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Nigella sativa as an antibiotic alternative to promote growth and enhance health of broilers challenged with *Eimeria* maxima and *Clostridium perfringens*

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ABSTRACT The poultry industry has significant coccidiosis and necrotic enteritis (**NE**) challenges, leading to high mortality and unacceptable growth without antibiotic treatment. This research explored supplementing *Nigella sativa* (black cumin) seed oil in poultry feed to mitigate coccidiosis and prevent or lessen NE in broilers. *In vivo* studies consisted of 384 and 320 Cobb 500 male broiler chicks distributed in a randomized complete block experimental design for trials 1 and 2, respectively. The first trial compared 3 concentrations (1, 2, and 5 mL/kg) of black cumin seed oil (**BCSO**), and trial 2 compared 2 concentrations (2 and 5 mL/kg) BCSO, with birds challenged with *Eimeria maxima* and *Clostridium perfringens* (**Cp**) strains Cp#6 and Cp#4, respectively. Broiler live performance, NE disease outcomes, and Cp populations were measured for both trials. A commercially available BCSO oil product, determined in a preliminary *in vitro* study to have the highest anti-Cp activity, was selected for *in vivo* studies. Gas chromatography-mass spectrometry analysis indicated the major bioactive compounds p-cymene, thymoquinone, carvacrol, and thymol were present in the BCSO. In trial 1 with strain Cp#6, BCSO concentrations of 2 and 5 mL/kg reduced NE lesion score and mortality rate to 1.6% compared with 7.8% for positive control, with no adverse impact on live performance. In trial 2 with strain Cp#4, BCSO reduced NE lesion scores and mortality rate to 35.9% compared with 51.6% for positive control and also improved weight gain when there was a Cp infection in broiler chickens. The current study compared NE in broilers challenged with 2 different Cp strains producing different levels of NE. Following Cp infection, both the population of vegetative cells and spores of Cp in cecal contents decreased for all treatments in trial 2. In conclusion, BCSO at concentrations of 2 and 5 mL/kg enhanced broiler live performance and alleviated NE and has potential as a natural, non-medication antimicrobial nutritional supplement for use as a feed additive in chickens.

Key words: broiler, coccidiosis, necrotic enteritis, *Nigella sativa*, *Clostridium perfringens*

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INTRODUCTION

Coccidiosis and necrotic enteritis (**NE**) are enteric diseases caused by the coccidial protozoan genus *Eimeria* and the bacterial pathogen *Clostridium perfringens* (**Cp**), leading to high mortality rates in the broiler industry. *Eimeria* spp. parasitizes the epithelial cells of the intestinal lining and causes pathological changes varying from local destruction of the mucosal barrier and underlying tissue to systemic effects such as blood loss, shock syndrome, and even death (Rose, 1980). Damage to the epithelium caused by coccidia is a major predisposing factor for NE, allowing Cp to replicate rapidly and produce toxins (Van Immerseel et al., 2009).

Cp, a gram-positive spore-forming anaerobe, is the causative agent of numerous histotoxic and enterotoxic diseases in humans and animals (Uzal et al., 2014). The key feature of these diseases is the response to the production of potent bacterial toxins, most of which are extracellular proteins. Cp is widely distributed in the environment, but some strains of this bacterial pathogen adversely affect poultry health. Coccidiosis in poultry

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often pre-exists or occurs concurrently with field outbreaks of NE. NE is characterized by the sudden onset of diarrhea and mucosal necrosis caused by the overgrowth of Cp in the small intestine with clinical signs of depression, dehydration, diarrhea, ruffled feathers, and lower feed intake (Songer, 1996). NE occurs in broilers aged between 2 and 6 wk and leading to a 10 to 40% total mortality rate. An estimated $90 million is spent in the US and over $3 billion is spent worldwide for coccidiosis prevention annually (Anonymous, 2013). NE caused US $6 billion loss on a worldwide scale as estimated in 2015 (Wade and Keyburn, 2015).

Globally, antibiotic use for growth promotion and disease prevention has become an integral part of the poultry production. In North America, antibiotics such as tetracycline, bacitracin, tylosin, salinomycin, and virginiamycin were often used in intensive poultry farming (Diarra and Malouin, 2014). However, the emergence of antibiotic-resistant bacterial strains and their transmission to humans can threaten food safety and public health. As of 1 Jan 2017, the U.S. Food and Drug Administration (**FDA**) implemented Guidance for Industry (**GFI**) #213, which requires antibiotics with importance in human medicine to be no longer used for growth promotion or feed efficiency in cows, pigs, chickens, turkeys, and other food animals (Schulz and Rademacher, 2017). Therefore, there is an urgent need to explore natural products as alternatives to antibiotics for the poultry industry.

Phytogenic feed additives (**PFA**) are substances derived from plants that cause improved livestock and poultry performance (Windisch et al., 2008). These PFA comprise a variety of herbs, spices, and essential oils that have been studied as alternatives to antibiotic growth promoters in poultry and swine nutrition. *Nigella sativa* L. (black cumin) seed is extensively grown and consumed in the Middle East, Southeast Asia, the Mediterranean, and Eastern Europe. Black cumin is an annual herbaceous plant belonging to the *Ranunculaceae* family and has been traditionally used for over 2,000 years in Middle Eastern folk medicine for treating various diseases (Phillips, 1992). Various chemical composition analyses have been carried out to identify compounds present in black cumin seeds. The results indicated the presence of volatile oils, alkaloids, and a variety of pharmacologically active compounds such as carvacrol, p-cymene, thymoquinone, thymohydroquinone, longifolene, and limonene (Dinagaran et al., 2016; Khalid and Shedeed, 2016). Black cumin seed and its essential oils have been shown to possess broad antimicrobial properties, including antibacterial, antiparasitic, antiviral, and antifungal activity. Several investigations have reported black cumin to have activity against a wide range of gram-positive and gram-negative bacteria in vivo and in vitro, and to increase the average body weight, feed intake, feed conversion ratio, and immunity in broilers (Osman and El-Barody, 1999; Swamy and Tan, 2000). The antibacterial activity of crude extracts of *N. sativa* has been reported against *Staphylococcus aureus*, *Pseudomonas aeruginosa, Escherichia coli*, and *Listeria monocytogenes,* but to date, there is a scarcity of studies on black cumin activity against anaerobes such as Cp (Morsi, 2000). In performance studies with broiler chickens, no significant effect of black cumin seed on average daily gain and feed conversion ratio was observed (Nasir and Grashorn, 2010).

To the best of our knowledge, no studies have evaluated the effect of supplementing feed of broiler chickens challenged with Cp with *N. sativa* oil as an alternative to antibiotics. The present study was, therefore, undertaken to investigate the effect of black cumin seed oil (**BCSO**) to inhibit coccidiosis and prevent or lessen NE in broilers challenged with 2 different Cp strains and to compare the effect of Cp strain variation on the severity of resulting NE in broiler chicks.

MATERIALS AND METHODS

Culture Conditions for C. perfringens *and* Eimeria maxima

The *C. perfringens* strains Cp#6 and Cp#4 used in this study were clinical isolates from field cases of NE in the United States (Thompson et al., 2006). Both isolates are alpha-toxin positive, but strain Cp#4 is also NetB-positive isolate (Lepp et al., 2010). The strains were kindly provided by Dr. Charles Hofacre at Southern Poultry Research Group (**SPRG**) (Nicholson, Georgia) and stored at -80°C in brain heart infusion (**BHI**) broth with 20% glycerol. *C. perfringens* isolates were sub-cultured twice and were grown on tryptose sulfite cycloserine (**TSC**) agar base (HiMedia, Mumbai, India) supplemented with D-cycloserine (Acros Organics, Fair Lawn, NJ) at 37°C for ~24 h in an anaerobic chamber model BACTRON EZ (Sheldon Manufacturing Inc., Cornelius, OR) before being used in the studies.

For broiler challenge trials, both Cp and *E. maxima* cultures were freshly prepared by SPRG using the established NE challenge method of Hofacre et al. (1998).

Antimicrobial Susceptibility Test

Five commercially available BCSO products (A, B, C, D, and E) were purchased online. *C. perfringens* strains Cp#6 and Cp#4 were tested for susceptibility to the commercial BCSOs using the disk diffusion method (CLSI, 2007). *C. perfringens* colonies were suspended in 0.85% saline and the OD at 600 nm was adjusted to ca. 0.5. The suspension was swabbed using a sterile cotton swab onto brucella blood agar (BD BBL, Franklin Lakes, NJ) plates supplemented with 5 μg/mL vitamin K1 (Alfa Aesar, Haverhill, MA) and 1 μg/mL hemin (MP Biomedicals, Santa Ana, CA). BCSO dilutions of 1:10, 1:20, and 1:30 were prepared using dimethyl sulfoxide (**DMSO**) (MP Biomedicals, Santa Ana, CA). Twenty μLof BCSO dilutions, thymoquinone (**TQ**) (LKT Laboratories Inc., St. Paul, MN) as the positive control, and DMSO as the negative control were each deposited onto a sterile 6 mm blank disk (BD BBL,

Franklin Lakes, NJ), then dried under a biosafety hood at room temperature for 60 min. The disks were then placed on the inoculated brucella blood agar plates. All the plates were incubated at 37°C for 24 h in BACTRON EZ anaerobic chamber and the diameters of the inhibition zones were recorded.

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The BCSO product (A) exhibiting the highest antimicrobial activity was subjected to GC-MS analysis at the Center for Applied Isotope Studies of Laboratory for Environmental Analysis, University of Georgia, Athens, Georgia. Triplicate replicates of 0.5 g oil were weighed and transferred to separate 10 mL volumetric flasks and filled to volume using hexane as the solvent. After thoroughly mixing, 0.1 mL of the solution was transferred into a 2 mL GC vial containing 0.9 mL of hexane and crimp-capped for GC-MS analysis using a selected ion monitoring method explicitly developed for thymoquinone quantitation using thymoquinone (Supelco, Bellefonte, PA) analytical standard at 100 ppm concentration.

Analysis of the volatile oil was conducted on a Hewlett-Packard model 6890 gas chromatograph coupled to a quadrupole mass spectrometer (model HP 5973) with a capillary column of HP-5MS (5% phenyl methylsiloxane, length = 30 m, inner diameter = 0.25 mm, and film thickness = 0.25 μm). GC-MS interphase, ion source, and selective mass detector temperatures were maintained at 280°C, 230°C, and 150°C, respectively. The carrier gas used was helium with a flow rate of 1.0 mL/min. The oven temperature program was set at an initial temperature of 35°C for 5 min, then increased to 280°C at the rate of 10°C/min and held at that temperature for 1 min. The compounds were identified by referring to the CAS Registry number on Wiley MS library and National Institute of Standards Technology libraries. The retention index (**RI**) was calculated for BCSO product A constituents by taking the average of RI of similarly identified compounds. The peak area percentages (Area%) were calculated for all BCSO constituents by taking the sums of Area% of similarly identified compounds.

Broiler Chickens, Experimental Design, Diets, and Housing

Animal trials were conducted at Southern Poultry Feed and Research, Inc. (**SPFR**) in Athens, Georgia. Cobb 500 male broiler chicks were purchased from Cobb-Vantress, Cleveland, Georgia. A total of 384 chicks were used for trial 1 and 320 chicks for trial 2. The newly hatched male chicks (d 0) were distributed randomly in a randomized complete block experimental design among 6 treatments for trial 1 and 5 treatments for trial 2. Each treatment was replicated eight times with 8 chicks per replicate (Table 1).

Table 1. Treatments and treatment schedule for animal trials 1 and 2.

Treatments	*E. maxima*	*C. perfringens*[1]	Cages/ Treatment
Trial 1:			
1. Nonmedicated	DOT 11[2]	No	8
2. Nonmedicated	DOT 11	DOT 16, 17,18	8
3. BMD 50 g/t (antibiotic control)	DOT 11	DOT 16, 17, 18	8
4. Black cumin seed oil, 1 mL/kg	DOT 11	DOT 16, 17, 18	8
5. Black cumin seed oil, 2 mL/kg	DOT 11	DOT 16, 17, 18	8
6. Black cumin seed oil, 5 mL/kg	DOT 11	DOT 16, 17, 18	8
Trial 2:			
1. Nonmedicated	DOT 14	No	8
2. Nonmedicated	DOT 14	DOT 19, 20, 21	8
3. BMD 50 g/t (antibiotic control)	DOT 14	DOT 19, 20, 21	8
4. Black cumin seed oil, 2 mL/kg	DOT 14	DOT 19, 20, 21	8
5. Black cumin seed oil, 5 mL/kg	DOT 14	DOT 19, 20, 21	8

[1]Strain Cp#6 was used for trial 1 and strain Cp#4 for trial 2.
[2]DOT refers to the day of trial.

The 6 treatments of animal trial 1 included 3 control groups and 3 treatment groups (Table 1). The controls included a nonmedicated-no Cp challenge (negative control), nonmedicated with Cp#6 challenge (positive control), and a bacitracin methylene disalicylate (**BMD**) 50 grams/ton (g/t) antibiotic control. All groups were fed the same basal diet taking into account their specific dietary needs (NRC, 1994) except for the antibiotic control, which received the basal diet supplemented with BMD antibiotic (50 g/t), and BCSO treatment groups, which received the basal diet supplemented with BCSO at 1, 2, or 5 mL/kg.

The experimental design of animal trial 2 was similar to trial 1 except for excluding 1 treatment (Table 1), a 3-d delay in *E. maxima* and Cp#4 challenges (which was a more virulent strain than Cp#6 used in animal trial 1). The chicks were fed with the basal diet supplemented with BCSO at concentrations of 2 and 5 mL/kg (treatments 4 and 5, respectively).

Chicks were raised in Petersime battery cages in an insulated, concrete-floored, metal structure building with temperature control. The experimental birds were randomly assigned to diets and fed ad libitum. Diet composition and the analyzed nutrient contents, analyzed by Agricultural and Environmental services laboratory at University of Georgia, are presented in Table 2.

NE Induction

On the day of trial (**DOT**) 11 in trial 1 and DOT 14 in trial 2, all birds were orally inoculated with ~5,000 oocysts of *E. maxima*. Starting on DOT 16 for trial 1 and DOT 19 for trial 2, all birds, except the negative control group (treatment 1), were orally given 1 mL of an overnight broth culture of *C. perfringens* ~10^8 CFU/

Table 2. Composition and nutrient contents of basal diet.

Ingredient	%
Corn, yellow, grain	54.969
Soybean meal dehulled, solvent	38.002
Vegetable fat	2.852
Dicalcium phosphate	2.040
Calcium carbonate	1.186
Salt (NaCl)	0.436
Methionine MHA	0.313
Trace mineral premix[1]	0.075
Vitamin premix[2]	0.065
Calculated nutrients	
Crude protein	22.5
Metabolizable energy, kcal/kg	3,054.00
Digestible methionine	0.6
Digestible lysine	1.2

[1]Trace mineral mix provides the following (per kg of diet): 60 mg of manganese ($MnSO_4 \cdot H_2O$); 30 mg of iron ($FeSO_4 \cdot 7H_2O$); 50 mg of zinc (ZnO); 5 mg of copper ($CuSO_4 \cdot 5H_2O$); 0.15 mg of iodine (ethylene diamine dihydroiodide); 0.3 mg of selenium ($NaSeO_3$).

[2]Vitamin mix provided the following (per kg of diet): 2.4 mg of thiamine mononitrate; 44 mg of nicotinic acid; 4.4 mg of riboflavin; 12 mg of D-Ca pantothenate; 12.0 μg of vitamin B_{12} (cobalamin); 4.7 mg of pyridoxine·HCl; 0.11 mg of D-biotin; 5.5 mg of folic acid; 3.34 mg of menadione sodium bisulfite complex; 220 mg of choline chloride; 27.5 μg of cholecalciferol; 6,306.6 IU of trans-retinyl acetate; 11 IU of all-rac α-tocopheryl acetate; 125 mg of ethoxyquin.

mL (strain Cp#6 for trial 1 and strain Cp#4 for trial 2) daily on 3 consecutive days.

Animal Care, Management, and NE Analysis

During the experimental period (4 wk in total), the live performance parameters of the birds were evaluated. All birds were weighed on DOT 0, 11, 18, and 28 for trial 1 and DOT 0, 14, 21, and 28 for trial 2. Feed was weighed on DOT 0 for both the trials, and the remaining feed was weighed on DOT 11, 18, and 28 for trial 1, but on DOT 14, 21, and 28 for trial 2.

On DOT 18 for trial 1 and DOT 21 for trial 2, 3 birds from each cage were randomly selected, sacrificed by cervical dislocation, weighed, and examined for the presence and degree of NE lesions. In the meantime, the cecal samples were aseptically collected, placed in a sterile centrifuge tube, and transported on ice to the laboratory for the following microbiological analysis. The NE scoring was based on a 0 to 3 scale, with 0 being normal and 3 being the most severe (lesion score 0 = normal; lesion score 1 = slight mucus covering small intestine; lesion score 2 = necrotic small intestine mucosa; lesion score 3 = sloughed and bloody small intestine mucosa and contents) (Hofacre et al., 1998). Animal care and management were performed according to SPFR's Institutional Animal Care and Use Committee (**IACUC**) including animal care and usage protocols presented in the guide for the care and use of laboratory animals (NRC, 2010).

Microbiological Analysis of Cecal Samples

On DOT 21 and DOT 28 for trial 2, cecal contents were collected aseptically from chickens sacrificed by manual cervical dislocation according to SPFR's IACUC protocols and processed within 24 h to enumerate Cp, Cp spores, and total aerobic bacteria. Contents from the ceca collected above were aseptically removed and pooled together as a composite sample. From each composite cecal sample, approximately 1 g of cecal content was weighed, mixed with 9 mL of 0.1% sterile peptone water, vortexed, and then serially diluted for microbiological culturing of Cp, Cp spores, and total bacterial count (**TBC**).

C. perfringens counts were determined by spread plating in duplicate using TSC agar base supplemented with 400 mg of D-cycloserine per liter and overlaid with the same agar by pour plating. For spore enumeration, cecal suspensions in 0.1% peptone were exposed to heat shock treatment at 75°C for 20 min to induce germination of the spores and kill vegetative cells. After heat treatment, the suspensions were cooled in a crushed ice water bath and then plated on TSC agar in duplicate as described above. Total bacteria were cultured on tryptic soy agar (**TSA**) (BD Difco, Franklin Lakes, NJ). The plates of both Cp vegetative cells and Cp spores for each treatment were incubated anaerobically while TSA plates were incubated aerobically. All plates were incubated for 24 h at 37° C.

Measurement of Physicochemical Parameters of Cecal Samples

Physical parameters such as moisture content, pH, and electrical conductivity of cecal samples were measured in duplicate. Moisture content was determined using the oven drying method by placing 1g gram samples in 43 mm diameter aluminum crinkle dish (VWR, Radnor, PA) and heating at 105°C for 24 h. Both the pH and electrical conductivity were measured in duplicate using an Orion Versa Star Pro benchtop meter (Thermo Fisher Scientific Inc., Waltham, MA) by resuspending 1g of cecal samples in 9 mL of deionized water.

Statistical Analysis

All data were calculated as cage average. Feed Intake (**FI**) was reported by the total feed consumed for the 0-to-18-, 11-to-18-, 0-to-28-, and 11-to-28-d periods for trial 1 and for the 0-to-21-, 14-to-21-, 0-to-28-, and 14-to-28-d periods for trial 2. Average body weight gain (**BWG**) was calculated as total weight gained by live birds divided by the total number of birds weighed for a given period for the 11-to-18-, 0-to-18-, 11-to-28-, and 0-to-28-d periods for trial 1 and for the 14-to-21-, 0-to-21-, 14-to-28-, and 0-to-28-d periods for trial 2. Feed conversion ratio (**FCR**) was calculated as total feed consumed divided by sum of total bird weight gained including dead and removed bird weights for the 11-to-18-, 0-to-18-, 11-to-28-, and 0-to-28-d periods for trial 1 and for the 14-to-21-, 0-to-21-, 14-to-28-, and 0-to-28-d periods for trial 2. NE lesion scores were determined for d 18 for trial 1 and d 21 for trial 2. The percentages of NE

mortality were calculated for the 0-to-28-d period for both trials.

The challenge studies were conducted using a randomized complete block design. There were 6 treatments, each with eight blocks, resulting in 48 cages for trial 1 and there were 5 treatments each with eight blocks, for a total of 40 cages for trial 2. At the beginning of each trial (d 0), 8 birds were placed in each cage. Battery cage location within the cage unit was the blocking factor. The cage of birds was the experimental unit for statistical analysis. The model included blocks and treatments. Data were analyzed by ANOVA using STATISTIX 10.0 data analysis software for researchers and JMP Pro 14 (SAS Institute, Cary, NC). Multiple mean comparisons were made using Tukey's HSD all pairwise comparisons test. The alpha level for the determination of significance was 0.05.

RESULTS

In Vitro *Antimicrobial Testing of BCSO*

A total of 5 commercial BCSO products (A, B, C, D, and E) purchased in the US market were tested for anti-Cp activities using the disk diffusion assay. A clear zone of inhibition of an average of 12.35 mm in diameter for Cp#6 and 7.55 mm for Cp#4 were observed for thymoquinone (TQ - positive control) (Table 3). BCSO dilutions in DMSO of 1:10 and 1:20 had a clear zone of inhibition against Cp#6 for all oil products except for product E. Only products A and B of dilutions 1:10 and 1:20 had a clear zone of inhibition against Cp#4. At a higher dilution of 1:30, BCSO products A and B had a clear zone for Cp#6, whereas both products did not have antimicrobial activity against Cp#4. Based on the product labels, all the BCSOs except E (1.5% TQ) contain ca. 0.95% TQ. By calculation, the tested BCSO dilutions of 1:10, 1:20, and 1:30 should have contained ca. 19, 9.5, and 6.3 μg TQ/disk, respectively. BCSO products A and B, therefore, possessed strong antimicrobial activity against *C. perfringens* strain Cp#6 at a concentration of 6.3 μg TQ/disk and against strain Cp#4 at 9.5 μg TQ/disk. Based on the zone of inhibition obtained from antimicrobial testing results, oil product A exhibited the highest antibacterial activity against both *C. perfringens* strains and, thus, was chosen for GC-MS analysis and animal trials.

Table 4. Chemical composition of black cumin seed oil product A analyzed by GC-MS.

RI[1] (min)	Area %[2]	Compounds Identified
4.30 ± 0.05[a]	2.54	Cymene, p-cymene, m-cymene, o-cymene
7.04 ± 0.27	50.78	Thymoquinone, duroquinone, α-pinene, α-terpinene, isoterpinolene, 2-carene, α-fenchene, sabinene, γ-elemene, δ-eiemene, germacrene b, camphene, allo-ocimene, isopseudocumenol, dimethyloctatriene, 2,3-dimethylanisole, benzene, benzyl alcohol, 1,5,5-trimethyl-6-methylene-cyclohexene
8.22 ± 0.05	2.20	Carvacrol, thymol, 2,3,4,6-tetramethylphenol, methoxymesitylene
11.18 ± 0.65	21.7	(+)-Valencene, 5-decene, cyclodecene, 2-hydroxy-6-methoxyacetophenone, 1-(2,4-dihydroxy-3-methylphenyl) ethanone, p-tert-butylcatechol, cis-δ4-tetrahydrophthalimide, 5-nitro-m-xylene, methyl carbanilate, [s-(e,e)]-3,8-dimethyldeca-4,6-diene, 2-chloro-2-methylpentane, hexadecylene oxide
12.62 ± 0.73	18.95	Nerolidol, (R)-(+)-β-citronellol, citronellyl formate, 1-penatdecyne, 1-tridecyne, 1-eicosyne, 7-dodecenol, 1-hexadecyne, 1-heptadecyne, methyl linolelaidate, 1,3-diethylallene, cis-hexahydroindan, 1,2-dimethylcyclohexene, cis-9-tetradecen-1-ol, fleximel, 2-hydroxycyclopentadecanone, chloromethyl 7-chlorododecanoate, tetradec-13-enal

[a]Mean ± S.D. is average of similarly identified compounds.
[1]The retention index (RI) is average of RI of similarly identified compounds.
[2]The area percentages (Area %) are the sums of Area% of similarly identified compounds.

GC-MS Analysis

The components in BCSO product A were identified by GC-MS analysis (Table 4). The identified major components in BCSO represented 96.17% of the total composition of the product. The major compounds with potential antimicrobial activities in BCSO were p-cymene, thymoquinone, α-terpinene, carvacrol, and thymol. Other compounds such as duroquinone, germacrene B, nerolidol, (R)-(+)-β-citronellol, and camphene also were present in the product. Thymoquinone was quantified since it is one of the major antimicrobial compounds present in BCSO. TQ was measured to be 2.13 ± 0.07% in BCSO A, which is more than the 0.95% TQ claimed on the label by product A.

Table 3. Anti-*C. perfringens* activity of commercially available *Nigella sativa* oil (BCSO) products.

Black cumin seed oil products	Inhibition zone (mm) for BCSO dilutions tested against Cp#6[1]			Inhibition zone (mm) for BCSO dilutions tested against Cp#4		
	1:10	1:20	1:30	1:10	1:20	1:30
A	16 ± 2.12[a,2]	9.5 ± 0[a]	7.5 ± 0.71[a]	11 ± 0.71[a]	8.5 ± 0.71[a]	0 ± 0[a]
B	13.75 ± 0.35[a,b]	7.75 ± 0.35[b]	7 ± 0[a]	9 ± 0[b]	7 ± 0[b]	0 ± 0[a]
C	8.25 ± 1.06[c]	7.75 ± 0.35[b]	0 ± 0[b]	0 ± 0[c]	0 ± 0[c]	0 ± 0[a]
D	10.25 ± 0.35[b,c]	7.75 ± 0.35[b]	0 ± 0[b]	8.25 ± 0.35[b]	0 ± 0[c]	0 ± 0[a]
E	0 ± 0[d]	0 ± 0[c]	0 ± 0[b]	0 ± 0[c]	0 ± 0[c]	0 ± 0[a]

[a,b,c]Different letters in the same column indicate significant difference ($P < 0.05$), Tukey's test.
[1]*C. perfringens* strain Cp#6 and strain Cp#4.
[2]Mean ± S.D. is average of 3 replicates, with TQ (3.2 μg/disk concentration) as positive control (zone of 12.35 ± 0.34 mm against Cp#6, zone of 7.55 ± 0.11 mm against Cp#4) and DMSO as negative control.

Table 5. Effect of supplementation of *Nigella sativa* oil in feed against strain Cp#6 on feed intake (trial 1).

	Total cage feed intake (FI) (kg)			
Treatment	Days 11−18	Days 0−18	Days 11−28	Days 0−28
1. No additive, no Cp	3.10 ± 0.31[a,1]	4.85 ± 0.46[a]	8.49 ± 0.48[a]	10.23 ± 0.58[a]
2. No additive, Cp	3.04 ± 0.18[a]	4.61 ± 0.32[a]	8.15 ± 0.38[a]	9.72 ± 0.44[a]
3. BMD 50 g/t, Cp	3.07 ± 0.24[a]	4.65 ± 0.30[a]	8.24 ± 0.59[a]	9.82 ± 0.65[a]
4. BCSO 1 mL/kg, Cp	3.09 ± 0.25[a]	4.82 ± 0.38[a]	8.18 ± 0.66[a]	9.90 ± 0.72[a]
5. BCSO 2 mL/kg, Cp	2.96 ± 0.08[a]	4.68 ± 0.43[a]	8.23 ± 0.36[a]	9.94 ± 0.50[a]
6. BCSO 5 mL/kg, Cp	2.89 ± 0.21[a]	4.50 ± 0.26[a]	7.80 ± 0.63[a]	9.40 ± 0.63[a]

[a]The same letter in the same column indicats no significant difference ($P > 0.05$), Tukey's test.
[1]Mean ± S.D. is average of 8 cages.

Table 6. Effect of supplementation of *Nigella sativa* oil in feed against strain Cp#6 on weight gain (trial 1).

	Body weight gain (BWG) (kg/bird)			
Treatment	Days 11−18	Days 0−18	Days 11−28	Days 0−28
1. No additive, no Cp	0.27 ± 0.03[a,1]	0.41 ± 0.05[a]	1.00 ± 0.09[a]	1.14 ± 0.11[a]
2. No additive, Cp	0.22 ± 0.01[b]	0.36 ± 0.01[a]	0.94 ± 0.09[a]	1.08 ± 0.09[a]
3. BMD 50 g/t, Cp	0.24 ± 0.02[a,b]	0.39 ± 0.03[a]	0.99 ± 0.10[a]	1.13 ± 0.11[a]
4. BCSO 1 mL/kg, Cp	0.23 ± 0.01[b]	0.37 ± 0.02[a]	0.93 ± 0.09[a]	1.07 ± 0.09[a]
5. BCSO 2 mL/kg, Cp	0.24 ± 0.02[b]	0.37 ± 0.03[a]	0.94 ± 0.05a	1.07 ± 0.06[a]
6. BCSO 5 mL/kg, Cp	0.24 ± 0.03[a,b]	0.37 ± 0.04[a]	0.96 ± 0.07[a]	1.09 ± 0.08[a]

[a,b]Different letters in the same column indicate significant difference ($P < 0.05$), Tukey's test.
[1]Mean ± S.D. is average of 8 cages.

Table 7. Effect of supplementation of *Nigella sativa* oil in feed against strain Cp#6 on feed conversion ratio (trial 1).

	Feed conversion ratio (FCR = FI:BWG)			
Treatment	Days 11−18	Days 0−18	Days 11−28	Days 0−28
1. No additive, no Cp	1.44 ± 0.03[a,b,1]	1.50 ± 0.06[a]	1.44 ± 0.09[c]	1.47 ± 0.09[a,b]
2. No additive, Cp	1.74 ± 0.09[a]	1.60 ± 0.10[a]	1.61 ± 0.03[a]	1.57 ± 0.05[a]
3. BMD 50 g/t, Cp	1.58 ± 0.10[b,c]	1.50 ± 0.10[a]	1.46 ± 0.03[c]	1.44 ± 0.05[b]
4. BCSO 1 mL/kg, Cp	1.66 ± 0.13[a,b]	1.64 ± 0.12[a]	1.58 ± 0.09[a,b]	1.58 ± 0.09[a]
5. BCSO 2 mL/kg, Cp	1.58 ± 0.12[b,c]	1.60 ± 0.18[a]	1.48 ± 0.07[b,c]	1.51 ± 0.09[a,b]
6. BCSO 5 mL/kg, Cp	1.51 ± 0.08[c]	1.52 ± 0.11[a]	1.49 ± 0.08[b,c]	1.50 ± 0.07[a,b]

[a,b,c]Different letters in the same column indicate significant difference ($P < 0.05$), Tukey's test.
[1]Mean ± S.D. is average of 8 cages.

Live Performance of Broiler Chickens

The same basal diet was used for both animal trials. The composition and nutrient contents shown in Table 2 meet the requirement for Cobb 500 broiler chicken growth (www.cobb-vantress.com)

Trial 1. Live performance data for feed intake (**FI**), body weight gain (**BWG**), and feed conversion ratio (**FCR**) for trial 1 are summarized in Tables 5−7, respectively. No significant difference in FI was observed among treatments ($P > 0.05$) for the 11-to-18-, 0-to-18-, 11-to-28-, and 0-to-28-d periods (Table 5).

In terms of BWG for the 11-to-18-d period, BCSO treatments exhibited a similar BWG as the antibiotic and positive controls (Table 6). There was no significant difference ($P > 0.05$) in BWG among all the treatments for 0-to-18-, 11-to-28-, and 0-to-28-d periods.

During the 11-to-18-d period, there was no significant difference ($P > 0.05$) in FCR between BCSO treatments and antibiotic control (Table 7). No significant difference ($P > 0.05$) in FCR was observed among treatments for 0-to-18- d period, whereas for 11-to-28-d period, FCR for BCSO-treated groups was higher than antibiotic (treatment 3) and negative (treatment 1) controls, with the 1 mL/kg BCSO treated group having the most negligible feed conversion difference ($P < 0.05$). There was no significant difference ($P > 0.05$) between the BCSO treated groups (2 and 5 mL/kg) and the BMD control during the 11-to-28- d period. Additionally, over the timeframe of 0-to-28- d, negative (treatment 1) and antibiotic (treatment 3) controls had the best FCR and there was no significant difference ($P > 0.05$) between the BCSO treated groups (2 and 5 mL/kg) and the BMD control, indicating that the inclusion levels of 2 and 5 mL/kg BCSO were as effective as the antibiotic.

Trial 2. Live performance data in terms of FI, BWG, and FCR for trial 2 are summarized in Tables 8−10, respectively. During the 14-to-21- d period, feed intake of BCSO treated groups, antibiotic (treatment 3) and positive (treatment 2) controls were not significantly different, and FI of the antibiotic control was similar to that of the negative control (treatment 2) (Table 8). Feed intake for the 0-to-21- d period in BCSO treatment groups was greater than positive control (treatment 2) and lower than the antibiotic-treated group, but there

Table 8. Effect of supplementation of *Nigella sativa* oil in feed against strain Cp#4 on feed intake (trial 2).

	Total cage feed intake (FI) (kg)			
Treatment	Days 14–21	Days 0–21	Days 14–28	Days 0–28
1. No additive, no Cp	3.60 ± 0.28[a,1]	6.32 ± 0.46[a,b]	7.14 ± 0.37[a]	9.86 ± 0.46[a]
2. No additive, Cp	3.22 ± 0.11[b]	5.89 ± 0.31[b]	3.87 ± 0.34[d]	6.54 ± 0.46[c]
3. BMD 50 g/t, Cp	3.48 ± 0.48[a,b]	6.68 ± 0.69[a]	6.16 ± 1.05[b]	9.36 ± 1.16[a]
4. BCSO 2 mL/kg, Cp	3.25 ± 0.34[b]	6.41 ± 0.47[a,b]	5.23 ± 1.20[c]	8.39 ± 1.09[b]
5. BCSO 5 mL/kg, Cp	3.23 ± 0.24[b]	5.98 ± 0.45[b]	3.81 ± 0.39[d]	6.56 ± 0.55[c]

[a,b,c,d]Different letters in the same column indicate significant difference ($P < 0.05$), Tukey's test.
[1]Mean ± S.D. is average of 8 cages.

Table 9. Effect of supplementation of *Nigella sativa* oil in feed against strain Cp#4 on weight gain (trial 2).

	Body weight gain (BWG) (kg/bird)			
Treatment	Days 14–21	Days 0–21	Days 14–28	Days 0–28
1. No additive, no Cp	0.31 ± 0.03[a,1]	0.52 ± 0.05[a]	0.83 ± 0.07[a]	1.04 ± 0.09[a]
2. No additive, Cp	0.19 ± 0.02[d]	0.40 ± 0.03[d]	0.56 ± 0.19[c]	0.77 ± 0.19[c]
3. BMD 50 g/t, Cp	0.25 ± 0.03[b]	0.47 ± 0.05[b]	0.66 ± 0.10[b,c]	0.87 ± 0.12[b,c]
4. BCSO 2 mL/kg, Cp	0.20 ± 0.03[c,d]	0.42 ± 0.03[c,d]	0.68 ± 0.08[b,c]	0.90 ± 0.10[a,b,c]
5. BCSO 5 mL/kg, Cp	0.21 ± 0.03[c,d]	0.42 ± 0.05[c,d]	0.68 ± 0.21[b,c]	0.90 ± 0.22[a,b,c]

[a,b,c,d]Different letters in the same column indicate significant difference ($P < 0.05$), Tukey's test.
[1]Mean ± S.D. is average of 8 cages.

Table 10. Effect of supplementation of *Nigella sativa* oil in feed against strain Cp#4 on feed conversion ratio (trial 2).

	Feed conversion ratio (FCR = FI:BWG)			
Treatment	Days 14–21	Days 0–21	Days 14–28	Days 0–28
1. No additive, no Cp	1.43 ± 0.05[d,1]	1.51 ± 0.11[d]	1.40 ± 0.08[d]	1.46 ± 0.09[d]
2. No additive, Cp	2.18 ± 0.16[a]	1.85 ± 0.09[a,b]	2.11 ± 0.14[a]	1.86 ± 0.12[a,b]
3. BMD 50 g/t, Cp	1.75 ± 0.04[c]	1.81 ± 0.17[a,b,c]	1.57 ± 0.16[c]	1.66 ± 0.14[c]
4. BCSO 2 mL/kg, Cp	1.94 ± 0.12[b]	1.89 ± 0.08[a]	1.95 ± 0.18[b]	1.90 ± 0.13[a]
5. BCSO 5 mL/kg, Cp	1.93 ± 0.18[b]	1.77 ± 0.09[b,c]	1.89 ± 0.12[b]	1.75 ± 0.08[b,c]

[a,b,c,d]Different letters in the same column indicate significant difference ($P < 0.05$), Tukey's test.
[1]Mean ± S.D. is average of 8 cages.

was no significant difference ($P > 0.05$) between 2 mL/kg BSCO and the BMD antibiotic control. Significantly higher ($P < 0.05$) feed intake was observed among treatments for the 14-to-28- d period, excluding the positive control (treatment 2) and BCSO 5 mL/kg treatment. Over the 0-to-28- d period, feed intake in treatment groups was lower than the negative control and the antibiotic-treated group, but the birds fed 2 mL/kg BCSO had the greatest feed intake among treatments, which was higher than the positive control (treatment 2) with no additive and inoculated with Cp.

For the 14-to-21- and 0-to-21- d periods, BWG of BCSO treated groups was significantly different ($P < 0.05$) compared to antibiotic control, whereas there was no significant difference ($P > 0.05$) in BWG of BCSO treatments and antibiotic control for the 14-to-28- d period (Table 9). Weight gains during the 0-to-28- d period for chickens treated with BCSO of 2 and 5 mL/kg were all relatively higher than positive (treatment 2) and antibiotic (treatment 3) controls but not statistically different ($P > 0.05$) from treatment 3. Broiler performance, especially in weight gain, was improved with BCSO as a supplement compared to non-medicated when there was a Cp infection (d 14-to-28) in broiler chickens.

FCR of BCSO treated groups for the 0-to-21-d period was not significantly different ($P > 0.05$) from the antibiotic-treated group, whereas FCR of BCSO treated groups for 14-to-21- and 14-to-28- d periods was significantly lower ($P < 0.05$) than the positive control, but less than the antibiotic control; there was no significant difference ($P > 0.05$) between BCSO treatments (Table 10). By 0-to-28- d, FCR for BCSO of 5 mL/kg treatment was lower than the positive control but similar to antibiotic control (BMD treatment), and 2 mL/kg BCSO treatment had the poorest feed conversion ratio. There was no significant difference ($P > 0.05$) between the FCR of the BMD control and 5 mL/kg BCSO, suggesting a 5 mL/kg BCSO diet is as effective as the antibiotic diet.

Lesion Score and Mortality Rates

Trial 1. The NE scores for BCSO treated groups were ≤ 0.21 in Table 11, which was lower than 0.38 of antibiotic control (treatment 3) and significantly lower ($p < 0.05$) than 0.54 of Cp positive control (treatment 2). All BCSO treated groups had mortality rates lower than the 7.8% of positive control (treatment 2). BCSO of

Table 11. Effect of supplementation of *Nigella sativa* oil in feed against strain Cp#6 on necrotic enteritis (NE) lesion scores and mortality in broiler chickens (trial 1).

Treatment	NE score (0−3)	% NE mortality
1. No additive, no Cp	$0.0 \pm 0^{c,1}$	0.0 ± 0^{b}
2. No additive, Cp	0.54 ± 0.17^{a}	7.81 ± 6.47^{a}
3. BMD 50 g/t, Cp	$0.38 \pm 0.21^{a,b}$	$1.56 \pm 4.42^{a,b}$
4. BCSO 1 mL/kg, Cp	$0.21 \pm 0.17^{b,c}$	$4.69 \pm 6.47^{a,b}$
5. BCSO 2 mL/kg, Cp	$0.21 \pm 0.35^{b,c}$	0.0 ± 0^{b}
6. BCSO 5 mL/kg, Cp	$0.17 \pm 0.25^{b,c}$	$1.56 \pm 4.42^{a,b}$

[a,b,c]Different letters in the same column indicate significant difference ($p < 0.05$), Tukey's test.
[1]Mean ± S.D. is average of 8 cages.

Table 12. Effect of supplementation of *Nigella sativa* oil in feed against strain Cp#4 on necrotic enteritis (NE) lesion scores and mortality in broiler chickens (trial 2).

Treatment	NE score (0−3)	% NE mortality
1. No additive, no Cp	$0.0 \pm 0^{c,1}$	0.0 ± 0^{c}
2. No additive, Cp	1.17 ± 0.40^{a}	51.56 ± 8.01^{a}
3. BMD 50 g/t, Cp	0.50 ± 0.36^{b}	1.56 ± 4.42^{c}
4. BCSO 2 mL/kg, Cp	0.96 ± 0.55^{a}	31.25 ± 17.68^{b}
5. BCSO 5 mL/kg, Cp	$0.88 \pm 0.64^{a,b}$	35.94 ± 4.42^{b}

[a,b,c]Different letters in the same column indicate significant difference ($p < 0.05$), Tukey's test.
[1]Mean ± S.D. is average of 8 cages.

2 mL/kg had no NE mortality, and 5 mL/kg was similar to that of antibiotic control (1.6%), but black cumin seed of 1 mL/kg group had higher mortality of 4.7% compared to antibiotic control. Overall, BCSO treatment at concentrations of 2 and 5 mL/kg successfully reduced *C. perfringens* infection and mortality to <1.6% comparable to the antibiotic treatment, with no adverse impact on broiler live performance. Therefore, BCSO concentrations of 2 and 5 mL/kg were selected for further testing in trial 2.

Trial 2. Lesion scores in BCSO treated groups (2 and 5 mL/kg) for trial 2 (Table 12) were lower than 1.17 of the positive control but slightly higher than 0.50 of the antibiotic control, and there was no significant difference ($P > 0.05$) between 5 mL/kg BCSO treatment (0.88) and the antibiotic treatment. Mortality rates of all treatment groups were lower ($P < 0.05$) than the positive control, which had the highest mortality of 51.6%. However, BMD-treated chickens had the lowest NE mortality rate of 1.6% which was more effective than 31.3% mortality rate of BCSO 2 mL/kg and 35.9% of BCSO 5 mL/kg treatments ($P < 0.05$). In conclusion, BCSO was found effective in reducing NE lesion scores and NE mortality to <35.9% compared with the positive control (treatment 2) but was less effective in reducing mortality than BMD treatment.

Analysis of Cecal Samples of Broiler Chickens

Physical parameters such as moisture content, pH, and electrical conductivity of cecal samples collected at d 18 and 28 for trial 1 (Table S1) and d 21 and 28 for trial 2 (Table S2), were measured. For both trials, cecal samples had a moisture content ranging from 81.17 to 83.14%, pH of 6.55 to 6.95, and electrical conductivity of 2.47 to 3.33 ms/cm with no significant trends observed. Results indicated BCSO did not alter these physicochemical properties of cecal contents.

For trial 2, microbiological analyses (Cp vegetative cells, Cp spores, and TBC) were conducted for cecal samples collected at d 21 and 28 (Table S3). Results indicated no significant difference ($P > 0.05$) in TBC which were in the range of 9.81 to 9.95 $\log_{10}$ CFU/g among the control and BCSO treatment groups for cecal samples. No significant trend was observed with Cp vegetative cells for cecal samples. Cecal samples on d 21 and 28 declined in the number of Cp spores with increasing concentrations of BCSO and there was a significant reduction ($P < 0.05$) of both spores and vegetative cells of Cp in BCSO treatment groups on d 28 as compared with the positive control (treatment 2). With BCSO treatments, both Cp vegetative cells and Cp spore populations were lower or significantly lower ($P < 0.05$) than BMD and positive control.

DISCUSSION

Plant-derived extracts are natural, low-cost, and environment-friendly and are thought to be ideal feed additives, in contrast to the use of synthetic chemicals in food animal production (Wang et al., 1998). Several studies have evaluated the benefits of essential oils and plant extracts, including *N. sativa* oil, on broiler growth performance and meat quality. However, no studies have evaluated the effect of supplementing feed with *N. sativa* oil for broiler chickens challenged with NE. In previously published animal studies, basal diet supplemented with BCSO was tested in the range of 0.05 to 1% for nutritional and antimicrobial evaluation purposes (Attia and Al-Harthi, 2015; Siddiqui et al., 2015; Ghaly et al., 2017; Demirci et al., 2019). In the current study, basal diet supplemented with BCSO concentrations of 1, 2, and 5 mL/kg (0.1%, 0.2%, and 0.5%) were investigated. Product A BCSO exhibited the highest antibacterial activity against Cp in vitro and thus was chosen for the animal trials. Further, the antimicrobial testing results indicate that strain Cp#4 was less sensitive to BCSO than strain Cp#6.

Black cumin seed comprises essential (volatile) and fixed (stable) oils, and the active components in the volatile oils confer several beneficial health effects to this herb. Khalid and Shedeed (2016) reported components in black cumin essential oil such as p-cymene (59.5−60.3%), α-thujen (6.9−7.2%), β-pinene (2.4−2.6%), γ-terpinene (3.5−3.8%), terpinen-4-ol (2.12.5%), thymoquinone (3.0−3.3%) and carvacrol (2.4−2.7%). These results are slightly different from those reported by Dinagaran et al. (2016), who reported that linoleic acid (13.48%) followed by palmitic acid (9.68%), p-cymene (2.54%), thymoquinone (1.86%), and cs-7-dodecen-1-yl

acetate (1.11%) as the major components of black cumin essential oil. Results of the current study indicated components such as thymoquinone, carvacrol, and p-cymene were present in the BCSO product A, which agrees with the above studies. However, owing to the qualitative nature of the analysis, further quantitative investigation needs to be done for accurate identification and determination of the concentration of individual compounds.

Thymoquinone was identified in the BCSO in this study. Thymoquinone is one of the major antimicrobial compounds in *N. sativa* and the concentration of thymoquinone was stated on the product label. However, in the present study 2.13 ± 0.07% of TQ was identified in BCSO product A, which is more than the claim (0.95% TQ) made but is similar to the above-stated studies. The difference in percentage compositions of the main constituents detected in *N. sativa* seeds between the studies may be due to the different extraction methods used to obtain the essential oil, seed cultivar, storage conditions, soil chemistry and structure, growing conditions, irrigation levels, and other factors specific to the region of growing. For example, Lutterodt et al. (2010) reported 0.35 to 0.87% TQ in cold-pressed BCSOs, which is lower than the TQ concentration of the cold-pressed oil product used in the present study.

The administration of *Eimeria* spp. oocysts in the NE challenge model was to predispose birds for the effective infection by Cp. The introduction of the clinical NE challenge was successful in this study, based on the observation of typical signs of clinical NE such as depressed live performance, lesions in the small intestine, and NE-related mortality. The disrupted nutrient absorption and utilization inflicted by damaged intestinal mucosa due to *Eimeria* and Cp infections could be responsible for reduced BWG and impaired FCR in the positive control in this study and as reported by other studies (Attia et al., 2012; Xue et al., 2018). The reduced FI in Cp challenged birds is believed to correlate to the immune system activation by cytokines, which can reduce FI and lower BWG in infected birds (Dantzer, 2004). The improvement in BWG and FCR and a significant increase in FI in chickens fed with antibiotics confirm the overall positive impact of antibiotic supplementation, as illustrated by several other studies and in this study (Ocak et al., 2008; Crisol-Martínez et al., 2017). It is generally agreed that antibiotics suppress the action of *Eimeria* to decrease the intestinal damage caused by oocytes, thus reducing the chance of birds being predisposed to NE.

The current poultry challenge studies revealed that supplementation of BCSO as PFA improved BWG and FCR of broilers inoculated with Cp isolates. The best FCR noted was in broiler chickens fed diets supplemented with 2 and 5 mL/kg BCSO in trial 1 and 5 mL/kg BCSO in trial 2 suggesting growth-promoting feed additives impact on FCR could be related to more efficient use of dietary nutrients, which in turn results in an improved FCR. This could be attributed to the greater BWG in treatment with 2 and 5 mL/kg BCSO as noted in trial 2, which might be due to the presence of bioactive components in the essential oil such as thymoquinone, carvone, carvacrol, and terpineol. These compounds have antimicrobial activities against pathogenic bacteria and parasites in the gut resulting in better feed utilization (Arslan et al., 2005; Guler et al., 2007). Previous data regarding the effects of black cumin seeds on BWG and FCR of broilers have been conflicting. In a study by Guler and Ertas (2006), supplementation of a diet with grounded black cumin seeds at the level of 10 g/kg (1%) improved weight gain and FCR. Another study (Nasir and Grashorn, 2010) did not demonstrate consistent results on weight gain and FCR in broilers fed diets containing grounded 10 g/kg black cumin seeds. In the current study, positive effects were seen in broiler performance after dietary administration of BCSO at 2 and 5 mL/kg than the previous studies. The differences in studies might be due to the adverse effects of components that are variable in BCSO and black cumin seeds such as volatile oils, saponin, alkaloids, and other antinutritional elements. Halle et al. (1999) examined the effects of diets supplemented with oilseed (10 or 50 g/kg) of black cumin and noted no positive impact on broilers' growth performance. The possible reason could be that a high concentration of the medicinal plant seed bioactive components affected both pathogenic and beneficial commensal microbes leading to counter production. Also, the inconsistencies among the studies may be due to differences in basal diet formulations, chickens, the virulence of Cp strains, inoculation time, duration of the study, and the quality and quantity of active components in the black cumin seeds or extracted oil.

For both animal trials in the current study, 2 and 5 mL/kg BCSO clearly reduced the NE lesion scores and mortality rate as compared with positive control but was less effective in reducing NE mortality than BMD treatment in trial 2. For the positive control (treatment 2), the NE mortality rate in trial 2 was significantly higher than that of trial 1, probably due to the difference in virulence of Cp strains and the physiological status of coccidia. The Cp strains, Cp#6 (trial 1) and Cp#4 (trial 2), resulted in different levels of NE severity in broiler chickens. This could also be explained by the antimicrobial activity results of the current study, which indicated that BCSO was more effective against Cp#6 than Cp#4. According to Thompson et al. (2006), strain Cp#6 is avirulent, and strain Cp#4 is virulent, as assessed by its ability to cause NE, thus leading to high mortality in the positive control group of trial 2. Both strains produced clinical NE; however, Cp#4 had a more severe impact on performance, resulting in impaired FCR and decreased BWG and FI. The effect of Cp#4 on lesion scores in the jejunum was significantly higher than Cp#6 in challenged birds. The results of this study indicate that Cp strains with different levels of virulence introduce different levels of NE severity in the chickens by compromising gut health, leading to poorer bird performance.

Extracellular toxins produced by different Cp strains are classified as A to G toxinotypes. All toxinotypes of the Cp strains produce alpha-toxin; however, the strains

that produce NetB toxin are classified as toxinotype G (Rood et al., 2018). The NetB toxin is pore-forming and is produced by most strains isolated from necrotic lesions but is less commonly found in Cp isolates from healthy birds (Keyburn et al., 2010). Although NetB is known for causing NE, other genomic regions have been identified that contribute to the virulence of the Cp strains (Parreira et al., 2017). The mechanism of virulence of Cp in inducing NE is still poorly understood but it has been known that different strains possess different levels of virulence.

Earlier studies on NE suggested that the alpha-toxin of *C. perfringens* is involved in the pathogenesis of the intestinal lesions in NE (Lovland et al., 2004; Kulkarni et al., 2007). However, a recent study by Keyburn et al. (2008) described NetB as a virulence factor likely involved in the pathogenesis of NE, although the importance of NetB in immunity to NE has not been demonstrated. In contrast, a few studies have reported that immunization of broiler chickens with antibodies to alpha-toxin provides a good degree of protection against NE. In agreement with Cooper and Songer (2010) the significant differences in NE scores and mortality caused by Cp#6 (alpha-toxin positive and NetB negative) and Cp#4 (alpha-toxin positive and NetB positive) in this study inferred that NetB production may be a significant factor in pathogenesis, but it is not an obligate requirement for NE virulence. Also, it emphasizes that other predisposing factors allow *C. perfringens* type A (strain Cp#6 in this study) to compete effectively in the gut and produce tissue damage consistent with NE.

The extracellular toxins such as alpha-toxin and NetB toxin produced by Cp can disrupt the phospholipid bilayer in cells causing a change in osmotic balance, resulting in cell lysis, and affecting the ability of birds to uptake nutrients (Uzal et al., 2014). Gharib-Naseri et al. (2019) reported that different strains of Cp affect the growth performance differently through modulating gut microbiota and immunity of chickens under challenge due to the difference in toxins produced, and the current study demonstrated similar effects. In Gharib-Naseri et al. (2019), the more virulent strains had a severe impact on growth performance and negatively affected the microbiota more than the less virulent strains. However, more detailed investigations on the mechanism that underlies the virulence and how the Cp strains differ in their impact on the gut health and integrity are needed.

In the current study, another reason for the significant difference in NE scores and mortality rate between the 2 trials was that *E. maxima* oocysts were administered on different dates in both studies since the timing of the chicks received for the trials was different. In the first trial, chicks were inoculated earlier than the usual timing used for the NE model; hence the birds were infected on DOT 11 so that the Cp for the following week could be prepared by the lab and be administered on d 5, 6, and 7 after infection of the coccidia. In the second trial, coccidial oocysts were administered on DOT 14 because the birds were inoculated at the usual time for the disease model, and Cp was administered 5-, 6-, and 7-d postcoccidial infection. *E. maxima* used for trial 2 was freshly passed from the host, which may be more virulent than the coccidia used in trial 1. In addition to use of a more virulent Cp stain (Cp#4), all the above changes may contribute to the higher NE scores and mortality of broiler chickens observed in trial 2 and the observed effectiveness of BCSO treatments.

There was no significant trend ($P > 0.05$) for the microbiological results in trial 2 with Cp vegetative cells and total bacterial counts for cecal samples, but BCSO treatments significantly reduced ($P < 0.05$) Cp spore counts during Cp infection. Owing to the wide distribution of *C. perfringens* in the poultry production environment, the culture-based methods used in this study were not effective in differentiating the inoculated Cp from indigenous *C. perfringens* in cecal samples. A real-time PCR assay needs to be explored to further investigate the origin of the *C. perfringens* in the birds.

It is well understood that intestinal microbiota and their metabolic activities considerably impact broiler health and performance. Gut microbiota are vital regulators of immune functions and inflammatory responses during disease outbreaks, playing a crucial role in the occurrence and severity of the disease. Growth promoters in the gut may not evoke any effect in the absence of an enteric challenge (Bedford, 2000) and unlike the antibiotics, PFA may require some time to bring any shift in gut microbiota (Hernandez et al., 2004). Plant extracts and essential oils were reported to improve broiler performance in other studies which corroborates the present findings (Osman and El-Barody, 1999; Hernandez et al., 2004). PFA are believed to decrease the production of growth depressing microbial metabolites such as ammonia and biogenic amines (Windisch et al., 2008), select for healthier microbial groups (Castillo et al., 2006) and increase nutrient availability to the host (Anderson et al., 1999). Changes in gut microbial groups also change the pattern of short-chain fatty acids concentrations in the gut which in turn affects intestinal function and integrity (Meimandipour et al., 2010). Overall, results from the current study indicates BCSO effectively improved live performance parameters during infection and reduced NE lesion scores and mortality but was less effective in reducing mortality than BMD treatment, especially when NE was more severe.

A limitation of the current study was the lack of consistency between the 2 animal trials conducted as 2 Cp strains with different levels of virulence were used. *E. maxima* oocysts of different host passages were administered on different dates in both the animal trials since there was a difference in the timing in which the chicks were gavaged for the trials. Further, the animal trials were conducted at different times to encompass such parameters as the effect of storage on volatile oxidation products in BSCO formulated feed and seasonal changes resulting in variations in experimental conditions.

CONCLUSIONS

Results of this study revealed that BCSO effectively reduced NE lesion scores and mortality compared with infected control but was less effective in reducing mortality compared with the antibiotic BMD treatment when NE infection was more severe. Also, broiler performance, especially in weight gain, was improved with BCSO supplementation when there was Cp infection in broiler chickens. BCSO may take a longer time to improve broiler health than BMD treatment. The broilers infected with strain Cp#4 and strain Cp#6 had subclinical NE; Cp#4 impaired performance and caused more severe disease outcomes than Cp#6. The impact of Cp strains on the disease and performance of the broiler chickens is probably due to the influence on the intestinal bacterial community dynamics and the changes in specific bacterial groups by Cp challenge which may impact gut health under certain conditions; this needs further investigation. However, the virulence of Cp strain is an important factor to consider while conducting an animal trial. Overall, it can be inferred that *N. sativa* as a phytogenic additive may be a potential replacement for commonly used in-feed antibiotics like BMD to enhance broiler performance, especially when the birds are at the risk of NE.

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DISCLOSURES

The authors have no conflicts of interest to declare.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2023.102831.

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