Original Research Article

Running title: Role of feed additives in enhancing broiler health challenged by *Eimeria*.

Effect of oregano essential oils and probiotics supplementation on growth performance, immunity, antioxidant status, intestinal microbiota, and gene expression in broilers experimentally infected with Eimeria.

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Abstract

The poultry industry has several disease challenges; coccidiosis is considered one of the most dangerous parasitic diseases. Six-hundred Ross 308 male broiler chicks (average initial body weight 41.5 g) were allocated to six groups: negative control uninfected (NCO); positive control infected with *Eimeria* (PCO); or positive control supplemented with diclazuril (0.2 g/kg diet, DIC); two-strains probiotic (2 g/ kg diet, PRO); oregano essential oil (300 mg/ kg diet, OEO); a combination of 2 g probiotic and 300 mg oregano essential oil (POE). At day 15, all groups except NCO were inoculated via oral gavage with oocysts of mixed *Eimeria* spp. Our results indicated that the infected chickens with mixed *Eimeria* (PCO) exhibited lower growth performance, higher mortality rate, and increased oocyst shedding ($P < 0.05$), in addition to deterioration of immune-oxidative status in broilers on 35 d. On the contrary, *Eimeria*-infected chickens fed on a diet supplemented with DIC, PRO, OEO, and POE showed improved growth performance ($P < 0.05$) and decreased mortality and oocyst shedding. Moreover, feeding chickens on POE enhanced body weight gain, feed conversion ratio, crude protein digestibility, and dressing. Furthermore, POE supplements supported immunity and antioxidant status by increasing IgA, IgG, IL-10, and SOD levels ($P < 0.05$) and reducing IL-6 and MDA levels. Additionally, adding POE altered the gut microbiota structure via an increasing *Lactobacillus* count and decreasing *C. perfringens*, *E. coli*, and *Coliforms* count. Notably, POE supplements also reduced oocyst shedding and lesion scores. Moreover, the up-regulated nutrient transporters, including the expressions of cationic amino acid transporter-1 (CAT-1) and mucin-2 (MUC-2) were up-regulated ($P<0.05$) in the POE group compared to other groups. Therefore, our study concluded that a combination of probiotics and oregano essential oils was preferred as an effective choice for protecting broilers against coccidiosis.

Keywords: Broiler, Performance, Anticoccidial, Oregano essential oils, Probiotics, Gene expression

1. Introduction

Over the past few years, the poultry industry has achieved high levels of productivity to meet the world's shortage of animal protein requirements through the production of high-yielding breeds and high feed efficiency. Feed additives are usually used to improve feed utilization and pathogen resistance such as enzymes, growth promoters, anticoccidials, etc. (Emam et al., 2024; Abdel-Moneim et al., 2024). The main target of these feed additives is to maintain the gut and immune health to promote rapid growth and counteract the hazard of infection such as avian coccidiosis, that negatively affects production performance (Abdelhady et al., 2021). Poultry nutrition plays an important role in solving many problems facing the poultry industry by manipulating the composition of the feed or using feed additives to meet many challenges (Abdel-Moneim et al., 2022; Elbaz et al., 2022). Coccidiosis is one of the most severe parasitic diseases in poultry resulting in significant economic losses. These *Eimeria* parasites (coccidia subclass) can multiply and infect the mucosal epithelia in different parts of the broiler gut causing damages, including inflammation, diarrhea, and hemorrhage, that leads to deterioration of performance and severe losses (Noack et al., 2019). Coccidiosis has been controlled through anticoccidial drugs whether in feed or water (Choi et al., 2018; Abdelhady et al., 2021). Recently, modern nutritional strategies were searched to find safe alternatives (probiotics, organic acids, plant extracts, etc.) that reduce the activity of pathogens and overcome the continuous and rapid development of anticoccidial drug resistance particularly, and the ban of European Union Regulation, E. U. 2005 on the use of antibiotics (Abdel-Moneim et al., 2022; Elbaz et al., 2021). Many studies have successfully used bio-additives as potential safe alternatives to antibiotics, which contain some beneficial microbes and natural medicinal products.

Essential oils have many quantities of flavonoids, phenolic compounds, and phytochemicals, which can help birds maintain commensal bacteria and latch large amounts of nitrogen (Pourali et al., 2014; Elbaz et al., 2022). Oregano essential oil is one of the plant extract by-products (*Origanum vulgare Spp*) that has been shown to have antimicrobial, anti-inflammatory, and antioxidative characteristics, through biologically active compounds such as thymol and carvacrol (Tauer et al., 2018; Gordillo Jaramillo et al., 2021). Several studies have shown that feed supplemented with oregano essential oil improves antioxidant status, and enhances nutrient digestion and immunity in broiler chickens (Franciosini et al., 2016; Yang et al., 2019), which is due to its containing several bioactive compounds that exhibit antifungal and antimicrobial activity (Ri et al., 2017; Betancourt et al., 2019).

Recently, the use of live microorganisms as a good alternative to antibiotics has increased due to their many benefits to the host. Probiotics have been confirmed to significantly enhance nutrition utilization, intestinal antioxidant capacity, and mucosa-related immune responses, improve the integrity and function of the intestine, regulate intestinal morphology, and increase the production of anti-microbial agents (Travers et al., 2011; Elbaz et al., 2021). Furthermore, many studies have demonstrated that probiotics effectively protect broilers against many pathogens (Abdelrahman et al., 2014; Ritzi et al., 2014; Elbaz, 2023). Although the antimicrobial activity of probiotics and essential oils against many pathogenic has been confirmed, there is still a lack of literature on their use in the control of coccidiosis in commercial broiler chickens. Thus, this study aimed to evaluate the potential effects of dietary oregano essential oil and probiotics on the growth performance, immune response, antioxidant capacity, gene expression, and microbial community structure of broilers infected with pathogenic *Eimeria* species.

2. Materials and methods

2.1. Birds, Diets, and Management

A total of 600 1-day-old Ross 308 male chicks with an average initial body weight of 41.50 ± 0.2 g were used in a 35-day feeding experiment. The broiler chicks were randomly allocated into 6 groups, 5 replications per group, and 20 chicks per replication pen. The pens with a surface area of 1.5 m^2 were littered with wood shavings with a depth of 5-6 cm. Dietary treatments consisted of NCO, chicks fed a basal diet not inoculated with mixed *Eimeria*; PCO, chicks fed a basal diet inoculated with mixed *Eimeria*; DIC, chicks fed a basal diet containing diclazuril (0.2 g/kg diet) inoculated with mixed *Eimeria*; PRO, chicks fed a basal diet containing two-strain probiotic (2 g/ kg diet) inoculated with mixed *Eimeria*; OEO, chicks fed a basal diet containing oregano essential oils (300 mg/ kg diet) inoculated with mixed *Eimeria*; POE, chicks fed a basal diet containing 300 mg oregano essential oils and 2 g probiotics inoculated with mixed *Eimeria*. Broiler chickens were fed a starter diet from 0 to 15 days and a grower diet from 16 to 35 days, as shown in Table 1. All diets were formulated according to NRC recommended requirements (1994). Experimental feed and fresh water were provided *ad libitum* and the broilers were raised in a temperature-controlled room, according to Ross strain guidelines, while the lighting program from 0 to 7 days was 24 L:0D and from 8 to 35 days was 23 L:1D. The temperature was set to 32 °C during the first and second days, and then it was gradually reduced by 0.3 °C every day until 21 °C.

2.2. *Coccidia challenge*

The two-strain probiotic mixture was composed of *Bacillus Subtilis* (1.8 × 10^9 CFU) and *Lactobacillus Acidophilus* (2 × 10^{10} CFU), which was supplied by Cairo MIRCEN, Faculty of Agriculture, Ain Shams University, Egypt. Oregano essential oil was purchased from Pure Live Company for Investment and Agricultural Development (Cairo, Egypt). Coccidia challenge was done on day 15 for all groups except NCO one using the commercial attenuated vaccine (Foshan Standard Bio-Tech Co., China) that contained live oocysts of *Eimeria Eacervulina*, *E. tenella*, and *E. maxima* (Wu et al., 2018).

2.3. Growth performance

Live body weight (LBW) and feed intake (FI) were recorded on days 21 and 35. Mortality was recorded daily throughout the experiment, and body weight gain (BWG), and feed conversion ratio (FCR) were calculated. At 35 days, ten broiler chicks were weighed and slaughtered from each group to estimate the carcass traits including dressing, liver, breast, thigh, abdominal fat, bursa of Fabricius, thymus, and spleen.

2.4. Nutrients digestibility

On day 35, fifteen broiler chickens (3 birds/ replicated) per group were placed in battery individual cages with a wire mesh bottom and trays to excreta collection. Chickens were initialized by starving for 12 hours before the start of the digestion experiment. Excreta were collected for 4 successive days, three times daily. The remaining feathers in the excreta trays were carefully removed, and feed and excreta were weighed and dried. The apparent nutrient retention percentage was calculated for each dietary treatment as follows: $\text{Apparent Nutrient Retention (\%)} = (\text{Nutrient intake(g)} - \text{Nutrient in Excreta(g)})/(\text{Nutrient intake}) \times 100.$ Dry matter (DM), crude protein (CP), ether extract (EE), and nitrogen-free extract (NFE) were analyzed using routine procedures (AOAC, 1990).

2.5. Serum analysis

At day 35, blood samples were collected from six chickens of each group randomly by wing puncture into vacuum tubes containing heparin and centrifuged at 1500×g for 10 min to get the serum. Serum samples were collected and stored in Eppendorf tubes at -10 °C. Serum malondialdehyde (MDA), superoxide dismutase (SOD), and total antioxidant capacity (TAC) were determined using commercial kits (Spinreact Co. Girona, Spain). Chicken serum-specific immunoglobulins (IgA, IgG, and IgM) concentrations were determined in diluted samples (1:100) by enzyme-linked immunosorbent assay (ELISA) using microtiter plates (NuncImmunoplate 96-well plates) as per manufacturer's ELISA quantitation kits (Bethyl Laboratories Inc., Montgomery, TX, USA). In addition, Serum concentrations of interleukin 10 (IL-10), interleukin 6 (IL-6), and tumor necrosis factor (TNF-α) at d 35 were analyzed with the commercially available ELISA kits (MyBioSource, San Diego, CA) by following the company's protocols. All samples were measured in duplicate according to Yan et al. (2020).

2.6. Oocyst counting

At 35 days, ten fresh excreta samples were collected from each group, homogenized thoroughly, and kept in airtight numbered plastic bags, then preserved at 4 °C, and transported to the laboratory for analysis. Samples were added to a centrifuge tube that contained a saturated salt solution for homogenization, and then loaded into a McMaster counting chamber. The oocysts were counted and expressed as the number of oocysts per gram of excreta (Arendt et al., 2016).

2.7. Coccidiosis lesion scores

To evaluate the anticoccidial effects of the experiment additives, five chickens from each group were collected and slaughtered randomly to examine the duodenum for Eimeria-induced lesions at 35 days of age, according to the method suggested by Song et al. (2017).

2.8. Enumeration of cecal microbiota

The fresh digesta samples from the ceca were collected during slaughter (35 d) for bacterial analyses. Digesta samples were serially diluted in sterile saline solution for enumeration of *Lactobacillus*, *Escherichia coli*, *Clostridium perfringens*, and *Coliforms* by conventional microbiological techniques, using selective agar media according to Engberg et al. (2004). The enumeration of microbiota was evaluated as log 10 colony-forming units per gram of cecal digesta.

2.9. Gene expression

Two genes were estimated in cecum tissue including mucin-2 (MUC-2) and cationic amino acid transporter-1 (CAT-1). According to the manufacturer's protocol, total RNA was extracted from the intestinal mucosa and homogenized in Trizol (Invitrogen, California, USA), then in nuclease-free ultrapure distilled water the RNA was re-suspended, visualized, and quantified in NanoDrop ND-2000 (Thermo Fisher Scientific, Massachusetts, USA). After the amplification step, the dissociation curve of the Real-time PCR (RT-qPCR) products was obtained to verify the amplification specificity. Specific MUC2 and CAT-1 genes primer pairs structures (Metabion, Germany) were formed as follows; forward: TATACCGCAAGCAGCCAGGT, reverse: GCAAGCAGGACACAGACCAG, and forward: CCAGTCTATTAGGTTCCATGTTCC, reverse: CGATTATTGGCGTTTTGGTC, respectively, with thermocycler protocol. The *GAPDH* gene was used as a constitutive normalization reference and the primer sequence was forward: AGTCAACGGATTTGGCCGTA-3′ and reverse: ACAGTGCCCTTGAAGTGTCC-3′ (Ghanem et al., 2024). Relative MUC2 and CAT-1 gene expression levels were determined by the $2^{\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) where; ΔCt for sample = Ct for sample—Ct for the reference (*GAPDH*) gene; ΔΔCt = ΔCt for sample—ΔCt for control.

2.10. Statistical analysis

All data were analyzed using a one-way analysis of variance, ANOVA procedure of SPSS (SPSS 19, 2022). The effectiveness of experimental group differences will be compared using Tukey's multiple comparison test. Results were expressed as mean and ± standard deviation (± SD), and differences were considered significant at $p < 0.05$. The statistical model: $Yij = \mu + Ti + eij$ Where; μ = overall mean, Ti = dietary treatment, eij = experimental error.

3. **Results**

3.1. Growth performance

The effects of supplementation of oregano essential oils and probiotics with coccidiosis challenge on growth performance, including BWG, FI, and FCR, are summarized in Table 2. Coccidiosis challenge without any supplementation increased FCR and decreased BWG and FI during the different experimental periods ($P < 0.05$). The results indicated that supplementation of a mixture of oregano essential oils and probiotics (PE) improved the BW and BWG with coccidiosis challenge ($P < 0.05$) compared with PCO, PRO, and OEO during the starter, grower, and overall experimental period. POE and DIC groups recorded higher BWG and lower FCR compared to the PCO, PRO, and OEO groups. Although diet supplementation had no significant effect on FI during the starter periods, there was increased FI in the group fed POE, PRO, DIC, and OEO ($P < 0.05$) compared with PCO in the other periods. However, the highest FI was in groups DIC and POE compared to the rest of the groups, while it was similar for group NCO. Furthermore, the mortality rate decreased in groups fed DIC, POE, PRO, and OEO compared with PCO, as shown in Figure 1.

3.2. Carcass traits

There was no effect of dietary treatments with a coccidiosis challenge on carcass traits, except for the noticeable improvement in dressing percentage, as shown in Table 3. Dressing percentage decreased in chickens fed on a diet without additives and exposed to the coccidiosis challenge ($P < 0.05$). Nevertheless, feeding on the experimental supplements led to a dressing increase compared to the PCO group ($P < 0.05$). However, the dressing rate increased in the chickens fed POE and NIC compared to the PRO and OEO groups during exposure to the coccidiosis challenge. Liver, breast, thigh, and abdominal fat were not affected ($P < 0.05$) by the experimental additives with a coccidiosis challenge.

3.3. Nutrient digestibility

Results showed a significant effect of supplement treatments on nutrient digestibility during exposure to the coccidiosis challenge, as shown in Table 4. The PCO group had a reduced nutrient digestibility ($P < 0.05$) compared to the other groups. Dry matter digestibility was increased in chickens fed POE, NCO, DIC, and PRO ($P < 0.05$) compared to OEO and PCO groups. Crude protein digestibility increased in chickens fed NCO ($P < 0.05$) compared to the other groups, nevertheless, the POE group had a higher ($P < 0.05$) CP digestibility than PCO, DIC, OEO, and PRO groups. However, EE and NFE digestibility were not affected ($P < 0.05$) by the experimental treatments.

3.4. Immune indices

The effect of dietary treatments on immune indices, including immune organs, immune cytokines, and antioxidative enzyme activities of broilers under coccidiosis challenge are shown in Tables 3 and 5. Dietary experimental supplements did not significantly affect the organs' relative weight, such as the bursa of

Fabricius, thymus, and spleen. Broilers fed POE and PRO had the highest IgA levels (P < 0.05), furthermore, broilers fed POE had the highest IgG levels (P < 0.05) compared to the other groups. Serum IgM levels did not significantly (P < 0.05) change among experimental groups.

Moreover, IL-6 levels decreased (P < 0.05) in broilers fed POE and NCO compared to the other groups. While IL-10 levels increased (P < 0.05) in broilers fed POE, DIC, and NCO compared to the other groups. Experimental additions did not impact serum tumor necrosis factor (TNF-α) concentrations (P < 0.05).

Furthermore, malondialdehyde (MDA) decreased in broilers fed POE and DIC, while serum superoxide dismutase (SOD) levels increased (P < 0.05) in broilers fed POE compared to the other group, however, total antioxidant capacity (TAC) was similar among treatments.

3.5. Duodenum lesion scores

Coccidiosis lesions in the ileum observed on 35 d are presented in Figure 2. Results indicated no lesion in the duodenum of broilers fed NCO (no coccidiosis challenged). At 35 d, the duodenum lesion score was significantly higher in the PCO group (P < 0.05) than in the DIC, PRO, OEO, and POE groups. Besides, the lesion score was significantly lower in the POE, DIC, and PRO groups than in other groups (P < 0.05).

3.6. Oocyst shedding

The total number of oocysts shedding per gram of excreta is shown in Figure 3. Compared to the group fed PCO, oocyst shedding decreased (P < 0.05) in the POE, DIC, OEO, and PRO groups. Nevertheless, the DIC and POE groups showed fewer oocyst shedding than the PRO and OEO groups under the coccidiosis challenge.

3.7. Enumeration of cecal microbiota

The effects of dietary treatments on the cecal microbiota in broilers under the infliction of coccidiosis are shown in Table 6. *Lactobacillus* counts increased in chickens fed POE, NCO, and PRO compared to other groups. While *E. coli* counts decreased in chickens fed POE and PRO (P < 0.05) compared to the other groups. As well, *C. perfringens* counts decreased in POE-fed chicken (P < 0.05) compared to other groups. However, there was a decrease (P < 0.05) in *Coliform* counts in chickens fed PRO and POE compared to other groups.

3.8. Gene expression

As shown in Figures 4 and 5, the mRNA abundance of MUC2 and CAT-1 was decreased in chickens fed PCO (P<0.05) compared to the other experimental groups. Nevertheless, increased MUC2 and CAT-1 mRNA abundance in broiler chickens fed POE compared with the other groups (P<0.05). Additionally, the mRNA expressions of MUC2 and CAT-1 increased in the OEO, PRO, NCO, and DIC groups compared with the PCO group (P<0.05).

4. **Discussion**

Avian coccidiosis is a parasitic disease that leads to significant intestinal tissue damage, ineffective nutrient absorption, and high mortality resulting in economic losses to the poultry industry, forcing the breeder to use antibiotics to combat it. Given the various recommendations to reduce antibiotics in poultry, the search for safe and effective nutritional alternatives to meet these challenges continues. Therefore, the current study was designed to investigate the potential role of combining probiotics with oregano essential oils as immunomodulatory, antioxidant, and growth promoters, as well as being a potential anti-coccidia.

The results of the present study indicated poor growth performance in broilers in the PCO group, while growth performance improved in the groups that received the experimental supplements under the coccidiosis challenge. Similar observations were reported by Jerzsele et al. (2012) and Wang et al., (2021), who observed an enhancement in the growth performance in broiler chickens fed a diet enriched with probiotics (1 kg/kg) or a blend of essential oils via involvement in the integrity and functionality of the intestine. Furthermore, several reports have indicated the positive role of essential oils (300 mg/kg diet) in enhancing the performance of broiler chickens, which may be due to their content of effective active compounds that act as immune enhancers, antioxidants, intestinal bacteria modifiers, and reduce intestinal inflammation (Abudabos et al., 2016; Elbaz et al., 2022). Additionally, its role in upregulating some intestinal genes that is associated with increasing the strength of the intestinal barrier against oxidative stress and inflammation and improving the regenerative capacity of epithelial cells (Zeng et al., 2015). Therefore, POE supplementation could be a suitable alternative for antibiotics (anticoccidial) due to their ability to improve intestinal health, support immune response, and enhance digestion and absorption of nutrients., which enhances the broiler's performance.

Even though there was a noticeable improvement in growth performance in broilers fed the experimental supplement, the carcass characteristics were not affected by the experimental treatments, except for the dressing percentage that increased in chickens fed POE and DIC compared to those chickens fed PCO. These results are in agreement with Elbaz et al. (2021) and Alagawany et al. (2021) who reported that adding essential oils (450 mg/kg diet) or probiotics (0.5 g/kg diet) enhanced carcass yield. The increased dressing percentage in chickens fed a combination of probiotics and oregano essential oils may be attributed to their role in enhancing nutrient digestion via enhancing the integrity and functionality of the intestine, increasing digestive enzyme activity (Alcicek et al., 2003; Huang et al., 2018), and modulating gut microbiota diversity (Xu et al., 2018; Elbaz, 2021), and thus improving carcass characteristics. Therefore, adding a mixture of natural supplements (POE) to broiler feed can support carcass characteristics during coccidiosis.

Our results showed a significant enhancement in DM digestibility in chickens fed POE, DIC, and PRO compared to positive control-fed chickens. The combination of a multi-strain probiotic mixture (2 g/kg) and oregano essential oils also significantly increased CP digestibility, these results were in agreement with the findings by Elbaz et al. (2023a) and Xu et al. (2018). The improvement in nutrient digestion may be due to the positive effects of essential oils (200-300 ppm) in enhancing digestive enzyme activity and modifying the microbial content of the chickens 'intestines, which enhances nutrient metabolism and improves the digestion and absorption of nutrients (Kırkpınar et al., 2011; Su et al., 2020). On the other hand, many studies showed that adding probiotics balanced the gut microbial ecosystem, improving the ability to digest nutrients (Abdel-Moneim et al., 2020). Furthermore, feeding chickens essential oil supplements enhanced the endogenous GIT enzyme secretion, which enhances nutrient digestion (Chandralekha et al., 2016). The current results study and previous reports show the effective role of adding a mixture of probiotics and essential oils in reducing the risk of coccidiosis by enhancing gut health, which enhances the digestion and utilization of nutrients.

Pathogens may depress the broiler immune system by invading the gastrointestinal tract, which impairs production performance. Probiotics and natural plants and their extracts are feed additives possessing multiple functions, including immunomodulatory response. In the current study, the experimental additives did not affect the lymphoid immune organs except for the relative weight of the bursa of Fabricius, which numerically increased in birds fed with PRO, OEO, and POE compared with birds fed PCO. In addition, the current study showed an increase in the levels of IgA and IgG in the groups fed on POE compared to the other groups. In agreement with our results, Sikandar et al., (2017) and Elbaz et al., (2022) found an increase in the relative weight of the bursa of Fabricius in chickens fed probiotics (1 g/kg) or essential oils (300 ppm). Increased bursa of Fabricius weight is known to be associated with immunostimulation (Villagrán-de et al., 2020). Adding POE can support the immune response via increasing immunoglobulin A (IgA) and G (IgG) levels by preventing the adhesion of pathogenic microorganisms to the mucosal membranes of epithelial cells in the intestinal lumen (Puvača et al., 2022). Our results showed that a significant reduction in IL-6 and significant increase in IL-10 levels in serum of broilers fed supplemented with POE. Similar results were reported by Nooreh et al. (2021), who demonstrated that essential oils (150 mg/kg) lowered the production of IL-6 in the intestinal-challenged group. The expressions of IL-6, TNF-α, and IL-10 are closely related to the immune status of broiler chickens and are the most investigated pro-inflammatory cytokines changes (Shini et al., 2010), and they can be expressed after invading pathogens, which enhance the disease resistance and immune response. Based on our results, supplementation of POE to the diet enhanced the immune system in broiler chickens, by enhancing the secretion of immunoglobulin, the relative weight of immune organs, and the expression of anti-inflammatory cytokines.

Exposure of the intestine to pathogenic bacteria impairs the antioxidant defense mechanisms of chickens (Gordillo Jaramillo et al., 2021). Therefore, feed additives with antioxidant properties can play an effective role as natural anticoccidials. Based on the present results, dietary supplementation of a mixture of probiotics and an essential oil enhanced serum antioxidant activity by increasing SOD levels and reducing MDA in coccidiosis-infected chickens. In agreement with our results, under the *Eimeria* challenge, essential oil addition enhanced the antioxidant status of broiler chickens (Deng et al., 2012; Moharreri et al., 2022). The enhanced oxidative stability of chickens fed the probiotics-essential oil mixture may be due to the more effective scavenging of free radicals, and thus enhanced cell health and metabolism (Naidoo et al., 2008; Moharreri et al., 2021; Elbaz et al., 2023b), subsequently reducing the negative effect of coccidiosis challenge and improving performance.

The intestinal microbial community is the primary controller of gut health and morphology and defense mainly against enteric pathogens (Yadav and Jha, 2019; Ahsan et al., 2021). Exploring cecal microbial composition, we found that adding a mixture of probiotics and oregano essential oils reduced the numbers of *E. coli* and *C. perfringens*, while *Lactobacillus* increased. Similar to the results of the current study, Zhang et al. (2021) and (Stojanov et al., 2020) found a noticeable improvement in the intestinal microbial diversity of chickens fed a diet that included oregano essential oil or probiotics (0.5 g/kg) supplements. The role of oregano essential oil in enhancing the microbial content may be due to a group of biologically active compounds that have antimicrobial properties, such as carvacrol and thymol (Burt, 2004), by penetrating the bacterial cell membrane and reaching the inner parts of the cell (Helander et al., 1998), and thus being responsible for its antibacterial activity. Our results indicate that POE supplementation maintained gut integrity by modifying the gut microbiota structure (Zeng et al., 2015), thus promoting nutrient absorption and growth performance.

In our trial, supplementing with a mixture of probiotics and oregano essential oils substances resulted in lower oocyst values compared to the other infected groups. However, the POE, DIC, and PRO groups presented significantly lower oocyst shedding than infected birds in the PCO group. Pertinent studies with coccidia-infected chickens showed that the administration of probiotics led to reduced oocyst values (Giannenas et al., 2014). This was further supported by reduced oocysts in chickens fed a diet containing essential oils compared to control-infected chickens (Jang et al., 2007; Molan and Faraj, 2015). The reduced oocyst count in birds receiving probiotics (1 g/kg) resulted in increasing production and performance of CD4+ and CD8+ T lymphocytes (Ritzi et al., 2014), which enhance host immunity, act as antioxidants, balance beneficial microbes, and thus improve intestinal health (Mohsin et al., 2021). Accordingly, the combined probiotics with oregano essential oils may act as an effective anticoccidial, as evidenced by reduced coccidian sporulation.

The results of the present study showed a reduced degree of ileum lesion score in broiler chickens fed diets containing combined probiotics with oregano essential oils. Similar to the results of the current study, Giannenas et al. (2014) reported that chickens fed the probiotics-supplemented diet exhibited reduced gut lesions (Mengistu et al., 2021). Furthermore, Alhotan and Abudabos, (2019) reported that adding essential oils led to decreasing intestinal lesions caused by the *Eimeria* species. This result was consistent with previous studies that indicated that the inclusion of dietary probiotics (400 mg /kg) or oregano essential oils led to lower lesion scores, oocysts number, and tissue necrosis, thus enhancing the growth performance of chickens susceptible to *E. tenella* infection (Abudabos et al., 2017; Mengistu et al., 2021). This may be because oregano essential oils and probiotics have anti-parasitic, antioxidant, and anti-inflammatory properties, which reduced lesion scores in broilers (Abudabos et al., 2017).

Gene expression is influenced by many factors, including microbial content and feed additives (Kamada et al., 2013; Farahat et al., 2020). Therefore, we studied gene expression to reveal the experimental therapeutic effects on intestinal mucosal integrity (MUC2) and nutrient transporter gene (CAT-1). In the current experiment, the coccidiosis challenge decreased the abundance of MUC2 and CAT-1. However, dietary supplementation with probiotics and oregano essential oils effectively modulated gene expression by up-regulating the expression of MUC2 and CAT-1 genes under the coccidia challenge. In agreement with our results, some reports have proven that probiotics have strengthened intestinal epithelia by regulating ZO-1, MUC2, CLDN-1, and CAT-1 (Abd El-Hamid et al., 2022). Similarly, Pu et al. (2018), found an increase in gene MUC2 expression in pigs fed a diet containing *Bacillus Coagulans*, and oregano oil (400 g/t). Our results suggested that a combination of oregano essential oils and probiotics could help restore intestinal barrier integrity and function (Aliakbarpour et al., 2012), as well it enhance nutrient digestion (Liu et al., 2019; Pu et al., 2018) in broilers during the coccidiosis challenge by regulating gene expression.

5. Conclusions

The study concluded that incorporating oregano essential oil supplements with probiotics into broiler diets was the drug of choice to protect birds against coccidiosis where they improved intestinal microbiota, increased the regulation of gene expression for MUC2 and CAT-1, which is responsible for intestinal integrity and nutrient absorption, furthermore, it fortified the immune system, and antioxidant activity, as well as reduced the excessive inflammatory response, and thus enhanced broilers' health. Hence, our results recommend that a mixture of oregano essential oil and probiotics supplements (300mg and 2g/kg diet, respectively) can be used as a practical alternative to antibiotics, to provide protection to broilers against coccidiosis infection.

Ethics approval and consent to participate

All animal handling procedures complied with the Institutional Animal Care and Use Research Ethics Committee’s guidelines at the Faculty of Agriculture, Benha University, Egypt, which approved this study under protocol # 5/00216, while the present experiment was conducted at a Poultry Production Farm, the Desert Research Center, Cairo, Egypt.

Acknowledgments

The authors acknowledge their respective universities and institutes for their cooperation.

CRediT authorship contribution statement

Ahmed M. Elbaz and Disouky M. Mourad: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Software, Conceptualization, Methodology**. Zangabel S. Mohamed and Eman S. Ashmawy**: Writing – review & editing, Supervision, Resources, Methodology, Investigation, Conceptualization. **Eman Kamel M. Khalfallah and Shimaa A. Amin**: Investigation, Formal analysis, Conceptualization.

Data accessibility statement

The dataset generated and analyzed during the current study is available in this article.

Funding source

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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Table 1. Composition of basal diet in this experiment.

Item	Starter (0 to 15 days)	Grower (16 to 35 days)
Corn	50.30	55.00
Soybean meal	36.10	30.65
Sunflower meal	3.00	3.00
Corn gluten meal	3.00	3.00
Soybean oil	3.55	4.30
Calcium carbonate	1.50	1.50
Dicalcium phosphate	1.70	1.80
DL-Methionine	0.15	0.15
Hcl Lysine	0.00	0.00
Salt	0.30	0.25
Premix*	0.30	0.25
Sodium bicarbonate	0.10	0.10
Calculated composition		
ME (kcal/kg)	3000	3100
Crude protein (%)	23	21
Crude fiber (%)	4.39	4.11
Calcium (%)	1.071	1.078
Available P (%)	0.453	0.461
Lysine	1.16	1.02
Methionine	0.52	0.50
Meth + Cys	0.90	0.85

* Premix provides per kg of diet: vitamin K, 0.004 g; vitamin A, 14,000 IU; vitamin E, 0.05 g; vitamin D3, 3000 IU; pyridoxine, 0.003 g; pantothenic acid, 0.02 g; cobalamin, 0.006 g; choline, 0.15 g; niacin, 0.06 g; folic acid, 0.0002 g; Ca, 0.048 g; P, 0.00032 g; Mn, 0.1 g; Zn, 0.08 g; Fe, 0.05 g; Cu, 0.01 g; iodine, 0.000015 g; Co, 0.000025 g.

Table 2. Effect of supplementing with oregano essential oils and two-strains probiotics on growth performance of broilers infected with mixed *Eimeria* at 35 days

Items	NCO	PCO	DIC	PRO	OEO	POE	P-value
Body weight (g)							
Initial (0 d)	41.3±0.02	41.7±0.05	41.5±0.03	41.6±0.07	41.4±0.02	41.5±0.05	0.311
21 d	981±5.31^{a}	765±8.04^{d}	932±4.52^{b}	847±6.33^{c}	826±7.12^{c}	918±5.64^{b}	0.024
35 d	2067±11.5^{a}	1528±13.7^{e}	1975±12.3^{b}	1784±14.2^{c}	1719±12.5^{d}	1947±13.8^{b}	0.001
BWG (g.bird.day^{-1})							
1-21d	44.7±1.20^{a}	34.4±0.94^{b}	42.4±1.08^{a}	38.3±1.54^{b}	37.3±1.21^{b}	41.7±0.87^{a}	0.005
22-35d	77.5±2.91^{a}	54.5±4.03^{d}	74.5±3.42^{b}	66.9±3.06^{c}	63.7±3.25^{c}	73.5±3.01^{b}	< 0.001
1-35d	57.8±2.07^{a}	42.4±2.41^{c}	55.2±2.73^{a}	49.7±2.14^{b}	47.9±1.98^{b}	54.4±2.87^{a}	< 0.001
FI (g.bird.day^{-1})							
1-21d	50.1±4.21	46.1±5.30	48.7±4.15	48.7±3.62	48.1±3.06	50.5±4.39	0.116
22-35d	134±5.57^{a}	105±6.74^{c}	132±6.11^{a}	123±4.84^{b}	119±5.16^{bc}	131±6.71^{a}	0.004
1-35d	82.7±5.16^{a}	69.4±6.34^{c}	80.9±5.44^{a}	77.6±5.03^{b}	75.7±4.76^{b}	81.6±5.14^{a}	0.001
FCR (g feed .g gain^{-1})							
1-21d	1.12±0.02^{c}	1.34±0.07^{a}	1.15±0.02^{c}	1.27±0.04^{b}	1.29±0.05^{b}	1.21±0.01^{b}	0.001
22-35d	1.74±0.001^{c}	1.93±0.010^{a}	1.78±0.006^{bc}	1.85±0.006^{b}	1.87±0.020^{b}	1.79±0.011^{bc}	0.003
1-35d	1.43±0.01^{c}	1.64±0.02^{a}	1.47±0.02^{c}	1.56±0.01^{b}	1.58±0.03^{b}	1.51±0.01^{bc}	< 0.001

The means with different superscripts within the rows differ significantly. FI feed intake, BWG body weight gain, FCR feed conversion ratio, NCO chicks fed a basal diet not inoculated with mixed *Eimeria*, PCO chicks fed a basal diet inoculated with mixed *Eimeria*, DIC chicks fed a basal diet containing diclazuril inoculated with mixed *Eimeria*, PRO chicks fed a basal diet containing two-strain probiotic inoculated with mixed *Eimeria*, OEO chicks fed a basal diet containing oregano essential oils inoculated with mixed *Eimeria*, POE chicks fed a basal diet containing oregano essential oils and probiotics inoculated with mixed Eimeria, ± SD - standard deviation. N = 600.

Table 3. Effect of supplementing with oregano essential oils and two-strains probiotics on carcass traits (%) of broilers infected with mixed *Eimeria* at 35 days.

Items	NCO	PCO	DIC	PRO	OEO	POE	P-value
Dressing	72.8±0.52^{a}	68.1±0.67^{c}	72.3±0.35^{a}	71.4±0.48^{b}	71.9±0.36^{b}	72.6±0.41^{a}	0.010
Liver	3.24±0.08	3.18±0.05	3.21±0.09	3.23±0.13	3.19±0.08	3.21±0.011	0.504
Breast	21.3±0.61	20.7±0.73	21.1±0.49	21.4±0.51	22.3±0.37	21.8±0.34	0.076
Thigh	15.4±0.28	13.8±0.39	15.2±0.41	14.7±0.46	14.5±0.37	15.1±0.51	0.105
A. fat	1.84±0.09	1.92±0.14	1.75±0.10	1.83±0.16	1.76±0.20	1.81±0.08	0.234
B. of Fabricius	0.131±0.03	0.124±0.04	0.128±0.02	0.136±0.03	0.133±0.02	0.142±0.03	0.062
Thymus	0.166±0.01	0.159±0.00	0.164±0.01	0.161±0.00	0.162±0.02	0.165±0.00	0.341
Spleen	0.158±0.01	0.161±0.03	0.147±0.02	0.153±0.01	0.151±0.01	0.157±0.01	0.090

The means with different superscripts within the rows differ significantly. A. fat abdominal fat, B. of Fabricius bursa of Fabricius, NCO chicks fed a basal diet not inoculated with mixed Eimeria, PCO chicks fed a basal diet inoculated with mixed *Eimeria*, DIC chicks fed a basal diet containing diclazuril inoculated with mixed *Eimeria*, PRO chicks fed a basal diet containing two-strain probiotic inoculated with mixed *Eimeria*, OEO chicks fed a basal diet containing oregano essential oils inoculated with mixed *Eimeria*, POE chicks fed a basal diet containing oregano essential oils and probiotics inoculated with mixed *Eimeria*, ± SD - standard deviation. N = 50.

Table 4. Effect of supplementing with oregano essential oils and two-strains probiotics on nutrients digestibility of broilers infected with mixed *Eimeria* at 35 days.

Items	NCO	PCO	DIC	PRO	OEO	POE	P-value
Dry matter	72.2±1.06^{a}	67.2±1.18^{c}	71.8±0.91^{a}	71.5±1.03^{a}	70.9±1.02^{b}	72.0±0.84^{a}	0.001
Crude protein	84.3±0.75^{a}	78.5±1.09^{f}	81.6±0.84^{c}	81.2±0.94^{c}	80.7±0.78^{d}	82.8±0.97^{b}	< 0.001
Ether extract	68.1±0.54	67.3±0.67	68.3±0.61	67.8±0.58	68.2±0.70	68.3±0.52	0.416
NFE	75.8±0.38	74.6±0.46	75.4±0.50	74.7±0.61	74.6±0.48	75.1±0.57	0.302

The means with different superscripts within the rows differ significantly. NFE nitrogen-free extract, NCO chicks fed a basal diet not inoculated with mixed *Eimeria*, PCO chicks fed a basal diet inoculated with mixed *Eimeria*, DIC chicks fed a basal diet containing diclazuril inoculated with mixed *Eimeria*, PRO chicks fed a basal diet containing two-strain probiotic inoculated with *Eimeria*, OEO chicks fed a basal diet containing oregano essential oils inoculated with mixed *Eimeria*, POE chicks fed a basal diet containing oregano essential oils and probiotics inoculated with mixed *Eimeria*, ± SD - standard deviation. N = 75.

Table 5. Effect of supplementing with oregano essential oils and two-strains probiotics on immune indices and antioxidant capacity of broilers infected with mixed *Eimeria* at 35 days.

Items	NCO	PCO	DIC	PRO	OEO	POE	P-value
Immune indices							
IgA (g l^{-1})	106±1.34^{ab}	92.3±1.20^{c}	98.4±0.87^{b}	117±1.06^{a}	97.2±0.93^{b}	113±1.08^{a}	0.010
IgM (g l^{-1})	53.5±0.85	51.7±1.08	52.6±0.96	50.3±0.79	53.1±1.13	52.4±0.94	0.504
IgG (g l^{-1})	342±5.22^{b}	308±6.07^{c}	361±5.04^{b}	356±4.26^{b}	332±4.67^{bc}	405±5.13^{a}	0.031
IL-10 (pg ml^{-1})	31.6±0.76^{a}	22.9±0.94^{c}	30.4±0.82^{a}	27.3±0.65^{b}	28.2±1.01^{b}	30.8±0.92^{a}	0.001
IL-6 (pg ml^{-1})	71.5±1.64^{c}	96.2±1.38^{a}	78.1±1.05^{bc}	82.4±1.12^{b}	85.3±1.37^{b}	73.1±1.24^{c}	0.020
TNF -α (pg ml^{-1})	231±0.77	243±0.54	237±0.68	228±0.51	233±0.69	224±0.61	0.345
Antioxidant capacity							
MDA (nmol ml^{-1})	1.41±0.03^{d}	2.37±0.02^{a}	1.54±0.02^{c}	1.73±0.01^{b}	1.85±0.04^{b}	1.57±0.03^{c}	< 0.001
SOD (U ml^{-1})	93.2±0.90^{b}	90.4±1.15^{b}	91.6±0.81^{b}	92.4±0.92^{b}	94.3±1.37^{b}	112.5±1.06^{a}	0.043
TAC (U ml^{-1})	2.05±0.83	2.11±0.75	1.97±0.54	2.02±0.67	2.14±0.72	2.06±0.43	0.115

The means with different superscripts within the rows differ significantly. MDA malondialdehyde, SOD superoxide dismutase, TAC total antioxidant capacity, IL-10 interleukin 10, IL-6 interleukin 6,TNF -α tumor necrosis factor, IgA Immunoglobulin A, IgM Immunoglobulin M, IgG Immunoglobulin G, NCO chicks fed a basal diet not inoculated with mixed *Eimeria*, PCO chicks fed a basal diet inoculated with mixed h *Eimeria*, DIC chicks fed a basal diet containing diclazuril inoculated with mixed *Eimeria*, PRO chicks fed a basal diet containing two-strain probiotic inoculated with mixed *Eimeria*, OEO chicks fed a basal diet containing oregano essential oils inoculated with mixed *Eimeria*, POE chicks fed a basal diet containing oregano essential oils and probiotics inoculated with mixed *Eimeria*, ± SD - standard deviation. N = 30.

Table 6. Effect of supplementing with oregano essential oils and two-strains probiotics on cecal microbiota enumeration of broilers infected with mixed *Eimeria* at 35 days.

Items	NCO	PCO	DIC	PRO	OEO	POE	P-value
Lactobacillus	8.73±0.14^{a}	6.46±0.09^{c}	7.05±0.07^{b}	8.37±0.08^{a}	7.42±0.10^{b}	8.84±0.12^{a}	0.001
E. coli	3.08±0.06^{b}	3.52±0.10^{a}	3.11±0.09^{b}	2.65±0.06^{c}	3.30±0.14^{ab}	2.76±0.04^{c}	< 0.001
C. perfringens	3.51±0.03^{b}	4.27±0.02^{a}	3.43±0.02^{b}	3.29±0.04^{bc}	3.56±0.01^{b}	3.05±0.03^{c}	0.020
Coliforms	5.94±0.51^{bc}	6.88±0.48^{a}	5.65±0.42^{c}	5.74±0.26^{c}	6.31±0.31^{b}	5.54±0.47^{c}	< 0.001

The means with different superscripts within the rows differ significantly. *E. coli Escherichia coli*, *C. perfringens Clostridium perfringens*, NCO chicks fed a basal diet not inoculated with *Eimeria*, PCO chicks fed a basal diet inoculated with mixed *Eimeria*, DIC chicks fed a basal diet containing diclazuril inoculated with mixed *Eimeria*, PRO chicks fed a basal diet containing two-strain probiotic inoculated with mixed *Eimeria*, OEO chicks fed a basal diet containing oregano essential oils inoculated with mixed *Eimeria*, POE chicks fed a basal diet containing oregano essential oils and probiotics inoculated with mixed *Eimeria*, ± SD - standard deviation. N = 50.

Figure 1. Effect of supplementing with oregano essential oils and two-strains probiotics on the mortality rate of broilers infected with mixed *Eimeria* at 35 days (n = 20/replicate; 5 replicates/group). NCO chicks fed a basal diet not inoculated with *Eimeria*, PCO chicks fed a basal diet inoculated with *Eimeria*, DIC chicks fed a basal diet containing diclazuril inoculated with mixed *Eimeria*, PRO chicks fed a basal diet containing two-strain probiotic inoculated with mixed *Eimeria*, OEO chicks fed a basal diet containing oregano essential oils inoculated with mixed *Eimeria*, POE chicks fed a basal diet containing oregano essential oils and probiotics inoculated with mixed *Eimeria*. Data are presented as the mean values with their standard errors. Values with different superscript letters are significantly different (P < 0.05). All data are expressed as the mean ± SD.

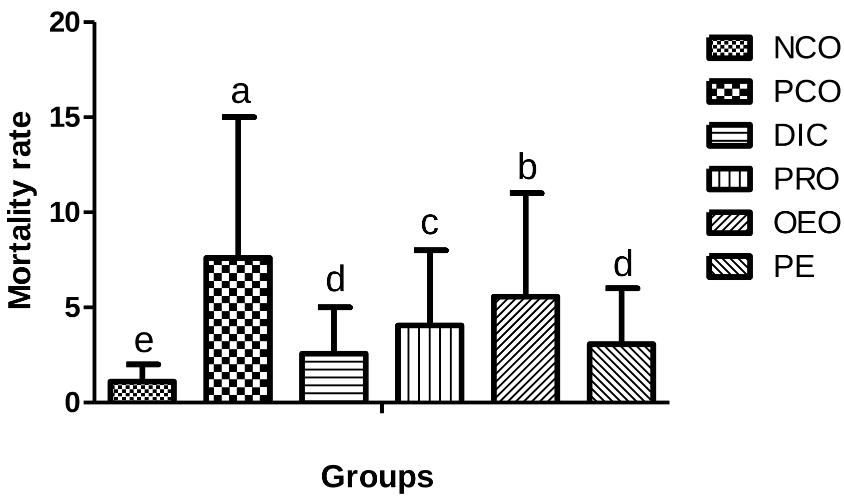

Figure 2. Effect of supplementing with oregano essential oils and two-strains probiotics on lesion scores of broilers infected with mixed *Eimeria* at 35 days (n = 1/replicate; 5 replicates/group). NCO chicks fed a basal diet not inoculated with mixed *Eimeria*, PCO chicks fed a basal diet inoculated with mixed *Eimeria*, DIC chicks fed a basal diet containing diclazuril inoculated with mixed *Eimeria*, PRO chicks fed a basal diet containing two-strain probiotic inoculated with *Eimeria*, OEO chicks fed a basal diet containing oregano essential oils inoculated with mixed *Eimeria*, POE chicks fed a basal diet containing oregano essential oils and probiotics inoculated with mixed *Eimeria*. Data are presented as the mean values with their standard errors. Values with different superscript letters are significantly different ($P < 0.05$). All data are expressed as the mean ± SD.

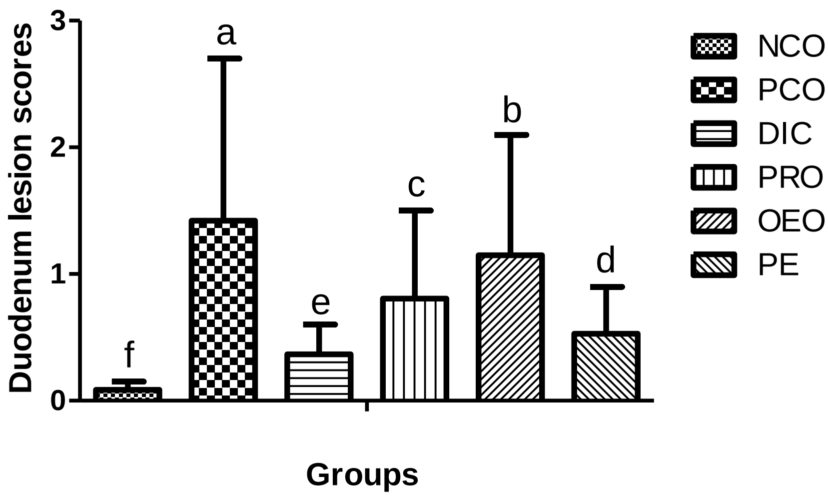

Figure 3. Effect of supplementing with oregano essential oils and two-strains probiotics on numbers of oocysts of broilers infected with mixed *Eimeria* at 35 days (n = 2/replicate; 5 replicates/group). NCO chicks fed a basal diet not inoculated with mixed *Eimeria*, PCO chicks fed a basal diet inoculated with *Eimeria*, DIC chicks fed a basal diet containing diclazuril inoculated with mixed *Eimeria*, PRO chicks fed a basal diet containing two-strain probiotic inoculated with *Eimeria*, OEO chicks fed a basal diet containing oregano essential oils inoculated with mixed *Eimeria*, POE chicks fed a basal diet containing oregano essential oils and probiotics inoculated with mixed *Eimeria*. Data are presented as the mean values with their standard errors. Values with different superscript letters are significantly different (P < 0.05). All data are expressed as the mean ± SD.

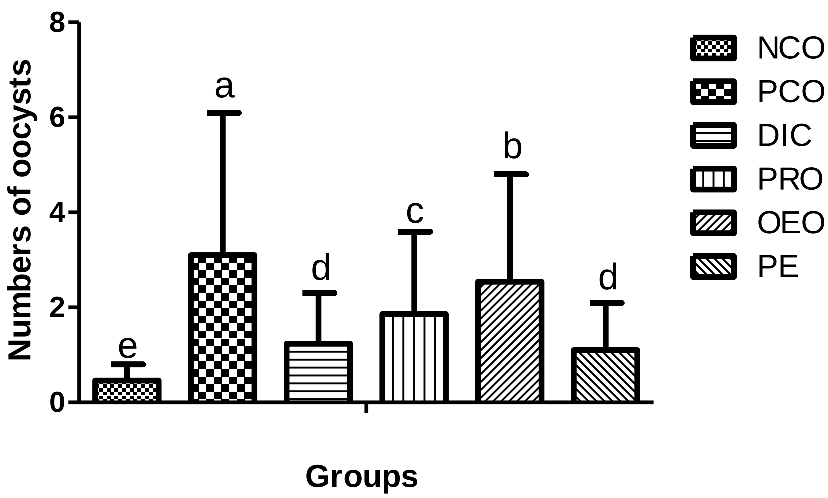

Figure 4. Effect of supplementing with oregano essential oils and two-strains probiotics on MUC2 gene expression of broilers infected with mixed *Eimeria* at 35 days (n = 1/replicate; 5 replicates/group). NCO chicks fed a basal diet not inoculated with *Eimeria*, PCO chicks fed a basal diet inoculated with *Eimeria*, DIC chicks fed a basal diet containing diclazuril inoculated with mixed *Eimeria*, PRO chicks fed a basal diet containing two-strain probiotic inoculated with mixed *Eimeria*, OEO chicks fed a basal diet containing oregano essential oils inoculated with mixed *Eimeria*, POE chicks fed a basal diet containing oregano essential oils and probiotics inoculated with mixed *Eimeria*. Data are presented as the mean values with their standard errors. Values with different superscript letters are significantly different ($P < 0.05$). All data are expressed as the mean ± SD.

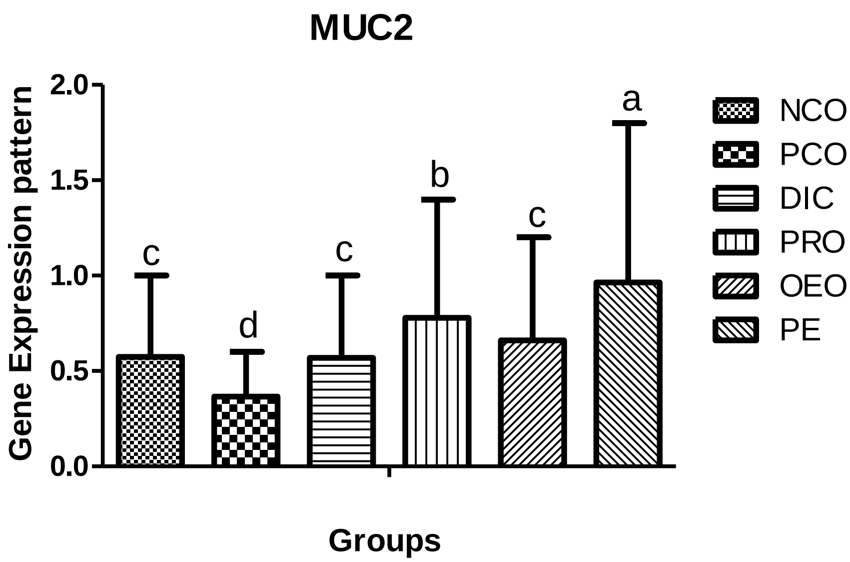

Figure 5. Effect of supplementing with oregano essential oils and two-strains probiotics on CAT-1 gene expression of broilers infected with mixed *Eimeria* at 35 days (n = 1/replicate; 5 replicates/group). NCO chicks fed a basal diet not inoculated with *Eimeria*, PCO chicks fed a basal diet inoculated with mixed *Eimeria*, DIC chicks fed a basal diet containing diclazuril inoculated with mixed *Eimeria*, PRO chicks fed a basal diet containing two-strain probiotic inoculated with mixed *Eimeria*, OEO chicks fed a basal diet containing oregano essential oils inoculated with mixed *Eimeria*, POE chicks fed a basal diet containing oregano essential oils and probiotics inoculated with mixed *Eimeria*. Data are presented as the mean values with their standard errors. Values with different superscript letters are significantly different ($P < 0.05$). All data are expressed as the mean ± SD.