Effects of methylsulfonylmethane and neutralizing anti–IL-10 antibody supplementation during a mild *Eimeria* challenge infection in broiler chickens

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ABSTRACT A 28-day experiment was conducted in broilers to study the effects of feeding methylsulfonyl-methane (**MSM**) and IL-10–neutralizing antibody from dried egg product (**DEP**) on the growth performance, immune responsivity, oxidative stress parameters, and gut health outcomes during a mild infection with mixed species of *Eimeria*. A total of 500 male Ross 308 chicks were allocated to five treatments: sham-inoculated (uninfected) chickens fed control diet (**UCON**), *Eimeria*-infected chickens fed control diet (**ICON**), and *Eimeria*-infected chickens fed control diet supplemented with 287 U/tonne of DEP (**I-DEP**), 0.4% MSM, or their combination (I-DEP-MSM), with 10 replicate cages of 10 birds per treatment. All infected groups received 1 mL of an oral inoculum containing *Eimeria acervulina* (10,000 oocysts), *Eimeria maxima* (5,000 oocysts), and *Eimeria tenella* (5,000 oocysts) on study days 7 and 14. Data were analyzed as a two-way ANOVA for all treatments including *Eimeria*-infected groups, in addition to a single degree of freedom contrast to compare uninfected and infected groups receiving the control diet. Mild *Eimeria* infection did not influence the growth performance in ICON compared with UCON at any time points. Overall (day 0–28) growth performance parameters were not influenced by either infection or dietary supplementation of MSM or DEP. However, birds in I-DEP-MSM showed improved ADG during study day 7 to 14 (i.e., 7 d after primary inoculation) indicating a beneficial effect immediately after *Eimeria* infection. Although MSM supplementation reduced thiobarbituric acid reactive substances (day 21 and 28), both MSM and DEP improved the total antioxidant capacity (day 21) in the plasma of infected birds. Histopathological outcomes were not influenced by treatments, and fecal oocyst output was higher in MSM- and DEP-supplemented groups than with ICON, indicating no beneficial effects. Similarly, expression of cecal inflammatory cytokines (IL-10, IL-1β, and interferon-γ) was not affected by MSM, DEP, or their combination. Overall, the current results suggest that both MSM and DEP supplementation may benefit birds during a mild *Eimeria* infection as indicated by improvements in ADG and oxidative stress outcomes.

Key words: IL-10 neutralizing antibody, Methylsulfonylmethane, Oxidative stress, *Eimeria*, Broiler

INTRODUCTION

Coccidiosis is a poultry parasitic disease caused by *Eimeria* spp. that affects the gastrointestinal tract of chickens. The disease has serious economic implications for the poultry industry worldwide as it affects growth and productivity of birds. Besides poor absorption, *Eimeria* infection reduces digestibility of nutrients along with an increase in nutrient demand to support host immunity (Rochell et al., 2016; Gautier et al., 2020). Because of better flock management strategies, severe outbreaks of coccidiosis are rare in modern poultry operations. However, subclinical infections continue to be a challenge as it affects the overall growth performance of a flock (Haug et al., 2008; Lehman et al., 2009). Prophylactic anticoccidials must be used judiciously, but immunomodulation using an appropriate dietary intervention may be a useful solution (Shanmugasundaram et al., 2013; Morris et al., 2015; Rochell et al., 2017).

Host immunity against *Eimeria* is predominantly mediated by cellular immunity, where cytokines and chemokines synthesized by immune cells effectively coordinate an immune response. Among various players of T cell immunity, IL-10 secreted 4 to 5 d after *Eimeria*

infection (Hong et al., 2006) has an inhibitory role (Shanmugasundaram et al., 2013), where it interferes with the Th1 response and reduces the host's ability to eliminate the parasite. Therefore, IL-10 may assist in evasion of the host immunity by *Eimeria* (Shanmugasundaram et al., 2013; Kim et al., 2019). As such IL-10 inhibits the transcription factor, nuclear factor kappa B, to suppress transcription of multiple proinflammatory cytokines that are involved in parasite clearance from intestinal epithelial cells (Dokka et al., 2001; Wu et al., 2012). It is also suggested that *Eimeria*-induced IL-10 primarily affects interferon-γ (**IFN-γ**) secretion from Th1 cells, thereby further reducing the host's ability to attract additional immune cells to the site of infection for effective parasitic elimination (Kim et al., 2019). Beneficial effects of reduced IL-10 activity using egg-derived anti–IL-10 antibody on the growth performance were observed in broilers experiencing a mild *Eimeria* infection (Sand et al., 2016). However, a recent study in our laboratory observed no beneficial effects during a severe infection, where broilers exhibited severe damage to the intestinal epithelium (Abdul Rasheed et al., *In press*). This supports the notion that suppression of anti-inflammatory IL-10 may promote a severe inflammatory reaction in the intestinal epithelium that may not ultimately benefit the gut health and performance. Therefore, a balance in the inflammatory response is needed to prevent severe damage to the intestinal epithelium.

Anti-inflammatory and antioxidant properties of methylsulfonylmethane (**MSM**), an organic, sulfur-containing compound, have been well established in both human and animal studies (Debbi et al., 2011; Pagonis et al., 2014; Van der Merwe and Bloomer, 2016; Rasheed et al., 2020). Diverse biological benefits of MSM, including inhibition of the inflammatory mediators, inducible nitric oxide synthase, cyclooxygenase 2, prostaglandin E2, IL-6, and tumor necrosis factor-alpha through downregulation of nuclear factor kappa B signaling (Kim et al., 2009), may help lessen inflammation during *Eimeria* infection in chickens. Oral administration of MSM at 2,000 mg/kg BW elicited no toxicological effects in broilers, suggesting that MSM is safe to be included in poultry diets (Abdul Rasheed et al., 2019). In addition, MSM has shown anti-inflammatory activities at the level of the intestinal epithelium, as evidenced by moderation of inflammation during a gastric mucosal injury in mice (Amirshahrokhi and Khalili, 2017). Inflammation is accompanied by oxidative stress because of excessive production of free radicals including nitric oxide, peroxides, and superoxides by immune cells (reviewed by Sorci and Faivre, 2006).

We hypothesized that while attenuation of the host IL-10 activity by an egg-derived anti–IL-10 antibody may favor a proinflammatory reaction to eliminate *Eimeria*, MSM, due to its anti-inflammatory and antioxidant capacity, may bring beneficial balance between the immune response and tissue injury during a mild, subclinical *Eimeria* infection in broilers. Therefore, the current experiment was conducted to evaluate the effects of feeding MSM and IL-10–neutralizing antibody from dried egg product (**DEP**) on the growth performance, cytokine response, oxidative stress parameters, and intestinal histopathological lesions in broilers during a mild infection with mixed species of *Eimeria*.

MATERIALS AND METHODS

All animal care and experimental procedures were approved by the University of Illinois Institutional Animal Care and Use Committee before initiation of the experiment.

Birds and Husbandry

Day-old Ross 308 male broiler chicks not vaccinated against *Eimeria* were obtained from a commercial hatchery (Hoover's Hatchery, Rudd, IA) and transported to the University of Illinois Edward R. Madigan Laboratory. Broiler chicks were housed in thermostatically controlled battery cages (model SB5T; Alternative Design Manufacturing, Siloam Springs, AR) with raised-wire flooring in an isolated, environmentally controlled room with continuous lighting. A standard corn–soybean meal–based starter diet (Table 1) that met or exceeded breeder recommendations (Aviagen, 2014) was used to prepare experimental diets, and DEP and MSM test articles were included individually and in combination on top of the formulation (i.e., no space reserved in the formulation) as having negligible nutritive contribution to the overall diet. All birds were provided continuous free access to water and their respective experimental diet, which was fed in a single phase throughout the experiment.

Eimeria *Infection*

Eimeria acervulina, *Eimeria maxima*, and *Eimeria tenella* used in this study were originally isolated from broiler farms in northwest Arkansas using single oocyst isolation by the agarose gel encapsulation method (Shirley and Harvey, 1996). After isolation, the strains have been maintained in the laboratory by periodical propagation of oocysts in 12-day-old broiler chicks. The procedures of propagation and harvesting of oocysts and preparation of oocysts for infection had been described elsewhere (Shirley, 1995). All oocysts used in this study were less than 6 wk old.

Experimental Treatments and Design

The DEP used herein was a proprietary product containing IL-10–neutralizing antibody incorporated in a whole egg (Elanco Animal Health, Greenfield, IN); the method of manufacture is described by Sand et al. (2016). The second test article, MSM (Cat. No.: 41867; Sigma Aldrich, St. Louis, MO), was purchased in the form of a pure, white crystalline powder.

Table 1. Formulation of common basal diet used to prepare experimental diets.

Ingredient, g/kg	Value
Corn	522.0
Soybean meal	400.0
Soy oil	31.3
Salt	4.0
Limestone	13.1
Dicalcium phosphate	18.5
Vitamin premix[1]	2.0
Mineral premix[2]	1.5
L-Lysine HCl	1.2
DL-Methionine	3.5
L-Threonine	0.8
Choline chloride	2.1
Calculated nutrient content, as-is basis	
CP, g/kg	233.7
Calcium, g/kg	10.5
Phosphorus (total), g/kg	7.6
Phosphorus (nonphytate), g/kg	5.0
AME_n, kcal/kg	3,025
SID amino acids, g/kg[3]	
Arg	14.6
His	5.9
Ile	9.0
Leu	17.6
Lys	12.8
Met	6.8
Met + Cys	9.9
Phe	10.5
Phe + Tyr	16.6
Thr	8.4
Trp	2.6
Val	9.7

[1]Provided per kg of diet: retinyl acetate, 4,400 IU; cholecalciferol, 25 μg; DL-α-tocopheryl acetate, 11 IU; vitamin B12, 0.01 mg; riboflavin, 4.41 mg; D-Ca-pantothenate, 10 mg; niacin, 22 mg; menadione sodium bisulfite complex, 2.33 mg.

[2]Provided as milligrams per kg of diet: Mn, 75 from MnO; Fe, 75 from $FeSO_4 \cdot 7H_2O$; Zn, 75 from ZnO; Cu, 5 from $CuSO_4 \cdot 5H_2O$; I, 0.75 from ethylene diamine dihydroiodide; Se, 0.1 from Na_2SeO_3.

[3]Standardized ileal digestible AA composition calculated using data acquired from AMINODat 4.0 (Evonik Industries AG, Hanau-Wolfgang, Germany).

A total of 500 day-old chicks were weighed, selected, wing-banded, and randomly allotted to 1 of 5 treatment groups each with 10 replicates of 10 birds per cage at study initiation, such that the average initial group weights and weight distributions were similar across treatments. The 5 treatments used were sham-inoculated (uninfected) chickens fed standard basal control diet (**UCON**), *Eimeria*-infected chickens fed basal control diet (**ICON**), *Eimeria*-infected chickens fed control diet supplemented with DEP at 287 U/tonne (**I-DEP**), MSM at 4 kg/tonne (0.4%; **I-MSM**), or the combination of DEP and MSM (**I-DEP-MSM**). Birds were given ad libitum access to their respective diets and water throughout the study. All birds in infected groups received 1 mL of an oral inoculum containing *E. acervulina* (10,000 oocysts), *E. maxima* (5,000 oocysts), and *E. tenella* (5,000 oocysts) on study day 7 and 14. The oocyst dosage, suspended in tap water, was individually administered by oral gavage using a repeater pipette fitted with a blunt-ended tip. Noninfected birds were handled similarly and received an oral gavage of tap water at a volume equal to that of the infected birds and were maintained apart from infected birds in a separate room.

Data and Sample Collection

Group bird and feeder weights were recorded on study day 0, 7, 14, 21, and 28 to calculate the body ADG, ADFI, and feed conversion ratio (**FCR**) to estimate the growth performance. Mortality and culls were monitored daily and used to adjust feed efficiency. Oocyst shedding was determined by oocyst counts (i.e., oocysts per gram [**OPG**]) in excreta samples from all treatment groups using the McMaster method (Levine et al., 1960) on study day 7, 14, 21, and 28. In addition, at each of these time points, one randomly selected bird in each cage was humanely euthanized with CO_2 gas to collect blood. After collection, separated plasma (2,200 $\times$ g at 4°C for 15 min) was stored at −80°C for further analysis. In the laboratory, plasma was used to quantify MSM concentrations by gas chromatography as previously described (Abdul Rasheed et al., 2019) and oxidative stress markers including thiobarbituric acid reactive substances (**TBARS**) [quantified as malondialdehyde (**MDA**)] and total antioxidant capacity (**TAC**) following Rasheed et al. (2020).

Intestinal Histopathology

On study day 14 and 21 (i.e., 7 days after primary and secondary *Eimeria* inoculations), samples of the duodenum, middle intestine (5 cm proximal to Meckel's diverticulum), and ceca were collected in 10% neutral buffered formalin from 1 bird per cage and stored in room temperature pending analysis. Tissue sections were prepared as described by Oelschlager et al. (2019) and examined for histopathological lesions and physical presence of parasites. The following lesion scores were recorded for severity as 0, normal; 1, minimal severity; 2, mild severity; 3, moderate; 4, marked and 5, severe; coccidia, villus shortening, crypt hyperplasia (increased depth), lamina propria lymphocytes and plasma cells, bacteria on tips/sides of villi and dysbacteriosis, cystic crypts, intraepithelial leukocytes, and lamina propria heterophils. In addition, a coccidia index was calculated by summing the coccidia scores from each section of the intestine. A cumulative (total) intestinal lesion index was calculated by summing all lesion scores for all sections of the intestine, and the total enteritis index was calculated by subtracting the coccidia index from the total lesion index, representing inflammation and repair.

Inflammatory Cytokine mRNA Expression

On study day 12 and 19 (i.e., 5 days after primary and secondary *Eimeria* inoculations), cecal tissue was collected, snap-frozen in liquid nitrogen, and stored at −80°C pending analysis by quantitative real-time PCR to measure relative gene expression of the proinflammatory cytokines IFN-γ, interleukin-1β (IL-1β), and anti-inflammatory IL-10 as previously described (Oelschlager et al., 2019). Briefly, tissue aliquots (50–100 mg) were homogenized in the TRIzol reagent (Cat. No.: 15596026; Invitrogen, Carlsbad, CA) using a tissue

disruptor (TissueLyser II, Qiagen, Valencia, CA) for 2 min at 30 Hz, and RNA was extracted. The extracted RNA was transcribed to cDNA using a high-capacity cDNA Reverse Transcription kit (Cat. No.: 4368814; Thermo Fisher Scientific, Waltham, MA) in a thermocycler (Bio-Rad, Hercules, CA) using the following sequence: 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min and then cooled at 4°C. The quantitative real-time PCR procedure was performed using TaqMan Gene Expression Assays (Thermo Fisher Scientific, Waltham, MA) to quantify relative gene expression of proinflammatory cytokines IFN-γ (NM_205149.1), IL-1β (NM_204524.1), and IL-10 (NM_001004414.2) (Life Technologies, Carlsbad, CA). Amplification was achieved by PCR for both target (IFN-γ, IL-1β, IL-10) and reference (glyceraldehyde-3-phosphate dehydrogenase, NM_204305.1; Life Technologies, Carlsbad, CA; Hong et al., 2006) chicken genes. Sample cDNA was amplified using TaqMan (Cat. No.: 4304437; Thermo Fisher Scientific, Waltham, MA) oligonucleotide probes containing 5′ fluorescent reporter dye (6-FAM) and 3′ nonfluorescent quencher dye, and fluorescence was determined using a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene expression was normalized through parallel amplification of endogenous glyceraldehyde-3-phosphate dehydrogenase for each sample. The relative gene expression was calculated using the comparative threshold cycle method (Livak and Schmittgen, 2001), and results were expressed as fold-change relative to sham-inoculated broilers fed the control diet (UCON).

Statistical Analyses

The experiment was conducted as a randomized complete block design with individual cage as the experimental unit. A total of 4 experimental diets were fed to *Eimeria*-infected chickens, whereas uninfected chickens received only the control diet. Thus, the two-way ANOVA described below involves effects between diets only within *Eimeria*-infected groups (i.e., only dietary treatments, DEP and MSM, and not infection status was included), whereas a separate contrast was used to compare UCON and ICON groups, and UCON is used only as a reference group. In all cases, interaction means are presented in data tables, whereas significant main effect means are presented in data figures.

All data were subjected to a two-way ANOVA using the MIXED procedure of SAS (version 9.4; SAS Institute, Cary, NC). All plasma MSM concentrations and gene expression outcomes were log-normal–transformed to stabilize variance structures before conducting the ANOVA (i.e., heteroscedasticity was confirmed and eliminated to ensure accurate statistical effects). The sample means presented in result tables represent the least square means for the raw data, but model *P*-values and means separation procedures were all derived from the transformed data sets for these outcomes. For all data, treatment means were separated using a Tukey-Kramer adjustment to protect the familywise error

rate when making pairwise means comparisons. Outliers were identified (and subsequently removed) as having an absolute studentized residual value of 3 or greater, and significance was accepted with a *P*-value of less than 0.05. For outcomes where there were 1 or more missing values, the highest SEM for any treatment was reported as the pooled SEM in results tables.

RESULTS

Growth Performance

Growth performance results are shown in Table 2. Birds in the UCON group had a lower (*P* = 0.006) ADG than those in the ICON group during the preinfection period (i.e., day 0–7). In addition, the ADG was decreased (*P* = 0.01) in the I-MSM group compared with the I-DEP-MSM group during this period. With the current dose of *Eimeria* infection at day 7 and 14, no difference (*P* > 0.05) in growth performance parameters were observed in the ICON compared with the UCON group throughout the postinfection period. However, within the infected groups, the I-DEP-MSM group showed a higher (*P* = 0.004) ADG than all other groups at day 7 to 14 (i.e., 7 d after primary inoculation). The ADG was not affected by either main effects or interaction effects of MSM and DEP at all other time points. Similarly, no main effect of DEP or interaction effect of MSM and DEP was observed for the ADFI at any time points. However, a main effect of MSM on the ADFI was observed during day 0 to 28 (overall) period, where the ADFI was increased (*P* = 0.03) in the MSM-supplemented groups. Finally, the FCR was worse (*P* = 0.002) in the I-DEP and I-MSM groups than in the ICON group during day 7 to 14 (i.e., 7 d after primary inoculation). In addition, the overall (day 0 to 28) FCR was poorer (*P* = 0.004) in the I-MSM group than in other infected groups. No interaction effect of MSM and DEP on the FCR was observed at any other time points. However, a main effect of MSM was observed during the period day 14 to 21 (i.e., 7 d after secondary inoculation) and day 0 to 28 (i.e., overall study period), where birds receiving MSM showed a poorer FCR than birds without MSM supplementation. No main effects of DEP supplementation were observed on the FCR at all time points.

Inflammatory Cytokine Gene Expression

Inflammatory cytokine mRNA expression was measured in cecal tissue on study day 12 and 19 (i.e., 5 d post-primary and secondary inoculations, respectively) and is reported as a fold-change relative to the sham-inoculated birds fed the control diet (Table 3). Expression of IFN-γ, IL-1β, and IL-10 was higher (*P* < 0.01) in the ICON group than in the UCON group at day 12, suggesting that *Eimeria* infection elicited a pronounced inflammatory response because of *Eimeria* inoculation. However, IFN-γ and IL-10, but not IL-1β, were elevated (*P* < 0.04) at day 19 compared with

Table 2. Effects of feeding MSM and DEP on the growth performance in broiler chickens experiencing mild repeated infection with 3 species of *Eimeria*[1].

	Uninfected		*Eimeria* infected[2]				Main effects		Interaction
Item	UCON	ICON	I-DEP	I-MSM	I-DEP-MSM	SEM	DEP	MSM	MSM × DEP
ADG, g/bird									
Day 0–7	131[3]	138[a,b]	137[a,b]	133[a]	139[b]	2.1	0.173	0.276	0.016
Day 7–14	247	224[b,c]	206[a,b]	203[a]	237[c]	8.4	0.336	0.535	0.004
Day 14–21	403	428	426	428	405	11.0	0.268	0.329	0.352
Day 21–28	481	471	506	494	519	18.4	0.099	0.318	0.799
Day 0-28	1263	1,261	1,253	1,257	1,298	27.8	0.552	0.470	0.385
ADFI, g/bird									
Day 0–7	142	148	148	142	144	2.8	0.818	0.050	0.661
Day 7–14	297	285	286	294	311	8.7	0.289	0.050	0.357
Day 14–21	536	537	556	576	558	13.3	0.966	0.093	0.128
Day 21–28	693	688	724	749	737	21.0	0.558	0.076	0.240
Day 0–28	1450	1,432	1,467	1,516	1,509	29.1	0.604	0.024	0.429
FCR, g:g									
Day 0–7	1.08	1.06	1.06	1.07	1.05	0.009	0.192	0.714	0.375
Day 7–14	1.21	1.29[a]	1.40[b]	1.41[b]	1.32[a,b]	0.033	0.736	0.553	0.002
Day 14–21	1.33	1.26	1.31	1.35	1.39	0.036	0.213	0.021	0.854
Day 21–28	1.45	1.39	1.46	1.53	1.42	0.045	0.671	0.321	0.072
Day 0–28	1.15	1.11[a]	1.17[a]	1.21[b]	1.16[a]	0.019	0.681	0.032	0.008

[a-c]Means lacking a common superscript letter in a row differ ($P < 0.05$).

Abbreviations: DEP, dried egg product; UCON, uninfected control; ICON, infected control; I-DEP, infected birds receiving 287 U/tonne of DEP; MSM, methylsulfonylmethane; I-MSM, infected birds receiving 0.4% of MSM; I-DEP-MSM, infected birds receiving 287 U/tonne of DEP plus 0.4% of MSM.

[1]Values represent the least square means derived from 10 replicate cages, n = 10. All birds received allotted treatment diet starting study day 0.

[2]Birds in infected groups received 1 mL of oral inoculum containing a mixture of *E. acervulina* (10,000 oocysts), *E. maxima* (5,000 oocysts), and *E. tenella* (5,000 oocysts) on each of study day 7 and 14.

[3]Difference ($P < 0.05$) between uninfected and infected groups fed the control diet.

UCON. No main effects of either MSM or DEP and no interaction effect of MSM and DEP were observed at both day 12 and 19.

OPG

No interaction effect of MSM and DEP was observed on OPG at day 14 (i.e., 7 d after primary inoculation) (Table 4). However, a main effect of MSM was observed at this time point. Birds supplemented with MSM had a higher ($P = 0.005$) OPG than birds receiving no MSM. At day 21 (i.e., 7 d after secondary inoculation), a main effect of DEP, but not MSM, was observed, where birds supplemented with DEP exhibited a higher ($P = 0.005$) OPG than birds receiving no DEP. In addition, due to an interactive effect of MSM and DEP, I-DEP had a higher ($P = 0.003$) OPG than ICON, and the other groups were intermediate at day 21. No main

Table 3. Effects of feeding MSM and DEP on cytokine gene expression in the ceca of broiler chickens experiencing mild repeated infection with 3 species of *Eimeria*[1].

	Uninfected		Eimeria infected[2]				Main effects		Interaction
Item	Control	Control	I-DEP	I-MSM	I-DEP-MSM	SEM	DEP	MSM	MSM × DEP
Day 12									
IL-10	1.76[3]	29.90	15.37	26.72	21.44	4.96	0.059	0.563	0.190
IL-1 β	1.47[3]	4.51	4.83	8.88	4.83	1.91	0.188	0.838	0.276
IFN-γ	1.00[3]	43.79	41.60	54.73	38.91	11.75	0.486	0.609	0.659
Day 19									
IL-10	0.99[3]	6.68	5.56	6.49	5.23	1.74	0.306	0.846	0.395
IL-1 β	1.01	1.55	2.16	2.16	1.79	0.30	0.505	0.550	0.039[4]
IFN-γ	1.00[3]	36.37	29.11	30.14	16.77	7.47	0.130	0.121	0.317

Abbreviations: DEP, dried egg product; UCON, uninfected control; ICON, infected control; I-DEP, infected birds receiving 287 U/tonne of DEP; MSM, methylsulfonylmethane; I-MSM, infected birds receiving 0.4% of MSM; I-DEP-MSM, infected birds receiving 287 U/tonne of DEP plus 0.4% of MSM; IFN, interferon.

[1]Values are the least square means initially derived from 1 bird per cage with 6 replicates per treatment (n = 6). Values are reported as fold-change. All birds received allotted treatment diet starting study day 0.

[2]Birds in infected groups received 1 mL of oral inoculum containing a mixture of *E. acervulina* (10,000 oocysts), *E. maxima* (5,000 oocysts), and *E. tenella* (5,000 oocysts) on study days 7 and 14.

[3]Difference ($P < 0.05$) between the uninfected and infected groups fed the control diet.

[4]Because the interaction P-value is close to 0.05 (given the Tukey-Kramer adjustment), no means separation was apparent or possible for this outcome.

Table 4. Effects of feeding MSM and DEP on OPG of broiler chickens experiencing mild repeated infection with 3 species of *Eimeria*[1].

	Uninfected	Eimeria infected[2]				SEM	P-values		
							Main effects		Interaction
Item	UCON[3]	ICON	I-DEP	I-MSM	I-DEP-MSM		DEP	MSM	MSM × DEP
Day 14	-	1.24×10^6	1.12×10^6	1.96×10^6	2.20×10^6	0.2×10^6	0.809	0.005	0.439
Day 21	-	$2.90 \times 10^{5,a}$	$8.09 \times 10^{5,b}$	$5.95 \times 10^{5,b}$	$5.48 \times 10^{5,a,b}$	0.81×10^5	0.005	0.776	0.001
Day 28	-	4.08×10^4	7.38×10^4	6.31×10^4	7.86×10^4	1.2×10^4	0.047	0.253	0.457

[a,b]Means lacking a common superscript letter in a row differ ($P < 0.05$).

Abbreviations: DEP, dried egg product; OPG, oocysts per gram; ICON, infected control; I-DEP, infected birds receiving 287 U/tonne of DEP; I-MSM, infected birds receiving 0.4% of MSM; I-DEP-MSM, infected birds receiving 287 U/tonne of DEP plus 0.4% of MSM; MSM, methylsulfonylmethane; UCON, uninfected control.

[1]Values represent the least square means (after a two-way ANOVA) derived from 10 replicates, n = 10. All birds received allotted treatment diet starting study day 0.

[2]Birds in infected groups received 1 mL of oral inoculum containing a mixture of *E. acervulina* (10,000 oocysts), *E. maxima* (5,000 oocysts), and *E. tenella* (5,000 oocysts) on each of study day 7 and 14.

[3]Excreta samples for the UCON group tested negative for *Eimeria* oocysts at all time points, thereby indicating successful biosecurity protocols were maintained.

effect of MSM or interaction effect of MSM and DEP was observed at day 28. However, birds supplemented with DEP had higher OPG than birds receiving no DEP in their diet because of the main effect of DEP.

Oxidative Stress Markers

Plasma measurements of oxidative stress, including TBARS and TAC, can be found in Table 5. Compared with UCON, the plasma TBARS (i.e., MDA) concentration was lower ($P = 0.04$) in ICON at day 14 and remain unaffected at all other time points. Within the infected groups, main effects of MSM or DEP and interaction

of MSM and DEP were not apparent at day 7 and 14. However, main effects were observed for MSM (day 21 and 28) and DEP (day 28), and an interactive effect was observed on study day 21. Birds in the I-MSM group exhibited lower ($P = 0.04$) plasma TBARS than those in the ICON at day 21. Compared with groups not receiving MSM, birds in MSM-supplemented groups had lower plasma TBARS both at day 21 ($P = 0.01$) and 28 ($P < 0.001$). Similarly, DEP supplementation reduced ($P = 0.02$) plasma TBARS at day 28 compared with unsupplemented groups.

There were no differences in plasma TAC between the UCON and ICON groups throughout the study period.

Table 5. Effects of feeding MSM and DEP on oxidative stress outcomes and MSM concentrations in the plasma of broiler chickens experiencing mild repeated infection with 3 species of *Eimeria*[1].

	Uninfected		Eimeria infected[2]			SEM	P-values		
							Main effects		Interaction
Item	UCON	ICON	I-DEP	I-MSM	I-DEP-MSM		DEP	MSM	MSM × DEP
MDA, μmol									
Day 7	10.49	11.63	10.68	11.61	12.01	0.633	0.649	0.284	0.275
Day 14	19.28[3]	17.08	15.74	16.04	15.56	1.154	0.269	0.456	0.599
Day 21	13.14	13.91	12.27	10.57	11.83	0.729	0.782	0.010	0.042
Day 28	18.44	18.44	16.93	15.18	13.37	0.753	0.028	<0.001	0.834
TAC, mmol trolox equivalence									
Day 7	2.04	1.83	1.84	1.68	1.77	0.166	0.703	0.454	0.790
Day 14	0.81	0.87	0.50	0.47	0.09	0.069	<0.001	<0.001	0.995
Day 21	1.37	1.51[b]	1.95[a]	2.04[a]	1.98[a]	0.076	0.003	<0.001	0.001
Day 28	1.27	1.29	1.20	1.14	1.02	0.096	0.077	0.005	0.759
MSM, μg/mL									
Day 7	113.78	47.56[b]	159.29[a,b]	4,700.49[a]	4,204.53[a]	790.37	0.007	<0.001	0.001
Day 14	170.84	81.61	86.88	3,397.03	2,926.34	292.46	0.467	<0.001	0.783
Day 21	184.41[3]	86.53	140.19	3,063.11	3,170.35	147.16	0.236	<0.001	0.287
Day 28	145.92	91.56	106.51	3,524.79	3,061.82	144.18	0.630	<0.001	0.351

[a-c]Means lacking a common superscript letter in a row differ ($P < 0.05$).

Abbreviations: DEP, dried egg product; ICON, infected control; I-DEP, infected birds receiving 287 U/tonne of DEP; I-MSM, infected birds receiving 0.4% of MSM; I-DEP-MSM, infected birds receiving 287 U/tonne of DEP plus 0.4% of MSM; MDA, malondialdehyde; TAC, total antioxidant capacity; MSM, methylsulfonylmethane; UCON, uninfected control.

[1]Values represent the least square means derived from 10 replicate cages, n = 10. All birds received allotted treatment diets starting study day 0.

[2]Birds in infected groups received 1 mL of oral inoculum containing a mixture of *E. acervulina* (10,000 oocysts), *E. maxima* (5,000 oocysts), and *E. tenella* (5,000 oocysts) on each of study day 7 and 14.

[3]Difference ($P < 0.05$) between the uninfected and infected groups fed the control diet.

Within the infected groups, an interaction effect of MSM and DEP were observed only at day 21, where birds in I-DEP, I-MSM, and I-DEP-MSM groups exhibited a higher ($P = 0.002$) TAC than those in the ICON group. However, a main effect of MSM (day 14, 21, and 28) and DEP (day 14 and 21) was observed. Groups receiving either MSM or DEP in their diets exhibited a lower ($P < 0.001$) TAC than unsupplemented groups at day 14. In contrast, either MSM or DEP supplementation improved ($P < 0.003$) TAC at day 21. However, at day 28, MSM supplementation reduced ($P = 0.005$) TAC compared with unsupplemented groups.

Histopathological Lesion Scoring

With the current dosage of mixed *Eimeria* infection, no relation between various lesion scores and dietary treatments was observed on multinomial logistic regression analysis (data not shown), suggesting that the dietary treatments did not influence histopathological scores during this mild *Eimeria* infection.

MSM Quantitation

Methylsulfonylmethane quantitation data are shown in Table 5. Except at day 21, no difference in plasma MSM concentrations were observed in the ICON group compared with the UCON group; ICON birds had lower ($P = 0.003$) plasma MSM than those in the UCON groups at day 21. Within the infected group, both main and interactive effects of MSM and DEP supplementation were observed at study day 7. Plasma MSM was higher ($P < 0.001$) in the I-MSM and I-DEP-MSM groups than in the ICON group, and I-DEP was intermediate. Groups supplemented with MSM had a higher ($P < 0.001$) plasma MSM concentration, whereas groups receiving DEP showed a lower ($P = 0.007$) plasma MSM than their respective unsupplemented groups day 7. No interaction effects were observed for plasma MSM concentration at day 14, 21, and 28. However, main effects of MSM supplementation but not DEP were observed at these time points, where birds receiving MSM in their diet showed a higher ($P < 0.001$) plasma MSM concentration than unsupplemented groups.

DISCUSSION

Striving for an appropriate immune response and proper nutrient support during a subclinical *Eimeria* infection may be helpful in achieving full production potential of a flock (Shanmugasundaram et al., 2013; Morris et al., 2015; Rochell et al., 2017). In the present study, we tested the effects of 2 immunomodulatory substances, MSM and egg-derived anti–IL-10 antibody in the form of DEP, during a mild repeated infection with 3 species of *Eimeria* commonly encountered in the poultry industry (Chapman et al., 2016). Although anti–IL-10 antibody is designed to reduce the activity of anti-inflammatory IL-10 to inhibit parasitic development by promoting tissue inflammation, MSM being an anti-inflammatory agent, may reduce the damage caused by excessive inflammation. Moreover, the free radical scavenging property of MSM (reviewed by Butawan et al., 2017) may further improve gut health as it reduces the free radical–induced damage to the gut epithelium. We observed that supplementation of MSM and DEP has beneficial effects on growth performance parameters and markers of oxidative stress but not gene expression pattern, histopathological lesions, or OPG during a mild repeated infection of mixed *Eimeria* species.

The experimental diets used in the study were analyzed for both MSM concentration (using gas chromatography; Abdul Rasheed et al., 2019) and anti–IL-10 antibody activity (by ELISA; Sand et al., 2016). The analysis confirmed no MSM or anti–IL-10 activity in the UCON and ICON groups. However, a higher than proposed inclusion level (287 U/tonne) of anti–IL-10 activity was detected by ELISA in the experimental diets (i.e., 478 in I-DEP and 553 in I-DEP-MSM). A main effect of DEP was observed, where DEP supplementation reduced plasma MSM concentration at day 7. This could be due to a lower MSM intake in DEP-supplemented group as the space for MSM in the feed is partially filled by DEP. In addition, an interaction effect of MSM and DEP (i.e., both ICON and I-DEP groups had lower plasma MSM than I-MSM and I-DEP-MSM groups) suggests that only MSM supplementation influences the plasma MSM concentration. Collectively, these data are used to validate the effects of MSM in groups supplemented with exogenous MSM via feed.

Accurate estimation of the impact of subclinical coccidiosis infection in a flock is challenging as it needs continuous evaluation of production performance and estimation of oocysts load in the poultry house (Haug at al., 2008). In a study to evaluate the impact of subclinical infection, Haug at al. (2008) observed a higher growth depression in birds when medium-to-large sized oocysts resembling *E. tenella* and *E. maxima* are predominant in poultry litter. In addition, birds were reinfected by the oocysts from litter over the entire production cycle. Infection with *E. acervulina* can also impact nutrient absorption (Augustine and Danforth, 1999) as it affects the upper portion of the small intestine where most of the nutrient absorption occurs. Moreover, these 3 species are commonly encountered in broiler production (Chapman et al., 2016). Therefore, in the present study we used all the 3 species of *Eimeria* (i.e., *E. acervulina*, *E. maxima*, and *E. tenella*) and used a repeated inoculation protocol to simulate the field condition where birds get multiple reinfections from litter. Similarly, the oocyst doses used with the current strains of *Eimeria* were aimed to impart up to 5% growth depression, as this may represent a true subclinical infection in the field. As such, we did not observe any growth reduction in the ICON group compared with the UCON group at any time points. With the UCON group being physically separated from the *Eimeria*-infected groups, we place emphasis on statistical comparisons between infected groups receiving test articles (i.e., MSM and

DEP), and as such, the UCON group was primarily used as a reference group.

Within the infected groups, birds fed MSM exhibited a lower ADG without affecting the FCR at day 0 to 7 (i.e., before infection) than other groups. Previous studies showed that MSM has no negative effects on the growth performance in broilers (Abdul Rasheed et al., 2019; Rasheed et al., 2020). Therefore, the depression in the ADG in the present study before infection may have been due to a nonsignificant depression in the ADFI in the I-MSM group. However, birds in the I-DEP-MSM group had the numerically highest ADG and an improved FCR, where both measures were statistically different from the I-MSM and I-DEP groups, but not the ICON group during the period immediately after primary *Eimeria* infection (i.e., day 7–14). Generally, growth reduction will be high during the first 7 d of *Eimeria* infection (Wallach et al., 1990; Allen and Fetterer, 2000), and the birds start to recover afterward depending on the severity of infection (Brake et al., 1997). In agreement to this observation, a secondary infection at day 14 did not affect any of the growth performance parameters in the present study as the severity of infection was designed to be low. Moreover, the overall (day 0–28) FCR was improved in I-DEP-MSM group, which was close to that of the UCON group. In the current infection model, the primary infection may have elicited an immune response (Brake et al., 1997) that may have protected the birds from a secondary infection. Overall, the current results suggest that birds supplemented with a combination of MSM and DEP in the diet had beneficial effects on the growth performance immediately after a challenge infection that was not observed with supplementation of either DEP or MSM alone.

Apart from growth performance, the effects of MSM and DEP on immune activation, inflammatory cytokine gene expression was quantified at the intestinal tissue level as development of immunity is key to improve growth performance during *Eimeria* infection. Expression of all 3 cytokines (IL-1β, IFN-γ, and IL-10) was elevated in the ICON group compared with the UCON group at both day 12 and 19 (i.e., 5 d after primary and secondary inoculations, respectively), suggesting a successful *Eimeria* infection that elicited a host immune response. However, neither MSM nor DEP, alone or in combination, influenced the expression of these cytokines. Interleukin-1β is mostly responsible for the sickness behavior during immune stimulation (Dantzer et al., 1993), which reduces feed intake during *Eimeria* infection to negatively affect the BW gain and feed efficiency (Laurent et al., 2001). Interferon-γ is primarily involved in mediating resistance against *Eimeria* infection and is secreted predominantly by Th1-type cells as part of adaptive immunity (i.e., CD4+ cells; Yun et al., 2000). Interferon-γ has been shown to inhibit *E. tenella* development and reduce OPG in feces (Lowenthal et al., 1997; Lillehoj and Choi, 1998). On the other hand, IL-10, produced mainly by activated macrophages or CD4+ and CD8+ T cells (Groux and

Powrie, 1999), has an anti-inflammatory role during *Eimeria* infection (Hong et al., 2006). By design, DEP ingestion should partially neutralize host IL-10 production to subvert the evasive mechanism that *Eimeria* species use to avoid detection and elimination by the host. Because the effect of DEP during *Eimeria* infection is expected to be mediated by IL-10 neutralization, measurement of the host IL-10 activity in the gut may have provided further insights on the efficacy of DEP. Several studies have explored the mechanism involved in the anti-inflammatory activity of MSM (Kim et al., 2009; Joung et al., 2016). As such MSM was expected to reduce the expression of proinflammatory cytokines. However, the current results do not support this observation as neither IL-1β nor IFN-γ expression was influenced by MSM. Overall, the current results on gene expression pattern of proinflammatory (IL-1β and IFN-γ) and anti-inflammatory (IL-10) cytokines show no evidence of beneficial effects of supplementation of either MSM or DEP alone or in combination when fed to birds experiencing a mild, mixed *Eimeria* infection.

As hypothesized, the beneficial effects on the growth performance could be due to a balance in inflammatory reaction by DEP and MSM at the gut epithelium. However, no change in histopathological lesion scores was observed between the treatment groups as most of the lesion scores were recorded as 0 or 1 with limited variation. In floor pens, infection with low doses of *E. acervulina* (3,000 oocysts per bird) elicited shortening of the duodenal villi and a lowered villi surface area after 21 d (Assis et al., 2010). However, multiple natural reinfections might have occurred from litter during this period, which may have delivered an adequate number of oocysts to impart histomorphometric changes in the gut. In the present study, birds were raised in battery cages, where birds did not have a direct contact with excreta, so opportunities for natural reinfection by oocysts were effectively nonexistent. In agreement to this, OPG counts were reduced in the later phases of the study because of the absence of reinfections from excreta. Moreover, a lower OPG is considered as a good indicator of the ability of birds to counteract *Eimeria* infection (Chapman, 1999; Parmentier et al., 2001; Lee et al., 2013). However, in the present study, neither MSM nor DEP, alone or in combination, lowered OPG compared with the ICON group. A similar result was observed previously, where DEP did not reduce OPG in broiler chicks infected with a 10× dose of live *Eimeria* vaccine (Sand et al., 2016). However, no data on the effect of MSM during *Eimeria* challenge are available for a comparison. These results suggest that the 2 inoculations (i.e., study day 7 and 14) used in the present study may not have been sufficient for birds to exhibit adequate lesions in battery cages, and MSM and DEP supplementation, alone or in combination, did not improve OPG.

The antioxidant properties of MSM have been well studied, especially during free radical–induced oxidative stress conditions such as inflammatory diseases (c.f., Butawan et al., 2017). The estimation of TBARS is

one of the most widely used indicators of oxidative stress in biological systems, and the TBARS assay measures the amount of a carbonyl compound (MDA) derived from processes leading to lipid peroxidation. In addition, MDA is formed during oxidative injury of DNA, proteins, or carbohydrates (Janero, 1990). In addition, increased TBARS production has been reported in several inflammatory conditions including obstructive pulmonary lung disorders and respiratory distress syndrome (Baldwin et al., 1986; Nowak et al., 1999). In a previous study conducted in our laboratory, 0.05% dietary MSM was found to be beneficial in reducing TBARS in the plasma and liver after diet-induced oxidative stress in broilers (Rasheed et al., 2020). In the present study, TBARS concentration in the plasma was reduced in MSM-supplemented groups at both day 21 and 28. Moreover, the I-MSM group had lower plasma TBARS than the ICON group at day 21. Collectively, these results suggest that MSM was beneficial in lowering oxidative stress during the mixed *Eimeria* infection. Except at day 14, where TBARS was higher in the UCON group, no difference in plasma TBARS level was observed in the ICON group compared with the UCON group. Like TBARS, TAC also provides a good indication of redox status. In the present study, both MSM and DEP supplementation improved plasma TAC at day 21, suggesting a beneficial effect. However, TAC was also lowered in the MSM and DEP groups at day 14, similar to the inconsistent effect of MSM observed in our previous study involving broilers during oxidized oil–induced oxidative stress (Rasheed et al., 2020). Hence, plasma TAC estimation may arguably not be as precise an estimator of redox potential in broilers as TBARS.

In conclusion, supplementation of a combination of MSM and anti–IL-10 antibody from DEP may be beneficial during a mild repeated infection with *E. acervulina*, *E. maxima*, and *E. tenella* in broilers evidenced by an improvement in growth performance parameters and oxidative stress markers in the plasma. However, validation of the current hypothesis (i.e., attenuation of host IL-10 activity by egg-derived anti–IL-10 antibody may favor a proinflammatory reaction to aid rapid parasitic elimination, and meanwhile, anti-inflammatory and antioxidant capacity of MSM may bring beneficial balance between the immune response and inflammatory tissue damage) needs further evaluation on the pattern of IL-10 and other proinflammatory activities in the gut using techniques such as ELISA along with characterization of immune cell infiltration and epithelial damage at the gut wall using methodologies such as immunohistochemistry and histomorphometry.

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DISCLOSURES

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