*Indian J. Anim. Nutr*. 2026. 43 (1): 93-101

doi: 10.56093/IJAN.v43i1.11

Indian Journal of
Animal Nutrition

# Zinc Oxide Nanoparticles: A Comprehensive Study of Its Effects on Growth Performance and Intestinal Barrier Functions in Broiler Chickens

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## ABSTRACT

Nano-trace elements have gained significant attention as a promising, emerging technology in the animal feed industry in recent years. Their minute size allows for enhanced absorption and efficiency compared to conventional zinc counterparts. The present study aimed to investigate the effects of Zinc oxide nanoparticles (ZnONPs) on the growth and intestinal barrier function of broiler chicks. A total of 150 one-day-old straight-run broiler chicks were randomly allotted to 5 dietary treatment groups are as follows. T1 (Control group) - 80 mg/kg of inorganic zinc (ZnO); T2 - Zn-Met group (60 mg/kg organic zinc- zinc methionine) T3, T4 and T5 - ZnONPs group (ZnONPs at doses of 60 mg/kg, 40 mg/kg and 20 mg/kg respectively. The chicks were reared for 35 days under standard conditions. Improved feed conversion ratio (FCR) was recorded in birds fed 20 and 40 mg/kg ZnONPs, among all the treatment groups. The different treatment groups did not affect the feed intake. Compared with the control group, the villus length (VL) and crypt depth (CD) in the duodenum and jejunum were increased in the 20 mg/kg ZnONPs group. In the ileum, CD was significantly higher in all studied doses of ZnONPs. Villi length to crypt depth ratio (VH/CD) was higher in ZnONPs groups in the duodenum, at 20 mg/kg ZnONPs in the ileum and in the jejunum. Dietary inclusion of ZnONPs showed no significant effects on mRNA expression of the Zona Occludens-1 (ZO-1) gene, while 20 mg/kg ZnONPs significantly upregulated the mucin-2 and claudin-3 genes in the ileal mucosa. The findings of the present study suggest that ZnONPs at a lower dose (20 mg/kg) promote growth and intestinal health.

**KEYWORDS:** Broiler, Growth and Intestinal Health, ZnONPs

Article received: 11 December 2025; Article accepted: 30 January 2026.

## INTRODUCTION

Trace minerals are essential feed additives in the broiler diet to ensure better productivity and health. In poultry feeds, zinc (Zn) is the most commonly added trace mineral (Abd El-Ghany et al., 2021). Zinc acts as an important co-factor for numerous metalloenzymes, which have effects on the metabolism of carbohydrates, proteins, lipids and nucleic acids (NRC, 2001; Mcdonald et al., 2010) as well as for transcription factors and hormones. In order to meet the zinc requirement of animals, inorganic Zn is added 20 to 30 folds higher than the usual requirement due to its low bioavailability in the body (Bratz et al., 2013; Yusof et al., 2023). Furthermore, higher levels of Zn supplementation may disrupt the mineral balance (Hazra et al., 2025) and may increase the cost of production (Mahmoud et al., 2020).

As a result, the research aiming at more bioavailable minerals that facilitate efficient utilization by poultry while minimizing its environmental excretion is gaining more importance worldwide (Rama Rao et al., 2025). One dietary tactic to minimize excessive trace mineral supplementation is replacing the inorganic sources with organic or nano zinc sources (El-Katcha et al., 2017). However, the usage of organic Zn is restricted due to its high cost. Therefore, by promoting the bioavailability of inorganic zinc, ZnONPs open an opportunity for augmenting the accessibility of this essential mineral (Qu et al., 2023).

In the form of nanoparticles, zinc has greater bioavailability even at a lower concentration than the inorganic and organic sources, having a better therapeutic impact on animals (Lail et al., 2023;

Hafez et al., 2020). It has been reported in several studies that nano forms of zinc surpass conventional zinc sources, even at lower doses, concerning growth (Zhao et al., 2014; Fathi et al., 2016; Fawaz et al., 2021) and intestinal barrier function (Hafez et al., 2017; Fawaz et al., 2021) of broiler chicks. Due to the limited availability of information regarding the optimal levels of ZnONPs in broiler chicken diets, this study aims to explore the effects of various levels of ZnONPs supplementation on growth performance, intestinal morphology and barrier function.

## MATERIALS AND METHOD

### Synthesis and characterization of ZnONPs

The ZnONPs were synthesized using the planetary ball milling technique (Mozhiarasi et al., 2023). Briefly, 5 g of food-grade Zinc oxide (Kesari Scientific Chemicals, India) was added to the zirconium jar (50 ml) along with 50 balls of 5 mm diameter. The ball mill was operated at a speed of 250 RPM for 9 hours. The particle size and shape of the ZnONPs used in this study were analyzed using High-Resolution Transmission Electron Microscopy (HR-TEM), as shown in figure 1. Results indicated that the particle size of the ZnONPs ranged from 36.28 to 47.48 nm and exhibited nearly hexagonal-shaped nanoparticles. X-Ray Diffraction (XRD) analysis confirms the existence of the hexagonal wurtzite structure of ZnONPs (Figure 2).

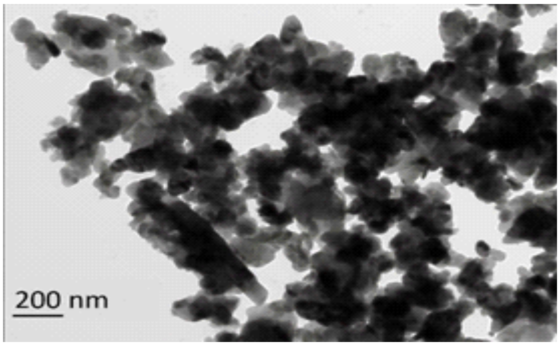


Fig 1. Transmission electron microscopic image of ZnONPs

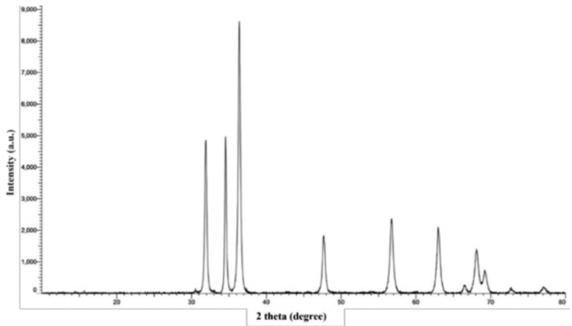


Fig 2. X-Ray Diffraction analysis of ZnONPs

### Experimental design and bird management

The feeding trial was conducted at the environmentally controlled poultry shed of Madras Veterinary College, Tamil Nadu Veterinary and Animal Sciences University, Chennai – 07. The experimental procedures were approved by the Institutional Animal Ethical Committee (Lr.No. 508/DFBS/IAEC/2022 dated on 05.05.2022). A total of 150 one-day-old broiler straight run chicks (Vencobb 400) were equally distributed into five dietary treatments randomly, with three replicates containing ten chicks in each pen replicate (each pen measures 6 × 3.5 ft). The birds were reared in a deep litter system up to 5 weeks of age. The experimental birds had *ad libitum* access to water and feed, and the light program was 23 hours of light per day until the experiment termination. Temperature was maintained at 34 ± 1 ºC during the first week and it was gradually decreased by 3 ºC during the second week to reach 31 ± 1 ºC; thereafter, the average temperature maintained was 28 ± 1 ºC. The relative humidity was 60-70%. All the chicks were vaccinated against Newcastle disease on the 7th day (*RDV- F strain)* by intra ocular route and on the 21st day (*Lasota strain)* by drinking water and against infectious bursal disease on the 14th day (*Georgia strain)* by intra ocular route.

### Experimental diets

The dietary treatments composed of different zinc sources per kg of maize soybean meal-based basal diets (Table 1) as follow: T1 (Control group) - 80 mg/kg of inorganic zinc (ZnO) at 100 % of BIS, 2007 requirement; T2 - Zn-Met group (60 mg/kg organic zinc (zinc methionine) at 75 % of the requirement); T3, T4 and T5 - ZnONPs group (ZnONPs at doses of 60 mg/kg (75 % of the requirement), 40 mg/kg (50 % of the requirement) and 20 mg/kg (25 % of the requirement) respectively). To prepare experimental diets and increased precision during mixing the ZnONPs in the basal diet, graded levels of ZnONPs were added into the premix which contained other trace elements as per the requirements and then the premix blend was thoroughly mixed with 5 kg of basal diet. The basal diets for broiler pre-starter (0 – 7 days), starter (8 - 21 days) and finisher (22 - 35 days) were formulated according to Bureau of Indian Standards (BIS, 2007) specifications.

Table 1 Composition of basal diets

| Ingredients (g/Kg) | Pre - Starter | Starter | Finisher |
|---|---|---|---|
| Maize | 495.60 | 503.50 | 542.50 |
| Soya bean meal | 425.20 | 401.00 | 350.20 |
| Palm Oil | 38.00 | 54.00 | 65.00 |
| Dicalcium Phosphate | 18.00 | 18.40 | 18.10 |
| Calcite | 12.20 | 12.20 | 13.00 |
| Salt | 3.00 | 3.00 | 3.00 |
| Choline chloride | 1.50 | 1.50 | 1.50 |
| Methionine | 1.50 | 1.40 | 1.20 |
| Threonine | 1.00 | 1.00 | 1.50 |
| Trace Mineral Mix* | 1.00 | 1.00 | 1.00 |
| Vitamin Premix** | 0.50 | 0.50 | 0.50 |
| Liver tonic | 0.40 | 0.40 | 0.40 |
| Antioxidant | 0.10 | 0.10 | 0.10 |
| Probiotic | 0.50 | 0.50 | 0.50 |
| Toxin binder | 1.00 | 1.00 | 1.00 |
| Coccidiostat | 0.50 | 0.50 | 0.50 |
| Total | 1000.00 | 1000.00 | 1000.00 |
| Nutrients (%) | | | |
| ME, kcal/kg | 3002.00 | 3103.00 | 3198.00 |
| CP, % | 23.05 | 22.08 | 20.02 |
| Calorie/protein ratio | 130.23 | 140.53 | 159.74 |
| Calcium, % | 1.00 | 1.00 | 1.00 |
| Available Phosphorus, % | 0.45 | 0.45 | 0.45 |
| Lysine, % | 1.56 | 1.48 | 1.31 |
| Methionine, % | 0.52 | 0.50 | 0.45 |

Trace mineral mix* - provided per kg of diet: Copper 12 mg, Iodine 1.6 mg, Iron 80 mg, Selenium 0.3 mg and Manganese 100 mg. Vitamin Premix** – provided per kilogram of diet: Vitamin A 10000 IU, Vitamin $D_3$ 3000 IU, Vitamin E 40 IU, Vitamin $K_3$ 1.5 mg, Vitamin $B_{12}$ 0.01 mg, Thiamine 2.5 mg, Choline 500 mg, Biotin 0.15 mg, Folic acid 1 mg, Niacin 40 mg, Pantothenic acid 15 mg, Pyridoxine 5.5 mg and Riboflavin 6.5 mg. CP – Crude Protein; ME- Metabolizable Energy; Kcal/kg – Kilo Calorie per Kilo gram.

All the experimental diets were chemically analyzed for their proximate composition as per AOAC (2012) methods (Table 2).

Table 2. Proximate composition (% DM) of experimental diet (Mean[#] ± SD)

| Parameter | Pre-starter feed | Starter feed | Finisher feed |
|---|---|---|---|
| Crude Protein | 23.14 ± 0.05 | 22.17 ± 0.58 | 20.19 ± 0.04 |
| Crude Fibre | 3.05 ± 0.18 | 3.89 ± 0.05 | 3.93 ± 0.10 |
| Ether Extract | 7.24 ± 0.14 | 7.38 ± 0.16 | 8.00 ± 0.01 |
| Total Ash | 6.16 ± 0.06 | 6.12 ± 0.12 | 5.819 ± 0.07 |
| Nitrogen Free Extract* | 60.40 ± 0.17 | 60.42 ± 0.19 | 62.32 ± 0.19 |

[#] Each value represents the mean of three observations; *Calculated by the difference

## Measurements

### Performance study

On the first day of the trial, the initial body weight of all chicks was recorded. The individual body weight of chicks and replicate pen-wise feed intake were recorded at weekly intervals throughout the experimental period (0 – 35 days) and the weekly FCR was calculated by dividing the respective feed intake (FI) by weight gain for each replicate pen. mRNA expression of ileal tight junction protein genes by quantitative real-time PCR.

To evaluate the gene expression of ileal tight junction proteins (ZO-1, Mucin-2 and Claudin-3), ileal tissues were collected from six birds per treatment on the day of slaughter. Total RNA from the ileum tissues were isolated using Trizol method (Rio et al., 2010). The concentration and purity of RNA were determined using spectrometry in a Nanodrop (Thermo Scientific NanoDrop™ One Microvolume UV-Vis Spectrophotometer). The iScript™cDNA synthesis kit (BIO-RAD) was used to synthesize cDNA from RNA as per the manufacturer's instructions. Real-time PCR analysis was carried out using a BIO-RAD, CFX Opus 96 Real-Time PCR system using iTaq Universal SYBR Green Supermix (BIO-RAD). The specific primers and thermal cyclic conditions used in Real-Time PCR are listed in table 3 and 4, respectively. The relative mRNA expressions were analyzed by $2^{-Ct}$ method (Livak and Schmittgen, 2001) and normalized to the level of 16s rRNA as a housekeeping gene.

Table 3. The sense and antisense primer sequences of genes used for Real-Time PCR study

| Gene | Orientation | Primers sequences (5' - 3') | Product size (bp) | Reference |
|---|---|---|---|---|
| Mucin-2 | Forward | TTCATGATGCCTGCTCTTGTC | 93 | |
| | Reverse | CCGTAGCCTTGGTACATTCTTGT | | |
| ZO-1 | Forward | GCCTGAATCAAACCCAGCAA | 197 | |
| | Reverse | TATGCGGCGGTAAGGATGAT | | |
| Claudin-3 | Forward | GAAGGGCTGTGGATGAACTG | 221 | |
| | Reverse | GAGACGATGGTGATCTTGGC | | |
| 16 S | Forward | GTAACGCAAGCGATCNCG | 130 | Xie et al., (2020) |
| | Reverse | AACCGCGACGCTTTCCAA | | |

Table 4. Thermal cyclic conditions used for Real-Time PCR study

| Thermal cyclic conditions | Genes (ZO –1, Mucin – 2 & Claudin – 3) |
|---|---|
| Initial denaturation temperature | 95 °C for 3 minutes |
| Final denaturation temperature | 95 °C for 30 seconds |
| Annealing temperature | 60 °C for 30 seconds |
| Extension temperature | 72 °C for 45 seconds |
| Number of Cycles | 40 |
| Melting curve | 65 °C – 95 °C |

## Histomorphometry parameters of the small intestine

On day 35, six birds were slaughtered per treatment and samples were collected from the midpoint of the duodenum, jejunum and ileum, fixed in 10 % of buffered formalin, dehydrated manually using ascending grades of alcohol, cleared using xylol, embedded in paraffin wax, cut to 3 µm thick, stained with hematoxylin and eosin (Uni et al., 1998). Histological indices measured using an image analyzer software (Magvision) included villus length (from the top of villi to the junction of the villus and crypt), crypt depth (defined as the depth of the invagination between adjacent villi) and villus height to crypt depth (VH/ CD) ratio.

## Statistical Analysis

The experimental data were analyzed by one-way ANOVA using SPSS v.20.0 statistical package and means of different treatments were computed using Duncan's multiple range test (Duncan, 1995). The obtained results were expressed as mean ± standard deviation and $p < 0.05$ was considered statistically significant.

## RESULTS AND DISCUSSION

### Growth Performance

The results for body weight gain (BWG), feed intake, and FCR are shown in Table 5.

Table 5. Effect of dietary addition of zinc oxide nanoparticles (ZnONPs) on broiler growth performances

| Age/Phase | Parameter | Control | Zn-met 60 mg/kg | ZnONPs (mg/kg) | | | Pooled SEM | p value |
|---|---|---|---|---|---|---|---|---|
| | BWG | 108.5 | 111.7 | 114.8 | 114.2 | 111.5 | 2.77 | 0.21 |
| I wk | FI | 147.6 | 154.4 | 152.0 | 153.1 | 144.7 | 4.03 | 0.16 |
| | FCR | 1.36$^{bc}$ | 1.38$^{c}$ | 1.32$^{ab}$ | 1.34$^{bc}$ | 1.30$^{a}$ | 0.01 | 0.09 |
| | BWG | 260.4 | 273.0 | 274.4 | 267.7 | 274.5 | 8.39 | 0.17 |
| II wk | FI | 527.8 | 516.7 | 516.4 | 537.3 | 526.3 | 15.40 | 0.64 |
| | FCR | 2.03 | 1.89 | 1.88 | 2.01 | 1.92 | 0.07 | 0.24 |
| | BWG | 434.9 | 434.3 | 440.0 | 444.3 | 433.9 | 12.92 | 0.85 |
| III wk | FI | 603.9 | 630.6 | 628.0 | 621.4 | 599.0 | 21.65 | 0.51 |
| | FCR | 1.39 | 1.45 | 1.43 | 1.40 | 1.38 | 0.04 | 0.74 |
| | BWG | 586.1$^{ab}$ | 593.0$^{ab}$ | 564.8$^{a}$ | 596.5$^{ab}$ | 610.7$^{b}$ | 12.06 | 0.20 |
| IV wk | FI | 888.8 | 913.4 | 919.2 | 898.3 | 910.1 | 22.40 | 0.67 |
| | FCR | 1.52 | 1.54 | 1.63 | 1.51 | 1.49 | 0.08 | 0.80 |
| | BWG | 475.5 | 475.2 | 508.5 | 510.3 | 521.2 | 17.85 | 0.41 |
| V wk | FI | 908.7 | 927.2 | 937.3 | 933.8 | 950.6 | 36.29 | 0.83 |
| | FCR | 1.91 | 1.95 | 1.84 | 1.83 | 1.82 | 0.03 | 0.88 |
| | BWG | 1856.0$^{a}$ | 1889.6$^{ab}$ | 1904.8$^{ab}$ | 1972.5$^{b}$ | 1945.1$^{ab}$ | 48.20 | 0.13 |
| 0 – V wk | FI | 3076.8 | 3142.8 | 3143.9 | 3152.3 | 3130.9 | 56.55 | 0.87 |
| | FCR | 1.66$^{b}$ | 1.66$^{b}$ | 1.65$^{b}$ | 1.60$^{a}$ | 1.61$^{a}$ | 0.01 | 0.02 |

Values bearing different superscript differed significantly; NS = non-significant ($P > 0.05$)

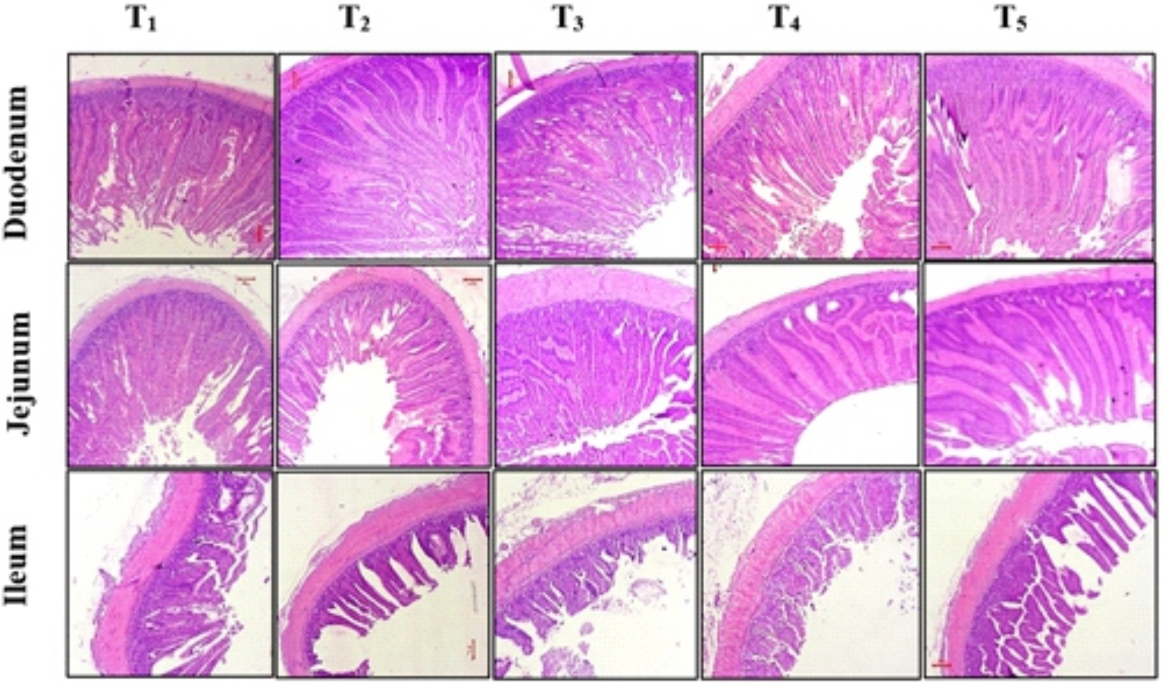
The body weight gain of the broilers was significantly (P 0.05) higher with better FCR fed with 40 mg/kg ZnONPs, followed by 20 mg/kg ZnONPs and 60 mg/kg ZnONPs, compared to the birds fed with the basal diet alone. In the overall trial, among the treatment groups, birds fed with 20 mg/kg ZnONPs exhibited better FCR values of 1.61 than other treatment groups. The different dietary treatments did not affect the feed intake of the broilers. Several reports stated positive effects of dietary supplementation of ZnONPs on broiler growth performances (Hafez et al., 2017; Zhao et al., 2014; Fathi et al., 2016; Ibrahim et al., 2017). However, Bami et al., 2018, Hazra et al., 2025 and Asheer et al., 2018 reported a non-significant result on BWG. Profitability of broiler farming mainly depends upon the enhanced FCR and growth rate (Bhalsing et al., 2026). In this study, birds fed with the 40mg/kg diet ZnONPs differed significantly ($p < 0.05$) in overall BWG compared with the control group. However, overall BWG did not differ between different levels of ZnONPs and Zn-met group. The results implied that the replacement of ZnO with Zn-met or ZnONPs did not affect average feed intake in broilers. However, FCR of 20 mg/kg of ZnONPs group has significant ($p< 0.05$) effects compared to conventional Zn at 1 week of age and overall period. This displays that the lower level (20mg/kg) of ZnONPs is equally efficient to conventional zinc sources at higher levels. The results show that replacement of conventional Zn sources with ZnONPs had no negative effects on broiler growth. A low FCR value implies better feed efficiency, thus demonstrating the efficiency of ZnO NPs in supplying sufficient Zn in the broilers even at lower dosages (Yusof et al., 2023). Likewise, Hafez et al., 2017 found that optimal levels of ZnONPs (40 and 80 mg/kg food) might improve feed conversion rate and increase body weight gain when compared to the control group (40 mg/kg diet); however, feed intake of broiler was not significant among all groups.

### Intestinal health status

### Histomorphometry parameters of the small intestine

Supplementation of zinc in different forms did not show any pathological alterations (Figure 3).

Fig 3. Effects of ZnONPs on histomorphometry of the small intestine of 35- day old broiler chicks. T1 – 80 mg/kg ZnO. T2 – 60 mg/kg Zn-methionine; T3 - 60 mg/kg ZnONPs; T4 - 40 mg/kg ZnONPs; T5 - 20 mg/kg



kg Zn-methionine; T3 - 60 mg/kg ZnONPs; T4 - 40 mg/kg ZnONPs; T5 - 20 mg/kg ZnONPs, showing significantly increased villi length in duodenum and jejunum

Interestingly, the villus length, crypt depth and villi height-to-crypt depth ratio in all segments of the small intestine is increased in a dose-dependent manner with decreasing levels of dietary ZnONPs (Table 6). Compared with control and Zn-Met groups, supplementation of the lowest level of ZnONPs in broiler diet at 20 mg/kg increased ($p < 0.05$) the villus length and crypt depth in the duodenum, while in the jejunum, in addition to 20 mg/kg, villus length and crypt depth was increased ($p < 0.05$) in 40 mg/kg ZnONPs. In the ileum, no significant ($p > 0.05$) changes in the villi length were observed among broiler chicks fed on different sources of zinc, while crypt depth was significantly ($p < 0.05$) higher in all studied doses of ZnONPs in the feed. Villi length to crypt depth ratio was higher at 20,40 and 60 mg/kg of ZnONPs in the duodenum, at 20 mg/kg ZnONPs in the ileum and in the jejunum, it didn't vary ($p > 0.05$) among all groups. Gut health is vital for poultry well-being and efficient production. Zinc is reported to be vital for developing intestinal cells, normal intestinal barrier function, absorptive capacity and regeneration of damaged gut epithelium (Barzegar et al., 2021). The highest VL, CD, and VH/CD in the duodenum, jejunum, and ileum were observed in 20 mg/kg of ZnONPs supplemented groups, suggesting the improvement of mucosal barrier functional capacity (El-Katcha et al., 2017). In addition, the increase in crypt depth of broiler chicken supplemented with different levels of ZnONPs could provide more surface area for nutrient absorption by increasing the proliferation of enterocytes and intestinal mucin secretion because mucin-producing goblet cells are present primarily in the crypts (Tsirtsikos et al., 2012) and the increased VL and VH/CD are appropriate indicators of mucosal integrity and intestinal function (El-Katcha et al. 2017; Lei et al. 2014). Also, several previous studies revealed the improvement of villi length or crypt depth or V/C in the jejunum (Barzegar et al. 2021) and all parts of the intestine (Ali et al., 2017; Hafez et al., 2017; Fawaz et al., 2021) with lower levels (10 – 40 mg/kg) of dietary ZnONPs, but Zhang et al., 2022 reported that supplementation with 80 mg/kg ZnONPs increased the villus width and height in the jejunum.

Table 6. Effect of supplementation of ZnONPs on histo-morphometric parameters of small intestine of 35-day-old broiler chicks (Mean# ± SD)

| Position | Indicator | T1 | T2 | T3 | T4 | T5 | P value |
|---|---|---|---|---|---|---|---|
| Duodenum | Villi length, *μm* | 554.30a ± 134.3 | 614.46ab ± 110.77 | 655.02b ± 132.53 | 681.40b ± 149.91 | 847.20c ± 138.32 | 0.00 |
| | Crypt depth, *μm* | 38.50a ± 15.31 | 41.37a ± 8.76 | 42.07ab ± 8.79 | 43.93ab ± 11.12 | 45.53b ± 9.73 | 0.08 |
| | VH/CD | 15.08a ± 4.21 | 15.70a ± 3.24 | 18.16b ± 6.29 | 18.63b ± 4.25 | 19.01b ± 5.04 | 0.00 |
| Jejunum | Villi length, *μm* | 548.41a ± 77.02 | 544.42a ± 141.51 | 591.54ab ± 71.91 | 616.40bc ± 115.19 | 650.27c ± 94.61 | 0.00 |
| | Crypt depth, *μm* | 39.15a ± 12.29 | 39.47a ± 7.42 | 43.44ab ± 9.67 | 44.73b ± 6.75 | 44.82b ± 9.92 | 0.01 |
| | VH/CD | 13.83 ± 3.11 | 13.47 ± 3.17 | 14.24 ± 3.12 | 14.37 ± 3.11 | 14.66 ± 3.13 | 0.55 |
| Ileum | Villi length, *μm* | 338.82± 80.36 | 346.79 ± 55.48 | 369.48 ± 97.35 | 349.07 ± 84.78 | 372.43 ±103.82 | 0.40 |
| | Crypt depth, *μm* | 32.85a ± 7.22 | 33.30a ± 5.80 | 36.83b ± 7.71 | 37.73b ± 6.78 | 39.69b ± 8.00 | 0.00 |
| | VH/CD | 8.98a ± 2.30 | 9.23a± 2.13 | 9.65ab± 3.32 | 9.94ab± 3.27 | 10.72b ± 2.81 | 0.10 |

#Each value represents the mean of thirty-six observations; V/C – Villi length to crypt depth ratio; Means bearing different superscripts in a row differ significantly with ($p < 0.05$); [T1 - 80 mg/kg ZnO; T2 - Zn met-60 mg/kg; T3 - ZnONPs- 60 mg/kg; T4 - ZnONPs - 40 mg/kg; T5 - ZnONPs - 20 mg/kg]

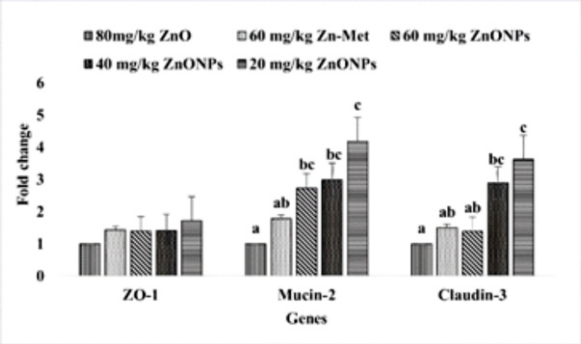


Fig 4. Effects of ZnONPs on mRNA expression of ileal tight junction protein of 35- day old broiler chicks (Mean# ± SD)

In the present study, dietary supplementation of ZnONPs in broiler chicks did not affect the mRNA expression of ZO-1 ($p>0.05$), but numerically, there was an upregulation in the 20 mg/kg ZnONPs, suggesting that nano zinc supplementation had not negatively affected the barrier function. Conversely, mucin-2 and claudin-3 genes were significantly ($p<0.05$) upregulated (4.18 and 3.63-fold respectively) in the 20 mg/kg diet of ZnONPs group compared with other groups (Figure. 4). Therefore, upregulation of these tight junction protein genes might strengthen the epithelial barriers by increasing intestinal integrity and restricting paracellular permeability, thus serving as a front line of defense against invading pathogens into the body (Bobíková et al., 2016). Accordingly, Zhang et al. (2022) reported that compared to negative control group, mucin-2 mRNA expression was higher in the 160 mg/kg ZnONPs group. Contrary to our result, several reports show addition of ZnONPs in the diet of broiler chicks increased the expression of the ZO-1 gene in the jejunum (Zhang et al., 2022; Barzegar et al., 2021; Fatholahi et al., 2021) and did not affect the Mucin-2 gene expression (Zhang et al., 2012, Jose et al., 2018). Taken together, the protective effects of ZnONPs supplementation on gut health might be attributable to the enhancement in the mRNA expression of genes related to ileal tight junction protein and small intestinal morphology of broiler chicks.

## CONCLUSION

ZnONPs exhibited superior effects compared to conventional zinc sources in terms of enhancing growth and intestinal barrier functions. These findings validate the potential of dietary supplementation with nano forms of zinc as a novel feed additive, capable of replacing both inorganic and organic forms of zinc. The results suggest that an optimal level of 20 mg/kg of ZnONPs in the broiler chick diet could be effective. Furthermore, additional research is warranted to unravel the underlying mechanisms of ZnONPs and their effects on diverse biological processes. It is imperative to address regulatory considerations and conduct thorough safety assessments to safeguard the well-being of broiler chickens.

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