Effect of anticoccidial monensin with oregano essential oil on broilers experimentally challenged with mixed *Eimeria* spp

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ABSTRACT Essential oil of oregano (**OEO**) has proven to be a potential candidate for controlling chicken coccidiosis. The aim of the current study is to determine whether OEO and an approved anticoccidial, monensin sodium (**MON**), as in-feed supplements could create a synergism when combined at low dosages. Day-old broiler chickens were separated into six equal groups with six replicate pens of 36 birds. One of the groups was given a basal diet and served as the control (**CNT**). The remaining groups received the basal diet supplemented with 100 mg/kg MON, 50 mg/kg MON, 24 mg/kg OEO, 12 mg/kg OEO, or 50 mg/kg MON + 12 mg/kg OEO. All of the chickens were challenged with field-type mixed *Eimeria* species at 12 d of age. Following the infection (i.e., d 13 to 42), the greatest growth gains and lowest feed conversion ratio values were recorded for the group of birds fed 100 mg/kg MON ($P < 0.05$), whereas results for the CNT treatment were inferior. Dietary OEO supplementations could not support growth to a level comparable with the MON (100 mg/kg). The MON programs were more efficacious in reducing fecal oocyst numbers compared to CNT and OEO treatments ($P < 0.05$). Serum malondialdehyde and nitric oxide concentrations were decreased ($P < 0.01$), whereas superoxide dismutase ($P < 0.05$) and total antioxidant status ($P < 0.01$) were increased in response to dietary medication with MON and OEO. All MON and OEO treatments conferred intestinal health benefits to chickens by improving their morphological development and enzymatic activities. The results suggest that OEO supported the intestinal absorptive capacity and antioxidant defense system during *Eimeria* infection; however, it displayed little direct activity on the reproductive capacity of *Eimeria*. This might be the reason for inferior compensatory growth potential of OEO compared to that MON following the challenge. Combination MON with OEO was not considered to show promise for controlling chicken coccidiosis because of the lack of a synergistic or additive effect.

Key words: Broiler chicken, *Eimeria*, oregano oil, digestive enzymatic activity, intestinal histomorphometry

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INTRODUCTION

Prophylactic chemotherapy using anticoccidial drugs, the traditional method for controlling coccidiosis in the rearing of broiler chickens, is still widely and routinely used (McDougald, 1990; McDougald et al., 1996; Chapman, 2001, 2014). However, drug resistance in *Eimeria* is common because of this extensive use of anticoccidial drugs for the control of avian coccidiosis (Chapman, 1997; Lillehoj and Lillehoj, 2000; Zhu et al., 2003). Because of drug resistance, pharmaceutical coccidiostats are becoming less effective in controlling coccidiosis in avian species (Peek and Landman, 2003; Abbas et al., 2011; Lillehoj and Lee, 2012).

The rising drug resistance of *Eimeria* field strains (Chapman, 1997) and public concerns about drug residues in poultry products (Atef et al., 1993; Elliott et al., 1998) are the main factors prompting the development and implementation of alternative strategies for coccidiosis control (Peek et al., 2013; Arczewska-Wlosek and Swiatkiewicz, 2014; Bozkurt et al., 2014). To date, there are no new anticoccidial drugs in development. It is, therefore, currently necessary to develop

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strategies to minimize the emergence of resistance in *Eimeria* strains. This includes exploring simple, practical, and sustainable dietary strategies for managing or mitigating the effects of coccidiosis in avian species.

The last decade has seen growing interest in the search for alternatives to the traditional methods of coccidiosis prophylaxis (Peek and Landman, 2011). Botanicals offer a potentially appealing alternative to be used in antiparasitic therapy, including coccidiosis (Wallace et al., 2010; Abbas et al., 2012; Bozkurt et al., 2013). A number of compounds derived from plants such as *Artemisia annua* (Allen, 1997), *Origanum vulgare* subsp. *hirtum* (Giannenas et al., 2003), *Curcuma*, *Capsicum*, and *Lentinus* spp. (Lee et al., 2010), and natural products such as prebiotics (Duffy et al., 2005), grape seed extract (Wang et al., 2008), and probiotics (Giannenas et al., 2012) appear to have anticoccidial activities against *Eimeria* species commonly found in poultry.

Earlier research was concerned with establishing the role of plant bioactives in lowering fecal oocyst output, intestinal lesions, and the performances of chickens (Giannenas et al., 2003; Oviedo-Rondón et al., 2005; Küçükyilmaz et al., 2012; Bozkurt et al., 2012, 2014). Extracts and steam-distillated essential oils (**EO**) of plants from the Labiate family (e.g., thyme, sage, and lavender), particularly oregano, are the most frequently investigated plants in broiler chickens infected with coccidiosis. Polyphenols, one of the most important constituents of these medicinal herbs and their EOs, have been found to exhibit anticoccidial activity against chicken coccidiosis. Carvacrol and thymol, the 2 main phenols that constitute about 70% to 80% of oregano OEO, are considered to exhibit anticoccidial activity (Economou et al., 1991; Giannenas et al., 2003). However, limited progress has been made in our understanding of the mechanism involved in anticoccidial activity mediated by phytonutrients, despite detailed investigations of the pathology, microbiology, biochemistry, intestinal morphology, and antioxidant defense system (Wang et al., 2008; Reisinger et al., 2011; Giannenas et al., 2012, 2014). The mode of action of the phytogenic substances seems related to a more complementary anticoccidial effect, but the underlying mechanisms require further in-depth characterization.

One objective of this study was to examine whether field isolates of mixed *Eimeria* spp. can be controlled by using dietary essential oil of oregano (**OEO**) and to compare it with one of the widely used ionophore anticoccidials, monensin (**MON**). Earlier investigations showed that each of these anticoccidials demonstrates a different mode of action via indirect or indirect route. It is most likely that the anticoccidial activity of the phytogenic compounds from OEO is not mainly related to one specific mechanism but is associated with their antimicrobial and antioxidant activities (Ultee et al., 2002; Abbas et al., 2011; Bozkurt et al., 2013), whereas MON affects the Eimerian parasites directly thereby promoting perturbations in the intracellular cation balance (Smith and Strout, 1979; Chapman, 1984).

However, no clear evidence is available regarding the use of botanicals and ionophore anticoccidials in a dietary combination. Hence, another purpose of this study was to examine whether a combination of MON with OEO might exert a synergistic effect in controlling coccidiosis while reducing the practical application dosages (in an attempt to reveal transient solutions until certain alternatives to ionophore anticoccidials are found). Thus, the synergism between MON and phytochemicals derived from OEO would enable reduction of the anticoccidial resistance, thereby lowering the dependence on traditional application dosages for carboxylic acid ionophores.

As a consequence, the potential protective effect of OEO and MON on growth performance, fecal oocyst shedding, oxidative stress, digestive enzymatic activity, and intestinal histomorphometry in broiler chickens challenged with field-type *Eimeria* spp. was assessed in an experimental setting. Both the industry and the consumer would welcome plant-based natural alternatives to traditional chemical methods of coccidiosis prophylaxis that controls *Eimeria* spp. without leaving residues in the product or the environment.

MATERIALS AND METHODS

Management

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Adnan Menderes University. The birds were obtained from commercial hatchery, Ege Tav, İzmir, Turkey. Bird sexing and vaccinating were carried out at the hatchery without administration of any coccidia vaccine. The chicks were vaccinated against infectious bursal disease virus and Newcastle disease virus with Gumbopest (Merial SAS®, Lyon-France). The experimental house was divided into 36 pens (replicate) of equal size, each having an area of 3.00 m^2, with fresh wood shavings as bedding with a thickness of approximately 6 cm on a concrete floor. Each replicate pen was equipped with three nipple drinkers, two tube-type feeders and electrical heater. From d 1 until 5, feed was also supplied on trays, directly placed on the litter. Rearing density was 12 birds per m^2 floor space. The room temperature was gradually reduced from 33°C on d 1 to 22°C on d 21 and then kept constant to trial termination on d 42, according to standard management procedure. Illumination was provided by fluorescent bulbs placed above the pens. The light regimen was as follows: d 1 to 3, 24L: 0D; d 4 to 7, 20L:4D; and d 8 to 42, 16L:8D. The house was naturally ventilated with adjustable windows and efforts were made to copy commercial conditions as much as possible. The health of the animals was monitored by a trained veterinarian. The experiment was terminated when the birds were 42 d old.

Birds and Experimental Design

A total of 1296 1-day-old broiler chickens (Ross 308) of mixed sex were allocated into 6 equal groups, 216 per group, with six replicates of 36 birds each (18 males and 18 females). The pen was the experimental unit. The groups consisted of 1) a basal diet with no anticoccidials or growth enhancers (**CNT**); 2) CNT+100 mg/kg of MON (**MON-1**); 3) CNT+50 mg/kg MON (**MON-2**); 4) CNT+24 mg/kg OEO (**OEO-1**); 5) CNT+12 mg/kg OEO (**OEO-2**); and 6) CNT+50 mg/kg MON+12 mg/kg OEO (**MON-2+OEO-2**).

Experimental Diets

The basal diet was a typical corn-soybean diet that was formulated to meet or exceed all nutrient recommendations published in the Ross rearing guideline (Aviagen, 2007). The experimental period was divided into three phases; a starter phase (1 to 12 d), a grower phase (13 to 27 d), and a finisher phase (28 to 42 d). The ingredient composition and nutrient content of the basal diets appropriate for the bird's growing stage are presented in Table 1. These diets contained no antibiotics, anticoccidials, or growth enhancers. All feeds were fed as mash and were mixed and issued fresh weekly. Each batch of feed was mixed and bagged separately and identified with the group number. Throughout, experimental diets and drinking water were available ad libitum. Chemical composition was determined according to the protocols stated by AOAC (1990). All of the feed samples were analyzed for dry matter (934.01), ash (942.05), nitrogen (Kjeldahl procedure: 988.05), ether extract (920.39), crude fiber (962.09), calcium (927.02) and total phosphorus (965.17). Treatment diets relative to experimental periods were also analyzed to guarantee that they were identical regarding chemical composition with the exception of the supplements.

The in-feed ionophore anticoccidial preparation (COXIDIN® 200 microGranulate; HUVEPHARMA, Sofia, Bulgaria), contains monensin sodium in a concentration of 20% (200 g/kg). The approved dose range is 100 to 125 mg/kg of complete broiler chicken feed in the EU. Thus, 500 g and 250 g MON preparation per ton feed mixture supplied 100 mg (MON-1) and 50 mg (MON-2) monensin sodium per kg diet. The commercial essential oil blend (WILDMIX®) was provided by İnan Tarım ECODAB® Ltd. Co., Antalya-Turkey. The essential oil is derived from the herb *Origanum minutiflorum*, growing wild in Turkey, by steam distillation. It contained carvacrol (81.69%), δ-3-caren (4.15%), thymol (2.06%), p-cymen (2.02%) and as the main active components. The essential oil preparation used 920 g of zeolite as a feed-grade inert carrier for each 80 g OEO. In order to supply 24 mg (OEO-1) and 12 mg (MON-1) oregano oil per kg diet, 300 and 150 g OEO preparation was added to one ton of feed mixture. Thus, the OEO-1 preparation provides 19.60 mg carvacrol, 1.00 mg δ-3-caren, 0.49 mg thymol, and 0.48 mg p-cymen per each kg of diet while corresponding to concentrations reduced by half in EOE-2 treatment.

The composition of the OEO was determined using the GC/MS (HP 6890GC/5973 MSD) system. The subsequent procedure regarding distillation of ground feed samples and dilution of the oil were conducted according to the procedure as mentioned by Bozkurt et al. (2014).

Feed-grade supplements of MON and OEO were in a form of a premix powder. Each preparation (i.e., MON-1, MON-2, OEO-1, OEO2, and MON-2+OEO2) was totaled up to 1 kg adding an amount of fine-ground soybean meal as required and homogenized by mixer, and then the pre-mixture was added to main mixture. When preparing experimental diets, sawdust was included in the control diet to match the addition of the corresponding supplements.

Table 1. Composition of the diets (as-fed basis): starter (d 1 to 12), grower (d 13 to 27), and finisher (d 28 to 42).

Ingredient, g/kg	Starter	Grower	Finisher
Corn, yellow, ground	532.72	559.53	606.63
Corn gluten (with 64% CP)	53.27	62.69	83.47
Soybean meal (with 48% CP)	347.51	290.95	226.81
Soy oil	32.01	46.61	46.27
Dicalcium phosphate	17.86	17.49	16.95
Limestone	11.56	9.71	8.04
Choline chloride	1.00	1.00	1.00
L-Lysine HCL	3.63	3.12	2.96
DL-Methionine	3.20	2.71	2.01
L-Threonine	0.61	0.54	0.24
Vitamin premix[1]	1.00	1.00	1.00
Trace mineral premix[2]	1.00	1.00	1.00
Sawdust	1.00	1.00	1.00
Contents by analysis[3],%			
Dry matter	88.63	88.43	88.00
Crude protein	22.93	21.12	19.44
Ether extract	5.15	6.64	6.70
Crude fiber	2.77	2.64	2.52
Crude ash	4.41	3.94	3.46
Ca	1.05	0.97	0.88
P (total)	0.70	0.67	0.64
Contents by calculation, %			
ME (kcal/kg)	3.013	3.161	3.203
P (Available)	0.44	0.42	0.40
Lysine	1.40	1.22	1.06
Methionine	0.68	0.61	0.54
Methionine + Cysteine	1.04	0.95	0.86
Threonine	0.91	0.83	0.74
Linoleic acid	2.76	3.68	3.68

[1]Vitamin mix provided the following (per kg of diet): *trans*-retinol (Vit. A) 3.6 mg; cholecalciferol (Vit. D_3) 0.1 mg; α-tocopherol acetate (Vit. E) 75 mg; menadione (Vit. K_3) 5 mg; thiamine (Vit. B_1) 3 mg; riboflavin (Vit. B_2) 6 mg; pyridoxine (Vit. B_6) 5 mg; cyanocobolamin (Vit. B_{12}) 0.03 mg; nicotinic acid 40 mg; pantothenic acid 10 mg; folic acid 0.75 mg; D-biotin 0.075 mg; choline chloride 375 mg.

[2]Trace mineral mix provides the following (per kg of diet): 80 mg of manganese ($MnSO_4 \cdot H_2O$); 40 mg of iron ($FeSO_4 \cdot 7H_2O$); 60 mg of zinc (ZnO); 5 mg of copper ($CuSO_4 \cdot 5H_2O$); 0.15 mg of iodine (ethylene diamine dihydroiodide); 0.3 mg of selenium ($NaSeO_3$).

[3]Analyzed values are referred to basal (control) diets.

Broiler Performance Responses

Chicks were weighed on a pen basis on d 1, 12, 27, and 42 to determine body weight (**BW**). Body weight gain (**BWG**) was determined as the difference between relevant experimental periods. Feed intake (**FI**) within each subgroup was calculated at d 12, 27 and 42 by subtracting residual feed plus the amount feed consumed by dead birds from the offered feed. The feed conversion ratio (**FCR**) was calculated as the ratio of FI to BWG (g feed/g gain). Mortality was recorded daily and expressed as a percentage of the initial number of chicks. The FCR was adjusted for mortality birds, and calculated on a per-pen basis. Any birds that died for sampling were weighed, and the FCR values were calculated by dividing total FI by BWG of live plus dead birds.

Eimeria *Infection and Fecal Oocysts Measurements*

Chicks were infected at 12 d of age with a standard oral inoculum containing 5×10^5 sporulated oocysts from field isolates of *E. acervulina*, *E. maxima*, *E. tenella*, *E. mitis*, *E. brunetti*, and *E. praecox*, respectively. The reference stocks in the current experiment were provided by the Department of Parasitology at the Veterinary Medicine Faculty of Ankara University, Turkey. These reference stocks were maintained by periodic passage through coccidia-free chicks and sporulated in 2% potassium dichromate by standard operation in Ankara University. The inoculum was washed several times with tap water to remove potassium dichromate then a 2-mL suspension of 5×10^5 sporulated oocysts administered directly into the crop by oral gavage by using a plastic syringe fitted with a plastic cannula.

Oocyst counts were determined in samples of excreta obtained from each subgroup at 10 d of age, i.e., before infection, and determined daily from d 18 (6 dpi) to d 27 (15 days post infection [**dpi**]. Random collection of the samples obtained from each pen was carried out according to the procedure as mentioned by Bozkurt et al. (2014). Briefly, total feces from each pen were collected and mixed thoroughly to ensure uniformity. Homogenized samples were diluted ten-fold with tap water and further diluted with saturated saline solution at a ratio of 1:10. Following work (i.e., saturation of sodium chloride solution, allowing the oocysts to float up) was accomplished according to the procedure outlined by Christaki et al. (2004). The number of oocysts was determined using a McMaster counting chamber (Hodgson, 1970) and the results are presented as per gram of excreta.

Collection of Samples

Six d after the inoculation (d 18), two birds (one male and one female) whose body weights were similar to the group mean were selected from each replicate pen (12 birds per treatment group) and wing tagged. The birds were bled via wing vein and blood samples were collected into tubes for blood biochemistry. After blood collection, the 72 sampled birds were electrically stunned and slaughtered. After 3 min suspension for exsanguination, birds were eviscerated, and pancreas and complete small intestines were immediately removed.

Antioxidant Indices in Serum

Lipid peroxidation was determined using the procedure described by Yoshoiko et al. (1979), in which malondialdehyde (**MDA**), an end-product of fatty acid peroxidation, reacts with tri-barbituric acid (**TBA**) to form a colored complex with a maximum absorbance at 532 nm. Total antioxidant status (**TAS**) of the serum was determined using an automated measurement method with a commercial available kit (Total Antioxidant Status Assay kit, Rel Assay Diagnostics, RL0017, Turkey). Using this method, the antioxidative effect of the sample is measured against the potent free radical reactions, initiated by the reduced hydroxyl radical. The results are expressed as mmol Trolox equiv/L. To measure superoxide dismutase (**SOD**) activity, serum is incubated with xanthine oxidase solution for 1 h at 37°C. Absorbance was read at 490 nm to generate superoxide anions. The SOD activity is determined as the inhibition of chromagen reduction. In the presence of SOD, superoxide anion concentration is reduced, yielding less colorimetric signal (OxiSelect^{TM}, Superoxide Dismutase Activity Assay, STA-340, Cell Biolabs, San Diego, CA). SOD activity was shown in inhibition%. Double sandwich ELISA kits were used to measure serum concentrations of nitric oxide (**NO**) (D2NO-100, Bioassay Systems, Hayward, CA).

Determination of Digestive Enzyme Activities

Sampling Procedure A homogenous intestinal digesta sample was collected by gentle finger stripping massaging the duedonal tract from both ends. The digesta samples were stored immediately at −80°C until used. The small intestinal digesta samples were diluted, homogenized and centrifuged using a protocol described by Jin et al. (2000). Then, the supernatants were divided into small portions and stored at −80°C for enzyme assays.

The pancreas was harvested from each bird within 5 min after death, placed in aluminum foil, snap frozen in liquid nitrogen, and stored at –80 °C. The following procedures were according to the method described by Chen et al. (2005).

Analyses Amylase activity (EC 3.2.1.1) was determined according to Bernfeld (1955), where the reducing groups liberated from starch are measured by the

reduction of 3,5-dinitrosalicylic acid (Sigma Chemical Co., St. Louis, MO). One unit liberates from soluble starch one micromole of reducing groups (calculated as maltose) per minute at 37 °C and pH 6.9 under the specified conditions.

Activation of chymotrypsinogen to chymotrypsin (EC 3.4.21.1) was based on method of Glazer and Steer (1977). Chymotrypsin activity was determined according to Hummel (1959) by measuring an increase in absorbency at 256 nm resulting from the hydrolysis of benzoyl-L-tyrosine ethyl ester (BTEE, Sigma Chemical Co.). One unit of chymotrypsin activity was defined as 1 mmol of BTEE hydrolysed per min in pH 7.8 phosphate buffer at 37°C.

The lipase activity was determined according to the method of Sigurgisladottir et al. (1993) with slight modification. Briefly, 0.1 mL of enzyme preparation mixed with 0.8 mL of Tris-HCl buffer (50 mM, pH 8.0) and 0.1 mL of p-nitrophenyl laurate (**pNPL**) (10 mM in ethanol) solutions. Reaction mixture was incubated for 30 min at 65°C and then mixed with 0.25 mL of Na_2CO_3 solution (0.1 M). After centrifugation at 10,000 × *g* for 15 min, absorbance of the supernatant was measured by using a spectrophotometer at 410 nm. One lipase activity was determined as the amount of enzyme that caused the release of 1.0 μmol of p-nitrophenol in 1 min under the experimental conditions. All enzyme activities were measured spectrophotometrically (Multiskan GO spectrophotometer, Thermo Scientific, Waltham, MA).

Intestinal Morphology

For intestinal morphology measurements, three cross-sections for each intestinal segment (duedonum, jejunum, and ileum) were removed. The preparation and fixation procedures and, comcomitant measurements for the villus and crypts dimension were carried out using a protocol outlined by Xu et al. (2003). The villus height was measured from the crypt-villus junction to the brush border at the tip. Villus width was measured between brushborders of opposing epithelial cells at the midpoint of the villus where possible. Crypt depths were taken at the level of the basement membranes of opposing crypt epithelial cells. Surface area was calculated using formula = $(2\pi) \times$ (villus width/2) × (villus height) as described by Sakamoto et al. (2000).

Statistical Analysis

All data were analyzed using SAS (SAS Institute Inc., Cary, NC). Data were subjected to ANOVA by using the GLM procedure. The pen was the experimental unit and a completely randomized statistical design was followed. Duncan's multiple-range test was carried out to detect differences among treatments. All differences were considered significant at $P < 0.05$. Since the oocyst yields and lesion scores were not distributed normally, the Kruskal-Wallis non-parametric analysis (SAS, 2001) was employed. Arcsin transformation was applied to the percentage values (i.e., mortality rate) before testing for differences.

RESULTS AND DISCUSSION

Performance Parameters

Data regarding performance indices during the pre-infection (starter) phase and post-infection phase are presented in Table 2. There were no treatment differences ($P < 0.05$) in growth performances responses [i.e., body weight gain (BWG), feed intake (FI), or feed conversion ratio (FCR)] during the starter phase (d 1 to 12), with the exception that the FCR of chicks fed MON-2 + EOM-2 was lower ($P < 0.01$) than those fed all other dietary treatments. Chickens fed MON-1 and MON-2 had heavier BWG as compared with those on the untreated control (**CNT**) treatment from d 13 to 27 ($P < 0.01$) and d 13 to 42 ($P < 0.05$). No significant difference in BWG was found between dietary treatments from d 28 to 42.

Corresponding increases in BWG were also reflected in improvements of feed-to-gain ratios for chicks fed 100 and 50 mg/kg MON, when compared with the CNT treatment. The lowest FCR values were noted in the MON-1 group throughout the post-infection periods, as compared with untreated and other medicated treatments. A diet providing OEO also reduced FCR in comparison with CNT treatment from d 13 to 27 dpi ($P < 0.01$), 28 to 42 dpi ($P = 0.08$) and 13 to 42 dpi ($P < 0.05$). The reduced FCR in OEO-treated chicks seemed to be a feed intake response because chicks treated with OEO tended to eat less.

No differences were observed when average feed intake data were compared between the treatments during the post-infection phases. The chickens remained exceptionally healthy throughout the trial, although they were challenged with a field mix of *Eimeria* spp. The percentage mortality was not associated with treatment in any of the experimental periods ($P > 0.05$; Table 2).

Improved feed efficiency during the post-infection periods (i.e., d 13 to 27 and d 13 to 42) could be attributed to the presence of the ionophore antibiotic, MON, and plant bioactives derived from OEO in the diet, which establish microbial balance throughout the intestines (Jeffers et al., 1988; Mitsch et al., 2004; Jang et al., 2007), which then enhances nutrient utilization and gut passage rates in chickens (Jamroz et al., 2003) and encourages secretions of endogenous digestive enzymes (Basmacioğlu et al., 2010).

Deteriorated performance as a consequence of coccidial challenge is associated with reduced absorptive surface area, malabsorption of nutrients, and inflammation (Cook, 1998; Giannenas et al., 2012, 2014). Based on the information that OEO, during periods of clinical coccidiosis, has been shown to improve broiler

Table 2. Body weight gain (BWG; g), feed intake (FI; g), feed conversion ratio (FCR; g feed/g gain) and mortality (%) after broilers were infected with an inoculum containing 5×10^5 oocysts of *Eimeria* spp. at 12 d of age and were provided with diets supplemented with MON and OEO, either alone or in combination.

	Diet[1]							
Age of chickens	CNT[1]	MON-1	MON-2	OEO-1	OEO-2	MON-2 + OEO-2	Pooled SEM[2]	*P*-value
Day 1 to 12								
BWG (g)	208	205	206	206	205	206	2.80	0.992
FI (g)	349	344	348	349	350	335	5.17	0.314
FCR	1.680^{a}	1.677^{a}	1.685^{a}	1.695^{a}	1.703^{a}	1.626^{b}	0.014	0.016
Mortality	0.00	0.00	0.00	0.00	0.55	0.00	0.22	0.4389
Day 13 to 27								
BWG (g)	671^{c,d}	709^{a}	692^{a,b}	665^{c,d}	654^{d}	681^{b,c}	7.65	0.0003
FI (g)	1393	1390	1383	1335	1318	1348	28.88	0.332
FCR	2.073^{a}	1.956^{c}	1.998^{b}	2.008^{b}	2.013^{b}	1.978^{b,c}	0.014	0.0001
Mortality	1.17	0.00	0.00	0.00	0.73	0.00	0.38	0.1644
Day 28 to 42								
BWG (g)	1,132	1,136	1,156	1,127	1,131	1,108	15.33	0.436
FI (g)	2,239	2,191	2,231	2,196	2,195	2,180	39.40	0.864
FCR	1.977	1.928	1.930	1.946	1.941	1.966	0.014	0.085
Mortality	0.62	0.62	0.62	1.25	0.62	0.62	0.65	0.9768
Day 13 to 42								
BWG (g)	1,803^{b}	1,846^{a}	1,848^{a}	1,792^{b}	1,786^{b}	1,790^{b}	16.05	0.019
FI (g)	3,633	3,581	3,629	3,531	3,514	3,542	63.73	0.675
FCR	2.013^{a}	1.938^{c}	1.963^{b,c}	1.970^{b,c}	1.966^{b,c}	1.976^{b}	0.013	0.014
Mortality	1.76	0.58	0.58	1.76	1.17	0.58	0.65	0.5784

$^{a–d}$Values within a row not sharing the same superscript are different at $P < 0.05$.

[1]The broilers were fed a control diet (CNT) that contained no anticoccidial or performance enhancer or a diet supplemented with preparations of anticoccidial monensin (100 mg/kg of diet; MON-1), anticoccidial monensin (50 mg/kg of diet; MON-2), oregano essential oil (24 mg/kg of diet; OEO-1), oregano essential oil (12 mg/kg of diet; OEO-2) or 50 mg/kg monensin + 12 mg/kg oregano essential oil (MON-2 + OEO-2).

[2]Data are means of 6 replicate pens with 216 chicks each per treatment.

performance (Saini et al., 2003; Oviedo-Rondón et al., 2006; Tsinas et al., 2011). In this study, in comparison with an unmedicated CNT program, the supplementation of OEO in diets is certainly to have been more effective with respect to FCR, and this could be ascribed to increased intestinal absorptive surface area, and digestive enzymatic activity. However, OEO, as a phytochemical with potential anticoccidial activity, was less effective in promoting growth of chickens following cocidial infection than that maintained by the conventional in-feed agent MON.

Oocyst Scoring and Anticoccidial Activity

Figure 1 shows the fecal oocyst yield at 18, 20, and 22 d of age (6, 8, and 10 dpi). When compared with the untreated CNT group, the number of oocysts per gram of excreta was lower ($P < 0.01$) in both MON treatments and the MON-2 + OEO-2 treatment during all challenge phases. Because the differences between OEO treatments and the CNT treatment did not reach statistical significance, the groups given 12 and 24 mg/kg OEO alone did not show a strong impact on oocyst shedding at all time intervals. This indicated that the supplementation of chickens with MON significantly reduced, and OEO only modestly reduced, fecal oocyst output in chickens infected with mixed *Eimeria* spp.

In contrast to our expectations, combination of MON and OEO failed to create a synergism to lower fecal oocyst output. When OEO (12 mg/kg; OEO-2) is supplemented with a half dose of MON (50 mg/kg; MON-2), oocyst production does not vary from the level produced by MON-2 alone supporting the fact that OEO exerts little activity on Eimerian parasites and does not influence the activity of the ionophore.

Again, the results identify MON as a valuable tool in the control of chicken coccidiosis with regard to reducing oocyst shedding, however, OEO is inferior in effect to anticoccidial drugs. Plant extracts had been previously reported to exert anticoccidial activity in association with reduced fecal oocyst excretion (Christaki et al., 2004; Abbas et al., 2012; Küçükyilmaz et al., 2012; Bozkurt et al., 2013). In agreement with our findings, the results of several previous studies (Giannenas et al., 2003; Saini et al., 2003; Tsinas et al., 2011; Bozkurt et al., 2012) designed to compare dietary supplementation of OEO with the ionophoric salinomycin, MON, or lasolacid sodium have shown that OEO has an inferior potential to reduce fecal oocyst excretion in comparison with conventionally recognized ionophore anticoccidial drugs.

Serum MDA, NO Concentrations, and Antioxidant Enzyme Activity

As shown in Table 3, a significant effect of treatment on serum concentrations of MDA, SOD, TAS, and NO was noted on d 18 (6 d post *Eimeria* infection). The results of this study showed that the serum MDA levels of all medicated treatments were significantly lower

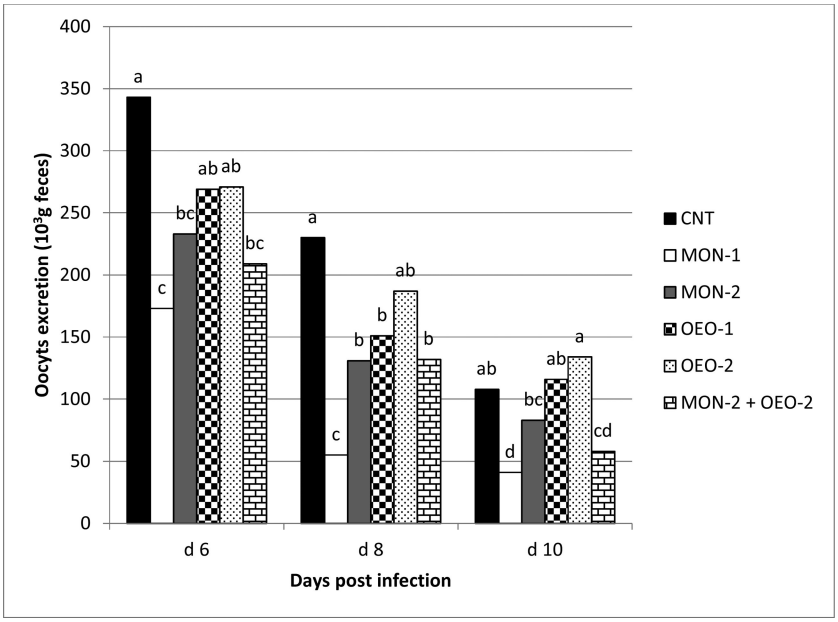

Figure 1. Daily (as measured at 6, 8 and 10 dpi) fecal oocyst output[1,2] in chicks given diet supplemented with anticoccidials[3] after broilers were infected with an inoculum containing 5×10^5 oocysts of *Eimeria* at 12 d of age. [a–c]Means within the same day, bars with different superscripts are differ significantly ($P < 0.05$). [1]Data are means of 6 measurements each per treatment. [2]Oocyst excretion, 10^3/g of feces. [3]The broilers were fed a control diet (CNT) that contained no anticoccidial or performance enhancer) or a diet supplemented with preparations of anticoccidial monensin (100 mg/kg of diet; MON-1), anticoccidial monensin (50 mg/kg of diet; MON-2), oregano essential oil (24 mg/kg of diet; OEO-1), oregano essential oil (12 mg/kg of diet; OEO-2) and 50 mg/kg monensin + 12 mg/kg oregano essential oil (MON-2 + OEO-2).

Table 3. The effects of chemical (MON) and botanical (OEO) methods of coccidiosis prophylaxis on malondialdehyde (MDA), superoxide dismutase (SOD), total antioxidant status (TAS) and nitric oxide (NO) concentration in blood serum of chickens at 6 d after infection (18 d of age) with mixed *Eimeria* spp.

Diet[1]	MDA (μmol/L)	SOD (Inhibition%)	TAS (mmol trolox Equiv./L)	NO (μM)
CNT	12.01^{a}	55.1^{b}	1.92^{c}	5.01^{a}
MON-1	10.41^{b,c}	60.3^{a,b}	2.16^{b,c}	0.94^{b}
MON-2	10.56^{b}	61.5^{a,b}	2.34^{b,c}	0.84^{b}
OEO-1	9.05^{d}	67.3^{a}	3.56^{a}	1.80^{b}
OEO-2	9.20^{c,d}	62.1^{a,b}	2.85^{a,b}	1.05^{b}
MON-2 + OEO-2	9.36$^{b–d}$	60.7^{a,b}	3.06^{a,b}	1.24^{b}
Pooled SEM[2]	0.318	2.617	0.228	0.493^{b}
P-value	0.0001	0.0130	0.0001	0.0001

[a–d]Values within a column not sharing the same superscript are different at $P < 0.05$.

[1]The broilers were fed a control diet (CNT) that contained no anticoccidial or performance enhancer, or a diet supplemented with preparations of anticoccidial monensin (100 mg/kg of diet; MON-1), anticoccidial monensin (50 mg/kg of diet; MON-2), oregano essential oil (24 mg/kg of diet; OEO-1), oregano essential oil (12 mg/kg of diet; OEO-2) or 50 mg/kg monensin + 12 mg/kg oregano essential oil (MON-2 + OEO-2).

[2]Data are means of 12 measurements each per treatment.

($P < 0.01$) than those of the unmedicated CNT group. The lowest MDA value was observed in the OEO-1 group, but this did not differ significantly from other the OEO-treated group. Birds in the OEO-1 group exhibited significantly higher ($P < 0.05$) serum SOD activity than those with the CNT treatment. SOD values obtained from other medicated treatments were at an intermediate position and did not differ from each other and the CNT treatment. The results of TAS followed a pattern quite similar to that in the SOD; however, the differences between the OEO-2 and CNT, and the MON-2 + OEO-2 and CNT treatments reached statistical significance ($P < 0.01$). A significant effect of treatment was evident for the serum NO concentration ($P < 0.01$). All of the medicated treatments showed markedly lower NO values compared with that of the CNT group, but there were no significant differences in the serum NO concentrations between the medicated groups.

The data indicate that plant bioactives have antioxidant properties that could reduce the harmful effects of oxidation and thus might be useful in controlling coccidiosis. Several authors (Giannenas et al., 2003; Tsinas et al., 2011; Bozkurt et al., 2012, 2014) have

Table 4. The effects of dietary anticoccidial strategies with MON and OEO on the intestinal morphology of 18-d-old broilers exposed to coccidial challenge at 12 d of age.

Item	Diet[1]							
	CNT	MON-1	MON-2	OEO-1	OEO-2	MON-2 + OEO2	Pooled SEM[3]	*P*-value
Duodenum								
Villous height (μm)	627^{c}	912^{a}	809^{b}	809^{b}	838^{b}	846^{b}	21.88	0.0001
Crypt depth (μm)	137^{a}	115^{c}	131^{a}	124^{b}	124^{b}	110^{c}	2.75	0.0001
Villous width	73^{d}	81^{a,b}	85^{a}	78^{b,c}	73^{d}	74^{c,d}	1.76	0.0001
VH/CD[2]	4.5^{c}	7.9^{a}	6.1^{b}	6.5^{b}	6.7^{b}	7.7^{a}	0.24	0.0001
Surface area (mm$^{2)}$	0.14^{d}	0.23^{a}	0.21^{a,b}	0.20^{b,c}	0.19^{c}	0.19^{c}	0.005	0.0001
Jejunum								
Villous height (μm)	634^{e}	794^{a}	733^{c}	676^{d}	745^{a,b}	771^{b,c}	12.50	0.0001
Crypt depth (μm)	152^{a}	145^{a-c}	143^{b,c}	147^{a,b}	138^{c}	128^{d}	2.75	0.0001
Villous width	59^{c}	66^{b}	66^{b}	66^{b}	74^{a}	63^{b}	1.31	0.0001
VH/CD[2]	4.1^{d}	5.4^{b}	5.1^{b}	4.6^{c}	5.4^{b}	6.0^{a}	0.14	0.0001
Surface area (mm$^{2)}$	0.11^{d}	0.17^{a}	0.15^{b,c}	0.13^{c}	0.17^{a}	0.15^{b}	0.004	0.0001
Ileum								
Villous height (μm)	552^{c}	716^{a}	654^{b}	560^{c}	649^{b}	640^{b}	12.20	0.0001
Crypt depth (μm)	129^{a}	96^{c}	117^{b}	100^{c}	118^{b}	99^{c}	2.40	0.0001
Villous width	57^{d}	64^{a,b}	68^{a}	61^{b,c}	60^{c,d}	60^{b-d}	1.44	0.0001
VH/CD[2]	4.2^{d}	7.4^{a}	5.5^{c}	5.6^{c}	5.5^{c}	6.4^{b}	0.14	0.0001
Surface area (mm^{2})	0.10^{c}	0.14^{a}	0.14^{a}	0.10^{c}	0.12^{b}	0.12^{b}	0.004	0.0001

[a–e]Values within a row not sharing the same superscript are different at $P < 0.05$.

[1]The broilers were fed a control diet (CNT) that contained no anticoccidial or performance enhancer, or a diet supplemented with preparations of anticoccidial monensin (100 mg/kg of diet; MON-1), anticoccidial monensin (50 mg/kg of diet; MON-2), oregano essential oil (24 mg/kg of diet; OEO-1), oregano essential oil (12 mg/kg of diet; OEO-2) and 50 mg/kg monensin + 12 mg/kg oregano essential oil (MON-2 + OEO-2).

[2]Villous height-to-crypt depth ratio.

[3]Mean represents 6 birds and average of 30 measurements per quantity per bird.

described the antioxidant-rich plant oregano as a potential candidate for controlling coccidiosis. They reported that phenolic compounds (e.g., carvacrol, thymol, and 1,8-cineol) with substantial antioxidant and antimicrobial activity found in plants from the Labiate family (Economou et al., 1991) might exhibit coccidiostatic action. In the current experiment, reduced serum MDA and NO concentrations in association with increased SOD and TAS activities in OEO-fed birds indicated that phytochemicals from oregano oil led to an improvement in the condition of birds. Although it has not previously been noted, the antioxidant activity exerted by MON was quite similar to that of OEO.

As in most cases of parasitic infection, the enzymatic antioxidant system of chickens with SOD was significantly decreased when infected with *Eimeria tenella* (Georgieva et al., 2006). However, serum concentrations of NO, which contributed to inflammatory injury, were increased with chicken coccidiosis (Allen et al., 1997). It appears that, in the present study, the effect of incorporating OEO into the chicken diet on the concentration of serum NO, MDA, and SOD indicated that OEO at inclusion levels of 12 and 24 mg/kg diet was able to reduce oxidative stress after infection with field-mix *Eimeria* spp.

Morphometric Analysis of the Gut

Table 4 shows morphometry measurements on small intestines performed at d 18 (6 dpi). The intestinal morphology (such as villus height, width, surface area, crypt depth, villus height-to-crypt depth ratio) of birds was significantly ($P < 0.01$) affected by OEO and MON compared with the unmedicated CNT treatment. In this study, higher villus height and decreased crypt depth of the duodenum, jejunum, and ileum were observed in chicks medicated with both MON and OEO. The significant improvements achieved by MON-1 were much more pronounced than those achieved by other coccidiosis prophylaxis procedures. This could be associated with the enhancements observed in BWG and FCR of chicks fed a diet with added MON-1.

Parasitic disease stress, such as coccidiosis, has a noxious effect on the intestinal microarchitecture, resulting in a reduction in the absorptive surface area, leading to inefficient digestion and absorption of nutrients (Fernando and McCraw, 1973; Ruff and Edgar, 1982). This situation may, however, be improved by using natural methods of coccidiosis prophylaxis (e.g., probiotic and prebiotic preparations) that alleviate the adverse effect on gut health and intestinal integrity, and enhance the digestibility of the diet (Giannenas et al., 2012, 2014). However, there is scarce information in the literature to indicate whether MON or OEO provides significant protection for the villous structure against lesions due to *Eimeria* spp. infection in chickens.

Longer and wider villi were observed in treated groups of birds, providing a greater surface area for nutrient digestion and absorption, thus contributing to increased mucosal enzymes, absorption, and the efficiency of the nutrient transport system (Amat et al., 1996). Therefore, in the present study, supplementation with MON and OEO enables birds to increase height and width of villi and thus compensate for the effects of

Table 5. The effects of dietary supplementation of MON and OEO on intestinal and pancreatic digestive enzyme activities in 18-day-old broilers after experimental infection with mixed *Eimeria* spp.

Item	Diet[1]							
	CNT	MON-1	MON-2	OEO-1	OEO-2	MON-2 + OEO-2	Pooled SEM[2]	*P*-value
Pancreas enzyme activity, unit/mg of protein of pancreas								
Amylase	12.6^{b}	34.0^{a}	22.9^{a,b}	27.3^{a}	33.5^{a}	33.1^{a}	4.6	0.0094
Chymotrypsin	0.40^{b,c}	0.47^{a,b}	0.46$^{a–c}$	0.51^{a}	0.39^{c}	0.40^{b,c}	0.027	0.0103
Lipase	7.7^{b}	29.2^{a}	12.1^{b}	11.7^{b}	11.4^{b}	11.2^{b}	1.6	0.0001
Intestinal enzyme activity, unit/g of wet intestinal contents								
Amylase	40^{c}	110^{a,b}	60^{c}	110^{a,b}	130^{a}	80^{b,c}	10.0	0.0006
Chymotrypsin	1.7^{b}	3.1^{a}	3.4^{a}	2.3^{b}	2.0^{b}	3.1^{a}	0.2	0.0001
Lipase	23.5^{c}	54.7^{a}	27.2^{b,c}	33.4^{b}	28.7^{b,c}	57.8^{a}	3.2	0.0001

$^{a–c}$Values within a row not sharing the same superscript are different at $P < 0.05$.

[1]The broilers were fed a control diet (CNT) that contained no anticoccidial or performance enhancer) or a diet supplemented with preparations of anticoccidial monensin (100 mg/kg of diet; MON-1), anticoccidial monensin (50 mg/kg of diet; MON-2), oregano essential oil (24 mg/kg of diet; OEO-1), oregano essential oil (12 mg/kg of diet; OEO-2) and 50 mg/kg monensin + 12 mg/kg oregano essential oil (MON-2 + OEO-2).

[2]Data are means of 12 measurements each per treatment.

a coccidia-induced depression of efficiency of feed conversion.

Enzymatic Activity

As shown in Table 5, amylase, lipase, and chymotrypsin activity in both the pancreas and small intestine generally presented a variable but positive response to the dietary anticoccidial procedures tested in the present study. Pancreatic amylase activity in all medicated groups, except for the MON-2 group, were higher ($P < 0.01$) than in the CNT group. A similar pattern to that of the pancreas was observed in the small intestine, with the exception that the amylase value in the MON-2 + OEO-2 group was slightly higher than that of the CNT group. The pancreatic chymotrypsin activity of birds fed OEO-1 was higher ($P < 0.05$) than those receiving the CNT diet, and no significant differences were observed between CNT and other treated groups. However, with regard to chymotrypsin activity in the intestines, the MON-1, MON-2, and MON-2 + OEO-2 treatments surpassed ($P < 0.01$) the CNT and remaining medicated treatments. When compared with the CNT and other medicated treatments, the MON-1 and MON-2 + OEO-2 treatments induced a significant rise ($P < 0.01$) in pancreatic and intestinal lipase activity, respectively.

In the present experiment, the reason for the higher BWG and lower FCR in birds fed MON and OEO supplements throughout the post-infection periods (i.e., d 13 to 27 and 13 to 42) was probably due to the slightly or significantly increased activities of corresponding enzymes that accompanied the increased absorption surface area (i.e., increased villus height and width). In agreement with the findings of this study, reports by Jamroz et al. (2005) and Basmacioğlu et al. (2010) indicated that the lipase and amylase activity increased in uninfected healthy birds fed a diet supplemented with plant extract consisting of carvacrol and OEO, respectively. Unfortunately, there is a dearth of literature regarding the role of phytogenic compounds from plant extracts in promoting intestinal enzymatic activity under the conditions of coccidial challenge. The base information in this regard reporting that a decrease in amylase (Major and Ruff, 1978) and disaccharides (Enigk and Dey-Hazra, 1976) dates back 40 years.

In conclusion, the data presented clearly point to the fact that anticoccidial activity is driven by the presence of MON. Also, this occured through its antioxidant effects, stimulation of enzymatic activity or enhanced nutrient absorption. However, OEO displayed little direct activity on the reproductive capacity of *Eimeria*, but may provide some ameliorative effect on the quality of intestines, secretion of digestive enzymes and particularly the antioxidant defence potential against *Eimeria* spp. Contrary to expectations, OEO failed to act synergistically with MON, as the effects of the two additives in combination were comparable with the effects of either fed separately. Overall, considering the definitive assessment of anticoccidial activity is a significant reduction in oocyst production, the questions remain about whether it could act as a certain replacement for MON, a widely used ionophore anticoccidial drug. This also begs the question of whether the anticoccidial activity of OEO would even occur under conditions of severe coccidial infection. Further research work is required to elucidate and confirm the efficacy of phytochemicals as potential in-feed anticoccidial agents.

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