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Research Article
Impact of Dietary Supplementation of Whole Flaxseed and Flaxseed Meal to Infected Broiler Chickens with *Eimeria tenella*

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Abstract

Background and Objective: Coccidiosis is the major parasitic disease affecting poultry industry. Drugs and live vaccines are the two main control measures for the disease. However, due to the prolonged drug usage and the high cost of vaccines, alternative strategies are needed for more effective and safer control of coccidiosis in chickens. This experiment was conducted to determine the effects of supplementation broiler diets with source of n-3 fatty acids as 10% grinding whole flaxseed (WF) or flaxseed meal (FM) during coccidian infection. **Materials and Methods:** A total of 121 days old Cobb chicks were randomly allocated to 4 treatments, 3 replicates containing 10 chicks in each replicate. The chickens in groups 1 and 2 were fed corn-soybean based diet (negative and positive control), chickens in group 3 were fed control diet supplemented with 10% WF and chickens in group 4 were fed 10% FM. Diets were fed from 8 days of age throughout 33 days of age. Chickens were inoculated by gavage with 40,000 sporulated oocysts of *Eimeria tenella* (*E. tenella*) at 21th day of age. Growth performance, oocysts count, cytokines and cecal histopathological changes were determined at the days 28 and 33 of age. **Results:** Feeding broilers in group 4 with 10% FM produced higher body weight (BW), body weight gain (BWG), plasma levels of interleukin (IL)-6 and tumor necrosis factor (TNF-α) with lower sporulated oocysts count ($p<0.05$), scores of histopathological lesions and parasitic densities when compared with other groups. **Conclusion:** Dietary supplementation of infected broilers with 10% FM improved growth performance, reduced oocyst excretion during oocysts shedding period and diminished the severity of *E. tenella* infection.

Key words: Whole flaxseed, flaxseed meal, coccidiosis, cecum, growth performance

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Avian coccidiosis is a worldwide intestinal disease caused by an intracellular protozoan parasite of the genus *Eimeria*. It was considered to be one of the most economically important diseases of domestic poultry due to morbidity and mortality losses[1]. There were various species of *Eimeria*, *E. tenella*, *E. necatrix*, *E. brunetti*, *E. praecox*, *E. acervulina*, *E. imitis* and *E. maxima*[2]. *E. tenella* was the most common pathogenic species affecting poultry industry resulting in extensive damage of the digestive tract[3,4].

Polyunsaturated fatty acids (PUFA) of the omega-3 (n-3) family, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found abundantly in fish oil and α-linolenic acid (ALA), being a major component of flaxseed oil. Several investigations such as those conducted by Allen *et al*.[5-8] and Allen and Danforth[9] showed that feeding diets rich with n-3 fatty acids from menhaden oil expressing flaxseed oil and flaxseed significantly reduced cecal lesions caused by *E. tenella* in broiler chickens from 1-21 days of age. It has been reported that omega-3 fatty acids possess anti-inflammatory or less inflammatory properties by decreasing the release of pro-inflammatory eicosanoids and cytokines[10].

Oil seeds such as flax are usually incorporated into poultry diets due to their nutritional value such as metabolizable energy and crude protein (CP) content. Flaxseed contains about 34% oil and a high content of ALA (>50%), which makes it a common feed ingredient for n-3 fatty acid enrichment of animal products[11]. Flaxseed meal (FM) contains much lower oil and ALA compared with full fat flaxseed and also contains some anti-nutritional factors such as mucilage, linatine and cyanogenic glucosides[12]. These compounds reduced enzymatic secretion from the pancreas and thereby decreased digestion and lowered bird performance[13,14]. The objective of this study was to determine the effect of dietary supplementation with 10% whole flaxseed (WF) or FM on growth performance, oocysts count and cecal lesions in infected broiler chickens with *E. tenella*.

MATERIALS AND METHODS

Experimental birds and dietary treatments: The WF and FM used in the current study were analyzed for gross energy (GS), CP, total lipid (%) and fatty acids at Oregon State University, Department of Animal Science (Table 1). The WF and FM were grind well to easy mix with the other ingredients. A total of 121 day old Cobb chicks were used. All the broiler chicks were fed a starter control diet from 1-7 days of age. At 8th day of age, the chicks were weighed and randomly allocated into four groups of equal body weights (BW), each of 30 chicks, in 3 replicates each of 10 chicks/cage (150×100 cm). The experiment was started from late August until first of October, 2016. The chicks were fed experimental starter diets from 8-14 days of age, experimental grower diets were fed from 14-28 days of age then experimental finisher diets were fed from 28-33 days of age (Table 2). The experimental isonitrogenous isocaloric corn-soybean meal based diets were supplemented with 3% corn oil for groups 1 and 2 (positive and negative control groups), 10% WF for group 3 and 10% FM for group 4. The chicks were not vaccinated against diseases. At day 21 (just before inoculation with *E. tenella*) and day 33 of the experiment, all of the experimental broilers were weighed and feed intake was measured. Body weight gain (BWG) and feed conversion ratio (FCR) (g feed g^{-1} gain) were calculated.

Table 1: Chemical composition and fatty acids (%) of FM and WF

Nutrient composition	FM	WF
CP (%)	34.30	23.00
GE (kcal kg^{-1})	4120.00	6000.00
NDF (%)	27.70	25.20
Total lipid (%)	2.45	28.85
Fatty acids (%)		
Palmitic acid (16:0)	9.54	5.56
Stearic acid (18:0)	2.76	4.15
Oleic acid (18:1)	23.33	18.52
Linolenic acid (18:2n6)	19.78	15.16
ALA (18:3n3)	43.07	55.65
Total saturated	12.50	9.80
Total monounsaturated	24.21	18.86
Total n-3	43.23	55.65
Total n-6	20.06	15.68

WF: Whole flaxseed, FM: Flaxseed meal, CP: Crude protein, GE: Gross energy, NDF: Neutral detergent fiber, ALA: α-linolenic acid

Infection with *Eimeria* and assessment of fecal oocysts: Before inoculation of the chickens with sporulated oocysts of *E. tenella*, necropsy was run on 3 chicks per each group to make sure that the birds were free from any kind of coccidiosis. The chickens in groups 2-4 were infected at the 21th day of age by oral inoculation with 40,000 sporulated oocysts of *E. tenella* per 1 mL for each chick using graduate adjustable insulin syringe introduced directly into the crop[15]. Samples of fecal material were collected from each cage tray (pool of three samples per tray in each replicate cage) at 6, 7, 8, 9, 10, 11, 12th day post infection (PI) and number of oocysts were assessed using a McMaster-chamber method[16]. The number of oocysts excreted was expressed as number of oocysts per gram of original sample and calculated according to the following equation:

$$\text{Number of oocysts g}^{-1}\text{ faeces} = \text{Number of oocysts in 2 chambers} \times 50$$

Table 2: Ingredients (%) and proximate composition of the experimental diets

	Experimental diets[1]					
	Grower			Finisher		
Ingredients (%)	Control	WF10%	FM10%	Control	WF10%	FM10%
Corn grain (8.5%)	60.10	55.00	56.50	66.20	60.40	61.50s
Soybean meal (44%)	27.60	28.00	17.00	22.00	22.10	10.90
Corn gluten (62%)	5.50	2.00	8.00	5.50	2.80	9.40
Whole flaxseed	0.00	10.00	0.00	0.00	10.00	0.00
Flaxseed meal	0.00	0.00	10.00	0.00	0.00	10.00
Oil	3.00	1.10	4.50	3.00	1.20	4.50
Lime stone	1.50	1.50	1.40	1.50	1.50	1.50
Dicalcium phosphate	1.70	1.65	1.75	1.30	1.20	1.25
Premix*	0.25	0.25	0.25	0.25	0.25	0.25
Salt	0.30	0.30	0.30	0.30	0.30	0.30
DL-methionine	0.12	0.14	0.11	0.12	0.14	0.11
DL-lysine	0.10	0.10	0.30	0.10	0.10	0.30
Proximate composition (calculated)						
CP (%)	21.17	21.11	21.04	19.01	19.25	19.43
ME (kcal kg^{-1})	3129.00	3122.00	3119.00	3197.00	3197.00	3193.00
Ca (%)	1.02	1.03	1.01	0.90	0.92	0.92
Available P (%)	0.45	0.45	0.45	0.35	0.35	0.35

[1]Control, WF and FM represent corn-soybean meal basal diet (control, positive or negative) or basal diets containing whole flaxseed at 10% (WF10%) or flaxseed meal at 10% (FM 10%),*Vitamins and minerals premix used to cover the required vitamins and minerals per each kilogram diet (Vit. A: 10000 I.U., Vit. D3: 1500 I.U., Vit. E: 10 mg, Vit. K3: 2 mg, Vit. B1: 2 mg, Vit. B2: 5 mg, Vit. B6, 3 mg, Vit. B12: 0.01 mg, Niacin: 27 mg, Folic acid: 1 mg, Biotin: 0.05 mg, Pantothenic acid: 10 mg, Mn: 60 mg, Zn: 50 mg, Cu: 10 mg, I: 0.1 mg, Se: 0.1 mg, Co: 0.1 mg, Fe: 50 mg

Total lipid and fatty acid analysis: About 2 g of WF and FM were taken for total lipid extraction using chloroform: Methanol (2:1) according to the method of Folch *et al.*[17]. Fatty acids methyl esters were prepared from lipid extracts as reported[18] and the analysis of fatty acid composition was performed with an Agilent 6890 gas chromatograph (Agilent Technologies Inc., Palo Alto, CA) equipped with an autosampler, flame-ionization detector and fused-silica capillary column, 30 m × 0.25 mm × 0.2 μm film thickness. Each sample (1 μL) was injected with helium as a carrier gas onto the column programmed for increased oven temperatures (the initial temperature of 110°C was held for 0.5 min, then increased at 20°C min^{-1} to 190°C, held for 7 min and then increased at 5°C min^{-1} to 210°C and held for 8 min). Inlet and detector temperatures were both 250°C. Peak areas and fatty acid percentages were calculated using Agilent Chem Station software. Fatty acid methyl esters were identified by comparison with retention times of authentic standards and were expressed as percentages of total fatty acid methyl esters.

Cytokines measurement: Blood samples were collected at 28th day of the broiler age (7 days of oocyst infection) from the brachial vein of 6 chickens per each experimental group using 3 mL sterile syringes. Plasma samples were centrifuged for 15 min at 1000 rpm at 2-8°C. Supernatant was stored at -20°C until analysis. Interlukin (IL)-1, IL-6 and tumor necrosis factor a (TNF-α) concentration were measured by IL-1, IL-6 and TNF-α kits (GENORISE SCIENTIFIC, INC) according to methods described by Brennan and McInnes[19].

Histopathological examination: At the day 28 and 33 of age, 3 birds/cage (9 birds per each group) were killed. The ceci were removed, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 mm and stained with hematoxylin and eosin (H&E)[20]. Six cecal sections from each chicken were mounted on a single slide and assigned a score for the histopathological lesions according to Korver and Klasing[21] as follows: 0 = No lesions, 1 = Mild inflammatory cell infiltrate in the mucosa with intact epithelium and no submucosal or muscularis involvement, 2 = Extensive mucosal inflammatory cell infiltration and submucosal edema, 3 = As described above in (2) with inflammatory cell infiltration extending into the muscularis and 4 = Destruction of mucosa with necrosis and hemorrhage. Another score was given microscopically for the parasite density (percentage parasitized epithelium) per cross section of the secum on a scale of 0-4: 1 = >0-25%, 2 = >25-50%, 3 = >50-75%, 4 = >75-100% according to Allen and Danforth[9]. The mean score was then determined for each slide.

Statistical analysis: The results were subjected to a one-way ANOVA to test the influence of WF and FM supplementation on growth performance, oocysts count, cytokines and cecal lesions of broilers infected by *E. tenella*. Data were analyzed using statistical SPSS version 20 (SPSS Inc., Chicago, IL, USA). Differences between means in four dietary groups were compared using Duncan's multiple range test. Differences due to dietary treatments were considered significant if p-value for the effect was <0.05.

RESULTS

Chemical composition and fatty acids percentage of the WF and FM were used in the experimental diets were presented in (Table 1). GE of WF (6000 kcal kg^{-1}) was higher than the FM (4120 kcal kg^{-1}). However, CP content was higher in FM (34.3%) than in WF (23%). The ALA and total n-3 fatty acid contents in WF were comparatively lower than those of FM. The calculated nutrient compositions of the experimental diets are shown in (Table 2).

Growth performance: It was observed that the negative control group was healthy showed normal dropping, good appetite and good feathering. The positive control group showed the most severe symptoms, depression, ruffled feather, anorexia and bloody diarrhea. The onset of clinical signs was at the 4th day PI. Growth performance of broiler chickens fed basal isocaloric-isonitrogenous control diets containing 3% corn oil and those supplemented with 10% WF or FM were presented in (Table 3). Prior to coccidian infection, no significant differences were observed in growth performance among treatment groups. Supplementation of the basal diets with 10% FM significantly improved ($p<0.05$) body weight (BW) and BWG at (21-33 days) in group 4 PI with *E. tenella*. However, there was no significant difference in FCR between 10% WF and negative control.

Plasma cytokines: A significant decrease was observed in levels of measured plasma cytokines in negative control group when compared to other treatment groups ($p<0.05$) (Table 4). Levels of IL-6 and TNF-α were the highest in group 4 fed 10% FM ($p<0.05$). However, there was a significant ($p<0.05$) increase in level of IL-1 in positive control group.

Oocysts shedding: The effect of 10% WF or FM dietary supplementation on oocyst numbers in the fecal material was presented in (Table 5). No oocysts were detected in fecal material obtained from the cages of the broiler chickens in negative control group. There were significant increases in oocysts counts among treatment groups starting from the day 6 PI until the day 12 PI ($p<0.05$). The highest fecal oocysts count was detected in the positive control group. During the oocysts shedding period, the infected birds in group 4 showed significant lower peaks of oocysts excretion when compared with the positive control group (Table 5).

Table 3: Effect of inclusion of broiler diets with WF and FM on growth performance of broiler chickens infected with *E. tenella*

	Experimental diets[1]			
Items	Negative control	Positive control	WF 10%	FM 10%
Pre-coccidial infection (8-21 days)				
BW (g) at 8 day	107.00±1.2	107.00±1.4	104.00±1.00	107.30±0.9
BW (g) at 21 day	505.50±10.82	508.00±17.90	504.00±21.66	509.75±19.48
BWG (g)	398.00±10.82	401.50±17.90	400.00±21.66	402.75±19.4
FCR	1.45±0.06	1.43±0.09	1.44±0.10	1.40±0.09
Post-coccidial infection (21-33 days)				
BW (g) at 35 day	1220.50±29.37^{ab}	1177.50±22.22^{ab}	1216.50±25.85^{ab}	1280.25±21.46^{a}
BWG (g) at 21-35 days	715.50±11.55^{b}	669.00±12.14^{c}	717.75±9.39^{b}	770.50±10.00^{a}
FCR	1.80±0.03^{b}	1.90±0.040^{a}	1.92±0.03^{a}	1.83±0.02^{b}

^{a,b}Means within a row with no common superscript differ significantly ($p<0.05$). [1]Control, WF and FM represent corn-soybean meal basal diet (control, positive or negative) or basal diets containing WF at 10% (WF 10%) or FM at 10% (FM 10%)

Table 4: Influence of WF and FM supplementation on cytokines of plasma of broiler chickens infected *E. tenella*

	Experimental diets[1]			
Cytokine	Negative control	Positive control	WF 10%	FM 10%
IL-1 pg mL^{-1}	26.43±0.23^{d}	47.73±0.37^{a}	42.83±0.44^{b}	35.80±0.41^{c}
IL-6 pg mL^{-1}	102.93±93^{c}	125.83±0.44^{a}	111.76±0.39^{b}	124.86±0.46^{a}
α-TNF pg mL^{-1}	38.83±0.44^{d}	50.76±0.39^{c}	52.96±0.54^{b}	60.73±0.37^{a}

^{a,b}Means within a row with no common superscript differ significantly ($p<0.05$). [1]Control, WF and FM represent corn-soybean meal basal diet (control, positive or negative) or basal diets containing WF at 10% (WF 10%) or FM at 10% (FM 10%)

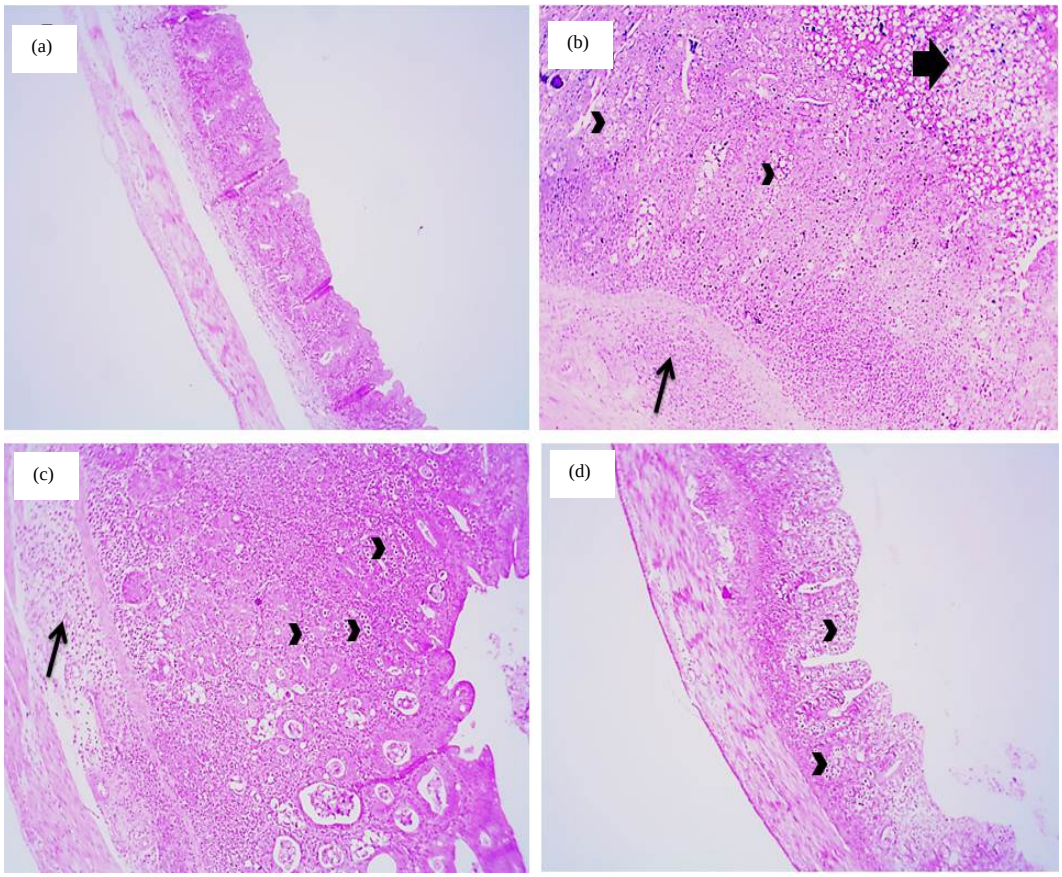

Fig. 1(a-d): Microscopic picture of cecum at age of 28 days shows (a) Score 0 for cecal lesions and parasitic density in control negative group, (b) Score 4 for cecal lesions and parasitic density in control positive group, (c) Score 3 for cecal lesions and parasitic density in group 3 supplemented with 10% WF and (d) Score 2 for cecal lesions and parasitic density in group 4 supplemented with 10% FM. Arrowheads point to parasitic infestation in epithelial cells. Thick arrow points to parasitic overload in cecal lumen. Thin arrows point to inflammatory cells in submucosa. H and E, X: 100

Table 5: Effects of dietary supplementation of WF and FM on oocyst counts in the fecal material* of broiler chickens infected with *E. tenella*

Post infection day	Positive control	WF 10%	FM 10%
6th day	1267±6.5^{a}	1213±5.8^{b}	1107±8.9^{c}
7th day	1396±11.5^{a}	1381±14.49^{b}	1257±10.6^{c}
8th day	1343±8.68^{a}	1314±11.76^{b}	1139±5.86^{c}
9th ay	1224±11.62^{a}	1203±15.5^{b}	904±17.57^{c}
10th day	1166±6.12^{a}	998±8.68^{b}	866±5.7^{c}
11th day	951±5.78^{a}	803±6.95^{b}	659±8.15^{c}
12th day	738±11.55^{a}	658±11.71^{b}	422±9.55^{c}

^{a,b}Means within a row with no common superscript differ significantly ($p<0.05$). [1]Control, WF and FM represent corn-soybean meal basal diet (control, positive or negative) or basal diets containing whole flaxseed at 10% (WF10%) or flaxseed meal at 10% (FM 10%)

Histopathological examination: In this study, the control negative group showed normal histological appearance characterized by normal mucosa, submucosal layer and crypt cells at both days 28 or 33 of age. Meanwhile, *E. tenella* infection in the groups 2-4 produced cecal lesions with variable severity degrees at the day 28. The most severe lesions were detected in the positive control group that scored 4 including: Marked proliferation of epithelial cells of intestinal crypts, dilation and necrosis of submucosal glands, discrete foci of hemorrhage, multifocal areas of severe inflammatory infiltrate at the submucosa, multifocal and discrete interstitial edema at the submucosa and muscular layers. The previous lesions were associated with heavy parasitic density (score 4) characterized by various intra-lesional forms of the parasite transmurally located throughout the mucosa associated with more severe necrosis in the epithelial cells (Fig. 1). In groups 3 and 4, lesser degrees of cecal lesions (scored 3-4) were recognized due to lower

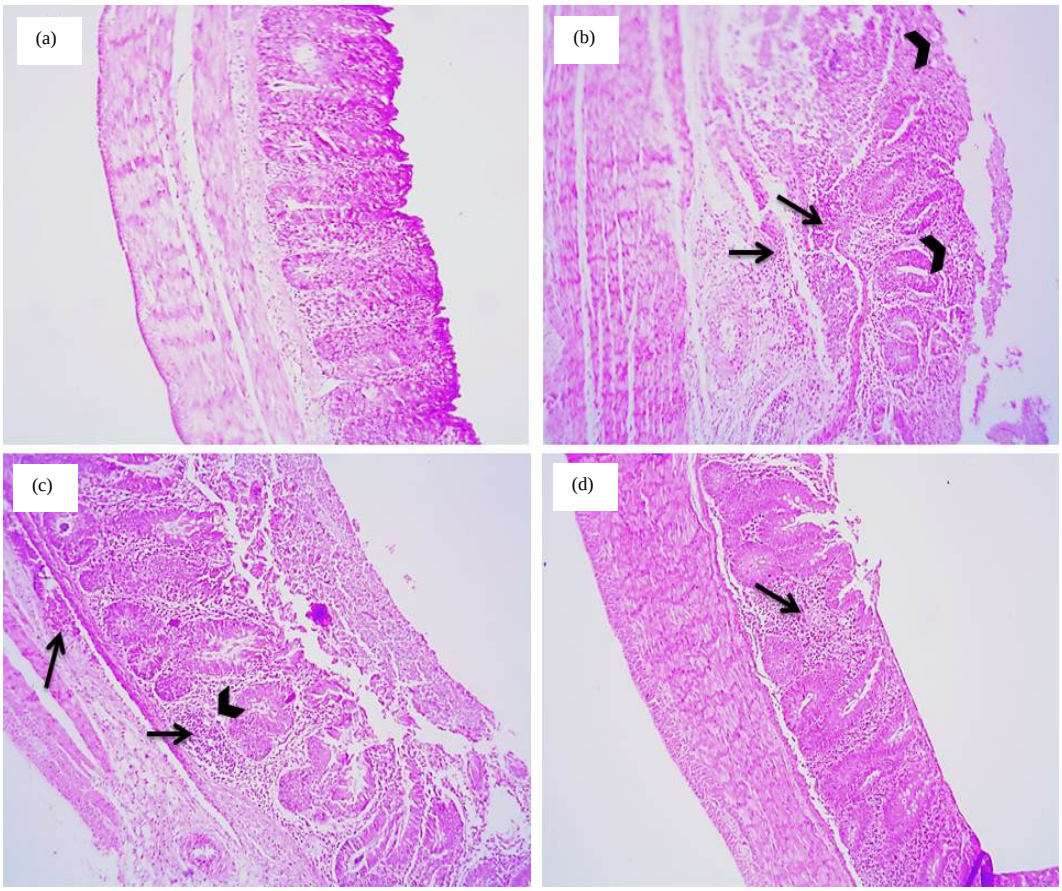

Fig. 2(a-d): Microscopic picture of cecum at age of 33 days shows (a) Score 0 for cecal lesions and parasitic density in control negative group, (b) Score 3 for cecal lesions and score 2 for parasitic density in control positive group, (c) Score 2 for cecal lesions and score 1 for parasitic density in group 3 supplemented with 10% WF and (d) Score 1 for cecal lesions and score 0 for parasitic density in group 4 supplemented with 10% FM (D). Arrowheads point to parasitic infestation in mucosa. Thin arrows point to inflammatory cells aggregations. H and E, X: 100

parasitic density (scored 2-3) (Fig. 1). At the day 33, scores of cecal lesions due to *E. tenella* infection decreased in all infected groups (scored 1-2) due to diminished scores of parasitic density (scored 0-1). The lowest scores of both cecal lesions and parasitic density were identified in group 4 supplemented with 10% FM (Fig. 2). Statistical analysis of scores of cecal lesions and parasitic density in 4 groups at the days 28 and 33 of age was shown in (Fig. 3a, b).

DISCUSSION

Coccidiosis is transmitted among broilers by a fecal to oral route through the ingestion of sporulated oocysts[22]. There were many factors affecting the infectivity of sporulated oocysts including: Number of oocysts present in the litter, chick density, susceptibility of birds and immunogenicity[23]. Feeding diets supplemented with 10% WF or FM showed anti-parasitic activity due to the oxidative stress that is detrimental to parasite development. Thus, the oxidation of the highly unsaturated fatty acids produced high concentration of easily oxidized double bonds that incorporated into the tissues of the host and cells of the parasite[7]. Counting of fecal oocysts was a useful way to determine the level of *E. tenella* infection[24]. In this study, the lowest oocyst shedding was observed in broiler chicken fed 10% FM. In the same trend, fecal oocysts count decreased in the chickens fed diet supplemented with 3% linseed oil throughout the oocysts counting period[25]. Dietary supplementation of 10% FM also improved BW and BWG of the infected broiler chickens with *E. tenella*. On contrary, no protection was exerted against weight gain depression, increased FCR or lesions in cockerels infected with *E. tenella* and fed diet supplemented with different levels (0, 2, 5 or 10%) of stabilized FM[26] observed. Researchers

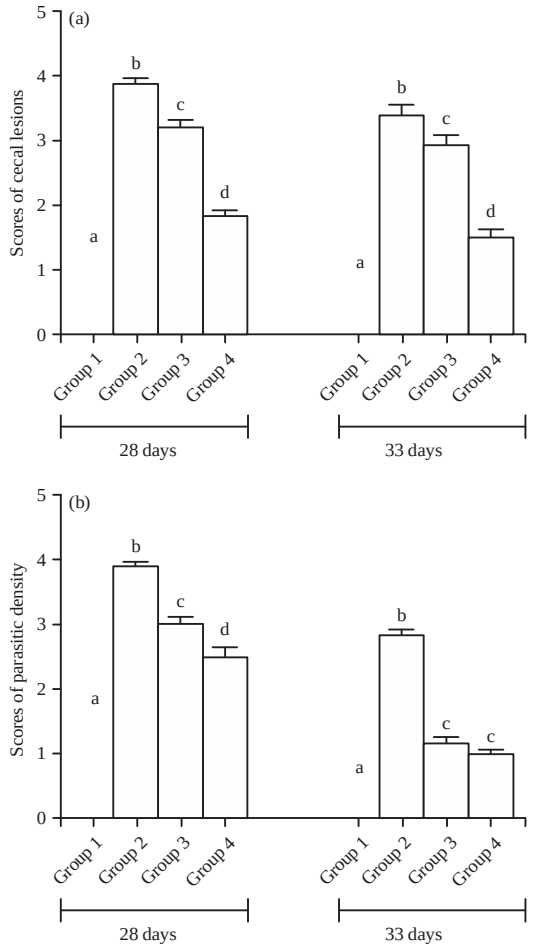

Fig. 3(a-b): Statistical analysis of scores of (a) Cecal lesions and (b) Parasitic density at age of 28 and 33 days to compare between different groups. Small alphabetical letters means significant when ($p<0.05$)

attributed their result to the low levels of linolenic acid and the suppressed oxidative potentials by the added vitamin E. Moreover, there was no significant difference in BW between positive and negative control groups in our study. Previous reports showed that the infection by *E. tenella* did not significantly reduce BW[6]. It has been postulated that *E. tenella* infection that took place in the ceca was not accompanied by a significant reduction in BWG[27].

The cytokines are synthesized and secreted by leukocytes played important regulatory roles during the immune response to infection[28]. Previous studies demonstrated that several pro- and anti-inflammatory cytokines were produced in chickens due to experimental *Eimeria* infections[29,30]. In this study, a significant increase in plasma level of IL-1 was recorded in positive control group as previously reported[25]. Levels of IL-6 and TNF-α were the highest in group 4 fed 10% FM ($p<0.05$). The IL-6 is a necessary component of resistance to primary infections[31]. Fish oil supplementation increased serum levels of IL-6 in infected chickens with *E. tenella* when compared with poultry oil at 25 or 45 g kg^{-1} and corn oil at 25 g kg^{-1} [32].

A number of natural products or feedstuffs have been tested as anti-coccidial dietary additives. Sources of fats containing high concentrations of n-3 fatty acids such as fish oils, flaxseed oil and WF, when added to starter rations and fed to chicks from 1 day of age, effectively reduced lesions resulting from challenge infections with *E. tenella*[6,7] but not *E. maxima*[7]. Diets supplemented with fish oil and flaxseed oil significantly reduced the degree of parasitization and development of *E. tenella*[6]. These results suggested that these diets induced a state of oxidative stress[8]. The pathological changes occurred mainly due to the second generation schizonts[12]. A reduction in parasitic density was associated with lowered caecal lesion scores[33]. Similar finding was noticed in group fed 10% FM in this study. Diets supplemented with 5% fish oil or 5% linseed oil significantly ($p<0.05$) reduced cecal lesions and parasitic density scores caused by *E. tenella*[25]. In addition, diets consisting of broiler starter ration supplemented with 2.5-10% fish oil, 10% flax seed oil or 10% linseed oil showed reduced parasitic invasion and development, significantly decreased cecal lesions and maintained weight gains as compared to un-supplemented diets[6].

CONCLUSION

It can be concluded that the 10% FM has the potential to lower the severity of the infection and at the same time maintain the oocysts production, which is important for the re-infection and the maintenance of the immunity stimulated by the initial infection.

SIGNIFICANCE STATEMENT

This study discovered that dietary supplementation of infected broilers with 10% flaxseed meal improved growth performance, reduced oocyst excretion during oocysts shedding period and diminished the severity of *E. tenella* infection. Flaxseed meal is safe and provides a good source of omega 3 to healthy and infected broilers. Such nutritional management of diet saves costs of mortality, treatment of coccidiosis and improves meat quality of broilers. Further study was recommended to investigate the effect of dietary supplementation of 10% flaxseed meal on coccidiosis in broilers raised on poultry litter which is the most common raising system.

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