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A Field Study Evaluating the Effects of Diclazuril and Oregano Oil for the Prevention of Coccidiosis in Fattening Rabbits

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Abstract: For years, there has been an increasing interest in natural alternatives to the conventional coccidiostats applied as feed additives, which have been used for decades to prevent coccidiosis in poultry and fattening rabbits. This study aimed to compare the possible anticoccidial effects of oregano oil to the established substance diclazuril in growing rabbits. The control group (CG) received a non-supplemented basal compound feed, to which either diclazuril (1 mg/kg; DG) or oregano oil (75 mg/kg; OG) was added. In each of the three trials, subgroups of 50 rabbits each were assigned to one of the three experimental groups (CG, DG and OG). Natural *Eimeria* infection was monitored weekly by fecal oocyst counts and *Eimeria* species identification following sporulation. Additionally, the performance parameters were determined at the middle and the end of the trials, and the deceased rabbits were subjected to necropsy. Neither oocyst excretion nor the performance parameters differed significantly between the three experimental groups. *Eimeria media*, *Eimeria magna*, *Eimeria perforans* and *Eimeria exigua* were identified as the occurring species. The highest animal losses (16.0%) occurred in the OG, while the losses were 12.7% in the DG and 12.0% in the CG. However, these differences were not statistically significant. Overall, neither diclazuril nor oregano oil was superior to the non-supplemented feed. This underlines the importance of diagnostics, as this study's results indicate that in the absence of the highly pathogenic *Eimeria* species, economic rabbit rearing and fattening is achievable without the use of coccidiostats.

Keywords: *Eimeria*; coccidia; coccidiosis prevention; coccidiostat; feed additives; *Origanum vulgare*; carvacrol; thymol

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1. Introduction

Coccidia of the genus *Eimeria* are common parasites in rabbits and one of the main causes of intestinal disorders on conventional rabbit farms [1,2]. Young animals in the post-weaning phase are particularly susceptible to coccidiosis, which may lead to high morbidity or even mortalities in rabbit production, accompanied by substantial economic losses, whereas adult animals are mostly asymptomatic carriers of the *Eimeria* spp. [3,4]. Coccidia are ubiquitous pathogens in all forms of rabbit husbandry, i.e., not only in intensive rabbit breeding, but also in backyard breeding, and are impossible to eliminate [1,5]. The way rabbits are kept has a decisive influence on the occurrence of coccidiosis. For example, rabbits kept on the floor or in litter housing have shown a higher incidence of coccidiosis compared to rabbits kept in cages with perforated floors [2,6]. In addition, high stocking densities are conducive to the infection extensity and the spread of the parasites [7]. Environmental factors, such as humidity, oxygenation and temperature, also contribute to a higher infection pressure as they influence sporulation [8]. Coudert et al. [9] reported that

the sporulation duration of all the rabbit *Eimeria* species is shorter at 26 °C than at 18 °C or 22 °C, whereas temperatures above 28 °C have adverse effects on oocyst viability.

Eleven different *Eimeria* species are known in rabbits, ten of which infect the intestinal tract, while one (*Eimeria stiedai*) multiplies in the bile ducts [9]. Intestinal coccidia cause more or less severe diseases in rabbits, depending mainly on the *Eimeria* species, infection intensity, immune status and age of the animals, leading to reduced feed intake, weight loss, diarrhea and even death [10]. In addition to their own pathogenicity, coccidia contribute significantly to the occurrence of other intestinal diseases, such as epizootic rabbit enteropathy (ERE) [11]. Treatment after the onset of clinical symptoms often does not result in the desired success. Thus, control measures to prevent coccidiosis are of the utmost importance [12]. Improved hygiene management alone is not sufficient to prevent coccidial diseases in rabbit breeding, so the use of chemical feed additives is a common control measure [10]. In general, the routine use of coccidiostats leads to the rapid development of resistance, so that new active substances with a different mode of action have to be constantly sought [13,14]. Regulation (EC) No. 1831/2003 on additives for use in animal nutrition regulates the use of coccidiostats in animal feed in the European Union [15]. Currently, only two coccidiostats, diclazuril and robenidine, are authorized as feed additives for rabbits [16]. As a result, rotation programs to reduce the development of anticoccidial resistance, as carried out in commercial poultry, are hardly feasible in the long term. In addition to increasing the anticoccidial resistance of both poultry and rabbit *Eimeria* spp. [10,17–20], there are also food safety and public health concerns due to drug residues in foods of animal origin, as well as concerns due to the accumulation of these substances in the environment [21–24]. These concerns have prompted the search for alternative control and prevention strategies [24,25].

Anticoccidial effects have been reported for a number of phyto-additives. Sage (*Salvia of-ficinalis* L.) plant extract, oregano (*Origanum vulgare*) plant extract and extract mixtures of oregano (*Origanum* spp.), cinnamon (*Cinnamonum* spp.) and Mexican pepper (*Capsicum annuum*) have shown anticoccidial effects against intestinal *Eimeria* spp. in naturally infected rabbits [26,27]. Other studies have shown partial anticoccidial effects. For example, Strychalski et al. [28] found reduced oocyst excretions only in the middle of the fattening period for rabbits fed a proportion of black cumin (*Nigella sativa*) seed meal. Similar results have been reported for moringa (*Moringa oleifera*) seed oil, thyme (*Thymus vulgaris*) oil, artemisinin (*Artemisia annua*) extract, cinnamon oil and clove oil against hepatic coccidia (*E. stiedai*) in experimentally infected rabbits [29,30]. Under in vitro conditions, it has been shown that certain bioactive compounds of some essential oils exhibit the ability to destroy *Eimeria* oocysts [31], inhibit their sporulation [32] or impair the invasion of *Eimeria* sporozoites [33].

In in vivo studies, experimentally *Eimeria*-infected broilers fed an oregano oil-supplemented feed showed reduced oocyst excretion [34–37] and fewer manifestations of intestinal lesions [37] than the control animals. Reduced oocyst excretion, as well as improved feed conversion, were also observed in broilers with a natural *Eimeria* infection to which oregano oil-supplemented diets were fed [38]. These authors concluded that oregano oil might be an alternative to conventional coccidiostats for the control of avian coccidiosis due to its anticoccidial effects. Farinacci et al. [39] evaluated various medicinal plants based on the results of 39 studies with a total of 78 in vivo experiments on gastrointestinal protozoa, and concluded that *O. vulgare* is among the most promising plants with which to replace anticoccidials in poultry production. Most coccidia research is focused on poultry because of its economic importance [10], whereas rabbit coccidiosis does not receive similar attention. In fattening rabbits fed with garlic–oregano mixtures as a feed additive, reduced oocyst excretion compared to rabbits fed with coccidiostat additives or non-supplemented feed were repeatedly observed [40,41]. The same results were observed when oregano was administered to weaned rabbits via drinking water [26,27].

Antibiotic growth promoters have been banned as feed additives in the EU since 2006. A future ban on coccidiostats in feed cannot be excluded. If the reliable anticoccidial effects of oregano oil were established, a natural alternative to control rabbit coccidiosis would

be available. Therefore, the aim of this study was to investigate the possible anticoccidial effects of oregano oil as a feed additive for fattening rabbits in comparison to a classical coccidiostat and a non-supplemented diet.

2. Results

2.1. Oocyst Excretion and the Differentiation of the Occurring Eimeria Species

The mean total oocyst excretion per gram of feces (opg) for all three diet variant groups (control groups [CGs], diclazuril groups [DGs] and oregano oil groups [OGs]) in each of the three trials is presented in Table 1, while the mean opg for all three groups on the seven days of fecal sampling is shown in Table 2. The mean oocyst excretion for each dietary treatment on the different collection days is shown in Table 3. Overall, the OGs and DGs showed lower oocyst excretion than the CGs, with the OGs' values slightly lower than those of the DGs; however, there were no significant differences among the groups at any time point.

Table 1. The mean (± standard deviation, *n* = 21 pooled fecal samples per trial) oocyst excretion for the three consecutive trials (*n* = 21 pooled fecal samples per trial).

	Trial			Kruskal–Wallis Test (*p*-Value)
	1	2	3	
Oocyst excretion (10^3/g feces)	47.6 ± 55.0	68.9 ± 59.0	66.8 ± 91.0	0.587

Table 2. Mean (± standard deviation, *n* = 9 pooled fecal samples per study day) oocyst excretion for each examination day.

	Study Day							Kruskal–Wallis Test (*p*-Value)
	1	6	13	20	27	34	41	
Oocyst excretion (10^3/g feces)	0.25 ± 0.58	22.6 ± 13.4	128 ± 112	98.4 ± 58.4	85.2 ± 48.9	29.9 ± 15.9	64.0 ± 66.7	<0.001

Dunn Post Hoc Test FDR-adjusted *p*-values

1 vs. 6	1 vs. 13	1 vs. 20	1 vs. 27	1 vs. 34	1 vs. 41	6 vs. 13	6 vs. 20	6 vs. 27	6 vs. 34	6 vs. 41	13 vs. 20	13 vs. 27	13 vs. 34	13 vs. 41	20 vs. 27	20 vs. 34	20 vs. 41	27 vs. 34	27 vs. 41	34 vs. 41
0.109	<0.001	<0.001	<0.001	0.033	0.001	0.001	0.001	0.023	0.677	0.243	0.877	0.691	0.025	0.109	0.775	0.033	0.142	0.066	0.243	0.544

Table 3. The mean (± standard deviation, *n* = 21 pooled fecal samples per group) oocyst excretion for the three different diet variant groups.

Oocyst Excretion (10^3/g Feces)	Diet Variant			*p*-Value
	Control Groups	Diclazuril Groups	Oregano Oil Groups	
Study day 1	0.0 ± 0.0 [1]	0.17 ± 0.15	0.59 ± 1.03	0.500
Study day 6	29.1 ± 19.3	18.5 ± 13.8	20.4 ± 7.26	0.647
Study day 13	188.4 ± 172.5	101.9 ± 92.8	93.8 ± 62.3	0.586
Study day 20	148.2 ± 69.4	72.9 ± 31.8	74.0 ± 47.0	0.205
Study day 27	85.3 ± 51.8	73.9 ± 40.8	96.5 ± 69.5	0.884
Study day 34	39.9 ± 14.4	34.1 ± 17.5	15.6 ± 3.11	0.140
Study day 41	64.5 ± 70.2	79.0 ± 94.7	48.5 ± 56.6	0.561

[1] Below detection limit of 10 opg.

While oocyst excretion did not differ significantly among the trials or diet variants (Tables 1 and 3), the factor "Study day" had a significant effect on oocyst excretion (Tables 1–3). The highest oocyst excretion occurred between the 13th and 27th study day (Table 2).

The course of oocyst excretion in the individual trials is demonstrated in Figure 1. In the first trial, the highest excretion was observed in the CG, with 215,700 opg on study day

[SD] 20, while the values for the OG remained below 100,000 opg throughout the whole fattening period (maximum of 75,210 opg). This also applies to the DG during the first five weeks, but at the end of fattening (SD 41), this group exhibited the highest excretion, with 188,400 opg. In the second trial, the highest values were found for the OG (174,200 opg on SD 27) and CG (152,000 opg on SD 20). In these groups, there was a marked decrease after the excretion maximum, followed by a renewed increase at the end of the fattening period (113,800 and 145,300 opg, respectively), while excretion in the DG remained below 100,000 opg throughout (maximum: 88,940 opg). In the third trial, all the groups showed the highest excretion on SD 13 (CG: 386,000; DG: 209,100; OG: 83,330 opg). As in the first trial, the OG exhibited the lowest oocyst excretion with less than 50,000 opg on six of the seven examination days.

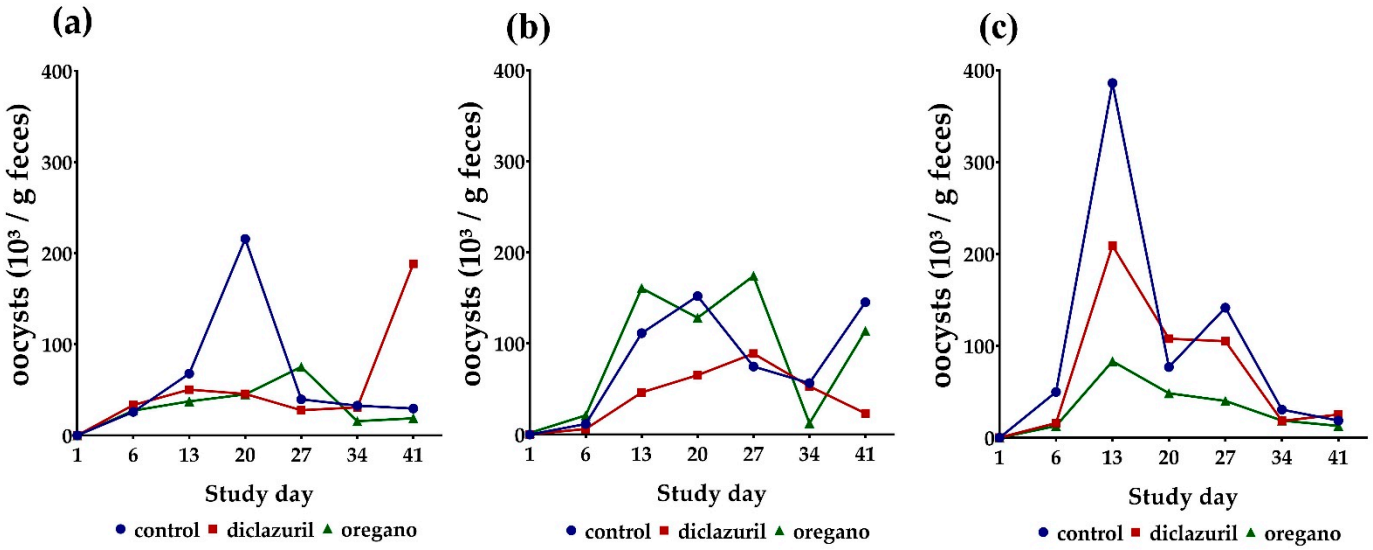

Figure 1. The oocysts per gram of feces for the three individual trials: (**a**) trial 1, (**b**) trial 2 and (**c**) trial 3.

The differentiation of the occurring *Eimeria* species after sporulation revealed four species, with *E. media* as the most frequent one, followed by *E. magna*, *E. perforans* and *E. exigua*. The relative shares of each species detected in the individual fecal samples were summarized, and the calculated mean proportions reveal a similar *Eimeria* species distribution among the three different diet variant groups, while the proportion of each species among the excreted oocysts was significantly different (Table 4).

Table 4. The mean proportion (± standard deviation) of the determined *Eimeria* species excreted by the three diet variant groups.

Group	*Eimeria* Species (%)				*p*-Value
	E. media	*E. magna*	*E. perforans*	*E. exigua*	
Control	61.39 ^{a} ± 5.07	27.88 ^{b} ± 5.23	9.77 ^{c} ± 2.29	0.97 ^{d} ± 0.37	<0.001
Diclazuril	56.65 ^{a} ± 5.02	30.89 ^{b} ± 7.35	11.50 ^{c} ± 2.76	0.96 ^{d} ± 0.09	<0.001
Oregano	60.31 ^{a} ± 2.56	29.66 ^{b} ± 4.93	9.27 ^{c} ± 2.94	0.77 ^{d} ± 0.06	<0.001
p-value	0.784	0.710	0.826	0.291	

^{a,b,c,d} The means within the same row with different superscripts differ significantly ($p \leq 0.05$).

2.2. Feed Intake and Performance

The feed intake and feed conversion rates were calculated for each trial (trial 1–3) for each feed variant group (CG, DG and OG) for two different phases (SD 0–20 and SD 21–41) and for the entire fattening period (SD 0–41). The mean values for all three trials calculated, including the experimental days and the body weight gains of the dead rabbits, are presented in Table 5. There were no significant differences among the different groups

for each phase analyzed ($p > 0.05$). For the OG, only 147 or 146 rabbits, respectively, were available for the calculations. The missing rabbits escaped through a hole in the fence during the first trial, which was mended before the following trials.

Table 5. The mean values for the daily feed intake per animal (g ± standard deviation) and the mean feed conversion ratio (kg/kg, ± standard deviation) for the three diet variant groups calculated, including the experimental days and the weight gains of the dead rabbits.

Study Days	Control Groups	Diclazuril Groups	Oregano Oil Groups	*p*-Value
Daily feed intake (g)				
0–20	105.7 ± 11.33	105.0 ± 5.12	103.7 ± 13.12	0.804
21–41	150.3 ± 9.24	151.0 ± 4.45	154.2 ± 6.34	0.488
0–41	128.0 ± 5.68	127.8 ± 0.87	129.5 ± 4.31	0.645
Feed conversion ratio (kg/kg)				
0–20	2.81 ± 0.05	2.73 ± 0.05	2.86 ± 0.19	0.600
21–41	4.86 ± 0.10	4.62 ± 0.06	4.73 ± 0.30	0.426
0–41	3.73 ± 0.03	3.59 ± 0.10	3.71 ± 0.05	0.792

The body masses of all the live rabbits measured at the different time points (SD 0, 21 and 41), as well as the daily body weight gains for the two different phases (SD 0–20 and SD 21–d 41) and over the entire fattening period (SD 0–41), are presented in Table 6. Again, there were no significant differences among the different groups at any time point or phase analyzed. The body weight of the rabbits in the CGs and DGs was significantly higher than in the OGs at the beginning of the trials. These significant differences disappeared towards the middle and end of the fattening period.

Table 6. The mean values for body weight (g ± standard deviation) and daily body weight gain (g ± standard deviation) of the live rabbits fed with the different diet variants over the course of all three trials.

Study Day	Age	Control Groups		Diclazuril Groups		Oregano Oil Groups		*p*-Value
	Days	*n*	g	*n*	G	*n*	g	
Body weight								
0	35	150	1033 ^{a} ± 103	150	1012 ^{ab} ± 96	150	997 ^{b} ± 107	0.009
20	55	148	1796 ± 198	145	1799 ± 183	138	1766 ± 249	0.340
41	76	132	2498 ± 266	131	2539 ± 206	122	2533 ± 272	0.347
Daily weight gain								
0–20	35–55	148	38.1 ± 7.9	145	39.2 ± 7.6	138	38.5 ± 10.3	0.169
21–41	56–76	132	33.4 ± 9.5	131	34.5 ± 7.3	122	35.1 ± 7.7	0.687
0–41	35–76	132	35.8 ± 5.8	131	37.1 ± 4.2	122	37.4 ± 5.5	0.060

n: number of rabbits. ^{a,b} The means within the same row with different superscripts differ significantly ($p \leq 0.05$).

2.3. Mortality and Pathologic Examinations

Over the course of all three trials, the animal losses were highest in the OG (16.0%), followed by the DG (12.7%) and the CG (12.0%), but these differences were not statistically significant ($p = 0.555$).

Out of the total of 61 dead rabbits, 60 were necropsied, as one animal was not frozen by the farm staff. Macroscopically, mucus accumulations were found in the colons of 22 animals. Neither coccidia-typical lesions nor other macroscopic changes were found in the gastrointestinal tracts. Furthermore, no macroscopic alterations were observed in the livers. Overall, the pathological examinations were complicated by autolytic processes due to the duration from the animal's death to freezing, as well as the thawing process prior to the necropsy.

3. Discussion

The presented field study did not show significant effects of the feed additives diclazuril and oregano oil on the health or performance parameters of the fattening rabbits naturally infected with low or moderately pathogenic Eimeria species. The evaluated parameters are discussed in detail below.

3.1. Oocyst Excretion

The increasing opg values observed in the first weeks after weaning, with the maxima reached in the second to fourth week (range: 50,330–386,000 opg), are in line with previous studies [42,43], and confirm that rabbits are particularly susceptible to *Eimeria* infections after weaning until sufficient immunity has developed [3,4]. Only the diclazuril subgroup in trial 1 deviated from this pattern by showing the highest oocyst excretion at the end of this study. Overall, the opg values for this field study were quite variable, which was also noted by Papeschi et al. [42]. In coccidia research, it is known that even under identical experimental conditions, the *Eimeria* oocyst excretion of rabbits often varies greatly [44], for which individual reactions to stressors may be responsible [10]. This could be an explanation for the observed variable excretion pattern.

Compared to numerous other fattening rabbit studies with natural *Eimeria* infections [2,40,41,45], the excretion maxima determined in the present study are quite high. However, a few studies are available with even higher opg values of up to more than 400,000 opg in fattening rabbits [46,47]. Coudert et al. [48] observed the highest opg values, in the range of 10,000–50,000, in some out of a total of 96 French rabbit farms. Nonetheless, the respective fecal samples were collected from young animals aged 43 ± 4 days. These farms had concomitant ERE symptoms and high mortality rates of 14.5–16.0% during the fattening period. Both the opg values for that age and the level of losses are comparable to those of the present study.

When comparing the opg values of the present study to those of previous investigations, it needs to be taken into account that in previous studies the rabbits were presumably kept on the usual wire mesh flooring with often only narrow (a few mm in width) and/or rounded slats, so good preventive hygienic conditions could be assumed with regard to the ingestion of infective oocysts. The rabbits in the present study were kept on flooring with a significantly lower degree of perforation, as required by the German Regulation on the Protection of Animals and the Keeping of Production Animals [49]. The slat and slot width of 10 mm each, and the flat flooring (slats not rounded), resulted in the considerable soiling of the floor and the soles of the rabbits. Other authors have also reported more heavily soiled rabbit soles on similar flooring depending on the degree of perforation [50,51]. The prevailing hygienic conditions probably resulted in a higher numbers of ingested oocysts, which consequently led to increased *Eimeria* infection intensity and oocyst excretion.

Diclazuril and oregano oil supplementation in the compound feed did not significantly reduce *Eimeria* oocyst excretion compared to that of the control group. Nosal et al. [41] compared a diet supplemented with garlic and oregano extracts, and a diet supplemented with the coccidiostat robenidine, to a control diet in growing rabbits. They found the lowest opg values for the rabbits fed the herbal additives, but did not mention any significancies. In other studies, significantly reduced oocyst excretion was found in rabbits that received drinking water supplemented with oregano or sage extract, or feed supplemented with a mixture of different plant extracts (oregano, cinnamon and Mexican pepper) [26,27]. The lack of significant differences in *Eimeria* oocyst excretion of the diclazuril groups compared to the control animals in the present study is in contrast to several previous investigations [47,52–55], while Gugołek et al. [56] also found no significant differences in the oocyst counts of rabbits fed diets with or without robenidine as the coccidiostat. Similarly, Papeschi et al. [43] studied a rabbit diet supplemented with robenidine and a diet without a coccidiostat in an alternative housing system, and found very similar oocyst excretion values during fattening for the different animal groups. They concluded that fattening rabbits without coccidiostats is possible without any adverse effects. In the

present study, conducted on a commercial farm, the non-supplemented compound feed variant also had no negative effects on the health or performance of the control animals compared to the rabbits fed the supplemented diets and, thus, underlines this statement.

3.2. The Differentiation of the Occurring Eimeria Species

Eimeria media, *E. magna* and *E. perforans* were most frequently detected in the present study, while the fourth identified species, *E. exigua*, was rather rare. The first three species mentioned are also among the most common *Eimeria* in fattening rabbit flocks found in previous studies in different countries, such as France, Belgium, Italy, and England [2,48,53,57]. The pathogenicity of *Eimeria* differs markedly between the species [9,10]. Therefore, their identification plays an important role in coccidiosis management [1]. The *Eimeria* species diagnosed in this study can be classified as low (*E. perforans*, *E. exigua*) to moderately pathogenic (*E. media*, *E. magna*) according to Coudert et al. [9], while the two highly pathogenic species (*E. intestinalis* and *E. flavescens*) were not found.

3.3. Feed Intake and Performance Parameters

The feed intake and fattening performance observed in this study are comparable with investigations by Chrenková et al. [58], who used the same genetic rabbit line and found quite similar values for feed intake, weight gain and feed conversion rate at almost identical ages.

The feed additives diclazuril and oregano oil did not result in significant improvements in feed intake or performance compared to the non-supplemented diet. The same applies to the comparison of diclazuril with oregano oil. A tendentious, but not significant, advantage in the feed conversion rate could only be found for diclazuril. Cardinali et al. [59] observed significantly higher weight gains in rabbits fed oregano extract or a combination of oregano and rosemary extracts compared to a control group. In a study on rabbit diets supplemented with fennel leaves, oregano leaves and a mixture of fennel and oregano leaves, all three diets resulted in a significantly improved final body weight, weight gain, and feed conversion rate compared to a control group [60]. On the other hand, other studies have reported no effects of oregano oil on fattening performance [61–63]. With regard to diclazuril, the performance results obtained in the present study contradict previous investigations showing the positive effects of this coccidiostat on weight gain and feed conversion rates when compared to untreated control rabbits [53–55]. Gugołek et al. [56] investigated the performance of rabbits fed a diet supplemented with the coccidiostat robenidine compared to a diet without a coccidiostat, and found no significant differences in weight gain. Also, in a study by Szabóová et al. [27], which investigated an oregano and sage extract in growing rabbits, no significant differences in weight gain were found compared to the non-supplemented control group. The lack of significantly higher body masses in the diclazuril group in the present study could be due to resistant coccidia or the only low pathogenicity of the *Eimeria* species.

Body weight gain is a reliable criterion with which to assess the health status of growing rabbits [10]. This is particularly important in investigations of coccidiosis, because it often occurs subclinically [2,41], and causes growth retardation and poorer feed conversion [2,53]. From this point of view, the two feed additives did not improve the health status of the rabbits compared to that of the control animals.

3.4. Mortality and Pathologic Examinations

The use of diclazuril as a feed additive did not reduce the losses compared to those of the control group. The oregano oil group had the highest losses, contrary to Benlemlih et al. [64], who found a significantly lower mortality rate in rabbits fed a diet with dried oregano leaves compared to control animals.

It is worth mentioning that the mortality rates were not associated with *Eimeria* oocyst excretion, especially when comparing the control to the oregano group. Despite the highest excretion values, losses were lowest in the control group, while the opposite was true for

the oregano group. These results confirm that the opg values do not correlate with the occurrence of clinical symptoms and mortality in rabbits [12,41,65].

Regarding the mortalities of the growing rabbits, other pathogens, such as enteropathogenic *E. coli* (EPEC) and clostridia, need to be considered in addition to *Eimeria* infections [1,66]. In particular, ERE, the etiology of which has not yet been fully clarified, has been increasingly occurring in fattening rabbit flocks since 1997, and can lead to high loss rates, of up to more than 30% [67–70]. The absence of macroscopic inflammation or obstructions in the intestine is one of the main features of ERE. Occasionally, mucus can be found, primarily in the colon [1,67,71]. This could be a reason for the missing pathological alterations in the present study, except for the findings of mucus accumulations in the gastrointestinal tract of the dissected rabbits, and would also explain the high loss rates.

4. Conclusions

In this field study, the feed additives diclazuril and oregano oil did not have any significant effect on *Eimeria* oocyst excretion or the performance and health status of young rabbits compared to animals fed a non-supplemented diet. Similarly, there were no significant differences between the coccidiostat and the plant oil groups. The present results suggest that in the absence of the highly pathogenic *Eimeria* species, the omission of coccidiostats in fattening rabbits is possible without negative effects on animal health and performance. The focus in diagnostics should be on oocyst counting or, rather, on *Eimeria* species identification. Overall, this field study did not provide evidence for the suitability and efficacy of oregano oil as a plant-based coccidiostat. To substantiate this result, further studies under standardized conditions would be desirable.

5. Materials and Methods

5.1. Animals and Husbandry

This study was carried out on a commercial rabbit farm in southern Germany (Neuenstein, Baden-Württemberg) where breeding and growing rabbits are kept. The young rabbits included in this study were hybrids born on the farm and the offspring of breeding rabbits of the French genetic line Hycole. A total of 450 weaned, clinically healthy, 35-day-old rabbits were included in the three trials, i.e., 150 rabbits were included in each trial, with three groups of 50 animals each kept in separate pens per group. The same three pens were used in all three trials. The animals were housed in a barn with a total of 1600 fattening rabbits on plastic slatted flooring (slat and slot width 10 mm each). Each pen had 4.25 m^2 (2.5 × 1.7 m) of ground floor and an additional area of 1.6 m^2 on three elevated platforms (one measuring 2.0 × 0.5 m and two measuring 0.5 × 0.6 m) placed 30 cm above the ground. The stocking density was 11.76 animals/m^2 ground floor or 8.55 animals/m^2 including the elevated platforms. As further enrichment measures, plastic tubes (inner diameter: 16 cm, length: 35 cm) were placed in the pens and gnawing material (untreated spruce wood) was attached to the walls of the pens.

5.2. Study Design and Diet Variants

Three variants of a pelleted compound feed for weaned rabbits were tested in the three experimental groups. Each group included a total of 150 weaned rabbits, receiving either a basal diet (control group, CG) or the basal diet supplemented with diclazuril (diclazuril group, DG) or oregano (*Origanum vulgare*) oil (oregano group, OG). The composition of the three diet variants is given in Table 7.

This study was carried out with three trials, including a total of 450 rabbits. In each trial 150 rabbits were included, and were divided into three subgroups of 50 animals per feed variant (CG, DG and OG), resulting in a total of nine subgroups in this study. Between the trials, the feed variant administered changed in the respective pen, so that each feed variant was administered in each pen (Figure 2). Each trial lasted for a fattening period of 41 days and ended at slaughter. Three days before the end of each trial (SD 38), the animals received a conventional fattening rabbit diet without coccidiostats to meet the legal

withdrawal period for diclazuril. To ensure equal conditions between the subgroups, all the animals were subjected to this change in diet.

Table 7. The composition of the three diet variants according to the declaration and calculated chemical composition per kg feed as original substance (oS).

Ingredients	Diet Variant (g/kg oS [1])		
	Control Group	Diclazuril Group	Oregano Oil Group
Alfalfa	260	258	259
Wheat bran	225	225	225
Sugarbeet pulp	150	150	150
Barley	95	95	95
Oat hull meal	63	63	63
Rapeseed meal	50	50	50
Sunflower meal	49	49	49
Flax	47	47	47
Sugar beet molasses	35	35	35
Vitamins and mineral premix [2]	26	26	26
Diclazuril premix [3]	-	2	-
Oregano oil premix [4]	-	-	1

[1] original substance; [2] per kg feed: 15,000 IU vitamin A, 1125 IU vitamin D3, 100 mg vitamin E, 112.5 mg vitamin C, 0.30 mg Se, 14 mg Cu, 84 mg Zn, 42 mg Mn, 70 mg Fe, 1.4 mg I and 0.35 mg Co; [3] per kg premix: 0.5 g diclazuril, inert carrier—calcium carbonate; [4] per kg premix: 75 g natural oregano oil, inert carrier—wheat flour.

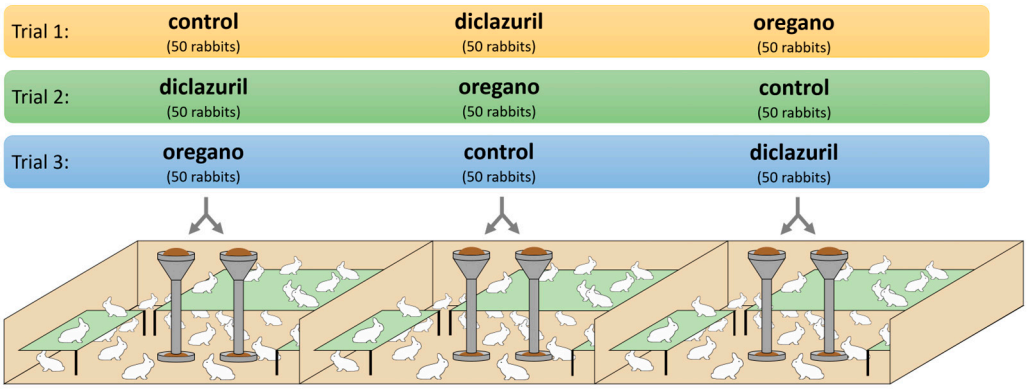

Figure 2. The feeding scheme of the three different diet variants (basal diet, control group; basal diet supplemented with diclazuril; basal diet supplemented with oregano oil) in the three trials.

The three diet variants were fed ad libitum using two automatic feeders per pen that were refilled twice daily. Water was also offered ad libitum through nipple drinkers. The supplemented feed variants contained 1 mg diclazuril (DG) and 75 mg oregano oil (OG) per kg feed variant, respectively. The oregano oil was mixed in as a powdery premix containing 7.5% natural oil of *O. vulgare* and wheat flour as the inert carrier. Oregano oil is approved as a sensory feed additive in the EU [16], so there were no concerns about using the carcasses for human consumption.

The chemical composition of the diet variants (Table 8) were analyzed at the Institute of Animal Nutrition, University of Veterinary Medicine Hannover, Foundation, Hannover, Germany. The investigations were carried out using a double approach in accordance with the official methods of the VDLUFA (Association of German Agricultural Analytic and Research Institutes) [72]. The dry matter content was determined by drying it to a weight constancy at 103 °C.

Table 8. The chemical composition of the three diet variants.

Chemical Composition	Diet Variant (g/kg oS [1])		
	Control Group	Diclazuril Group	Oregano Oil Group
Dry matter [a]	907	906	911
Crude protein [a]	160	160	161
Crude fat [a]	23.2	23.6	24.8
Crude fiber [a]	151	150	150
Crude ash [a]	78.2	78.6	79.1
Starch [c]	113	113	113
Sugar [c]	61	61	61
Calcium [c]	9.0	9.6	9.0
Phosphorus [c]	5.9	5.9	5.9

[1] original substance; [a] analyzed; [c] calculated.

5.3. Analyzed Study Parameters

5.3.1. Oocyst Excretion and Occurring Eimeria Species

A pooled fecal sample was collected weekly from each subgroup. The sampling was carried out by placing a plastic box under the perforated floors for 24 h. From these boxes, 500 g of feces was collected, mixed, and two samples of 200 g each were analyzed to determine the number of oocysts per gram of feces (opg). The fecal samples were examined according to the method of Coudert et al. [9], modified by using 200 g of feces each and examining five McMaster counting grids (100× magnification). A saturated sodium chloride solution (specific gravity: 1.2) was used for flotation. A portion of the feces–water suspension prepared for the opg determination was stored at 26 °C for at least one week and aerated daily through pipettes to induce sporulation of the oocysts. After a flotation procedure with saturated sodium chloride solution (specific gravity: 1.2), the *Eimeria* species present in each sample were determined by microscopic differentiation (400× magnification, or 1000× magnification if necessary) of 500 sporulated oocysts according to the morphological keys by Eckert et al. [73].

5.3.2. Feed Intake and Performance

In all three trials, the feed intake was determined on a subgroup basis. For this purpose, the consumed mass of feed was documented and the residuals from the opened feed bags, as well as from the automatic feeders, were weighed back on SD 20 and SD 41. A mean value for the daily intake of the compound feed was calculated, taking into account the deceased rabbits with the following formula:

$$\text{Daily feed intake} = \frac{\text{feed consumptiom}}{\text{sum of experienced fattening days}}$$

The feed conversion was also calculated, taking into account the body weight gains of the deceased rabbits. To assess the weight gain, the rabbits were tattooed on their left ears with a consecutive number prior to the start of each trial, and weighed at three different times. The first weighing was carried out directly before the start of the trial (SD 0). The second weighing was performed in the middle of the trial (SD 20), and the last weighing at the end of the trial (SD 41) immediately before slaughter. All the weighings were performed by placing the rabbits individually in a plastic box on a digital platform scale. Using individual animal identification, the body weight gains were individually calculated by the difference in body weights.

5.3.3. Mortality Rate and Pathologic Findings

In all three trials, the mortalities were recorded daily for each subgroup. The mortality rate of each experimental group (CG, DG and OG) was calculated as the percentage of deaths to the number of rabbits housed.

The rabbits lost during the trials were weighed and frozen at −20 °C. After each trial, the carcasses were thawed and necropsied. The entire gastrointestinal tract and liver were removed and examined for pathologic alterations.

5.4. Statistical Analysis

A statistical analysis was carried out using R (version 4.1.2) [74]. Pearson's chi-squared test was used to determine whether the proportion of animal losses in the three diet variant groups differed significantly from each other. The Shapiro–Wilk normality test was used for the data normality check by analyzing the model residuals with the function shapiro_text() implemented with the package "rstatix" (version 0.7.2) [75]. The normally distributed data were tested for significant differences with a one-way analysis of variance (ANOVA) and subsequent multiple pairwise comparisons using a pairwise t-test (the used functions were the anova_test() and pairwise_*t*_test() from the package "rstatix"). The non-normally distributed data were tested with the Kruskal–Wallis rank sum test, using the function kruskal_test() from the package "rstatix". Following the Kruskal–Wallis test, Dunn's test was performed for multiple pairwise comparisons with the function dunn_test(), also implemented from the package "rstatix". To control for the false discovery rate (FDR), the *p*-values were adjusted using the method of Benjamini and Hochberg [76].

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Institutional Review Board Statement: An ethical review and approval were not required because all the animals received a standard diet with approved feed additives at recommended rates and were sampled by non-invasive procedures (fecal sampling). Animal welfare concerns for this work were reviewed by the Animal Welfare Officer of the University of Veterinary Medicine Hannover, Foundation, Germany, code AZ: TVG-2019-V-23.

Informed Consent Statement: Informed consent was obtained from the owner of the rabbit farm.

Data Availability Statement: Data supporting the reported results are contained within the article.

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