Livestock Studies 63(2), 92-99
http://doi.org/10.46897/livestockstudies.1418212

LIVESTOCK STUDIES

RESEARCH PAPER

Growth and Immune Responses, Oxidative Stress Biomarkers, and Antioxidative Enzymes of Broilers Fed with Supplementation of *Chenopodium ambrosioides L.* and *Crassocephalum crepidioides* leaf meals

Andrew Bamidele FALOWO[1,*]

[1]Adekunle Ajasin University, Faculty of Agriculture, Department of Animal Science, Akungba-Akoko, Nigeria.

Article History
Received: 08 June 2023
Accepted:20 December 2023

Corresponding Author
E-mail: andrew.falowo@aaua.edu.ng
Anddele2013@gmail.com
Tel: +2348065320285

Keywords
Broiler
Immune system
Leaf meal
Supplement
Stress biomarkers

Abstract

This study assessed the effect of dietary supplementation of Crassocephalum crepidioides (CCLM) and Chenopodium ambrosioides L. (CALM) leaf meals on relative growth rate, oxidative stress biomarkers, immune response, serum and meat antioxidative enzymes of broilers. A total of 120 one-day-old Cobb 500 chicks were randomly assigned to four dietary treatments (three replicates of 10 birds/treatment) as follows, Diet 1 (basal/control diet), Diet 2 (basal +200mg/kgVitC), Diet 3 (basal +2500mg/kgCCLM) and Diet 4 (basal +2500mg/kgCALM) for 42 days. The result revealed that supplementation of CCLM and CALM significantly reduced ($P < 0.05$) the concentration of heat shock protein and hydroxy-2-deoxyguanosine in broilers compared to the control group. Broilers fed diet containing CCLM and CALM exhibited higher ($P < 0.05$) serum glutathione peroxidase content compared to the control group. The level of immunoglobulins were significantly higher in birds fed diet containing CCLM, CALM and Vit C compared to control group. However, supplementation of CCLM and CALM did not influence ($P > 0.05$) the relative growth rate and antioxidant content of breast muscle compared to control group. It's concluded that CCLM and CALM could be used as natural additive to alleviate oxidative damage, improve immune system and serum antioxidant content of broilers.

Introduction

Commercial poultry is often subject to a range of stressors, from environmental to nutritional factors, that could impair performance during growing period, despite advances in management, genetic selection, and biosecurity [1]. In terms of nutrition, poultry production requires a balanced diet to support the immune system and inhibit cell damage. However, stresses that occur due to nutritional imbalances or deficiency may induce the production of free radicals, which in turn could weaken the immune system, create health problems, and result in significant production-related financial losses [1-3]. In order to avoid this, the inclusion of natural antioxidants in diets has been encouraged [4-5]. This is because natural antioxidants contain inherent bioactive compounds that can activate the production of immunoglobulin [6], increase endogenous antioxidants [7], and inhibit the oxidative process that is caused by an imbalance between the production of reactive oxygen species (ROS) and endogenous antioxidant defense mechanisms in the body [4,8]. In addition, studies have shown that natural antioxidants can be used to improve feed intake, enhance body weight gain, and improve meat quality and the overall performance of poultry during production [7, 9, 10].

Interestingly, most natural antioxidants are obtained from plant materials, such as herbs (flowers, leave, fruits, seed, stem, root, etc.), vegetables, and spices, as well as their extracts such as essential oils [4, 11]. Its utilization has been encouraged in animal diets because they are easily affordable, accessible and safe when used at low concentrations compared to

antibiotic growth promoters [12]. Natural antioxidants possessed enormous phytochemicals such as phenols, flavonoid, alkaloid, tannin, saponin, and other bioactive compounds. Chenopodium ambrosioides L. and Crassocephalum crepidioides plants are among the natural antioxidants with a lot of potential that can be utilized as feed additives in poultry production.

Chenopodium ambrosioides L. is an aromatic herbaceous medicinal/vegetable plant that belongs to the family of Chenopodiaceae and the genus Chenopodium. The plant is extensively found in West Africa, particularly in Nigeria, and is generally known as Mexican tea [13]. In traditional medicine, the leaf of the plant is used to treat uterine fibroids as well as bacterial, fungal, parasitic, and viral diseases [14-15]. According to research by Maldonado-Garcia *et al.* [16] and Reyes-Becerril *et al*. [17], fish fed a diet supplemented with C. ambrosioides L. at 0.5, 1.0, and 2.0% (w/w) had stronger immune cell and antioxidant enzyme activities than fish without the supplements. Crassocephalum crepidioides is a succulent annual leafy vegetable and herb that belongs to the family Asteraceae (Compositae). It is widely distributed in tropical and subtropical regions of the world, including Africa, Asia and Australia [18]. Every part of a plant is known to have abundant secondary metabolites with the ability to elicit antibacterial, hypoglycemic, and antioxidant actions [18-19]. According to a preliminary investigation by Falowo *et al*. [19], both Chenopodium ambrosioides L. and Crassocephalum crepidioides plants are readily available and their leaves possessed moderate nutritional content, high bioactive compounds and antioxidant properties, which qualifies them as potential natural additives. In fact, the dietary supplementation of leaf meal of Crassocephalum crepidioides plant has been reported to improve the performance, immune systems and meat quality of broiler chicken [20]. However, to our knowledge the dietary utilization of Chenopodium ambrosioides L. leaf meal as natural additives on boiler performance has not been studied. Therefore, this study was designed to examine the effect of Chenopodium ambrosioides L. and Crassocephalum crepidioides leaf meals on oxidative stress biomarkers, immune response, serum and meat antioxidative enzymes of broiler chickens.

Materials and Methods

Plant collection and extract preparation

Freshly harvested leaves of Chenopodium ambrosioides L. and Crassocephalum crepidioides were purchased from the commercial markets in southwestern Nigeria. The leaves were cleaned and air-dried in an open shade. The dried leaves were ground using an electric blending machine and the powdered samples were packed into a black polyethylene bag for further analysis.

Experimental site

The study was conducted at the Poultry Unit of the Teaching and Research Farm, Federal College of Agriculture, Akure, Nigeria. The experimental site is located at 7°25' N and 5°19' E with average annual temperature and annual rainfall of 25.3°C and 1455 mm, respectively. The entire study was carried out for six weeks following the research ethics and guidelines of the Animal Health and Prodution Technology Department of the institution.

Experimental diets and animals

Two basal diets [starter (0-21 days) and finisher phase (22-42days)] were formulated to meet the broiler's nutritional requirement as recommended by NRC [21] (Table 1). At each phase, the experimental diets were divided into four treatments and designated as Diet 1 (basal/control diet), Diet 2 (basal + 200mg/kg Vitamin C as positive control), Diet 3 (basal +. 2500mg/kg CALM) and Diet 4 (basal + 2500mg/kg CCLM). One hundred and twenty 1-day-old Cobb 500 unsexed broiler chicks were randomly distributed to four dietary treatments. Each treatment was replicated three times. Thirty birds were assigned to each treatment (10 birds/replicate) in a completely

Table 1. The Experimental Basal Diets' Composition.

Ingredients	Starter feed	Finisher diet
Maize	52.33	59.32
Maize bran	7.02	0.00
Rice bran	0.00	6.03
Fish meal	3.00	3
Soybean meal	30	24
Premix	0.30	0.30
Bone meal	3.00	3.00
Soy oil	3.00	3.00
Methionine	0.30	0.30
Limestone	0.50	0.50
Salt	0.30	0.30
Lysine	0.25	0.25
Analyzed composition (g/kg)		
Crude fibre	3.55	3.63
Crude fat	4.47	3.94
Crude protein	22.19	20.09
Calculated composition (g/kg)		
Calcium	1.02	0.97
Available phosphorus	0.44	0.41
Methionine	0.68	0.65
Lysine	1.36	1.24
Metabolizable energy (Kcal/kg)	3018.93	3108.16

randomized design (CRD). The birds were housed in their respective pen (200 x 100 cm) with the floor covered with wood shavings. The temperature of the house was maintained within 31oC ± 2 for the first 7 days and reduced by 2°C after each consecutive 7 days until the house temperature was 26 ± 2 oC. The feed and water were provided ad libitum throughout the six weeks feeding trial. The lights were left on for 24 hours on the first day, and then reduced by 1 hour until 7th days, and later reduced to 20 hours per day til the end of the feeding trial.

Growth, Oxidative Stress Biomarkers, Immune and antioxidant enzymes analysis

The body weights of broilers were measured every seven days. The relative growth rate (RGR) was calculated using the following formula [22]:

RGR= [(w2-w1)/ ((w1+w2))/2] *100

Where w1= Bodyweight when the trial began, and w2= Bodyweight at the conclusion of the study

At 42 weeks of age, three birds per treatment were randomly selected and humanely slaughtered. Before slaughter, feeds were withdrawn from the birds overnight. During slaughtering, blood samples were obtained from jugular veins into a plain blood sample bottle for antioxidant enzymes (catalase and glutathione peroxidase). The blood sample in each of the plain bottles was centrifuged for 10 min at 3000 rpm to obtain clean supernatant serum. The harvested supernatant serum was kept at -20 °C before analysis. The serum enzymatic activities of the catalase (CAT) and glutathione peroxidase (GPx) activities were determined according to the method of Aebi [21,3] and Rotruck *et al.* [22,4], respectively. Also, the fresh centrifuged serum was used to determine the concentration of immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM) and immunoglobulin E (IgE) of the broiler chickens. The concentrations of IgE, IgM, and IgA were determined by mixing serum with buffer solution and antibody reagents to form an antigen/antibody complexes which, following agglutination, were measured turbidimetrically, using commercial ELISA quantitation kits (Fortress Diagnostics Limited, United Kingdom): IgG (Catalog No BXC0721A), IgM (Cat No BXC0731A), IgE (Cat No BXCF751A) and IgA (Cat No BXC0701A).

The levels of oxidative stress biomarkers were analyzed in the collected serum. The concentration of 8-hydroxydeoxyguanosine (8-OHdG) was determined in the blood serum using a commercial ELISA Kit (Elabscience Biotechnology Inc, USA) with catalog No E-EL-0028 96T, based on the manufacturer's instructions. The level of heat shock protein (HSP)/heat shock factor (HSP) was also determined using ELISA kit, according to the manufacturer's instructions (Bioassay Technology Laboratory, Shanghai, China). For antioxidant meat enzyme analysis, about 100g of fresh meat samples were excised from the breast muscle of broiler chicken after slaughter and evisceration for determination of catalase activity and superoxide dismutase, by the methods described by Hadwan and Khabt [23, 5] and Marhlund and Marklund [24, 6]

Statistical analysis

Data obtained on growth and immune responses, oxidative stress biomarkers and antioxidative enzymes of broilers were analyzed using PROC GLM procedures of Statistical Analysis System (SAS, version 9.1.3 of 2007). Differences in mean values were computed using Duncan's Multiple Range Test for multiple comparisons. For all statistical tests, significance was determined at $P < 0.05$.

Results

The result of the effect of CCLM and CALM supplements on serum oxidative biomarkers of broilers is presented in Table 2. The result revealed significant ($P < 0.05$) differences in heat shock protein (HSP) and 8-Hydroxy-2-deoxyguanosine (8OHDG) content of broilers among the treatments. Broilers fed the diet supplemented with CCLM (0.50pg/mL), CALM (0.46pg/mL) and Vit C (0.48 pg/mL) recorded lower heat shock protein content compared to the control group (0.65 pg/mL). Similarly, the 8-Hydroxy-2-deoxyguanosine content was significantly lowered in broiler chicken fed diet supplemented with CCLM (95.06 ng/mL), CALM (83.01 ng/mL) and Vit C (81.82 ng/mL) compared to the control group (122.06 ng/mL).

Table 3 shows the result of the serum antioxidant enzymes of broiler chickens fed diet supplemented with CCLM and CALM. The analysis revealed that broiler chickens fed diet containing CCLM (45.79 mg/ml), CLAM (32.20 mg/ml) and Vit C (46.01 mg/ml) had a higher concentration of glutathione peroxidase

Table 2: Serum oxidative stress biomarkers of broiler chicken fed diets supplemented with *Chenopodium ambrosioides L. and Crassocephalum crepidioides leaf meal*.

Parameters	Diet 1 Control	Diet 2 200mg/kg Vit C	Diet 3 2500mg/kg CCLM	Diet 3 2500mg/kg CALM	SEM	*P value*
HSP (pg/mL)	0.65^{a}	0.48^{b}	0.50^{b}	0.46^{b}	0.02	0.00
8OHDG (ng/mL)	122.06^{a}	81.82^{b}	95.06^{ab}	83.01^{b}	9.01	0..03

Means within a row with different letters and significantly different ($P < 0.05$). SEM Standard error. CALM = *Chenopodium ambrosioides* leaf meal. HSP = Heat shock protein. 8OHDG= 8-Hydroxy-2-deoxyguanosine

Table 3: Serum antioxidant enzymes of broiler chicken fed diets supplemented with *Chenopodium ambrosioides L. and Crassocephalum crepidioides leaf meal*

Parameters	Diet 1 Control	Diet 2 200mg/kg Vit C	Diet 3 2500mg/kg CCLM	Diet 3 2500mg/kg CALM	SEM	*P value*
GPX (mg/ml)	29.72[b]	46.01[a]	45.79[a]	32.20[b]	2.13	0.01
Catalase (mg/g)	135.01	160.78	160.94	170.53	9.27	0.13

Means within a row with different letters and significantly different (*P* < 0.05). SEM Standard error. CALM = *Chenopodium ambrosioides* leaf meal, GPX = Glutathione peroxidase, LDH = Lactate dehydrogenase

compared to the control group (29.72 mg/ml) (P < 0.05). The concentration of lactate dehydrogenase was significantly higher (P < 0.05) in broiler chickens fed diet containing CALM compared to other treatments. However, there was no significant influence (p > 0.05) of supplemented diets (CCLM and CALM) on the catalase content of the broiler chicken compared to the control group.

Table 4 shows the result of the immune response of broiler chicken fed the diet supplemented with CCLM and CALM. The results revealed significant differences (P< 0.05) in the value of immunoglobulin A (IgA) and M (IgM) across the experimental treatments. Broiler chicken fed diet supplemented with CCLM (277.55 mg/dl), CALM (259.65 mg/dl) and Vit C (253.79 mg/dl) had higher IgA content compared to the control group (209.58). Similarly, broiler chicken fed a diet containing CCLM, CALM and Vit C had higher IgM content at 382.92, 456.41 and 365.12 mg/dl respectively, compared to the control group (357.51 mg/dl). However, the result did not show any significant effect of supplemented diets (CCLM and CALM) on immunoglobulin E (IgE) and G (IgG) of the broiler chicken across the experimental treatments.

As presented in Table 5, supplementation of CCLM and CALM did not significantly influence the meat antioxidant content (catalase and superoxide dismutase) of the broiler chicken (P > 0.05) across treatments. The result of the effect of CCLM and CALM supplements on relative growth rate of the broilers is shownin Figure 1. The result did not show any significant (P > 0.05) effect of CCLM and CALM supplements on relative growth rate of the broilers compared to control group in both the starter and finisher phases of the feeding trial.

Discussion

Oxidative stress biomarkers

Natural antioxidants are commonly used in animal nutrition to promote growth, enhance endogenous antioxidants, inhibit oxidative stress, and boost the immune response of broilers [4, 6]. In this study, supplementation of CCLM and CALM significantly decreased the expression of heat shock protein in the broilers compared to the control. HSPs are specific proteins that are produced by cells in response to a variety of oxidative stressors such as oxidants, toxins, heavy metals, free radicals, and microbes [27]. They are produced as molecular chaperones to ameliorate the adverse effect of oxidative stress during animal production [28]. HSps have been implicated in a diverse range of cellular activities, including senescence, endoplasmic stress, cell death signaling and inflammation [29]. The result of this study is suggesting that CCLM and CALM possessed the ability to attenuate the production of HSPs and alleviate oxidative damage in the cell. Different studies have shown that the dietary supplementation of natural antioxidants that is rich in phytochemicals can be used to modulate or inhibit the expression of heat shock protein in broilers under heat stress [30-32]. Conversely, the observed reduction in serum concentration of 8-Hydroxy-2-deoxyguanosine (8OHDG) of broiler chicken fed the diet supplemented with CCLM and CALM is further buttressing the ability of the CCLM and CALM to inhibit oxidative damages in poultry during production. Many 8-Hydroxy-2'-deoxyguanosine (8-OhdG) are produced in the cells as a product of DNA damage or oxidation, which is caused

Table 4: Immune response of broiler chicken fed diets supplemented with *Chenopodium ambrosioides L. and Crassocephalum crepidioides leaf meal.*

Parameters (mg/dl)	Diet 1 Control	Diet 2 200mg/kg Vit C	Diet 3 2500mg/kg CCLM	Diet 3 2500mg/kg CALM	SEM	*P value*
IgA	209.58[b]	253.79[a]	277.55[a]	259.65[a]	11.70	0.001
IgE	1082.15	1075.88	1077.37	1100.25	25.67	0.88
IgG	303.24	318.50	344.67	318.58	20.91	0.73
IgM	357.51[b]	365.12[b]	382.92[b]	456.41[a]	9.57	0.00

Means within a row with different letters and significantly different (*P* < 0.05). SEM Standard error. CALM = *Chenopodium ambrosioides* leaf meal, IgA =Immunoglobulin A, IgE= =Immunoglobulin E, IgG =Immunoglobulin G, IgM =Immunoglobulin M

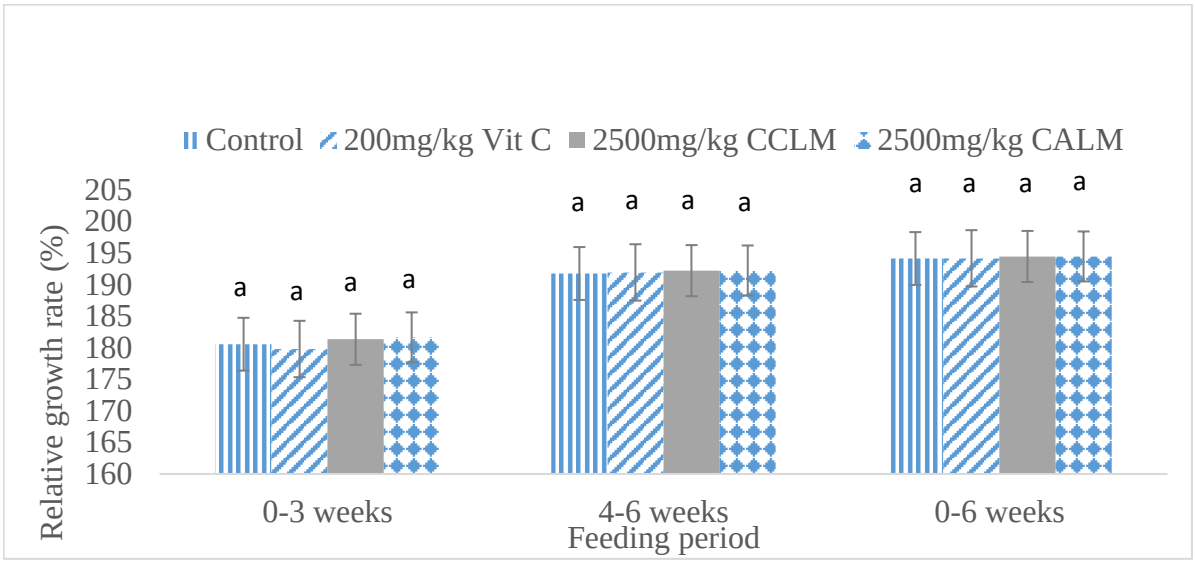

Figure 1: The effects of *Chenopodium ambrosioides L. and Crassocephalum crepidioides leaf meal* on the relative growth rate of broilers.

by hydroxyl radical, superoxide, hydrogen peroxide, singlet oxygen or direct photodynamic action [33]. This result is similar to the findings of Prima *et al*. [34] who found that broiler chickens fed the diet supplemented with olive leaves and marigold petal extract significantly reduce the plasma concentration of 8-Hydroxy-2-deoxyguanosine compared to the control. Liu *et al.* [35] in their study also found that the dietary inclusion of Macleaya cordata extracts significantly reduced hepatic contents of 8-hydroxy-2'-deoxyguanosine in broiler chickens compared to the control group.

Antioxidant enzymes

The result of serum antioxidant enzymes revealed that supplementation of CCLM and CALM significantly increased the concentration of glutathione peroxidase compared to control. Glutathione peroxidase is one of the endogenous enzymatic antioxidants that help to protect the cell against oxidative damage by inactivating or scavenging or removing free radicals and other reactive oxygen species in cells [36]. This result is in agreement with the findings of Adeyeye *et al.[*36] who reported a significant increase in glutathione peroxidase content of broiler chickens fed the diet supplemented with wild sunflower and goat weed leaf meals composite-mix compared to the control group. The increase in GPX in diets supplemented with CCLM, CALM and Vit C is indicating that they could exert higher antioxidative activity against oxidative damage in broiler chicken compared to the control. However, the observed similarity in the value of catalase of the broiler chicken across experimental treatments is in contrast with the report of Adu *et al.* [7] who found a significant increase in catalase content of broiler chicken fed the diet supplemented with Syzygium aromaticum leaf meal compared to control.

Immune response

Besides the potential of plant leaf meal as a natural antioxidant to inhibit oxidative damage and increase endogenous antioxidants, several studies have reported their ability to induce or modulate the immune response and increase immunoglobulin secretion in broiler chicken during production 37-38]. Immunoglobulins (IgA, IgM, IgE and IgG) are produced by plasma cells as part of the body's adaptive humoral immune response against a foreign pathogen [39]. In this study, the observed increase in the immune responses of the broiler chickens has shown that supplementation of CCLM and CALM could be used to stimulate the production of immunoglobulin M and A

Table 5: Meat antioxidant content of broiler chickens fed diets supplemented with *Chenopodium ambrosioides L. and Crassocephalum crepidioides leaf meal.*

Parameters	Diet 1 Control	Diet 2 200mg/kg Vit C	Diet 3 2500mg/kg CCLM	Diet 3 2500mg/kg CALM	SEM	*P value*
Catalase (mM/ml/min)	48.53	48.58	46.91	46.17	0.57	0.15
Superoxide dismutase (%)	91.22	89.75	93.66	94.63	1.88	0.31

Means within a row with different letters and significantly different ($P < 0.05$). SEM Standard error.

during production. Immunoglobulin (Ig) M is known as the first antibody isotype that helps to protect the host against infections and regulate other immune responses and tolerance [39]. While IgA plays an important role in inhibiting macromolecule absorption or binding of allergens to mucosal target cells, neutralizing bacterial toxins and enhancing nonspecific defense mechanisms in animals [41-42]. The result is similar to the findings of Osman *et al.* [43] and Cheng *et al.* [44] who found that boiler chickens fed the diet supplemented with turmeric powder and lotus leaf extract as natural antioxidants, respectively, had higher IgA and IgM concentrations compared to control. On the contrary, this result agreed with the report of Adeyemi *et al.* [20] who found no significant difference in the value of serum IgM of broiler chicken supplement with Crassocephalum crepidioides leaf meat at 1000 and 2000 ppm compared to the control. However, the observed similarity in concentration of IgE and IgG is indicating that the dietary inclusion of CCLM and CALM at 2500mg/kg could not improve their content compared to the control. This result is in contrast with the findings of Osman *et al.* [43] and Yao *et al.* [45] who reported a significant improvement in Immunoglobulins (IgA, IgM, IgE and IgG of chicken supplemented with turmeric powder and sea buckthorn extract as a natural antioxidant, respectively, compared to control.

Meat antioxidant content

The observed similarity in the meat catalase and superoxide dimutase across experimental treatments is indicating that the inability of dietary inclusion of CCLM and CALM at 2500mg/kg to meat antioxidant content of the broiler chicken during production. This result is in line with the report of Adu *et al.* [7] and Gbore *et al.* [46] who found that dietary supplementation of Syzygium aromaticum and moringa leaf meal at 2500mg/kg each, respectively did not increase the catalase content of breast meat of broiler chicken compared to control.

Relative growth rate

The observed similarity in the relative growth rate between the broilers fed leaf meals and control diet is indicating that the utilization of CCLM and CALM at 2500mg/kg could not improve the growth performance of broilers during production. This result is in contrast with report of Oloruntola *et al.* [22] and Adu *et al.* [7] who found that dietary inclusion of plant leaf meal as feed additives at 2500mg/kg significantly increased the growth performance (body weight) of broilers compared to control group.

Conclusion

The findings of the present study reveal the potential of Chenopodium ambrosioides L. and Crassocephalum crepidioides leaf meal to inhibit oxidative damage, enhance endogenous antioxidant enzymes, improve the immune system and maintain the meat quality of broiler chickens when used as a natural antioxidant in the diet. It was shown that the inclusion of CCLM and CALM at 2500mg/kg reduced the production of heat shock protein and Hydroxy-2-deoxyguanosine content, increased the level of serum glutathione peroxidase and catalase content, enhance the production of immunoglobulin IgA and IgM, and maintain the breast meat antioxidant of broiler chicken during production. Further research is required to assess CCLM and CALM effects on the meat fatty acid profile of broiler chickens.

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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