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RESEARCH ARTICLE

Effects of Natural Zeolite on Growth Performance, Antioxidant Status, and *NRF2* and *TGF-β1* Gene Detection in Broilers

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ABSTRACT

Natural zeolite has been proposed as a dietary additive to enhance broiler performance through modulation of the gastrointestinal environment, detoxification, and improved nutrient utilization. This study evaluated the impact of graduated addition of natural zeolite on growing performance, digestive enzyme activity, serum biochemistry, antioxidant status, hematological parameters, humoral immunity, and detection of *NRF2* and *TGF-β1* genes in broiler chickens. Three hundred one-day-old unsexed broiler chicks were divided into four food categories at random for 40 days: a control group and three groups supplemented with natural zeolite at 5, 10, and 15 g/kg diet. Zeolite supplementation significantly improved growth performance in a dose-dependent manner, as evidenced by increased live weight and body weight growth, reduced feed consumption, improved conversion of feed rate, and enhanced European Production Efficiency Factor ($P<0.05$). Digestive enzyme activities (amylase, lipase, and trypsin) were significantly increased ($P<0.05$). Serum biochemical analysis revealed improved protein metabolism (increased total protein and albumin) and reduced metabolic waste indicators (uric acid and bilirubin) ($P<0.05$). Antioxidant status was enhanced, with increased total antioxidant capacity and antioxidant enzyme activities (SOD, GSH-Px, and catalase), alongside reduced lipid peroxidation (MDA) ($P<0.05$). Moreover, the presence of *NRF2* and *TGF-β1* genes was confirmed in all experimental groups, indicating their potential involvement in antioxidant defense and immune regulation. Hematological parameters showed significant increases in hemoglobin, red blood cell count, and packed cell volume ($P<0.05$), while white blood cell counts remained stable. Humoral immunity was also improved, as indicated by increased IgM and IgA levels, with IgY showing optimal response at moderate inclusion levels ($P<0.05$). In conclusion, graded dietary supplementation of natural zeolite improves growth performance, antioxidant capacity, hematological status, and immune function in broiler chickens, potentially through enhancement of intestinal health, nutrient utilization, and modulation of antioxidant and immune-related pathways.

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INTRODUCTION

Poultry production is an important subsector of animal agriculture. It is an important source of economic growth, employment and food supply (Ali *et al.*, 2025). The poultry business is a readily available source of high-quality animal protein and a substantial contributor to the national diet and food security (Elshafae *et al.*, 2025). The sector also provides direct and indirect employment to millions of individuals involved in farming, feed production, distribution and processing (Zaki *et al.*, 2026). The current grill industry is challenged to improve efficiency, reduce the use of antimicrobial growth boosters, and address environmental challenges such as ammonia emissions and mycotoxin levels (Elbarbary *et al.*, 2026).

Natural zeolites, particularly clinoptilolite, have gained attention as functional feed additives due to their unique physicochemical properties. These porous aluminosilicates possess high cation-exchange capacity and adsorption potential, enabling them to bind ammonium, toxins, and other harmful compounds while modulating the gastrointestinal environment and improving nutrient utilization (Abdelrahman *et al.*, 2023; Elsherbeni *et al.*, 2024). Their structural stability and ion-exchange characteristics have supported their application in various agri-food systems, including poultry nutrition (Rodríguez-Iznaga *et al.*, 2022; Elmelegy *et al.*, 2025).

Previous studies have reported that dietary zeolite supplementation can enhance broiler performance by improving digestive enzyme activity, modulating gut microbiota, enhancing antioxidant status, and optimizing blood biochemical parameters (Wang *et al.*, 2021; Abdel-Kader *et al.*, 2025). In addition, zeolite-based aluminosilicates are widely used as mycotoxin binders, contributing to improved performance under contaminated feed conditions (Albarki *et al.*, 2024). However, most studies have focused on individual aspects of performance or health, with limited integrated evaluations encompassing growth, biochemical, antioxidant, hematological, and immune responses within a single experimental framework.

Despite these developments, there are still gaps in integrated, dose-graded assessments that evaluate digestive enzymes, serum biochemistry, hematological, antioxidant status, and humoral immunity in addition to growth and feed efficiency within a single experimental setup. Thus, we hypothesized that zeolite would elicit a dose-dependent improvement in live body weight, feed efficiency, and European Production Efficiency Factor, accompanied by enhanced digestive enzymes, positive hepato-renal and protein metabolism indices, bolstered antioxidant capacity, healthier hematological profiles, and increased immunoglobulins.

Thus, this research pointed to evaluate the dose-dependent impact of dietary natural zeolite on growth performance, digestive enzyme activity, serum biochemistry, antioxidant status, hematological factors, and humoral immunity in broilers. This integrative approach seeks to clarify the relationships among these physiological responses and to establish practical inclusion levels for clinoptilolite in broiler diets.

MATERIALS AND METHODS

Birds, diets, and experimental design: All of the experiments were done following the international standards for the proper handling and care of lab animals. The experiment was conducted from January to March 2024. Three hundred one-day-old unsexed broiler chicks (Cobb-500) were obtained from a commercial hatchery and used in this study. Chicks with similar initial body weights (42 ± 1 g) were randomly allocated into four experimental groups. Each group consisted of three replicates, with 25 chicks per replicate. The control (C) group was provided with a basal diet that was designed to meet the nutritional needs of broilers (Cobb-Vantress, 2018). The second group (Z1), third group (Z2) and fourth group (Z3) were supplemented with zeolite at 5, 10 and 15 g/kg respectively. The chicks were kept in semi-closed housing with straw litter in floor cages. Each enclosure had a floor area of 2 m2 and provided space for 25 birds. Each enclosure has three feeders and three waterers to give enough area for the birds to eat and drink. The illumination schedule consisted of the following: Total 23 hours light, every day (days 1–7) there is one hour of darkness followed by 18 hours of illumination. There are six hours of darkness each day (days eight through forty). The temperature was kept at 33°C ± 1°C from 0 to 7 days and gradually decreased to 25°C ± 1°C by 35 days of age, with an average relative humidity of 60-65% (Cobb-Vantress, 2018).

In accordance with the broiler management guide, three basal diets consisting of maize and soybean meal (starter 1–10 days of age, grower 11–24 days of age, and finisher 25–40 days of age) were produced to provide sufficient nutrients for chicks. The constituents and chemical assessment of baseline diets were presented in Table 1. Water and mash-based feed were given freely. Natural zeolite powder was incorporated with feed at doses of 5, 10, and 15 g/kg.

Table 1: Components and chemical assessment of the baseline diets

Components (%)	Starter diet	Grower diet	Finisher diet
Yellow corn	58.50	64.00	68.00
Soybean meal (46% CP)	28.10	26.00	17.00
Vegetable oil	0.41	0.41	2.15
Corn gluten (60% CP)	8.52	5.60	9.00
Limestone	1.10	1.02	1.00
Di-calcium phosphate	2.01	1.63	1.41
Sodium chloride	0.35	0.30	0.30
Methionine	0.15	0.13	0.13
Lysine	0.55	0.60	0.70
Vitamin and mineral premix*	0.31	0.31	0.31
Total	100.00	100.00	100.00
Chemical analysis			
Crude fiber%	3.32	3.38	2.68
Crude protein (CP) %	22.87	21.08	19.06
Metabolizable energy (kcal/kg)	2967	3045	3211
Ether extract%	3.22	3.41	5.72
Methionine%	0.61	0.56	0.47
Lysine %	1.39	1.23	1.13
Calcium%	0.96	0.84	0.78
Available Phosphorus %	0.52	0.50	0.43

Performance and Production Efficiency: Birds were individually weighed at 1 and 40 days of age to determine their live body weight (LBW). Feed intake (FI) per pen was calculated during the study period (1 to 40 days). The difference between the final and initial body weights was used to calculate body weight gain (BWG). The feed conversion ratio (FCR) was determined by dividing feed consumption by weight gain (Hamed *et al.,* 2025). The mortality rate of the birds was recorded daily. The following formula was used to determine the growth rate (GR) and the European Production Efficiency Factor (EPEF) at day 40:

$$EPEF = [\text{Final LBW} \times \text{Survival rate\%}] / [\text{FCR} \times 40 \text{ (Marketing age)}] \times 100 \quad (1)$$

$$GR_{1-40} = [(BW_{40} - BW_1) \times 100] / [0.5(BW_{40} + BW_1)] \quad (2)$$

Biochemical analyses:

Serum and plasma collection: Ten birds from each group (n = 40) were chosen at random at the end of the experiment

(day 40), slaughtered by cutting the jugular vein in accordance with Islamic law, and blood samples were taken. Each blood sample was divided into two portions. Serum was obtained by centrifuging the initial portion at 3000 rpm for 15 minutes after it was collected in plain containers (without anticoagulant) and allowed to clot at room temperature. Plasma was separated by centrifuging the second quantity at 3000rpm for 15 minutes in containers that contained the anticoagulant ethylene diamine tetraacetic acid (EDTA). Serum and plasma samples were properly prepared and maintained at -20°C for biochemical tests (Ali *et al.*, 2025). In addition, specimens from the liver were stored at -20°C to detect gene expression related to oxidative stress responses using the conventional polymerase chain reaction (PCR) technique.

Determination haematological parameters: The plasma of each sample was used for the measurement of hemoglobin concentration (Hb) using a complete reagent kit (Merckotest®) and packed cell volume (PCV) using a Micro-Capillary Reader (USA). Measurements of RBC and WBC are performed using a hemocytometer (Londok and Rompis, 2021). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) values were calculated:

MCV in femolitres (fL) = 10 x PCV (%)/RBC counts (millions/µl) (3)

MCH in pg/cell= 10 x haemoglobin (g/100 ml)/ RBC counts (millions/µl) (4)

MCHC in g/dl= haemoglobin (g/100 ml) x 100/PCV (%) (5)

Oxidative/Antioxidant markers: Total antioxidant capacity (T-AOC), malondialdehyde (MDA; Cat. No. 2529), catalase (CAT; Cat. No. CA2517), glutathione peroxidase (GSH-Px) activity, and superoxide dis-mutase (SOD) in the serum were analyzed colorimetrically by a commer-cial kit following manufacturer guidelines (Bio Diagnostic Co) and an Infinite F50 spectrophotometer (T80 + UV/VIS; PG Instruments Ltd, UK) (Abo-Aziza *et al.*, 2022; Elbarbary *et al.*, 2023).

Detection of *TGF-β1* and *NRF2* Gene by PCR: The Aurum™ Total RNA Mini Kit (Cat. No. 7326820, Bio-Rad, USA) was employed to extract total RNA from the samples in accordance with the directions provided by the manufacturer. The cDNA Reverse Transcription Kit (Cat. No. 170-8843, Bio-Rad, USA) was employed to synthesize complementary DNA (cDNA) from the RNA obtained in accordance with the manufacturer's methodology. The presence of TGF-β1 and NRF2 genes (Malila *et al.*, 2022; Wang *et al.*, 2023) was evaluated using specific primers (Table 2).

The reaction quantity for PCR amplification was 25 µL, with 12.5 µL of ABT 2X Red Mix, 1 µL of forward and reverse primers, 1 µL of synthesized cDNA, and 9.5 µL of nuclease-free water. The 1.5% agarose gel (Applichem GmbH, Germany) was prepared in 1× TBE buffer at room temperature for electrophoresis to separate the amplified PCR products. Electrophoresis was carried out at a voltage gradient of 5 V/cm, and 15 µL of each PCR product was loaded into individual gel wells. The gel was stained with ethidium bromide (0.5 µg/mL), the DNA bands were pictured under a UV-transilluminator, and photographed using a digital imaging system (Elbarbary *et al.*, 2024).

Table 2: The oligonucleotide primer sequences used in the PCR

Primer	Oligonucleotide (5'-3')	Amplicon (bp)	Reference
NRF2	F: CTGCCCAAAACTGCCGTA R: TCAAATCTTGCTCCAGTTCCA	285 bp	Malila *et al.*, (2022)
TGFβ1	F: GACGATGAGTGGCTCTCCTTC R: GTGCTTCTTGGCAATGCTCT	195 bp	Wang *et al.*, (2023)

Determination of the serum biochemical parameters: Serum biochemical parameters include alanine aminotransferase (ALT; Cat. No. 2650052), aspartate aminotransferase (AST; Cat. No. 260002), total protein (Cat. No. 310001), albumin (Cat. No. 2110001), uric acid (Cat. No. 320004), creatinine (Cat. No. 2370002), and total bilirubin levels, which were measured colorimetrically using appropriate commercial diagnostic kits manufactured by Spectrum with a UV-visible spectrophotometer (T80 + UV/VIS; PG Instruments Ltd, UK). Globulin was estimated as the variance between total proteins and albumin (Ahmed *et al.*, 2025).

Determination of digestive enzymes, include amylase and lipase enzymes were analyzed by Friedman and Young (2005). The trypsin enzyme was measured by the Bovine Trypsin ELISA Kit MBS706461 accord-ing to Zhou *et al.* (2014).

Humoral immune status: Serum levels of immunoglobulins, include A(IgA), Y(IgY), and M(IgM), were determined by ELISA via appropriate kits (Elsherbeni *et al.*, 2024).

Statistical analysis: The results were reported as mean ± SD. Using GraphPad Prism Software (GPPS), one-way analysis of variance (ANOVA) was used to study variability within a data set. Then, if significant, a post hoc test (Tukey's HSD) identifies which groups differ. A value of $P<0.05$ was determined as statistically significant.

RESULTS

Productive performance: Table 3 shows that the analysis of live body weight showed no significant biological differences among groups on Day 1, confirming uniform starting conditions. From Day 10 onward, significant increases ($P<0.001$) were observed in all treated groups compared with the control, with values progressively increasing through Day 24 and reaching the highest level at Day 40. Final LBW followed the order Z3 > Z2 > Z1 > C, and Tukey post-hoc comparisons showed significant variances ($P<0.001$) among all groups.

Body-weight gain data demonstrated the same trend. Significant improvements ($P<0.001$) were recorded during all growth periods (1–10, 11–24, 25–40, and 1–40 days). Early gains increased from 194.9 g in the control to 276.3 g in Z3, while cumulative gains rose from 2325.4 g to 2575.0 g, reflecting a clear dose-dependent enhancement. The growth rate ($GR_{1–40}$ %) also showed a significant difference ($P<0.001$), with the highest rate noted in Z3.

Figure 1 shows the Effect of treatment on FI, FCR, and EPEF over 1–40 days in broiler chickens. Across days 1–40, the treatment produced a clear, dose-dependent improvement in production efficiency. Total FI declined from 3488.4 ± 6.4 g in controls to 3217.9 ± 7.6 g in Z3, while the FCR ratio improved from 1.50 ± 0.02 to 1.25 ± 0.02 ($P<0.05$). At the same time, the EPEF went up in steps from 347.3 ± 2.2 (C) to 523.4 ± 3.8 (Z3). One-way ANOVA confirmed highly significant among-group differences for FI, FCR, and EPEF ($P<0.001$), consistent with a graded efficiency response (Z3 > Z2 > Z1 > C). In practical terms, birds receiving the highest level (Z3) ate less feed, converted it more efficiently, and achieved the best overall productivity, indicating a strong biological and economic advantage of the treatment over the 40 days.

Mean values are represented as means ± SD. GR: Growth rate during 1–40 days of age. [a-c]Means bearing different superscripts within the same column are significant differences at $P<0.05$ by one-way ANOVA and Tukey's HSD. The results show a clear, dose-dependent increase in live-body weight and weight gain across all treatment groups.

Digestive enzyme activities: Digestive enzyme activities (Figure 2) increased dose-dependently in treated groups (C → Z3), with amylase rising from 289.3 ± 2.4 to 387.5 ± 4.2 U/L, lipase from 18.4 ± 0.6 to 21.8 ± 0.6 U/L, and trypsin from 22.5 ± 0.2 to 24.3 ± 0.5 U/L. One-way ANOVA revealed significant treatment effects on pancreatic/intestinal enzymes. Tukey's HSD showed a graded rise across C < Z1 < Z2 < Z3 for all enzymes, with each treated group exceeding the control ($P<0.001$), indicating enhanced capacities for carbohydrate, lipid, and protein hydrolysis in a dose-dependent manner, and are concordant with the observed improvements in feed conversion and production efficiency.

Table 3: Effect of Zeolite treatment on live weight and body-weight growth of broilers during the experimental period (*n* = 25, triplicates/group)

Group	Live weight (g)				Body weight gain (g)				GR_{1-40} %
	1 d	10 d	24 d	40 d	1-10 d	11-24 d	25-40 d	1-40 d	
C	42.3±0.26[a]	237.2±5.4[c]	854.3±5.6[c]	2367.7±9.3[c]	194.9±3.6[c]	617.1±4.2[c]	1513.4±3.3[c]	2325.4±5.4[c]	192.9±1.1[b]
Z1	42.5±0.31[a]	267.6±4.7[b]	893.4±7.2[b]	2448.3±10.5[b]	225.1±5.2[b]	625.8±3.5[b]	1554.9±5.4[b]	2405.8±6.3[b]	193.2±1.2[a]
Z2	42.7±0.46[a]	296.9±6.2[a]	944.8±7.6[a]	2523.6±8.8[b]	254.2±2.1[b]	647.9±3.6[b]	1578.8±3.4[b]	2480.9±4.7[b]	193.3±1.1[a]
Z3	42.2±0.34[a]	318.5±7.8[a]	982.2±6.3[a]	2617.2±11.7[a]	276.3±3.3[a]	663.7±5.1[a]	1635.0±5.2[a]	2575.0±7.2[a]	193.7±1.1[a]
p-value	< 0.001	< 0.001	< 0.001	< 0.0001	< 0.001	< 0.001	< 0.001	< 0.0001	< 0.001

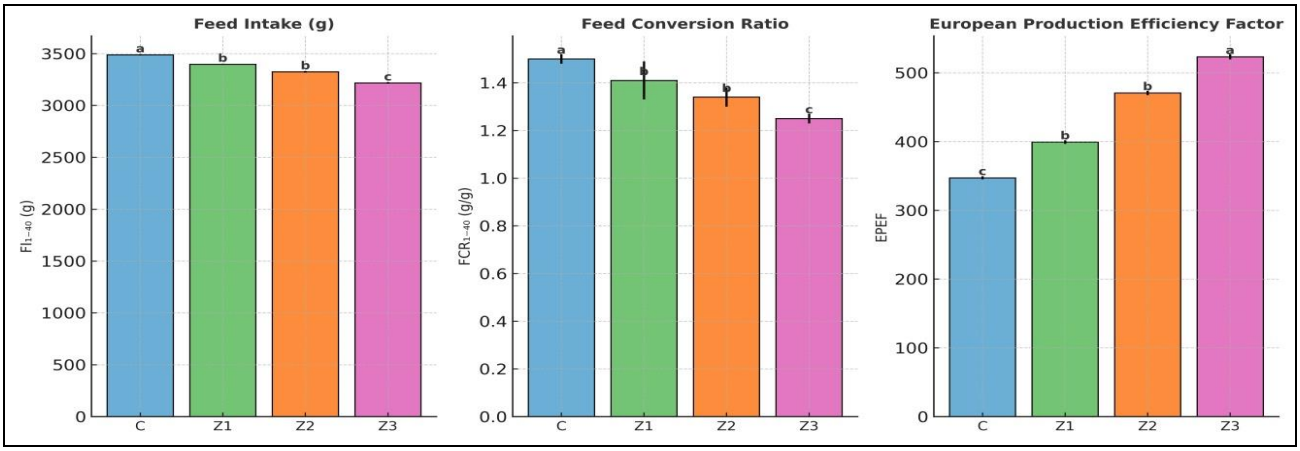

Fig. 1: Effect of dietary zeolite treatment on feed intake (FI_{1-40}), feed conversion ratio (FCR_{1-40}), and European Production Efficiency Factor (EPEF) of broiler chickens during the experimental period. The data is reported as mean ± SD. Bars with varying superscript letters (a–c) throughout each variable exhibit a significant difference at $P<0.05$ by one-way ANOVA followed by Tukey's HSD.

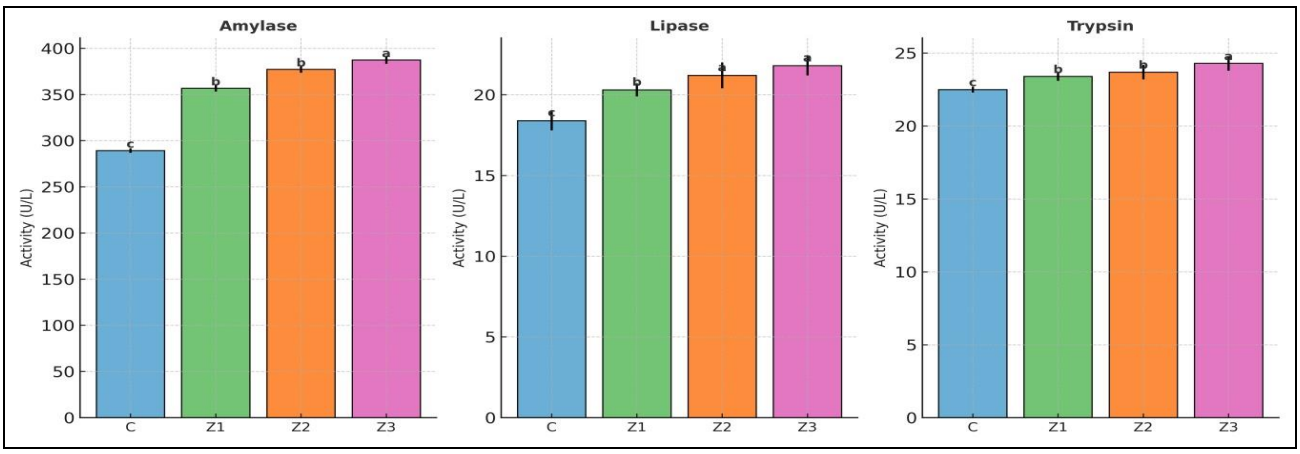

Fig. 2: Effect of dietary zeolite supplementation on digestive enzyme activities (amylase, lipase, and trypsin) of broiler chickens during the experimental period. The data is reported as mean ± SD. Bars with varying superscript letters (a–c) throughout each variable exhibit a significant difference at $P<0.05$ by one-way ANOVA followed by Tukey's HSD.

Serum biochemical parameters: Concerning Table 4, across 1–40 d, serum markers showed a consistent, dose-dependent shift with treatment (P<0.05). Hepatic enzymes increased in the order C < Z1 ≈ Z2 < Z3, with ALT rising from 14.75 ± 0.01 to 21.21 ± 0.03 U/L (~+44%) and AST from 92.96 ± 2.1 to 112.84 ± 4.6 U/L (~+21%), indicating greater hepatocellular enzyme activity in treated birds. In parallel, indices commonly associated with nitrogen/renal handling and heme turnover declined: creatinine fell from 0.46 ± 0.31 to 0.35 ± 0.31 mg/dl (~−24%), uric acid from 6.74 ± 1.1 to 5.64 ± 1.6 mg/dl (~−16%), and total bilirubin from 0.89 ± 0.11 to 0.75 ± 0.11 mg/dl (~−16%), suggesting more favorable metabolic/clearance status with increasing dose. The total protein level went up from 3.76 ± 1.7 to 4.43 ± 1.3 g/dl (~+18%), which was due to increases in albumin (1.17 ± 0.06 → 1.52 ± 0.07 g/dl, ~+30%) and globulin (2.59 ± 0.41 → 2.91 ± 0.73 g/dl, ~+12%). This is consistent with an increase in synthetic capacity and the humoral protein pool. One-way ANOVA indicated significant treatment effects for ALT, AST, uric acid, total bilirubin, total protein, albumin, and globulin (all P<0.05), with a non-significant trend for creatinine (p = 0.084); post-hoc patterns were dose-ordered (C < Z1 < Z2 < Z3 for ALT/AST/proteins; inverse for uric acid/bilirubin).

The data is reported as mean ± SD. Bars with varying superscript letters (a–c) throughout each variable exhibit a significant difference at p<0.05 by one-way ANOVA followed by Tukey's HSD. ALT: Alanine aminotransferase. AST: Aspartate aminotransferase.

Oxidative/antioxidant status: Table 5 shows the influence of various treatments of dietary zeolite on oxidative/antioxidant parameters in the serum of 40-day-old Cobb broilers. Antioxidant capacity improved dose-dependently across treatments, with T-AOC rising from 13.38 ± 1.7 (C) to 14.83 ± 1.4 (Z3), indicating improved overall antioxidant status. In addition, enzymatic defenses show the same graded pattern: SOD increased from 134.31 ± 2.6 to 174.33 ± 3.7 U/mL, indicating improved enzymatic defense against superoxide radicals; GSH-Px increased from 97.26 ± 3.4 to 135.43 ± 3.7 U/mL, indicating a strong dose-effect treatment that stimulates glutathione-dependent detoxification of peroxides; and catalase increased from 52.64 ± 2.2 to 72.47 ± 3.3 U/mL, confirming improvement that complements SOD and GSH-Px in reducing oxidative load. In parallel, the lipid peroxidation marker MDA showed a slight numerical decrease (0.112 ± 0.66 to 0.093 ± 0.73 mmol/L); however, this change was not statistically significant (p = 0.912). One-way ANOVA revealed significant treatment effects for T-AOC, SOD, GSH-Px, and catalase (all p<0.001). These findings indicate that zeolite supplementation enhanced antioxidant enzyme activity, while no significant effect was observed on MDA levels.

The data is reported as mean ± SD. Bars with varying superscript letters (a–c) throughout each variable exhibit a significant difference at p<0.05 by one-way ANOVA followed by Tukey's HSD. T-AOC: Total antioxidant capacity. MDA: Malondialdehyde. CAT: Catalase. GSH-Px: Glutathione peroxidase. SOD: Superoxide dismutase. Data indicate that treatment groups, especially Z2 and Z3, significantly enhanced antioxidant enzyme activities and reduced oxidative stress compared to control.

Detection of *TGF-β1* and *NRF2* Genes: PCR analysis confirmed the successful amplification of *TGF-β1* and *NRF2* genes, as evidenced by distinct DNA bands at the expected sizes in all experimental samples (Fig. 3). Visual inspection of band patterns suggested no marked qualitative differences among the control and zeolite-supplemented groups.

Table 4: Effect of zeolite treatment on serum biochemical parameters of broilers over the experimental period

Group	ALT (U/L)	AST (U/L)	Creatinine (mg/dl)	Uric acid (mg/dl)	Total bilirubin (mg/dl)	Total protein (g/dl)	Albumin (g/dl)	Globulin (g/dl)
C	14.75±0.01^{c}	92.96±2.1^{c}	0.46±0.31^{a}	6.74±1.1^{a}	0.89±0.11^{a}	3.76±1.7^{c}	1.17±0.06^{c}	2.59±0.41^{c}
Z1	19.22±0.01^{b}	98.65±3.4^{b}	0.41±0.31^{b}	6.04±1.2^{b}	0.81±0.14^{b}	3.95±1.3^{b}	1.33±0.03^{b}	2.62±0.33^{b}
Z2	20.58±0.02^{b}	105.76±3.8^{b}	0.36±0.31^{c}	5.82±1.4^{c}	0.78±0.16^{c}	4.25±1.5^{a}	1.46±0.02^{b}	2.79±0.36^{b}
Z3	21.21±0.03^{a}	112.84±4.6^{a}	0.35±0.31^{c}	5.64±1.6^{c}	0.75±0.11^{c}	4.43±1.3^{a}	1.52±0.07^{a}	2.91±0.73^{a}
p- value	< 0.0001	< 0.001	0.084	0.052	0.051	0.013	< 0.001	0.052

Table 5: Impact of various zeolite supplements on broiler chicks' oxidative and antioxidant state

Group	T-AOC (U/mL)	MDA (mmol/L)	SOD (U/mL)	GSH-Px (U/mL)	Catalase (U/mL)
C	13.38±1.7^{c}	0.112±0.66^{a}	134.31±2.6^{c}	97.26±3.4^{c}	52.64±2.2^{c}
Z1	13.87±1.3^{b}	0.108±0.36^{b}	142.61±2.2^{b}	110.41±2.8^{b}	61.53±2.7^{b}
Z2	14.35±1.6^{a}	0.102±0.43^{b}	163.27±3.1^{a}	122.36±3.5^{b}	68.22±3.2^{b}
Z3	14.83±1.4^{a}	0.093±0.73^{c}	174.33±3.7^{a}	135.43±3.7^{a}	72.47±3.3^{a}
p- value	0.007	0.912	< 0.001	< 0.001	< 0.001

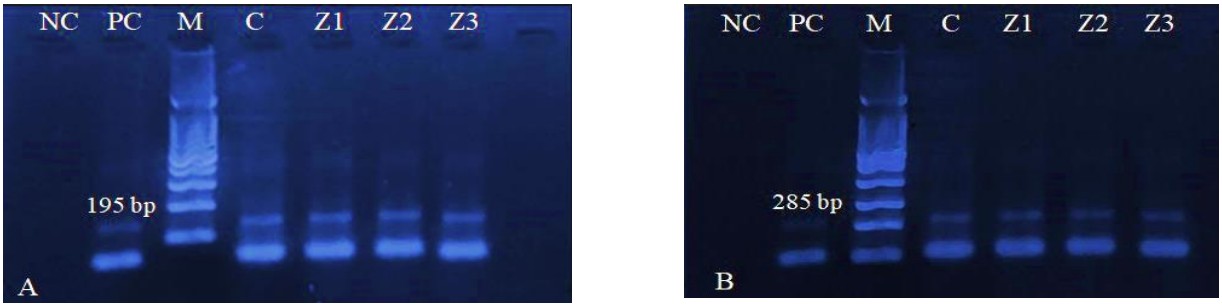

Fig. 3: The amplification of the *TGF-β1* gene (A) at 195 bp and the *NRF2* gene (B) at 285 bp in broiler tissue samples was demonstrated by agarose gel electrophoresis of PCR products. Lane M: 100 bp DNA ladder; Lane NC: negative control; PC: positive control; C: control group; Lanes Z1, Z2, and Z3: broilers supplemented with natural zeolite at 5, 10, and 15 g/kg diet, respectively. Distinct bands corresponding to the expected amplicon sizes of *TGF-β1* and *NRF2* were observed, indicating successful amplification of the target genes.

Hematological indices: On the other hand, the influence of zeolite treatment on hematological indices of broiler chickens over the experimental period (Table 6) shows that the zeolite supplementation produced a dose-responsive erythroid shift. Hemoglobin (Hb) increased from 11.14 ± 1.2 g/dL (C) to 12.32 ± 1.3 g/dL (Z3), and RBC count increased from 2.68 ± 0.52 to 3.53 $\times 10^6/mm^3$, with PCV rising from 32.43 ± 1.2% to 36.67 ± 1.5% ($P<0.05$). In contrast, red-cell indices declined with dose: MCV fell from 121.01 ± 1.2 to 103.88 fL, and MCH from 41.57 ± 2.1 to 34.91 pg, indicating a shift toward more numerous, smaller erythrocytes carrying slightly less hemoglobin per cell. MCHC showed a non-linear pattern (C ≈ 34.35% → Z1/Z2 ≈ 35% → Z3 = 33.59%), suggesting that at higher inclusions, the increase in cell number and PCV outpaced hemoglobin concentration per cell. Importantly, WBCs remained stable (~22.3–22.4 $\times 10^3/mm^3$ across groups), indicating no overt systemic leukocytic response ($p = 0.613$). Overall, the profile (Hb↑, RBC↑, PCV↑ with MCV/MCH↓, and stable WBCs) is consistent with improved erythropoiesis and oxygen-carrying ability without leukocytosis. Simultaneously, the microcytic tendency at higher concentrations (lower MCV/MCH, slightly lower MCHC in Z3) suggests that erythrocytes are more compact, rather than a reduction in the total oxygen-transport capacity. This is in accordance with the enhanced growth and efficiency that have been reported in the performance attributes.

The data is reported as mean ± SD. Bars with varying superscript letters (a–c) throughout each variable exhibit a significant difference at $p<0.05$ by one-way ANOVA followed by Tukey's HSD. Hb: Hemoglobin (g/dl), RBC: Red blood cell ($10^6/mm^3$), WBC: White blood cell ($10^3/mm^3$), PCV: packed cell volume, MCV: mean corpuscular volume, MCH: mean corpuscular haemoglobin, MCHC: mean corpuscular haemoglobin concentration.

Humoral immune status: The humoral immune state was frequently enhanced by the dietary treatment, frequently in a dose-dependent manner (Figure 4). IgM rose sharply from 1.32 ± 0.42 (C) to 2.46 ± 0.71 (Z1) and 2.78 ± 0.62 (Z2), then plateaued at 2.96 ± 0.56 (Z3); superscripts indicate Z2–Z3 > Z1 > C ($p<0.05$), consistent with an augmented primary antibody response. IgA increased steadily (0.37 ± 0.18 → 0.64 ± 0.22), with Z3 > Z1–Z2 > C ($p<0.05$), suggesting improved mucosal immunity. IgY (the avian version of IgG) went up from control (4.87 ± 0.28) to Z1 (5.35 ± 0.34) and Z2 (5.89 ± 0.62), then went down slightly at Z3 (5.21 ± 0.41). The letters show that Z1–Z2 > Z3 > C ($P<0.05$). In brief, the profiles of IgM, IgA, and IgY increased up to Z2, then slightly decreased at Z3, but remained higher than the control, indicating that supplementation improves systemic and mucosal antibody responses. On the other hand, there could be a ceiling or modulatory effect for IgY at the highest level. However, the overall humoral competence is still better than that of the control in all treated groups.

Table 6: Impact of zeolite treatment on hematological factors of the broilers over the trial period

Group	Hb (g/dl)	RBC ($10^6/mm^3$)	WBC ($10^3/mm^3$)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (%)
C	11.14±1.2^c	2.68±0.52^c	22.25±1.2^a	32.43±1.2^c	121.01±1.2^a	41.57±2.1^a	34.35±1.8^a
Z1	11.76±1.2^b	2.86±0.41^b	22.32±1.3^a	33.55±1.8^b	117.31±1.2^b	41.12±1.5^a	35.05±1.5^b
Z2	12.11±1.1^a	3.26±0.33^a	22.37±1.2^a	34.27±1.2^b	105.12±1.2^c	37.15±1.3^b	35.33±1.4^b
Z3	12.32±1.3^a	3.53±0.72^a	22.42±1.2^a	36.67±1.5^a	103.88±1.4^c	34.91±1.3^c	33.59±1.1^c
p- value	< 0.01	< 0.001	0.613	< 0.001	< 0.0001	< 0.001	< 0.01

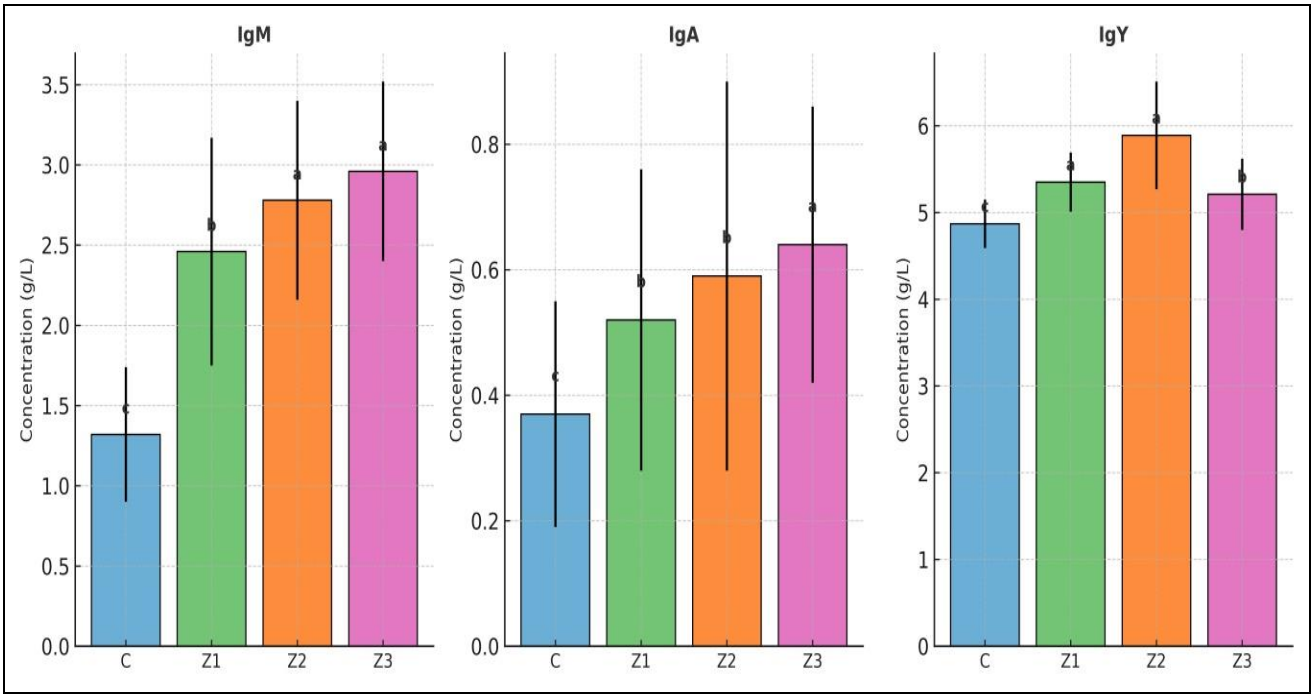

Fig. 4: The impact of zeolite treatment on the humoral immunity state (IgM, IgA, and IgY concentrations) of broilers during the study period. The data is reported as mean ± SD. Bars with varying superscript letters (a–c) throughout each variable exhibit a significant difference at $P<0.05$ by one-way ANOVA followed by Tukey's HSD.

DISCUSSION

Natural zeolite has gained increasing attention as a sustainable feed additive capable of improving poultry performance while reducing reliance on synthetic growth promoters (Abdel-Kader *et al.,* 2025). Its physicochemical properties, particularly high cation-exchange capacity and adsorption potential, enable binding of ammonia and toxic metabolites, thereby improving the intestinal environment and nutrient utilization efficiency (Al-Waheeb and Al-Shukri 2022).

In the present study, dietary zeolite supplementation resulted in a clear dose-dependent improvement in growth performance, as reflected by increased live body weight and cumulative gain, alongside reduced feed intake and improved feed conversion ratio. These findings suggest that zeolite enhances feed efficiency rather than simply increasing feed consumption (Abdelrahman *et al.,* 2023). Similar improvements have been reported in recent studies, where zeolite supplementation improved broiler growth performance and overall production efficiency, likely through improved gut conditions and nutrient availability (Elsherbeni *et al.*, 2024; Abdel-Kader *et al.*, 2025). The observed improvements may be attributed to reduced ammonia toxicity, improved intestinal morphology, and enhanced microbial balance, all of which contribute to better nutrient absorption (Abdelrahman *et al.,* 2023; Fotouh *et al.,* 2025; Hamed *et al.,* 2025). Additional trials show zeolite improving BWG, FCR, and EPEF across inclusion levels, again concordant with the current efficiency gains; however, they note a practical dose window rather than an unlimited linear response (Saed *et al.,* 2024). These outcomes were also agreed with by Alharthi *et al.* (2022), AL-musawy *et al.* (2023), Dunislawska *et al,* (2022), Emam *et al.* (2019), and Pavlak *et al.* (2023), who reported significant influence on FI and FCR when broiler diets were supplemented with various amounts of zeolite (2% - 6% per Kg).

The enhancement of digestive enzyme activities (amylase, lipase, and trypsin) provides further mechanistic support for the improved performance observed. Increased enzyme activity indicates enhanced digestive capacity and nutrient breakdown, which directly contributes to improved feed conversion and growth (Surai *et al.,* 2029). This is consistent with previous reports suggesting that zeolite improves digestive efficiency by modulating gut microenvironment and enzyme secretion. An investigation showed that the zeolite (1.5–3%) enhanced growth while modulating the gut (cecal microbiota, digesta viscosity) and digestive enzymes, providing a direct link between zeolite, enzyme up-regulation, and performance gains (Abdel-Kader *et al.,* 2025). The application of zeolite to both feed and litter in field trials improved growth parameters and housing conditions, including a decrease in NH_3 and an improvement in litter pH/moisture. This is consistent with the notion that increased enzyme activity indicates greater efficacy (Elsherbeni *et al.*, 2024). Earlier controlled studies and reviews consistently attribute zeolite-driven performance to adsorptive detoxification (e.g., mycotoxins, ammonia), improved intestinal milieu and morphology, and enhanced digestive enzyme activity—including rises in amylase and trypsin—all of which plausibly underpin the present enzyme and productivity outcomes (Zhou *et al.,* 2014; Ciszewski *et al.,* 2022). The improved enzymatic profile observed in the present study likely contributed to the more efficient utilization of dietary nutrients (Zaki *et al.,* 2024).

Zeolite supplementation also improved serum biochemical parameters, as evidenced by increased total protein and albumin levels and decreased uric acid, bilirubin, and creatinine concentrations. These changes suggest enhanced protein metabolism and improved hepatic and renal function. The increase in protein-related parameters reflects improved nutrient assimilation and metabolic efficiency, while reductions in metabolic waste indicators indicate improved physiological status (Kaya *et al.,* 2024). Similar findings have been reported in previous studies, where zeolite improved liver function and metabolic profiles through detoxification and improved nutrient utilization. A study by Abdel-Kader *et al.* (2025) found that adding 1.5–3% dietary zeolite increased AST, total protein, and albumin and reduced uric acid, creatinine, and total bilirubin, attributing the improvements to zeolite's adsorptive detoxification (ammonia/mycotoxin binding), gut environment modulation, and better nutrient utilization—mechanisms that align with the current dataset's direction of effect (Aldawood *et al.,* 2026).

Simultaneously, responses of biochemical markers are inconsistent across investigations. The ALT variations were not consistently significant, as reported by Pavlak *et al.* (2023). Despite improvements in nutrient utilization and environmental conditions, other studies have demonstrated minimal effects on ALT, AST, and serum proteins across varying inclusion levels, particle sizes, or zeolite sources. This diversity emphasizes the significance of zeolite properties and the dietary environment. The current study demonstrated that increases in ALT and AST were accompanied by improvements in protein status measures (total protein and albumin) and decreases in markers of metabolic waste (creatinine, uric acid, and bilirubin). Despite elevated ALT and AST levels typically indicating hepatic stress, concurrent improvements in other biochemical markers and performance indices suggest that these changes may be associated with increased metabolic activity rather than pathological liver injury. Several investigations had identified comparable patterns of modest increases in liver enzymes without any indications of diminished liver function (Abdel-Kader *et al.,* 2025). In addition, the decrease in creatinine and urea levels observed in reports of zeolite supplementation suggests that the liver and kidneys may experience less metabolic stress due to improved gastrointestinal conditions and adsorptive detoxification (AlMaswari *et al.,* 2024; Aldawood *et al.*, 2026). Therefore, while the observed increase in ALT and AST should be interpreted cautiously, the overall biochemical profile suggests improved metabolic efficiency rather than hepatic dysfunction. Nevertheless, further studies incorporating histopathological or liver function assessments are recommended to confirm these interpretations.

Antioxidant status is a key indicator for evaluating the effects of dietary additives in poultry (Vakili *et al.*, 2023). In the present study, antioxidant capacity improved dose-dependently with zeolite supplementation, as

evidenced by significant increases in T-AOC, SOD, GSH-Px, and catalase activities. In contrast, MDA values showed a numerical decrease, although this change was not statistically significant. These findings suggest an overall improvement in redox balance primarily driven by enhanced enzymatic antioxidant defenses rather than changes in lipid peroxidation (Fotouh *et al.,* 2026).

This pattern is consistent with recent studies demonstrating that dietary zeolite (≈1.5–3%) enhances antioxidant capacity in broilers by increasing enzymatic defenses and improving gut conditions through reduced digesta viscosity, modulation of intestinal microbiota, and adsorptive detoxification of harmful compounds such as ammonia and mycotoxins (Ahmed *et al.,* 2024;Abdel-Kader *et al.,* 2025). Similarly, previous studies on clinoptilolite have reported increased activities of antioxidant enzymes (SOD, GSH-Px, and catalase), linking these changes to improved growth performance and intestinal health (Jomova *et al., 2024*). From a mechanistic perspective, the coordinated increase in antioxidant enzymes observed in this study aligns with the classical antioxidant defense system, where SOD converts superoxide radicals into hydrogen peroxide, which is subsequently detoxified by catalase and GSH-Px. The enhancement of these enzymatic pathways supports improved oxidative balance and may contribute to the observed improvements in physiological status and performance (Oke *et al.,* 2024; Jomova *et al.*, 2024).

The detection of *NRF2* and *TGF-β1* genes in broiler chickens confirms their biological relevance in antioxidant defense and immune regulation. *NRF2* is a critical transcription factor that controls cellular antioxidant pathways by activating genes that express antioxidant enzymes, including SOD, CAT, and GSH-Px, which protect cells against oxidative stress (Ali *et al.,* 2025). Previous studies in poultry have demonstrated that activation of the *NRF2* pathway enhances antioxidant capacity and improves physiological resilience under stress conditions (Oke *et al.,* 2024; Tran *et al.,* 2024). Similarly, *TGF-β1* is a multifunctional cytokine that is essential to immune modulation, cell proliferation, and tissue repair, contributing to the maintenance of immune homeostasis and control of inflammatory responses (Deng *et al.,* 2024).

In the current research, although conventional PCR revealed the occurrence of these genes, no quantitative conclusions can be drawn regarding their expression or regulation. Therefore, the improvements observed in antioxidant enzyme activities (SOD, CAT, and GSH-Px) and overall physiological status may be indirectly associated with these pathways, but this relationship cannot be confirmed at the transcriptional level (Mohamed *et al.,* 2026). Future studies employing quantitative techniques such as real-time PCR are required to elucidate the potential role of *NRF2* and *TGF-β1* signaling in mediating the effects of dietary zeolite.

In the present study, zeolite supplementation induced a dose-dependent improvement in erythroid parameters, as reflected by increased Hb, RBC count, and PCV, accompanied by reduced MCV and MCH, indicating a higher number of smaller erythrocytes. This hematological profile suggests enhanced oxygen-carrying capacity without evidence of leukocytosis, as WBC counts remained within normal ranges (Abdel-Kader *et al.,* 2025). These findings are consistent with previous studies reporting increased erythrocyte indices in broilers supplemented with zeolite, likely due to improved nutrient utilization and reduced exposure to toxic metabolites such as ammonia and mycotoxins (Abdelrahman *et al.*, 2023; Elsherbeni *et al.*, 2024). However, variations across studies highlight that hematological responses may depend on zeolite source, inclusion level, and physicochemical characteristics.

In the same vein, Emam *et al.* (2019) noted that the hematological markers (RBCs, Hb, and HCT) of the avian were improved by the addition of 3% and 6% zeolite to the meal. The enhancement suggests that zeolite likely plays a significant role in protecting poultry by reducing harmful elements, such as heavy metal salts, and appears to have a beneficial impact on the health of chickens in challenging environments (Zha *et al.,* 2025). Similarly, Al-Musawy *et al.* (2023) reported that adding zeolite to broiler chicken diets at concentrations of 1, 2, and 3% significantly increased red blood cell (RBC) and hemoglobin (Hb) levels. Nevertheless, not all studies offer the same level of detail. Some trials demonstrate numerical performance benefits with dose- and particle-size effects but limited blood changes. In contrast, others report little impact on serum constituents at specific inclusion rates, underscoring the dependence of zeolite responses on formulation, source, and dose (Abdelrahman *et al.,* 2023; Pavlak *et al.,* 2023).

Zeolite supplementation also improved humoral immunity, as indicated by progressive increases in IgM and IgA levels, with IgY peaking at moderate inclusion levels. This pattern suggests enhanced primary and mucosal immune responses, potentially linked to improved intestinal health and reduced systemic stress (Abdel-Kader *et al.,* 2025). Similar immunomodulatory effects have been reported in broilers receiving clinoptilolite, where improved gut environment and microbial balance were associated with enhanced antibody production (Pirzado *et al.*, 2022; Elsherbeni *et al.*, 2024). Mechanistically, zeolite may act as a gut-modulating agent that indirectly supports immune function through improved barrier integrity and nutrient absorption (Lim *et al.,* 2023). The primary avian immunoglobulins identified in the literature are IgA, IgM, and IgY. Our findings are consistent with the evidence that dietary immunomodulators, particularly mineral sorbents such as zeolite, can improve antibody-mediated defenses (Wlaźlak *et al.,* 2023). The weight of evidence (and related aluminosilicate work showing restoration of mucosal/serum IgGsIg levels) supports a context-dependent, but generally positive, effect of optimized zeolite use on humoral immunity, despite some trials reporting limited or non-significant immunoglobulin changes at specific inclusion levels or particle sizes (Saed *et al.,* 2024; Zha *et al.,* 2025).

Despite these promising results, significant restrictions must be addressed. The use of a single set of experimental conditions, a single broiler strain, and a single zeolite source in the study may limit generalizability. Furthermore, the zeolite's

physicochemical properties (such as purity, particle size, and cation-exchange capacity) were not well specified, and only a narrow range of inclusion levels was examined. Future research should include multi-environment trials, comprehensive material characterization, and larger dose-response designs to help discover effective application strategies.

Conclusions: The results of this study indicate that the addition of natural zeolite to broiler rations was associated with dose-dependent improvements in growth performance and a variety of physiological indicators during the 1–40 day rearing season. The production efficiency was enhanced at higher inclusion levels as a result of the reduction in the feed intake and feed conversion ratio of poultry and the improvement of live body weight and cumulative gain through the addition of zeolite to the diet. These enhancements were accompanied by changes in the antioxidant status (increased T-AOC, SOD, GSH-Px, and catalase), digestive enzyme activity, serum biochemical indexes, haematological parameters, and humoral immune response. Natural zeolite has the potential to enhance development efficiency by regulating intestinal circumstances, nutrient consumption, and antioxidant capacity, as evidenced by observed findings. Nevertheless, it is crucial to consider the research design when interpreting the results. The investigation was conducted using a single broiler strain and a restricted range of zeolite inclusion levels in a single set of experimental settings. Furthermore, the *NRF2* and *TGF-β1* genes were identified; however, their expression or regulatory functions cannot be determined as a result of the conventional PCR method. Future studies employing quantitative molecular approaches, along with multi-environment trials and economic evaluations, are required to confirm these findings and optimize practical application strategies. Long-term studies are also recommended to assess the safety and efficacy of higher inclusion levels of zeolite in broiler nutrition.

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Ethical approval: Every trial was carried out in compliance with international and institutional standards for the use and care of laboratory animals. All possible effort was done to avoid animal duress and verify proper handling.

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