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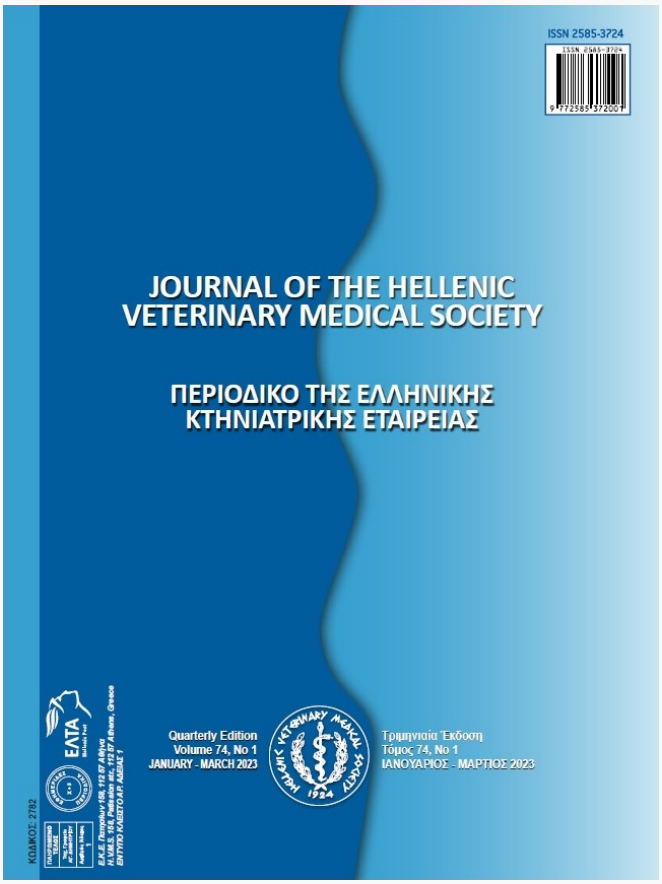


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# Impact of stocking density and dietary nano-zinc supplementation on stress indicators, immunity, and DNA damage in broiler chickens

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**ABSTRACT:** Effects of stocking density (SD) and nano zincon oxidative status, immune function, and DNA damage in broilers were examined in the current study. A total number of 480 one-day-old male broilers (Ross 308) were randomly divided into 4 treatments each contained 8 replicates.A 2 ×2 factorial arrangement with two groups for dietary zinc(Zn) form (inorganic or nano) and two groups for SD (low = 12 birds/$m^2$ and high = 18 birds/$m^2$) was conducted. Basal diets based on the corn-soybean meal were formulated.Relative liver weight was lower ($P<0.05$) in broiler subjected high stocking density (HSD) but other lymphoid organ weights have not been affected. Relative lymphoid organ weights were not affected by dietary nano zinc.HSD increased the serum corticosterone level of broiler ($P<0.05$) but did not affect the blood heterophil:lymphocyte ratio, serum glutathione, superoxide dismutase, and malondialdehyde levels. Dietary nano zinc had no effect on these stress indicators. Serum interferon-gamma, interleukin 12, and interleukin 18 levels were not affected by HSD.Dietary nano zinc tended to reduce the interferon-gamma level ($P=0.05$) but did not affect interleukin 12, and interleukin 18 levels in broiler. Either HSD or nano zinc had no effect on DNA damage in lymphocyte.In conclusion, HSD may decrease the relative lymphoid organ weights and increase the stress indicators but did not affect the immunity and DNA damage. Dietary nano zinc had no effect on stress indicators, immunity, and DNA damage in broiler.

***Keywords:*** Broiler; nano zinc; high stocking density;s tress indicators; immunity; DNA damage

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## INTRODUCTION

Many factors may affect the cost and quality of products in poultry, and stocking density (SD) is one of the major factors. High stocking density (HSD) reduces production costs and increases live weight per floor space in broiler (Dozier et al., 2006). However, HSD is a stress factor and adversely affects performance, product quality, oxidative status, and immunity of broiler due to decreased air and litter quality and increased number of animals per feeder and drinker (Cengiz et al., 2015; Cai et al., 2019).

Zinc (Zn) belongs to the class of essential trace elements required for growth and metabolism in animals. It participates in the structure of superoxide dismutase (SOD), which is an antioxidant enzyme and decreases the formation of free radicals (Sloup et al., 2017). It also has functions on immune system elements such as T cells, thymocyte development, and thymic integrity (Rossi et al., 2007).

Nanotechnology is used in many fields such as engineering, health, food, medicine, information technologies, agriculture, and animal nutrition in recent years. In animal nutrition, nanotechnology is mostly used for the production of nano minerals. Nano minerals are characterized by a particle size of 1 to 100 nm. Because of its larger surface area, nano minerals are absorbed more easily and effectively from the digestive system compared to other forms (Anwar et al., 2019).

There have been some studies about the effects of nano Zn on stress indicators and immunity of broilers (Dukare Sagar et al., 2018; Ramiah et al., 2019; Hafez et al., 2020).However, there is no previous study has been reported on the impact of dietary nano zinc in broiler under HSD. As far as our knowledge, there is no published data about the effect of nano Zn on DNA damage in lymphocytes with the comet assay. Therefore, the present study aimed to investigate the impact of dietary nano Zn on stress indicators, immunity, and DNA damage of lymphocytes in broiler under HSD.

## MATERIALS AND METHODS

The study was approved by Aydın Adnan Menderes University Local Ethics Committee (No. 64583101/2017/042) and carried out at the Poultry Research Unit of the Veterinary Faculty.

### Experimental design and diets

A total number of 480 Ross 308 one-day-old male broiler chickens were assigned to 4 experimental groups with 8 subgroups as a completely randomized design with 2 × 2 factorial arrangement of dietary Zn forms (inorganic and nano) and stocking densities (low = 12 birds/$m^2$ or high = 18 birds/$m^2$). Broilers were housed in floor pens that have 1 $m^2$ space (feeders and drinker excluded). Wood shavings were used as litter material. A 23L:1D lighting program was implemented up to 7 days and 18L:6D thereafter until d 42. The temperature of 32 °C was maintained during the first week followed by a reduction of 3 °C per week until d 21 and a temperature of 24-26 °C was maintained afterwards. The duration of the experiment was 42 days and broilers had free access to feed and water.

Corn-soybean meal based diets (starter d 1 to 10, grower d 11 to 24, and finisher d 25 to 42) were formulated according to Aviagen (2014) in mash form (Table 1). Zinc oxide (ZnO) with 99.0% purity was used as the Zn source. Inorganic ZnO (Sigma-Aldrich Co., USA) and nano ZnO (US Research Nanomaterials Inc., USA) were purchased from the market. ZnO was supplemented at 111 mg/kg to provide 80 mg/kg Zn in all diets.

### Chemical analysis of feed

The dry matter (DM), crude protein (CP), ether extract (EE), calcium (Ca), and phosphorus (P) levels of diets were analyzed according to AOAC (2000). To find out the neutral detergent fiber (NDF) values of the diets Van Soest (Van Soest et al., 1991) method was used. The Zn content of feeds was determined by inductively coupled plasma optical emission spectrometry (ICP-OES). Feed sample (0.3 g) was treated at 180 °C with nitric acid (8 mL 65%) and hydrochloric acid (2 mL 37%) in a microwave (MARS 6, CEM Corporation, Matthews, NC) for 20 min. The digested samples filtered and diluted to 25 mL with deionized water.

### Lymphoid organ relative weights

Three chickens from each replicate were slaughtered by decapitation at the end of the experiment. Liver, spleen, and bursa of Fabricius were weighed individually and the relative weights were calculated as a percent of live weight.

### Stress indicators and cytokine levels

On the last day of the experiment, blood samples from the wing vein (v. subcutanea ulnaris) were collected from chickens (two from each replicate). The

**Table 1.** Ingredient and nutrient composition of diets

| Ingredients (g/kg) | Starter (1-10 d) | Grower (11-24 d) | Finisher (25-42 d) |
|---|---|---|---|
| Corn | 555.2 | 560.1 | 589.5 |
| Soybean meal (48% CP) | 375.1 | 360.5 | 334.4 |
| Vegetable oil | 25.1 | 41.6 | 42.4 |
| Limestone | 8.8 | 8.4 | 8.1 |
| Dicalcium phosphate | 23.1 | 20.1 | 17.2 |
| Salt | 3.6 | 3.4 | 3.6 |
| DL-Methionine | 3.6 | 2.4 | 1.3 |
| L-Lysine | 2.0 | - | - |
| Vitamin premix[1] | 2.5 | 2.5 | 2.5 |
| Mineral premix[2] | 1.0 | 1.0 | 1.0 |
| **Nutrient composition (calculated)** | | | |
| Metabolisable energy, kcal/kg | 3033 | 3151 | 3191 |
| Crude protein, CP | 229.6 | 220.0 | 210.0 |
| Calcium, Ca | 10.0 | 9.1 | 8.2 |
| Available phosphorus | 5.0 | 4.5 | 4.0 |
| Methionine-cystine | 10.9 | 9.5 | 8.0 |
| Lysine | 14.2 | 12.1 | 1.5 |
| Zinc, mg/kg | 109.22 | 108.57 | 107.86 |
| **Nutrient composition (analyzed)** | | | |
| Dry matter | 906 | 915 | 914 |
| CP | 229.6 | 220.8 | 211.3 |
| Ether extract | 62.8 | 80.9 | 81.7 |
| NDF | 93.2 | 86.6 | 83.4 |
| Ca | 11.0 | 10.5 | 10.2 |
| Total phosphorus | 8.7 | 8.0 | 7.3 |
| Zinc, mg/kg | 112.3 | 110.9 | 109.5 |

[1]Vitamin premix (per kilogram diet): vitamin A, 12 000 IU; vitamin $D_3$,3 000 IU; vitamin E,50 mg; vitamin $K_3$,5 mg; vitamin $B_1$,3 mg; vitamin $B_2$,6 mg; niacin,30 mg;pantothenic acid, 10 mg; vitamin $B_6$,5 mg; vitamin $B_{12}$,0.03 mg; d-biotin, 0.1 mg; folic acid, 1 mg; choline chloride, 400 mg.
[2]Mineral premix (per kilogram diet): manganese,100 mg; iron,60 mg; copper,5 mg; cobalt, 0.2 mg; iodine, 1 mg; selenium, 0.3 mg.

serum and plasma samples were collected by centrifugation and stored at −20 °C until analyzed.

To determine the heterophil:lymphocyte (H:L)ratio, the smear was made on the glass slide from each blood sample and stained with Wright-Giemsa. Cells were counted with the microscope and the H:L ratio was calculated (Campbell, 1988).

The glutathione (GSH) level in the blood was analyzed as spectrophotometrically (Shimadzu UV-1601, Kyoto, Japan) and the data were determined as µmol/g haemoglobin (Hb) by comparison with the standard GSH solution (Beutler et al., 1963). Hb (U/g Hb) analysis was also performed from the blood samples for the GSH level in the spectrophotometer (Fairbanks and Klee, 1999). SOD activity was measured by spectrophotometer and expressed as U/g protein (Sun et al., 1988). The malondialdehyde (MDA) absorbance was measured spectrophotometrically and its concentration was calculated with the coefficient of absorbance (ε=1.56x105/M/cm) (Yoshioka et al., 1979).

Serum corticosterone, interferon-gamma, interleukin 12, and interleukin 18 (Cusabio Biotech Co. Ltd. Wuhan, Hubei, China, Cat. No: CSB-E11991C, CSB-E08550Ch, CSB-E12836C, and CSB-E10070Ch, respectively) levels were determined by Enzyme-linked immunosorbent assay (ELISA) using a commercial kit, following the manuals.

**Comet assay**

Comet assay was carried out according to the

method of Collins et al.(1997)which was modified by Boyacioglu et al.(2014). Lymphocytes were isolated from the blood taken into the tubes containing sodium heparin and tail density and tail moment parameters (%) were examined to determining DNA damage. Preparation of the slides and embedding of the cells in agarose, lysis, dissolution of the DNA helix, electrophoresis, neutralization, and staining steps were performed. Finally, damaged and undamaged DNA images were obtained by evaluating the slides under a fluorescence microscope. The evaluation was carried out through computer software of Comet Assay v4.3 (Perceptive Instruments, UK).

**Statistical analysis**

The impact of SD and dietary Zn form was evaluated using GLM procedures of SPSS (version 22.0; IBM Corp., Armonk, NY, US). The statistical model of variance analysis used was as follows.

$$Y_{jkl} = \mu + s_j + l_k + e_{jkl}$$

Where:

$Y_{jkl}$ is the phenotypic value of the trait for the $l^{th}$ group of broilers belonging to $j^{th}$ SD level and $k^{th}$ Zn form in the diet;

$\mu$ = mean value of the trait for a given population;

$s_j$ = effect of $j^{th}$ SD level (j = 1, 2);

$l_k$ = effect of $k^{th}$ Zn form in the diet (k = 1, 2,);

$e_{jkl}$ = effect of experimental error.

For comparison of the means Duncan's Multiple Range test was performed. The P-values less than 0.05 were assumed significant.

## RESULTS

The average live weight was determined as 34.9 kg and 51.3 kg kg/m$^2$ in low stocking density (LSD) and HSD groups, respectively at the end of the experiment.

The interaction was not determined between SD and dietary Zn form for relative lymphoid organ weights. HSD decreased liver weight (P<0.05) but did not affect the weight of spleen and bursa of Fabricius. The impact of dietary nano zinc on relative lymphoid organ weights of broiler was not significant (Table 2).

In the present study, no interaction was found between SD and dietary Zn form for H:L, serum corticosterone, GSH, SOD, and MDA levels of broilers.

**Table 2.** Effects of stocking density (SD) and dietary zinc form on relative lymphoid organ weights of broiler, % of body weight

| Item | Liver | Spleen | Bursa of Fabricius |
|---|---|---|---|
| LSD[1] | | | |
| Inorganic zinc | 1.89 | 0.08 | 0.19 |
| Nano zinc | 1.85 | 0.08 | 0.20 |
| HSD[2] | | | |
| Inorganic zinc | 1.73 | 0.08 | 0.19 |
| Nano zinc | 1.78 | 0.08 | 0.18 |
| SEM[3] | 0.05 | 0.00 | 0.01 |
| | | | |
| SD | | | |
| Low | 1.87 | 0.08 | 0.20 |
| High | 1.76 | 0.08 | 0.18 |
| Zinc form | | | |
| Inorganic | 1.81 | 0.08 | 0.19 |
| Nano | 1.81 | 0.08 | 0.19 |
| SEM | 0.04 | 0.00 | 0.01 |
| | | | |
| *p*-values | | | |
| SD | 0.04 | 0.80 | 0.19 |
| Zinc form | 0.94 | 0.72 | 0.83 |
| SD × Zinc form | 0.40 | 0.20 | 0.33 |

[1]LSD: Low stocking density
[2]HSD: High stocking density
[3]SEM: Standard error mean

**Table 3.** Effects of stocking density (SD) and dietary zinc form on blood heterophil:lymphocyte (H:L) ratio, serum corticosterone (ng/ml), glutathione (GSH, μM/g Hb), superoxide dismutase (SOD, U/mg protein), and malondialdehyde (MDA, nmol/g protein) levels in broiler

| Item | H:L | Corticosterone | GSH | SOD | MDA |
|---|---|---|---|---|---|
| LSD[1] | | | | | |
| Inorganic zinc | 0.89 | 7.97 | 384.75 | 0.12 | 553.57 |
| Nano zinc | 0.92 | 7.96 | 412.74 | 0.11 | 530.94 |
| HSD[2] | | | | | |
| Inorganic zinc | 1.01 | 8.41 | 430.14 | 0.11 | 562.76 |
| Nanozinc | 1.02 | 1.21 | 392.77 | 0.11 | 523.69 |
| SEM[3] | 0.07 | 0.55 | 56.10 | 0.01 | 28.67 |
| | | | | | |
| SD | | | | | |
| Low | 0.90 | 7.97 | 398.74 | 0.12 | 542.26 |
| High | 1.01 | 9.31 | 411.45 | 0.11 | 543.22 |
| Zinc form | | | | | |
| Inorganic | 0.95 | 8.19 | 407.44 | 0.12 | 558.17 |
| Nano | 0.97 | 9.09 | 402.75 | 0.11 | 527.31 |
| SEM | 0.05 | 0.39 | 39.67 | 0.01 | 20.27 |
| | | | | | |
| *p*-values | | | | | |
| SD | 0.12 | 0.02 | 0.82 | 0.64 | 0.97 |
| Zinc form | 0.76 | 0.11 | 0.93 | 0.47 | 0.29 |
| SD ×Zinc form | 0.88 | 0.10 | 0.56 | 0.93 | 0.78 |

[1]LSD: Low stocking density
[2]HSD: High stocking density
[3]SEM: Standard error mean

**Table 4.** Effects of stocking density (SD) and dietary zinc form on serum interferon-gamma (IFN-γ), interleukin 12 (IL-12), and 18 (IL-18) levels in broiler, pg/ml

| **Item** | **IFN-γ** | **IL-12** | **IL-18** |
|---|---|---|---|
| LSD[1] | | | |
| Inorganic zinc | 56.40 | 59.06 | 5.92 |
| Nano zinc | 52.16 | 51.89 | 5.03 |
| HSD[2] | | | |
| Inorganic zinc | 56.55 | 61.25 | 5.58 |
| Nano zinc | 55.74 | 60.00 | 5.42 |
| SEM[3] | 1.27 | 3.01 | 0.32 |
| | | | |
| SD | | | |
| Low | 54.28 | 55.47 | 5.48 |
| High | 56.14 | 60.63 | 5.50 |
| Zinc form | | | |
| Inorganic | 56.48 | 60.16 | 5.75 |
| Nano | 53.94 | 55.95 | 5.22 |
| SEM | 0.90 | 2.13 | 0.23 |
| | | | |
| *p*-values | | | |
| SD | 0.15 | 0.09 | 0.94 |
| Zinc form | 0.05 | 0.17 | 0.10 |
| SD ×Zinc form | 0.18 | 0.33 | 0.25 |

[1]LSD: Low stocking density
[2]HSD: High stocking density
[3]SEM: Standard error mean

HSD increased serum corticosterone level (P<0.05) however not affect other stress indicators. Dietary nano zinc did not affect theH:L, serum corticosterone, GSH, SOD, and MDA levels significantly (Table 3).

No interaction was determined between SD and dietary Zn form for serum IFN-γ, IL-12, and IL-18 levels of broiler. The IFN-γ, IL-12, and IL-18 levels of broiler was not effected either HSD or dietary nano zinc. However, IFN-γ level tended to reduce by the dietary nano zinc (Table 4).

In this study no interaction was determined between SD and dietary Zn form for DNA damage of lymphocytes. Either HSD or dietary nano zinc had no significant effect on DNA damage in lymphocytes (Table 5).

## DISCUSSION

In the present study, HSD decreased the relative liver weight and did not affect the relative weights of spleen and bursa of Fabricius. Similar to our results, Qaid et al.(2016) reported that HSD reduced the relative weight of the liver but did not affect relative weights of spleen and bursa of Fabricius in the 14-day old broiler. Similarly, Simitzis et al.(2012) found that HSD decreased the relative weight of the liver and bursa of Fabricius but had no significant effect on spleen weight. HSD might be resulting in less developed liver depend on the stressor effect.

This is the first report that investigates the effect of dietary nano zinc on lymphoid organ weights of broilers reared HSD. However, some previous studies stated that dietary nano zinc had no significant effect on lymphoid organ weights of broiler (Mohammadi et al., 2015a; 2015b; Hussan and Kirishna 2018). Depend on these results, it may be concluded that dietary nano zinc had no effect on relative lymphoid organ weights of broiler.

In the current study, HSD increased the serum corticosterone level but did not affect other stress indicators. There are varied reports on the effect of HSD on stress indicators. Houshmand et al. (2012) demonstrated that HSD (16 birds/$m^2$) had no significant effect on H:L ratio and corticosterone levels in broiler. Likewise, Cengiz et al.(2015) found that H:L ratio, corticosterone, MDA and nitric oxide levels did not differ in broiler reared at LSD or HSD (20 birds/$m^2$). However, Uzum and Oral Toplu (2013) determined that H:L ratio increased and corticosterone levels did not affect in broiler under HSD (18 birds/$m^2$). In another study, HSD (27.2 kg weight/$m^2$) was

**Tablo 5.** Effects of stocking density (SD) and dietary zinc form on DNA damage in lymphocytes by comet assay

| Item | Tail intensity | Tail moment |
|---|---|---|
| LSD[1] | | |
| Inorganic zinc | 31.96 | 16.72 |
| Nano zinc | 33.98 | 16.96 |
| HSD[2] | | |
| Inorganic zinc | 36.58 | 18.58 |
| Nano zinc | 35.36 | 18.86 |
| SEM[3] | 4.41 | 3.37 |
| | | |
| SD | | |
| Low | 32.97 | 16.84 |
| High | 35.97 | 18.72 |
| Zinc form | | |
| Inorganic | 34.27 | 17.65 |
| Nano | 34.67 | 17.91 |
| SEM | 3.12 | 2.38 |
| | | |
| *p*-values | | |
| SD | 0.50 | 0.58 |
| Zinc form | 0.93 | 0.94 |
| SD ×Zinc form | 0.72 | 0.99 |

[1]LSD: Low stocking density
[2]HSD: High stocking density
[3]SEM: Standard error mean

increased the H:L ratio and decreased the GSH level (Simitzis et al., 2012). The physiological changes that occur during stress and the biological defences and responses against it may change, so the effects of varied stocking densities on different stress parameters may also change. However, it is possible that the inconsistency between studies in stress indicators could be the result of the research protocol, housing, and management variations. A previous study reported that broiler welfare was affected by housing conditions rather than SD (Simitzis et al., 2012).

The dietary nano zinc had no significant effect on stress indicators. This is the first report about the effects of dietary nano zinc in broilers reared at HSD. There is some inconsistency between the results of previous reports on the effect of nano zinc. Zhao et al. (2012) found that dietary nano zinc (60 and 100 mg/kg) did not change serum SOD activity to the 21st and 42nd days of the experiment, but increased it on the 28th and 35th days in broiler. Hafez et al. (2020) stated that dietary nano zinc (40 and 80 mg/kg) increased serum SOD and catalase (CAT) activity and decreased MDA level in broiler chickens reared without stress. However, Ramiah et al. (2019) reported that dietary nano zinc (60 and 100 mg/kg) increased the thigh meat MDA level on the 0 and 1 day, but decreased the level on the 7th day in broiler under heat stress. These variable results may arise from the level of dietary nano zinc, research protocol, housing, and management differences between among experiments.

In the present study,it was determined that HSD had no significant effect on cytokine levels (IFN-γ, IL-12, and IL-18 levels). Similar results were found by Jang et al.(2014) who reported that HSD (0,023 $m^2$/bird) had no effect on IFN-γ, IL-6, and IL-18 levels in broiler. However, Kang et al.(2011) stated that combined stress (HSD and feed restriction) reduce the levels of inducible nitric oxide synthase expression and hepatic lipopolysaccharide-induced tumor necrosis factor-α but did not affect IL-4 and IL-6 levels in laying hens. The differences between results may depend on the source and severity of stress.

According to results, dietary nano zinc had no significant effect on cytokine levels. As far as we know, there is no data about the effects of dietary nano zinc on cytokine levels in broiler. However, some researchers such as Mohammadi et al. (2015a) and Asheer et al. (2018) reported that dietary nano zinc had no significant effect on antibody titer against Newcastle disease virus. Nevertheless, Hafez et al.(2020) stated that dietary nano zinc increased serum immunoglobulin Y, total lymphocyte count, and macrophage levels in broiler.

Although there are very few literature on the effect of environmental stress sources (such as SD or temperature) on DNA damage in poultry (Sohn et al., 2012; Jastrebski et al., 2017). Sohn et al.(2012) stated that stress (HSD and feed restriction) increased the DNA damage in laying hens. In this study, HSD did not affect DNA damage (tail intensity and tail moment) of lymphocytes. This inconsistency may arise from source and severity of stress.

To the best of our knowledge, there is no literature describing the effect of dietary nano zinc on DNA damage in broiler. However, recently Attia et al.(2018) reported that oral exposure of nano zinc (100 mg/kg daily for 7 days) induced oxidative DNA damage in rats. Also, it is known that Zn deficiency caused DNA damage in peripheral blood cells and Zn is essential for DNA repair (Song et al., 2009). In the current study, the level of dietary nano zinc neither inadequate nor toxic therefore the possible effect of nano zinc could not be seen.

## CONCLUSION

High stocking density decreased relative liver weight and increased serum corticosterone levels but did not affect other lymphoid organ weights and stress indicators. Likewise, HSD had no effect on cytokine levels and DNA damage. Dietary nano zinc had no effect on relative lymphoid organ weights, stress indicators, cytokine levels, and DNA damage but IFN-γ level tended to reduce by the effect of dietary nano zinc. Further research is needed to understand the effects and determine the optimum dietary level of nano zinc in broiler.

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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