

Response of broiler chickens fed diets supplemented with a bioactive olive pomace extract from *Olea europaea* to an experimental coccidial vaccine challenge

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ABSTRACT This study aimed to investigate an experimental procedure of coccidial challenge in battery cages and the anticoccidial effect of a bioactive olive pomace extract from *Olea europaea* (OE) in broiler chickens. To this end, four hundred 1-day-old male chicks were randomly assigned to 5 experimental treatments (10 cages/treatment; 8 birds/cage). One group was fed the control diet without any additives and not challenged (NCU). The other 4 groups were challenged and fed the control diet with no additives (NCC) or supplemented with 500 ppm of coccidiostat or with 500 or 1,500 ppm of OE. At 0, 7, and 14 d, all challenged birds, except the NCC group, were orally gavaged with a live *Eimeria* spp. oocyst vaccine at 1x, 4x, and 16x of the manufacturer's recommended dose, respectively. Feed intake (FI), body weight gain (BWG), and feed conversion ratio (FCR) were determined at 7, 14, 20, and 28 d. At 20 d of age, 1 bird per

cage was euthanized to analyze duodenum and jejunum morphology, ileal mucosa gene expression, and plasma cytokine, alpha-1-acid glycoprotein, and carotenoid (CAR) concentrations. Coccidial vaccine challenge lowered BW ($P < 0.05$) throughout the trial, and reduced FI and BWG, except from 20 to 28d, and increased FCR from 0 to 7, 0 to 14, and 0 to 20 d. Birds in the NCC group had higher ($P < 0.05$) oocyst counts and lower ($P < 0.05$) CAR and villus height to crypt depth ratios compared with NCU birds. Overall, coccidia challenge caused the expected reductions in growth performance and gut integrity. While the coccidiostat reduced oocysts excretion, dietary OE or coccidiostat had no effects on performance or gut integrity. The attenuated inflammatory response observed for all the treatments following the third infection can be attributed to the adaptation or immunization to the repetitive exposure to *Eimeria* spp.

Key words: broiler chicken, coccidiosis, olive pomace extract, *Eimeria*, vaccine

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INTRODUCTION

Coccidiosis causes large economic losses in the poultry industry by reducing growth performance, increasing mortality, and driving up production costs associated with the use of antibiotics and coccidiostats (Peek and Landman, 2011). Furthermore, the presence of microbial resistance to antibiotics has triggered the ban of most of these medications in the European Union (Huyghebaert et al., 2011). This directive has reduced the drug application in the poultry industry (EMA and ESVAC, 2019),

which has led to the search for alternatives to reduce coccidiosis and necrotic enteritis prevalence (Ferket, 2004; Gadde et al., 2017).

In this regard, new strategies such as increased vaccine use, better management and biosecurity practices at farms, and novel nutritional approaches to prevent coccidiosis and maintain gut health in poultry are being implemented (Ferket, 2004; Oviedo-Rondón, 2019). Among these, prebiotics, probiotics, and phytogenics utilization have shown potential for reducing the impact of coccidiosis in poultry (Peek and Landman, 2011; Oviedo-Rondón, 2019). Among phytogenics, bioactive compounds including polyphenols, oleuropeosides, flavonoids, and phenolics naturally occurring in olives and olive oil have potential as alternatives to antibiotics as growth promoters due to their anti-inflammatory, anti-oxidant, and antibacterial properties (Benavente-García et al., 2000; Romero et al., 2017). As such,

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various extracts rich in the aforementioned bioactives have been tested in broiler chickens and other animal species and have elicited positive responses in growth performance, gut health, and immune modulation (King et al., 2014; Gisbert et al., 2017; Morrison et al., 2017; Liehr et al., 2017; Herrero-Encinas et al., 2020a,b). Moreover, in vivo and in vitro studies with bioactive compounds derived from olive fruits, in particular maslinic acid, quercetin, and oleuropein, have shown promising anticoccidial activity against *Eimeria* spp. strains in broiler chickens (de Pablos et al., 2010; Debbou-Iouknane et al., 2019).

Previous studies have used coccidiosis models based on single, large doses of *Eimeria* spp. vaccines to induce subclinical coccidiosis and elicit growth depression and immune responses in broiler chickens (Adedokun and Adeola, 2017; Osho et al., 2019). However, such experimental models, especially when conducted in battery cages that reduce exposure to excreted and sporulated oocysts, do not reflect the multiple cycles of *Eimeria* infection and reinfection that occur under field conditions. Therefore, it may be advantageous to utilize models that better mimic the natural cycling of *Eimeria* by inoculating the birds several times with increasing doses of vaccine (Williams, 2002). Thus, the first of objective of the present study was to investigate the impact of a novel mixed *Eimeria* spp. challenge model for broiler chickens reared in cages to better mimic vaccinal oocyst cycling that occurs in the field. The second objective aimed to evaluate the effects of a bioactive pomace extract from *Olea europaea* (OE) supplementation on growth performance, gut histology, function, and immunity in broiler chickens challenged with this novel experimental model of coccidiosis.

MATERIALS AND METHODS

All animal care and experimental procedures were approved by the University of Arkansas Institutional Animal Care and Use Committee before initiation of the experiment (IACUC protocol #19005).

Housing and Experimental Animals

A feeding trial was carried out at the University of Arkansas Poultry Research Farm (Department of Poultry Science and The Center of Excellence for Poultry Science). A total of four hundred 1-day-old male by-product chicks from a Cobb 500 female line were obtained from a commercial hatchery (Cobb-Vantress Hatchery, Fayetteville, AR). Chicks were randomly distributed among 50 battery cages (0.61 × 0.61 m) with 8 birds each (37.6 ± 0.4 g) in thermostatically controlled rooms. Cages were equipped with wire flooring, 2 nipple drinkers, and a trough-style feeder. The temperature of the rooms was set at 33°C at the placement and gradually decreased to 22°C by 28 d post-hatch. A photoperiod of 23L:1D was set from the first 7 d of age and 18L:4D until the end of the experiment at 28 d of age.

Diets and Experimental Design

Birds were fed experimental diets ad libitum across 2 feeding phases consisting of a starter phase from 1 to 14 d of age and a grower phase from 15 to 28 d of age. Test additives were added to the basal starter and grower diets that were corn and soybean meal-based and formulated to meet breeder recommendations (Cobb, 2014) and in mash form. Feeds were mixed at the University of Arkansas Poultry Feed Mill (Fayetteville, AR). Basal diets were supplemented with 0, 500 (OE500 C), or 1,500 ppm (OE1500 C) of a bioactive olive pomace extract (OE; Lucta SA, Spain) or with 500 ppm of a commercial coccidiostat (PCC; Clinacox, Huvepharma, Peachtree City, GA; 0.2% diclazuril) in powdered form (Table 1).

At the beginning of the trial, all birds were weighed and randomly allotted to 5 experimental treatments (10 cages/treatment, with 8 chicks/cage) in a randomized complete block design. One group was fed the control diet without any additives and was not challenged (NCU). The other 4 groups were orally challenged with *Eimeria* spp. and fed their respective experimental diets as follows: control basal diet with no additives or coccidiostat (NCC), basal diet supplemented with PCC (Clinacox, 0.2% diclazuril), or with OE500 C or OE1500 C of an olive pomace extract. Supplemented OE was a standardized olive pomace extract containing ≥6% of triterpenes and ≥1% of polyphenols, quantified by HPLC-UV as oleanolic acid and hydroxytyrosol equivalents, respectively.

Coccidia Infection

Upon arrival, all birds except those in the NCU group were orally gavaged with a live oocyst vaccine (Cocci-vac-B52, Merck Animal Health, Millsboro, DE). An oral gavage (0.25 mL/bird diluted in distilled water) was used to provide uniform administration and animals of the NCU group received 0.25 mL of distilled water. At 7 and 14 d of age, birds in the challenge treatments (NCC, OE500 C, OE1500 C, and PCC) were orally inoculated with the same vaccine (1 mL) at 4x and 16x, respectively, of the manufacturer's recommendation, while chickens in the NCU group received 1 mL of distilled water (Figure 1). According to the manufacturer, the vaccine contained live sporulated oocysts of *Eimeria maxima*, *E. maxima* MF, *Eimeria acervulina*, *Eimeria tenella*, and *Eimeria mivati*.

Productive Traits and Sampling

Body weight and feed consumption were determined by cage at 7, 14, 20, and 28 d of age to calculate the body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR). At the end of the experiment at 28 d of age, chickens were euthanized by inhalation of CO₂. To determine the number of oocysts shed in the excreta, feces from each cage (excreted feces 24 h before sampling) were sampled at 7, 14, and 21 d post-hatch. At

Table 1. Ingredients and chemical composition (% , as fed basis, unless otherwise indicated) of starter (1–14 d) and grower diets (15–28 d).

Item	Starter diet				Grower diet			
	NCU/NCC	OE500 C	OE1500 C	PCC	NCU/NCC	OE500 C	OE1500 C	PCC
Ingredients								
Corn	60.87	60.87	60.87	60.87	64.80	64.80	64.80	64.80
Soybean meal	34.32	34.32	34.32	34.32	29.77	29.77	29.77	29.77
Soy oil	1.15	1.15	1.15	1.15	2.03	2.03	2.03	2.03
Limestone	1.11	1.11	1.11	1.11	1.07	1.07	1.07	1.07
Dicalcium phosphate	1.05	1.05	1.05	1.05	0.92	0.92	0.92	0.92
Sodium chloride	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39
DL-methionine	0.32	0.32	0.32	0.32	0.27	0.27	0.27	0.27
L-lysine·HCl	0.21	0.21	0.21	0.21	0.18	0.18	0.18	0.18
Inert filler (sand)	0.20	0.15	0.05	0.15	0.20	0.15	0.05	0.15
Trace mineral premix ¹	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Vitamin premix ²	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
L-threonine	0.085	0.085	0.085	0.085	0.073	0.073	0.073	0.073
Choline chloride (60%)	0.05	0.05	0.05	0.05	0.050	0.050	0.050	0.050
Phytase ³	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
Selenium premix, 0.06%	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Clinacox	-	-	-	0.050	-	-	-	0.050
OE	-	0.050	0.15	-	-	0.050	0.15	-
Calculated composition								
AME, kcal/kg	3,008	3,008	3,008	3,008	3,100	3,100	3,100	3,100
Crude protein	21.45	21.45	21.45	21.45	19.5	19.5	19.5	19.5
Ash	2.73	2.73	2.73	2.73	2.50	2.50	2.50	2.50
Ether extract	2.83	2.83	2.83	2.83	2.88	2.88	2.88	2.88
dLys	1.18	1.18	1.18	1.18	1.05	1.05	1.05	1.05
dTSAA	0.89	0.89	0.89	0.89	0.80	0.80	0.80	0.80
dThr	0.77	0.77	0.77	0.77	0.69	0.69	0.69	0.69
Calcium	0.90	0.90	0.90	0.90	0.84	0.84	0.84	0.84
Available phosphorus	0.45	0.45	0.45	0.45	0.42	0.42	0.42	0.42
Sodium	0.18	0.18	0.18	0.18	0.18	0.18	0.18	0.18

Abbreviations: dLys, digestible Lysine; dThr, digestible Threonine; dTSAA, digestible total sulphur amino acids; NCC, basal diet containing no additives and challenged; NCU, negative control with no additives and no challenge; OE500 C and OE1500 C, basal diet containing 500 and 1,500 ppm of olive pomace extract and challenged, respectively; OE, *Olea europaea*; PCC, basal diet containing 500 ppm of Clinacox and challenged.

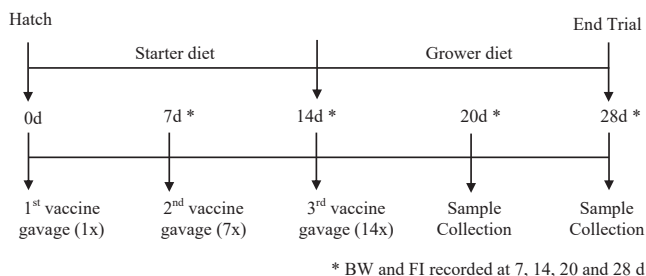
¹Supplied the following per kg of diet: manganese, 100 mg; zinc, 100 mg; copper, 10.0 mg; iodine, 1.0 mg; iron, 50 mg; magnesium, 27 mg.

²Supplied the following per kg of diet: vitamin A, 6,173 IU; vitamin D3, 4,409 IU; vitamin E, 44 IU; vitamin B12, 0.01 mg; menadione, 1.20 mg; riboflavin, 5.29 mg; D-pantothenic acid, 7.94 mg; thiamine, 1.23 mg; niacin, 30.86 mg; pyridoxine, 2.20 mg; folic acid, 0.71 mg; biotin, 0.07 mg; manganese, 24 mg; zinc, 14.4 mg; selenium, 0.04 mg; copper, 0.68 mg; iodine, 0.47 mg.

³OptiPhos 2000 PF (Huvepharma, Peachtree City, GA) added to provide 250 FTU/kg of phytase.

20 d of age, 2 birds per pen were randomly selected and euthanized to analyze ileal mucosa gene expression, duodenal and jejunal morphology, and plasma concentrations of carotenoids (**CAR**), interleukin 1 β (**IL-1 β**) and interleukin 8 (**IL-8**), and alpha-1-acid glycoprotein (**AGP**). For gene expression analysis, 200 mg of ileal mucosal scrapings were collected in RNA stabilization solution (RNAlater, Invitrogen, Carlsbad, CA) following the manufacturer's instruction and subsequently stored at -80°C . For intestinal morphology, 3-cm long segments of the middle part of duodenum and jejunum (proximal to the Meckel's diverticulum) were

stored in 10% neutral buffered formaldehyde solution for 24 h and then transferred into 70% of ethanol solution. Blood samples were collected immediately post-mortem via cardiac puncture into tubes containing K2 EDTA (BD Vacutainer, Franklin Lakes, NJ), held in ice, and centrifuged at $1,300 \times g$ for 15 min and 4°C to collect plasma. Samples were stored at -80°C for further analysis of CAR, IL-1 β , IL-8, and AGP plasma concentrations. At the end of the trial (28 d of age), the remaining animals were slaughtered by CO₂ inhalation and 2 birds per cage were randomly selected to analyze CAR plasma concentrations as previously described.

**Figure 1.** Diagram of the experimental design and *Eimeria* challenge of the trial. Abbreviation: FI, feed intake.

Oocyst per Gram of Excreta

Representative samples of 30 g of excreta from each cage were sampled and stored at 4°C . All samples were processed within 1 wk of collection. After recording the standardized excreta weight (10 g), 40 mL of water was added and the samples were soaked overnight. Then, 60 mL of water was added and mixed for approximately 10 min. The solubilized sample (1 mL) was added to 9 mL of a saturated salt solution in a test tube and mixed. The solution was pipetted (1 mL) into

2 chambers of a McMaster counting slide. The McMaster slide was examined under a light microscope at 10× magnification. A total of 2 slides was counted and averaged, and the oocyst per gram (**OPG**) was calculated based on the following formula:

$$\text{OPG} = \frac{\text{OAC} \times \text{DF} \times \text{FSV}}{\text{CVV} \times \text{WE}}$$

where, OAC is the oocyst average count, DF is the dilution factor (10), FSV is the fecal sample volume (mL), CVV is the counting chamber volume (0.15 mL), and WE is the weight of the excreta (g).

Intestinal Morphology Analysis

Samples collected at 20 d of age were embedded in paraffin using a tissue processor. Sections of 2.5 µm were stained with hematoxylin and eosin and subsequently imaged with an Olympus BX-40 (Olympus Optical Co., Ltd., Tokyo, Japan) digital camera and the Softsoftware version 3.2 C4040 Z (Olympus Soft Imaging Solutions, Hamburg, Germany). Images were analyzed by the same investigator who was blinded to the sample treatment. Villus height (**VH**) and crypt depth (**CD**) of 9 intact villi per section were recorded for each animal.

Carotenoids, Cytokine, and Alpha-1-Acid Glycoprotein Plasma Concentration

Carotenoids plasma concentrations of 2 birds per cage were analyzed at 20 and 28 d of age. Blood processing and CAR analysis were conducted under yellow light. Plasma was pooled, aliquoted, and stored at −80°C until further analysis. CAR was analyzed following the spectrophotometry method as described by Allen (1987). Quantification in plasma of cytokines was performed with commercial ELISA according to manufacturer's instructions (IL-1β, SEA565 Ga; IL-8, SEA080 Ga; Wuhan USCN Business Co. Ltd., Wuhan, China). Kit standards and test sample optical densities were read at 450 nm by an ELISA plate reader (Sunrise Microplate Reader, Tecan Trading AG, Switzerland). Plasma concentration of AGP was measured using an ELISA kit (ab157690; Abcam, Cambridge, MA) according to the manufacturer's instructions.

Gene Expression Analysis

Total RNA was extracted from approximately 50 mg of ileal mucosal scraping with TRIzol reagent (Invitrogen), disrupted with a mixer mill MM-400 (Retsch, Stuttgart, Germany), and isolated by using the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). To prevent genomic DNA contamination, an "in column" DNase step was performed by using the RNase-Free DNase Set (Qiagen, Australia). Extracted RNA yield and quality were measured by spectrophotometry (Epoch, BioTek,

Winoosky, VT) combined with the Take3 Micro-Volume Plate (BioTek, Santa Barbara, CA) by absorbance at wavelengths of 260 and 280 nm.

Reverse transcription of around 2,400 ng of extracted RNA was performed with the SuperScript VILO Master Mix (Invitrogen). Quantitative real-time PCR analysis was performed in a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA). Primers and PCR conditions for the chicken ubiquitin (house-keeping), toll-like receptor 4 (**TLR4**), IL-8 and 1β (IL-1β), interferon gamma (**IFN-γ**), B cell marker, and claudin 1 were obtained from the literature (Table 2). Samples were analyzed in duplicate using the right amount of each primer, ultra-purified water, and SYBR Green Master Mix (Applied Biosystems).

Statistical Analysis

All data were subjected to ANOVA using the GLM procedure of SAS (release 9.2; SAS Institute, Cary, NC) with diets and block as fixed effects. Normality distribution was checked using Shapiro-Wilk and Kolmogorov-Smirnov tests, and Levene's tests were used to confirm homogeneity of variance of data. For growth performance and oocyst counts, the experimental unit was the cage and for the intestinal morphology, gene expression and plasma concentration of AGP, IL-1β, IL-8, and CAR of the animal was the experimental unit. The effect of *Eimeria* spp. challenge on the studied variables was determined by single degree of freedom contrasts comparing the NCU and NCC groups. Proc Interactive Matrix Language of SAS was used to generate the polynomial coefficients for the unequally spaced treatments which were included in polynomial contrasts to test the linear and nonlinear effect of the OE inclusion. To analyze differences among challenge groups, Tukey's test at $P < 0.05$ was used. Oocysts per gram were analyzed using a Poisson model (GENMOD procedure and considering a Poisson distribution) and differences among challenge treatments were analyzed using LSMeans. Results are presented in tables as means and SEM except for the oocyst counting results, which are presented as means of natural logarithmic transformation and the standard error. For gene displaying efficiencies different from 2 ($E \neq 2$), cycle threshold values were adjusted according to the previous model described by other authors (Steibel et al., 2009). Cytokine IL-8 and IL-1β plasma concentrations were natural log transformed before being statistically analyzed to satisfy population normality and variance homogeneity assumptions.

RESULTS

Body Weight Gain, Feed Intake, Feed Conversion, and Mortality

Growth performance data are shown in Table 3. Body weight was significantly lower ($P < 0.05$) for birds in the NCC group compared with those in the NCU group at 7,

Table 2. Genes, forward and reverse primers, and efficiency for gene expression analysis by quantitative real-time PCR.

Gene ¹	5'-primer sequence forward-3'	5'-primer sequence reverse-3'	Efficiency
UB ²	GGGATGCAGATCTTCGTGAAA	CTTGCCAGCAAAGATCAACCTT	1.94
TLR4 ³	AGTCTGAAATTGCTGAGCTCAAAAT	GCGACGTTAAGCCATGGAAG	1.94
IL-8 (CXCLi2) ⁴	CCTGGTTTCAGCTGCTCTGT	GCGTCAGCTTCACATCTTGA	1.98
Bu-1 ⁵	GGTGTCCAGTGAAGGTGTG	GATGCAAAGGATGGGTGTC	1.94
IL-1 β ⁶	TGGGCATCAAGGGCTACA	TCGGGTTGGTTGGTGATG	2.01
IFN- γ ⁶	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTC	1.94

¹UB, ubiquitin; TLR4, toll like receptor 4; IL-8, interleukin 8 (CXCLi2 former); IL-1 β , interleukin 1 β ; IFN- γ , interferon-gamma; Bu-1, chicken B-cell marker chB6.

²de Boever et al., 2008.

³Chen et al., 2015.

⁴Rasoli et al., 2015.

⁵Bar-Shira et al., 2003.

⁶Hong et al., 2006.

14, 20, and 28 d of age. Throughout the experimental period, NCC birds had lower ($P < 0.05$) FI and BWG compared to NCU, except from 20 to 28 d of age. Furthermore, FCR was higher ($P < 0.05$) in NCC birds compared with NCU birds during the periods from 0 to 7, 0 to 14, and 0 to 20 d, but there was no difference ($P > 0.05$) in FCR between the NCC and NCU groups from 20 to 28 d. No differences ($P > 0.05$) were observed in BW, FI, BWG, and FCR during the experiment

among the challenged treatments. Mortality was less than 5% and unrelated to treatment (data not shown).

Oocyst per Gram of Excreta

Results of OPG are shown in Table 4. The OPG were significantly ($P < 0.001$) higher in NCC birds compared to NCU on days 7, 14, and 21. Among the challenged treatments, the PCC group had lower ($P < 0.05$) OPG

Table 3. Effect of experimental diets on *Eimeria* spp. challenged broiler chickens growth performance from 0 to 28 d of age.¹

							<i>P</i> -values ³		
Item	NCU	NCC	OE500 C	OE1500 C	PCC	SEM ²	NCU vs. NCC	OE linear	OE nonlinear
BW (g/bird)									
0 d	38.8	38.4	38.4	38.4	38.7	0.15	0.075	0.76	0.98
7 d	145	126	130	128	128	4.10	0.003	0.87	0.53
14 d	405	327	355	345	347	14.5	<0.001	0.52	0.22
20 d	779	646	683	685	663	23.6	<0.001	0.32	0.41
28 d	1,435	1,301	1,327	1,311	1,242	36.9	0.016	0.93	0.62
FI (g/bird)									
0–7 d	126	114	114	116	117	3.42	0.027	0.63	0.72
7–14 d	313	268	297	277	275	11.7	0.011	0.86	0.082
14–20 d	503	440	458	461	443	12.5	0.001	0.30	0.48
20–28 d	995	951	972	969	919	26.5	0.25	0.69	0.64
0–14 d	439	383	410	393	393	13.9	0.008	0.79	0.16
0–20 d	942	823	869	855	835	25.0	<0.001	0.50	0.26
0–28 d	1,937	1,774	1,841	1,824	1,754	45.1	0.016	0.54	0.37
BWG (g/bird)									
0–7 d	106	87.6	91.3	89.2	89.3	4.09	0.004	0.88	0.53
7–14 d	260	201	225	217	219	11.0	<0.001	0.44	0.17
14–20 d	374	319	328	340	316	11.9	0.003	0.23	0.89
20–28 d	656	655	644	626	580	22.2	0.97	0.37	0.95
0–14 d	366	288	317	306	308	14.5	<0.001	0.52	0.22
0–20 d	741	608	645	647	624	23.6	<0.001	0.32	0.41
0–28 d	1,397	1,263	1,289	1,272	1,203	36.9	0.016	0.93	0.62
FCR (g/g)									
0–7 d	1.20	1.34	1.25	1.31	1.34	0.034	0.006	0.80	0.068
7–14 d	1.26	1.36	1.32	1.29	1.27	0.038	0.066	0.18	0.82
14–20 d	1.36	1.41	1.42	1.45	1.43	0.027	0.22	0.29	0.85
20–28 d	1.52	1.46	1.52	1.55	1.60	0.038	0.22	0.10	0.50
0–14 d	1.24	1.35	1.30	1.29	1.29	0.031	0.012	0.21	0.43
0–20 d	1.30	1.38	1.36	1.37	1.36	0.024	0.017	0.79	0.51
0–28 d	1.39	1.41	1.43	1.43	1.46	0.024	0.49	0.53	0.67

Abbreviations: BWG, body weight gain; FCR, feed conversion ratio; FI, feed intake; OE, *Olea europaea*.

¹NCU, negative control with no additives and no challenge; NCC, OE500 C, OE1500 C, basal diet containing 0, 500, and 1,500 ppm of olive pomace extract and challenged, respectively; PCC, basal diet containing 500 ppm of Clinacox and challenged.

²SEM (n = 10).

³Challenged treatments were not significantly different by Tukey's test.

Table 4. Effect of experimental diets on *Eimeria* spp. challenged broiler chickens on OPG of excreta at 7, 14, and 21 d of age.¹

Item	NCU	NCC	OE500 C	OE1500 C	PCC	P-values		
						NCU vs. NCC	OE linear	OE nonlinear
7 d	0.95 ± 0.95	5.11 ± 0.12 ^a	5.40 ± 0.10 ^a	5.29 ± 0.11 ^a	1.75 ± 0.62 ^b	<0.001	0.47	0.092
14 d	0.25 ± 0.97	5.46 ± 0.08 ^a	5.50 ± 0.07 ^a	5.30 ± 0.08 ^a	0.23 ± 1.00 ^b	<0.001	0.10	0.29
21 d	0.41 ± 0.92	4.42 ± 0.13 ^a	4.17 ± 0.14 ^a	4.30 ± 0.13 ^a	-0.34 ± 1.27 ^b	<0.001	0.65	0.17

^{a,b}Challenged treatments means with different letters differ statically by LSMMeans ($P < 0.05$).

Abbreviations: NCC, basal diet containing no additives and challenged; NCU, negative control with no additives and no challenge; OE500 C and OE1500 C, basal diet containing 500 and 1,500 ppm of olive pomace extract and challenged, respectively; OE, *Olea europaea*; OPG, oocyst per gram; PCC, basal diet containing 500 ppm of Clinacox and challenged1..

¹Values expressed as Ln ($\times 1,000$ oocyst OPG) $\pm$ SE.

compared to NCC, OE500 C, and OE1500 C on days 7, 14, and 21. Furthermore, the inclusion of OE showed a nonlinear trend ($P = 0.092$), displaying higher OPG in OE500 C compared to NCC and OE1500 C at 7 d of age.

Intestinal Morphology

No differences ($P > 0.05$) were observed in the duodenum and jejunum VH and CD among NCU and NCC treatments at 20 d of age (Table 5). However, the VH to CD ratio (VH/CD) of duodenum and jejunum was reduced ($P < 0.05$) in NCC compared to NCU. Additionally, no differences ($P > 0.05$) in the duodenum and jejunum morphology were observed among challenge treatments.

Cytokine, Alpha-1-acid Glycoprotein, and Carotenoid Plasma Concentration

No differences ($P > 0.05$) in plasma concentrations of IL-1 β , IL-8, and AGP were observed among experimental treatments at 20 d of age (Table 6). However, CAR was reduced ($P < 0.001$) in the NCC treatment compared to NCU at 20 d of age. Moreover, CAR tended ($P = 0.080$) to be lower in NCC compared to NCU at 28 d of age. Furthermore, CAR was significantly ($P < 0.05$) higher in PCC animals compared to NCC at 20 d of age.

Gene Expression

Results of ileum gene expression at 20 d of age are shown in Table 7. The expression of TLR4, IL-8, IL-1 β , IFN- γ , B cell marker, and claudin 1 was not different ($P > 0.05$) between NCU and NCC groups at 20 d of age. However, the expression of IFN- γ tended ($P = 0.077$) to linearly increase with the inclusion of OE. Among challenged treatments, no differences ($P > 0.05$) were observed in the gene expression of broilers at 20 d of age.

DISCUSSION

Eimeria spp. Challenge Effect

Vaccination programs against coccidiosis using live *Eimeria* oocysts are a current practice in poultry production. However, such programs can cause temporary

decline in growth performance and gut intestinal health (Williams, 2002). In this context, it is necessary to establish repeatable experimental models to mimic vaccinal oocyst cycling to aid in the development of nutritional strategies that can ameliorate the transient intestinal disruption derived from the coccidiosis vaccine in poultry. Thus, the first objective of this study was to evaluate the impact of a novel experimental model of the vaccinal oocyst cycling in broiler chickens on growth performance and gut health and function. The selected timing of the repeated inoculations and increased oocyst number with each inoculation were targeted to mimic the time required for oocyst shedding (4–6 d) and the sporulation in the environment (1–2 d), as well as oocysts amplification that occurs in the field (Chapman et al., 2010).

Growth performance and oocyst counts in litter or excreta are the main parameters to evaluate the severity of *Eimeria* infections in poultry (Holdsworth et al., 2004). In the current study, the coccidia challenge reduced BW in broiler chickens at 7, 14, 20, and 28 d of age, which resulted from lower FI and poorer FCR compared with the non-challenged birds. Moreover, the effects of coccidia challenge were apparent in the OPG of excreta which were higher in NCC compared to NCU at 7, 14, and 21 d of age. These results are in agreement with those reported by Leung et al. (2019) who observed a reduction in BWG and BW and increased oocyst shedding in animals challenged with *E. maxima* and *E. acervulina* at 7 d post-challenge. In addition, Wang et al. (2018) reported impaired FCR (from 15 to 40 d post-hatch) in broiler chickens challenged at 21 d of age with 10x of Coccivac-B52 vaccine. Furthermore, Persia et al. (2006) showed a reduction in FI and BWG in broiler chickens aged 9 to 22 d challenged with multiple *E. acervulina* inoculations (5.0×10^5 sporulated oocyst) at 9, 12, 15, and 18 d of age. Thus, it can be concluded that the novel replicable model of coccidia challenge used in this experiment exerted the expected response of growth depression and high OPG counts. Further, the reduction in BWG and increase in oocyst shedding between NCU and NCC groups were slightly less from 14 to 20 d of age following the 16 x inoculation than from 7 to 14 d following the 4x inoculation, possibly reflecting that some degree of immunity had developed with the repeated inoculations. This would also allow lesions to resolve by the sampling time point of 20 d and,

Table 5. Effect of experimental diets and *Eimeria* spp. challenged broiler chickens on intestinal morphology at 20 d of age.¹

							<i>P</i> -values ³		
Item	NCU	NCC	OE500 C	OE1500 C	PCC	SEM ²	NCU vs. NCC	OE linear	OE nonlinear
Duodenum									
VH, μm	1,851	1,774	1,719	1,795	1,770	61.5	0.40	0.71	0.40
CD, μm	148	178	196	195	159	13.7	0.14	0.47	0.49
VH/CD	13.2	10.1	9.50	9.62	11.6	0.90	0.026	0.78	0.70
Jejunum									
VH, μm	993	942	995	973	948	60.8	0.55	0.79	0.58
CD, μm	128	152	149	153	145	12.1	0.17	0.91	0.84
VH/CD	8.06	6.41	6.85	6.67	6.74	0.48	0.021	0.78	0.57

Abbreviations: CD, crypt depth; OE, *Olea europaea*; VH, villus height; VH/CD, villus height: crypt depth ratio

¹NCU, negative control with no additives and no challenge; NCC, OE500 C, OE1500 C, basal diet containing 0, 500, and 1,500 ppm of olive pomace extract and challenged, respectively; PCC, basal diet containing 500 ppm of Clinacox and challenged.

²SEM (n = 10).

³Challenged treatments were not significantly different by Tukey's test.

though not measured in this experiment, no severe *Eimeria* lesions were observed during gut sampling.

Plasma CAR concentrations are an indicator of intestinal mucosa integrity in poultry and can be markedly impacted by coccidiosis infections in chickens (Conway et al., 1993; Holdsworth et al., 2004). In previous studies, vaccinated birds against coccidiosis or experimentally infected with *Eimeria* showed a reduction in CAR plasma concentration (Rochell et al., 2016; Leung et al., 2019; Gautier et al., 2020). These previous findings agree with the results of this experiment as NCC showed a lower CAR concentration compared to the NCU group after 6 d of the last oral gavage vaccine (20 d of age). However, CAR concentration was similar at 28 d of age (14 d post-challenge), which may indicate that the gut integrity of challenged birds was restored.

Increase of CD and lower VH/CD ratio have been observed during coccidiosis vaccination or infection, presumably due to the increased epithelial turnover and to replace damaged enterocytes (Morris et al., 2004). In this regard, Leung et al. (2019) observed morphological damage in the jejunum with a reduction of VH, CD, and VH/CD ratio in chickens challenged with *Eimeria* spp. (5 d post-challenge). Furthermore, Wang et al. (2018) showed deeper crypts in the ileum and higher VH/CD values after 5 d post-challenge (26 d of age) with

Coccivac-B52. These previous reports are in line with the results of the current experiment as the VH/CD ratio in duodenum and jejunum was reduced in challenged birds after 6 d post-challenge. This response combined with the reduced plasma CAR concentration reflect a damaged mucosa in response to the *Eimeria* spp. challenge employed in the current experiment.

Coccidiosis in poultry induces an immune response in the host that results in inflammation and activation of intraepithelial T lymphocytes in which an upregulation of IFN- γ expression is a hallmark response to *Eimeria* infection (Rothwell et al., 2000; Yun et al., 2000). Moreover, an upregulation of different markers has been observed in coccidia-infected chickens, including IL-1 β and several other cytokines of the interleukin (IL) family, toll-like receptors (TLRs), and transforming growth factors β 1–4 (Hong et al., 2006; Kim et al., 2019). The host gut immune response depends on the *Eimeria* spp. and strain, the age of the flocks, and the environmental conditions and feed quality in poultry (Conway and McKenzie, 2007). In this context, utilization of an overdose (20x) of Coccivac-B52 in broiler chickens (at 14 d of age) caused increased gene expression of IFN- γ and IL-10 after 6 d post-challenge (Oxford and Selvaraj, 2019). Moreover, other authors have similarly shown that a single overdose of Coccivac-B52 vaccine

Table 6. Effect of experimental diets on AGP, IL-1 β , and IL-8 at 20 d of age and carotenoids at 20 and 28 d of age in broiler chicken plasma.¹

Item	NCU	NCC	OE500 C	OE1500 C	PCC	SEM ²	P-values ³		
							NCU vs. NCC	OE linear	OE nonlinear
IL-8 (pg/mL)	82.7	58.2	67.3	83.1	88.0	16.0	0.37	0.32	0.63
IL-1 β (ng/mL)	1.58	1.32	1.64	1.43	1.68	0.23	0.69	0.95	0.41
AGP ($\mu\text{g/mL}$)	117	116	110	113	109	6.36	0.91	0.81	0.51
Carotenoids ($\mu\text{g/mL}$)									
20 d	1.82	1.24 ^b	1.30 ^{a,b}	1.31 ^{a,b}	1.57 ^a	0.10	<0.001	0.67	0.74
28 d	1.23	1.01	1.10	1.20	1.19	0.084	0.080	0.13	0.81

^{a,b}Challenged treatments were significantly different by Tukey's test at 5%.

Abbreviations: AGP, alpha-1-acid glycoprotein; IL-8, interleukin 8; IL-1 β , interleukin 1 β ; OE, *Olea europaea*.

¹NCU, negative control with no additives and no challenge; NCC, OE500 C, OE1500 C, basal diet containing 0, 500, and 1,500 ppm of olive pomace extract and challenged, respectively; PCC, basal diet containing 500 ppm of Clinacox and challenged.

²SEM (n = 20).

³P-values of IL-1 β and IL-8 are from natural logarithmic data transformation analysis.

Table 7. Effect of experimental diets and *Eimeria* spp. challenge on broiler chicken ileum gene expression at 20 d of age.¹

Item	NCU	NCC	OE500 C	OE1500 C	PCC	SEM ²	P-values ³		
							NCU vs. NCC	OE linear	OE nonlinear
TLR4	9.49	9.76	9.59	10.1	9.75	0.24	0.44	0.25	0.36
IL-1 β	12.5	12.0	12.3	11.9	12.1	0.26	0.24	0.68	0.38
IL-8	8.27	8.73	7.90	8.29	8.51	0.43	0.46	0.63	0.20
IFN- γ	12.1	11.2	10.3	10.1	10.7	0.38	0.11	0.077	0.27
Bu-1	6.45	5.98	5.85	6.01	6.36	0.26	0.22	0.86	0.67
Claudin 1	11.3	11.9	11.7	11.9	11.5	0.40	0.30	0.98	0.66

Abbreviations: Bu-1, B-cell marker; IL-1 β , interleukin 1 β ; IL-8, interleukin 8; IFN- γ , interferon gamma; OE, *Olea europaea*; TLR4, toll-like receptor.

¹NCU, negative control with no additives and no challenge; NCC, OE500 C, OE1500 C, basal diet containing 0, 500, and 1,500 ppm of olive pomace extract and challenged, respectively; PCC, basal diet containing 500 ppm of Clinacox and challenged. Values are increments of cycle threshold (Δ Ct) of target gene relative to housekeeping. Ct values are inversely related to RNA abundance.

²SEM (n = 10).

³Challenged treatments were not significantly different by Tukey's test.

in broilers induced an upregulation of several IL including IL-1 β , IL-6, IL-10, IFN- γ , TLR4 and downregulation of other gene markers related to gut integrity such as claudin 1 or occludin (Osho and Adeola, 2019; Osho et al., 2019). According to Williams (2002), a single *Eimeria* spp. exposure might activate the primary immune response but does not generate an acquired immunization. In the present study, the expression of all immune system-related markers in the plasma and ileum was similar between challenged and non-challenged birds at 20 d of age (6 d after the third inoculation). Therefore, this attenuated inflammatory response along with the reduced impact in growth performance following the third infection can be attributed to the adaptation or immunization to the repetitive exposure to *Eimeria*.

Effect of Olive Pomace Extract and Coccidiostat

Natural alternatives to anticoccidial chemical drugs and ionophores are being developed to reduce coccidiosis prevalence in the poultry industry (Quiroz-Castañeda and Dantán-González, 2015). Several products have been described as potential alternatives against coccidiosis, but their mechanism of action and effectiveness have not been completely described or validated (Muthamilselvan et al., 2016). The second objective of this study was to evaluate the potential anticoccidial effect of an olive pomace extract in broiler chickens.

Dietary supplementation with different concentrations of OE showed no significant growth performance effects in birds challenged with the 3 cycles of live oocyst vaccine throughout the trial. These findings are not aligned with those of de Pablos et al. (2010), who observed better BWG but no FI differences at 21 d post-infection with *E. tenella* in chickens that were fed diets supplemented with 90 ppm of maslinic acid, a bioactive compound derived from olive oil by-products. These authors reported that the OPG from 6 to 10 d post-infection were significantly reduced with maslinic

acid. In addition, a recent study showed that quercetin and oleuropein substances derived from OE were capable of lysing *Eimeria* oocysts in vitro (Debbou-Iouknane et al., 2019), though in vivo or ex vivo activities were not tested. Further, in our previous studies, OE inclusion in broiler diets exerted a positive effect on performance because of its anti-inflammatory properties (Herrero-Encinas et al., 2020a). In the present study, the OPG were not affected by OE. Moreover, the selected markers of immune response in plasma and ileum, and the intestinal morphology in the duodenum and jejunum were not affected by OE. Additionally, coccidiostat (Clinacox, 0.2% diclazuril) did not improve growth performance during the experiment despite the significantly lower OPG observed at 7, 14, and 21 d of age and the higher plasma CAR concentrations at 20 d of age (6 d post third oocyst cycling). As with the OE, no significant effects of diclazuril were observed on the selected markers of immune response and the intestinal morphology in the duodenum and jejunum. In-feed administration of diclazuril at 0.5 ppm is almost entirely effective against *E. tenella*, *E. acervulina*, and *Eimeria mitis* (McDougald et al., 1990). However, in case of *E. maxima* infection, diclazuril is not effective until the sexual stages after some intestinal damage already occurs, allowing some growth depression even if the *Eimeria* cycle is subsequently disrupted (McDougald et al., 1990). These findings might explain the lack of expected beneficial effects of diclazuril on broiler chicken growth performance. Moreover, the beneficial coccidiostat activity of bioactive compounds derived from olive by-products in broiler chickens, including the OE extract, seems to depend on the concentration and type of bioactive compounds, the *Eimeria* strain, and the age and developed immune system of birds.

It can be summarized that under the present study conditions, the coccidiosis challenge decreased growth performance and gut integrity as indicated by oocyst excretion, and reduced plasma CAR and VH/CD ratio. Bearing in mind the effects of the *Eimeria* spp. challenge and the lack of beneficial effects of inclusion of OE or Clinacox on performance, gut integrity, and immune

system might be indicative of an immunity development response against *Eimeria* spp. Considering our previous results of OE positive immunomodulatory effects, it is plausible that this extract could be used in combination with a vaccine program for OE anti-inflammatory effects without compromising the intended development of immunity with the vaccination. Disclosures: The authors declare no conflict of interest.

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DISCLOSURES

The authors declare no conflict of interest.

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