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Impact of Zinc Hydroxychloride and Oxide Nanoparticles on Broiler Chicken Growth, Gut Microbiota, Immunity and Serum Biochemistry

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ABSTRACT

Background: Use of more efficient sources of zinc (Zn) in corn–soybean basal diet improves the productivity in the absence of growth promoters.

Objective: This study aimed to compare the effects of different forms of Zn (hydroxychloride: OHCl, oxide nanoparticles: ONPs) on performance and physiological parameters in broiler chickens.

Methods: Treatments included control (without use of ZnOHCl and ZnONPs) and three levels of zinc (40, 80 and 110 mg/kg of diets) as either ZnOHCl or ZnONPs.

Results: The body weight gain and feed conversion ratio for broilers fed with all levels of ZnOHCl, respectively, were greater and smaller than those of ZnONPs and control groups in the entire experiment ($p < 0.05$). Broilers fed with 110 mg ZnOHCl/kg benefited from the highest anti-Newcastle antibody titre and the lowest breast drip loss, whereas the heaviest relative weight of lymphatic organs and the smallest number of breast muscle pH were relevant to chickens fed with 110 mg ZnONPs/kg ($p < 0.05$). All levels of either ZnOHCl or ZnONPs caused better humoral immunity and the villus height:crypt depth than the control group ($p < 0.05$). However, all treatments had a similar effect on the relative weights of internal organs, the heterophil:lymphocyte, anti-influenza antibody titre, percentages of fat, dry matter and crude protein of breast meat, intestinal microbiota and serum biochemistry of broilers ($p > 0.05$).

Conclusion: To sum up, supplementation of ZnOHCl in broilers' diets up to 110 mg was more efficient than ZnONPs in most responses relevant to immunology, growth performance and meat quality.

1 | Introduction

Zinc (Zn) deficiency is one of the noticeable reasons for suffering from some ailments in developing countries (Shrimpton et al. 2005). Although Houshiar-Rad et al. (2013) reported that in spite of the frequent intake of poultry meat, this product contains low levels of Zn for human consumers such as Iranians. Then, the regular feeding of Zn is needed to increase its storage dosage in animal body (Swain et al. 2016). Zn is important in metabolism and immunity (Jankowski et al. 2019), and it can improve antioxidant status and deposit in either muscle or liver (Dukare et al. 2021).

The first form of Zn source that includes the aforementioned importance is inorganic zinc hydroxychlorides (ZnOHCl), which is generated via the reaction between high-purified metal shapes and water + hydrochloric acid (Leisure et al. 2014). Genther et al. (2015) explained that ZnOHCl is more soluble at the more acidic pH of the gastrointestinal tract, thereby resulting in high bioavailability and absorption. Refined bioavailability might be anticipated to efficiently improve the antioxidant defence of immune cells, for instance, heterophils (Perez et al. 2017).

The second form is cost-effective inorganic zinc oxide nanoparticles (ZnONPs) with higher bioavailability than zinc oxide (ZnO), which its overconsumption causes environmental contamination stems from excessive excretion of the heavy metal, and nanotechnology facilitates the production of tinnier particle sizes of ZnONPs as nutritional supplementation to refine either bioavailability or intestinal absorption of Zn, mitigating Zn excretion to potentially impact their corporal distribution in poultry (Mohd Yusof et al. 2023). ZnONPs might be considered a potential alternative to antibiotics in diet under either in vitro or in vivo conditions because of their antibacterial effects (Mohd Yusof et al. 2023). Furthermore, Dosoky et al. (2022) found that the use of ZnONPs might be dose-dependent because it made either benefits or toxicities for poultry at different dosages.

Therefore, according to the importance of either nanoparticle sizes or chemical formulas of Zn sources in poultry's diets and their human consumers, the major objective of the present study was to compare the effects of ZnONPs and ZnOHCl on growth performance, microbial population and some physiological parameters of broiler chickens to know which bioavailable shape of these inorganic Zn forms might be more preferable than others.

2 | Materials and Methods

These trials were conducted at the base of the comprehensive guideline of animal welfare as accepted by the Federation of Animal Science Societies (FASS) (2020).

2.1 | Birds, Diets, Vaccination and Experimental Procedures

A total of 420 one-day-old male broiler chickens (Ross 308) had been bought from a regional hatchery to randomly be either housed at 35 clean floor pens or distributed into 7 treatments, with 5 replicates (12 chicks) for each treatment in a completely randomized design; then after this, all one-on-one birds were weighed on the 1st day of age, and the mean weight was 41 g. Treatments included control (without use of ZnOHCl and ZnONPs) and three amounts of Zn (40, 80 and 110 mg/kg of diets) in the forms of ZnOHCl or ZnONPs. Birds received corn–soybean starter (1–14 days), grower (15–28 days) and finisher (29–42 days) diets. The diets of three rearing phases were formulated to supply the nutritional requirements of broilers according to recommended nutritional requirements of ROSS 308 (Aviagen 2019). Either water or mash feed was supplied ad libitum during the trial for all birds. Moreover, vaccination programmes and all diets are shown in Tables 1 and 2, respectively.

2.2 | Growth Performance

Body weight (BW), BW gain (BWG), feed conversion ratio (FCR) and feed intake (FI) were measured and calculated for different rearing phases. In addition, FCR equals FI/BWG.

2.3 | Relative Weights of Internal Organs

On the final day of trial, two broiler chickens were randomly chosen and sacrificed from each replicate to calculate the partial weights of empty carcass, heart, gizzard, proventriculus, liver and abdominal fat at the base of the live BW of broiler chickens.

2.4 | Breast Meat Quality

One bird per replicate was sacrificed to be measured for its breast meat samples, including pH, dry matter, percentages of either fat or protein and drip loss in 42 days of trial (AOAC International 2005).

2.5 | Serum Biochemical Parameters

At the end of trial and before slaughter, blood samples were taken from the wing vein of two opted male broilers belonging to each pen. Thus, serum samples after separation with utilization of a sampler were assessed by an autoanalyser and Pars Azmoon kits to measure the concentrations of total cholesterol, triglyceride, total protein, high-density lipoprotein (HDL), low-density lipoprotein (LDL), albumin, globulin and glucose of serum (Kececi et al. 1998).

2.6 | Immune Responses

2.6.1 | Antibody Titres Against Influenza, Newcastle and Sheep Red Blood Cell (SRBC)

Dual injection of 0.2 mL antigens of Influenza–Newcastle per bird was conducted in Day 9 of age (Table 1). Moreover, oral vaccination of Newcastle was solely carried out on Day 19 of experiment (Table 1). Taking blood from the brachial vein of two birds per replicate was conducted on Day 28 of age to determine antibody titres against Newcastle and influenza separately through haemagglutination inhibition manner (Wegmann and Smithies 1966).

TABLE 1 | Vaccination programs for broiler chickens.

Day	Name of vaccine	Vaccination method
1	Bronchitis-Newcastle	Spray
8	Newcastle-influenza	Injection
8	Bronchitis-Newcastle	Eye drop
15	Gambro	Consumed with water
19	Newcastle	Consumed with water
30	Newcastle	Consumed with water

TABLE 2 | Composition and nutrient contents of the basal diets over three phases.

Ingredients (%)	Starter (Days 1–14)	Grower (Days 15–28)	Finisher (Days 29–42)
Corn	56.5	63.4	65.7
Soybean meal	39.1	32.5	30.5
Soybean oil	0.9	1	1.2
Common salt	0.23	0.23	0.22
Sodium bicarbonate	0.1	0.1	0.1
Dicalcium phosphate	0.88	0.69	0.47
Calcium carbonate	1.2	1.1	1.1
L-Lysine hydrochloride	0.26	0.24	0.13
DL-Methionine	0.33	0.29	0.22
L-Threonine	0.14	0.11	0.04
Vitamin and mineral premix[a]	0.15	0.14	0.13
Mineral premix[b]	0.15	0.14	0.13
Choline chloride	0.05	0.05	0.05
Rovabio multi-enzyme phytase	0.01	0.01	0.01
Calculated analysis (% unless stated otherwise)			
ME (kcal/kg)	3000	3090	3130
Crude protein	23	19.93	19
Methionine + cysteine	0.95	0.85	0.78
Threonine	0.86	0.76	0.67
Lysine	1.28	1.13	1
Calcium	0.96	0.87	0.79
Available phosphorus	0.48	0.435	0.395

[a]Per 1.3 kg vitamin premix contains 9,000,000 units of retinol, 4,000,000 units of cholecalciferol, 55,000 units of tocopherol, 2200 mg menadione, 2200 mg thiamin, 5400 mg riboflavin, 15,000 mg pantothenic acid, 45,000 mg niacin, 2200 mg pyridoxine, 11 mg B12 (cobalamin) and 150 mg biotin.
[b]Per 1 kg mineral premix: Mn, 120 g; Zn, 110 g; Fe, 20 g; I, 1.25 g; Se, 0.3 g.

In addition, 1 mL of a 1% SRBC suspension diluted with phosphate-buffered saline (PBS) was injected intraperitoneally into two birds from each replicate on Days 24 and 28 (in the morning) of age. Then, the selected birds were bled from their brachial vein 5-day post-injection to measure antibody titre against SRBC on Days 28 and 32 of age with the use of haemagglutination assay and microtitre plates. Lastly, all of the measured antibody titres were transformed at the base of Log_2 (Wegmann and Smithies 1966).

2.6.2 | Partial Weights of Lymphoid Organs

Two broilers were sacrificed on Day 42 to measure the relative weights of the bursa of Fabricius and spleen based on the percentage of live BW.

2.6.3 | Blood Haematological Parameters

Two birds via their brachial vein were bled from each replicate to provide smears from the gathered blood samples containing heparin in Day 28 of trial. Then, the provided duplicated smears were stained with the Giemsa method (Gajendra et al. 2015) for differential counting of either heterophils (H) or lymphocytes (L) among 100 white blood cells per slide under light microscope with a magnification of ×1000. Finally, heterophil-to-lymphocyte proportion was calculated (Gross and Siegel 1983).

2.7 | Intestinal Morphology

First, two male broilers were randomly separated from each pen to be slaughtered on Day 21 of age. Then, 1 cm of Jejunal segment was separated from the middle of Jejunum to be washed by use of 0.1 M phosphate-buffered saline. Subsequently, it was immersed in 10% formalin buffer. After 24 h, formalin buffer was swapped with the new one to find a suitable opportunity for doing the next steps. In the next steps, the selected sections were dehydrated and embedded in paraffin wax. Subsequently, 5 mm colourful slices were provided after cutting either transverse or longitudinal parts by microtome, staining with haematoxylin–eosin. After this, the fixed two samples per pen under a light microscope, which was well-equipped with either digital camera (BA400 Digital, McAudi Industrial Group Co. Ltd.) or 4× and 10× gridded eyepiece lens (micro-measure glass), were examined. Moreover, all photographed images were stored in a computer to choose 10 well-oriented villi per image for scoring villi length (VL)

and crypt depth (CD) with the help of the Motic Advanced 3.2 digital image analysis software. Lastly, the VL/CD was calculated (Liu et al. 2022; Sakamoto et al. 2000).

2.8 | Intestinal Microbial Population

Two birds per replicate were slaughtered to transfer 1 g of digestive content from their ceca into 9 mL of sterile physiological serum, and both were mixed by shaker to obtain a 0.1 mL diluted solution on Day 42 of experiment. Then, 1 mL of the latest produced solution was serially nine times transferred into 9 mL of sterile physiological serum to create a final solution containing a 1×10^{-10} mL dilution of digesta. Then after this, 0.5 mL of the last seven times of diluted solutions were cultivated on either De Man–Rogosa–Sharpe (M.R.S.) agar or MacConkey (MC) agar to be incubated at 37°C for 48 h in an anaerobic chamber and for 24 h in an aerobic chamber to grow *Lactobacillus* and *Escherichia coli*, respectively (Mohebodini et al. 2021). All bacteria on duplicated plates were counted by bacterial colony counter. Microbiological counts were subjected to base-10 logarithm transformation before analysis (Koch 2007).

2.9 | Statistical Analysis

Data were analysed in a completely randomized design with seven treatments and five replicates. The following model procedure of SAS (SAS Institute 2001) was used to compare treatments together:

$$Y_{ij} = \mu + a_i + e_{ij}$$

where Y_{ij} is the observed value for a special character, μ is the overall mean, a_i is the effect of the ith treatment and e_{ij} is the random error pertained to ijth recording. Data were analysed considering the pen of birds as the experimental unit about performance parameters, and the one-on-one chicken was measured as the experimental unit for the rest of the parameters. The mean values were compared using the Tukey test. Statistical significance was detected at $p < 0.05$.

3 | Results and Discussion

3.1 | Growth Performance

According to Table 3, an increase of either ZnONPs or ZnOHCl from 40 to 110 mg/kg of starter diet could significantly enhance both FI and BWG during the starter phase; meantime, the lowest FCR was recorded for chicks that ate 40 mg ZnONPs/kg of basal diet ($p < 0.05$). A reason for this might be relevant to these subjects: nanoparticles have very extensive surface areas, which might elevate their bioavailability and absorption (Mohd Yusof et al. 2023), and this might be helpful as Ibrahim et al. (2017) pointed out that Zn can refine the growth rate of broilers through regulation of appetite and enhancement of the action of insulin-like growth factors and genes which are responsible for the production of growth hormone. In agreement with our study, 40 mg ZnONPs/kg of corn–wheat–soybean basal diets refined BWG and overall FCR of broilers (Akhavan-Salamat and Ghasemi 2019).

In addition, feeding either ZnONPs or ZnOHCl could not influence FI of broiler chickens in the grower, finisher periods, and the whole of experiment ($p > 0.05$). However, BWG and FCR of broiler chickens fed with ZnOHCl in the grower and finisher periods were better than BWG and FCR of those groups fed with ZnONPs or the control diet in the same times ($p < 0.05$). This happened first because adding ZnOHCl to the diets of broiler chickens presumably was more efficient in supplying their physiological requirements, encompassing protein synthesis or its metabolism, enzymatic and co-enzymatic activities. All these hypotheses have been supported that the paucity of Zn in diet potentially leads to deregulation of appetite, metabolism and growth rate of animals (Ibrahim et al. 2017). Then, the growth performance of broilers potentially is at stake due to the negative repercussion of Zn paucity because physiological functions primarily depend on Zn as an activator, which molecularly plays essential roles either in the proteins or in enzymatic, co-enzymatic structures (Nguyen et al. 2021; Prasad and Lall 2022). The next reason presumably may be considered for better absorption and stems from hydroxychloride properties, as Hawthorne and Sokolova (2002) showed that the crystalline structure of OHCl trace elements allows for a slow release throughout digestion. In addition, this might be contributed to the less potential reactivity between nutritional ingredients and the crystalline complex of ZnOHCl (basic zinc chloride) added to the corn–soybean basal diet in upper parts of gastrointestinal tract (Yu et al. 2022). The third reason might seem to be regarding the positive interaction between 0.01% phytase and ZnOHCl in broiler's diet (Table 2) to cause a better utilization of energy and nutritional ingredients than other treatments. As this was supported by Akter et al. (2017) who proved that with enhancement of Zn levels (30, 40 and 50 mg/kg) in maize–soybean-based diets containing 0 and 500 U/kg exogenous microbial phytase (0, 0.01 g/kg of diet), the growth performance improved due to refined use of nutrients and energy without an adverse effect on broilers. In consensus with our study, over 15–28 days of age when the trial occurred, the average BWG and FCR of broilers fed 120 mg/kg ZnOHCl + 7 mg/kg toltrazuril (anticoccidial drug) were noticeably better than the unmedicated control (Nonkookhetkhong and Chalala 2023).

3.2 | Meat Quality, Relative Weights of Internal Organs and Serum Biochemical Parameters

Results displayed in Table 4 indicate that all treatments had similar effects on breast muscle quality parameters encompassing dry matter, fat and crude protein of broilers ($p > 0.05$). In the present study, the highest numbers for pH and drip loss of meat quality, respectively, were pertained to the significant effects of 80 ZnOHCl and 40 ZnONPs (mg/kg of diet), whereas the lowest were those of 110 ZnONPs and 110 ZnOHCl (g/ton of diet) ($p < 0.05$). In contrast to our study, Dukare et al. (2021) evaluated a 3 × 3 factorial arrangement, consisting of three sources (inorganic, green nanoparticles and market nanoparticles) and three levels (40, 60 and 80 ppm) of ZnO, and they found that the main effects of either 80 ppm ZnO or market ZnONPs source brought about the lowest percentage of fat in breast meat of broiler chickens, whereas the main effects of either 80 ppm ZnO or green ZnONPs

TABLE 3 | The effect of treatments on growth performance traits of broilers at different phases.

	Treatments								
		ZnONPs (mg/kg of diets)			ZnOHCl (mg/kg of diets)				
Parameters	**Control[1]**	**40**	**80**	**110**	**40**	**80**	**110**	**SEM**	***p* value**
Starter phase (1–14 days)									
BW (g/bird/day)	355.6^{b}	378.8^{a}	377.8^{a}	376.7^{a}	361.1^{ab}	385.3^{a}	386.8^{a}	13.86	0.04
FI (g/bird/day)	26.90^{b}	27.32^{ab}	28.14^{ab}	29.14^{a}	27.23^{ab}	28.80^{a}	29.14^{a}	0.65	0.04
BWG (g/bird/day)	22.68^{c}	24.03^{ab}	23.96^{ab}	23.88^{b}	22.77^{ab}	24.50^{ab}	24.60^{a}	1.04	0.03
FCR (g/g)	1.18^{bc}	1.13^{c}	1.17^{bc}	1.21^{b}	1.19^{b}	1.17^{bc}	1.18^{bc}	0.04	0.05
Grower phase (15–28 days)									
BW (g/bird)	1076.5^{d}	1120.5^{bc}	1124.3^{bc}	1128.9^{b}	1091.7^{cd}	1173.7^{a}	1152.4^{ab}	31.29	0.02
FI (g/bird/day)	89.55	92.58	92.99	93.50	89.62	91.02	90.22	1.04	0.16
BWG (g/bird/day)	51.48^{d}	52.98^{cd}	53.31^{bc}	53.73^{bc}	53.61^{bc}	56.31^{a}	54.69^{b}	1.32	0.01
FCR (g/g)	1.73^{a}	1.74^{a}	1.74^{a}	1.73^{a}	1.67^{b}	1.61^{b}	1.64^{b}	0.03	0.05
Finisher phase (29–42 days)									
BW (g/bird)	1991.6^{e}	2129^{d}	2181.2^{cd}	2257.9^{ab}	2216.2^{bc}	2300.5^{a}	2304.9^{a}	50.35	0.01
FI (g/bird/day)	161.19	166.66	167.39	168.30	162.32	163.84	162.39	1.78	0.21
BWG (g/bird/day)	65.36^{d}	72.03^{c}	75.49^{b}	80.64^{a}	80.32^{a}	80.48^{a}	82.32^{a}	1.49	0.02
FCR (g/g)	2.46^{a}	2.31^{ab}	2.21^{b}	2.08^{c}	2.02^{c}	2.03^{c}	1.97^{d}	0.04	0.02
Experiment overall (1–42 days)									
FI (g/bird/day)	92.28	95.52	96.18	96.98	90.06	94.55	93.92	0.97	0.28
BWG (g/bird/day)	46.51^{e}	49.68^{d}	50.925^{d}	52.75^{ab}	51.75^{bc}	53.76^{a}	53.87^{a}	1.20	0.02
FCR (g/g)	1.98^{a}	1.92^{ab}	1.88^{bc}	1.83^{cd}	1.74^{e}	1.75^{de}	1.74^{e}	0.03	0.01

Note: Different letters (a–e) in the same row indicated significant difference ($p < 0.05$).

Abbreviations: BW, body weight; BWG, body weight gain; FCR, feed conversion ratio; FI: feed intake; ZnOHCl, zinc hydroxychloride; ZnONPs, nanoparticles of zinc oxide.

[1]Control: Without use of ZnONPs and ZnOHCl.

source (its particle size with spherical and rod shapes ranged between 12 and 53 nm produced by *Catharanthus roseus* plant) led to the smallest amount (mg/100 g) of cholesterol in breast meat of broiler chickens. In another study, the percentages of water retention capacity and water loss for broilers fed 50 g ($ZnCl_2$ + lignans)/ton of diet were, respectively, higher and lower than those of 100 g ($ZnCl_2$ + lignans)/ton of diet and the control, whereas the number of pH was not influenced by all Zn sources and the control (Galli et al. 2022). One potential reason for the contradictory results between our study and other studies might be relevant to dosage, size of nanoparticles, and either oxidized or hydroxychloride forms of Zn sources.

In the current study, neither the partial weights of carcass, abdominal fat, internal organs nor the concentration of serum biochemical parameters of broilers were influenced by all treatments ($p > 0.05$). In consensus with our findings, Van Kuijk et al. (2021) declared that there was not a significant difference in the percentage of carcass yield between broilers fed 80 and 20 mg ZnOHCl/kg at the base of wheat–soybean diet. Similarly, some studies reported that the disparate levels of ZnONPs had no striking effect on the relative weights of carcass, liver of Japanese quails on Day 35 (Reda et al. 2021), abdominal fat pad, gizzard, heart and proventriculus of broilers in 21st day of age (Ahmadi et al. 2013).

Some studies about the haematology of broiler chickens reported that different quantities of ZnOHCl could not affect the concentrations of mean corpuscular haemoglobin (MCH), haemoglobin (Hb), total protein of plasma (Santos et al. 2023) and albumin (Yu et al. 2022). There was no a significant difference in the serum biochemical variables, which include total protein, albumin, globulin, triglyceride and glucose of broilers fed the additive blend of zinc chloride ($ZnCl_2$) and lignans at disparate amounts of 50 and 100 g/t of corn–soybean diets (Galli et al. 2022). Similarly, feeding 10, 50 and 100 mg ZnO/kg or 10, 50 and 100 mg ZnONPs (its size was 25 nm)/kg of diet could not affect the doses of glucose and triacylglycerols of turkey hens (Jankowski et al. 2019). Moreover, feeding 0, 0.1, 0.2, 0.3 and 0.4 g ZnONPs/kg of diet could not influence the doses of cholesterol, triglycerides, LDL, HDL, total protein, albumin and globulin of Japanese quails (Reda et al. 2021).

3.3 | Immunity

As shown in Table 5, although antibody titres against Newcastle vaccine for broilers fed 0.01% phytase with (40, 80 mg/kg of diet) Zn forms of ONPs were nearly equal to those of OHCl in Day 28, respectively, it is obvious that enhanced levels of both ZnONPs and ZnOHCl from 40 to 110 (mg/kg) could significantly increase

TABLE 4 | The effects of treatments on relative weights of organ, meat quality and serum biochemical parameters of broilers in Day 42.

	Treatments								
		ZnONPs (mg/kg of diets)			ZnOHCl (mg/kg of diets)				
Parameters	**Control**[1]	**40**	**80**	**110**	**40**	**80**	**110**	**SEM**	***p* value**
Partial weights of disparate organs (% of live BW)									
Carcass	74.5	73.9	74.2	72.8	73.8	74.5	74.6	0.85	0.21
Abdominal fat	1.30	1.20	1.25	1.35	1.30	1.20	1.20	0.21	0.16
Liver	2.22	2.32	2.35	2.5	2.34	2.22	2.11	0.03	0.12
Heart	0.51	0.50	0.49	0.50	0.51	0.53	0.52	0.03	0.23
Gizzard	2.40	2.50	2.10	2.60	2.00	2.40	2.50	0.01	0.19
Proventriculus	0.40	0.50	0.55	0.50	0.49	0.54	0.52	0.07	0.28
Breast meat quality parameters									
Drip loss (%)	7.62^{ab}	7.65^{a}	6.95^{bc}	7.22^{abc}	7.42^{abc}	7.26^{abc}	6.94^{c}	0.31	0.03
DM[2] (%)	22.80	21.70	22.50	22.50	22.10	21.90	22.60	0.56	0.27
Fat (%)	1.67	1.58	1.60	1.65	1.62	1.60	1.59	0.04	0.31
CP[3] (%)	20.32	20.25	21.00	20.14	20.50	20.70	20.53	0.36	0.22
pH	5.02^{b}	5.35^{ab}	5.07^{b}	5.02^{b}	5.04^{b}	5.98^{a}	5.88^{a}	0.47	0.04
Serum biochemical parameters (mg/dL, unless stated otherwise)									
Cholesterol	131.93	124.83	122.63	120.99	128.01	125.22	123.75	0.006	0.41
Triglyceride	79.408	78.824	75.170	79.568	86.304	85.510	80.838	0.26	0.35
LDL[4]	23.808	22.724	23.014	23.792	23.800	23.122	21.964	0.04	0.29
HDL[5]	75.312	75.268	79.955	85.272	78.368	76.037	85.828	1.32	0.33
Glucose	181.54	184.76	182.59	183.86	185.46	181.29	180.65	2.40	0.50
TP[6] (g/dl)	2.976	2.866	2.837	2.879	2.816	2.866	2.793	0.09	0.18
Al[7] (g/dL)	1.785	1.680	1.666	1.689	1.686	1.731	1.646	0.06	0.15
Glo[8] (g/dL)	1.190	1.185	1.170	1.189	1.130	1.135	1.147	0.03	0.12

Note: Different letters (a–c) in the same row indicated significant difference ($p < 0.05$).

Abbreviations: BW, body weight; HDL, high-density lipoprotein; LDL, low-density lipoprotein; ZnOHCl, zinc hydroxychloride; ZnONPs, nanoparticles of zinc oxide.

[1]Control: Without use of ZnONPs and ZnOHCl.

[2]Dry matter.

[3]Crude protein.

[4]Low-density lipoprotein.

[5]High-density lipoprotein.

[6]Total protein.

[7]Albumin.

[8]Globulin.

the antibody titres against Newcastle vaccine ($p < 0.05$) without influencing antibody titres against influenza at the same time ($p > 0.05$). Our results were supported by Mohammadi et al. (2015), who reported that the antibody titre against Newcastle vaccine in broiler chickens fed with 80 mg Zn-NPs-Max (Bonza Zn Metabolism Optimizer)/kg of diet was greater than that of the control diet.

In the current study, for primary response to SRBC administered on Day 28 of age, either ZnOHCl or ZnONPs had similar effects on anti-SRBC antibody titers of broilers ($p > 0.05$), whereas both significantly had higher anti-SRBC antibody titers than the control either at primary or at secondary response to SRBC assay ($p < 0.05$). It is worth noting that three levels of feeding ZnONPs had a similar effect on the secondary anti-SRBC antibody titre of broiler chickens ($p > 0.05$), whereas the secondary anti-SRBC antibody titre of broilers fed with 80 or 110 mg ZnOHCl/kg of diet was significantly smaller than the secondary anti-SRBC antibody titre of those groups fed with 40 mg ZnOHCl/kg of diet or three levels of ZnONPs ($p < 0.05$). One reason for this might be attributed to these subjects that the size of ZnONPs was smaller than the size of ZnOHCl, and this in turn caused the bioavailable speed of ZnOHCl (absorption speed of Zn by immune cells) to be less than the bioavailable speed of ZnONPs in response to the secondary SRBC injection. As Mohd Yusof et al. (2023) declared, ZnONPs through enhancement of Zn bioavailability either modulate or refine the immunity of broilers. In agreement with our study, utilization of ZnONPs and zinc methionine in

TABLE 5 | The effects of treatments on immune system, jejunal morphology, and cecal microbial population of broilers.

Parameters	Treatments							SEM	*p* value
		ZnONPs (mg/kg of diets)			ZnOHCl (mg/kg of diets)				
	Control[1]	40	80	110	40	80	110		
Antibody titres and humoral immunity (log_2)									
AI[2] (Day 28)	6.54	6.50	6.45	6.55	6.50	6.45	6.48	0.02	0.14
ND[3] (Day 28)	8.22^{c}	8.11^{c}	9.00^{b}	9.40^{ab}	8.12^{c}	9.00^{b}	10.50^{a}	0.72	0.01
Anti-SRBC[4] antibody assay (Day 28)	7.41^{b}	9.22^{a}	9.21^{a}	9.00^{a}	9.20^{a}	8.80^{a}	9.20^{a}	0.87	0.04
Anti-SRBC antibody assay (Day 32)	8.12^{b}	10.10^{a}	10.12^{a}	10.20^{a}	10.10^{a}	9.98^{ab}	9.87^{ab}	1.02	0.04
Lymphoid organs (% of live BW in Day 42)									
Bursa of Fabricius	0.180^{de}	0.168^{e}	0.190^{cd}	0.270^{a}	0.200^{c}	0.230^{b}	0.200^{c}	0.04	0.01
Spleen	0.088^{b}	0.091^{b}	0.095^{ab}	0.102^{a}	0.101^{a}	0.100^{a}	0.101^{a}	0.007	0.04
Serum haematology parameters (% in Day 28)									
Heterophil	15	16	16	15	16	16	15	0.45	0.21
Lymphocyte	83	83	82	83	83	82	82	0.47	0.38
H/L ratio	0.180	0.192	0.195	0.180	0.192	0.195	0.182	0.007	0.29
Jejunal morphology (Day 21) (μm)									
VL	1037^{b}	1239^{a}	1237^{a}	1240^{a}	1105^{ab}	1202^{a}	1255^{a}	105.5	0.04
CD	289^{a}	250^{b}	249^{b}	239^{b}	263^{ab}	252^{b}	249^{b}	20.32	0.03
VL/CD	3.580^{b}	4.952^{ab}	4.962^{ab}	5.017^{a}	4.191^{ab}	4.765^{ab}	5.038^{a}	0.72	0.04
Microbial population (log CFU/g of digesta in Day 42)									
Lactobacillus	8.90	8.60	9.10	8.60	8.60	9.00	8.40	0.31	0.29
Escherichia Coli[5]	6.20	5.60	5.80	5.80	6.20	5.40	5.60	0.37	0.18
Lactobacillus/*Escherichia Coli*	1.43	1.53	1.56	1.48	1.38	1.66	1.50	0.28	0.23

Note: Different letters (a–e) in the same row indicated significant difference ($p < 0.05$).

Abbreviations: BW, body weight; CD, the crypt depth; H/L, heterophil/lymphocyte ratio; VL, the villi length; VL, villi length; VL/CD, the villi length/crypt depth ratio; ZnOHCl, zinc hydroxychloride; ZnONPs, nanoparticles of zinc oxide.

[1]Control: Without use of ZnONPs and ZnOHCl.

[2]AI: Antibody titre against Influenza vaccine.

[3]ND: Antibody titre against Newcastle disease vaccine.

[4]Sheep red blood cell.

[5]*Escherichia coli*.

the diet of broilers enhanced initial total antibody titres and IgG antibody titres against SRBC (Akhavan-Salamat and Ghasemi 2019). Furthermore, nanoparticles of Zn, due to their high bioavailability, cause remarkable Zn absorption, which stems from a high percentage of Zn retention; thus, this circumstance refines immunity and the relative weight of the spleen of broilers (Mohd Yusof et al. 2023).

In our study, the relative weights of bursa of Fabricius and spleen of broilers that fed on diets with 40, 80 and 110 mg ZnOHCl/kg of diet were strikingly heavier than those of 40 and 80 mg ZnONPs/kg of diet and the control on Day 42 ($p < 0.05$). The weight of lymphoid organs is a usual index, which informs us about the immunity situation and body ability of broiler chickens in the production of lymphoid cells (Heckert et al. 2002). In one study, Fatholahi et al. (2021) explained that the relative weights of either bursa of Fabricius on Day 42 or spleen on Day 14 for broiler chickens fed by Nano-bio Zn (each kg of diet was enriched with 120 mg Zn, which was selected from a source containing 40 g Nano-bio Zn/kg of Zn) were slighter than the control; conversely, the relative weight of spleen for broilers fed by Nano-bio Zn (120 mg/kg of diet) on Day 42 was heavier than the control. Similarly, hydroxychloride form of a salt contained $Zn_5(OH)_8Cl_2.H_2O$+ (disparate forms of other trace minerals) significantly increased the percentage of spleen of broilers' live BW without a significant effect on their bursa of Fabricius (% of live BW) on Day 25 (M'Sadeq et al. 2018). In one study, Perez et al. (2017) reported that an increase of ZnOHCl level in broilers' diets could potentially influence the spleen to synergistically enhance the superoxide dismutase activity and mRNA amounts in immune cells, including heterophils and monocytes.

In our study, all treatments had similar effects on the percentages of heterophils, lymphocytes and the heterophils/lymphocytes proportion of broilers ($p > 0.05$). In agreement with our findings, 80 mg Zn-NPs-Max/kg at the base of maize–soybean diet caused a similar effect on the white blood cell, lymphocyte counts and heterophil:lymphocyte ratio in broilers; however,

it led to a larger percentage of heterophil than the control group (Mohammadi et al. 2015). Similarly, Nonkookhetkhong and Chalala (2023) observed that either 120 mg ZnOHCl/kg or 120 mg ZnOHCl/kg + 7 mg toltrazuril (anticoccidial drug)/kg of diet had not a significant impact on the disparate leukocyte counts such as heterophils of broilers, although the lymphocytes' count of broilers fed diets supplemented with 120 mg ZnOHCl/kg + 7 mg toltrazuril/kg was noticeably smaller than other treatments, which include the infected-unmedicated control, the infected and treated with 120 mg ZnOHCl/kg, the infected and medicated with 7 mg toltrazuril/kg, and the uninfected–unmedicated controls. They reported that the lower lymphocyte might be attributed to less gut damage, which means refinement of the immune system in broilers has undergone stress, inflammatory illness and infection such as *Eimeria tenella* as an intestinal detrimental microorganism (Nonkookhetkhong and Chalala 2023). In our study, unchanged leukocyte numbers among all treatments potentially mean that the intestinal status of broilers treated with ZnOHCl was as healthy as the intestines of other groups without undergoing infection, inflammation and stress.

3.4 | Jejunal Morphology and Caecal Microbial Population

As Table 5 displays, the largest proportion of the jejunal VL/CD was related to broilers fed with 110 mg of either ZnOHCl or ZnONPs, whereas the smallest VL:CD ratio was pertained to the control group ($p < 0.05$). This means that both 110 mg ZnOHCl and ZnONPs probably, through enhancement of longevity and resistance of jejunal epithelial cells against damage stemming from intestinal health problems, could noticeably refine the VL for better absorption and digestion of nutritional ingredients than other treatments. Our results have been supported that intestinal capacity to digest and absorb nutritional ingredients can be enhanced when the VL increases surface area (Awad et al. 2017). In another study, constant mitosis of epithelial cells can regularly provide either an absorption capacity for substituted villus cells or a normal apoptosis of obsolete villus cells (Van der Flier and Clevers 2009) when the epithelial cells covering the intestinal lumen can ceaselessly be renewed by new epithelial cells, which migrate since the basic point of crypt until the tip of villi, as this turns out that the migrated epithelial cells of villi not only find opportunities to grow in length, but also they might be at stake of damage, which leads to enhanced death of epithelial cells of villi relevant to health problems of intestine (Awad et al. 2017; De Grande et al. 2020). In our study, Zn sources might cater to the physiological needs of broilers through enhancement of VL, which probably led to a lower toxicity stemming from the penetration of pathogenic bacteria into gut and then improvement of immunity and growth performance. Our hypothesis was supported by Horst et al. (2020), who explained that ileal VL tended to enhance in Holstein cows fed ZnOHCl with feed restriction (40% of ad libitum diet, which was enriched with 75 mg ZnOHCl/kg) compared with that of the control diet with feed restriction (40% of ad libitum, the control diet, which was enriched with 75 mg zinc sulphate/kg). In fact, feeding ZnOHCl tends to cope with either reduction of mucosal surface area in ileum and jejunum or induction of intestinal hyperpermeability to provide gut barrier integration (Horst et al. 2020). Moreover, Mohd Yusof et al. (2023) declared that the tinnier size of ZnONPs results in an enhanced bioavailability that stems from convenient intestinal membrane penetration, and consequently, this causes higher absorption of ZnONPs in the body and more accessibility of ZnONPs for the function of some physiological parts. In another study, Fatholahi et al. (2021) supported our results that Zn sources, especially 120 mg Nano-bio Zn/kg of diet with nearly crystalline spherical shapes and sizes between 80 and 350 nm, could lead to either refinement of the VL (in Day 28) or reduction of CD, CD/VL proportion, and this indicates that Nano-bio Zn brought about lower loss of villous epithelial cells at the end point of villus, and less penetration of detrimental bacteria such as *E. coli* to the inside of circulation through intestine stem from benefiting a healthier gut barrier integrity than the control group nourished without Nano-bio Zn.

Although various quantities of either ZnOHCl or ZnONPs resulted in a lower population of *E. coli* than *Lactobacillus* counts, Table 5 is clearly exhibiting that both Zn sources compared with the control group could not negatively affect the population of *Lactobacillus* as a beneficial bacterium ($p > 0.05$). Similarly, Nguyen et al. (2021) reported that different levels of ZnOHCl in diet could not negatively influence either *Lactobacillus* or lactic acid generation in ceca, whereas those of dropped either *Bacillus* or total bacterial counts. Of course, the connection between healthy status of gut and microbial population was investigated by Mohd Yusof et al. (2023), who highlighted that the antibacterial properties of the 29.7 nm spherical oval form of ZnO at levels of either 100 or 10 mg/kg of diet refined intestinal health because it noticeably alleviated the *E. coli* population without having an adverse effect on *Lactobacillus* count. As there were worries regarding toxic effects of high concentration (100 mg/kg of diet) of ZnONPs and disruption of gut (beneficial/detrimental) microbial balance, ZnONPs/kg of 10 mg of diet as a safest level was suggested to include in broiler's diet to overcome detrimental bacteria (Mohd Yusof et al. 2023). According to the negative effect of Zn sources, particularly ZnONPs, on *E. coli*, we presumably might infer that *E. coli* population due to the interaction of lipopolysaccharide molecules existing in their outer membrane with ZnONPs tends to be more sensitive to the toxicity of ZnONPs than *Lactobacillus* and this toxicity potentially led to more death and a lower population of *E. coli* than *Lactobacillus* in the intestine of broilers. As our report was supported by Leung et al. (2016), who proved that interaction between antibacterial ZnONPs and molecules of lipopolysaccharide presenting in the external membrane of *E. coli* can upregulate the reactive oxygen species (ROS)-pertained proteins to generate ROS as a common symptom, which indicates that the ZnONPs toxicity brought about peroxidation of lipid structures for the commencement of either oxidative stress or bacterial membrane damage.

4 | Conclusion

Feeding both dose-dependent ZnONPs and ZnOHCl not only did not have a toxic effect, but also significantly brought about better immunity, growth performance, jejunal morphology and some breast meat quality indexes of broilers up to 110 mg/kg of corn–soybean diet than the control group. Among these parameters, moreover, most of the physiological capacity of broilers was positively influenced by ZnOHCl to be catered to their physiological needs better than groups fed with ZnONPs. Thus, it sounds like

the dietary inclusion of ZnOHCl up to 110 mg/kg of diet can be utilized instead of ZnONPs by broilers.

Author Contributions

Ahmad Taregh Hosein was related to data gathering and conceptualization. Majid Toghyani, with a supervisory role, was concerned with conceptualization, investigation, methodology and data curation. Mehdi Shahsavan was responsible for the context interpretation, using softwares, formal analysis, methodology, study validation, conceptualization, information gathering, searching or finding references, editing, writing, preparing, revising, and proofreading the original research manuscript.

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Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Research data are not shared.

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