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Dietary supplementation with ZN-loaded montmorillonite: influence on physiological responses and health indicators in broiler chickens

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Abstract

A 35-day feeding trial was conducted to evaluate the effects of zinc-loaded montmorillonite clay (Zn-MMT) on growth performance, physiological responses, antioxidant capacity, immunity, and bone mineralization in Ross 308 broiler chickens. Zn-MMT was prepared by ion exchange of sodium montmorillonite with zinc sulfate solution, yielding 78.5 mg Zn/g. A total of 300 one-day-old male broilers were randomly assigned to five dietary treatments containing 0, 40, 80, 120, or 160 mg supplemental Zn/kg feed, with six replicates of ten birds each. Dietary supplementation with 80–120 mg Zn/kg as Zn-MMT significantly improved final body weight, body weight gain, and feed conversion ratio, while reducing feed intake. Hematological parameters, including erythrocyte count, hemoglobin, packed cell volume, and total leukocyte count, were significantly enhanced ($P < 0.01$). Zn-MMT also increased serum total protein, albumin, and globulin, while decreasing cholesterol, glucose, ALT, and AST levels compared with the control group. Antioxidant enzyme activities, including superoxide dismutase, glutathione peroxidase, and catalase, increased with Zn-MMT supplementation, whereas malondialdehyde levels decreased. Antibody titers against Newcastle disease and infectious bursal disease viruses were highest at 80–120 mg Zn/kg. Tibia ash, Zn, Ca, and P contents also improved, with a breakpoint for tibia Zn deposition estimated at 103.2 mg Zn/kg. Pearson correlation analysis revealed positive relationships among growth performance, immunity, antioxidant status, and bone mineralization. Overall, supplementation with 80–120 mg Zn/kg as Zn-MMT enhanced growth performance and health status in broiler chickens.

Keywords: Zn-MMT; growth performance; antioxidant status; bone mineralization

Introduction

Zinc (Zn) is the most physiologically versatile trace mineral required by broiler chickens that is a structural, catalytic, and regulatory component of over 300 metalloenzymes and over 2000 transcription factors [1]. These enzymes and transcription factors are involved in carbohydrate, protein, lipid and nucleic-acid metabolism, antioxidant defense, skeletal mineralization and immune competence [2]. Given that zinc cannot be stored in large amounts and there are no specific zinc stores in the body, a continuous and adequate supply of zinc in the diet is essential for supporting growth and tissue function of fast-growing modern broiler genotypes [3].

Traditional Zn supplementation in poultry diets predominantly involves the use of inorganic salts of Zn, such as zinc sulphate and zinc oxide [4]. Nevertheless, Zn in poultry diets is supplemented at inclusion levels between 60 to 120 mg/kg of complete feed. However, these easily soluble sources have many practical limitations [5]. The presence of phytate-rich plant ingredients causes their dissociation and absorption to suffer, as do antagonistic interactions with the other divalent cations, copper, iron and calcium [6]. A large amount of the supplemented Zn ends up in the litter. This contributes to environmental loading while also raising waste management costs. Moreover, the quick disintegration of inorganic Zn salts in the proventriculus and gizzard minimizes their presence time within the proximal small intestine, where the key Zn transporters are expressed [7].

Poultry producers around the world are increasingly using mineral clays mainly of the smectite group (montmorillonite, MMT) as multicomponent feed additives [8]. Their unique 2:1 layered aluminosilicate structure, expandable interlayer distance, large specific surface area and high cation-exchange capacity lend adsorptive activity toward mycotoxins, bacterial endotoxins, ammonia and other intestinal irritants, while simultaneously safeguarding gut mucosal integrity and microbial community stability [9]. The interlayer cations of MMT which is generally either Na+ or Ca2+ can be exchanged for Zn2+ meaning the montmorillonite (MMT) will release Zn slowly throughout the GI tract as well as providing the gut stabilisation provided by the clay matrix [10].

While Zn-MMT appears conceptually as a beneficial zinc supplementation for both nutrition and environment, assessment of its effects on the entire physiological network of broiler chickens is still wanting. Earlier investigations were mainly directed toward a single endpoint, which is growth performance, mycotoxin adsorption or mucosal morphology [1]. More rarely attention was paid to the simultaneous response of these parameters with growth, haematology, serum biochemistry, antioxidant status, humoral immunity and bone mineralization in the same experiment [11]. An assessment of the interrelationships among these physiological domains, together determining bird health and productive efficiency, has rarely been made for Zn-MMT delivery [1].

The aim of the present experiment was therefore to (i) evaluate the dose-response of broiler chickens to graded concentrations of dietary Zn-MMT (0–160 mg supplemental Zn/kg), (ii) determine the magnitude of change in a widely used suite of physiological and health-relevant traits, (iii) estimate optimum supplementation levels as determined by linear and quadratic regression, and one-slope broken-line analysis, and (iv) determine inter-relationships among these traits based on Pearson correlation matrices.

Materials and Methods

Zn-loaded montmorillonite (Zn-MMT) was prepared through ion-exchange modification of food-grade sodium montmorillonite (Na-MMT; cation-exchange capacity 92 meq/100 g; particle size <75 μm) [12]. This is Na-MMT dispersion. 100 g of Na-MMT was dispersed in 1 L of deionized water and stirred for 1 h at room temperature to get a homogeneous suspension [13]. Following this, a 0.5 M aqueous solution of zinc sulfate monohydrate was added drop-wise at a Zn:CEC molar ratio of 3:1 and the suspension was kept at 80 °C with continuous magnetic stirring for 4 h [14]. The Zn-MMT product was centrifuged for 15 min at 4,000 × g. It was repeatedly washed with deionized water until the supernatant was free of sulfate (BaCl2 test). The oven-dried at 80 °C for 24 h, ground and sieved through a 75-μm mesh [15].

The total Zn concentration of the fabricated Zn-MMT determined by atomic absorption spectrophotometry after nitric–perchloric acid digestion was 78.5 mg/g (7.85% w/w). The spacing of the basal (d(001)) interlayer of the Na-MMT and Zn-MMT was confirmed as 1.24 nm and 1.46 nm with the aid of X ray diffractions test using Cu-Kα radiation. Our tests thus show that Zn^{2+} intercalation was a success. The Zn-MMT's Brunauer-Emmett-Teller specific surface area is 96.4 m^2/g (compared with 88.7 m^2/g for the parent Na-MMT). Moreover, Fourier-transform infrared spectroscopy data confirmed the characteristic Si–O stretching (1037 cm^{-1}) and Al–OH bending (915 cm^{-1}) bands resulting from the layered silicate framework are retained [1].

Birds, Housing and Experimental Design

Three hundred one-day-old male Ross 308 broiler chicks were sourced from a commercial hatchery and wing-banded individually. Initial body mass of each chick was taken with the value being 42.6 g ± 0.4 g. Moreover, chicks were allocated randomly to five treatments diets depending on a completely randomized design. In addition, there were six replicated floor pens of 10 birds each. Pens (1.0 × 1.2 m) received fresh wood-shaving litter, a tube feeder and a nipple drinker [1]. For the first three days, ambient temperature was maintained at 33 °C subsequently decreased by 3 °C every week finally reaching a temperature of 22 °C. Throughout the trial lasting 35 days, a photoperiod of 23L: 1 D was employed [16]. The chickens were given standard vaccinations against Newcastle disease (ND, day 7 and 21) and infectious bursal disease (IBD, day 14) via drinking water.

Diets and Treatments

The basal starter (1–14 d) and grower–finisher (15–35 d) diets composed was iso-nitrogenous and iso-energetic based on corn and soybean meal ingredients to meet or slightly exceed the nutrient requirements of Ross 308 broilers for all the necessary nutrients except Zn as per Table 1 [17]. The Zn concentration was analyzed to be 31.2 mg/kg of the basal diet in the starter and 28.7 mg/kg in the grower–finisher phase [18]. The basal diet was supplemented with Zn-MMT in 5 experimental diets at inclusion rates of 0, 0.51, 1.02, 1.53 and 2.04 g/kg to provide a nominal supplemental Zn content of 0, 40, 80, 120 and 160 mg Zn/kg of complete feed, respectively (T1, T2, T3, T4 and T5) [10]. As zinc-montmorillonite was given in the basal diet, the addition of Zn-MMT was done restricting its inert carrier (sand) equivalentlyto making iso-nutrient compositions across the treatments. Analysis confirmed that the total dietary Zn concentrations averaged, respectively, 30, 70, 110, 150 and 190 mg/kg in T1–T5 after Zn-MMT addition [1]. During the experiment, animals were allowed free access to feed and water.

Performance Measurements

Body weight and feed intake were recorded weekly on a pen basis. Mortality was monitored daily and dead birds were weighed and recorded. Body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR, corrected for mortality) were calculated for the overall 1–35 d period [19].

Blood Sampling and Hematological Analyses

At the age of 35 days, blood was taken from the brachial vein of two birds per pen (12 birds per treatment) of average pen body weight [20]. One aliquot was placed in EDTA tubes for haematological analysis. Another aliquot was collected in plain tubes, allowed to clot at room temperature, and centrifuged at 3,000 × g for 15 min to obtain serum, which was stored at −20 °C until analysis. Using an automated veterinary hematology analyzer (BC-2800Vet, Mindray, Shenzhen, China), the researcher determined the erythrocyte count (RBC), hemoglobin concentration (Hb), packed cell volume (PCV) and total leukocyte count (WBC) [21]. Manual differential leukocyte counting was performed on Wright-stained blood smears [22].

Serum Biochemistry and Antioxidant Status

The following tests were performed using commercial colorimetric kits (Biolabo SAS, Maizy, France) according to the manufacturer's instructions. They were performed on a UV–Vis spectrophotometer (UV-1900, Shimadzu, Kyoto, Japan). The tests include serum total protein, albumin, glucose, total cholesterol, triglycerides, ALT, and AST [23].

Globulin was worked out as total protein minus albumin. The commercial assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) will determine the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) and concentration of malondialdehyde (MDA) [24].

Humoral Immunity and Lymphoid Organs

The serum antibody titers against ND virus and IBD virus were determined by hemagglutination inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) using commercial kits (BioChek, Reeuwijk, the Netherlands) and expressed as log_2 titers [25]. After the blood was collected, sampled birds were killed humanely by dislocating the neck and the bursa of Fabricius, spleen and thymus were cut off, trimmed of adhered tissue and weighed individually. Relative organ weights were determined as g/100 g body weight [26].

Tibia Mineralization

The right tibia was removed, defleshed by gentle boiling, defatted in petroleum ether for 48 h, and oven-dried at 105 °C to constant weight to determine tibia weight [27]. Tibias were then ashed in a muffle furnace at 600 °C for 6 h. Tibia ash was expressed as a percentage of dry, fat-free bone weight [28]. The ash was acid-digested

(HNO_3:$HClO_4$, 4:1) and Ca, P and Zn were determined by atomic absorption spectrophotometry (AA-7000, Shimadzu, Kyoto, Japan) and colorimetry, as appropriate. Tibia breaking strength was measured using a universal testing machine equipped with a three-point bending fixture [29].

Statistical Analysis

Tests for normal distribution and homogeneity of variances were conducted before proceeding with the analysis. Using pen as the experimental unit (n = 6 per treatment), data were analysed by one-way analysis of variance (ANOVA) through the GLM procedure of SAS 9.4 (SAS Institute Inc., Cary, NC, USA) following the model [30].

$Y_{ij} = \mu + T_i + \varepsilon_{ij}$, here Y_{ij} is an observation, μ overall mean, T_i is the fixed effect of dietary Zn-MMT level (i = 1–5), while ε_{ij} is the residual error [31].

Duncan's multiple range test ($P < 0.05$) was used to separate treatment means as per F-test significance [32]. The linear and quadratic responses to growing Zn-MMT levels were assessed using orthogonal polynomial contrasts. Using PROC NLIN, optimum supplementary Zn requirements were estimated for selected response variables using two methods: (1) quadratic regression ($y = a + bx + cx^2$) and (2) one-slope broken-line regression ($y = L + U(R - x), x \leq R; y = L, x > R$).

Pearson correlation coefficients were obtained using PROC CORR to estimate inter-relationships among the character studied. The significance level was set at $P < 0.05$ whereas $0.05 \leq P < 0.10$ was set to indicate a tendency. The means as well as pooled standard error of the mean (SEM) are shown for all values.

Results and Discussion

The formulations and calculated nutrient compositions of experimental diets, that given to broiler chickens for the duration of the study (table1). The birds were fed starter and grower corn-soya meal-based feeds that were formulated to meet the nutritional need of Ross 308 broilers during the other stages of growth. The basal diets were used as a basis for determining the effect of dietary Zn-MMT supplementation on growth performance, physiological traits, and health status.

Table (1): Ingredient and chemical composition of the basal experimental diets (as-fed basis).

Item	Starter (1–14 d)	Grower (15–35 d)	Item	Starter / Grower
Ingredient (g/kg)			**Calculated composition**	
Yellow corn	548.2	590.5	ME (kcal/kg)	3010 / 3105
Soybean meal (48% CP)	370.0	320.0	Crude protein (g/kg)	225 / 205

Soybean oil	35.0	45.0	Lysine (g/kg)	13.5 / 11.8
Limestone	12.0	11.0	Met + Cys (g/kg)	10.2 / 9.0
Dicalcium phosphate	17.0	15.0	Calcium (g/kg)	9.5 / 8.7
DL-Methionine	2.5	2.0	Available P (g/kg)	4.6 / 4.2
L-Lysine·HCl	2.3	1.5	Analyzed Zn (mg/kg)	31.2 / 28.7
Salt (NaCl)	3.5	3.5		
Vitamin–mineral pre-mix[1]	5.0	5.0		
Choline chloride (60%)	0.5	0.5		
Sand (carrier)[2]	4.0	6.0		
Total	**1000.0**	**1000.0**		

[1] Provided per kg of diet: vitamin A, 12,000 IU; vitamin D_3, 3,000 IU; vitamin E, 50 mg; vitamin K_3, 3 mg; vitamin B_1, 2.5 mg; vitamin B_2, 6 mg; vitamin B_6, 4 mg; vitamin B_{12}, 0.02 mg; folic acid, 1 mg; biotin, 0.10 mg; niacin, 50 mg; pantothenic acid, 12 mg; Cu, 10 mg; Fe, 80 mg; Mn, 100 mg; I, 1 mg; Se, 0.30 mg. The premix supplied no Zn. [2] Sand was reduced equivalently in Zn-MMT-supplemented diets to maintain iso-nutrient composition: 4.00, 3.49, 2.98, 2.47 and 1.96 g/kg in T1–T5, respectively (starter), and 6.00, 5.49, 4.98, 4.47 and 3.96 g/kg (grower).

Growth Performance

Effects of graded levels of dietary Zn-MMT on growth performance of broilers during the 1–35 d period are summarized in Table 2. Increasing Zn-MMT supplementation linearly and quadratically increased final body weight ($P < 0.001$) and BWG ($P < 0.001$), and improved FCR (linear and quadratic, $P < 0.01$). Birds fed 80, 120 and 160 mg Zn/kg as Zn-MMT had statistically similar BWG, all of which were significantly higher than those of T1 (no Zn-MMT) and T2 (40 mg/kg). Feed intake was also increased by Zn-MMT supplementation (linear, $P = 0.011$), although the differences among supplemented groups (T2–T5) were small. Mortality was low (1.0–3.3%) and did not differ among treatments ($P = 0.41$). Quadratic regression of BWG on supplemental Zn ($R^2 = 0.97$) suggested that maximal BWG was reached at 117 mg/kg, while broken-line analysis indicated a breakpoint at 92.4 mg/kg.

Table (2): Effect of dietary Zn-MMT levels on growth performance of broiler chickens (1–35 d).

Item	T1 (0)	T2 (40)	T3 (80)	T4 (120)	T5 (160)	SEM	P-value
Initial BW (g)	42.5	42.6	42.7	42.6	42.5	0.21	0.984
Final BW (g)	1855[c]	2053[b]	2190[a]	2215[a]	2180[a]	15.4	<0.001

BWG (g/bird)	1812[c]	2010[b]	2147[a]	2172[a]	2137[a]	15.2	<0.001
Feed intake (g/bird)	3422[b]	3526[a]	3540[a]	3536[a]	3543[a]	22.8	0.011
FCR (g feed/g gain)	1.85[a]	1.72[b]	1.62[c]	1.60[c]	1.63[c]	0.018	<0.001
Mortality (%)	3.3	1.7	1.7	1.0	1.7	0.65	0.412

Means within a row with no common superscript differ significantly (P < 0.05; Duncan's multiple range test). Values are means of six replicate pens (10 birds/pen). T1–T5: basal diet supplemented with Zn-MMT providing 0, 40, 80, 120 and 160 mg supplemental Zn/kg of feed, respectively. BW: body weight; BWG: body weight gain; FCR: feed conversion ratio; SEM: standard error of the mean.

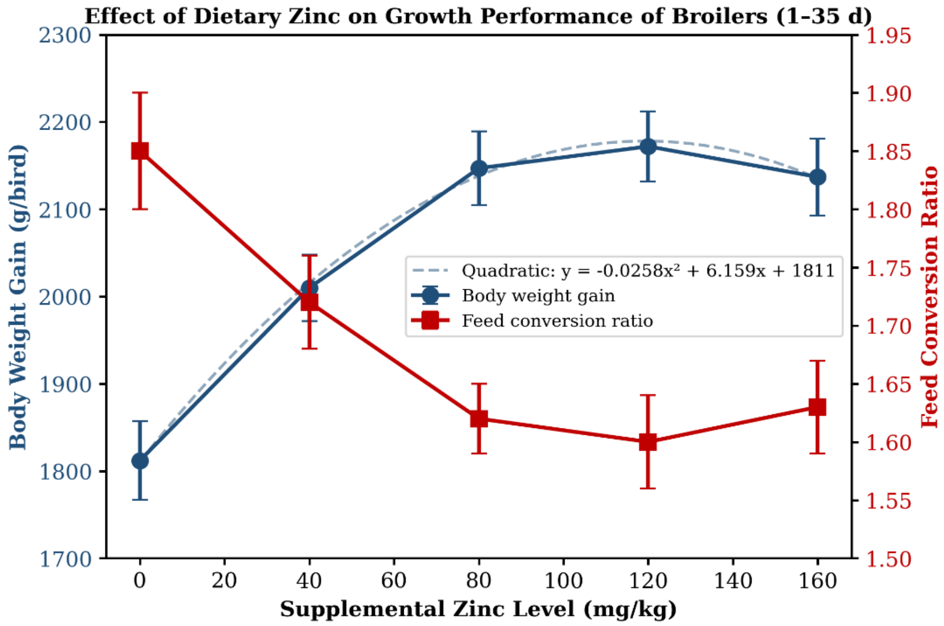

Figure (1): Effect of dietary Zn-MMT level on body weight gain and feed conversion ratio of broiler chickens during the 1–35 d period. Data are means ± SD (n = 6).

Hematological Parameters

Table 3 displays the hematological responses to graded Zn-MMT levels. Erythrocyte count, Hb concentration, and PCV increased linearly and quadratically (P < 0.001) with increasing Zn-MMT level. T1 birds showed lowest values, while T4 had highest values, and T3 and T5 were statistically similar to T4. The leukocyte count identified in the total showed a parallel value (P<0.001) indicating immunostimulant property. The percentage of lymphocytes (P = 0.003) was higher in the supplemented groups than in T1, while the heterophil:lymphocyte (H/L) ratio is an indicator of stress. Zn-MMT supplementation reduced the H/L ratio from 0.62 in T1 to 0.42-0.45 in T3-T5 (P = 0.005).

Table (3): Effect of dietary Zn-MMT levels on hematological parameters of broilers at 35 days of age.

Item	T1 (0)	T2 (40)	T3 (80)	T4 (120)	T5 (160)	SEM	P-value
RBC ($\times10^6/\mu L$)	2.45^c	2.68^b	2.85^a	2.92^a	2.88^a	0.034	<0.001
Hb (g/dL)	8.20^c	9.10^b	9.80^a	10.10^a	9.90^a	0.118	<0.001
PCV (%)	27.5^c	30.2^b	32.5^a	33.1^a	32.8^a	0.45	<0.001
MCV (fL)	112.2	112.7	114.0	113.4	113.9	1.05	0.872
MCH (pg)	33.5	34.0	34.4	34.6	34.4	0.42	0.521
WBC ($\times10^3/\mu L$)	18.5^c	21.2^b	23.4^a	24.1^a	23.6^a	0.31	<0.001
Lymphocytes (%)	58.4^b	63.2^a	66.5^a	67.1^a	66.0^a	0.95	0.003
Heterophils (%)	35.8^a	31.5^b	28.4^c	28.0^c	28.6^c	0.78	<0.001
H/L ratio	0.62^a	0.50^b	0.43^c	0.42^c	0.45^c	0.022	0.005

Means within a row with no common superscript differ ($P < 0.05$; Duncan). Values are means of 12 birds/treatment. RBC: red blood cell count; Hb: hemoglobin; PCV: packed cell volume; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; WBC: white blood cell count; H/L: heterophil:lymphocyte ratio.

Serum Biochemistry

Table 4 indicates that supplementation with Zn-MMT in the diet significantly influenced serum biochemical parameters. The concentrations of total protein, albumin and globulin increased (linear and quadratic, $P < 0.001$), reaching T4 maximum. In contrast, serum glucose, total cholesterol and triglycerides decline linearly with increasing Zn-MMT level ($P < 0.01$) indicating improvements in lipid and glucose metabolism. The hepatic markers ALT and AST activities were lowered ($P < 0.001$) in Zn-MMT supplemented birds as compared to T1 indicating lower hepatocellular stress. The treatment did not affect uric acid and creatinine ($P > 0.10$) suggesting that none of the tested Zn-MMT levels imposes a measurable load on protein catabolism and renal function.

Table (4): Effect of dietary Zn-MMT levels on serum biochemical parameters of broilers at 35 days of age.

Item	T1 (0)	T2 (40)	T3 (80)	T4 (120)	T5 (160)	SEM	P-value
Total protein (g/dL)	3.45^c	3.85^b	4.20^a	4.32^a	4.25^a	0.052	<0.001
Albumin (g/dL)	1.62^c	1.85^b	2.05^a	2.12^a	2.08^a	0.027	<0.001
Globulin (g/dL)	1.83^c	2.00^b	2.15^a	2.20^a	2.17^a	0.031	<0.001
A/G ratio	0.89	0.93	0.95	0.96	0.96	0.018	0.124
Glucose (mg/dL)	232^a	218^b	205^c	202^c	208^c	2.85	<0.001

Total cholesterol (mg/dL)	145[a]	132[b]	121[c]	118[c]	122[c]	1.95	<0.001
Triglycerides (mg/dL)	95.2[a]	88.5[b]	78.4[c]	75.6[c]	79.8[c]	1.78	<0.001
ALT (U/L)	28.5[a]	24.3[b]	21.0[c]	20.5[c]	21.2[c]	0.55	<0.001
AST (U/L)	220[a]	195[b]	175[c]	172[c]	178[c]	2.85	<0.001
Uric acid (mg/dL)	4.85	4.72	4.65	4.58	4.68	0.082	0.412
Creatinine (mg/dL)	0.42	0.40	0.41	0.39	0.40	0.012	0.685

Means within a row with no common superscript differ ($P < 0.05$; Duncan). A/G: albumin:globulin ratio; ALT: alanine aminotransferase; AST: aspartate aminotransferase.

Antioxidant Status

The dosage of Zn-MMT followed clear and consistent dose response serum antioxidant indices. As the dietary Zn-MMT concentration increased, activities of SOD, GSH-Px and CAT increased in a linear manner ($P < 0.001$) while the MDA, an end product of lipid peroxidation, decreased in a linear manner ($P < 0.001$). When compared to T1, providing Zn-MMT supplementation (120 mg Zn/kg) led to increases of 77.2%, 53.6% and 55.1% in the activities of SOD, GSH-Px and CAT, respectively. Also, it reduced MDA by 47.4%. The quadratic model yielding the strongest relationship between the level of Zn-MMT and the antioxidant response was SOD ($R^2 = 0.98$).

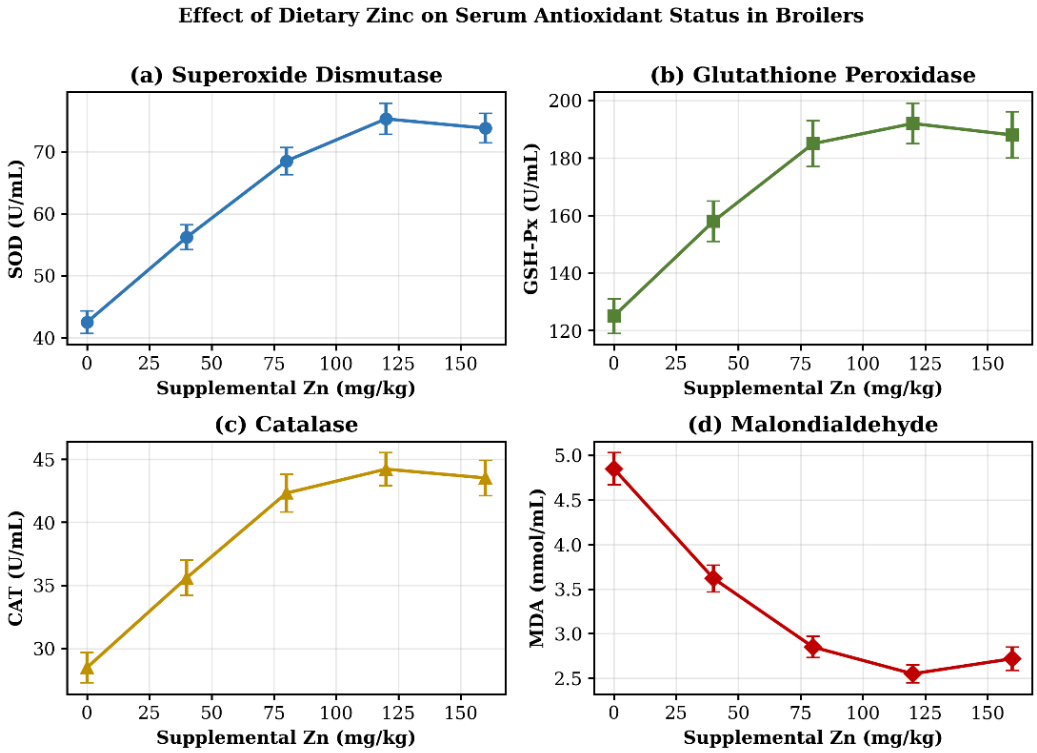

Figure (2): Effect of dietary Zn-MMT on serum antioxidant status of broilers at 35 d: (a) superoxide dismutase, (b) glutathione peroxidase, (c) catalase, and (d) malondialdehyde concentration. Data are means ± SD.

Humoral Immunity and Lymphoid Organs

With Zn-MMT supplementation, the antibody titers against ND virus and IBD virus significantly increased ($P < 0.001$; Figure 3) and peaked at 80 mg Zn/kg. The study revealed that the weights of the bursa of Fabricius, spleen, and thymus increased ($P < 0.01$) at the increased Zn-MMT level (Table 5). The highest value was observed in T4 (120 mg Zn/kg). The heightened weight of lymphoid organs corresponded with growing antibody titers, suggesting that dietary Zn-MMT intensifies both the humoral and structural aspects of the immune system.

Table (5): Effect of dietary Zn-MMT levels on antibody titers and relative weights of lymphoid organs of broilers at 35 days of age.

Item	T1 (0)	T2 (40)	T3 (80)	T4 (120)	T5 (160)	SEM	P-value
ND antibody titer (log_2)	5.20^c	6.50^b	7.80^a	8.10^a	7.90^a	0.115	<0.001
IBD antibody titer (log_2)	4.80^c	5.90^b	7.20^a	7.50^a	7.30^a	0.118	<0.001

Bursa of Fabricius (%)	0.150[c]	0.180[b]	0.210[a]	0.220[a]	0.210[a]	0.0048	<0.001
Spleen (%)	0.100[c]	0.120[b]	0.140[a]	0.150[a]	0.140[a]	0.0042	<0.001
Thymus (%)	0.280[c]	0.320[b]	0.360[a]	0.380[a]	0.360[a]	0.0085	<0.001

Means within a row with no common superscript differ ($P < 0.05$; Duncan). Relative organ weight = (organ weight / body weight) × 100. ND: Newcastle disease; IBD: infectious bursal disease.

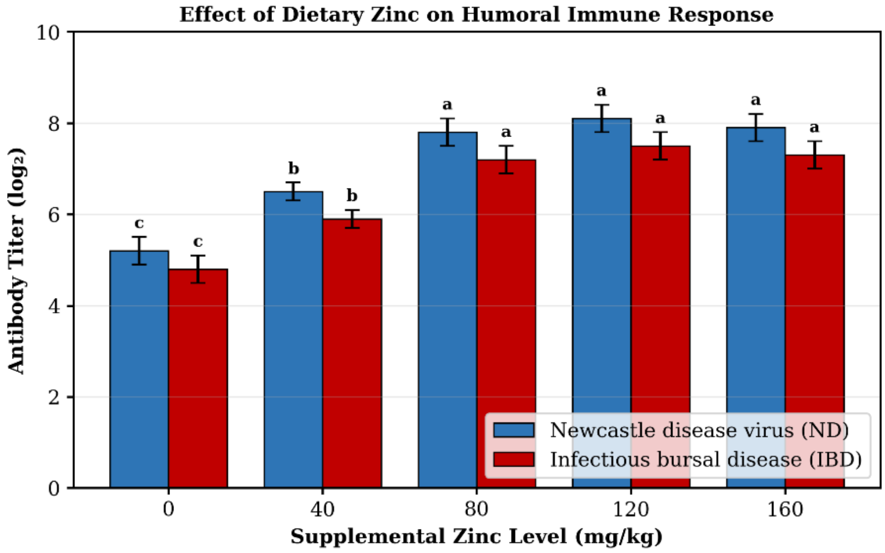

Figure (3): Antibody titers against Newcastle disease (ND) and infectious bursal disease (IBD) viruses in broilers fed graded levels of Zn-MMT. Bars with no common letters within a virus differ ($P < 0.05$). Error bars represent SD.

Tibia Mineralization

Bone responses to dietary Zn-MMT are summarized in Table 6 and Figure 4. Tibia weight, tibia ash, tibia breaking strength, and tibia Ca, P and Zn contents all increased significantly ($P < 0.001$) with increasing dietary Zn-MMT, but the responses were curvilinear and reached a plateau at 80–120 mg supplemental Zn/kg. One-slope broken-line regression of tibia Zn on supplemental Zn level identified a breakpoint of 103.2 mg/kg ($R^2 = 0.99$), which is consistent with the level required to optimize growth and antioxidant responses.

Table (6) Effect of dietary Zn-MMT levels on tibia mineralization of broilers at 35 days of age.

Item	T1 (0)	T2 (40)	T3 (80)	T4 (120)	T5 (160)	SEM	P-value
Tibia weight (g)	7.85[c]	8.95[b]	10.20[a]	10.45[a]	10.35[a]	0.135	<0.001
Tibia ash (%)	42.5[c]	45.8[b]	48.5[a]	49.2[a]	48.8[a]	0.42	<0.001

Tibia Ca (%)	18.5[c]	20.2[b]	22.5[a]	23.1[a]	22.8[a]	0.32	<0.001
Tibia P (%)	8.5[c]	9.2[b]	10.5[a]	10.8[a]	10.6[a]	0.18	<0.001
Tibia Zn (mg/kg DM)	185[d]	245[c]	295[b]	325[a]	332[a]	5.95	<0.001
Tibia breaking strength (N)	215[c]	248[b]	275[a]	282[a]	276[a]	4.85	<0.001

Means within a row with no common superscript differ (P < 0.05; Duncan). DM: dry matter.

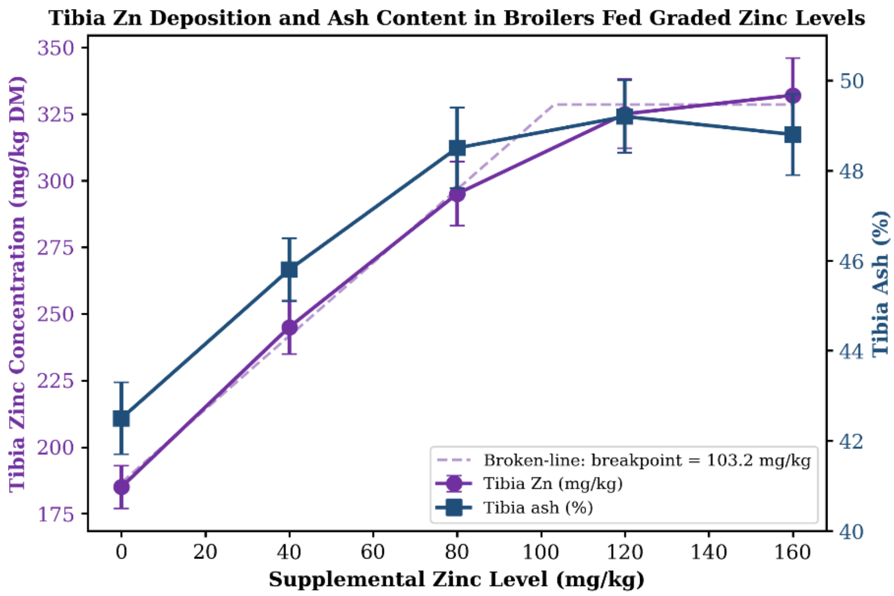

Figure (4): Tibia zinc concentration and tibia ash percentage of broilers fed graded levels of Zn-MMT. Broken-line regression for tibia Zn estimated a breakpoint of 103.2 mg supplemental Zn/kg.

Inter-relationships Among Physiological Variables

Presented in Table 7 are the Pearson correlation coefficients of the main response variables. Serum total protein, Hb, SOD, ND antibody titer and tibia Zn showed strong positive correlations with BWG (r = 0.78–0.96, all P < 0.01) and with each other and these traits showed strong negative correlations with serum MDA, cholesterol and AST (r = −0.74 to −0.93).

The strong correlation between Tibia Zn and the SOD activity, GSH-Px and ND antibody titer (r=0.96, 0.92, 0.91, respectively) indicates the significance of deposition of Zn in bones as an integrative indicator of physiological Zn status. The correlations observed provide evidence for a unified physiological network whereby elements involved in growth, redox balance, immunity and bone mineralisation respond simultaneously to Zn-MMT.

Table (7): Pearson correlation coefficients among selected physiological variables in broilers fed graded levels of dietary Zn-MMT (n = 30 pen means).

Vari-able	BWG	FCR	Hb	TP	SOD	MDA	NDAb	Tibia Zn
BWG	1.000	−0.92**	0.88**	0.91**	0.93**	−0.89**	0.86**	0.90**
FCR		1.000	−0.81**	−0.84**	−0.88**	0.82**	−0.79**	−0.85**
Hb			1.000	0.85**	0.87**	−0.83**	0.78**	0.84**
TP				1.000	0.89**	−0.86**	0.84**	0.88**
SOD					1.000	−0.91**	0.90**	0.96**
MDA						1.000	−0.85**	−0.93**
NDAb							1.000	0.91**
Tibia Zn								1.000

** Correlation significant at $P < 0.01$. BWG: body weight gain; FCR: feed conversion ratio; Hb: hemoglobin; TP: serum total protein; SOD: superoxide dismutase; MDA: malondialdehyde; NDAb: Newcastle disease antibody titer.

This study assessed the impact of Zn-loaded montmorillonite clay (Zn-MMT) levels on growth performance, hemato-biochemical parameters antioxidant potential and immune response in Ross 308 broilers The study concluded that 30Zn-MMT was the optimal inclusion level based on all the studied parameters The fact that all measured physiological domains have a similar optimum range of 80–120 mg supplemental Zn/kg together with an estimated tibia-Zn breakpoint of 103.2 mg/kg demonstrates that Zn-MMT is an excellent vehicle for delivering bioavailable zinc to broiler chickens and that that level of supplementation optimizes physiological performance and health status [1].

Through its variable structure and other behavior, clay carrier appears to have organized several mechanisms. The clay mutadas on the other hand could help to control the physiological processes of the chickens which may impact the account expressing better performance and feed efficiency [9]. The Zn-MMT's multilayer aluminosilicate matrix can preserve Zn in the upper digestive tract, where it is primarily absorbed by enterocytes and transported by Zn transporters. As a result, it works as a “controlledrelease system". The release of Zn from organic Zn salts occurs at a steady rate. This is in contrast to the pronounced release of inorganic Zn salts [33]. In addition, the high specific surface area and a prominent cation-exchange capacity of montmorillonite can give rise to adsorptive activity towards mycotoxins, bacterial endotoxins and ammonia [34]. Therefore, subclinical enteric stress which often limits feed conversion in intensive rearing can be alleviated. Moreover, Zn itself is a component of structure and catalyst of more than 300 metalloenzymes playing a part in protein synthesis, cell proliferation and intermediary metabolism together enhancing lean tissue accretion [35].

Gains made in hematological parameters are indicative that Zn has an established involvement in hematopoiesis, can stabilize the erythrocyte membrane via Cu/Zn-superoxide dismutase activity and can support the differentiation of bone-marrow-derived leukocytes [36]. The drop in ratio of heterophils to lymphocytes – a robust indicator of physiological stress on poultry – indicates that Zn-MMT can mitigate the chronic low-grade inflammatory responses incurred in intensive broiler production possibly due to the combined effect bioavailable Zn and the gut-stabilizing effect of the clay carrier [37]. The concurrent rise in lymphocyte percentage additionally reflects a more favorable shift in leukocyte profile, suggesting an improved health status [38].

The rise in total protein, albumin and globulin concentrations, as shown by blood tests, illustrates an enhancement of liver protein synthesis [39]. Furthermore, it indicates a structural role of Zn in several serum proteins including transferrin, ceruloplasmin and immunoglobulins [40]. The improvement in insulin signaling may account for low serum glucose levels, low cholesterol and low triglyceride concentrations. Zn is essential for the storage and secretion of insulin in the pancreatic β-cells [41]. Reduced serum ALT and AST activities further signify that sufficient Zn-MMT supplementation preserves hepatocyte membrane integrity probably by the Z-thionein system, decreased hepatic oxidative damage, and by the adsorption of hepatotoxic substance of clay matrix in the lumen[15].

The significant increase of SOD, GSH-Px, and CAT activities, along with a substantial decrease in MDA, indicates that Zn-MMT can strengthen the antioxidant defense system in broilers [42]. Zinc is a structural co-factor of Cu/Zn-SOD and stabilizes cellular and mitochondrial membranes, binding to thiol groups. It competes with redox-active metals (iron, copper, etc.) for binding and inhibits their biological activity [43]. A strong inverse correlation ($r = -0.93$) of tibia Zn with serum MDA observed in the present study indicates that bone Zn deposition is closely related to systemic redox status and corroborates the suitability of tibia Zn as a physiological indicator of zinc adequacy under field conditions [44].

The addition of Zn-MMT also increased the humoral immunity, as indicated by the increased ND and IBD antibody titers and increased relative weights of bursa of Fabricius, spleen and thymus [1]. The above statement shows that Zn is essential both for the development of T- and B-lymphocyte as well as for the action of the thymic hormone thymulin and for the expression of cytokines like IL-2 and IFN-γ. The plateau in immune responses at an inclusion of 80 mg Zn/kg supports that suboptimal Zn intake is likely to be a particularly sensitive nutritional constraint for immune competence, and Zn-MMT can adequately meet this requirement at moderate inclusions [45].

The tibia mineralization responses indicated bone-specific physiological effect of Zn-MMT. Zinc is needed for proper chondrocyte and osteoblast functioning, enzyme stimulating collagenase and alkaline phosphatase activity, and enables the stabilization of the bone matrix [46]. The finding from broken-line regression shows that the tibia Zn deposition suppresses around 103.2 mg/Kg (on Zn-MMT) which confirms that supplemental Zn from Zn-MMT in the concentration of around 100 mg/Kg will maximize skeletal Zn retention in fast-growing broilers [1]. Zn-MMT supplementation enhances not only chemical composition but also biomechanical competence of the skeleton, as shown by the parallel increase in tibia breaking strength.

The strong inter-correlations among growth, hematology, antioxidant capacity, immunity and bone mineralization (Table 7) indicate that Zn-MMT does not act upon individual traits but rather coordinates these attributes to act as a functional block. The very high correlations of tibia Zn with SOD ($r = 0.96$), MDA ($r = -0.93$) and ND antibody titer ($r = 0.91$) suggest that bone Zn could serve as a single integrative biomarker of the overall Zn status and health of broiler flocks under field conditions. Such a proposal is a useful observation, which has considerable applied value.

The Zn-MMT ingredient presented by a team of Chinese scientists is seen to have superiour functionalities as compared to inorganic Zn sources like zinc oxide [10]. The montmorillonite matrix slows down the release of Zn in the gastro-intestinal tract. It thereby permits the chelation of soluble Zn with phytate and restricted the formation of insoluble Zn–phytate complexes [47]. Further, it avoids antagonistic interactions with competing divalent cations like copper, iron and calcium. Through the absorption of intestinal toxins and stabilization of the mucin layer, the clay carrier itself contributes to gut-barrier integrity. These combined effects help account for the consistently strong physiological results obtained in the present study at supplementation levels that appear comparable to those usually required from sulfate or oxide sources. Zn-MMT supplementation may lead to less total Zn that must be supplied to achieve physiological adequacy. If true, Zn-MMT may lower the Zn load that is excreted to the environment in commercial broiler production [1].

There was study limitation in that only a single batch of Zn-MMT (and its defined Zn-loading capacity and interlayer composition) was used; further work is warranted to characterize the effects of Zn-loading capacity, particle size and ion-exchange conditions on the in-vivo physiological responses of broilers. In order for Zn-MMT to find its place alongside other zinc supplements for poultry, it will have to go through direct head-to-head comparisons against zinc sulfate, zinc oxide, organic Zn sources and Zn nanoparticles, as well as under heat stress or pathogen challenge.

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