CPCR) were calculated according to the following formulae (Arias et al., 2013b):

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

$$CPCR\ (\%) = [1 - (\text{number of positive cattle}_{post\text{-}treatment} / \text{positive cattle}_{pre\text{-}treatment})] \times 100$$

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$$CPCR\ (\%) = [1 - (\text{number of positive cattle}_{post\text{-}treatment} / \text{positive cattle}_{pre\text{-}treatment})] \times 100$$

Side effects

Heifers provided pellets enriched with chlamydospores of parasiticide fungi (G-F2) were examined for the possibility of signs reflecting damage or disorders affecting the reproductive, digestive or respiratory systems. The skin and oral mucosa were also carefully scrutinized for the presence of disorders.

Because the commercial pelleted feed has an expiration period of three months, pellets manufactured with the chlamydospores were stored for longer and then examined for abnormal odor, color, consistency or appearance. Attention was also paid to pellets showing fungal growth on the surface.

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Welfare and ethics

The authors confirm that legal and ethical requirements were met regarding the humane treatment of animals described in the study. The ethical review committee from the University of Santiago de Compostela (Spain) approved the current study, which fulfils the national and international guidelines for animal research.

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Statistical analysis

According to the Kolmogorov-Smirnov test, data were not normally distributed (Z= 4.672, *P*= 0.001 for the trematodes egg-output, and Z= 2.852, *P*= 0.001 for the GIN). By means of Levene's test, homogeneity of variances was excluded (Z= 27.241, *P*= 0.001 for the trematodes egg-output, and Z= 30.171, *P*= 0.001 for the GIN). The non-parametric tests Kruskal-Wallis and Mann-Whitney U were then performed at a significance level of $P < 0.05$ (Thrusfield, 2007).

The values of FECR and CPCR are shown as percentages and 95% confidence intervals. The kinetics of the egg-output are represented as the means and ranges (defined as the difference between the maximum and minimum values). All the tests were carried out using the statistical package SPSS, version 20 (IBM SPSS Inc., Chicago, IL, USA).

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The values of FECR and CPCR were shown as the percentage and the 95% confidence interval. The kinetics of the egg-output was represented as the mean and the range. All the tests were carried out by using the statistical package SPSS, version 20 (IBM SPSS Inc., Chicago, IL, USA).

Results

Helminths identified

Eggs of trematodes and gastrointestinal nematodes (GIN) were detected in the feces of all cattle at the beginning of the trial. Trematodes were identified as *Calicophoron daubneyi*; third stage larvae belonging to the genera *Trichostrongylus*, *Ostertagia*, *Chabertia*, *Bunostomum*, *Cooperia* and *Oesophagostomum* were recovered from the fecal patscoprocultures.

Efficacy of deworming

As summarized in Table 1, during the respective drying periods, anthelmintic treatment was administered 48 times to the cows belonging to G-L1 and the same number to the cows belonging to G-L2.

Fourteen days after every treatment, the FECR values were 94-100% (average FECR: 98%) against *C. daubneyi* and GIN (Table 2), while the percentage of reduction of cattle shedding eggs was 50-100% (average CPCR: 77% and 82%, respectively).

The heifers in G-F1 and G-F2 were treated at the beginning of the study only, and fourteen days later, the efficacy resulted in 100% FECR and CPCR against *C. daubneyi* (Tables 3 & 4); all heifers in G-F1 were positive to the sedimentation by the 8th month after treatment (m.a.t.), whereas eggs of *C. daubneyi* were never observed in G-F2 after the administration of anthelmintics (Table 4). The average efficacies of albendazole against GIN were 96% FECR and 83% CPCR (Tables 3 & 4); all heifers were positive to the flotation test five months after treatment in G-F1 and by the 9th m.a.t. in G-F2 (Tables 3 & 4).

Cattle managed as leaders (G-L1 and G-L2) received anthelmintic treatment during the drying period, resulting in a total of 48 applications in G-L1, and the same in G-L2. The efficacy was 94-100% (average FECR: 98%) against *C. daubneyi* and GIN (Table 1), while the percentage of reduction of cattle shedding eggs was 50-100% (average CPCR: 77% and 82%, respectively).

Deworming of the followers (heifers belonging to G-F1 and G-F2) performed at the beginning of the study resulted in 100% FECR and CPCR against the rumen fluke (Tables 2 & 3); all heifers in G-F1 were positive to the sedimentation by the 8th month after treatment (m.a.t.), whereas eggs of *C. daubneyi* were never observed in G-F2 after the administration of anthelmintics (Table 3). The average efficacy of albendazole against GIN was 96% FECR and 83% CPCR (Tables 2 & 3); five months after treatment, all heifers in G-F1 were positive to the flotation test (0% CPCR), and by the 9th m.a.t. in G-F2.

Effect of rotational grazing

In the leaders of trial 1 (G-L1), the numbers of eggs of *C. daubneyi* did not decrease during the first three pasture rotations (sixth m.a.t.) (Fig. 2) and then diminished between 15% (four rotations) and 3% (seven rotations) (Z= 1.316, *P*= 0.059). In the followers (G-F1), the highest values of reduction (94-96%) were attained during the first two full rotations (fourth m.a.t.) and then decreased to 31-45% from the fifth rotation (10th m.a.t.) (Z= 7.098, *P*= 0.011).

The counts of GIN in G-L1 decreased by 3% (2nd rotation) and 13% (12th rotation) (Fig. 2) (Z= 1.305, *P*= 0.065). In G-F1, a reduction from 96% (first rotation) to 51% (fifth rotation) was obtained after the first five rotations were completed (eighth m.a.t.) and then dropped to 9-29% until the end of the trial (Z= 1.620, *P*= 0.011). Significant differences were observed between both groups regarding *C. daubneyi* (Z= -9.188, *P*= 0.001) and GIN (Z= -6.085, *P*= 0.001).

In assay 1, the numbers of eggs of *C. daubneyi* ranged from 350 to 264 EPG in the leaders (G-L1), and decreased by 15% after pasture rotation was fully completed four times (eight months) (Fig. 2), then values close to 10% were observed (Z= 1.316, *P*= 0.059). In the followers (G-F1), the numbers of *trematode* egg-output ranged from 331 to 13 EPG, and the highest values of reduction (94-96%) were attained after the first two full rotations (four months), then decreased to 30-40% (Z= 7.098, *P*= 0.011).

The counts of GIN in G-L1 oscillated between 330 and 215 EPG, lessening by -13% throughout the assay (Fig. 2) (Z= 1.305, *P*= 0.065). In G-F1, numbers ranging from 336 to 13 EPG were recorded; a reduction higher than 50% was obtained after the first four rotations were completed (eight months after treatment), then dropped to 12-27% until the end of the trial (Z= 1.620, *P*= 0.011). Significant differences were proven between both groups regarding the counts of eggs of *C. daubneyi* (Z= -9.188, *P*= 0.001) and GIN (Z= -6.085, *P*= 0.001).

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Effect of rotational grazing + and ingestion of fungal chlamydospores

In the leaders of trial 2 (G-L2) an important reduction in the eggs of *C. daubneyi* eggs was noted during the trial (Z= -4.348, *P*= 0.001), with values between 4% (first rotation) and 56% (sixth rotation, 12th m.a.t.), which increased to 83% at the end of the trial (12 rotations, 24th m.a.t.) (Fig. 3). In the followers (G-F2), the values of *C. daubneyi* egg-output dropped by 100% after the administration of successful deworming, and eggs were never detected from that point forward.

Regarding the GIN, percentages of reduction higher than 50% were achieved in the G-L2 from the 3rd rotation (sixth m.a.t.) and > 80% from the 9th rotation (18th m.a.t.) (Fig. 3) (Z= -3.040, *P*= 0.001). In the G-F2, significant reductions were noted throughout the trial, with percentages of 78-93% (Z= -2.683, *P*= 0.001). Significant differences between the two groups of ruminants were observed for *C. daubneyi* (Z= -13.880, *P*= 0.001) and GIN (Z= -5.974, *P*= 0.001).

In the leaders of assay 2 (G-L2) excretion of eggs of the rumen fluke fluctuated between 343 and 51 EPG, and an important reduction was noted along the assay (Z= -4.348, *P*= 0.001), with values up to 56% after six rotations (12 months) were completed, and 83% at the end of the assay (12 rotations, 24 months) (Fig. 3). In the followers (G-F2), ruminants receiving pellets with fungal spores, the values of *C. daubneyi* egg-output dropped by 100% (from 334 to 0) and eggs were not detected since that timeafter the administration of successful deworming.

Numbers of GIN between 321 and 36 EPG were recorded in the leaders (G-L2), and between 329 and 14 EPG in G-F2. Percentages of reduction higher than 50% were recorded in the G-L2 from the 3rd rotation (six months), and 80% from the 9th rotation (18 months) (Fig. 3) (Z= -3.040, *P*= 0.001). In the G-F2, a significant reduction was noted throughout the assay, with percentages of 78-90% (Z= -2.683, *P*= 0.001). Statistical differences in the numbers of eggs of *C. daubneyi* (Z= -13.880, *P*= 0.001) and GIN (Z= -5.974, *P*= 0.001) between the two groups of ruminants were observed.

Comparison between the two assaystrials

The lowest values of reduction of eggs of *C. daubneyi* and GIN were observed in the leaders of trial 1 (G-L1) and were highest in the followers of trial 2 (G-F2) (Figs. 2 & 3).

Significantly higher values of *C. daubneyi* FECR were observed in the leaders of trial 2 (G-L2) than those in trial 1 (G-L1) (Z= -7.329, *P*= 0.001), especially from the fifth rotation, when percentages 7.5 times higher than those of G-L1 were observed (Table 5). Values of GIN FECR 2.8 to 31.1 times higher in G-L2 than in G-L1 were recorded, mainly from the third rotation (6th m.a.t.) (Z= -10.382, *P*= 0.001) (Table 5).

Regarding the followers, eggs of *C. daubneyi* were never detected in G-F2 after their deworming at the beginning of the study. From the fourth rotation, the values of GIN FECR in G-F2 were 1.5 – 8.7 times higher than in G-F1 (Z= -9.536, *P*= 0.001).

The highest values of eggs of *C. daubneyi* and GIN were observed in the leaders of assay 1 (G-L1) and the lowest in the followers of assay 2 (G-F2). Significantly lower values of *C. daubneyi* egg-output were recorded in G-L2 than in G-L1 (Z= -7.329, *P*= 0.001), especially from the sixth rotation, when numbers half than those of G-L1 were observed (Table 4). In G-F2, eggs of the rumen fluke were not detected till the end of the assay.

The EPG values of GIN in G-L2 were significantly lower than in G-L1 (Z= -10.382, *P*= 0.001), and counts were half or less than in G-L1 from the third rotation (6th m.a.t.) (Table 4). In the G-F2, EPG values halved with regard to G-F1 from the second rotation (Z= -9.536, *P*= 0.001).

Analysis of adverse effects in the cattle receiving chlamydospores of parasiticide fungi

The pellets manufactured with the parasiticide chlamydospores were normally taken by the heifers in G-F2;, and rejection was not detected at any time. Clinical signs concerning the digestive system or respiratory apparatus were not recorded, and heifers cycled normally. No alterations affecting the skin or fur were identified.

The analysis of pellets manufactured with chlamydospores of *M. circinelloides* and *D. flagrans* and stored for longer than three months revealed the absence of abnormal odor, consistency or physical appearance, nor hyphal growth on the surface.

Discussion

Infections by the ruminal fluke *C. daubneyi* and gastrointestinal nematodes (GIN) were detected among two groups of dairy Friesian cattle managed under a leaders/followers rotational grazing regime. Accordingly, closantel and albendazole were administered to the cows throughout the study during the corresponding drying periods, whereas the heifers were dewormed once at the beginning of each trial only. The anthelmintic treatment was successful, in concordance with data collected by administering the salicylanilide against the rumen fluke (Rolfe & Boray, 1993; Arias et al., 2013b), although a lack of or reduced efficacies have been reported (Malrait et al., 2015; Nzalawahe et al., 2018). A similar efficacy of albendazole on GIN was reported previously (Demeler et al., 2009).

Resting periods (weeks - months) are observed in rotational pasturing regimes to allow the pasture to regrow (Flack, 2016). It has been noted that this interval could also be useful to reduce the survival of infective stages in the soil (Chandrawathani et al., 2004). In the current study, a pasture resting period of two months was maintained over an interval of 24 months, with the most-needing cattle (leaders: milking cows) taking the best feed, then replaced four days later by individuals with lower nutritional requirements (followers: dried cows and heifers). In the leaders, a slight reduction in the number of *C. daubneyi* eggs was noted, whereas the number of eggs in the followers decreased markedly (≥ 94%) after completing two rotations (four months after treatment), then by one-third to half until the end; the values of GIN egg-output showed minor variations in the leaders but halved in the followers after four rotations and then increased. Despite the fact that beneficial effects have been reported by feeding first season grazing calves for a period of 20 weeks on grasslands fed previously by second-season grazing animals (Larsson et al., 2006), a high increment of the counts of GIN EPG was recorded in grazing cattle a few weeks after turnout, followed by a slow progressive decrease (Nogareda et al., 2006). In the present study, the control of parasites involved the deworming of the heifers at the beginning of the trial only; once the leaders attained the drying period, they were also treated and managed as followers (together with the heifers) for two months, after which they were brought back to the leader group. Because anthelmintic treatment cannot be simultaneously administered to the leaders, the presence of infected cattle (milking cows) seemed to maintain the contamination of the grasslands. Four days later, the followers (successfully dewormed at the beginning of the trial) fed on a pasture with low levels of infective stages ("cleaned" by the action of the leaders). It should be noted that the time elapsed (four days) is insufficient for new infective parasites to originate from the eggs excreted previously by the leaders; however, new infective stages can appear after two months, improving the possibility of the leaders becoming infected when returning to the prairie. Hence, this rotational pasturing regime benefits the followers during the first rotations only. The eggs or metacercariae of trematodes may remain viable up to several months, and larvae of GIN for one to three months in tropical areas or six to twelve months in temperate zones (Torres-Acosta & Hoste, 2008; Moazeni et al., 2010). Accordingly, pasture resting periods of approximately three to six months have been determined to be helpful in diminishing the risk of infection (Craig, 2009; Kumar et al., 2013).

On considering the difficulty and low likelihood of using resting periods longer than three months because large numbers of pasturing areas would be needed, another interesting approach might include avoiding the onset and survival of infective stages in the feces and soil. Prior investigations have reported that the chlamydospores of *M. circinelloides* and *D. flagrans* are able to survive the mechanized production of pellets without losing their biological activity; the analysis of pellets demonstrated that the chlamydospores retained their antagonistic effect without changes beyond 6 months (Arias et al., 2015; Arroyo et al., 2016). Because dairy cows are commonly supplemented with nutritional pelleted feed, in the present study, a second trial involved the supplementation of a group of heifers in the followers with nutritional pellets manufactured with a blend of chlamydospores ($3x10^5$/kg pelleted feed) of two soil saprophytic filamentous fungi: *Mucor circinelloides* (ovicide) and *Duddingtonia flagrans* (larvicide) (Hernández et al., 2018a). The fact that the numbers of *C. daubneyi* eggs in the feces of the leaders dropped by 56% after six rotations and by 83% by the end of the trial, together with their absence in the followers after their successful treatment, suggests that the possibility of infection with snails acting as intermediate hosts decreased significantly; therefore, cercariae are not released, and metacercariae do not emerge. Moreover, though metacercariae are highly resistant in the environment, their numbers seem to be reduced by the action of the group of leaders feeding four days earlier than the followers. Regarding the GIN, at the end of the trial (24 months), the fecal counts diminished by 78% in the leaders and 85% in the followers, which appears to indicate a significant reduction in the contamination levels of the grasslands; likewise, numbers ≤100 EPG were recorded in the followers until the end of the research, so the need of additional deworming was discarded. Previous studies on grazing cattle given mycelium of the ovicide fungus *Pochonia chlamydosporia* for 18 months reported a 67% reduction of the eggs of *Fasciola hepatica* passed in the feces (Dias et al., 2013). In spite of the absence of significant differences regarding the GIN egg-output values (Dimander et al., 2003), the numbers of third-stage larvae (L3) of GIN decreased by 73.8% in the feces of cattle orally given chlamydospores of *D. flagrans* and by 58-73.5% in pasturing zebu (Ortiz et al., 2017; Mendoza de Gives et al., 2018). It has also been demonstrated that the daily administration of *D. flagrans* chlamydospores to cattle decreased the trichostrongylid larval population on grass by 61-97% (Grønvold et al., 1993; Hertzberg et al., 2007; Jobim et al., 2008). More recently, significant reductions of the numbers of GIN infective larvae on pasture 6-8 weeks after cattle received chlamydospores of *D. flagrans* have been reported (Healey et al., 2018).

Herbivores are routinely exposed to several helminth species while pasturing, and combined actions are required against different infective stages, eggs or larvae. In the current study, significant reductions in the egg-output values of *C. daubneyi* and GIN were recorded by giving daily fungal chlamydospores to the heifers belonging to the group of followers, with respect to those maintained under rotational pasturing only and receiving commercial pellets without chlamydospores (trial one). Additionally, a significant decrease was also established in the leaders of the second trial regarding those in the first trial. These results are in agreement with data collected in horses maintained under rotational grazing and given pellets enriched with fungal chlamydospores, where the counts of strongyle eggs were reduced by two thirds in relation to those kept under rotational pasturing only (Hernández et al., 2018a).

Most of the previous experiences regarding the administration of parasiticide fungi to cattle encompassed the use of high amounts of *D. flagrans* chlamydospores focused on the control of gastrointestinal nematodes and, to a lesser extent, of *Pochonia chlamydosporia* against liver trematodes (Assis et al., 2013; Dias et al., 2013; Ortiz et al., 2017). The presence of different infective stages in the soil and herbage requires a combined action. In the present study, chlamydospores of an ovicide fungus and other larvicide species were added during the manufacturing of pelleted feed, confirming that this is a very advantageous strategy to achieve a very suitable formulation for spreading in the feces/soil (Hernández et al., 2016). The dosage and frequency of providing fungal chlamydospores are critical points to consider when performing biological control measures. Administration two to three days a week, or even daily, guarantees their regular presence in feces and the substantial diminution of helminth infective stages (Santurio et al., 2009; Dias et al., 2013; Mendoza de Gives et al., 2018). This allows the doses of chlamydospores to be decreased and improves their utilization, as lately highlighted (Hernández et al., 2016, 2018a; Healey, 2018). The absence of adverse effects in cattle taking pellets with fungal chlamydospores concerning skin, digestive, respiratory or reproductive systems corroborates the innocuousness stated in investigations carried out among other herbivorous or even carnivorous species (Hernández et al., 2016, 2018a, 2018b).

This is the first long-term field study analyzing a program for the control of helminths affecting dairy cattle that integrates their successful deworming plus preventing their infection by developing a leaders/followers rotational grazing regime, together with supplementation with pellets containing chlamydospores of the saprophytic filamentous fungi *M. circinelloides* and *D. flagrans*. It should be highlighted that this strategy is not restricted to dried cows, as occurs when anthelmintics are applied; thus, a withdrawal period is not mandatory. In addition, the lack of residues in the milk avoids its destruction.

The analysis of feces of two groups of dairy Friesian cattle managed under a leaders/followers rotational grazing regime demonstrated their infection by the ruminal fluke *C. daubneyi* and gastrointestinal nematodes (GIN). The group of leaders was composed of the most-needing individuals taking the best feed (milking cows), and four days later the group of followers formed by cattle with lower nutritional requirements (heifers, dried cows) replaced them; nutritional pellets were provided daily and the resting period was of two months. The administration of closantel + albendazole was successful, in concordance with data collected by administering the salicylanilide against the rumen fluke (Rolfe & Boray, 1993; Arias et al., 2013b), although lack or reduced efficacy have been reported (Malrait et al., 2015; Nzalawahe et al., 2018). A similar efficacy of albendazole on GIN was priory reported (Demeler et al., 2009).a slight reduction on the numbers of eggs of *C. daubneyi* and GIN was noted in the leaders. After completing two rotations (four months), a marked decrease occurred in the followers. The eggs of the trematodes dropped from the fifth rotation till the end, whereas the GIN egg-output numbers halved after four rotations and then increased. These results point that the presence of infective stages lessened through rotational grazing, but notable levels of contamination look to remain. Despite beneficial effects have been reported by feeding first season grazing calves, during a period of 20 weeks, on grasslands priory fed by second season grazing animals (Larsson et al., 2006), a high increment of the counts of GIN EPG was recorded in grazing cattle a few weeks after turnout, followed by a slow progressive decrease (Nogareda et al., 2006).

Rotational pasturing regimes consist of a short grazing phase (hours - days) followed by a resting period (weeks - months) to allow the pasture to regrow (Flack, 2016). The eggs or metacercariae of trematodes may remain viable up to several months, and larvae of nematodes for one-three months under tropical areas or six-twelve months in temperate zones (Torres-Acosta & Hoste, 2008; Moazeni et al., 2010), thus resting periods around 3-6 months have been stated helpful to diminish the risk of infection (Chandrawathani et al., 2004, Craig, 2009; Kumar et al., 2013). In the current investigation, a resting period of two months resulted in a weak influence on the helminths egg-output among the leaders; after five rotations were completed, half of the followers passed eggs of trematodes and all did it for GIN. Hence, this rotational pasturing regime benefits the followers during the first rotations only. The control of parasites involved the deworming of the heifers at the beginning of the assay only; once the leaders attained the drying period, they were also treated and managed as followers (together with the heifers) for two months, then brought back to the leader group. Due to anthelmintic treatment can not be simultaneously administered to the leaders, the presence of parasitized cattle (milking cows) maintains the contamination of the grasslands. Four days later, the followers (successfully dewormed at the beginning of the assay) fed on a pasture with low levels of infective stages ("cleaned" by the action of the leaders), because of the time elapsed (four days) is insufficient to new infective parasites originate from the eggs excreted previously by the leaders; however, new infective stages can appear after two months, improving the possibilities of the leaders infect when returning to the prairie.

On considering the difficult and unlikely of resting periods longer than 3-6 months because of large numbers of pasturing areas would be needed, other interesting approach might include avoiding the onset and survival of infective stages in the soil (and feces). The compliance of preventive policies depends heavily on their easiness of application, taking care of extra tasks are not added to animal keepers. Dairy cows are commonly supplemented with nutritional pelleted feed, and the addition of fungal spores during the industrial manufacturing appears very advantageous to get a very suitable formulation for spreading them on the feces/soil (Hernández et al., 2016). Prior investigations reported the spores of *M. circinelloides* and *D. flagrans* are able to survive to the mechanized production of pellets without losing their biological activity; the analysis of pellets demonstrated that the spores retain their parasiticide effect without changes beyond 6 months (Arias et al., 2015; Arroyo et al., 2016). Based on these premises, in the present study a second assay comprised the supplementation of a group of followers (heifers) with nutritional pellets manufactured with a blend of spores ($3x10^5$ / Kg pelleted feed) of two soil saprophytic filamentous fungi, *Mucor circinelloides* (ovicide) and *Duddingtonia flagrans* (larvicide) (Hernández et al., 2018a). The finding of the numbers of eggs of *C. daubneyi* in the feces of the leaders dropped by 82%, together with their absence in the followers, points that the possibilities of infection of the snails acting as intermediate hosts decreases significantly, therefore cercariae are not released and metacercariae do not emerge. Regarding the GIN, the fecal counts diminished by 69% in the leaders and 88% in the followers, which means a significant lessening of the contamination levels of the grasslands; likewise, numbers ≤100 EPG were recorded in the followers until the end of the research, so the need of additional deworming was discarded. Previous studies on pasturing cattle given mycelium of the ovicide fungus *Pochonia chlamydosporia* for 18 months reported a 67% reduction of the eggs of *Fasciola hepatica* passed in the feces (Dias et al., 2013). In spite of the absence of significant differences regarding the GIN egg-output values (Dimander et al., 2003), the numbers of third stage larvae (L3) of GIN reduced by 73.8% in the feces of cattle given orally chlamydospores of *D. flagrans*, and by 58-73.5% in pasturing zebu (Ortiz et al., 2017; Mendoza de Gives et al., 2018). It has been also demonstrated that the daily administration of *D. flagrans* spores to cattle decreased the trichostrongylid larval population on grass by 61-97% (Grønvold et al., 1993; Hertzberg et al., 2007; Jobim et al., 2008). More recently, significant reductions of the numbers of GIN infective larvae on pasture by 6-8 weeks after cattle received chlamydospores of *D. flagrans* have been reported (Healey et al., 2018).

Herbivores are exposed routinely to several helminth species while pasturing, and a combined action is required against different infective stages, eggs or larvae for instance. In the current investigation, a reduction of 100% of the egg-output values of *C. daubneyi* and 64% of GIN was recorded in heifers under rotational grazing (followers of assay two) and receiving daily fungal spores, in respect to the followers maintained under rotational pasturing only (assay one). Besides this, a significant decrease was also established in the leaders of the second assay (43% trematodes and 52% GIN, respectively) regarding those in the first assay. In horses maintained under rotational grazing and given pellets enriched with fungal spores, the counts of strongyle eggs reduced by 66% in relation to those kept under rotational pasturing only (Hernández et al., 2018a).

Most of the previous experiences regarding the administration of spores of parasiticide fungi to cattle encompassed the use of high amounts of *D. flagrans* chlamydospores focused on the control of gastrointestinal nematodes, and to a lesser extent, of *Pochonia chlamydosporia* against liver trematodes (Assis et al., 2013; Dias et al., 2013; Ortiz et al., 2017). Dosage and frequency of providing fungal spores are critical points to consider when performing biological control measures. The administration two-three days a week, or even daily, guarantees their regular presence in feces and the substantial diminution of helminth infective stages (Santurio et al., 2009; Dias et al., 2013; Mendoza de Gives et al., 2018). This allows to decrease the doses of spores and improves their utilization, as lately pointed (Hernández et al., 2016, 2018a; Healey, 2018).

The absence of adverse effects in cattle taking pellets with fungal spores concerning the skin, fur, digestive, respiratory or reproductive apparatus, corroborates the innocuousness stated in investigations carried out among other herbivorous species or even carnivorous (Hernández et al., 2016, 2018a, 2018b).

This is the first long-term field study dealing on the analysis of a program for the control of helminths affecting dairy cattle which integrates their successful deworming plus preventing their infection by developing a leaders/followers rotational grazing regime, together with supplementation with pellets containing spores of the saprophytic filamentous fungi *M. circinelloides* and *D. flagrans*. It should be highlighted that this strategy is not restricted to dried cows, as occurs when parasiticide drugs are applied, and thus a withdrawal period is not mandatory. In addition, the lack of residues in the milk avoids its destruction.

Conclusions

It is concluded that long-term ingestion of chlamydospores of *M. circinelloides* and *D. flagrans* provides a safe and valuable tool to improve the effect of rotational grazing to prevent helminth (by *C. daubneyi* and GIN) infections in grazing dairy cattle. This also constitutes a very helpful strategy to develop programs for the integrated control of helminths.

A ~~It is concluded that long-term ingestion of spores of *M. circinelloides* and *D. flagrans* provides a safety and valuable tool for the integrated control of *C. daubneyi* and GIN by improving the effect of rotational grazing and lessening the risk of infection in grazing dairy cattle.~~

Declaration of interest

The authors declare that they have no conflict of interest.

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References

Araújo, J.V., Guimarães, M.P., Campos, A.K., de Sá, N.C., Sarti, P., Assis, R.C.L., 2004. Control of bovine gastrointestinal nematode parasites using pellets of the nematode-trapping fungus *Monacrosporium thaumasium*. Cienc. Rural 34, 457-463.

Arias, M.S., Cazapal-Monteiro, C.F., Suárez, J., Miguélez, S., Francisco, I., Arroyo, F.L., Suárez, J.L., Paz-Silva, A., Sánchez-Andrade, R., Mendoza de Gives, P., 2013a. Mixed production of filamentous fungal spores for preventing soil-trasmitted helminth zoonoses: a preliminary analysis. Biomed Res. Int. http://dx.doi.org/10.1155/2013/567876.

Arias, M.S., Sanchís, J., Francisco, I., Francisco, R., Piñeiro, P., Cazapal-Monteiro, C., Cortiñas, F.J., Suárez, J.L., Sánchez-Andrade, R., Paz-Silva, A., 2013b. The efficacy of four anthelmintics against *Calicophoron daubneyi* in naturally infected dairy cattle. Vet. Parasitol. 197, 126-129.

Arias, M.S., Arroyo, F.L., Cazapal-Monteiro, C., Hernández, J.A., Suárez, J., Francisco, I., López-Arellano, M.E., Sánchez-Andrade, R., Mendoza de Gives, P., Paz-Silva, A., 2015. Formulating *Duddingtonia flagrans* in nutritional pellets for the sustainable control of equine strongyles. J. Sci. Technol. Environm. 1, 1-16.

Arroyo, F.L., Arias, M.S., Cazapal-Monteiro, C.F., Hernández, J.A., Suárez, J., Miguélez, S., Romasanta, A., Sánchez-Andrade, R., Paz-Silva, A., 2016. The capability of the fungus *Mucor circinelloides* to maintain parasiticidal activity after the industrial feed pelleting enhances the possibilities of biological control of livestock parasites. Biol. Control 92, 38-44.

Arroyo, F., Hernández, J.A., Cazapal-Monteiro, C.F., Pedreira, J., Sanchís, J., Romasanta, Á., Sánchez-Andrade, R., Paz-Silva, A., Arias, M.S., 2017. Effect of the filamentous fungus *Mucor circinelloides* on the development of eggs of the rumen fluke *Calicophoron daubneyi* (Paramphistomidae). J. Parasitol. 103, 199-206.

Assis, R.C., Luns, F.D., Araújo, J.V., Braga, F.R., Assis, R.L., Marcelino, J.L., Freitas, P.C., Andrade, M.A., 2013. Comparison between the action of nematode predatory fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium* in the biological control of bovine gastrointestinal nematodiasis in tropical southeastern Brazil. Vet. Parasitol. 193, 134-140.

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Cazapal-Monteiro, C.F., Hernández, J.A., Arroyo, F.L., Miguélez, S., Romasanta, A., Paz-Silva, A., Sánchez-Andrade, R., Arias, M.S., 2015. Analysis of the effect of soil saprophytic fungi on the eggs of *Baylisascaris procyonis*. Parasitol. Res. 114, 2443-2450.

Chandrawathani, P., Jamnah, O., Waller, P.J., Larsen, M., Gillespie, A.T., 2004. Field studies on the biological control of nematode parasites of sheep in the tropics, using the microfungus *Duddingtonia flagrans*. Vet. Parasitol. 120, 177–187.

Cortiñas, F.J., Cazapal-Monteiro, C.F., Hernández, J.A., Arroyo, F.L., Miguélez, S., Suárez, J., López de Arellano, M.E., Sánchez-Andrade, R., Mendoza de Gives, P., Paz-Silva, A., Arias, M.S., 2015. Potential use of *Mucor circinelloides* for the biological control of certain helminths affecting livestock reared in a care farm. Biocontrol Sci. Techn. 25, 1443-1452.

Craig, T.M., 2009. Helminth Parasites of the Ruminant Gastrointestinal Tract. In: Anderson, D.E., Rings, M. (Eds.), Veterinary Therapy: Food Animal Practice (5th edition). Saunders Publishing, Philadelphia, pp. 78-90.

Demeler, J., Van Zeveren, A.M., Kleinschmidt, N., Vercruysse, J., Höglund, J., Koopmann, R., Cabaret, J., Claerebout, E., Areskog, M., von Samson-Himmelstjerna, G., 2009. Monitoring the efficacy of ivermectin and albendazole against gastro intestinal nematodes of cattle in Northern Europe. Vet. Parasitol. 160, 109-115.

Dias, A.S., Araújo, J.V., Braga, F.R., Puppin, A.C., Perboni, W.R., 2013. *Pochonia chlamydosporia* in the biological control of *Fasciola hepatica* in cattle in Southeastern Brazil. Parasitol. Res. 112, 2131-2136.

Dias Luns, F., Lopes Assis, R.C., Pinheiro Costa Silva, L., Magri Ferraz, C., Ribeiro Braga, F., Victor de Araújo, J., 2018. Coadministration of nematophagous fungi for biological control over nematodes in bovine in the South-Eastern Brazil. Biomed Res Int. https://doi.org/10.1155/2018/2934674.

Dimander, S.O., Höglund, J., Uggla, A., Spörndly, E., Waller, P.J., 2003. Evaluation of gastro-intestinal nematode parasite control strategies for first-season grazing cattle in Sweden. Vet. Parasitol. 111, 193-209.

Flack, S., 2016. The art and science of grazing. Chelsea Green Publishing, Vermont, pp. 240.

Grønvold, J., Wolstrup, J., Larsen, M., Henriksen, S.A., Nansen, P., 1993. Biological control of *Ostertagia ostertagi* by feeding selected nematode-trapping fungi to calves. J. Helminthol. 67, 31-36.

Francisco, I., Arias, M., Cortiñas, F.J., Francisco, R., Mochales, E., Sánchez, J.A., Uriarte, J., Suárez, J.L., Morrondo, P., Sánchez-Andrade, R., Díez-Baños, P., Paz-Silva, A., 2009. Silvopastoralism and autochthonous equine livestock: analysis of the infection by endoparasites. Vet Parasitol. 164, 357-362.

Healey, K., Lawlor, C., Knox, M.R., Chambers, M., Lamb, J., Groves, P., 2018. Field evaluation of *Duddingtonia flagrans* IAH 1297 for the reduction of worm burden in grazing animals: Pasture larval studies in horses, cattle and goats. Vet. Parasitol. 258, 124-132.

Hernández, J.Á., Arroyo, F.L., Suárez, J., Cazapal-Monteiro, C.F., Romasanta, Á., López-Arellano, M.E., Pedreira, J., de Carvalho, L.M.M., Sánchez-Andrade, R., Arias, M.S., de Gives, P.M., Paz-Silva, A., 2016. Feeding horses with industrially manufactured pellets with fungal spores to promote nematode integrated control. Vet. Parasitol. 229, 37-44.

Hernández, J.Á., Sánchez-Andrade, R., Cazapal-Monteiro, C.F., Sanchís, J.M., Arroyo, F.L., Paz-Silva, A., Arias, M.S., 2018a. A combined effort to avoid strongyle infection in horses in an oceanic climate region: rotational grazing and parasiticidal fungi. Parasit. Vectors 11, 240-247.

Hernández, J.Á., Cazapal-Monteiro, C.F., Sanchís, J., Sánchez-Andrade, R., Paz-Silva, A., Arias, M.S., 2018b. Potential usefulness of filamentous fungi to prevent zoonotic soil-transmitted helminths. Vector Borne Zoonotic Dis. 18, 690-696.

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Hertzberg, H., Maurer, V., Heckendorn, F., Wanner, A., 2007. Control of gastrointestinal nematodes in cattle under dry pasture conditions. Rev. Suisse Agric. 39, 89-93.

Jobim, M.B., Santurio, J.M., de la Rue, M.L., 2008. *Duddingtonia flagrans*: biological control of cattle nematodes in the field. Cienc. Rural 38, 2256-2263.

Kumar, N., Rao, T.K., Varghese, A., Rathor, V.S., 2013. Internal parasite management in grazing livestock. J. Parasit. Dis. 37, 151-157.

Larsson, A., Dimander, S.O., Rydzik, A., Uggla, A., Waller, P.J., Höglund, J., 2006. A 3-year field evaluation of pasture rotation and supplementary feeding to control parasite infection in first-season grazing cattle--effects on animal performance. Vet Parasitol. 142, 197-206.

Malrait, K., Verschave, S., Skuce, P., Van Loo, H., Vercruysse, J., Charlier, J., 2015. Novel insights into the pathogenic importance, diagnosis and treatment of the rumen fluke (*Calicophoron daubneyi*) in cattle. Vet. Parasitol. 207, 134-139.

Mendoza de Gives, P., López-Arellano, M.E., Aguilar-Marcelino, L., Olazarán-Jenkins, S., Reyes-Guerrero, D., Ramírez-Vargas, G., Vega-Murillo, V.E., 2018. The nematophagous fungus *Duddingtonia flagrans* reduces the gastrointestinal parasitic nematode larvae population in faeces of orally treated calves maintained under tropical conditions-Dose/response assessment. Vet. Parasitol. 263, 66-72.

Moazeni, M., Ansari-Lari, M., Masoodfar, M., Hosseinzadeh, S., Mootabi Alavi, A., 2010. Lethal effect of high temperatures on the eggs of *Fasciola hepatica*. Iran. J. Vet. Res. 11, 168-173.

Nogareda, C., Mezo, M., Uriarte, J., Lloveras, J., Cordero del Campillo, M., 2006. Dynamics of infestation of cattle and pasture by gastrointestinal nematodes in an Atlantic temperate environment. J. Vet. Med. B Infect. Dis. Vet. Public Health 53, 439-444.

Nzalawahe, J., Hannah, R., Kassuku, A.A., Stothard, J.R., Coles, G., Eisler, M.C., 2018. Evaluating the effectiveness of trematocides against *Fasciola gigantica* and amphistomes infections in cattle, using faecal egg count reduction tests in Iringa Rural and Arumeru Districts, Tanzania. Parasit. Vectors 11, 384-392.

Ortiz Pérez, D.O., Sánchez Muñoz, B., Nahed Toral, J., Orantes Zebadúa, M.Á., Cruz López, J.L., Reyes García, M.E., Mendoza de Gives, P., 2017. Using *Duddingtonia flagrans* in calves under an organic milk farm production system in the Mexican tropics. Exp. Parasitol. 175, 74-78.

Rolfe, P.F., Boray J.C., 1993. Comparative efficacy of moxidectin, an ivermectin/clorsulon combination and closantel against immature paramphistomes in cattle. Aust. Vet. J. 70, 265-266.

Santurio, J.M., Zanette, R.A., Da Silva, A.S., De La Rue, M.L., Monteiro, S.G., Alves, S.H., 2009. Improved method for *Duddingtonia flagrans* chlamydospores production for livestock use. Vet. Parasitol. 164, 344-346.

Saumell, C.A., Fernández, A.S., Fusé, L.A., Rodríguez, M., Sagüés, M.F., Iglesias, L.E., 2015. Nematophagous fungi from decomposing cattle faeces in Argentina. Rev. Iberoam. Micol. 32, 252-256.

Silva, M.E., Braga, F.R., Borges, L.A., Oliveira, J.M., Lima, W. dos S., Guimarães, M.P., Araújo, J.V., 2014. Evaluation of the effectiveness of *Duddingtonia flagrans* and *Monacrosporium thaumasium* in the biological control of gastrointestinal nematodes in female bovines bred in the semiarid region. Vet. Res. Commun. 38, 101-106.

Smith, M.C., Sherman, D.M., 2009. Goat Medicine, second ed. WileyBlackwell, Ames, IA.

United States Department of Agriculture (USDA). March 25, 2011. Animal and Plant Health Inspection Service (APHIS), Policy #Farm17, Regulation of Agricultural Animals. Available at: <http://www.aphis.usda.gov/animal_welfare/policy.php?policy=17>.

Szmidt-Adjidé V, Abrous M, Adjidé CC, Dreyfuss G, Lecompte A, Cabaret J, Rondelaud D., 2000. Prevalence of *Paramphistomum daubneyi* infection in cattle in central France. Vet Parasitol. 87, 133-138.

Thornton, P.K., 2010. Livestock production: recent trends, future prospects. Philos. Trans. R. Soc. Lond. B Biol. Sci., 365, 2853-2867.

Thrusfield, M., 2007. Veterinary epidemiology. Blackwell Science Ltd, Oxford (3rd Edition), pp. 624.

Torres-Acosta, J.F.J., Hoste, H., 2008. Alternative or improved methods to limit gastro-intestinal parasitism in grazing sheep and goats. Small Ruminant Res. 77, 159-173.

Undersander, D., Albert, B., Cosgrove, D., Johnson, D., Peterson, P., 2002. Pastures for Profit: A guide to rotational grazing (A3529). 2002. Cooperative Extension Publishing, University of Wisconsin-Extension [Online] Available: http://learningstore.uwex.edu/pdf/A3529.pdf.

van Wyk, J.A., Mayhew, E., 2013. Morphological identification of parasitic nematode infective larvae of small ruminants and cattle: a practical lab guide. Onderstepoort J. Vet. Res. 80, 539. DOI: 10.4102/ojvr.v80i1.539.

~~Araújo, J.V., Guimarães, M.P., Campos, A.K., de Sá, N.C., Sarti, P., Assis, R.C.L., 2004. Control of bovine gastrointestinal nematode parasites using pellets of the nematode-trapping fungus *Monacrosporium thaumasium*. Cienc. Rural 34, 457-463.~~

~~Arias, M.S., Sanchís, J., Francisco, I., Francisco, R., Piñeiro, P., Cazapal-Monteiro, C., Cortiñas, F.J., Suárez, J.L., Sánchez-Andrade, R., Paz-Silva, A., 2013b. The efficacy of four anthelmintics against *Calicophoron daubneyi* in naturally infected dairy cattle. Vet. Parasitol. 197, 126-129.~~

~~Arias, M.S., Arroyo, F.L., Cazapal-Monteiro, C., Hernández, J.A., Suárez, J., Francisco, I., López-Arellano, M.E., Sánchez-Andrade, R., Mendoza de Gives, P., Paz-Silva, A., 2015.~~

Formulating *Duddingtonia flagrans* in nutritional pellets for the sustainable control of equine strongyles. J. Sci. Technol. Environm. 1, 1-16.

Arias, M.S., Cazapal-Monteiro, C.F., Suárez, J., Miguélez, S., Francisco, I., Arroyo, F.L., Suárez, J.L., Paz-Silva, A., Sánchez-Andrade, R., Mendoza de Gives, P., 2013a. Mixed production of filamentous fungal spores for preventing soil-trasmitted helminth zoonoses: a preliminary analysis. Biomed Res. Int. 2013a. doi: 10.1155/2013567876.

Arroyo, F., Hernández, J.A., Cazapal-Monteiro, C.F., Pedreira, J., Sanchís, J., Romasanta, Á., Sánchez-Andrade, R., Paz-Silva, A., Arias, M.S., 2017. Effect of the filamentous fungus *Mucor circinelloides* on the development of eggs of the rumen fluke *Calicophoron daubneyi* (Paramphistomidae). J. Parasitol. 103, 199-206.

Arroyo, F.L., Arias, M.S., Cazapal-Monteiro, C.F., Hernández, J.A., Suárez, J., Miguélez, S., Romasanta, A., Sánchez-Andrade, R., Paz-Silva, A., 2016. The capability of the fungus *Mucor circinelloides* to maintain parasiticidal activity after the industrial feed pelleting enhances the possibilities of biological control of livestock parasites. Biol. Control 92, 38-44.

Assis, R.C., Luns, F.D., Araújo, J.V., Braga, F.R., Assis, R.L., Marcelino, J.L., Freitas, P.C., Andrade, M.A., 2013. Comparison between the action of nematode predatory fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium* in the biological control of bovine gastrointestinal nematodiasis in tropical southeastern Brazil. Vet. Parasitol. 193, 134-140.

Ball, D.M., Hoveland, C.S, Lacefield, G.D., (Eds.), 2007. Southern Forages. Norcross (GA): International Plant Nutrition Institute (Fifth Edition), 322 pp.

Barger, I.A., Siale, K., Banks, D.J.D., Le Jambre, L.F., 1994. Rotational grazing for control of gastrointestinal nematodes of goats in a wet tropical environment. Vet. Parasitol 53, 109-116.

Cazapal-Monteiro, C.F., Hernández, J.A., Arroyo, F.L., Miguélez, S., Romasanta, A., Paz-Silva, A., Sánchez-Andrade, R., Arias, M.S., 2015. Analysis of the effect of soil saprophytic fungi on the eggs of *Baylisascaris procyonis*. Parasitol. Res. 114, 2443-2450.

Chandrawathani, P., Jamnah, O., Waller, P.J., Larsen, M., Gillespie, A.T., 2004. Field studies on the biological control of nematode parasites of sheep in the tropics, using the microfungus *Duddingtonia flagrans*. Vet. Parasitol. 120, 177–187.

Cortiñas, F.J., Cazapal-Monteiro, C.F., Hernández, J.A., Arroyo, F.L., Miguélez, S., Suárez, J., López de Arellano, M.E., Sánchez-Andrade, R., Mendoza de Gives, P., Paz-Silva, A., Arias, M.S., 2015. Potential use of *Mucor circinelloides* for the biological control of certain helminths affecting livestock reared in a care farm. Biocontrol Sci. Techn. 25, 1443-1452.

Craig, T.M., 2009. Helminth Parasites of the Ruminant Gastrointestinal Tract. In: Anderson, D.E., Rings, M. (Eds.), Veterinary Therapy: Food Animal Practice (5th edition). Saunders Publishing, Philadelphia, pp. 78-90.

Demeler, J., Van Zeveren, A.M., Kleinschmidt, N., Vercruysse, J., Höglund, J., Koopmann, R., Cabaret, J., Claerebout, E., Areskog, M., von Samson-Himmelstjerna, G., 2009. Monitoring the efficacy of ivermectin and albendazole against gastro intestinal nematodes of cattle in Northern Europe. Vet. Parasitol. 160, 109-115.

Dias, A.S., Araújo, J.V., Braga, F.R., Puppin, A.C., Perboni, W.R., 2013. *Pochonia chlamydosporia* in the biological control of *Fasciola hepatica* in cattle in Southeastern Brazil. Parasitol. Res. 112, 2131-2136.

Dias Luns, F., Lopes Assis, R.C., Pinheiro Costa Silva, L., Magri Ferraz, C., Ribeiro Braga, F., Victor de Araújo, J., 2018. Coadministration of nematophagous fungi for biological control over nematodes in bovine in the South-Eastern Brazil. Biomed Res Int. 2018: 2934674.

~~Dimander, S.O., Höglund, J., Uggla, A., Spörndly, E., Waller, P.J., 2003. Evaluation of gastro-intestinal nematode parasite control strategies for first-season grazing cattle in Sweden. Vet. Parasitol. 111, 193-209.~~

~~Flack, S., 2016. The art and science of grazing. Chelsea Green Publishing, Vermont, pp. 240.~~

~~Grønvold, J., Wolstrup, J., Larsen, M., Henriksen, S.A., Nansen, P., 1993. Biological control of *Ostertagia ostertagi* by feeding selected nematode-trapping fungi to calves. J. Helminthol. 67, 31-36.~~

~~Healey, K., Lawlor, C., Knox, M.R., Chambers, M., Lamb, J., Groves, P., 2018. Field evaluation of *Duddingtonia flagrans* IAH 1297 for the reduction of worm burden in grazing animals: Pasture larval studies in horses, cattle and goats. Vet. Parasitol. 258, 124-132.~~

~~Hernández, J.Á., Arroyo, F.L., Suárez, J., Cazapal-Monteiro, C.F., Romasanta, Á., López-Arellano, M.E., Pedreira, J., de Carvalho, L.M.M., Sánchez-Andrade, R., Arias, M.S., de Gives, P.M., Paz-Silva, A., 2016. Feeding horses with industrially manufactured pellets with fungal spores to promote nematode integrated control. Vet. Parasitol. 229, 37-44.~~

~~Hernández, J.Á., Cazapal-Monteiro, C.F., Sanchís, J., Sánchez-Andrade, R., Paz-Silva, A., Arias, M.S., 2018b. Potential usefulness of filamentous fungi to prevent zoonotic soil-transmitted helminths. Vector Borne Zoonotic Dis. 18, 690-696.~~

~~Hernández, J.Á., Sánchez-Andrade, R., Cazapal-Monteiro, C.F., Sanchís, J.M., Arroyo, F.L., Paz-Silva, A., Arias, M.S., 2018a. A combined effort to avoid strongyle infection in horses in an oceanic climate region: rotational grazing and parasiticidal fungi. Parasit. Vectors 11, 240-247.~~

~~Hertzberg, H., Maurer, V., Heckendorn, F., Wanner, A., 2007. Control of gastrointestinal nematodes in cattle under dry pasture conditions. Rev. Suisse Agric. 39, 89-93.~~

~~Jobim, M.B., Santurio, J.M., de la Rue, M.L., 2008. *Duddingtonia flagrans*: biological control of cattle nematodes in the field. Cienc. Rural 38, 2256-2263.~~

Kumar, N., Rao, T.K., Varghese, A., Rathor, V.S., 2013. Internal parasite management in grazing livestock. J. Parasit. Dis. 37, 151-157.

Larsson, A., Dimander, S.O., Rydzik, A., Uggla, A., Waller, P.J., Höglund, J., 2006. A 3-year field evaluation of pasture rotation and supplementary feeding to control parasite infection in first-season grazing cattle--effects on animal performance. Vet Parasitol. 142, 197-206.

Malrait, K., Verschave, S., Skuce, P., Van Loo, H., Vercruysse, J., Charlier, J., 2015. Novel insights into the pathogenic importance, diagnosis and treatment of the rumen fluke (*Calicophoron daubneyi*) in cattle. Vet. Parasitol. 207, 134-139.

Mendoza de Gives, P., López-Arellano, M.E., Aguilar-Marcelino, L., Olazarán-Jenkins, S., Reyes-Guerrero, D., Ramírez-Vargas, G., Vega-Murillo, V.E., 2018. The nematophagous fungus *Duddingtonia flagrans* reduces the gastrointestinal parasitic nematode larvae population in faeces of orally treated calves maintained under tropical conditions-Dose/response assessment. Vet. Parasitol. 263, 66-72.

Moazeni, M., Ansari-Lari, M., Masoodfar, M., Hosseinzadeh, S., Mootabi Alavi, A., 2010. Lethal effect of high temperatures on the eggs of *Fasciola hepatica*. Iran. J. Vet. Res. 11, 168-173.

Nogareda, C., Mezo, M., Uriarte, J., Lloveras, J., Cordero del Campillo, M., 2006. Dynamics of infestation of cattle and pasture by gastrointestinal nematodes in an Atlantic temperate environment. J. Vet. Med. B Infect. Dis. Vet. Public Health 53, 439-444.

Nzalawahe, J., Hannah, R., Kassuku, A.A., Stothard, J.R., Coles, G., Eisler, M.C., 2018. Evaluating the effectiveness of trematocides against *Fasciola gigantica* and amphistomes infections in cattle, using faecal egg count reduction tests in Iringa Rural and Arumeru Districts, Tanzania. Parasit. Vectors 11, 384-392.

Ortiz Pérez, D.O., Sánchez Muñoz, B., Nahed Toral, J., Orantes Zebadúa, M.Á., Cruz López, J.L., Reyes García, M.E., Mendoza de Gives, P., 2017. Using *Duddingtonia flagrans* in

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calves under an organic milk farm production system in the Mexican tropics. Exp. Parasitol. 175, 74-78.

Rolfe, P.F., Boray J.C., 1993. Comparative efficacy of moxidectin, an ivermectin/clorsulon combination and closantel against immature paramphistomes in cattle. Aust. Vet. J. 70, 265-266.

Sanchís, J., Sánchez-Andrade, R., Macchi, M.I., Piñeiro, P., Suárez, J.L., Cazapal-Monteiro, C., Maldini, G., Venzal, J.M., Paz-Silva, A., Arias, M.S., 2013. Infection by Paramphistomidae trematodes in cattle from two agricultural regions in NW Uruguay and NW Spain. Vet. Parasitol. 191, 165-171.

Santurio, J.M., Zanette, R.A., Da Silva, A.S., De La Rue, M.L., Monteiro, S.G., Alves, S.H., 2009. Improved method for *Duddingtonia flagrans* chlamydospores production for livestock use. Vet. Parasitol. 164, 344-346.

Saumell, C.A., Fernández, A.S., Fusé, L.A., Rodríguez, M., Sagüés, M.F., Iglesias, L.E., 2015. Nematophagous fungi from decomposing cattle faeces in Argentina. Rev. Iberoam. Micol. 32, 252-256.

Silva, M.E., Braga, F.R., Borges, L.A., Oliveira, J.M., Lima, W. dos S., Guimarães, M.P., Araújo, J.V., 2014. Evaluation of the effectiveness of *Duddingtonia flagrans* and *Monacrosporium thaumasium* in the biological control of gastrointestinal nematodes in female bovines bred in the semiarid region. Vet. Res. Commun. 38, 101-106.

Smith, M.C., Sherman, D.M., 2009. Goat Medicine, second ed. WileyBlackwell, Ames, IA.

United States Department of Agriculture (USDA). March 25, 2011. Animal and Plant Health Inspection Service (APHIS), Policy #Farm17, Regulation of Agricultural Animals. Available at: <http://www.aphis.usda.gov/animal_welfare/policy.php?policy=17>.

~~Szmidt-Adjidé V, Abrous M, Adjidé CC, Dreyfuss G, Lecompte A, Cabaret J, Rondelaud D., 2000. Prevalence of *Paramphistomum daubneyi* infection in cattle in central France. Vet Parasitol. 87, 133-138.~~

~~Thornton, P.K., 2010. Livestock production: recent trends, future prospects. Philos. Trans. R. Soc. Lond. B Biol. Sci., 365, 2853-2867.~~

~~Thrusfield, M., 2007. Veterinary epidemiology. Blackwell Science Ltd, Oxford (3rd Edition), pp. 624.~~

~~Torres-Acosta, J.F.J., Hoste, H., 2008. Alternative or improved methods to limit gastro-intestinal parasitism in grazing sheep and goats. Small Ruminant Res. 77, 159-173.~~

~~Undersander, D., Albert, B., Cosgrove, D., Johnson, D., Peterson, P., 2002. Pastures for Profit: A guide to rotational grazing (A3529). 2002. Cooperative Extension Publishing, University of Wisconsin-Extension [Online] Available: .~~

FIGURE CAPTIONS

Fig. 1.- Scheme of a rotational grazing regime developed over an interval of 24 months involving Friesian cattle (20 cows and 12 heifers) maintained under a leaders/followers system in which milking ruminants (leaders) pasture ahead of dried-off cows and heifers (followers). A pasture resting period of two months was observed.

Fig. 2.- Values of fecal egg count reduction (FECR) of *C. daubneyi* and GIN in cattle maintained under a leaders/followers grazing system and provided 2.5 kg of commercial nutritional pellets daily. G-L1: leaders (dewormed with closantel and albendazole during the drying periods); G-F1: followers (dewormed with closantel and albendazole at the beginning of the trial). Cd: *Calicophoron daubneyi*; GIN: gastrointestinal nematodes.

Fig. 3.- Values of fecal egg count reduction (FECR) of *C. daubneyi* and GIN in cattle maintained under a leaders/followers. G-L2: leaders (dewormed with closantel and albendazole during the drying periods and provided daily 2.5 kg commercial nutritional pellets); G-F2: followers (dewormed with closantel and albendazole at the beginning of the trial and provided 2.5 kg of commercial nutritional pellets containing chlamydospores of *Mucor circinelloides* and *Duddingtonia flagrans* - $3x10^5$ each/kg pellet) daily. Cd: *Calicophoron daubneyi*; GIN: gastrointestinal nematodes.

~~Fig. 1.- During two years, Friesian cattle (20 cows and 12 heifers) were maintained under a leaders/followers system consisting of milking ruminants (Leaders) pasture ahead of the dried-off cows and heifers (Followers).~~

Fig. 2.- Percentages of reduction of helminths egg-output in cattle maintained under a leaders/followers grazing system and provided daily 2.5 Kg commercial nutritional pellets. G-L1: leaders (dewormed with closantel + albendazole during the drying periods); G-F1: followers (dewormed with closantel + albendazole at the beginning of the assay). Cd: *Calicophoron daubneyi*; GIN: gastrointestinal nematodes.

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INTEGRATING THE CONTROL OF HELMINTHS IN DAIRY CATTLE: DEWORMING, ROTATIONAL GRAZING AND NUTRITIONAL PELLETS WITH PARASITICIDE FUNGI

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Abstract

Thirty-two Friesian cattle under a leaders/followers four-day rotation and passing eggs of trematodes and gastrointestinal nematodes (GIN) were studied in two trials for the integrated control of these helminths over two years. In the first trial, the effect of rotational pasturing was assessed on a group of leaders (milking cows, G-L1) and followers (dried-off cows and heifers, G-F1) supplemented daily with commercial nutritional pellets. In the second trial, leaders (G-L2) and followers (G-F2) were maintained under a rotational pasturing regime; the cows received daily commercial pelleted feed and heifers pellets manufactured with a blend of parasiticide fungi ($3x10^5$ chlamydospores of both *Mucor circinelloides* and *Duddingtonia flagrans*/kg pellet). Deworming via closantel and albendazole was performed in cows in each trial at the beginning of their drying periods, and fourteen days later, the fecal egg-count reductions (FECR) of *Calicophoron daubneyi* and GIN were from 94-100% (average 98%), while the percentages of reduction of cattle shedding eggs (CPCR) were from 50-100% (average 77% and 82%, respectively). The heifers were dewormed one time only, at the beginning of each trial, and the values of FECR and CPCR were 100% against *C. daubneyi* and 96% and 83%, respectively, against GIN. Over a period of 24 months, significantly higher numbers of helminth egg-output were observed in G-L1, with the lowest numbers in G-F2. *C. daubneyi* egg output was reduced by 5% (G-L1) and 42% (G-F1) at the end of trial 1 and by 83% (G-L2) and 100% (G-F2) at the end of trial 2; the numbers of GIN egg-output decreased by 13% (G-L1) and 18% (G-F1) at the end of trial 1, and by 72% (G-L2) and 85% (G-F2) at the end of trial 2. No adverse effects were detected in cattle taking pellets enriched with fungal spores (G-F2). It is concluded that long-term ingestion of spores of *M. circinelloides* and *D. flagrans* provides a valuable tool to improve the effect of rotational grazing and to lessen the risk of infection by *C. daubneyi* and GIN in dairy cattle, and accordingly, the performance of integrated control programs.

Key words: biological control, ruminants, *Mucor circinelloides*, *Duddingtonia flagrans*, chlamydospores, sustainability

Introduction

The numbers of grazing cattle have increased markedly, partly because this is a cost-effective regime, and partly because of the preoccupation on animal welfare in food production (Thornton, 2010). Trematodes belonging to Fasciolidae and Paramphistomidae and can frequently affect ruminants on humid habitats, where appropriate conditions are present for the development of the external phase in their life cycle (Szmidt-Adjidé et al., 2000). Gastrointestinal nematodes (GIN) are helminths that often infect grazing ruminants through the ingestion of third larvae (the infective stages) (Smith & Sherman, 2009; Craig, 2009).

Control of helminths in dairy cattle becomes especially difficult because deworming is only allowed during the dry period, which occurs consecutively on farms throughout the year. Therefore, treated and untreated cows can share the same pastures, and untreated ruminants continue to shed eggs in the feces, increasing the risk of all cows ingesting infective stages of helminths (e.g., eggs, larvae) while feeding on grass.

With the aim to diminish the presence of infective stages in the soil, rotational grazing regimes characterized by ruminants feeding on grass for a time interval (grazing period), followed by other rest periods, are advised (Undersander et al., 2002). In the last years, biological strategies for the control of helminths based on the use of certain soil saprophytic fungi have been reported. Some species (*Duddingtonia flagrans*, *Monacrosporium thaumassium*) are able to trap the infective stages of GIN (Araújo et al., 2004), and others can produce antagonistic effects on helminth eggs as trematodes, cestodes and nematodes (*Pochonia chlamydosporia*, *Mucor circinelloides*, *Purpureocillium lilacinum*) (Cazapal-

Monteiro et al., 2015; Cortiñas et al., 2015); they can also act against the miracidia of *C. daubneyi* (Arroyo et al., 2017). Former studies have stated the usefulness of providing cattle chlamydospores in water solutions (Assis et al., 2013; Silva et al., 2014; Saumell et al., 2015), mass mycelia of *D. flagrans* or *M. thaumassium* in alginate pellets (Dias et al., 2018), or as a blend of spores of *D. flagrans* and *M. circinelloides* in industrially manufactured pellets (Hernández et al., 2016). Recently, a commercial formulation containing chlamydospores of *D. flagrans* was registered (Healey et al., 2018). The efficacy and safety of combining rotation of grasslands with providing pellets enriched with chlamydospores of *M. circinelloides* and *D. flagrans* were successfully tested on horses (Hernández et al., 2018a).

In the current investigation, the helpfulness of the integrated control of helminths among dairy cattle based on anthelmintic treatment, pasture rotation and feeding on pellets manufactured with chlamydospores of *M. circinelloides* and *D. flagrans* was analyzed.

Material and methods

Area of study

The present research was carried out in Galegos (Riotorto, Lugo, NW Spain, 43º 15' 43.9" N - 7º 12' 36.0" W), an agricultural and mountainous area (250-734 m altitude) under an oceanic climate, which covers ca. 16 km^2 crossed by profuse streams watering the grasslands and is mostly dedicated to dairy farming and forestry production (pine and eucalyptus).

Fungal chlamydospores

A submerged culture (COPFr) was used to obtain chlamydospores of *M. circinelloides* and *D. flagrans* simultaneously (Arias et al., 2013a). The concentration was adjusted to $1x10^8$ chlamydospores of each fungus/L before manufacturing the pelleted feed.

Addition of fungal chlamydospores to nutritional pellets

Pelleted feed (NANFOR, Begonte, Lugo, Spain) containing corn, dehulled soya flour, barley, sunflower seed flour, soya hulls, rape seed flour, calcium carbonate, palm kernels as expeller, sugarcane molasses and sodium chloride was used in the current study. The analytical composition includes crude protein (20.5%), crude fat (2.3%), crude fiber (9.3%), ash content (7.9%), calcium (1.6%), phosphorus (0.55%), sodium (0.32%), magnesium (0.54%), vitamin A (20000 UI/kg), vitamin D3 (2750 UI/kg) and vitamin E (45 UI/kg).

According to previous investigations (Hernández et al., 2016), a blend of $3x10^5$ chlamydospores of *M. circinelloides* and $3x10^5$ chlamydospores of *D. flagrans* was added per kg of feedstuff during the mixing phase of the elaboration of the pellets, after which the feedstuff was conditioned by an injecting steam (75 ºC for 90 s) and passed through the pelletizer (Arroyo et al., 2016; Hernández et al., 2018a). The final product was cooled, dried, packed into 40-kg bags, and stored for a minimum of three months at room temperature, avoiding direct sunlight.

Cattle management

Friesian cattle reared in the current farm are stabled overnight and graze on perennial ryegrass and white clover during the daytime, under a leaders/followers rotational scheme consisting of the most-needing cattle (leaders: milking cows) taking the best feed, followed four days later by the individuals with lower nutritional requirements (followers: dried cows and heifers) (Fig. 1). With the aim to ensure that pasture plants can recover and regrow, the leaders do not come back to the same paddock after two months (pasture resting period). Hay is given when green forage is insufficient, from December to February. All cattle receive 2.5 kg commercial pelleted feed daily, distributed in the morning and in the night, and corn ensilage.

Every month, four cows are dried off, dewormed and managed as followers (with the heifers) until they calve (after approximately two months). Calving occurs year round, and calves are removed from the dam soon after birth and reared outdoors until they are weaned as heifer replacements.

Experimental scheme

Over two years, Friesian cattle were maintained into two trials (Table 1).

G-L1 (leaders): ten milking cows (4-6 yr) were grazed on a paddock during four days, after which they were moved to other pasture. Commercial pellets were given daily. Every month, two cows were dried off, dewormed (see below) and maintained with the followers (G-F1) until calving (approximately two months later).

G-F1 (followers): eight cattle (heifers and dried off cows) grazed a paddock four days after the leaders grazed. a) Six heifers (12-14 mo) received, at the beginning of the trial only, closantel (10 mg/kg bodyweight (bw) ENDOEX, SP Veterinaria, Spain) and albendazole (7.5 mg/kg bw ALBENDEX, SP Veterinaria, Spain); b) cows were treated at the start of their respective drying periods with closantel and albendazole as explained above. Supplementation consisting of commercial pellets was provided daily.

Trial 2: analyzing the effect of rotational grazing and the intake of pellets with fungal chlamydospores.

G-L2 (leaders): ten milking cows (4-6 yr) fed on a paddock for four days, after which they were brought to another pasture. Every month, two cows were dried off, dewormed and maintained in the group of followers (G-F2) until calving (approximately two months). Commercial pellets were given daily.

G-F2 (followers): eight cattle (heifers and dried off cows) grazed on a paddock four days after the leaders. a) Six heifers (12-14 mo) were treated one time only with closantel and albendazole as previously described at the beginning of the trial only; supplementation was given daily, consisting of commercial pellets manufactured with $3x10^5$ chlamydospores of *M. circinelloides* and $3x10^5$ *D. flagrans* per kg of pelleted feed. b) Cows were dewormed at the start of the drying period with closantel and albendazole as explained above. Commercial pellets (without fungal chlamydospores) were provided daily.

A total of thirty different areas pastured by cattle for more than 7 years, with flooded zones, were randomly divided into two lots of 15 each and were used for every trial. As explained before, a grazing interval of four days and a resting period of two months were observed. Accordingly, the pastures were completely rotated after two months in each trial (Fig. 1). It is important to note that every group of ruminants fed on the same 15 grasslands throughout the study, which were never shared by cattle belonging to the different trials.

Coprological probes

Fecal samples were collected monthly from the rectum of each ruminant and divided into two subsamples of five grams; one was analyzed through a flotation probe with saturated NaCl solution (specific gravity= 1.20) (Sensitivity= 10 eggs per gram feces, EPG) and the other by using the sedimentation test (Se= 10 EPG) (Francisco et al., 2009). The results are expressed as EPG.

For the purpose of identifying the gastrointestinal nematodes infecting cattle, feces were individually collected at the beginning of the trial; pools containing five grams from every ruminant were prepared for each group and incubated over 17 days at 22-25°C according to Hernández et al. (2016). The Baermann technique was applied to collect the third-stage larvae (L3), and identification was performed under an optical microscope employing morphological keys (van Wyk & Mayhew, 2013).

Evaluation of the efficacy

With the objective of assessing the efficacy of deworming, fecal samples were individually collected from the rectum of each ruminant at days 0 and 14 after treatment and analyzed by means of the flotation and sedimentation probes. The reductions in fecal egg counts (FECR) and in the numbers of cattle positive by coprological tests (CPCR) were calculated according to the following formulae (Arias et al., 2013b):

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

$$\text{CPCR (\%)} = [1 - (\text{number of positive cattle}_{\text{post-treatment}} / \text{positive cattle}_{\text{pre-treatment}})] \times 100$$

Side effects

Heifers provided pellets enriched with chlamydospores of parasiticide fungi (G-F2) were examined for the possibility of signs reflecting damage or disorders affecting the reproductive, digestive or respiratory systems. The skin and oral mucosa were also carefully scrutinized for the presence of disorders.

Because the commercial pelleted feed has an expiration period of three months, pellets manufactured with the chlamydospores were stored for longer and then examined for abnormal odor, color, consistency or appearance. Attention was also paid to pellets showing fungal growth on the surface.

Welfare and ethics

The authors confirm that legal and ethical requirements were met regarding the humane treatment of animals described in the study. The ethical review committee from the University of Santiago de Compostela (Spain) approved the current study, which fulfils the national and international guidelines for animal research.

Statistical analysis

According to the Kolmogorov-Smirnov test, data were not normally distributed ($Z= 4.672$, $P= 0.001$ for the trematodes egg-output, and $Z= 2.852$, $P= 0.001$ for the GIN). By means of Levene's test, homogeneity of variances was excluded ($Z= 27.241$, $P= 0.001$ for the trematodes egg-output, and $Z= 30.171$, $P= 0.001$ for the GIN). The non-parametric tests Kruskal-Wallis and Mann-Whitney U were then performed at a significance level of $P < 0.05$ (Thrusfield, 2007).

The values of FECR and CPCR are shown as percentages and 95% confidence intervals. The kinetics of the egg-output are represented as the means and ranges (defined as the difference between the maximum and minimum values). All the tests were carried out using the statistical package SPSS, version 20 (IBM SPSS Inc., Chicago, IL, USA).

Results

Helminths identified

Eggs of trematodes and gastrointestinal nematodes (GIN) were detected in the feces of all cattle at the beginning of the trial. Trematodes were identified as *Calicophoron daubneyi*; third stage larvae belonging to the genera *Trichostrongylus*, *Ostertagia*, *Chabertia*, *Bunostomum*, *Cooperia* and *Oesophagostomum* were recovered from the coprocultures.

Efficacy of deworming

As summarized in Table 1, during the respective drying periods, anthelmintic treatment was administered 48 times to the cows belonging to G-L1 and the same number to the cows belonging to G-L2.

Fourteen days after every treatment, the FECR values were 94-100% (average FECR: 98%) against *C. daubneyi* and GIN (Table 2), while the percentage of reduction of cattle shedding eggs was 50-100% (average CPCR: 77% and 82%, respectively).

The heifers in G-F1 and G-F2 were treated at the beginning of the study only, and fourteen days later, the efficacy resulted in 100% FECR and CPCR against *C. daubneyi* (Tables 3 & 4); all heifers in G-F1 were positive to the sedimentation by the 8th month after treatment (m.a.t.), whereas eggs of *C. daubneyi* were never observed in G-F2 after the administration of anthelmintics (Table 4). The average efficacies of albendazole against GIN were 96% FECR and 83% CPCR (Tables 3 & 4); all heifers were positive to the flotation test five months after treatment in G-F1 and by the 9th m.a.t. in G-F2 (Tables 3 & 4).

Effect of rotational grazing

In the leaders of trial 1 (G-L1), the numbers of eggs of *C. daubneyi* did not decrease during the first three pasture rotations (sixth m.a.t.) (Fig. 2) and then diminished between 15% (four rotations) and 3% (seven rotations) (Z= 1.316, *P*= 0.059). In the followers (G-F1), the highest values of reduction (94-96%) were attained during the first two full rotations (fourth m.a.t.) and then decreased to 31-45% from the fifth rotation (10^{th} m.a.t.) (Z= 7.098, *P*= 0.011).

The counts of GIN in G-L1 decreased by 3% (2^{nd} rotation) and 13% (12^{th} rotation) (Fig. 2) (Z= 1.305, *P*= 0.065). In G-F1, a reduction from 96% (first rotation) to 51% (fifth rotation) was obtained after the first five rotations were completed (eighth m.a.t.) and then dropped to 9-29% until the end of the trial (Z= 1.620, *P*= 0.011). Significant differences were observed between both groups regarding *C. daubneyi* (Z= -9.188, *P*= 0.001) and GIN (Z= -6.085, *P*= 0.001).

Effect of rotational grazing and ingestion of fungal chlamydospores

In the leaders of trial 2 (G-L2) an important reduction in the eggs of *C. daubneyi* eggs was noted during the trial (Z= -4.348, *P*= 0.001), with values between 4% (first rotation) and 56% (sixth rotation, 12^{th} m.a.t.), which increased to 83% at the end of the trial (12 rotations, 24^{th} m.a.t.) (Fig. 3). In the followers (G-F2), the values of *C. daubneyi* egg-output dropped by 100% after the administration of successful deworming, and eggs were never detected from that point forward.

Regarding the GIN, percentages of reduction higher than 50% were achieved in the G-L2 from the 3^{rd} rotation (sixth m.a.t.) and > 80% from the 9^{th} rotation (18^{th} m.a.t.) (Fig. 3) (Z= -3.040, *P*= 0.001). In the G-F2, significant reductions were noted throughout the trial, with percentages of 78-93% (Z= -2.683, *P*= 0.001). Significant differences between the two groups of ruminants were observed for *C. daubneyi* (Z= -13.880, *P*= 0.001) and GIN (Z= -5.974, *P*= 0.001).

Comparison between the two trials

The lowest values of reduction of eggs of *C. daubneyi* and GIN were observed in the leaders of trial 1 (G-L1) and were highest in the followers of trial 2 (G-F2) (Figs. 2 & 3).

Significantly higher values of *C. daubneyi* FECR were observed in the leaders of trial 2 (G-L2) than those in trial 1 (G-L1) (Z= -7.329, *P*= 0.001), especially from the fifth rotation, when percentages 7.5 times higher than those of G-L1 were observed (Table 5). Values of GIN FECR 2.8 to 31.1 times higher in G-L2 than in G-L1 were recorded, mainly from the third rotation (6^{th} m.a.t.) (Z= -10.382, *P*= 0.001) (Table 5).

Regarding the followers, eggs of *C. daubneyi* were never detected in G-F2 after their deworming at the beginning of the study. From the fourth rotation, the values of GIN FECR in G-F2 were 1.5 – 8.7 times higher than in G-F1 (Z= -9.536, *P*= 0.001).

Analysis of adverse effects in the cattle receiving chlamydospores of parasiticide fungi

The pellets manufactured with the parasiticide chlamydospores were normally taken by the heifers in G-F2; rejection was not detected at any time. Clinical signs concerning the digestive system or respiratory apparatus were not recorded, and heifers cycled normally. No alterations affecting the skin were identified.

The analysis of pellets manufactured with chlamydospores of *M. circinelloides* and *D. flagrans* and stored for longer than three months revealed the absence of abnormal odor, consistency or physical appearance, nor hyphal growth on the surface.

Discussion

Infections by the ruminal fluke *C. daubneyi* and gastrointestinal nematodes (GIN) were detected among two groups of dairy Friesian cattle managed under a leaders/followers rotational grazing regime. Accordingly, closantel and albendazole were administered to the cows throughout the study during the corresponding drying periods, whereas the heifers were dewormed once at the beginning of each trial only. The anthelmintic treatment was successful, in concordance with data collected by administering the salicylanilide against the rumen fluke (Rolfe & Boray, 1993; Arias et al., 2013b), although a lack of or reduced efficacies have been reported (Malrait et al., 2015; Nzalawahe et al., 2018). A similar efficacy of albendazole on GIN was reported previously (Demeler et al., 2009).

Resting periods (weeks - months) are observed in rotational pasturing regimes to allow the pasture to regrow (Flack, 2016). It has been noted that this interval could also be useful to reduce the survival of infective stages in the soil (Chandrawathani et al., 2004). In the current study, a pasture resting period of two months was maintained over an interval of 24 months, with the most-needing cattle (leaders: milking cows) taking the best feed, then replaced four days later by individuals with lower nutritional requirements (followers: dried cows and heifers). In the leaders, a slight reduction in the number of *C. daubneyi* eggs was noted, whereas the number of eggs in the followers decreased markedly (≥ 94%) after completing two rotations (four months after treatment), then by one-third to half until the end; the values of GIN egg-output showed minor variations in the leaders but halved in the followers after four rotations and then increased. Despite the fact that beneficial effects have been reported by feeding first season grazing calves for a period of 20 weeks on grasslands fed previously by second-season grazing animals (Larsson et al., 2006), a high increment of the counts of GIN EPG was recorded in grazing cattle a few weeks after turnout, followed by a slow progressive decrease (Nogareda et al., 2006). In the present study, the control of parasites involved the deworming of the heifers at the beginning of the trial only; once the leaders attained the drying period, they were also treated and managed as followers (together with the heifers) for two months, after which they were brought back to the leader group. Because anthelmintic treatment cannot be simultaneously administered to the leaders, the presence of infected cattle (milking cows) seemed to maintain the contamination of the grasslands. Four days later, the followers (successfully dewormed at the beginning of the trial) fed on a pasture with low levels of infective stages ("cleaned" by the action of the leaders). It should be noted that the time elapsed (four days) is insufficient for new infective parasites to originate from the eggs excreted previously by the leaders; however, new infective stages can appear after two months, improving the possibility of the leaders becoming infected when returning to the prairie. Hence, this rotational pasturing regime benefits the followers during the first rotations only. The eggs or metacercariae of trematodes may remain viable up to several months, and larvae of GIN for one to three months in tropical areas or six to twelve months in temperate zones (Torres-Acosta & Hoste, 2008; Moazeni et al., 2010). Accordingly, pasture resting periods of approximately three to six months have been determined to be helpful in diminishing the risk of infection (Craig, 2009; Kumar et al., 2013).

On considering the difficulty and low likelihood of using resting periods longer than three months because large numbers of pasturing areas would be needed, another interesting approach might include avoiding the onset and survival of infective stages in the feces and soil. Prior investigations have reported that the chlamydospores of *M. circinelloides* and *D. flagrans* are able to survive the mechanized production of pellets without losing their biological activity; the analysis of pellets demonstrated that the chlamydospores retained their antagonistic effect without changes beyond 6 months (Arias et al., 2015; Arroyo et al., 2016). Because dairy cows are commonly supplemented with nutritional pelleted feed, in the present study, a second trial involved the supplementation of a group of heifers in the followers with nutritional pellets manufactured with a blend of chlamydospores ($3x10^5$/kg pelleted feed) of two soil saprophytic filamentous fungi: *Mucor circinelloides* (ovicide) and *Duddingtonia flagrans* (larvicide) (Hernández et al., 2018a). The fact that the numbers of *C. daubneyi* eggs in the feces of the leaders dropped by 56% after six rotations and by 83% by the end of the trial, together with their absence in the followers after their successful treatment, suggests that the possibility of infection with snails acting as intermediate hosts decreased significantly; therefore, cercariae are not released, and metacercariae do not emerge. Moreover, though metacercariae are highly resistant in the environment, their numbers seem to be reduced by the action of the group of leaders feeding four days earlier than the followers. Regarding the GIN, at the end of the trial (24 months), the fecal counts diminished by 78% in the leaders and 85% in the followers, which appears to indicate a significant reduction in the contamination levels of the grasslands; likewise, numbers ≤100 EPG were recorded in the followers until the end of the research, so the need of additional deworming was discarded. Previous studies on grazing cattle given mycelium of the ovicide fungus *Pochonia chlamydosporia* for 18 months reported a 67% reduction of the eggs of *Fasciola hepatica* passed in the feces (Dias et al., 2013). In spite of the absence of significant differences regarding the GIN egg-output values (Dimander et al., 2003), the numbers of third-stage larvae (L3) of GIN decreased by 73.8% in the feces of cattle orally given chlamydospores of *D. flagrans* and by 58-73.5% in pasturing zebu (Ortiz et al., 2017; Mendoza de Gives et al., 2018). It has also been demonstrated that the daily administration of *D. flagrans* chlamydospores to cattle decreased the trichostrongylid larval population on grass by 61-97% (Grønvold et al., 1993; Hertzberg et al., 2007; Jobim et al., 2008). More recently, significant reductions of the numbers of GIN infective larvae on pasture 6-8 weeks after cattle received chlamydospores of *D. flagrans* have been reported (Healey et al., 2018).

Herbivores are routinely exposed to several helminth species while pasturing, and combined actions are required against different infective stages, eggs or larvae. In the current study, significant reductions in the egg-output values of *C. daubneyi* and GIN were recorded by giving daily fungal chlamydospores to the heifers belonging to the group of followers, with respect to those maintained under rotational pasturing only and receiving commercial pellets without chlamydospores (trial one). Additionally, a significant decrease was also established in the leaders of the second trial regarding those in the first trial. These results are in agreement with data collected in horses maintained under rotational grazing and given pellets enriched with fungal chlamydospores, where the counts of strongyle eggs were reduced by two thirds in relation to those kept under rotational pasturing only (Hernández et al., 2018a).

Most of the previous experiences regarding the administration of parasiticide fungi to cattle encompassed the use of high amounts of *D. flagrans* chlamydospores focused on the control of gastrointestinal nematodes and, to a lesser extent, of *Pochonia chlamydosporia* against liver trematodes (Assis et al., 2013; Dias et al., 2013; Ortiz et al., 2017). The presence of different infective stages in the soil and herbage requires a combined action. In the present study, chlamydospores of an ovicide fungus and other larvicide species were added during the manufacturing of pelleted feed, confirming that this is a very advantageous strategy to achieve a very suitable formulation for spreading in the feces/soil (Hernández et al., 2016). The dosage and frequency of providing fungal chlamydospores are critical points to consider when performing biological control measures. Administration two to three days a week, or even daily, guarantees their regular presence in feces and the substantial diminution of helminth infective stages (Santurio et al., 2009; Dias et al., 2013; Mendoza de Gives et al., 2018). This allows the doses of chlamydospores to be decreased and improves their utilization, as lately highlighted (Hernández et al., 2016, 2018a; Healey, 2018). The absence of adverse effects in cattle taking pellets with fungal chlamydospores concerning skin, digestive, respiratory or reproductive systems corroborates the innocuousness stated in investigations carried out among other herbivorous or even carnivorous species (Hernández et al., 2016, 2018a, 2018b).

This is the first long-term field study analyzing a program for the control of helminths affecting dairy cattle that integrates their successful deworming plus preventing their infection by developing a leaders/followers rotational grazing regime, together with supplementation with pellets containing chlamydospores of the saprophytic filamentous fungi *M. circinelloides* and *D. flagrans*. It should be highlighted that this strategy is not restricted to dried cows, as occurs when anthelmintics are applied; thus, a withdrawal period is not mandatory. In addition, the lack of residues in the milk avoids its destruction.

Conclusions

It is concluded that long-term ingestion of chlamydospores of *M. circinelloides* and *D. flagrans* provides a safe and valuable tool to improve the effect of rotational grazing to prevent helminth (by *C. daubneyi* and GIN) infections in grazing dairy cattle. This also constitutes a very helpful strategy to develop programs for the integrated control of helminths.

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Declaration of interest

The authors declare that they have no conflict of interest.

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References

Araújo, J.V., Guimarães, M.P., Campos, A.K., de Sá, N.C., Sarti, P., Assis, R.C.L., 2004. Control of bovine gastrointestinal nematode parasites using pellets of the nematode-trapping fungus *Monacrosporium thaumasium*. Cienc. Rural 34, 457-463.

Arias, M.S., Cazapal-Monteiro, C.F., Suárez, J., Miguélez, S., Francisco, I., Arroyo, F.L., Suárez, J.L., Paz-Silva, A., Sánchez-Andrade, R., Mendoza de Gives, P., 2013a. Mixed production of filamentous fungal spores for preventing soil-trasmitted helminth zoonoses: a preliminary analysis. Biomed Res. Int. http://dx.doi.org/10.1155/2013/567876.

Arias, M.S., Sanchís, J., Francisco, I., Francisco, R., Piñeiro, P., Cazapal-Monteiro, C., Cortiñas, F.J., Suárez, J.L., Sánchez-Andrade, R., Paz-Silva, A., 2013b. The efficacy of four anthelmintics against *Calicophoron daubneyi* in naturally infected dairy cattle. Vet. Parasitol. 197, 126-129.

Arias, M.S., Arroyo, F.L., Cazapal-Monteiro, C., Hernández, J.A., Suárez, J., Francisco, I., López-Arellano, M.E., Sánchez-Andrade, R., Mendoza de Gives, P., Paz-Silva, A., 2015. Formulating *Duddingtonia flagrans* in nutritional pellets for the sustainable control of equine strongyles. J. Sci. Technol. Environm. 1, 1-16.

Arroyo, F.L., Arias, M.S., Cazapal-Monteiro, C.F., Hernández, J.A., Suárez, J., Miguélez, S., Romasanta, A., Sánchez-Andrade, R., Paz-Silva, A., 2016. The capability of the fungus *Mucor circinelloides* to maintain parasiticidal activity after the industrial feed pelleting enhances the possibilities of biological control of livestock parasites. Biol. Control 92, 38-44.

Arroyo, F., Hernández, J.A., Cazapal-Monteiro, C.F., Pedreira, J., Sanchís, J., Romasanta, Á., Sánchez-Andrade, R., Paz-Silva, A., Arias, M.S., 2017. Effect of the filamentous fungus *Mucor circinelloides* on the development of eggs of the rumen fluke *Calicophoron daubneyi* (Paramphistomidae). J. Parasitol. 103, 199-206.

Assis, R.C., Luns, F.D., Araújo, J.V., Braga, F.R., Assis, R.L., Marcelino, J.L., Freitas, P.C., Andrade, M.A., 2013. Comparison between the action of nematode predatory fungi *Duddingtonia flagrans* and *Monacrosporium thaumasium* in the biological control of bovine gastrointestinal nematodiasis in tropical southeastern Brazil. Vet. Parasitol. 193, 134-140.

Cazapal-Monteiro, C.F., Hernández, J.A., Arroyo, F.L., Miguélez, S., Romasanta, A., Paz-Silva, A., Sánchez-Andrade, R., Arias, M.S., 2015. Analysis of the effect of soil saprophytic fungi on the eggs of *Baylisascaris procyonis*. Parasitol. Res. 114, 2443-2450.

Chandrawathani, P., Jamnah, O., Waller, P.J., Larsen, M., Gillespie, A.T., 2004. Field studies on the biological control of nematode parasites of sheep in the tropics, using the microfungus *Duddingtonia flagrans*. Vet. Parasitol. 120, 177–187.

Cortiñas, F.J., Cazapal-Monteiro, C.F., Hernández, J.A., Arroyo, F.L., Miguélez, S., Suárez, J., López de Arellano, M.E., Sánchez-Andrade, R., Mendoza de Gives, P., Paz-Silva, A., Arias, M.S., 2015. Potential use of *Mucor circinelloides* for the biological control of certain helminths affecting livestock reared in a care farm. Biocontrol Sci. Techn. 25, 1443-1452.

Craig, T.M., 2009. Helminth Parasites of the Ruminant Gastrointestinal Tract. In: Anderson, D.E., Rings, M. (Eds.), Veterinary Therapy: Food Animal Practice (5th edition). Saunders Publishing, Philadelphia, pp. 78-90.

Demeler, J., Van Zeveren, A.M., Kleinschmidt, N., Vercruysse, J., Höglund, J., Koopmann, R., Cabaret, J., Claerebout, E., Areskog, M., von Samson-Himmelstjerna, G., 2009. Monitoring the efficacy of ivermectin and albendazole against gastro intestinal nematodes of cattle in Northern Europe. Vet. Parasitol. 160, 109-115.

Dias, A.S., Araújo, J.V., Braga, F.R., Puppin, A.C., Perboni, W.R., 2013. *Pochonia chlamydosporia* in the biological control of *Fasciola hepatica* in cattle in Southeastern Brazil. Parasitol. Res. 112, 2131-2136.

Dias Luns, F., Lopes Assis, R.C., Pinheiro Costa Silva, L., Magri Ferraz, C., Ribeiro Braga, F., Victor de Araújo, J., 2018. Coadministration of nematophagous fungi for biological control over nematodes in bovine in the South-Eastern Brazil. Biomed Res Int. https://doi.org/10.1155/2018/2934674.

Dimander, S.O., Höglund, J., Uggla, A., Spörndly, E., Waller, P.J., 2003. Evaluation of gastro-intestinal nematode parasite control strategies for first-season grazing cattle in Sweden. Vet. Parasitol. 111, 193-209.

Flack, S., 2016. The art and science of grazing. Chelsea Green Publishing, Vermont, pp. 240.

Grønvold, J., Wolstrup, J., Larsen, M., Henriksen, S.A., Nansen, P., 1993. Biological control of *Ostertagia ostertagi* by feeding selected nematode-trapping fungi to calves. J. Helminthol. 67, 31-36.

Francisco, I., Arias, M., Cortiñas, F.J., Francisco, R., Mochales, E., Sánchez, J.A., Uriarte, J., Suárez, J.L., Morrondo, P., Sánchez-Andrade, R., Díez-Baños, P., Paz-Silva, A., 2009. Silvopastoralism and autochthonous equine livestock: analysis of the infection by endoparasites. Vet Parasitol. 164, 357-362.

Healey, K., Lawlor, C., Knox, M.R., Chambers, M., Lamb, J., Groves, P., 2018. Field evaluation of *Duddingtonia flagrans* IAH 1297 for the reduction of worm burden in grazing animals: Pasture larval studies in horses, cattle and goats. Vet. Parasitol. 258, 124-132.

Hernández, J.Á., Arroyo, F.L., Suárez, J., Cazapal-Monteiro, C.F., Romasanta, Á., López-Arellano, M.E., Pedreira, J., de Carvalho, L.M.M., Sánchez-Andrade, R., Arias, M.S., de Gives, P.M., Paz-Silva, A., 2016. Feeding horses with industrially manufactured pellets with fungal spores to promote nematode integrated control. Vet. Parasitol. 229, 37-44.

Hernández, J.Á., Sánchez-Andrade, R., Cazapal-Monteiro, C.F., Sanchís, J.M., Arroyo, F.L., Paz-Silva, A., Arias, M.S., 2018a. A combined effort to avoid strongyle infection in horses in an oceanic climate region: rotational grazing and parasiticidal fungi. Parasit. Vectors 11, 240-247.

Hernández, J.Á., Cazapal-Monteiro, C.F., Sanchís, J., Sánchez-Andrade, R., Paz-Silva, A., Arias, M.S., 2018b. Potential usefulness of filamentous fungi to prevent zoonotic soil-transmitted helminths. Vector Borne Zoonotic Dis. 18, 690-696.

Hertzberg, H., Maurer, V., Heckendorn, F., Wanner, A., 2007. Control of gastrointestinal nematodes in cattle under dry pasture conditions. Rev. Suisse Agric. 39, 89-93.

Jobim, M.B., Santurio, J.M., de la Rue, M.L., 2008. *Duddingtonia flagrans*: biological control of cattle nematodes in the field. Cienc. Rural 38, 2256-2263.

Kumar, N., Rao, T.K., Varghese, A., Rathor, V.S., 2013. Internal parasite management in grazing livestock. J. Parasit. Dis. 37, 151-157.

Larsson, A., Dimander, S.O., Rydzik, A., Uggla, A., Waller, P.J., Höglund, J., 2006. A 3-year field evaluation of pasture rotation and supplementary feeding to control parasite infection in first-season grazing cattle--effects on animal performance. Vet Parasitol. 142, 197-206.

Malrait, K., Verschave, S., Skuce, P., Van Loo, H., Vercruysse, J., Charlier, J., 2015. Novel insights into the pathogenic importance, diagnosis and treatment of the rumen fluke (*Calicophoron daubneyi*) in cattle. Vet. Parasitol. 207, 134-139.

Mendoza de Gives, P., López-Arellano, M.E., Aguilar-Marcelino, L., Olazarán-Jenkins, S., Reyes-Guerrero, D., Ramírez-Vargas, G., Vega-Murillo, V.E., 2018. The nematophagous fungus *Duddingtonia flagrans* reduces the gastrointestinal parasitic nematode larvae population in faeces of orally treated calves maintained under tropical conditions-Dose/response assessment. Vet. Parasitol. 263, 66-72.

Moazeni, M., Ansari-Lari, M., Masoodfar, M., Hosseinzadeh, S., Mootabi Alavi, A., 2010. Lethal effect of high temperatures on the eggs of *Fasciola hepatica*. Iran. J. Vet. Res. 11, 168-173.

Nogareda, C., Mezo, M., Uriarte, J., Lloveras, J., Cordero del Campillo, M., 2006. Dynamics of infestation of cattle and pasture by gastrointestinal nematodes in an Atlantic temperate environment. J. Vet. Med. B Infect. Dis. Vet. Public Health 53, 439-444.

Nzalawahe, J., Hannah, R., Kassuku, A.A., Stothard, J.R., Coles, G., Eisler, M.C., 2018. Evaluating the effectiveness of trematocides against *Fasciola gigantica* and amphistomes infections in cattle, using faecal egg count reduction tests in Iringa Rural and Arumeru Districts, Tanzania. Parasit. Vectors 11, 384-392.

Ortiz Pérez, D.O., Sánchez Muñoz, B., Nahed Toral, J., Orantes Zebadúa, M.Á., Cruz López, J.L., Reyes García, M.E., Mendoza de Gives, P., 2017. Using *Duddingtonia flagrans* in calves under an organic milk farm production system in the Mexican tropics. Exp. Parasitol. 175, 74-78.

Rolfe, P.F., Boray J.C., 1993. Comparative efficacy of moxidectin, an ivermectin/clorsulon combination and closantel against immature paramphistomes in cattle. Aust. Vet. J. 70, 265-266.

Santurio, J.M., Zanette, R.A., Da Silva, A.S., De La Rue, M.L., Monteiro, S.G., Alves, S.H., 2009. Improved method for *Duddingtonia flagrans* chlamydospores production for livestock use. Vet. Parasitol. 164, 344-346.

Saumell, C.A., Fernández, A.S., Fusé, L.A., Rodríguez, M., Sagüés, M.F., Iglesias, L.E., 2015. Nematophagous fungi from decomposing cattle faeces in Argentina. Rev. Iberoam. Micol. 32, 252-256.

Silva, M.E., Braga, F.R., Borges, L.A., Oliveira, J.M., Lima, W. dos S., Guimarães, M.P., Araújo, J.V., 2014. Evaluation of the effectiveness of *Duddingtonia flagrans* and *Monacrosporium thaumasium* in the biological control of gastrointestinal nematodes in female bovines bred in the semiarid region. Vet. Res. Commun. 38, 101-106.

Smith, M.C., Sherman, D.M., 2009. Goat Medicine, second ed. WileyBlackwell, Ames, IA. United States Department of Agriculture (USDA). March 25, 2011. Animal and Plant Health Inspection Service (APHIS), Policy #Farm17, Regulation of Agricultural Animals. Available at: <http://www.aphis.usda.gov/animal_welfare/policy.php?policy=17>.

Szmidt-Adjidé V, Abrous M, Adjidé CC, Dreyfuss G, Lecompte A, Cabaret J, Rondelaud D., 2000. Prevalence of *Paramphistomum daubneyi* infection in cattle in central France. Vet Parasitol. 87, 133-138.

Thornton, P.K., 2010. Livestock production: recent trends, future prospects. Philos. Trans. R. Soc. Lond. B Biol. Sci., 365, 2853-2867.

Thrusfield, M., 2007. Veterinary epidemiology. Blackwell Science Ltd, Oxford (3rd Edition), pp. 624.

Torres-Acosta, J.F.J., Hoste, H., 2008. Alternative or improved methods to limit gastro-intestinal parasitism in grazing sheep and goats. Small Ruminant Res. 77, 159-173.

Undersander, D., Albert, B., Cosgrove, D., Johnson, D., Peterson, P., 2002. Pastures for Profit: A guide to rotational grazing (A3529). 2002. Cooperative Extension Publishing, University of Wisconsin-Extension [Online] Available: http://learningstore.uwex.edu/pdf/A3529.pdf.

van Wyk, J.A., Mayhew, E., 2013. Morphological identification of parasitic nematode infective larvae of small ruminants and cattle: a practical lab guide. Onderstepoort J. Vet. Res. 80, 539.

FIGURE CAPTIONS

Fig. 1.- Scheme of a rotational grazing regime developed over an interval of 24 months involving Friesian cattle (20 cows and 12 heifers) maintained under a leaders/followers system in which milking ruminants (leaders) pasture ahead of dried-off cows and heifers (followers). A pasture resting period of two months was observed.

Fig. 2.- Values of fecal egg count reduction (FECR) of *C. daubneyi* and GIN in cattle maintained under a leaders/followers grazing system and provided 2.5 kg of commercial nutritional pellets daily. G-L1: leaders (dewormed with closantel and albendazole during the drying periods); G-F1: followers (dewormed with closantel and albendazole at the beginning of the trial). Cd: *Calicophoron daubneyi*; GIN: gastrointestinal nematodes.

Fig. 3.- Values of fecal egg count reduction (FECR) of *C. daubneyi* and GIN in cattle maintained under a leaders/followers. G-L2: leaders (dewormed with closantel and albendazole during the drying periods and provided daily 2.5 kg commercial nutritional pellets); G-F2: followers (dewormed with closantel and albendazole at the beginning of the trial and provided 2.5 kg of commercial nutritional pellets containing chlamydospores of *Mucor circinelloides* and *Duddingtonia flagrans* - $3x10^5$ each/kg pellet) daily. Cd: *Calicophoron daubneyi*; GIN: gastrointestinal nematodes.

Table

Table 1.- Management of cattle involved into two trials maintained under a leaders/followers grazing system.

	Trial 1		**Trial 2**	
	Cows (*n*= 10)	Heifers (*n*= 6)	Cows (*n*= 10)	Heifers (*n*= 6)
Dewormer	Closantel and Albendazole	Closantel and Albendazole	Closantel and Albendazole	Closantel and Albendazole
Time of deworming	At the beginning of every dry period	At the beginning of the trial only	At the beginning of every dry period	At the beginning of the trial only
Nutritional supplementation	Commercial pellets	Commercial pellets	Commercial pellets	Commercial pellets manufactured with spores of *Mucor circinelloides* and *Duddingtonia flagrans*

Table

Table 2.- Efficacy of anthelmintic treatment (closantel and albendazole) on dried-off dairy cows maintained under a leaders/followers grazing system and provided 2.5 kg commercial nutritional pellets daily.

		Calicophoron daubneyi				Gastrointestinal nematodes			
Month	Dried cows (*n*)	EPG_0 (R)	EPG_{14} (R)	% FECR (CI 95%)	% CPCR (CI 95%)	EPG_0 (R)	EPG_{14} (R)	% FECR (CI 95%)	% CPCR (CI 95%)
1	4	318 (101)	0	100	100	316 (110)	0	100	100
2	4	321 (97)	8 (14)	98 (96-99)	75 (33-100)	367 (107)	15 (15)	95 (94-98)	50 (1-100)
3	4	294 (80)	0	100	100	308 (84)	8 (30)	98 (96-100)	75 (33-100)
4	4	275 (111)	0	100	100	317 (174)	0	100	100
5	4	284 (152)	4 (7)	99 (97-100)	50 (1-100)	354 (129)	15 (30)	95 (94-98)	50 (1-100)
6	4	309 (92)	0	100	100	323 (197)	0	100	100
7	4	314 (84)	4 (7)	99 (95-100)	75 (33-100)	339 (103)	0	100	100
8	4	307 (109)	0	100	100	306 (54)	0	100	100
9	4	295 (113)	17 (29)	94 (92-97)	50 (1-100)	348 (155)	8 (30)	97 (96-99)	75 (33-100)
10	4	302 (107)	8 (14)	97 (93-100)	50 (1-100)	309 (161)	0	100	0
11	4	311 (86)	0	100	100	323 (148)	15 (15)	95 (93-98)	75 (33-100)
12	4	306 (95)	13 (12)	96 (94-98)	75 (33-100)	320 (99)	0	100	100
13	4	319 (117)	0	100	100	325 (128)	0	100	100
14	4	308 (93)	0	100	100	333 (119)	8 (30)	98 (96-99)	50 (1-100)

15	4	324 (148)	12 (18)	96 (93-99)	50 (1-100)	390 (127)	0	100	100
16	4	313 (73)	13 (12)	96 (92-100)	50 (1-100)	352 (150)	8	98 (96-99)	75 (33-100)
17	4	298 (136)	13 (12)	96 (92-98)	75 (33-100)	301 (125)	15 (30)	96 (93-98)	75 (33-100)
18	4	312 (84)	10 (14)	97 (92-100)	50 (1-100)	350 (98)	0	100	100
19	4	304 (112)	15 (21)	95 (90-100)	75 (33-100)	307 (102)	0	100	100
20	4	308 (79)	0	100	100	341 (142)	8 (15)	98 (96-99)	75 (33-100)
21	4	296 (85)	15 (29)	95 (92-97)	50 (1-100)	309 (101)	0	100	100
22	4	298 (92)	15 (21)	95 (92-97)	50 (1-100)	317 (95)	0	100	100
23	4	309 (117)	0	100	100	303 (110)	0	100	100
24	4	303 (82)	8 (14)	97 (92-100)	75 (33-100)	352 (101)	8 (30)	98 (96-99)	75 (33-100)
Average		306 (101)	6 (9)	98 (96-100)	77 (69-85)	330 (126)	5 (15)	98 (97-100)	82 (75-90)

EPG_0: eggs per gram of feces at deworming; EPG_{14}: eggs per gram of feces 14 days after deworming;

FECR: fecal egg count reduction; CPCR: reduction in the numbers of cattle positive by coprological tests.

CI: confidence interval. R: range.

Table

Table 3.- Efficacy of control of helminths on heifers of trial 1 belonging to the group of followers (G-F1), dewormed with closantel and albendazole at the beginning of the study (month 0), and provided 2.5 kg commercial nutritional pellets daily.

Month	*Calicophoron daubneyi*						Gastrointestinal nematodes					
	EPG		FECR		CPCR		EPG		FECR		CPCR	
	Mean	Range	%	CI 95%	%	CI 95%	Mean	Range	%	CI 95%	%	CI 95%
0	331	100					332	175				
0.5	*0*	*0*	*100*		*100*		*13*	*50*	*96*	*91-100*	*83*	54-100
1	13	50	96	92-100	83	54-100	13	50	96	91-100	83	54-100
2	21	50	94	90-100	83	54-100	13	50	96	91-100	83	54-100
3	13	50	96	92-100	83	54-100	31	150	91	85-96	67	29-100
4	19	100	94	91-98	67	29-100	75	450	77	46-100	33	0-71
5	107	200	68	60-77	50	10-90	100	350	70	30-100	0	
6	157	150	53	33-73	17	0-46	114	200	65	27-100	0	
7	175	250	47	20-74	17	0-46	129	150	61	25-97	0	
8	171	250	48	14-83	0		132	250	60	26-94	0	

EPG: eggs per gram of feces; FECR: fecal egg count reduction; CPCR: reduction in the numbers of cattle positive by coprological tests.

Table

Table 4.- Efficacy of control of helminths on heifers of trial 2 belonging to the group of followers (G-F2), dewormed with closantel and albendazole at the beginning of the study (month 0), and provided 2.5 kg commercial nutritional pellets containing spores of *Mucor circinelloides* and *Duddingtonia flagrans* ($3x10^5$ each/kg pellet) daily.

Month	*Calicophoron daubneyi*						Gastrointestinal nematodes					
	EPG		FECR		CPCR		EPG		FECR		CPCR	
	Mean	Range	%	CI 95%	%	CI 95%	Mean	Range	%	CI 95%	%	CI 95%
0	334	350					329	285				
0.5	*0*	*0*	*100*		*100*		*14*	*50*	*96*	*92-100*	*83*	*54-100*
1	0	0	100		100		14	50	96	92-100	83	54-100
2	0	0	100		100		18	150	95	90-100	83	54-100
3	0	0	100		100		29	150	91	88-94	67	29-100
4	0	0	100		100		33	50	90	81-100	67	29-100
5	0	0	100		100		36	150	89	62-100	50	10-90
6	0	0	100		100		71	250	78	45-100	50	10-90
7	0	0	100		100		100	200	70	36-100	33	0-71
8	0	0	100		100		106	100	68	35-100	17	0-46
9	0	0	100		100		71	200	78	51-100	0	

EPG: eggs per gram of feces; FECR: fecal egg count reduction; CPCR: reduction in the numbers of cattle positive by coprological tests.

Table

Table 5.- Ratios between the fecal egg count reduction (FECR) values in cattle maintained under a leaders/followers grazing system. G-L1: leaders in trial 1; G-L2: leaders in trial 2; G-F1: followers in trial 1; G-F2: followers in trial 2.

Number of pasture rotations completed	*Calicophoron daubneyi*		Gastrointestinal nematodes	
	$FECR_{G-L2}/FECR_{G-L1}$	$FECR_{G-F2}/FECR_{G-F1}$	$FECR_{G-L2}/FECR_{G-L1}$	$FECR_{G-F2}/FECR_{G-F1}$
1	-	1	2.8	0.9
2	-	1.1	-	1.2
3	-	1.9	8.7	12
4	1.6	2.1	19.5	1.5
5	7.5	3.2	-	1.6
6	7.5	3.2	-	3.1
7	21.3	2.4	6.3	3.5
8	6.9	2.8	7.9	2.9
9	10.3	2.2	31.1	3.4
10	5.5	2.5	6.6	8.7
11	17.6	2.3	6.7	4.1
12	16.2	2.4	6	4.7

Trial 1: deworming and rotational grazing; Trial 2: deworming, rotational grazing and commercial nutritional pellets containing spores of *Mucor circinelloides* + *Duddingtonia flagrans* ($3x10^5$ each / Kg pellet). FECR: fecal egg count reduction.

Figure
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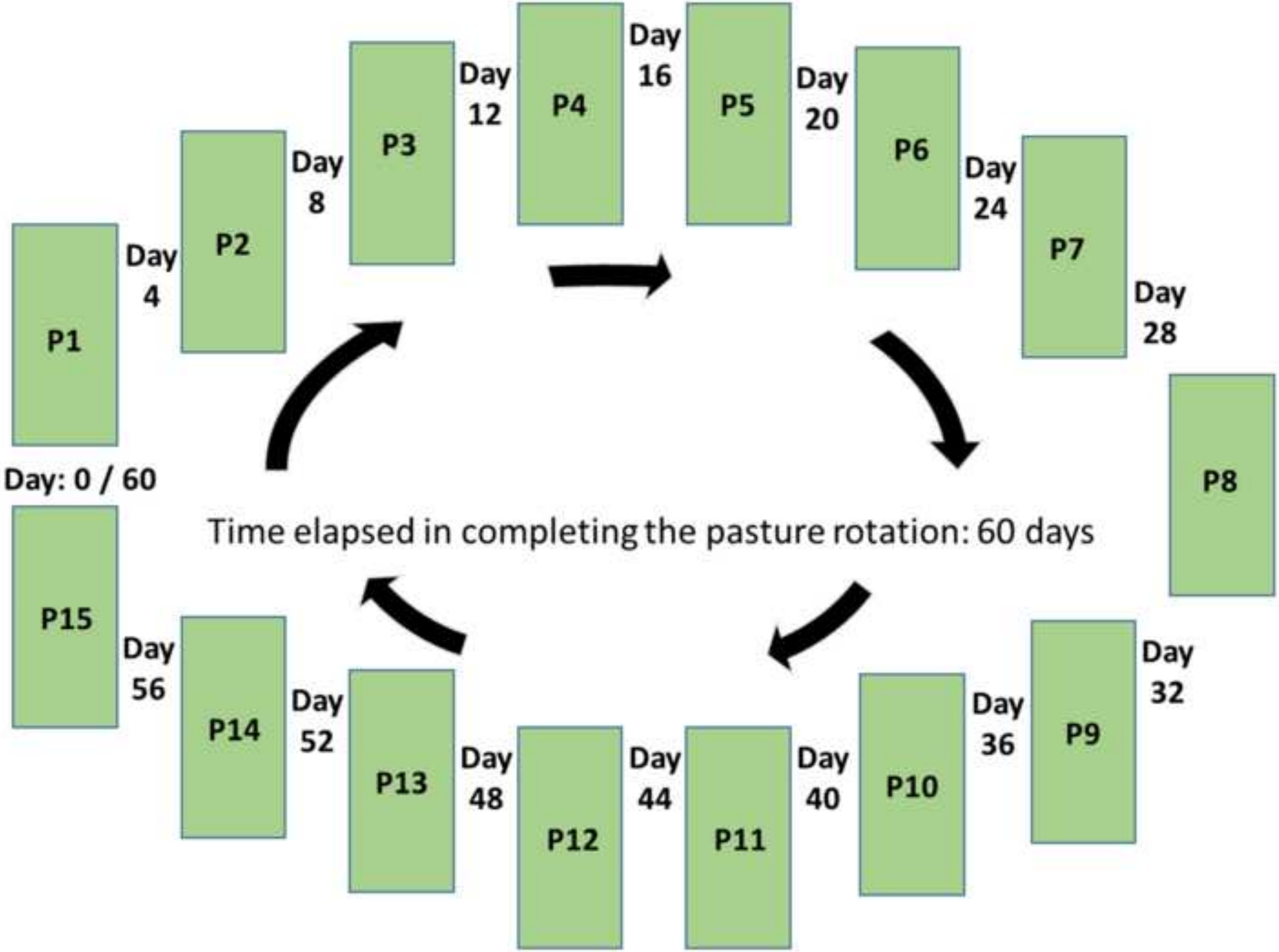

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Figure
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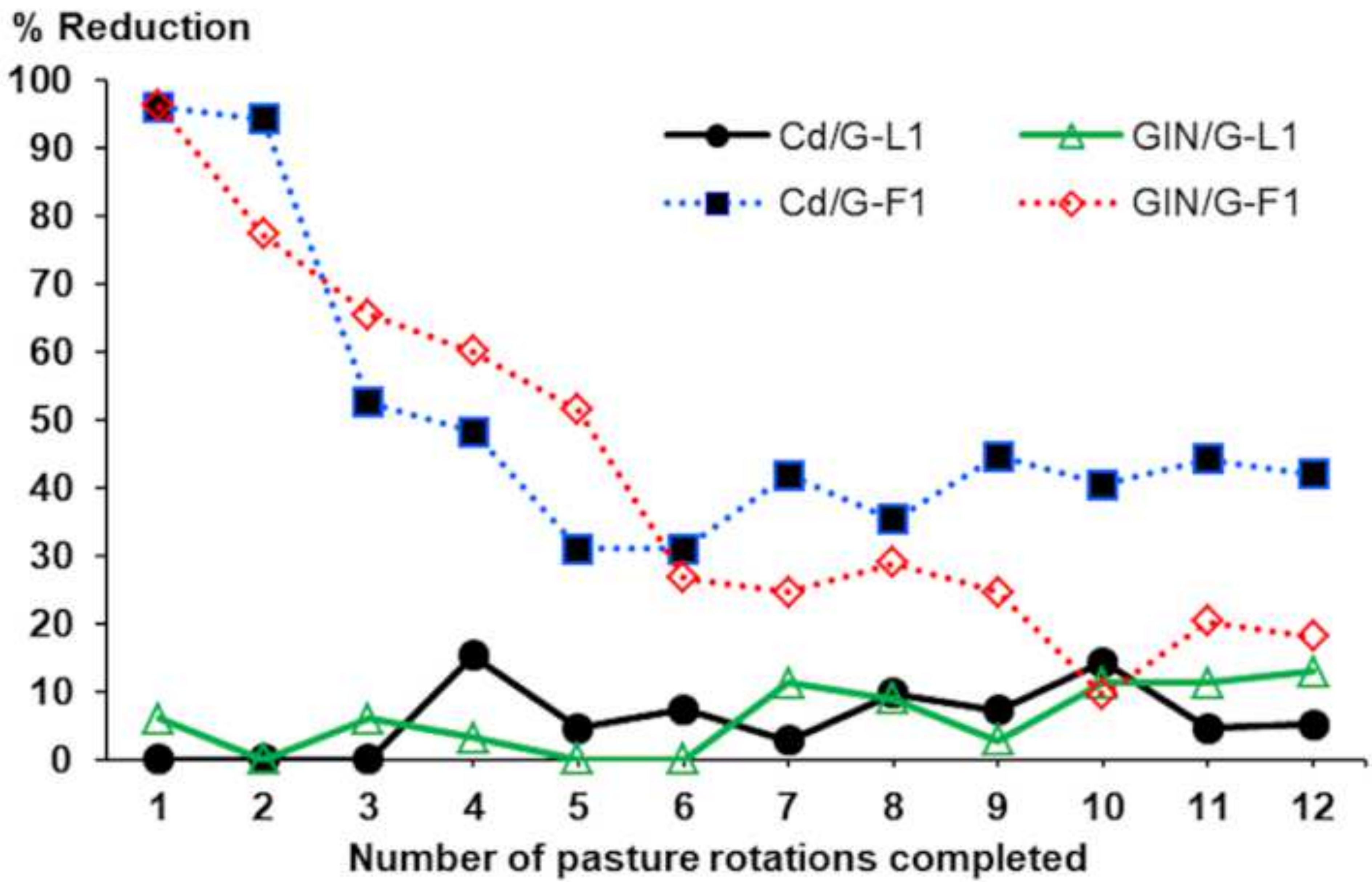

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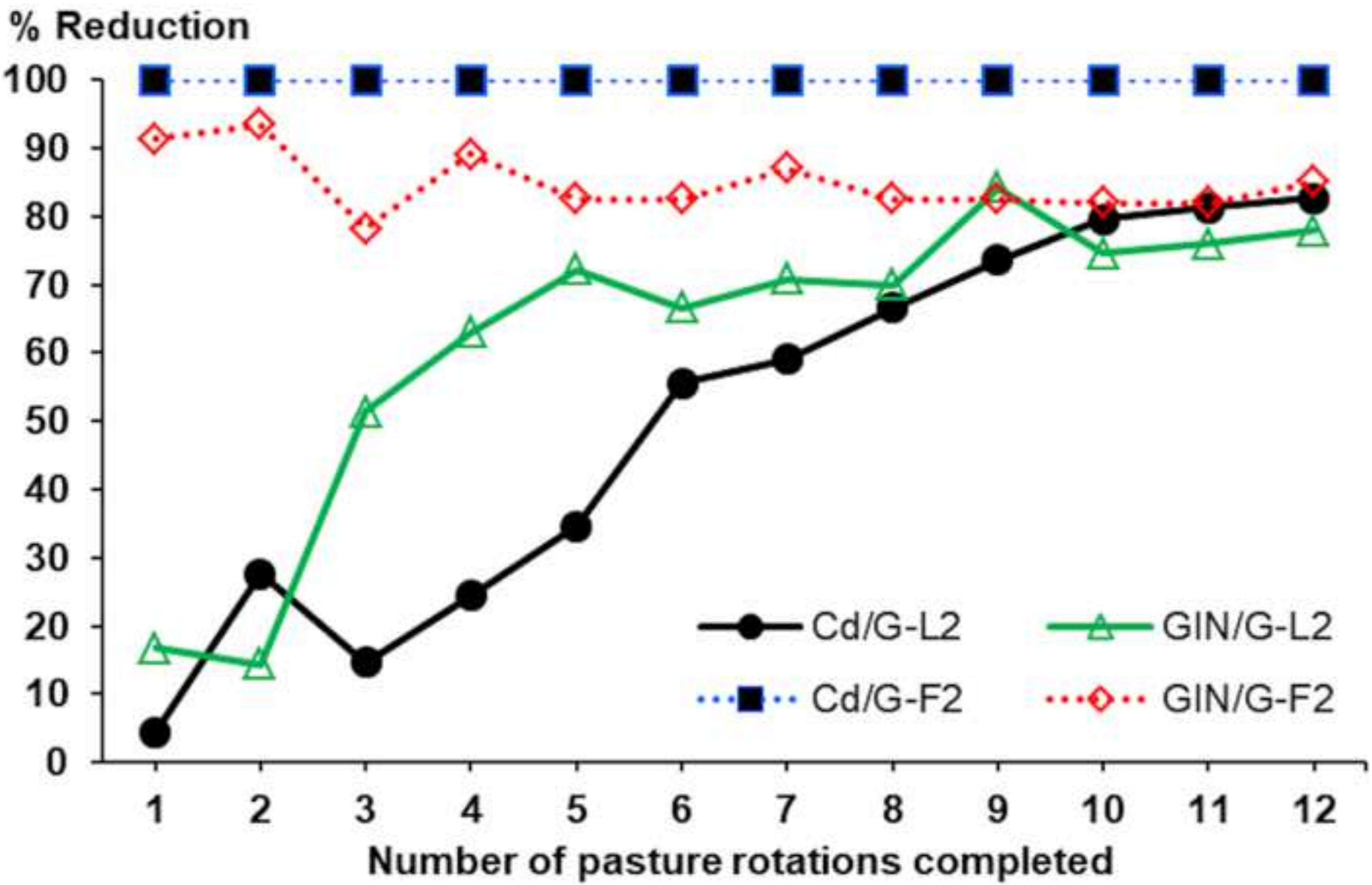
% Reduction
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
Number of pasture rotations completed
Cd/G-L2
GIN/G-L2
Cd/G-F2
GIN/G-F2

*Conflict of Interest

Declaration of interest

The authors declare that they have no conflict of interest.