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**RESEARCH ARTICLE**
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# ZINC OXIDE NANOPARTICLES AND CHITOSAN OLIGOSACCHARIDE SUPPLEMENTATION ALLEVIATE HEAT STRESS INDUCED EFFECTS ON GROWTH PERFORMANCE, DUODENUM HISTOMORPHOMETRY, ANTIOXIDANT ACTIVITY AND SERUM MINERAL PROFILES OF BROILER CHICKENS

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## Abstract

This study aimed to evaluate the effects of zinc oxide nanoparticles (ZnO-NPs) and chitosan oligosaccharide (COS) on heat-stressed broiler chickens. D-old 336 birds were divided into seven groups, comprising 6 replicates with eight birds per replicate. G-Group (NC) Negative Control, fed basal diet only; (PC) HS + Positive Control; (HZ) HS + Zinc oxide 60 mg/kg (ZnO); (HZN) HS + ZnO-NPs 60 mg/kg; (HC) HS + COS 200 mg/kg; (HZC) HS + ZnO 60 mg/kg + COS 200 mg/kg; (HZNC) HS + ZnO-NPs 60 mg/kg + COS 200 mg/kg. An interaction effect ($p \leq 0.05$) was observed between ZnO-NPs and COS on parameters such as feed conversion ratio (FCR), body weight gain (BWG), feed consumption, and the relative weight of immune and visceral organs. G-HZNC showed excellent improvements ($p \leq 0.05$) in BWG, FCR, and feed conversion ratio. Similarly, the G-HZNC displayed significant improvement ($p \leq 0.05$) in the weights of the small intestine, bursa, and cecal tonsil. Furthermore, G-HZNC significantly ($p \leq 0.05$) enhanced the duodenum villus structure and goblet cell count ($p \leq 0.05$) compared to G-PC. Among the groups, the best results were found in G-HZNC, where the thickness of the lamina propria, muscularis mucosa, and muscularis externa significantly increased ($p \leq 0.05$). The antioxidant profile, including enzymes, superoxide dismutase SOD significantly increased ($p \leq 0.05$) in G-HZNC compared to G-PC, while glutathione peroxidase GPx and malondialdehyde MDA concentration significantly reduced ($p \leq 0.05$) in all dietary groups

compared to G-PC. This study concluded that 60 mg/kg of ZnO-NPs with 200 mg/kg of COS mitigated the detrimental impacts of heat stress. This combination enhanced growth performance, duodenal histomorphometry, increased antioxidant enzymatic activity, and serum mineral profile in broiler chickens.

**Keywords:** Heat stress, Zinc Oxide Nanoparticles, Chitosan oligosaccharide, Growth performance, Goblet cells

## INTRODUCTION

The elevated ambient temperature serves as a significant stress factor impacting the physiological well-being and growth of animals, leading to notable morbidity and mortality within the poultry sector (Hu et al., 2024). Heat stress detrimentally affects the microbial population within the intestines and compromises the integrity of the duodenum's structure, ultimately hampering growth performance (Ashraf et al., 2013). In times of stress, intraepithelial lymphocytes and goblet cells play a pivotal role in safeguarding against pathogens and enhancing gut immunity (Shah et al., 2020). Conversely, increased heat stress results in reduced growth performance, feed intake, and immune response in birds (Quinteiro-Filho et al., 2010). Moreover, heightened heat stress triggers an escalation in the body's oxidative response, posing a more complex physiological challenge. This leads to diminished body growth rate, compromised intestinal integrity, altered muscle pH, decreased water holding capacity, and cellular damage, attributed to the degradation of proteins, lipids, and DNA (Huang et al., 2015). Goblet cells produce mucin, which acts as a protective layer over the duodenum's mucosal lining. However, heat stress adversely affects these goblet cells, resulting in a weakened intestinal defensive response and increased susceptibility to various pathogens (Cornick et al., 2015). The disruption caused by heat stress to the intestinal barrier leads to damage to the epithelial tissue. Therefore, a more concerted effort is needed to manage the health of the intestinal epithelium in broilers (Chand et al., 2016).

Zinc, a trace mineral, cannot be stored in the animal body. Consequently, it is vital for over 300 enzymatic reactions and plays a critical role in maintaining intestinal integrity and mounting immune protective responses. Additionally, it assumes an inflammatory role in the gut and exhibits bactericidal activity, potentially serving as an alternative to antibiotics (Shah et al., 2020). According to the NRC, the recommended dose of zinc in the basal diet is between 45 to 75 mg/kg, sufficient to meet the growth and performance requirements of broiler chickens. Antibiotics have historically been employed to enhance growth performance; the increasing use of antibiotics over the past 70 years has driven a demand for antibiotic-free meat within the commercial poultry market (Haque et al., 2020; Mak et al., 2022).

Nanotechnology introduces a novel dimension to dietary supplementation, involving atoms with sizes ranging from 1 to 100 nm and notable chemical stability. Zinc oxide nanoparticles are utilized due to their superior bioavailability and are also found in applications such as transduction electronics, cosmetics, and food packaging (Ali et al., 2017). These nanoparticles possess antimicrobial, immune-boosting, and antioxidant properties, showing significant potential for enhancing growth performance during heat stress in broiler chickens (Zhao et al., 2014). Zinc oxide nanoparticles improved growth performance, physiological status, antioxidant status, and serum indices in broiler chickens (Hatab et al., 2023).

Chitosan, an affordable and readily available prebiotic agent derived from chitin, is commonly used, often in the form of chitosan oligosaccharides, to meet European Union standards (Li et al., 2019). The inclusion of prebiotics in the diet can improve animal growth performance, the intestinal barrier, serum indices, and amino acid digestibility in broiler chickens (Lan et al., 2020). However, dietary COS can reduce the activity of antioxidant enzymes during oxidative stress, negatively affecting intestinal integrity and promoting the growth of harmful bacteria. This, in turn, leads to reduced production efficiency in broiler chickens (Li et al., 2019).

The present study aims to investigate the potential of using either ZnO or nano-zinc oxide, alone or in combination with a prebiotic, to mitigate the detrimental effects of heat stress, provide antibiotic-free meat, and enhance growth performance, intestinal villus structure, serum mineral concentration, and antioxidant enzyme activity in broiler chickens subjected to cyclic heat stress.

## Materials and Methods

### Zinc oxide Nanoparticles Preparation

Zinc oxide nanoparticles were synthesized at the National Center of Excellence in Analytical Chemistry, University of Sindh, Jamshoro (Ramiah et al., 2020).

### Birds' management

The study focused on Ross day-old 336 broilers obtained from a commercial hatchery, with duration of 42 days. Upon arrival, each bird was weighed and distributed. The study involved day-old (Cobb 500) 336 birds, divided into seven groups comprising 6 replicates with eight birds per replicate. G-Group (NC) Negative Control, fed basal diet only, (PC) HS + Positive Control, (HZ) HS + Zinc oxide 60 mg/kg (ZnO), (HZN) HS + ZnO-NPs 60 mg/kg, (HC) HS + COS 200 mg/kg, (HZC) HS + ZnO 60 mg/kg + COS 200 mg/kg, (HZNC) HS + ZnO-NPs 60 mg/kg + COS 200 mg/kg.

### Heat Stress Management

Upon arrival, the shed temperature was maintained at 35℃ with 65% humidity. Over an 8-day interval, the temperature was gradually lowered by 2.8℃ until it reached 26℃, considered a thermoneutral zone, with 65% humidity for the first 21 days. On day 22, the negative control group remained at normal temperature (control), while the other six groups were exposed to 35℃ heat stress for 8 hours (Lochi et al., 2023).

### Growth Assessment and Sampling

The experiment was conducted over a span of 42 days within controlled environmental conditions in experimental sheds situated at the Department of Poultry Science, Sindh Agriculture University, Tandojam, Pakistan. Daily weight gain and feed consumption were recorded using leftover feed. Feed conversion ratio (FCR) was calculated weekly using FCR

### Feed consumed / body weight gain

After 42 days, 12 birds from each group were randomly selected, weighed, and blood samples were collected from the wing vein. Various immune and visceral organs were weighed. A portion of the small intestine duodenum mid-portion was taken, washed with normal saline, and then stored in 10% formalin solution for histomorphometry study.

### Intestinal morphology analysis

Tissues were processed for light microscopy by paraffin embedding technique. All samples were washed overnight and dehydrated through an ascending series of ethyl alcohol concentrations of 70%, 80%, 90%, and 100% for two hours (Spencer et al., 2012).

### Duodenum and goblet cells morphology

### Alcian blue periodic acid-Schiff (AB-PAS)

Histo morphometry analysis, slides were observed under a microscope, and a specific software program (Prog Res®2.1.1 Capture Prog Camera Control Software) was used to measure various parameters in the small intestine, including villus height, width, crypt depth, thickness of the lamina propria, and thickness of the tunica muscularis. Five well-oriented villi with intact lamina propria in each intestinal cross section were selected for measuring villus height, width, and crypt depth. The villus height (μm) was measured from the tip of villus crypt, width of villus was recorded at three

points, i.e., at the tip of the villus, at the midpoint, and at the base of the villus. The average of these three values was used as width of villus. The surface area of the villus (μm²) was calculated by the following formula: (*VW/2) × (VL) × (2π).*
While counting goblet cells, the slides were stained with combined Alcian blue-PAS to be observed under bright field microscope at 10X (Labomed USA). Goblet cells, which have acidic mucin stained blue; magenta is the result of neutral mucin stained; and mixed goblet cells containing both neutral mucins stained purple and acidic (Ali et al., 2017).

**Antioxidant profile and hormonal analysis**
Antioxidant status was evaluated using blood samples. Serum was obtained after centrifugation, and the activity of MDA through the ELISA kit (DEIA,3918), SOD (BCI-70-50T/24S), and GPx enzymes was determined using Salarbio/ BC 1190 ELISA kit, and test method was spectrophotometry and size 50T/24J Beijing, China. Serum hormone concentrations (cortisol and cholesterol) were measured using commercially available ELISA kits.

**Serum mineral digestion**
For mineral concentrations in the serum, around 1ml of serum sample was placed in a Schott bottle, to which a mixture of HClO4 (70%) and H2O2 (30%) in a 2:1 ratio (2 ml) was added. These tubes were subjected to digestion on a hot plate. The digested samples were then filtered using Whatman filter paper No (X). Serum mineral content of Zn, Cu, Mn, Ca, P, and Fe was quantified utilizing an automated analyzer (Thermo Electron Corporation, England; Serial No: GE650228) of M6 Mk2 AA system, operating at 100-240 V~, 50-60 Hz, and 300 VA.

**Statistical Analysis**
Data were analyzed using one-way ANOVA in SPSS, with results presented as means ± standard error. Statistical differences were determined using Duncan's multiple range tests, with significance set at ($p \leq 0.05$).

**Results - Growth Performance**
**Live body weight gain**
During the initial three weeks of the study, body weight gain was nearly identical across all groups due to consistent management practices. However, in the fourth week, a significant improvement ($P<0.05$) in body weight gain was observed in all supplemented groups. G-PC displayed reduced body weight gain compared to G-NC. Moving into the fifth week, a decrease in weight gain was noted in G-PC compared to supplemented groups. Body weight gain was evident in the sixth week of the G-HZNC as compared to what the G-PC demonstrated. Among all supplemented groups, G-HZNC has the best body weight gain (Table 1).

**Feed intake**
In the fourth week, the groups G-HZNC had higher feed consumption with a significant level of ($p \leq 0.05$) compared to G-PC. Particularly, the G-HZNC displayed higher feed consumption compared to all other treated groups. In the fifth and sixth weeks, the G-HZNC displayed highly significant feed intake ($p \leq 0.05$) compared to the G-PC, and similarly, the G-HZNC displayed high significance ($p \leq 0.05$) feed intake compared to other treated groups. (Table 1).

**Feed FCR**
Throughout the initial three weeks, there were non-significant differences in feed conversion ratio (FCR) among all groups. However, on day 22, when cyclic heat stress was initiated, a significant increase in FCR with a significance of ($p \leq 0.05$) was observed in the G-HZNC compared to the G-PC. In the fifth week, a significant difference in FCR was observed in the G- HZNC ($p \leq 0.05$), in contrast

to the G-PC. In the sixth week, the FCR was notably higher ($p \leq 0.05$) in the G- HZNC compared to other treated groups (Table 1).

**Weight of Visceral and Immune Organs**

The weight of the bursa was significantly greater ($p \leq 0.05$) in all supplemented groups compared to G-PC. The weights of the thymus and cecal tonsil exhibited significant improvement ($p \leq 0.05$) across all dietary groups compared to G-PC. The weight of the small intestine was notably higher ($p \leq 0.05$) in group G-HZNC compared to G-PC and G-NC. The most favorable outcomes were observed in the G-HZNC. The weight of the gizzard was significantly higher ($p \leq 0.05$) in G-HZNC as compared to G-PC. (Table 3).

**Duodenum Histo-Morphometry**

The study's findings indicated a significant increase ($p \leq 0.05$) in duodenal villus height across all supplemented groups. Particularly, G-HZNC has the best villus height as compared to G-PC. The G-HZNC also showed better results in terms of villus surface area, crypt depth, and villus width. The thickness of the intestinal lamina propria was significantly higher ($P \leq 0.05$) in the G-HZNC compared to the G-PC. Muscularis mucosa and muscularis externa exhibited a significant increase ($p \leq 0.05$) in G- HZNC compared to G-PC. VH:CD (villus height to crypt depth ratio) was consistently higher in all supplemented groups in comparison to G-PC. Overall, the most favorable duodenum morphometric results were observed in the G-HZNC (Table 2).

**Goblet Cells Count**

The count of goblet cells in the duodenum exhibited a significant increase ($p \leq 0.05$) in G-HZNC compared to the G-PC (Table 3).

**Antioxidant Profile**

Across all supplemented groups, there was a highly significant increase ($P \leq 0.05$) in the antioxidant profile of glutathione peroxidase (GPx). On the other hand, the concentration of malondialdehyde (MDA) was notably reduced in the G-HZNC compared to the G-PC. Superoxide dismutase (SOD) also exhibited a significant increase ($p \leq 0.05$) in all supplemented groups in relation to both G-PC and G-NC. Particularly positive outcomes were observed in G-HZNC corelate to G-PC (Table 3).

**Serum Minerals Profile**

The concentrations of zinc, selenium, calcium, phosphorus, iron, and copper demonstrated significant improvement ($p \leq 0.05$) across all supplemented groups compared to G-PC. The most favorable outcomes were observed in G-HZNC (Table 4).

**Discussion**

Zinc oxide cannot be retained within the body, necessitating an increased intake of zinc during various stressful conditions, as noted by Fatima et al., (2024). The European Union prohibited antibiotic usage in the animal industry in 2006 due to antibiotic resistance and consumer demand for antibiotic-free meat. To address this, ZnO-NPs, a trace mineral feed additive, has been explored as an alternative to antibiotics under heat stress conditions (Fatima et al., 2024; Wu, 2024).

Zinc functions as a co-factor for over 300 enzymes essential for processes such as production, reproduction, hormone regulation, antioxidant activity, and enhanced mineral absorption, which have been linked to improve mineral absorption with zinc supplementation (Prasad and Bao, 2019). This study is aligned with the findings of Fathi et al., (2016). Notably, the addition of single or combined ZnO-NPs with probiotics resulted in significant increases in body weight gain and feed conversion ratio (FCR) over the last three weeks, contrasting non-significant results in the positive control group due to potential oxidative stress impacting growth, as suggested by (Shah et al., 2020).

The positive outcomes in growth, FCR, and feed intake in the prebiotic group could be attributed to the antimicrobial and immune response properties of chitosan, which, as per Lochi et al., (2023) have been shown to enhance growth performance and FCR in heat-stressed broiler chickens. Oxidative stress may lead to intestinal injuries and digestive problems, affecting visceral and immune organs. Zinc's potential to enhance growth hormone and insulin-like growth factor actions could influence growth, production, and feed efficiency, as described by (Ibrahim et al., 2022). Consistent with Sagar et al. (2018) findings, the present study shows increased relative weights of immune organs, liver, and gizzard, possibly due to the antimicrobial properties of zinc oxide. Prebiotics, being beneficial undigestible feed ingredients, can improve weight gain and production by countering undesirable bacterial populations during critical periods, as indicated by (MH et al., 2022).

The concurrent use of zinc oxide nanoparticles and Bacillus coagulans exhibited improved body weight gain, FCR, and feed conversion ratio, which aligns with the results of (Khajeh Bami et al., 2020). Heat stress triggers the production of free radicals, detrimental to immunological and intestinal functions, potentially leading to reduced feed intake and productivity, as noted by (Shah et al., 2020). Histological changes in the small intestine, like crypt hyperplasia and villus atrophy, could compromise absorption, which corresponds with (Khan and Islam, 2012). However, the present study demonstrated enhanced villus morphology in all supplemented groups, consistent with (Sohail et al., 2012).

Prebiotics positively influence gut morphology and microflora, like (Bami et al. 2022) findings. Additionally, prebiotic-induced enhancements in duodenal morphology have been observed, consistent with (Ashraf et al., 2013). Oxidative stress can diminish small intestine absorptive activity, but the present study's results are associated with (Zhao et al., 2014). Showing increased thickness of lamina propria and muscularis mucosa, possibly due to zinc's bactericidal effects and reduction of free radicals, as explained by Wu et al., (2018). Zinc's involvement in maintaining gut health is evident in the linkage between intestinal epithelium and immune cells. Prebiotic-induced improvements in intestinal morphology align with Ahmadi et al., (2013) findings. In the context of heat stress, serum cholesterol and cortisol concentrations were found to increase, potentially due to hypothalamic-pituitary-adrenal axis activation. This corresponds with Piray et al., (2022) research and (Reza et al., 2014) report on zinc's cholesterol and cortisol-reducing effects.

Zinc's antioxidant properties contribute to decreased cholesterol and cortisol levels, combating oxidative stress. The present study's results align with these findings, highlighting the role of zinc in reducing oxidative damage. Serum mineral concentrations increased due to longer villi, which allowed better nutrient absorption. However, heat stress can affect mineral absorption in the small intestine, as observed by (Sirelkhatim et al., 2015; Wang et al., 2016).

The study also showcased alterations in antioxidant enzyme activity, such as reduced malondialdehyde and improved superoxide dismutase activity. These findings reflect a complex interplay of enzymatic reactions during heat stress, consistent with (Fathi et al., 2016) study. Enhanced glutathione peroxidase activity could be an adaptive response to oxidative stress, while zinc's role as an antioxidant and its influence on enzyme systems is highlighted by (El-Bahr et al., 2020).

The synergistic effects of zinc and prebiotics could explain the reduction in MDA and SOD concentrations. The study underscores the importance of zinc in promoting antioxidant defense mechanisms and increasing the absorption of minerals from small intestine. Notably, the present study provides insights into the relationship between zinc and antioxidant enzyme activities, aligning with prior research (Zhang et al., 2022).

In conclusion, the study offers a comprehensive examination of zinc's multifaceted roles in growth performance, gut health, antioxidant activity, and mineral homeostasis under cyclic heat stress conditions. The findings corroborate and expand upon existing knowledge in the field, shedding light on the intricate interplay between these factors in broiler chicken physiology.

## Conclusion

Dietary supplementation of Nano-Zinc oxide 60 mg/kg alone or combined with prebiotic (chitosan) 200 mg/kg; ameliorated the harmful effects of heat stress, and provided antibiotic-free meat with improved growth performance, intestinal villus structure, and enhanced antioxidant enzymatic activity. Therefore, a combination of ZnO-NPs with COS has a synergistic effect and could serve as an antibiotic free additive on commercial farms for improving meat quality.

## Ethical Endorsement

The process of sample collection received official authorization from the Faculty of Animal Husbandry and Veterinary Science, Sindh Agriculture University, Tandojam. The established protocols obtained approval from the Directorate of Advanced Study and Research at Sindh and Animal Welfare Committee Agriculture University under reference No-DAS- 1728/2021.

## Participation Consent

All authors actively contributed to the ongoing research effort. The research methodology was formulated by J. A Gandahi, S. A Hadi, M. G Shah, Saima Masood, while data collection and analysis were executed by G. Lochi, T. Farooq, M Hayat. Data preparation was performed by S.A M. Hayat, N. S Gandahi, M Nawaz, A.A Farooq, M.U Saleem, the final version of the manuscript was reviewed by M.A Javid, M.F Hassan and approved by J.A Gandahi and S.A Hadi. All authors collectively endorsed and ratified the final manuscript.

## Conflict of Interests

The authors confirm the absence of any conflicting financial or non-financial concerns.

## Acknowledgments

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**Table 1.** Zinc Oxide Nanoparticle with prebiotic effect on growth performance under heat- Stressed broiler chicken (Mean ± SEM)

| GROUPS | NC | PC | HZ | HZN | HC | HZC | HZNC | P-value |
|---|---|---|---|---|---|---|---|---|
| **Feed intake** | | | | | | | | |
| 3rd | 1002.20±4.9 | 1010.10±4.7 | 1005.60±3.3 | 988.50±3.6 | 1008.10±4.5 | 998.60±5.9 | 1010.11±3.3 | 0.12 |
| 4th | 1922.50±8.3[a] | 1650.33±4.5[d] | 1820.8±5.4[c] | 1850.22±6.1[b] | 1830.60±7.4[c] | 1890.24±6.3[b] | 1930.90±7.6[a] | 0.01 |
| 5th | 3276.77±7.1[a] | 2830.33±4.2[d] | 3107.70±6.3[b] | 3150.99±9.10[b] | 3002.60±10.0[c] | 3210.78±4.3[a] | 3310.55±19.3[a] | 0.05 |
| 6th | 3780.55±3.3[a] | 3410.78±5.4[c] | 3628.88±3.5[b] | 3670.44±9.1[b] | 3596.43±3.2[b] | 3750.80±1.9[b] | 3790.33±8.4[a] | 0.04 |
| **Body weight growth** | | | | | | | | |
| 3rd | 800.70±3.2 | 804.22±4.0 | 807.10±2.6 | 810.11±2.4 | 799.12±2.2 | 806.30±3.5 | 808.33±4.3 | 0.07 |
| 4th | 1418.4±3.2[a] | 1280.34±3.1[d] | 1340.33±2.5[bc] | 1390.33±3.6[bc] | 1330.11±4.1[bc] | 1440.54±3.6[b] | 1465.42±3.5[a] | 0.03 |
| 5th | 2014.4±13.5[a] | 1790.20±13.7[d] | 1890.60±7.1[c] | 1920.0±10.8[b] | 1880.60±11.6[c] | 1980.60±9.9[a] | 2040.60±7.8[a] | 0.00 |
| 6th | 2420.20±1.3[a] | 2020.26±1.5[d] | 2170.0±1.7[c] | 2210.6±1.5[b] | 2150.43±6.1[c] | 2367.21±2.7[b] | 2460.24±3.2[a] | 0.02 |
| **Feed conversion ratio** | | | | | | | | |
| 3rd | 1.28 ±.029 | 1.29±.060 | 1.26±.011 | 1.28±.023 | 1.24±.037 | 1.28±.032 | 1.25±.019 | 0.13 |
| 4th | 1.39±.015 | 1.51±.015 | 1.41±.035 | 1.40±.018 | 142±.031 | 1.37±.049 | 1.33±.019 | 0.01 |
| 5th | 1.55±.038 | 1.73±.049 | 1.68±.013 | 1.60±.037 | 1.66±.048 | 1.54±.008 | 1.52±.037 | 0.00 |
| 6th | 1.72±.03 | 1.99±.09 | 1.83±.028 | 1.74±.028 | 1.79±.041 | 1.70±.015[d] | 1.69±.044[cd] | 0.01 |

[a–d] means with different superscripts are significantly different($P<0.05$) within the same row, Values represent the Mean ± SEM of Five Replicates. Weight in gram HS= Heat stress; PB = Prebiotic; Zn = Zinc NC* Represents Negative Control and PC* Represents Positive Control: NPs* Nanoparticle; BWG= Body Weight

**Table 2.** Zinc Oxide Nanoparticle and prebiotic effect on Duodenum Histo-morphometry of heat-stressed broiler chicken (Mean ± SEM)

| Groups | NC | PC | HZ | HZN | HC | HZC | HZNC | P-value |
|---|---|---|---|---|---|---|---|---|
| VH | 1160±7.3$^{b}$ | 970±4.7$^{c}$ | 1020±10.5$^{c}$ | 1180±8.6$^{b}$ | 1140±5.3$^{b}$ | 1215±6.5$^{a}$ | 1280±46.2$^{a}$ | 0.023 |
| VW | 120.3±3.6$^{c}$ | 90.8±2.3$^{d}$ | 120.8±3.4$^{c}$ | 141.1±2.3$^{b}$ | 125.4±2.3$^{c}$ | 150±4.3$^{a}$ | 165±3.1$^{a}$ | 0.000 |
| VSA | 0.28±0.5$^{a}$ | 0.15±0.6$^{c}$ | 0.20±0.9$^{b}$ | 0.22±0.1$^{b}$ | 0.18±82$^{c}$ | 0.26±0.6$^{a}$ | 0.34±0.6$^{a}$ | 0.012 |
| CD | 190.8±1.6$^{b}$ | 110.3±1.3$^{d}$ | 130.6±5.4$^{c}$ | 140.2±3.3$^{c}$ | 130.5±1.3$^{c}$ | 165.4±4.3$^{b}$ | 205.7±1.1$^{a}$ | 0.000 |
| LPT | 125.2±1.6$^{c}$ | 105.8±1.3$^{d}$ | 125.8±5.4$^{c}$ | 135.5±2.3$^{b}$ | 118.4±1.3$^{c}$ | 149.2±4.3$^{a}$ | 155.0±1.1$^{a}$ | 0.000 |
| MM | 31.8±4.52$^{b}$ | 21.0±2.95$^{d}$ | 26.3±2.2$^{c}$ | 30.6±1.5$^{b}$ | 25.4±2.1$^{c}$ | 33.8±1.4$^{b}$ | 40.8±1.3$^{a}$ | 0.016 |
| ME | 190±5.5$^{a}$ | 110.4±2.9$^{d}$ | 140.7±2.2$^{c}$ | 156.8±1.5$^{b}$ | 138.8±2.1$^{c}$ | 170.2±1.4$^{b}$ | 205.2±1.3$^{a}$ | 0.006 |
| VH:CD | 6.3±1.6$^{b}$ | 8.3±1.2$^{a}$ | 7.8±1.3$^{a}$ | 8.4±1.6$^{a}$ | 8.7±1.1$^{a}$ | 8.1±1.7$^{a}$ | 6.2±1.3$^{b}$ | 0.001 |

$^{a-e}$Within the same row, means with different superscripts are significantly different($P<0.05$).

Values represent the Mean ± SEM of five replicates, HS: Heat stress; PB: Prebiotic; Zn: Zinc (VH, $\mu$m) Villus height (VW, $\mu$m) Villus width (VSAmm$^{2}$) Villus surface area; CD: Crypt depth; (LPT, $\mu$m) Thickness of lamina propria (MM, $\mu$m) Muscularis mucosa; (ME, $\mu$m) Muscularis externa (VH: CD) indicates ratio of villus height and crypt depth (NC)$^{*}$Negative control (PC$^{*}$) Positive control. : NPs* Nanoparticle.

**Table 3.** Zinc Oxide Nanoparticle and prebiotic effect on goblet cells and antioxidant status of heat-stressed broiler chicken (Mean ± SEM)

| Groups | NC | PC | HZ | HZN | HC | HZC | HZNC | P-value |
|---|---|---|---|---|---|---|---|---|
| AGC | 70.60±5.4$^{c}$ | 58.30±2.9$^{c}$ | 65.60±3.4$^{c}$ | 78.60±3.5$^{b}$ | 70.60±3.4$^{c}$ | 79.60±3.4$^{b}$ | 86.20±2.8$^{a}$ | 0.01 |
| MGC | 89.90±1.3$^{a}$ | 60.30±4.4$^{c}$ | 62.30±2.1$^{a}$ $^{b}$ | 68.90±1.3$^{b}$ | 64.10±2.1$^{a}$ $^{b}$ | 72.60±2.1$^{b}$ | 82.30±1.4$^{a}$ | 0.01 |
| TGC | 160.5±6.5$^{a}$ | 118.60±6.1$^{d}$ | 147.9±5.4$^{a}$ $^{b}$ | 157.5±5.4$^{a}$ | 134.7±5.1$^{b}$ $^{c}$ | 142.6±4.8$^{b}$ | 168.5±4.8$^{a}$ | 0.01 |
| MDA | 1.12$^{d}$ | 6.212$^{a}$ | 3.11$^{b}$ | 2.64$^{c}$ | 3.46$^{b}$ | 2.36$^{b}$ | 1.26$^{d}$ | 0.02 |
| SOD | 340.51$^{b}$ | 280.54$^{c}$ | 314.21$^{b}$ | 331.32$^{b}$ | 308.54$^{b}$ | 355.45$^{b}$ | 371.23$^{a}$ | 0.02 |
| GPx | 128.32$^{c}$ | 115.21$^{c}$ | 127.21$^{b}$ | 136.21$^{b}$ | 125.86$^{b}$ | 143.32$^{a}$ | 152.32$^{a}$ | 0.01 |
| Thymus | 0.12 | 0.14 | 0.16 | 0.18 | 0.17 | 0.19 | 0.21 | 0.03 |
| Bursa | 0.06 | 0.04 | 0.05 | 0.06 | 0.05 | 0.07 | 0.07 | 0.03 |
| CT | 0.04 | 0.02 | 0.04 | 0.04 | 0.03 | 0.04 | 0.04 | 0.05 |
| Gizzard | 2.68 | 2.53 | 2.64 | 2.66 | 2.63 | 2.65 | 2.82 | 0.04 |
| SM | 10.62 | 8.16 | 9.52 | 9.65 | 9.39 | 10.20 | 10.90 | .005 |

$^{a-d}$means with different superscripts are significantly different($P<0.05$) within the same row, Values represent the Mean ± SEM of six replicates. Visceral organ weight and Immune organ weight are in percentage (%) GC= Goblet cells; AGC= Acidic Goblet Cell; MGC= Mixed Goblet Cell; TGC= Total Goblet Cell. NC$^{*}$ represents negative control and PC$^{*}$ represents positive control: NPs* Nanoparticle, CT= Cecal tonsil; SM= small intestine MDA; nmol mL$^{-1}$ = Malondialdehyde; SOD ; $\mu$molg$^{-1}$Hb= Superoxide Dismutase; GPx; $\mu$molg$^{-1}$Hb= Glutathione Peroxidase.

**Table 4.** Zinc Oxide Nanoparticle and prebiotic supplementation on serum indices under heat-stressed broiler chicken (Mean ± SEM)

| Groups | NC | PC | HZ | HZN | HC | HZC | HZNC | P- V |
|---|---|---|---|---|---|---|---|---|
| Zn ug/dl | 86.50±2.4$^{ab}$ | 45.56±2.3$^{d}$ | 65.56±1.4$^{c}$ | 83.45±2.3$^{ab}$ | 61.45±2.2$^{c}$ | 94.34±2.5$^{b}$ | 116.89±2.6$^{a}$ | 0.01 |
| Se mg/l | 0.34±0.03$^{b}$ | 0.11±0.04$^{d}$ | 0.21±0.07$^{c}$ | 0.27±0.03$^{c}$ | 0.20±0.03$^{c}$ | 0.42±0.03$^{b}$ | 0.55±0.07$^{a}$ | 0.01 |
| Ca mg/dl | 14.34±.51$^{a}$ | 6.24±.62$^{d}$ | 9.34±.33$^{c}$ | 11.34±.49$^{bc}$ | 9.11±.43$^{c}$ | 13.34±.63$^{b}$ | 15.78±.69$^{a}$ | 0.01 |
| P mg/dl | 6.34±0.34$^{b}$ | 3.78±0.51$^{d}$ | 5.45±0.42$^{b}$ | 6.34±0.31$^{b}$ | 5.11±0.43$^{c}$ | 6.98±.46$^{b}$ | 7.98±.56$^{a}$ | 0.02 |
| Fe ug/dl | 20.23±.53$^{b}$ | 14.56±.44$^{d}$ | 16.36±.83$^{c}$ | 18.43±.63$^{ab}$ | 16.11±.53$^{c}$ | 20.34±.73$^{b}$ | 22.21±.37$^{a}$ | 0.01 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Cu ug/dl | 1.85±0.032 [a] | .989±0.045 [d] | 1.23±0.023 [c] | 1.65±0.032 [b] | 1.25±0.024 [c] | 1.78±0.033 [b] | 1.93±0.071 [a] | 0.03 |
| Mn ug/dl | 1.94±0.022 [a] | 1.12±0.025 [c] | 1.43±0.043 [b] | 1.55±0.032 [b] | 1.41±0.036 [b] | 1.68±0.023 [b] | 1.89±0.071 [a] | 0.01 |
| CHO mg/dl | 132.3±3.4 [b] | 210±6.4 [a] | 140.6±5.4 [b] | 130.7±6.3 [b] | 145±3.1 [c] | 120±5.6 [d] | 111.6±4.6 [d] | 0.03 |
| CRT ng/ml$^{-1}$ | 1.08±0.021 [b] | 2.12±0.021 [a] | 1.45±0.071 [b] | 1.35±0.024 [b] | 1.50±0.021 [b] | 1.24±0.012 [b] | 1.13±0.018 [c] | 0.01 |

[a–d] means with different superscripts are significantly different($P<0.05$) within the same row, values represent the Mean ± SEM of six replicates. Se* selenium, Ca* calcium, P* phosphorus, Fe* iron, Cu* cupper, CHO* Cholesterol, CRT* Cortisol

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