Long-term survey of *Eimeria* spp. prevalence and faecal shedding in a traditional Portuguese free-range broiler farm *

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In free-range broiler production, animals are highly exposed to gastrointestinal parasitism, namely by coccidia, due to their long contact with the outdoor environment. Also, the prevalence of gastrointestinal parasites in extensive poultry production, is frequently influenced by biotic and abiotic factors. The current national pioneer long-term survey aimed to assess the prevalence and faecal shedding of *Eimeria* spp. oocysts in a traditional Portuguese free-range broiler farm, on different production conditions. Between July 2018 and March 2019, a total of 350 faecal samples were collected from different groups of free-range broilers at the fattening phase, in a poultry farm located in North-western Lisbon district, Portugal. Quantitative and qualitative coprological techniques were performed, aiming to quantify the *Eimeria* spp. prevalence and faecal shedding level. Coccidia prevalence was higher in Summer and Autumn groups (72% and 80%, respectively), although faecal shedding levels were higher in the Autumn and Winter groups (1191 and 1562 Oocysts Per Gram, OPG). Animals showed clinical signs of coccidiosis during the survey, especially in Autumn's 1^{st} sampling, in which 42% of the faecal samples had blood. The lack of sanitary depopulations, length of the fattening phase (1 month), short pre-patent period of coccidia, exposure to different environmental conditions and irregular drug treatments with amprolium, were key factors responsible for the different parasitic scenarios observed during this survey.

Keywords: Free-range broilers; Gastrointestinal parasitism; *Eimeria* spp.; Portugal.

1 Introduction

Coccidiosis by *Eimeria* spp. is the most prevalent and economically important gastrointestinal parasitic disease in poultry industry. In 2016, it is estimated that the global cost of coccidiosis in worldwide chicken production, reached values of near 10.36 billion £, based on the production losses and costs of treatment for this disease (Blake et al., 2020; Györke et al., 2016). A total of nine *Eimeria* species have already been described in chicken production and are commonly characterized according to the invasion of specific sites in the intestine, pathogenicity and type of lesion, being *E. tenella*, *E. necatrix* and *E. brunetti* the most pathogenic ones (Kaboudi et al., 2016; López-Osorio et al., 2020; McDougald & Fitz-Coy, 2008). Most studies in free-range poultry systems concluded

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that *Eimeria* spp. were the most prevalent gastrointestinal parasites, ranging from 23% to 90% (Chalchisa & Deressa, 2016; Lozano et al., 2018; Tomza-Marciniak et al., 2014).

Several authors have described the impact of biotic and abiotic factors, such as flock age and density, type of production system, weather and parasites life cycles, in the evolution of gastrointestinal infection in poultry farms (Ahad et al., 2015; Chengat Prakashbabu et al., 2017; Gharekhani et al., 2014; Kaboudi et al., 2016). However, there is still a lack of research regarding regional long-term studies on poultry gastrointestinal parasitism in traditional free-range systems. The main objective of this long-term survey was to track the evolution of coccidiosis in a traditional Portuguese free-range poultry farm, on different production scenarios.

2 Material and methods

2.1 Farm location and production conditions

For this study, a poultry farm located in north-western Lisbon district (39°13'59.5"N, 9°17'15.2"W) was selected, in which broilers are produced in a traditional free-range system and therefore contact with the outdoor environment in more than 50% of the production cycle. At 3-4 weeks of age, animals start to be partially exposed to the outdoor environment, until they reach 7-8 weeks. At this age, the flock is moved permanently to the outdoor park, where they are fattened until the slaughtering age of three months (Figure 1).

Figure 1: Photograph of free-range broilers in the fattening park of the chosen farm (original).

During this study. four different groups of slow-growing naked-neck broilers were analysed in different timeframes and fattening conditions (Table 1). The farm followed the all-in/all-out policy, despite not performing sanitary depopulations in the fattening park. Although previously to this study drug treatments were not included in the farm's health program policy, by the 2^{nd} sampling in the Autumn group, the farm owner decided to start treating animals with amprolium (Amproline®), via drinking-water (10 ml per day per 100 animals) and during four consecutive days. Afterwards, this treatment started to be applied whenever broilers reached the fattening phase. Groups sizes ranged from 80 to 200 animals, whose density in the fattening park reached values of 0.13 to 0.18 animals/m^2. Birds did not receive any other drug or probiotic, neither were vaccinated.

Table 1: Production conditions and animal health management in each group, during the research period

Group/Size	Date	Flock's age	Density (animals/m^2)	Amprolium treatment
G1	09/07/2018	66 days	0.17	NA[a]
200 chickens	23/07/2018	80 days		NA
G2	10/10/2018	80 days	0.14	NA
86 chickens	22/10/2018	92 days		3^{rd} day of treatment
G3	07/01/2019	64 days	0.13	4^{th} day of treatment
80 Chickens	21/01/2019	79 days		NA
G4	11/03/2019	68 days	0.18	NA
110 chickens	18/03/2019	75 days		NA

[a]NA - Not applicable.

2.2 Sampling procedure

This cross-sectional study had the least interference in the normal animal husbandry and management of the poultry facility, and therefore sampling periods were defined according to the farm's agenda. The characteristics of the flocks were not controlled and standardized by the authors, having been assessed the gastrointestinal parasitism of flocks, in parallel with the farm's normal activity. Between July 2018 and March 2019, a total of 350 faecal samples from free-range broilers, were collected directly from the fattening park's soil, after excretion, in each of the 4 groups selected for this study: Group 1 (G1) in July 2018 (Summer), Group 2 (G2) in October 2018 (Autumn), Group 3 (G3) in January 2019 (Winter) and Group 4 in March 2019 (Spring). Two samplings were performed in each group, with 1-2 weeks of interval.

Samples were inserted in plastic bags of 50 ml, and then transported in a cooling bag to the Laboratory of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Lisbon, where they were stored at 4-5 °C before analyses.

2.3 Coprological techniques

Faecal samples were analysed for the assessment of *Eimeria* spp. oocysts prevalence and number of oocysts per gram of faeces (OPG) (McMaster method), as well as species characterization (Willis-Flotation) and oocysts sporulation (faecal culture). McMaster and Willis-Flotation protocols were adapted and modified from Madeira de Madeira de Carvalho (2002), Vadlejch et al. (2013) and Zajac and Conboy (2012). Each sample was mixed with saturated solution of sucrose (specific gravity 1.2) and the filtrated suspension was transferred to a McMaster slide and visualized in a light microscope (10x), to calculate the *Eimeria* spp. prevalence and oocyst shedding level, using an analytic sensitivity of 50 OPG. The remaining suspension was transferred to a test tube until the formation of a convex meniscus, which was then covered by a coverslip. After 15 minutes, the coverslip was visualized in a light microscope (10x and 40x) for the identification of *Eimeria* species (McDougald & Fitz-Coy, 2008; Zajac & Conboy, 2012).

The faecal culture protocol was adapted from Zajac and Conboy (2012) and Kaboudi et al. (2016). Filter paper was placed in a Petri dish and 5-6 drops of potassium dichromate (2%) were added, as well as, 5 g of faecal samples positive for coccidia. Samples were incubated at 26-27°C,

for 7 days, and flotations were periodically performed every 24 hours, to assess oocysts sporulation and species identification.

2.4 Weather data collection

Since this study was conducted in four different timeframes, during the 2018-2019 period, and in order to evaluate a possible role of the outdoor temperature and relative humidity, in the parasitic scenarios detected in each group, data regarding average daily temperatures and relative humidity recorded in Lisbon district, was collected from Weather Underground (2019). A total of eight weather timeframes were analysed: from the beginning of the month until the 1^{st} sampling of the respective groups (Summer 1, Autumn 1, Winter 1 and Spring 1 samples) and between the two samplings of each season (Summer 2, Autumn 2, Winter 2 and Spring 2 samples).

2.5 Statistical analysis

The software GraphPad Prism®, version 8.4.3 for Windows (GraphPad Software, 2020) was used for statistical analysis and chart editing. Data Normality was assessed with the Kolmogorov-Test and, for every group included in this study, faecal oocysts shedding data failed the Normality Test ($p < 0.0001$). These results determined the statistical analysis of the data with the following tests: Mann-Whitney (intra-group OPG comparison), Fisher (intra-group species prevalence comparison and inter-group overall *Eimeria* spp. prevalence comparison), Chi-square (overall species prevalence comparison) and Kruskal-Wallis (inter-group OPG comparison), being $p < 0.05$ the significance level for the four tests. The software Microsoft®Excel®, for Microsoft 365, was also used for data compilation and chart editing.

3 Results

This study identified a global *Eimeria* spp. prevalence of 67% and an overall oocyst shedding level of 704.9 OPG, with G2 animals, produced in Autumn, having the highest values of *Eimeria* spp. prevalence (80%, $p = 0.0002$), while G3 ones, produced in Winter, had the highest OPG levels (Figure 2). In terms of coccidia species prevalence, *E. mitis* averaged the highest frequency of the study (27%, $p < 0.0001$), followed by *E. maxima* (18%) and *E. tenella* (17%) (Table 2). *Eimeria* spp. shedding levels differed significantly between groups ($p < 0.05$) and there was a significant correlation between the prevalence of *Eimeria* spp. and OPG levels all over the study ($p = 0.0072$).

Table 2: Global and per group/season *Eimeria* spp. prevalence (%) and faecal oocyst shedding (OPG)

Species	G1 Summer (n=100)	G2 Autumn (n=100)	G3 Winter (n=50)	G4 Spring (n=100)	Averages (n=350)
E. mitis	27[a]	32[a]	34[a]	19[a]	27[b]
E. maxima	30[a]	23	14	2	18
E. brunetti	16	13	8	3	10
E. praecox	11	23	12	5	13
E. tenella	13	24	26	9	17
E. mivati	9	4	6	1	5
E. acervulina	11	11	14	4	9
E. necatrix	17	7	16	6	11
Overall *Eimeria* spp. prevalence	72	80[a]	58	46	65
Faecal oocyst shedding (OPG)	419.5	1191	1562	75.5	704.9

[a]Fisher test ($p < 0.05$); [b]Chi-square test ($p < 0.05$).

Faecal oocyst shedding (OPG)

In the Summer group (G1), the most frequent coccidian species were *E. maxima* and *E. mitis*, with prevalence values of 18% and 16% in the 1^{st} sampling, and 42% and 38% in the 2^{nd} collection, respectively. In the 2^{nd} sampling, were also identified oocysts of *E. acervulina* (22%) and *E. necatrix* (34%) and all faecal samples tested positive for *Eimeria* spp. A statistically significant increase in the oocysts faecal shedding was observed between the two samplings ($p < 0.0001$), averaging a total of 419.5 OPG, as well as *E. maxima* and *E. mitis* were the most frequent species ($p = 0.0005$ and $p = 0.006$, respectively). Coproculture of oocysts from faecal samples collected in G1 only allowed the identification of *E. maxima* and E. tenella, under sporulating activity in the 1^{st} sampling, although in the 2^{nd} faecal sampling of this group, all coccidia species documented for chickens sporulated within two days of incubation, with exception to *E. mivati* and *E. acervulina* which did not sporulate. In this group, the global prevalence of coccidia reached 72%.

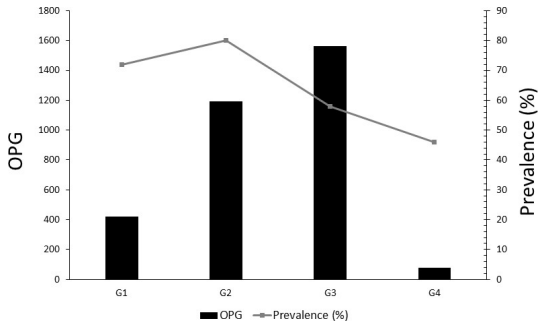

Figure 2: Overall prevalence and OPG of *Eimeria* spp. in each group, during the 2018-2019 period, displayed by the grey line and black bars, respectively.

In the Autumn group (G2), *E. mitis* (42%), *E. maxima* (38%) and *E. tenella* (34%) were the most frequent species in the 1^{st} sampling, as well as, *E. mitis* (22%), *E. praecox* (22%) and *E. tenella* (14%) were the most prevalent in the 2^{nd} sampling. A significant decrease in the oocysts

shedding was observed between the two samplings ($p < 0.0001$), as well as almost all species of coccidia suffered a reduction on their frequencies, except *E. mivati*, which maintained its prevalence (4%). In the 1^{st} sampling, 21 out of 50 samples (42%) had blood and tested positive for *E. necatrix* and/or *E. tenella* ($p < 0.0001$). In the coproculture of oocysts, almost all species of *Eimeria* spp. sporulated in the 1^{st} sampling, within 7 days of incubation. In addition, *E. mitis*, *E. maxima* and *E. tenella* were identified in the 1^{st} day of incubation already under sporulation. In samples from the 2^{nd} collection, a delay in the sporulating activity was observed, being only identified *E. maxima* and *E. praecox* under Table 1. Production conditions and animal health management in each group, during the research period, sporulation, after 5 and 7 days of incubation, respectively. The global prevalence of coccidia for this group was significant and reached 80% ($p = 0.0002$), the highest of the study.

In the Winter group (G3), *E. tenella* (4%) and *E. brunetti* (2%) were the only coccidian species identified in the 1^{st} sampling, a parasitic scenario that completely changed until the 2^{nd} sampling, in which all *Eimeria* spp. species were identified, with highlight to *E. mitis* (34%), *E. tenella* (22%) and *E. necatrix* (16%). The overall prevalence of coccidia in Winter's group totalized 58%, reaching 20% in the 1^{st} sampling and 96% in the 2^{nd} sampling. In terms of oocysts faecal burden, a significant increase was observed between samplings (8 to 3116 OPG, $p < 0.0001$). In the coproculture of oocysts, samples from the 1^{st} Winter collection did not exhibit *Eimeria* spp. sporulating activity, whereas in samples from the 2^{nd} collection, only *E. mitis* and *E. mivati* were detected under sporulation.

In the Spring group (G4), *E. mitis* was the most frequent coccidia in both collections. A change in the parasitic scenario was observed between the two samplings, specially related to *E. maxima*, *E. praecox* and *E. mivati*, which have only been identified in the 1^{st} sampling. In this group, the most frequent coccidia was *E. mitis* ($p < 0.0001$) and the overall prevalence of *Eimeria* spp. reached 46%, which was the lowest of the study. The faecal shedding of oocysts totalized 98 OPG and 53 OPG, in the 1^{st} and 2^{nd} sampling collections and did not differ significantly ($p = 0.7398$). In the coproculture of oocysts, only *E. mitis* and *E. acervulina* sporulated in samples from the 1^{st} and 2^{nd} collections, respectively.

Regarding the weather data recorded for Lisbon district, the highest value of average relative humidity during this study, was identified in G3 (73.4%), while average daily temperatures were higher in G2 (20.2°C) (Figure 3).

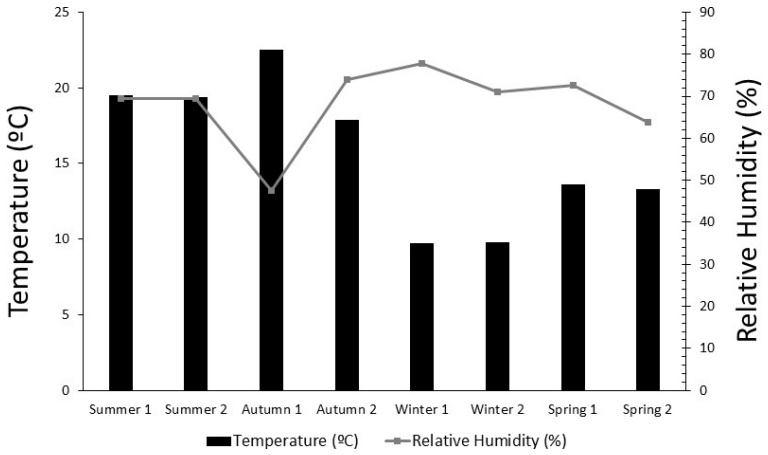

Figure 3: Average daily temperature (°C) and relative humidity (%) recorded in each sampling, during the 2018-2019 period.

4 Discussion

This was the 1st national long-term study evaluating the prevalence of coccidia in free-range broilers, reared in a traditional Portuguese poultry farm. Different parasitic scenarios regarding coccidiosis were recorded in each studied group, being coincident with different fattening phase conditions in which animals were produced. This study obtained a higher and alarming overall prevalence of *Eimeria* spp., in comparison with recent literature published in free-range chicken production (Ahad et al., 2015; Fatoba & Adeleke, 2018; Gharekhani et al., 2014), which in turn confirms in Portugal the wide distribution of coccidia as seen in worldwide poultry farms and its potential consequences for domestic birds' health.

The overall prevalence of *Eimeria* spp. was higher in Summer and Autumn groups, which was coincident with the highest values of average daily temperatures recorded in this study. *E. mitis* averaged the highest prevalence in this study, which allows to consider possible future health concerns in this farm. Despite being commonly described as less pathogenic and prevalent, when compared to other species like *E. tenella* (Ahad et al., 2015; Ola-Fadunsin et al., 2019), infections by *E. mitis* are frequently asymptomatic and responsible for weight losses and feed efficiency reductions in chicken production (McDougald & Fitz-Coy, 2008; Meireles & and R. F. Riera, 2005). These results are in accordance with the ones previously obtained by Chengat Prakashbabu et al. (2017), whose study in India also concluded that *E. mitis* was one of the most prevalent coccidian, in free-range chickens.

Although no correlations were performed between average temperature and relative humidity with the OPG levels for each group (since samplings were conducted twice in each season and therefore the amount of weather data was not suitable for correlation analyses), the OPG levels for each group still differed significantly, suggesting a possible role of the outdoor temperature and relative humidity in stimulating oocysts sporulation on soil and their consequent dissemination in the flock, as previously proposed by Ahad et al. (2015) and Kaboudi et al. (2016).

Considering the results obtained in each group, the significant increase in the *Eimeria* spp. shedding in G1, between the two samplings, could have been due to the cumulative effect of the high temperatures identified in this sampling period and the short pre-patent period of coccidia, which allowed their fast sporulation on soil and consequent reinfections, as referenced by McDougald and Fitz-Coy (2008) and Lozano et al. (2018). It is also important to highlight the identification of some species with notable pathogenicity, such as *E. necatrix* (Chapman, 2014), which was only identified in the 2nd sampling of this group.

The highest *Eimeria* spp. prevalence of the study was detected in G2, which was reared under Autumn weather conditions, in which was recorded the highest average temperature of the study (20.2°C). This may suggest a possible role of outdoor temperature in stimulating oocysts sporulation on soil, as previously proposed by Kaboudi et al. (2016). However, in order to avoid possible confounding influences, other factors must be assessed when discussing these results. Group 2 was also the oldest of the study and therefore was exposed for a longer time to coccidia, in comparison to the other groups, allowing to suggest animal's age and time of permanence in the fattening park, as key factors that may have influenced the overall results obtained in this group, as previously stated by Gharekhani et al. (2014) and Lozano et al. (2018). In G2 1st sampling, 42% of the samples had diarrhoea and blood, and all tested positive for *E. necatrix* and/or *E. tenella*, which is coincident with the common clinical signs of coccidiosis by these two species (Chapman, 2014; Zhou et al., 2020). The lack of sanitary depopulations associated with disinfectants, which is an elementary measure to reduce the risk of infection by *Eimeria* spp. in poultry production (Myung-Jo, 2014), may have contributed to the accumulation of oocysts shed by the previous groups fattened in the

same outdoor area, which in conjugation to the lack of a drug treatment program, allowed a higher shedding of *Eimeria* spp. contaminating the soil and its dissemination among the flock (Györke et al., 2016; Lozano et al., 2018; Peek & Landman, 2011). Despite the prevalence of coccidia in the 2^{nd} sampling was still high (72%), results obtained after amprolium administration revealed a reduction on the oocysts shedding, as well as an interruption in their sporulating activity, allowing to conclude about the coccidiostatic activity of amprolium against coccidia in this free-range chicken production (DGAV, 2020; Kant et al., 2013).

The highest oocysts shedding of the study was recorded in G3, which was fattened in Wintertime. Considering that this period had the lowest average daily temperatures of the study, values of relative humidity above 70% could have been a key-factor in stimulating oocysts sporulation on the environment, countering the adverse effect of low temperatures, and allowing the dissemination of coccidia among the flock, as proposed by Ahad et al. (2015). Considering that at the time of the 1^{st} sampling, animals from G3 were at the end of a 4-day drug administration program, the significant increase in the *Eimeria* spp. faecal shedding between samplings may suggest a possible lack of efficacy or even the development of drug resistance, despite no specific study on this sense was performed and no control groups were available to be included in this study to accurately conclude about this aspect. The long permanence of infected hosts in the same area and the consequent soil's recontamination with resistant oocysts, can be an explanation to the results obtained in this group, being in accordance with previous research from Gharekhani et al. (2014) and Lozano et al. (2018). Group 4 recorded the lowest values of OPG and prevalence of the study. Although the majority of *Eimeria* species were diagnosed in the 1^{st} sampling for Group 4, the coproculture of oocysts only revealed *E. mitis* under sporulation, which suggests again a possible suppressive activity by amprolium (Kant et al., 2013).

5 Conclusions

Regular parasitological surveys in poultry farms are of relevant importance, aiming to assess flock's health status, parasite dynamics and understand the potential role of biotic and abiotic factors in animal's overall gastrointestinal parasitism.

coccidia belonging to the genus *Eimeria* are indeed ubiquitous in avian hosts, and *E. mitis* was the most prevalent coccidia in this study, which is an interesting result, since most of the studies in poultry farms from other countries, often identify *E. tenella* and *E. maxima* as the most frequent coccidia.

Although the groups from Summer and Autumn had the highest *Eimeria* spp. prevalence of the study, oocysts shedding levels followed a different pattern, since they were higher in Autumn and Winter groups. This discrepancy alerts to the fact that coccidia prevalence and shedding levels should not be analysed separately, as well as without measuring the impact of other biotic and abiotic factors, since a higher prevalence of *Eimeria* spp. oocysts in a flock may not be directly linked with higher shedding levels and higher pathogenicity to the hosts.

Although different rearing conditions were found during the study, it can be globally concluded that the lack of sanitary depopulations, length of the fattening phase (1 month), short pre-patent period of coccidia, exposure to different environmental conditions and irregular drug treatments with amprolium, were key factors responsible for the different parasitic scenarios observed during this long-term survey.

More parasitological assays should be conducted in free-range poultry farms and the search for alternative parasite control programs must be a priority, due to animals' long exposure to

gastrointestinal parasites and the high risk of reinfection in this production system.

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