Calcium Montmorillonite-Based Dietary Supplement Attenuates Necrotic Enteritis Induced by *Eimeria maxima* and *Clostridium perfringens* in Broilers [‡]

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Necrotic enteritis (NE) is a poultry disease caused by *Clostridium perfringens* and characterized by severe intestinal necrosis. The incidence of avian NE has been progressively increasing following the removal of antibiotics from poultry feed. We evaluated the effect of diets supplemented with the thermally-processed clays, calcium montmorillonite (CaMM) on clinical signs, immunopathology, and cytokine responses in broiler chickens using an experimental model of NE consisting of co-infection with *Eimeria maxima* and *C. perfringens*. In Trial 1, Ross/Ross chickens were fed from hatch with a normal basal diet or a CaMM-supplemented diet with or without a fermentable fiber, an organic acid, and/or a plant extract, and co-infected with *E. maxima* and *C. perfringens* under conditions simulating clinical infection in the field. Chickens fed a diet supplemented with CaMM plus a fermentable fiber and an organic acid had increased body weight gain, reduced gut lesions, and increased serum antibody levels to *C. perfringens* α-toxin and NetB toxin compared with chickens fed the basal diet alone. Levels of transcripts for interleukin-1β (IL-1β), IL-6, inducible nitric oxide synthase, and tumor necrosis factor-α superfamily-15 were significantly altered in the intestine and spleen of CaMM-supplemented chickens compared with unsupplemented controls ($p < 0.05$). In Trial 2, Cobb/Cobb chickens were fed an unsupplemented diet or a diet supplemented with CaMM or Varium®, each with a fermentable fiber and an organic acid, and co-infected with *E. maxima* and *C. perfringens* under subclinical infection conditions. Compared with unsupplemented controls, broilers fed with CaMM plus a fermentable fiber and an organic acid had increased body weight gain, and reduced feed conversion ratio, mortality, and intestinal lesions, compared with chickens fed an unsupplemented diet ($p < 0.05$). Dietary supplementation of broiler chickens with CaMM plus a fermentable fiber and an organic acid might be useful to control avian NE in the field.

Key words: calcium montmorillonite, *Clostridium perfringens,* coccidiosis, *Eimeria maxima,* necrotic enteritis, organic acid

Introduction

Necrotic enteritis (NE) is an economically important intestinal infectious disease caused by the common gut bacterium, *Clostridium perfringens*, under conditions favoring *in vivo* bacterial proliferation (Drew *et al*., 2004; Jia *et al*., 2009). NE is estimated to cost U.S. commercial poultry producers up to $3 billion annually. Even mild subclinical outbreaks in a flock can cost more than $0.07 per bird (Skinner *et al*., 2010). The incidence of NE has been increasing globally following the reduction in use of in-feed antibiotics, such as virginiamycin, that are used to control enteric diseases in poultry (Williams, 2005). Thus, there is a

timely need to develop novel methods to reduce the negative effects of NE in broilers.

C. perfringens produces several exotoxins, including α-toxin and NE toxin B (NetB), that disrupt the intestinal epithelium causing necrotizing lesions that constitute the characteristic sign of NE (Songer, 1996; Williams, 2005; Keyburn *et al*., 2006). A thermally-processed, highly-refined clay, calcium montmorillonite (CaMM; Calibrin-Z®, Processed by Amlan International, Chicago, IL 60611), ameliorates the negative biological effects of several mycotoxins, including aflatoxin, fumonisin, and zearalenone, when fed to broilers and other livestock, presumably through its adsorptive and dipolar properties that sequester the toxins in the gastrointestinal tract, thereby decreasing their systemic bioavailability (Ledoux *et al*., 2009; Jiang *et al*., 2012; Wang *et al*., 2012). CaMM is comprised of a soft phyllosilicate group of minerals that typically form in microscopic crystals. Our recent unpublished studies demonstrated that CaMM also binds to *C. perfringens* α-toxin and NetB toxin *in vitro*. Therefore, we hypothesized that CaMM, or its blends with other materials known to improve gut health (e.g. fermentable fibers, organic acids, and/or phytonutrients) might decrease the negative effects of avian NE. To test this hypothesis, the current study evaluated two different trials utilizing CaMM- or Varium® (manufactured and formulated by Amlan International, Chicago, IL 60611)-based dietary products on the growth performance, clinical signs, immunopathology, and cytokine responses of young broilers using an experimental model of avian NE.

Materials and Methods

Feed and Treatments

All basal diets were corn/soybean-based and formulated to the experimental design (Trial 1) or meet or exceed National Research Council (1994) recommendations (Trial 2). Trial 1 was carried out at the USDA Beltsville Agricultural Research Center (BARC, Beltsville, MD) by HSL and approved by the BARC Institutional Animal Care and Use Committee. Two-hundred-twenty one-day-old Ross/Ross broilers (Longenecker's Hatchery, Elizabethtown, PA) were randomly divided into 11 groups (20 birds/group). The chickens were fed *ad libitum* from hatch to day 18 post-hatch with either a non-medicated commercial basal ration containing 18% (wt/wt) crude protein (Table 1) or the same basal ration supplemented with 0.25% or 0.50% (wt/wt) CaMM (group B), 0.25% or 0.50% CaMM plus citric acid and an extract of Yucca plant (group Y), 0.25% or 0.50% CaMM plus a fermentable fiber (group C), 0.25% or 0.50% CaMM plus organic acid and a fermentable fiber (group D), or 22 mg/kg virginiamycin (VM) (Table 3). Yucca extract containing 10% saponin was added into the feed formula as much as possible.

At day 18 post-hatch, the protein content of all diets was increased to 24% (wt/wt) (Table 1) until the end of the experiment.

Trial 2 was carried out at the Southern Poultry Research Facility by GFM and was approved by the Southern Poultry

Table 1. **Ingredient composition of basal diet (Trial 1)**

Ingredients (%)	Low Protein Diet	High Protein Diet
Corn	69.01	55.78
Soybean meal	23.99	37.03
Soybean oil	2.75	2.97
Dicalcium phosphate	2.00	1.80
Calcium carbonate	1.40	1.51
Salt	0.35	0.38
Poultry Vit Mix	0.20	0.22
Poultry Mineral Mix	0.15	0.15
DL-Methionine	0.10	0.10
Choline-chloride, 60%	0.05	0.06
Total	100	100
Calculated values (DM basis, %)		
Crude Protein, %	18.00	24.00
Ca, %	1.19	1.20
Available P, %	0.54	0.51
Lys, %	1.00	1.40
Met, %	0.42	0.49
Cys + Met, %	0.65	0.80
TMEn, kcal/kg	3585	3450

[1] Vitamin mixture provided the following nutrients per kg of diet: vitamin A, 2,000 IU; vitamin D_3, 22 IU; vitamin E, 16 mg; vitamin K, 0.1 mg; vitamin B_1, 3.4 mg; vitamin B_2, 1.8 mg; vitamin B_6, 6.4 mg; vitamin B_{12}, biotin, 0.17 mg; pantothenic acid, 8.7 mg; folic acid, 0.8 mg; niacin, 23.8 mg.
[2] Mineral mixture provided the following nutrients per kg of diet: Fe, 400 mg; Zn, 220 mg; Mn, 180 mg; Co, 1.3 mg; Cu, 21 mg; Se, 0.2 mg.

Table 2. Ingredient composition of basal diet (Trial 2)

Ingredient	%, as is basis
Corn	57.547
Soybean meal (dehulled)	35.283
Animal by-product (55% protein)	3.000
Fat, vegetable	1.828
Calcium carbonate	0.847
Dicalcium phosphate	0.565
Salt	0.430
Methionine hydroxy analog	0.286
Vitamin premix[1]	0.065
Trace mineral premix[2]	0.075
L-Lysine	0.056
RonozymeP	0.018
Total	100.000
Calculated nutrient composition	
ME poultry, kcal/kg	3,067
Crude protein, %	23.35
Lysine, %	1.35
Methionine, %	0.62
Digestible lysine, %	1.2
Digestible TSAA, %	0.9
Calcium, %	0.9
Phosphorus, %	0.61
Sodium, %	0.21

[1] Vitamin mix provided the following (per kg of diet): thiamin·mononitrate, 2.4 mg; nicotinic acid, 44 mg; riboflavin, 4.4 mg; D-Ca pantothenate, 12 mg; vitamin B_{12} (cobalamin), 12.0 μg; pyridoxine·HCL, 4.7 mg; D-biotin, 0.11 mg; folic acid, 5.5 mg; menadione sodium bisulfite complex, 3.34 mg; choline chloride, 220 mg; cholecalciferol, 27.5 ug; trans-retinyl acetate, 1,892 ug; all-rac α tocopheryl acetate, 11 mg; ethoxyquin, 125 mg.

[2] Trace mineral mix provided the following (per kg of diet): manganese ($MnSO_4 \cdot H_2O$), 60 mg; iron ($FeSO_4 \cdot 7H_2O$), 30 mg; zinc (ZnO), 50 mg; copper ($CuSO_4 \cdot 5H_2O$), 5 mg; iodine (ethylene diamine dihydroiodide), 0.15 mg; selenium (NaSe03), 0.3 mg.

Research Institutional Animal Care and Use Committee in accordance with the principles and specific guidelines of the Federation of Animal Science Societies (FASS, 2010). Three-hundred-twenty one-day-old Cobb/Cobb male broilers (Cobb-Vantress, Cleveland, GA) were randomly divided into 5 groups (64 birds/group). The chickens were fed *ad libitum* from hatch to day 18 post-hatch with either a non-medicated commercial basal ration containing 17% (wt/wt) crude protein or the same basal ration (Table 2) supplemented with 0.25% CaMM plus organic acid and a fermentable fiber (group D), 0.25% Varium®, or 22 mg/kg VM (Table 4). The scheme of experimental protocol of Trial 1 and 2 are illustrated in Fig. 1.

Chickens and Experimental Models of Avian NE

In Trial 1, the chickens were initially housed in Petersime starter brooder pens (Petersime Incubator Co, Gettysburg, OH) in a disease-free facility. At 14 day post-hatch, the broilers were transferred to large hanging cages in a separate location where one group was uninfected while the remaining groups were co-infected with *E. maxima* and *C.*

perfringens under conditions simulating clinical infection in the field as described (Park *et al*., 2008; Jang *et al*., 2012). Briefly, the birds were given an oral infection of 1.0×10^4 sporulated oocysts of *E. maxima* (strain 41A) at day 14 post-hatch, followed 4 days later by oral infection with 1.0×10^9 colony forming units (cfu) of a field strain of *C. perfringens* (strain Del-1). *E. maxima* and *C. perfringens* were maintained and propagated as previously described (Lee *et al*., 2011). In Trial 2, chickens were housed in Petersime battery cages in 8 pens with 8 birds per pen. At day 14 post-hatch, the chickens were uninfected or orally inoculated under conditions simulating subclinical infection with 5.0×10^3 oocysts of *E. maxima*, followed 5 days later by 1.0×10^8 cfu of *C. perfringens* in 1.0 m*l* of broth culture for 3 consecutive days (days 19, 20 and 21 post-hatch) as described (Zhang *et al*., 2010).

Intestinal Lesion Scoring

In Trial 1, chickens (5 birds/group) were randomly selected on day 2 following *C. perfringens* infection (day 20 post-hatch), weighed, and euthanized by cervical dislocation. Approximately 20 cm of the intestine extending 10 cm anterior and posterior from the Meckel's diverticulum was removed and cut longitudinally. Lesion scoring was performed by 3 independent observers in a blinded fashion as described (Prescott, 1979; Park *et al*., 2008). Lesions were scored as 0 (normal, no lesion), 1 (thin-walled minor lesions), 2 (moderate focal necrotic lesions), 3 (severe necrotic lesion patches), or 4 (dead or moribund). In Trial 2, three birds from each cage were randomly selected on day 1 following the final *C. perfringens* infection (day 22 post-hatch) and sacrificed, and intestines examined for lesion score as described (Zhang *et al*., 2010). Lesions were scored as 0 (normal, no lesion), 1 (thin-walled or friable), 2 (focal necrosis or ulceration), or 3 (severe lesions).

Serum Collection

In Trial 1, blood was collected from chickens (3 birds/group) by cardiac puncture immediately following euthanasia on day 2 following *C. perfringens* infection (day 20 post-hatch) for measuring circulating α-toxin and NetB toxin levels, and on days 7 and 14 following *C. perfringens* infection (days 25 and 32 post-hatch) for measuring serum antibodies to α-toxin and NetB toxin. Sera were prepared by centrifugation at 3,000 rpm for 10 min at 4℃ and stored at −20℃ until analysis.

Serum Antibodies to α-Toxin and NetB Toxin

Recombinant *C. perfringens* α-toxin and NetB toxin were expressed in *E. coli* as described (Lee *et al*., 2012). Ninety-six well microtiter plates were coated overnight with 1.0 μg/well of each of the recombinant toxins. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with PBS containing 1.0% bovine serum albumin (PBS-B). Sera were diluted 1:20 (vol:vol), 100 μl were added to each well, and incubated with continuous gentle shaking for 2 h at room temperature. The wells were washed with PBS-T and bound antibodies were detected with peroxidase-conjugated rabbit anti-chicken IgG antibody (Sigma, St. Louis, MO) and 3,3′,5,5′-tetramethylbenzidine substrate.

Table 3. **Experimental scheme of Trial 1 (Clinical Infection)**

Treatment Group	Product Inclusion	Diet Supplementation	Bacterial Challenge	
			E. maxima	*C. perfringens*
Control	—	None	—	—
NE	—	None	+	+
B	0.25%	CaMM	+	+
Y	0.25%	CaMM + OA + Yucca extract	+	+
C	0.25%	CaMM + Fermentable fiber	+	+
D	0.25%	CaMM + Fermentable fiber + OA	+	+
B	0.50%	CaMM	+	+
Y	0.50%	CaMM + OA + Yucca extract	+	+
C	0.50%	CaMM + Fermentable fiber	+	+
D	0.50%	CaMM + Fermentable fiber + OA	+	+
VM	22 mg/kg	Virginiamycin	+	+

Ross/Ross broiler chickens, except the control group, were infected with 1.0×10^4 oocysts of *E. maxima* on day 14 post-hatch followed by 1×10^9 cfu of *C. perfringens* on day 18 post-hatch. Crude protein content of the basal diet was 18% between days 0 and 18 post-hatch and 24% from days 18 to 25 (Refer to Table 1). Birds (20 birds/group) were randomly divided into 11 groups and fed from day 0 with an unsupplemented diet or diets supplemented with 0.25% or 0.50% CaMM (Calcium Montmorillonite, Calibrin-Z®, processed by Amlan International, Chicago, IL 60611) with or without a fermentable fiber, an organic acid (OA), or Yucca plant extract containing 10% saponin into the feed formula, or with 22 mg/kg virginiamycin.

Table 4. **Experimental scheme of Trial 2 (Subclinical Infection)**

Treatment Group	Product Inclusion	Diet Supplementation	Bacterial Challenge	
			E. maxima	*C. perfringens*
Control	—	None	—	—
NE	—	None	+	+
D*	0.25%	CaMM + Fermentable fiber + OA	+	+
Varium®	0.25%	CaMM + Fermentable fiber + OA	+	+
VM	22 mg/kg	Virginiamycin	+	+

* D and Varium® were formulated differently by Amlan International (Chicago, IL 60611). Cobb/Cobb male broiler chickens, except the control group, were infected with 5.0×10^3 oocysts of *E. maxima* on day 14 post-hatch followed by 1×10^8 cfu of *C. perfringens* on days 19, 20, and 21 post-hatch. Birds (64 birds/group) were randomly divided into 5 groups and fed from day 0 with an unsupplemented diet or diets supplemented with 0.25% CaMM or Varium® with a fermentable fiber and an organic acid (OA), or with 22 mg/kg virginiamycin (Refer to Table 2).

Optical density values at 450 nm (OD_{450}) were measured using a microplate reader (Bio-Rad, Richmond, CA) and corrected for background reactivity in the absence of recombinant toxins.

Serum α-Toxin and NetB Toxin Levels

Ninety-six well microtiter plates were coated overnight with 0.5 μg/well of monoclonal antibody to α-toxin or NetB toxin, washed with PBS-T, and blocked with PBS-B. Sera were diluted 1:2 (vol:vol) in PBS-T and 100 μl were added to the wells. The wells were incubated for 2 h at room temperature with continuous gentle shaking, washed with PBS-T, and the bound α-toxin or NetB toxin detected with peroxidase-conjugated rabbit anti-α-toxin or anti-NetB toxin antibodies, respectively, and 3,3′,5,5′-tetramethylbenzidine substrate. OD_{450} values were measured and serum toxin levels were determined by comparison with a standard curve generated with known concentrations of each purified recombinant toxin.

Quantitative RT-PCR

The levels of transcripts for proinflammatory cytokines and inducible nitric oxide synthase (iNOS) were measured in spleen and intestine as described (Park *et al*., 2007, 2008; Xu *et al*., 2015). At day 2 following *C. perfringens* infection, spleens and intestinal jejunum located proximal to the Meckel's diverticulum were collected (5 birds/group). Single cell suspensions of spleen were prepared by gently flushing with a cell strainer to remove clumps. Intestinal jejunum tissues were cut open longitudinally, gently washed 3 times with ice-cold Hank's Balanced Salt Solution (Sigma) containing 100 U/ml of penicillin and 100 μg/ml of streptomycin (Sigma). The mucosal layer was carefully scraped off using a surgical scalpel and intraepithelial lymphocytes
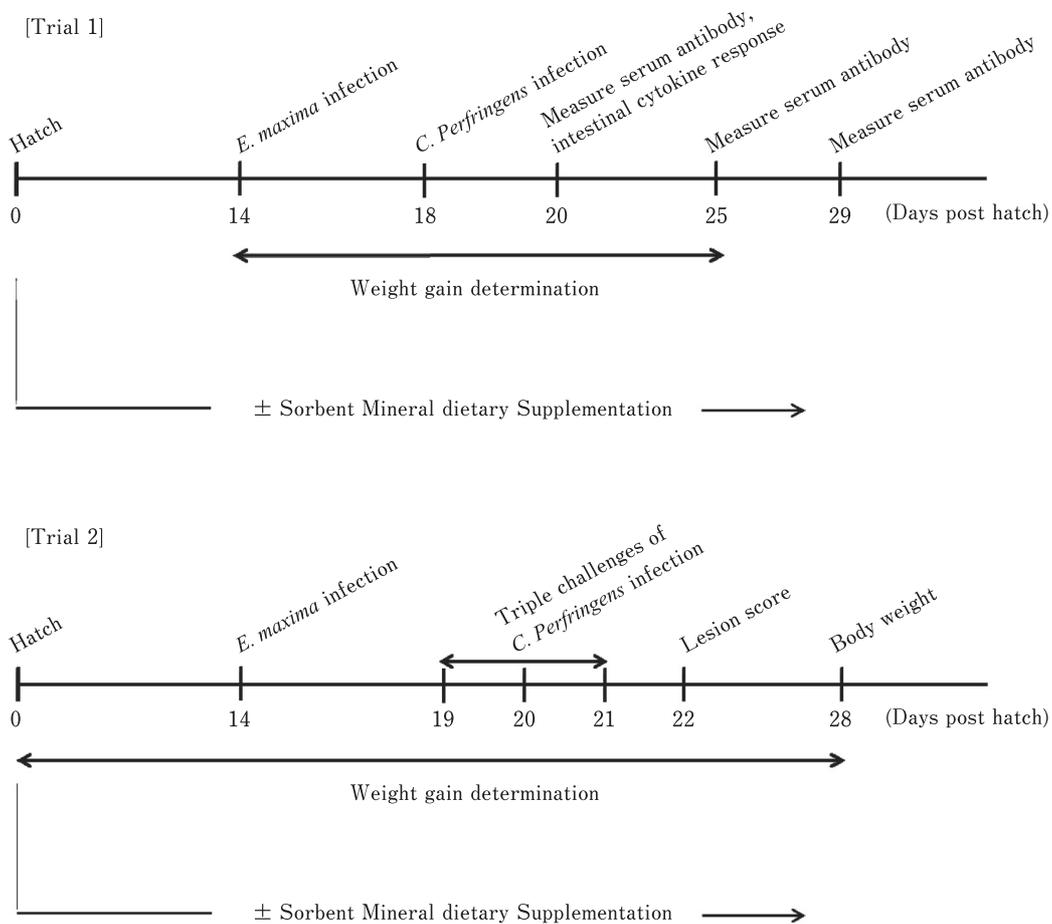
Fig. 1. **Schematic illustration of the experimental protocol.** Ab, antibody; CP, *C. perfringens*; DCPI, days following *C. perfringens* infection; EM, *E. maxima*.

(IELs) were isolated by density gradient centrifugation. Total RNA from spleen and intestinal IELs was extracted using TRIzol (Invitrogen, Carlsbad, CA). Five micrograms of total RNA were treated with 1.0 U of DNase I and $1.0\,\mu l$ of 10X reaction buffer (Sigma) and incubated for 15 min at room temperature. One μl of stop solution was added to inactivate DNase I and the mixture was heated for 10 min at 70℃. RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer's recommendations. Quantitative RT-PCR oligonucleotide primers for chicken interleukin-1β (IL-1β), IL-6, iNOS, tumor necrosis factor superfamily 15 (TNFSF15), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control are listed in Table 5. Amplification and detection were carried out with the Mx3000P system and Brilliant SYBR Green qPCR master mix (Stratagene). The reverse transcription product was diluted 1:10 (vol:vol), and $5\,\mu l$ was used for PCR amplification. PCR conditions were as follows: denaturation at 95℃ for 10 min followed by amplification at 72℃ for 1 min for 40 cycles.

Standard curves were generated using $\log_{10}$ diluted standard RNA to calculate the amplification efficiency and the levels of individual transcripts were normalized to those of GAPDH by the Q-gene program as described (Muller *et al.*, 2002; Lee *et al.*, 2012, 2013). Each sample was analyzed in triplicate. To normalize individual replicates, the logarithmic-scaled threshold cycle (C_t) values were transformed to linear units of normalized expression prior to calculating means and SEM for the references and individual targets, followed by the determination of mean normalized expression using the Q-gene program.

Statistical Analysis

For Trial 1, mean±SEM values were compared between different treatment groups by the Duncan's multiple range test following ANOVA using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). For Trial 2, mean±SEM values were compared between different treatment groups using Duncan's multiple range test using JMP 11.0.0 for Windows (SAS Institute Inc., Cary, NC). In both trials, differences between means were considered significant at $p < 0.05$. Brooder pens

Table 5.   **Oligonucleotide primer sequences used for quantitative RT-PCR**

Gene		Primer Sequence	Size (bp)	Genebank Accession no.
IL-1β	F	5′-TGGGCATCAAGGGCTACA-3′	244	Y15006
	R	5′-TCGGGTTGGTTGGTGATG-3′		
IL-6	F	5′ CAAGGTGACGGAGGAGGAC-3′	254	AJ309540
	R	5′ TGGCGAGGAGGGATTTCT-3′		
iNOS	F	5′ TGGGTGGAAGCCGAAATA-3′	241	U46504
	R	5′ GTACCAGCCGTTGAAAGGAC-3′		
TNFSF15	F	5′-CCTGAGTATTCCAGCAACGCA-3′	292	NM_01024578
	R	5′-ATCCACCAGCTTGATGTCACTAAC-3′		
GAPDH	F	5′-GGTGGTGCTAAGCGTGTTAT-3′	264	K01458
	R	5′-ACCTCTGTCATCTCTCCACA-3′		

F, forward primer; R, reverse primer. IL-1β, chicken Interleukin-1β; IL-6, chicken Interleukin-6; iNOS, chicken inducible nitric oxide synthease; TNFSF15, chicken Tumor necrosis factor superfamily 15; GAPDH, chicken Glyceraldehyde 3-phosphate dehydrogenase as an internal control.

Table 6.   **Effects of dietary sorbent minerals on body weight gain and lesion score in Ross/Ross broilers following clinical *E. maxima*/*C. perfringens* co-infection (Trial 1)**

Treatment Group#	Body Weight Gain (g)		Lesion Score
	Days 0–6 following *E. maxima*	Days 0–7 following *C. perfringens*	
Control	279.9±43.7[a]	503.0±31.9[a]	Not taken
NE	203.4±3.7[b]	431.5±52.0[b]	2.63±0.52[a]
B (0.25%)	228.1±43.1[b]	467.3±71.1[ab]	2.38±0.52[ab]
Y (0.25%)	226.0±49.5[b]	488.8±57.6[ab]	2.50±0.53[a]
C (0.25%)	239.7±41.9[ab]	489.8±58.2[ab]	2.38±0.52[ab]
D (0.25%)	264.0±37.0[a]	502.8±51.5[a]	2.13±0.35[b]
B (0.50%)	230.8±49.6[ab]	497.2±54.3[ab]	2.38±0.52[ab]
Y (0.50%)	254.8±46.8[ab]	473.4±55.4[ab]	2.13±0.35[b]
C (0.50%)	250.6±45.7[ab]	490.8±49.5[ab]	2.50±0.53[a]
D (0.50%)	261.5±42.2[a]	496.3±47.3[ab]	2.13±0.35[b]
VM	227.5±45.8[b]	488.2±56.3[ab]	2.38±0.52[ab]

Body weight gains were determined between days 0 and 6 after *E. maxima* infection, and between days 0 and 7 following *C. perfringens* infection. Each value represent the mean weight gain±SEM (n=20). Intestinal lesion scores were determined at day 2 following *C. perfringens* infection by 3 independent observers in a blinded manner on a scale from 0 to 4. Each value represent the mean score±SEM (n=5). Within each column, values with different superscripts are statistically different according to the Duncan's multiple range test ($P<0.05$). #See Table 3 for treatment group. Values in boxes are significantly different compared with the co-infected NE group given the unsupplemented diet ($p<0.05$).

were used for experimental units in repeated experiments in both trials.

Results

Trial 1
Body Weight Gain and Intestinal Lesion Score

Chickens co-infected with *E. maxima* and *C. perfringens* and fed an unsupplemented basal diet had significantly ($p<0.05$) reduced body weight gain between days 0 and 6 following *E. maxima* infection (days 14 and 20 post-hatch) and between days 0 and 7 following *C. perfringens* infection (days 18 and 25 post-hatch) compared with the uninfected controls ($p<0.05$, Table 6). In contrast, co-infected chickens that were fed a diet containing 0.25% of a blend of CaMM, a fermentable fiber, and an organic acid (group D) had significantly increased body weight gain between both time spans compared with co-infected birds fed with the unsupplemented diet (group NE) ($p<0.05$). In fact, body weight

Table 7. Effects of sorbent mineral on serum *Clostridium perfringens* α-toxin- and NetB toxin-specific antibody levels in Ross/Ross broilers following clinical *E. maxima*/*C. perfringens* co-infection (Trial 1)

Treatment Group#	Serum Antibody Response			
	α-Toxin		Net B Toxin	
	7 DCPI	14 DCPI	7 DCPI	14 DCPI
Control	0.489 ± 0.030^c	0.509 ± 0.035^d	0.474 ± 0.012^c	0.501 ± 0.020^c
NE	0.645 ± 0.024^b	0.725 ± 0.040^c	0.621 ± 0.027^b	0.689 ± 0.036^b
B (0.25%)	0.684 ± 0.018^b	0.708 ± 0.014^c	0.650 ± 0.013^b	0.672 ± 0.013^b
Y (0.25%)	0.677 ± 0.040^b	0.679 ± 0.052^c	0.648 ± 0.027^b	0.678 ± 0.010^b
C (0.25%)	0.646 ± 0.036^b	0.675 ± 0.042^c	0.668 ± 0.026^b	0.666 ± 0.027^b
D (0.25%)	0.750 ± 0.032^a	0.897 ± 0.009^a	0.728 ± 0.015^a	0.860 ± 0.056^a
B (0.50%)	0.714 ± 0.015^b	0.734 ± 0.048^c	0.684 ± 0.019^b	0.714 ± 0.073^b
Y (0.50%)	0.678 ± 0.049^b	0.708 ± 0.048^c	0.691 ± 0.016^b	0.691 ± 0.016^b
C (0.50%)	0.687 ± 0.027^b	0.695 ± 0.036^c	0.691 ± 0.024^b	0.715 ± 0.012^b
D (0.50%)	0.728 ± 0.014^{ab}	0.828 ± 0.013^b	0.704 ± 0.013^b	0.777 ± 0.012^{ab}
VM	0.694 ± 0.039^b	0.754 ± 0.025^c	0.698 ± 0.027^b	0.712 ± 0.014^b

Antibody levels were measured at day 7 (7 DCPI) and day 14 (14 DCPI) following *C. perfringens* infection. Each value represents the mean OD_{450} value$\pm$SEM corrected for background ($n=3$). Within each column, values with different superscripts are statistically different according to the Duncan's multiple range test ($P<0.05$). #See Table 3 for treatment group. Values in boxes are significantly different compared with the co-infected NE group given the unsupplemented diet ($p<0.05$).

Table 8. Effect of dietary supplementation of sorbent minerals on *C. perfringens* α-toxin and NetB toxin levels in sera of Ross/Ross broilers following clinical *E. maxima*/*C. perfringens* co-infection (Trial 1)

Treatment Group#	Serum Concentration (ng/m*l*)	
	α-Toxin	NetB Toxin
NE	248.9 ± 6.8^a	199.0 ± 54.9
B (0.25%)	255.2 ± 29.1^a	172.4 ± 27.8
Y (0.25%)	244.4 ± 31.0^a	197.8 ± 38.2
C (0.25%)	239.9 ± 22.4^{ab}	194.2 ± 27.8
D (0.25%)	178.1 ± 27.1^c	167.5 ± 23.4
B (0.50%)	198.7 ± 15.0^{bc}	173.6 ± 38.3
Y (0.50%)	246.2 ± 32.1^a	163.9 ± 45.5
C (0.50%)	271.3 ± 21.0^a	163.9 ± 45.2
D (0.50%)	255.2 ± 20.3^a	183.3 ± 25.8
VM	250.7 ± 27.0^a	170.0 ± 25.2

Toxin levels were measured at day 2 following *C. perfringens* infection. Each value represents the mean toxin concentration$\pm$ SEM ($n=3$). Within each column, values with different superscripts are statistically different according to the Duncan's multiple range test ($P<0.05$). #See Table 3 for treatment group. Values in boxes are significantly different compared with the co-infected NE group given the unsupplemented diet ($p<0.05$).

gains in the 0.25% group D chickens were equal to those of the uninfected controls. Birds in the 0.50% group D also had significantly ($p<0.05$) increased body weight gains between days 0 and 6 following *E. maxima* infection compared with co-infected chickens given the unsupplemented diet. Chickens in the 0.25% or 0.50% group D, as well as those in the 0.50% group Y, had significantly ($p<0.05$) decreased intes-

tinal lesion scores at day 2 following *C. perfringens* infection (day 20 post-hatch) compared with co-infected birds given the unsupplemented diet (Table 4).

Serum Antibody Levels to *C. perfringens* α-Toxin and NetB Toxin

Serum antibody levels against α-toxin and NetB toxin were significantly ($p<0.05$) greater at days 7 and 14 following *C. perfringens* infection (days 25 and 32 post-hatch) in birds fed the unsupplemented diet compared with uninfected controls ($p<0.05$, Table 7). Co-infected chickens in 0.25% group D had further increased antibody levels against both toxins at both time points compared with co-infected chickens given the unsupplemented basal diet. Co-infected birds in the 0.50% group D also had increased antibodies against α-toxin at the later time point compared with co-infected chickens given the unsupplemented diet.

Serum α-Toxin and NetB Toxin Levels

Co-infected chickens in the 0.25% group D and 0.50% group B had significantly ($p<0.05$), decreased α-toxin levels in sera at day 2 following *C. perfringens* infection (day 20 post-hatch) compared with co-infected chickens given the unsupplemented diet (Table 8). However, none of the dietary treatments influenced NetB toxin levels in sera.

Proinflammatory Cytokine and iNOS Transcript Levels

In intestinal IELs as shown in Fig. 2, increased levels of IL-1β transcripts normalized to GAPDH transcripts were seen in the 0.50% group C, in the 0.50% group D for IL-6 transcripts, and in the 0.25% group C for TNFSF15 transcripts. Decreased levels of IL-1β transcripts were seen in the 0.25% group C, in the 0.25% groups B and C for IL-6 transcripts, in the 0.25% groups Y and C, and 0.50% group D, for iNOS transcripts, and in the 0.25% groups B and Y,
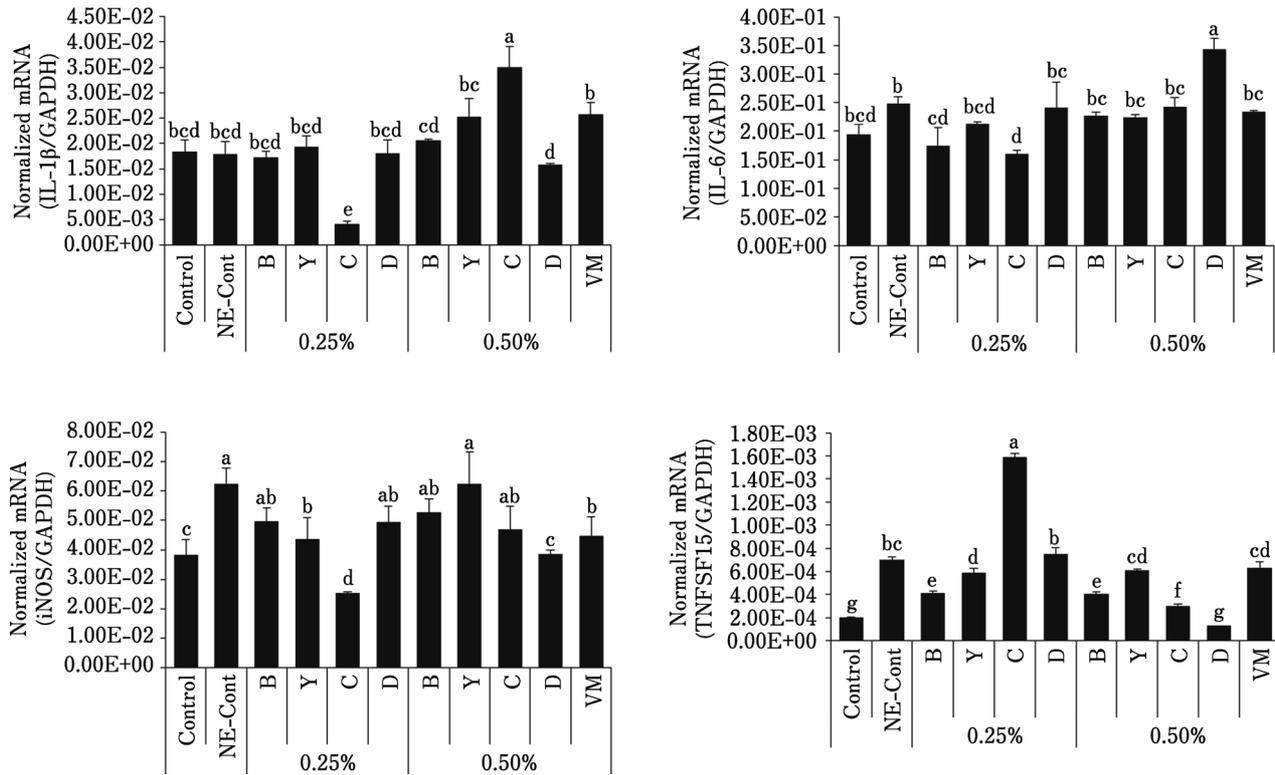
Fig. 2. **Levels of transcripts for proinflammatory cytokines and iNOS in intestinal jejunum IELs.** Intestinal IELs were collected at day 2 following *C. perfringens* infection and the levels of transcripts for IL-1β, IL-6, iNOS, and TNFSF15 were measured by quantitative RT-PCR. Individual transcript levels were normalized to GAPDH transcript levels. Each bar represents the mean normalized transcript level$\pm$SEM ($n=5$). Bars with different superscripts are statistically different according to the Duncan's multiple range test ($p<0.05$).

and 0.50% groups B, C, and D, for TNFSF15 transcripts. In the spleen as shown in Fig. 3, increased levels of IL-6 transcripts were seen in the 0.25% group B, and in the 0.25% group Y for TNFSF15 transcripts. Decreased levels of IL-1β and iNOS transcripts were seen in all treatment groups, in the 0.25% groups Y and D, and 0.50% group B, for IL-6 transcripts, and in the 0.25% and 0.50% groups B and D for TNFSF15 transcripts.

Trial 2

Body Weight Gain, Feed Conversion, Mortality, and Intestinal Lesion Score

Chickens co-infected with *E. maxima* and *C. perfringens* and fed an unsupplemented diet had significantly ($p<0.05$) reduced body weight gain, and increased feed conversion ratio (FCR), mortality, and lesion score, between days 0 and 14, and between days -14 and 14, following *E. maxima* infection (days 14−28 and 0−28 post-hatch, respectively) compared with the uninfected controls (Table 9). In contrast, co-infected chickens that were fed a basal diet containing 0.25% Varium® had significantly ($p<0.05$) increased body weight gain, and decreased FCR, mortality, and lesion score, be-

tween both time spans compared with co-infected birds fed the unsupplemented diet (group NE). Co-infected chickens in the 0.25% group D only had decreased FCR compared with co-infected birds given the unsupplemented diet. Dietary VM increased weight gain between days 14 and 28, and reduced FCR and mortality, compared with unsupplemented, co-infected controls.

Discussion

In both the clinical and subclinical models of experimental avian NE, co-infected chickens given the basal unsupplemented diet had significantly ($p<0.05$) reduced body weight gain compared with uninfected birds. Decreased growth performance is a prominent hallmark of NE and is responsible for the majority of economic loss for poultry producers (Branton *et al.*, 1997; Kaldhusdal *et al.*, 2001; Lensing *et al.*, 2010; Cravens *et al.*, 2013). Forty-one percent of the loss due to avian NE in the UK in 1995 was from reduced weight gain (Williams, 2005). Much of this decreased weight gain is attributable to the appearance of intestinal lesions and other gut pathologies that can vary from mild with by a thin,
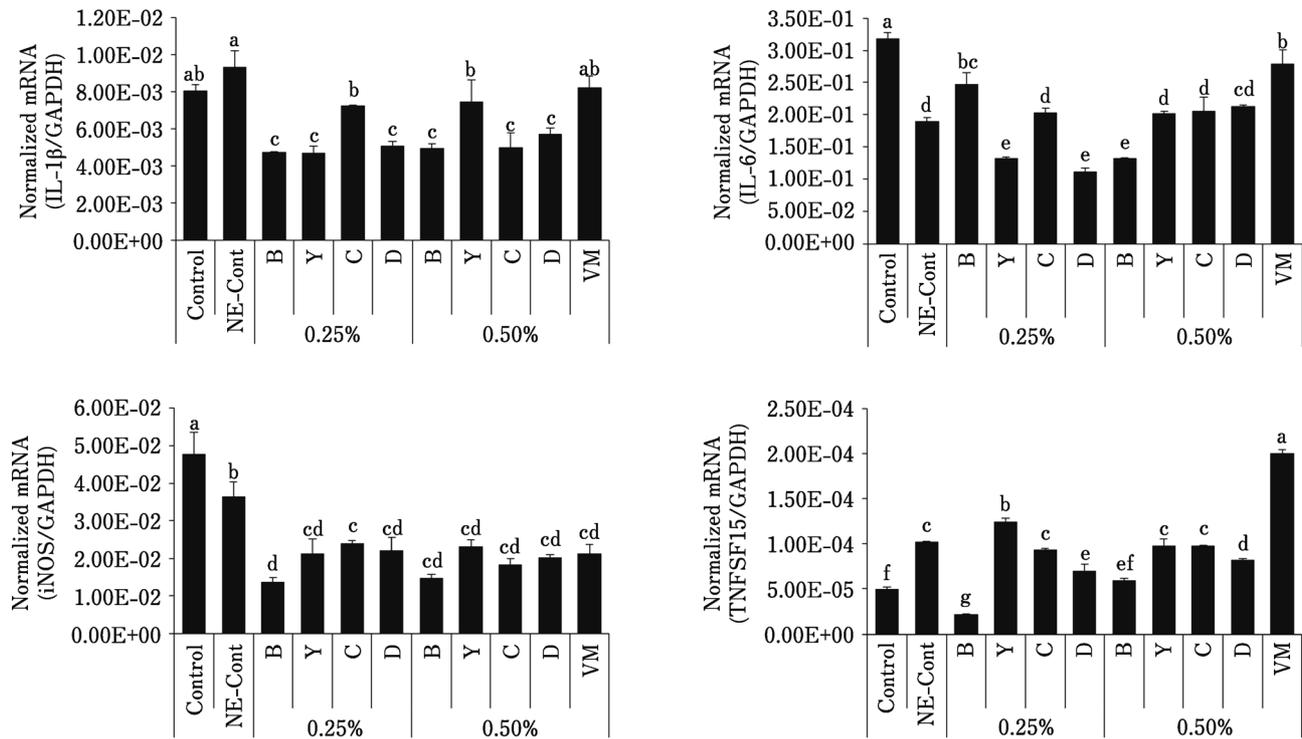
Fig. 3.   **Levels of transcripts for proinflammatory cytokines and iNOS in spleen cells.** Spleen cells were collected at day 2 following *C. perfringens* infection and the levels of transcripts for IL-1β, IL-6, iNOS, and TNFSF15 were measured by quantitative RT-PCR. Individual transcript levels were normalized to GAPDH transcript levels. Each bar represents the mean normalized transcript level$\pm$SEM ($n=5$). Bars with different superscripts are statistically different according to the Duncan's multiple range test ($p<0.05$).

flaccid gut wall and thickened mucus layer, to extensive areas of necrosis and ulceration with significant hemorrhage, typically resulting in death (Jia *et al*., 2009). In the current study, NE-induced chickens on an unsupplemented basal diet in both the clinical and subclinical infection trials showed the greatest lesion scores and lowest weight gains. During clinical infection, treatment groups B, Y, and C showed a trend toward reduced lesion scores and increased weight gains, although none were statistically significant compared with unsupplemented and co-infected controls. However, significantly ($p<0.05$) greater body weight gain and reduced intestinal lesions were observed in co-infected chickens fed a diet supplemented with CaMM plus a fermentable fiber and an organic acid (group D) compared with unsupplemented, co-infected controls. In fact, these beneficial effects seen in group D-treated broilers were not seen in clinically-infected chickens given VM, an antibiotic commonly used to control NE in the field (Williams, 2005). Under the subclinical infection conditions in Trial 2, where a beneficial effect of infeed VM on experimental NE was observed, the 0.25% Varium® group, and to a lesser extent the 0.25% group D, were equivalent to VM in reducing the negative consequences of this disease. These results suggest that the CaMM-

based feed additives, at the specified level of supplementation, may be useful for improving gut health and growth performance in commercial broilers under field conditions.

Antibodies to *C. perfringens* α-toxin and NetB toxin were increased in NE-challenged chickens fed an unsupplemented diet relative to the uninfected controls, and were further increased in co-infected group D animals compared with the unsupplemented, co-infected controls. Increased serum antibodies to the major toxins of *C. perfringens* may reflect reduced gut damage contributing to improved body weight gain. In other studies, birds fed a potato protein-supplemented diet had increased anti-α-toxin antibody levels along with increased intestinal hemorrhage and liver lesions compared with chickens fed a soy protein-supplemented diet (Palliyeguru *et al*., 2010). Higher anti-*Salmonella* antibody titers were seen in bacteria-infected chickens fed an arginine- and vitamin E-supplemented diet compared with birds given an unsupplemented diet (Liu *et al*., 2014). Previous work from our laboratory showed higher anti-toxin antibody levels in the sera of healthy chickens raised on farms with endemic NE compared with NE-diseased birds in the same flock, suggesting a protective role for these antibodies against infection in the field (Lee *et al*., 2011). Further, when improvements

Table 9. **Effects of dietary sorbent minerals on body weight gain, feed conversion ratio, mortality, and lesion score in Cobb/Cobb broilers following subclinical *E. maxima*/*C. perfringens* co-infection (Trial 2)**

Treatment Group#	Body Weight Gain, g		Feed Conversion Ratio		% Mortality Day 0−28	Lesion Score
	Day 14−28	Day 0−28	Day 14−28	Day 0−28		
Control	447 ± 13^a	621 ± 24^a	1.78 ± 0.08^c	1.81 ± 0.05^c	0 ± 0^c	0 ± 0^c
NE	303 ± 5^c	465 ± 15^c	2.47 ± 0.09^a	2.25 ± 0.07^a	10.9 ± 1.6^a	0.54 ± 0.09^a
D (0.25%)	333 ± 23^{bc}	482 ± 30^{bc}	1.99 ± 0.08^{bc}	1.98 ± 0.06^b	6.3 ± 2.4^{ab}	0.33 ± 0.13^{ab}
Varium® (0.25%)	367 ± 15^b	541 ± 17^b	2.13 ± 0.08^b	2.03 ± 0.06^b	3.1 ± 2.0^{bc}	0.25 ± 0.08^{bc}
VM	367 ± 19^b	512 ± 28^{bc}	1.80 ± 0.03^c	1.93 ± 0.03^{bc}	1.6 ± 1.6^{bc}	0.46 ± 0.09^{ab}

Body weight gain, feed conversion ratio (feed intake/weight gain), and mortality were determined between days 0 and 14 after *E. maxima* infection (days 14 and 28 post-hatch), and/or between days -14 and 14 after *E. maxima* infection (days 0 and 28 post-hatch). Each value represent the mean weight gain, feed conversion ratio, or % mortality$\pm$SEM ($n=20$). Intestinal lesion scores were determined at day 1 following final *C. perfringens* infection (day 22 post-hatch) on a scale from 0 to 3. Each value represent the mean lesion score$\pm$SEM ($n=8$). Within each column, values with different superscripts are statistically different according to the Duncan's multiple range test ($P<0.05$). #See Table 4 for treatment group. Values in boxes are significantly different compared with the co-infected NE group given the unsupplemented diet ($p<0.05$).

in other response criteria (growth, lesion score, serum α-toxin concentration) were considered, it is likely that the increased levels of these antibodies is due, in part, to an increase in the ability of the avian immune system to respond to bacterial infection. While dietary probiotics, phytochemicals, and yeast-derived compounds all have been shown to increase plasma or serum antibody concentrations in chickens (Haghighi *et al*., 2005, 2006; Gao *et al*., 2008; Kim *et al*., 2013), to the best of our knowledge, this is the first report to document that dietary treatment with CaMM plus a fermentable fiber and an organic acid enhances the avian humoral immune response to a combined *E. maxima*/*C. perfringens* challenge.

At the level of cellular immunity, the levels of transcript for the cytokines, IL-1β, IL-6, and TNFSF15, and iNOS, were significantly ($p<0.05$) altered in the intestine and spleen of CaMM-treated chickens following co-infection with *E. maxima* plus *C. perfringens*. In mammals, IL-1β is involved in a variety of cellular activities, including proliferation, differentiation, and apoptosis (Thornberry and Molineaux, 1995), while IL-6 acts as either a proinflammatory cytokine or an anti-inflammatory myokine (Kamimura *et al*., 2003). iNOS catalyzes the synthesis of the cell signaling molecule, nitric oxide, particularly in response to IL-1β (Green *et al*., 1994), and TNFSF15 is a proinflammatory cytokine that is increased in chickens in response to *Eimeria* infection (Park *et al*., 2007). In the *E. maxima* plus *C. perfringens* co-infection model used here, prior studies showed a mixed response in TNFSF15 transcript levels in intestial IELs, being upregulated on day 1 but downregulated on day 2, following *C. perfringens* infection compared either with uninfected birds or chickens infected with *E. maxima* or *C. perfringens* alone (Park *et al*., 2008). In the current study, TNFSF15 transcript levels in intestinal IELs and spleen also were increased or decreased, but in this case in response to the different dietary supplements used. Of the four cytokines/mediators examined here, TNFSF15 transcripts in-

creased to the greatest extent in both the intestine and spleen of the unsupplemented, NE-challenged group compared with the uninfected controls. However, with the exception of the 0.25% groups C and Y, dietary supplementation of co-infected chickens with any of the other CaMM-based treatments either had no effect or decreased TNFSF15 transcripts compared with the unsupplemented, co-infected controls. Park *et al*. (2008) reported that intestinal IEL levels of IL-1β transcripts were decreased in chickens co-infected with *E. maxima* and *C. perfringens* compared with single infection by either pathogen alone, while IL-6 transcript levels were not affected following infection with either or both microorganisms. Similarly, Yitbarek *et al*. (2012) reported that IL-6 expression in the intestinal ileum or cecal tonsils was unaffected in *C. perfringens*-infected chickens compared with uninfected controls, leading these authors to theorize that IL-6 is not involved in Toll-like receptor regulation of *C. perfringens*-induced NE. *Eimeria* infection of chickens also induces iNOS expression both *in vitro* and *in vivo* (Dalloul *et al*., 2005; Lee *et al*., 2014). In the current study, iNOS transcripts were either unaffected or decreased in co-infected chickens fed the supplemented diets compared with unsupplemented NE controls.

In conclusion, two separate trials carried out at different research facilities using either a clinical or a subclinical experimental model of avian NE showed that dietary supplementation with the thermally-processed clays, CaMM or Varium®, in combination with a fermentable fiber and an organic acid improved the growth performance and mitigated the negative effects of the disease. Future studies are needed to further characterize the CaMM-regulated physiological and immunological mechanisms that are activated in response to avian NE.

Acknowledgments

Conflict of Interests

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