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Effects of Nano Zinc on Growth Performance, Health Status, and Cecal Microbiota in Broiler Chickens Challenged with *Salmonella* Kentucky

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ABSTRACT

Public concern with the incidence of antibiotic-resistant bacteria, particularly among foodborne pathogens, such as *Salmonella*, has been challenging the poultry industry to find alternative means of control. The present study was conducted to investigate the effect of dietary replacement of inorganic zinc oxide (ZnO) by different levels of zinc nanoparticles on growth performance, blood serum biochemical changes, immune response, cecal microbiota, and some internal organs histopathology of *Salmonella* Kentucky (SK) challenged broiler chickens. A total of 180 one-day-old broiler chicks were used in the present experiment. The chicks were randomly allotted into six equal groups (30 chicks/group), with 3 subgroups containing 10 chicks as a replicate. The first group fed on the basal diet supplemented by 100 mg ZnO/kg diet, while the second and the third groups fed on the basal diet with replacement of ZnO by 100 and 50 mg of zinc oxide nanoparticles (ZnONPs)/kg diet, respectively. Moreover, the fourth, fifth, and sixth groups fed as the first three groups with SK challenge on the third day of age. Results showed that supplementation of 100 mg ZnONPs/kg diet instead of ZnO reduced the severity of the clinical signs, post-mortem lesions, mortality, and SK fecal shedding of SK challenged chicks. Replacement of ZnO by 100% or 50% of ZnONPs increased cecal total bacterial counts and lactobacillus bacterial count while reducing total coliform counts. On the other hand, the SK challenge increased cecal total bacterial counts and lactobacillus bacterial counts, compared to the broiler chicks group fed on the diet without SK challenge. The SK challenge with inorganic zinc addition reduced body gain and feed conversion ratio, while 100 or 50 mg ZnONPs/kg diet supplementation instead of ZnO improved growth performance, feed efficiency parameters. It was observed that the replacement of inorganic zinc (serum ZnO) by 100 mg /kg diet significantly increased lysosomal and phagocytic activity by about 261.5% and 17.9%, respectively. Moreover, 100% or 50% of ZnONPs instead of inorganic zinc significantly ZnONPs increased liver, spleen, and thymus gland relative weights of SK-challenged broiler chickens, compared to broiler chickens group fed on the same diet without challenge or compared to chicks group fed on ZnO supplemented diet with SK challenge, while replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet reduced the adverse effect.

Keywords: Broiler chicken, Growth performance, Nano zinc particles, Immune response, *Salmonella* challenge

INTRODUCTION

Despite numerous technological and sanitary improvements, poultry products continue to cause an increasing number of cases of human salmonellosis (Shinohara et al., 2008), generating economic loss and posing a threat to public health. The consumption of chicken and eggs represents the main cause of human infection by this pathogen (Baumler et al., 2000). *Salmonella* is a member of the Enterobacteriaceae family which causes food infections in humans and animals all over the world (Pasmars et al., 2008; Lan et al., 2009). One of the most common diseases is salmonellosis, which is caused by different serotypes of *Salmonella* bacteria, and there are concerns about the contamination of poultry and its products by a microorganism, moreover, *Salmonella* is one of the major sources of foodborne diseases in many parts of the world (Akbarmehr, 2010). There are more than 2500 serovars of *Salmonella enterica*, *Salmonella* Kentucky (SK) has been identified as one of the most prominent *Salmonella* serovars isolated from broilers causing diarrhea and high mortalities (Mahmoud et al., 2018).

Recently, many types of bacteria have become highly resistant to antibiotic treatments. Bacterial antibiotic resistance may be related to the overuse of these drugs and there are no alternative new medications (Gould and Bal, 2013; Wright, 2014). Consequently, scientists are trying to find new practical approaches to control bacterial infections in broiler productions. One way to prevent the spread of infectious agents is to replace antibiotics and consequently prevent antibiotic-bacterial resistance. Nanotechnology is a promising new approach that has the potential to substitute antibiotics as an antibacterial agent (Abd El-Ghany, 2019)

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Zinc (Zn) is considered an important nutrient for broiler chickens and plays a vital role in maintaining many physiological and metabolic processes in the living tissues (Bao et al., 2009). Adequate Zn supplementation and availability are essential for growth performance and reproduction as well as improvement of meat quality and immune response against pathogen challenge (Salim et al., 2008). Moreover, Zn was shown to have other important functions, such as controlling infectious diseases, improving wound healing, and keeping the epithelial tissue healthy (Vallee and Falchuk, 1993).

In recent years, high levels of inorganic Zn supplementation in broilers' diets to improve growth performance and immune response have led to higher Zn excretion and environmental pollution. Many previous studies have indicated that organic Zn sources have higher bioavailability, compared to inorganic Zn sources which are related to an organic compound that protects Zn from reacting with phytates (Star et al., 2012; Sahraei et al., 2013; Swain et al., 2016). In addition, in order to find low-cost alternatives to zinc sources, researchers have focused on nano-Zn sources in the animal feed industry (Lee et al., 2017; Kumar et al., 2021). The available data which clarify the influence of nano zinc on broiler performance and health status are limited. Generally, nano-sized mineral characters are different from inorganic sources due to their smaller particles size, larger surface area, and higher availability (Mahmoud et al., 2016).

Zinc nanoparticles are characterized by antimicrobial activity, especially against foodborne pathogenic bacteria, such as *Escherichia coli*, *Listeria monocytogenes*, *Salmonella*, and *Staphylococcus aureus* (Jones et al., 2008). Previous studies stated that the major processes of nanoparticles elements are disruption and penetration of the bacterial cell membrane (Wu et al., 2010).

Therefore, this study aimed to investigate the effect of replacement of inorganic zinc by different levels of zinc nanoparticles on growth performance, serum biochemical alterations, immune response, intestinal microbiota, and histopathological changes of some internal organs of broiler chickens challenged by SK.

MATERIALS AND METHODS

Ethical approval

The present study was affirmed by the Ethics of Animal Experiments Committee, Agricultural Research Center, Egypt.

Birds accommodation and management

A total of 180 one-day-old Ross-308 broiler chickens were used in this experiment. The chicks were obtained from a local Egyptian private hatchery at Kafr El-Sheikh Governorate, Egypt. The chicks were randomly allotted into six equal groups (30 chicks/group), with 3 subgroups containing 10 chicks as a replicate. The chickens were housed in a clean well-ventilated room previously fumigated with formalin and potassium permanganate. The temperature of the house adjusted according to the chick's age using electric heaters (temperature was around 33^{o}C on day one and reduced 2-3^{o}C each week until reach 24-26^{o}C). The prepared litter on the floor was 4 cm in depth using clean wheat straw. Different types of vaccine, including Hitchner B_{1} (at day 7 of chick's age), Gumboro intermediate (day 12), Gumboro weak (day 23), and cloned (days 18 and 28) were used to protect the broiler chicks against Newcastle and infectious bronchitis diseases. To confirm the absence of *Salmonella* infection, three Ross chicks per group were slaughtered via neck cutting at hatching before the challenge to confirm no presence of SK. The intestinal contents were collected and samples were placed in tetrathionate broth for 24 hours at 37^{o}C. The isolation was done on XLD agar plates which were incubated for 24 hours at 37^{o}C according to Janet et al. (2003). No *Salmonella* species was detected.

Feeding program

Experimental diets were formulated to meet the nutrient requirement of broiler chickens according to National Research Council (NRC, 1994, recommendation). Diet formulated with two different zinc sources using zinc oxide (ZnO) supplemented according to NRC recommendation (100 mg/kg diet) and replaced by nano zinc at two concentrations (100%, 50%) of the NRC recommendation to formulate three different experimental diets. The ingredient composition and chemical analysis of the experimental basal diets used for the starter, grower, and finisher are presented in Table 1.

Salmonella Kentucky challenge

A chicken *Salmonella entrica* serovar Kentucky field strain isolated from a clinical case of salmonellosis was obtained from Agricultural Research Center, Egypt. To determine the number of colony-forming units (CFUs), the inoculum was diluted and plated on XLD agar (Oxoid) for 24 h at 37^{o}C. Bacterial culture was diluted in sterile saline solution using McFarland standard 0.5 to make 10^{8} CFU/ml. At 1 day of age, all chicks in challenged groups (IV, V, and VI) were orally gavaged with the actively growing culture of *Salmonella* (0.5×10^{8} CFU/ml, 1.0 ml/bird), and the non-challenged group chicks were mock challenged with 1 ml sterile buffered peptone water.

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Table 1. Ingredient composition of the used basal diet in broiler chickens

Ingredients (%)	Feed type		
	Starter diet (0-2 weeks)	Grower diet (2-4 weeks)	Finisher diet (4-5 weeks)
Yellow corn	53.65	58.15	61.65
Soybean meal	32.6	29.5	26.6
Corn gluten	8.0	6.5	5.5
Vegetable oil[1]	2.0	2.0	2.5
DCP[2]	1.7	1.5	1.7
Limestone[3]	1.3	1.6	1.3
Lysine[4]	0.05	0.05	0.05
DL-Methionine[5]	0.15	0.15	0.15
Salt	0.3	0.3	0.3
Premix (vitamin)[6]	0.15	0.15	0.15
Mineral premix [7]	0.1	0.1	0.1
Total	100	100	100
Chemical composition			
Moisture (%)	11.95	11.55	11.76
Crude protein (%)	22.77	20.8	17.95
Ether extract (%)	3.85	4.55	4.94
Crude fibre (%)	3.08	2.95	3.02
Ash (%)	5.99	5.77	6.09
NFE (%)*	50.75	51.45	53.26
Calcium (%)	1.10	1.09	0.98
Total phosphorus (%)	0.73	0.68	0.69
ME Kcal/kg diet**	3039.8	3058.7	3096.46

[1]Vegetable oil: Mixture of sunflower oil and cottonseed oil, [2]DCP: Dicalcium phosphate (contain 18% P and 25% Ca). [3]Limestone (contain 34% calcium). [4]Lysine = lysine hydrochloride (contain 98.5% Lysine). [5]DL-Methionine (Produced by Evonic Co and contain 99.5% methionine). [6]The premix used was Heromix produced by Heropharm and composed of (per 1.5 kg) vitamin A 12000000 IU, vitamin D3 2500000 IU, vitamin E 10000 mg, vitamin K3 2000 mg, thiamin 1000 mg, riboflavin 5000 mg, pyridoxine 1500 mg, cyanocobalamin 10 mg, niacin 30000 mg, biotin 50 mg, folic acid 1000 mg, pantothenic acid. [7] mineral premix: formulated and (each 1 kg) composed of 70000 mg Mn, 100000mg Zn (Using zinc oxide (ZnO) and replaced by z nano zinc particles according to experimental design), 8000mg Cu, 1000mg I, 250mg Se, and 150mg Co. * NFE: Nitrogen free extract (calculated by difference "100- (moisture% + CP% + EE % + CF% + ash%)". **Calculated according to Lodhi et al. (1976) as Metabolizable energy MJ/Kg = 1.549+ (crude protein%*0.102) + (ether extract %*0.275) + (nitrogen free extract%*0.148) + (crude fiber%*0.034). The results multiply by 0.239 X 1000 = Kcal/kg.

Experimental design

The experimental design is presented in table 2.

Growth performance

Individual chick body weight and feed intake were recorded weekly and at the beginning of the experiment. Weight gain, feed conversion ratio (FCR), protein efficiency ratio (PER), and performance index (PI) were calculated according to McDonald et al. (1987) and North (1981).

Chemical composition

Analytical dry matter (DM) contents of feed samples were determined by oven-drying them at 105^{o}C for 8 hours (AOAC, 1985). Ash contents were determined by incineration at 550°C overnight. Crude protein and ether extract were determined according to Tinnimit and Thomas (1976) and Bligh and Dyer (1959), respectively.

Table 2. Experimental design outline

Group no.	Experimental diet	Zinc source & level		*Salmonella* Kentucky challenge
		Inorganic zinc[1]	Nano zinc[2]	
1	Basal diet	100 mg/kg diet	--	--
2	Basal diet	--	100 mg/kg diet	--
3	Basal diet	--	50 mg/kg diet	--
4	Basal diet	100 mg/kg diet	--	+
5	Basal diet	--	100 mg/kg diet	+
6	Basal diet	--	50 mg/kg diet	+

[1]: Zinc oxide (ZnO) as fed basis produced by El-Gomhoria Co., Egypt with a guaranteed minimum of 80% Zn); [2]: Zinc oxide nanoparticles "ZnONPs" produced by Mknano Co., Canada" with 30 nm.

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Assessment of some blood parameters and i**mmune response measurements**

Six blood samples were collected from each group of the experimental chicks (two from each replicate) at end of the experimental period (35 days of age) in clean dry vials containing anticoagulant (0.1 ml sodium citrate 3.8%) for the determination of phagocytic activity, phagocytic index according to Kawahara et al. (1991). Some blood pictures (total leukocytic count (WBCs), red blood cells (RBCs) counts, hemoglobin, packed cell volume (PCV) were recorded according to Maxine and Benjamin (1985). Other blood samples were collected without anticoagulant and separation of serum for the determination of serum total protein, globulin, albumin, GOT, GPT, ALP, uric acid, creatinine, calcium, phosphorus, serum lipids concentrations (cholesterol, triglyceride, HDL, LDL, and VLDL). Glucose and antioxidant enzymes (GSH-Px and MAD) were estimated using specific commercial kits (Roche Diagnostica, Basel, Switzerland) and lysosomal activity according to Engstad et al. (1992).

Lymphoid organs weight and some carcass traits

At the end of the experimental period, four birds from each dietary treatment (two from each replicate) were randomly taken, fasted for 6 hours then weighed (g/chick), and slaughtered to complete bleeding and weighed to determine dressing percentage and relative weight of immune organs (spleen, bursa, and thymus gland).

***Salmonella* identification**

Cloacal swabs from each bird (group IV, V, and VI) were aseptically collected on days 1, 3, and 7, and then weekly after the infection until the birds were 35 days old post-infection. Swabs were kept in a tube containing 2 mL Selenite F Broth (HiMedia Laboratories, Mumbai- India). Broth tubes were incubated at 37°C/24 hours, each broth was separately plated on XLD agar (Oxoid) for 24 hours at 37^{o}C (Gast et al., 1993). Suspected colonies from all samples were biochemically examined (urease test, H2S productions, TSI, motility test, oxidase test, indole test, Methyl Red test, and citrate test) according to Grimont and Weill (2007). All isolates were further identified by the serological method using *Salmonella* poly "O" antiserum and *Salmonella* monovalent "O and H" antiserum (SINIF Co., Germany) according to Mallinson and Snoeyenbos (1989).

***Salmonella* enumeration**

Samples of voided feces from each bird group (group IV, V, and VI) were aseptically collected 1 day and 3 days post-infection then weekly until the chickens were 35 days of age. Then, 1 gram of samples was diluted in 9 volumes of 0.85% saline followed by 10-fold dilutions. All dilutions were plated onto XLD agar for 24 hours at 37^{o}C. After incubation, typical *Salmonella* colonies were counted (Gast et al., 1993).

Intestinal microflora enumeration

The intestinal bacterial population was measured at the end of the experiment (35 days). Two chicks from each experimental replicate (6 chicks from each group) were selected and sacrificed. Pooled samples of the cecal contents were collected in sterile dishes. Collected samples were immediately put on ice, transferred to the laboratory to determine microbial population using 1 g of pooled cecal samples and serially diluted then 10 µl of each dilution was spot on each plate containing plate count agar and MacConkey agar was used to count total aerobes bacteria and Coliforms bacteria, respectively (Behnamifar et al., 2015) and incubated at 37°C for 48 hours. T was read and expressed as a colony-forming unit (CFU) per gram of cecal contents. De Man-Rogosa-Sharpe agar (MRS) was used for lactobacilli, cultivated in a 3% CO_2 atmosphere at 37ºC for 48 hours (Guban et al., 2006). After incubation, the bacteria were counted in Petri dishes, and the number of bacteria in the initial volume was calculated using the formula of Number of bacteria = Number of colonies × (1/Dilution factor) × Cultured volume. Then, the logarithms to base 10 of the obtained values were used in CFU/g for later analyses.

Intestinal and some internal organs histopathology

During slaughtering on day 35, about 2.5 cm of the ileum portion was sectioned and parts from the liver and spleen were collected. The tissues were collected and submerged in 10% neutral-buffered formalin for 3 days for tissue fixation. The collected samples were dehydrated and rinsed several times in absolute ethanol alcohol, and then embedded in paraffin. Serial 5-µm longitudinal sections were cut on Leica Rotary Microtome (RM 2145, Leica Microsystems, Wetzlar, Germany) and mounted on glass slides. Then, slides were routinely stained with hematoxylin and eosin (H&E). The histomorphometric analysis was performed using Image J analysis software (National Institutes of Health, MD, USA), whereas the villus height (measured from the tip of the villus to the villus- crypt junction), villus width from the mid of the villus, and crypt depth (measured from the crypt-villus junction to the base of the crypt) following Law et al. (2007).

Statistical analysis

Statistical analysis was made using Analysis of Variance (ANOVA) two-way analysis of variance for study the effect of different treatment groups on the different studied variables that includes (growth performance parameters,

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hematological, biochemical, and gut morphology) variables using statistical analysis system (SAS, 2004). Duncan's test was chosen for finding the level of significance ($p < 0.05$).

RESULTS

Clinical signs and post-mortem of broiler chickens

During the whole experimental period, no gastrointestinal or respiratory disorders were observed in *Salmonella* non-challenged broiler chickens. Evaluation of health parameters of SK challenged groups (Tables 3-4 and Figures 1-2) showed that using ZnONPs (100 mg/kg diet) instead of ZnO reduced the severity of the clinical signs, post-mortem (P/M) lesions, and mortality percentage, compared to broiler chicken group fed on ZnO supplemented diet (control). However, replacement of ZnO by ZnONPs (50 mg/kg diet percent) had no effect on clinical signs or post mortem changes of broiler chickens.

Table 3. Clinical signs of *Salmonella* challenged broiler chickens affected by replacement of ZnO by ZnONPs

Signs and diseases	Zinc source and levels		
	ZnO (100 mg/kg)	ZnONPs (100 mg/kg)	ZnONPs (50 mg/kg)
First post-challenge			
General signs of illness	+++*	++**	+++
Respiratory distress	+++	++	+++
Whitish diarrhea	++	+***	++
Second post challenge			
General signs of illness	+++	++	+++
Respiratory distress	+++	++	+++
Whitish diarrhea	++	+	++
Third post-challenge			
General signs of illness	++	+	++
Respiratory distress	++	+	++
Whitish diarrhea	++	+	+

General signs of illness (decreased appetite, loss of weight, general activity, depression, ruffled feather or drooped wings; *: Severe symptoms; **: Moderate symptoms; ***: Mild symptoms

Figure 1. White pasty diarrhea appeared in one-day-old chickens challenged with *Salmonella*

Table 4. Post mortem lesions of *Salmonella* challenge dead broiler chickens affected by replacement of ZnO by ZnONPs

Lesions	Zinc source and levels		
	ZnO (100 mg/kg)	ZnO (100 mg/kg)	ZnO (100 mg/kg)
First day post-challenge			
Airsacculitis	+++*	++**	+++
Congested liver and spleen	+++	++	+++
Pericarditis and perihepatitis	++	+***	++
Mortality	**1**	**1**	**1**
Second day post-challenge			
Airsacculitis	+++	-	+++
Congested liver and spleen	+++	-	+++
Pericarditis and perihepatitis	++	-	++
Mortality	**1**	-	**1**

*: Severe symptoms; **: Moderate symptoms; ***: Mild symptoms

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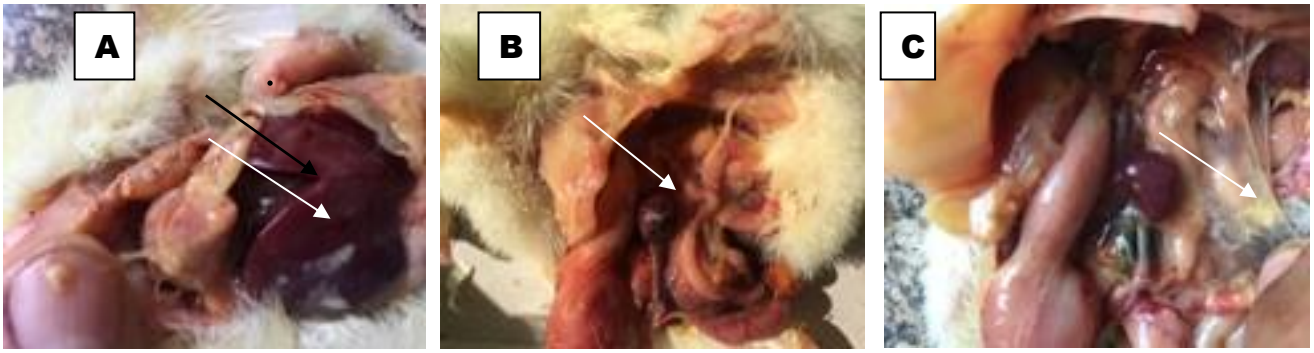

Figure 2. Congested liver and spleen (A and B) and airsacculitis with the congested spleen (C) in one-day-old chickens challenged with *Salmonella*

Growth performance and feed efficiency parameters

As shown in Table 5, replacement of inorganic zinc (ZnO) by 100 or 50 mg zinc oxide nano-particles (ZnONPs/kg) diet non-significantly increased final body weight and total gain by about (5.5% and 5.6%) and (0.1% and 0.3%), respectively, as compared to broiler chicks group fed on a diet supplemented by 100mg ZnO/kg ($p > 0.05$). However, ZnONPs supplementation instead of inorganic source increased feed intake and did not affect the average FCR, PER, and PI. Moreover, replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet with SK challenge non-significantly deteriorate final weight, FCR, PER, and PI compared to broiler chicks group fed on the diet without SK challenges ($p < 0.05$).

Hematological parameters

Table 6 represents the hematological parameters of broiler chicks fed on different experimental diets. No significant differences were observed for RBCs and WBCs counts between different experimental groups ($p > 0.05$). The same direction was observed for hemoglobulin (Hb percentage) and packed cell volume (PCV%). On the other hand, replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet with SK challenge non-significantly reduced the above-mentioned hematological parameters compared to broiler chicks group fed on the diet without SK challenge ($p > 0.05$).

Table 5. Effect of replacement of inorganic zinc by nano zinc on growth performance of broilers chicken challenged by *Salmonella* Kentucky

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Initial body weight (g/chick)	ZnO (100 mg/kg)	47.67 ± 1.60^{ax}	48.27 ± 1.28^{ax}
	ZnONPs (100 mg/kg)	48.73 ± 0.80^{ax}	48.82 ± 1.50^{ax}
	ZnONPs (50 mg/kg)	46.64 ± 1.75^{ax}	48.82 ± 0.82^{ax}
Final body weight (g/bird)	ZnO (100 mg/kg)	1820.33 ± 45.05^{ax}	1781.11 ± 38.31^{ax}
	ZnONPs (100 mg/kg)	1920.50 ± 51.45^{ax}	1874.44 ± 123.53^{ax}
	ZnONPs (50 mg/kg)	1822.14 ± 54.61^{ax}	1752.44 ± 125.07^{ax}
Total gain (g/bird)	ZnO (100 mg/kg)	1772.67 ± 43.48^{ax}	1734.22 ± 176.60^{ax}
	ZnONPs (100 mg/kg)	1872.10 ± 50.72^{ax}	1827.33 ± 122.50^{ax}
	ZnONPs (50 mg/kg)	1778.71 ± 53.06^{ax}	1704.44 ± 203.58^{ax}
Total feed intake (g/bird)	ZnO (100 mg/kg)	2673.23 ± 102.66^{bx}	2838.57 ± 82.74^{ax}
	ZnONPs (100 mg/kg)	2921.17 ± 89.34^{ax}	3007.06 ± 90.92^{ax}
	ZnONPs (50 mg/kg)	2793.91 ± 91.21^{abx}	2898.98 ± 67.91^{ax}
Average feed conversion ratio	ZnO (100 mg/kg)	1.52 ± 0.04^{ax}	1.64 ± 0.04^{ax}
	ZnONPs (100 mg/kg)	1.57 ± 0.04^{ax}	1.73 ± 0.16^{ax}
	ZnONPs (50 mg/kg)	1.58 ± 0.05^{ax}	1.80 ± 0.18^{ax}
Average protein efficiency ratio	ZnO (100 mg/kg)	3.01 ± 0.07^{ax}	3.03 ± 0.07^{ax}
	ZnONPs (100 mg/kg)	3.20 ± 0.09^{ax}	3.02 ± 0.20^{ax}
	ZnONPs (50 mg/kg)	3.17 ± 0.10^{ax}	2.92 ± 0.21^{ax}
Performance index	ZnO (100 mg/kg)	121.73 ± 5.94^{ax}	109.21 ± 13.85^{ax}
	ZnONPs (100 mg/kg)	123.80 ± 12.71^{ax}	117.93 ± 18.19^{ax}
	ZnONPs (50 mg/kg)	116.62 ± 18.23^{ax}	107.32 ± 16.90^{ax}

Values are means ± Standard Error. Superscript letters at the same column ($^{a-b}$) represent significant differences between different levels and sources of zinc while superscript letters at the same row ($^{x-z}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

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Table 6. Effect of replacement of inorganic zinc by nano zinc on some blood pictures of broilers chicken challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
RBCs count (10^3)	ZnO (100 mg/kg)	2.23 ± 0.18^{ax}	2.14 ± 0.11^{ax}
	ZnONPs (100 mg/kg)	2.43 ± 0.06^{ax}	2.30 ± 0.03^{ax}
	ZnONPs (50 mg/kg)	2.44 ± 0.11^{ax}	2.16 ± 0.11^{ax}
WBCs count (10^6)	ZnO (100 mg/kg)	18.52 ± 1.88^{ax}	17.94 ± 2.11^{ax}
	ZnONPs (100 mg/kg)	22.81 ± 2.05^{ax}	20.65 ± 1.09^{ax}
	ZnONPs (50 mg/kg)	19.56 ± 1.54^{ax}	18.47 ± 2.85^{ax}
Hb (%)	ZnO (100 mg/kg)	11.19 ± 0.90^{ax}	10.73 ± 0.55^{ax}
	ZnONPs (100 mg/kg)	12.15 ± 0.28^{ax}	11.54 ± 0.17^{ax}
	ZnONPs (50 mg/kg)	12.24 ± 0.57^{ax}	10.8 ± 0.55^{ax}
PCV (%)	ZnO (100 mg/kg)	36.93 ± 2.97^{ax}	36.39 ± 1.83^{ax}
	ZnONPs (100 mg/kg)	40.11 ± 0.92^{ax}	38.08 ± 0.56^{ax}
	ZnONPs (50 mg/kg)	40.39 ± 1.89^{ax}	35.64 ± 1.82^{ax}

Values are means ± Standard Error. RBCs: Red blood cells, WBCs: White blood cells, Hb%: Hemoglobin percentage, PCV%: Packed cell volume percentage. Superscript letters at the same column ($^{a-d}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x-z}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

Serum lipid profile

The effect of inorganic zinc by nano zinc on lipid serum profile including triglyceride, total cholesterol, HDL, LDL, and VLDL serum concentrations are presented in Table 7. Serum lipid parameters were not statistically ($p \geq 0.05$) influenced by replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet, except the lower level of ZnONPs significantly ($p < 0.05$) reduced serum triglyceride and VLDL concentrations, compared to other treatments. Moreover, using 100 or 50mg ZnONPs/kg diet instead of inorganic zinc of broiler chicken diet non-significantly reduced CHO/HDL ratio by about 11% and 7.3% respectively ($P > 0.05$).

Liver and kidney function

Liver and kidney function of broiler chicken as affected by dietary treatments are presented in Table 8. Results indicated that serum creatinine or uric acid concentrations and serum GOT or GPT activities were not statistically ($p \geq 0.05$) influenced by replacement of inorganic zinc (ZnO) by 100 or 50mg ZnONPs/kg diet, except higher level (100mg/kg) of ZnONPs which significantly ($p < 0.05$) reduced serum GOT or GPT activities compared to other treatments. On the other hand, using ZnONPs instead of ZnO with SK challenge increased serum GOT or GPT activities, compared to the broiler chicks group fed the same diet without SK challenge.

Table 7. Effect of replacement of inorganic zinc by nano zinc on serum lipid profile of broilers chicken challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Triglyceride (mg/dl)	ZnO (100 mg/kg)	215.13 ± 2.86^{ax}	211.06 ± 1.99^{ax}
	ZnONPs (100 mg/kg)	216.31 ± 0.27^{ax}	212.95 ± 2.30^{ax}
	ZnONPs (50 mg/kg)	195.10 ± 9.07^{by}	210.27 ± 0.97^{ax}
Cholesterol (mg/dl)	ZnO (100 mg/kg)	287.68 ± 2.73^{ax}	276.86 ± 4.48^{bx}
	ZnONPs (100 mg/kg)	280.37 ± 4.25^{ax}	279.05 ± 1.46^{abx}
	ZnONPs (50 mg/kg)	283.42 ± 4.28^{ax}	290.62 ± 3.55^{ax}
HDL (mg/dl)	ZnO (100 mg/kg)	55.73 ± 2.58^{ax}	53.43 ± 1.84^{ax}
	ZnONPs (100 mg/kg)	60.76 ± 1.24^{ax}	53.40 ± 3.21^{ax}
	ZnONPs (50 mg/kg)	59.03 ± 1.08^{ax}	52.16 ± 2.39^{ax}
LDL (mg/dl)	ZnO (100 mg/kg)	188.92 ± 2.51^{ax}	181.21 ± 6.62^{bx}
	ZnONPs (100 mg/kg)	176.34 ± 4.01^{ax}	183.06 ± 2.46^{abx}
	ZnONPs (50 mg/kg)	185.37 ± 2.60^{ax}	196.40 ± 4.64^{ax}
vLDL (mg/dl)	ZnO (100 mg/kg)	43.02 ± 0.57^{ax}	42.21 ± 0.40^{ax}
	ZnONPs (100 mg/kg)	43.26 ± 0.05^{ax}	42.59 ± 0.46^{ax}
	ZnONPs (50 mg/kg)	39.02 ± 1.81^{by}	42.05 ± 0.19^{ax}
CHO/HDL ratio	ZnO (100 mg/kg)	5.18 ± 0.21^{ax}	5.19 ± 0.27^{ax}
	ZnONPs (100 mg/kg)	4.61 ± 0.09^{ax}	5.25 ± 0.28^{ax}
	ZnONPs (50 mg/kg)	4.80 ± 0.11^{ax}	5.59 ± 0.28^{ax}

Values are means ± Standard Error; CHO: Cholesterol, HDL: High-density lipoprotein, LDL: Low-density lipoprotein. vLDL: Very low-density lipoprotein. Superscript letters at the same column ($^{a-b}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x-y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

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Table 8. Effect of replacement of inorganic zinc by nano zinc on serum parameters related to liver and kidney functions of broilers chicken challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Creatinine (mg/dl)	ZnO (100 mg/kg)	2.80 ± 0.07^{ax}	2.77 ± 0.16^{ax}
	ZnONPs (100 mg/kg)	2.73 ± 0.04^{ax}	2.72 ± 0.05^{ax}
	ZnONPs (50 mg/kg)	2.63 ± 0.20^{ax}	2.90 ± 0.06^{ax}
Uric acid (mg/dl)	ZnO (100 mg/kg)	8.38 ± 0.14^{ax}	7.94 ± 0.06^{ax}
	ZnONPs (100 mg/kg)	7.90 ± 0.03^{ax}	7.93 ± 0.41^{ax}
	ZnONPs (50 mg/kg)	8.47 ± 0.09^{ax}	8.23 ± 0.18^{ax}
SGOT (um/ml)	ZnO (100 mg/kg)	8.45 ± 0.18^{ax}	8.60 ± 0.26^{ax}
	ZnONPs (100 mg/kg)	8.00 ± 0.58^{bx}	8.76 ± 0.15^{ax}
	ZnONPs (50 mg/kg)	9.16 ± 0.30^{ax}	9.10 ± 0.15^{ax}
SGPT (um/ml)	ZnO (100 mg/kg)	6.80 ± 0.06^{ax}	7.26 ± 0.18^{bx}
	ZnONPs (100 mg/kg)	5.80 ± 0.29^{by}	7.93 ± 0.20^{ax}
	ZnONPs (50 mg/kg)	6.70 ± 0.17^{ax}	7.10 ± 0.21^{bx}

Values are means ± Standard Error; GOT: Glutamic-Oxaloacetic Transaminase. GPT: Glutamic-Pyruvic Transaminase. Superscript letters at the same column ($^{a- b}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x- y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

Immune response

Lysosomal activity and phagocytosis

Table 9 showed that replacement of inorganic zinc (ZnO) by 100 mg ZnONPs/kg diet significantly ($P < 0.05$) increased lysosomal and phagocytic activity by about 361.5% and 66.7% respectively while using 50mg ZnONPs/kg diet instead of ZnO non-significantly improved the mentioned immune parameters of broiler chickens ($p > 0.05$). On the other hand, using ZnONPs instead of ZnO with SK challenge reduced lysosomal and phagocytic activities compared to the broiler chicks group fed the same diet without SK challenge.

Immune organs index

Table 10 showed that replacement of ZnO by 100 mg or 50 mg of ZnONPs/kg in broiler chickens diet had no significant effect on dressing percentage or relative weight of liver, spleen, bursa of Fabricius, and thymus gland ($p \geq 0.05$). On the other hand, it was observed that using 100 mg or 50 mg of ZnONPs/kg diet instead of inorganic zinc significantly increased liver, spleen, and thymus gland relative weights of SK challenged broiler chickens, compared to broiler chickens group fed on the same diet without challenge or compared to chicks group fed on ZnO supplemented diet with *Salmonella* challenge ($p < 0.05$).

***Salmonella* Kentucky shedding**

Cloacal swabs taken from chicks during the first-week post-challenge revealed that SK shedding was nearly similar between challenged groups (Table 11) and reached more than 80% of the sampled broiler chicks. Shedding of SK reduced starting from second-week post-challenge and the highest reduction was observed with the chicks group fed on ZnONPs (100mg/kg) supplemented diet. Concentrations of SK of cloacal swabs were significantly ($p < 0.05$) higher in the chicks group fed on ZnONPs (100 mg/kg) or ZnONPs (50 mg/kg) supplemented diets during the first two weeks post-challenge and reduced compared to broiler chicks group fed on inorganic zinc supplemented diet. The highest reduction of SK shedding was observed with the chicks group fed on ZnONPs (100 mg/kg) supplemented diet.

Cecal microbiota

Replacement of ZnO by 100 or 50mg of ZnONPs increased cecal total bacterial counts and *Lactobacillus* bacterial count while reducing total coliform count (Table 12). On the other hand, SK challenge increased cecal total bacterial counts and lactobacillus bacterial count, compared to the broiler chicks group fed on the diet without challenge.

Intestinal morphology

Histomorphometric measurements of the ileum are presented in Table 13 as well as Figures 3 and 4. Statistical analysis of the obtained data indicated that 100 mg of ZnO/kg diet replacement by 100 mg or 50 mg of ZnONPs/kg diet significantly ($p < 0.05$) improved ileum villi height, crypt depth and Villus height: Crypt depth (VH: CD) ratio, while non-significantly ($p \geq 0.05$) improved villi width. Moreover, using both levels ZnONPs instead of ZnO with SK challenge reduced all ileum histomorphometric measurements items compared to broiler chicks group fed the same diet without SK challenge.

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Table 9. Effect of replacement of inorganic zinc by nano zinc on immune response parameters of broiler chicken challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Lysozomal activity	ZnO (100 mg/kg)	0.13 ± 0.07^{bx}	0.09 ± 0.04^{ax}
	ZnONPs (100 mg/kg)	0.47 ± 0.04^{ax}	0.21 ± 0.07^{ay}
	ZnONPs (50 mg/kg)	0.27 ± 0.01^{bx}	0.11 ± 0.05^{ax}
Phagocytic activity	ZnO (100 mg/kg)	36.32 ± 0.98^{bx}	30.08 ± 1.02^{ay}
	ZnONPs (100 mg/kg)	42.82 ± 0.75^{ax}	35.45 ± 0.68^{ay}
	ZnONPs (50 mg/kg)	39.27 ± 1.05^{abx}	34.98 ± 0.88^{ax}
Phagocytic index	ZnO (100 mg/kg)	1.95 ± 0.07^{ax}	1.78 ± 0.06^{ax}
	ZnONPs (100 mg/kg)	2.18 ± 0.09^{ax}	1.94 ± 0.07^{ax}
	ZnONPs (50 mg/kg)	1.98 ± 0.05^{ax}	1.78 ± 0.04^{ax}

Values are means ± Standard Error. Superscript letters at the same column ($^{a-b}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x-y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

Table 10. Effect of replacement of inorganic zinc by nano zinc on dressing and some internal organs index of broiler chicken challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Dressing (%)	ZnO (100 mg/kg)	70.88 ± 2.17^{ax}	71.51 ± 3.38^{ax}
	ZnONPs (100 mg/kg)	72.60 ± 1.87^{ax}	71.93 ± 2.77^{ax}
	ZnONPs (50 mg/kg)	72.23 ± 3.27^{ax}	71.27 ± 2.39^{ax}
Liver (percentage of live weight)	ZnO (100 mg/kg)	2.52 ± 0.32^{ax}	1.92 ± 0.52^{cy}
	ZnONPs (100 mg/kg)	2.34 ± 0.29^{ax}	2.59 ± 0.31^{bx}
	ZnONPs (50 mg/kg)	2.24 ± 0.31^{ay}	3.03 ± 0.21^{ax}
Spleen (percentage of live weight)	ZnO (100 mg/kg)	0.13 ± 0.03^{ax}	0.12 ± 0.03^{cx}
	ZnONPs (100 mg/kg)	0.15 ± 0.04^{ay}	0.22 ± 0.06^{bx}
	ZnONPs (50 mg/kg)	0.15 ± 0.04^{ay}	0.29 ± 0.03^{ax}
Bursa (percentage of live weight)	ZnO (100 mg/kg)	0.16 ± 0.02^{ax}	0.18 ± 0.04^{ax}
	ZnONPs (100 mg/kg)	0.19 ± 0.03^{ax}	0.16 ± 0.03^{ax}
	ZnONPs (50 mg/kg)	0.14 ± 0.03^{ax}	0.15 ± 0.03^{ax}
Thymus (percentage of live weight)	ZnO (100 mg/kg)	0.22 ± 0.04^{ax}	0.24 ± 0.04^{bx}
	ZnONPs (100 mg/kg)	0.24 ± 0.03^{ay}	0.32 ± 0.06^{ax}
	ZnONPs (50 mg/kg)	0.21 ± 0.04^{ay}	0.29 ± 0.04^{abx}

Values are means ± Standard Error. Superscript letters at the same column ($^{a-c}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x-y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

Table 11. Effect of replacement of inorganic zinc by nano zinc on shedding of *Salmonella* Kentucky in broiler chickens at 35 days of age

Period post challenge	Sources and levels of Zinc					
	ZnO (100 mg/kg)	ZnONPs (100 mg/kg)	ZnONPs (50 mg/kg)	ZnO (100 mg/kg)	ZnONPs (100 mg/kg)	ZnONPs (50 mg/kg)
	Cloacal swab incidence			Log_{10} of *Salmonella* Kentucky/cloacal swab		
First day	15/30 (50%)	17/30 (56.7%)	18/30 (60%)	2.3 ± 0.20^{c}	4.2 ± 0.27^{b}	7.6 ± 0.41^{a}
Third day	25/28 (89.3%)	22/29 (75.9%)	23/29 (79.3%)	3.4 ± 0.21^{c}	5.3 ± 0.28^{b}	8.4 ± 0.59^{a}
First week	21/27 (77.8%)	23/28 (78.6%)	25/27 (96.6%)	5.2 ± 0.18^{c}	6.5 ± 0.31^{b}	9.2 ± 0.36^{a}
Second week	18/25 (72%)	16/28 (57.1%)	12/26 (46.2%)	2.8 ± 0.24^{c}	7.3 ± 0.26^{a}	3.3 ± 0.17^{b}
Third week	11/25 (44%)	10/27 (37%)	9/26 (34.7%)	2.2 ± 0.31^{b}	1.9 ± 0.31^{b}	8.1 ± 0.41^{a}
Fourth week	7/24 (29.2%)	8/27 (29.6%)	8/25 (32%)	6.4 ± 0.27^{a}	5.4 ± 0.28^{b}	4.6 ± 0.26^{c}
Fifth week	5/24 (20.8)	5/25 (20%)	8/25 (32%)	4.5 ± 0.28^{a}	1.6 ± 0.23^{c}	3.7 ± 0.24^{b}

Incidence of recovery expressed as positive/total chickens (%). Log_{10} *Salmonella* Kentucky (SK)/cloacal swab, data are expressed as mean ± standard error. Values within a row with no common superscript differ significantly ($p < 0.05$).

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Table 12. Effect of replacement of inorganic zinc by nano zinc on cecal bacterial counts of broiler chickens challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Total aerobic count (log9 CFU/g)	ZnO (100 mg/kg)	1.1 ± 0.18^{by}	7.9 ± 0.40^{ax}
	ZnONPs (100 mg/kg)	1.2 ± 0.18^{by}	3.4 ± 0.56^{bx}
	ZnONPs (50 mg/kg)	2.1 ± 0.20^{ay}	8.4 ± 0.29^{ax}
Total coliform count (log7 CFU/g)	ZnO (100 mg/kg)	8.4 ± 0.36^{ax}	8.2 ± 0.35^{ax}
	ZnONPs (100 mg/kg)	7.4 ± 0.29^{bx}	5.6 ± 0.32^{by}
	ZnONPs (50 mg/kg)	5.5 ± 0.36^{cx}	6.1 ± 0.36^{bx}
Lactobacillus bacterial count (log10 CFU/g)	ZnO (100 mg/kg)	1.7 ± 0.22^{cx}	2.3 ± 0.33^{cx}
	ZnONPs (100 mg/kg)	5.7 ± 0.25^{ay}	9.4 ± 0.43^{ax}
	ZnONPs (50 mg/kg)	3.4 ± 0.41^{by}	4.8 ± 0.31^{bx}

Values are means ± Standard Error. Superscript letters at the same column ($^{a-c}$) represent a significant difference between different levels and sources of zinc while superscript letters at the same row ($^{x-y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

Table 13. Effect of replacement of inorganic zinc by nano zinc on ileum morphology of broilers challenged by *Salmonella* Kentucky at 35 days of age

Parameters	Zinc source and levels	*Salmonella* Kentucky challenge	
		Non-challenged	Challenged
Villus height (VH/μm)	ZnO (100 mg/kg)	532.37 ± 17.8^{cx}	223.14 ± 8.25^{cy}
	ZnONPs (100 mg/kg)	694.63 ± 13.75^{ax}	526.16 ± 16.1^{ay}
	ZnONPs (50 mg/kg)	592.19 ± 15.04^{bx}	454.74 ± 44.16^{by}
Crypt depth (μm)	ZnO (100 mg/kg)	76.31 ± 4.28^{cx}	30.74 ± 2.19^{cy}
	ZnONPs (100 mg/kg)	105.87 ± 5.22^{ax}	81.17 ± 6.14^{ay}
	ZnONPs (50 mg/kg)	95.34 ± 5.71^{bx}	66.72 ± 2.74^{by}
VH:CD ratio	ZnO (100 mg/kg)	7.02 ± 0.56^{ax}	7.25 ± 0.28^{ax}
	ZnONPs (100 mg/kg)	6.56 ± 0.17^{abx}	6.59 ± 0.65^{ax}
	ZnONPs (50 mg/kg)	6.26 ± 0.30^{bx}	6.85 ± 0.51^{ax}
Villi width (μm)	ZnO (100 mg/kg)	94.77 ± 4.05^{ax}	72.69 ± 5.75^{by}
	ZnONPs (100 mg/kg)	102.80 ± 8.62^{ax}	63.69 ± 4.29^{cy}
	ZnONPs (50 mg/kg)	102.89 ± 6.23^{ax}	105.53 ± 3.54^{ax}

Values are means ± Standard Error. Superscript letters at the same column ($^{a-c}$) represent significant differences between different levels and sources of zinc while superscript letters at the same row ($^{x-y}$) represent the difference between nonchallenged and challenged groups ($p < 0.05$).

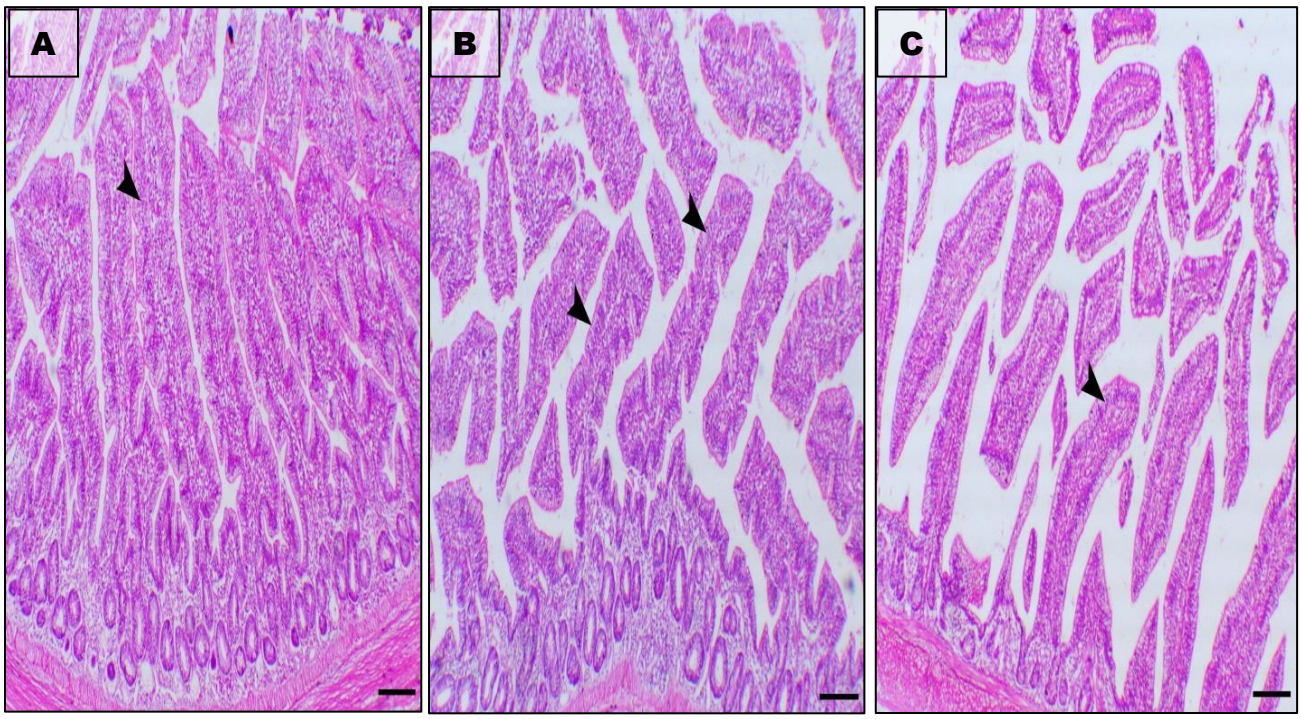

Figure 3. Ilium histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. Histopathology of the intestine (ilium portion) of broiler chickens showing normal intestinal villi fed on a diet containing inorganic zinc (A), while group fed on 100 mg/kg diet Nano zinc showing an increase in the intestinal villi length and marked decrease the space between the villi (B), moreover, the group fed 50 mg/kg diet Nano zinc indicating normal intestinal villi (C). H&E, X200, bar= 50 µm.

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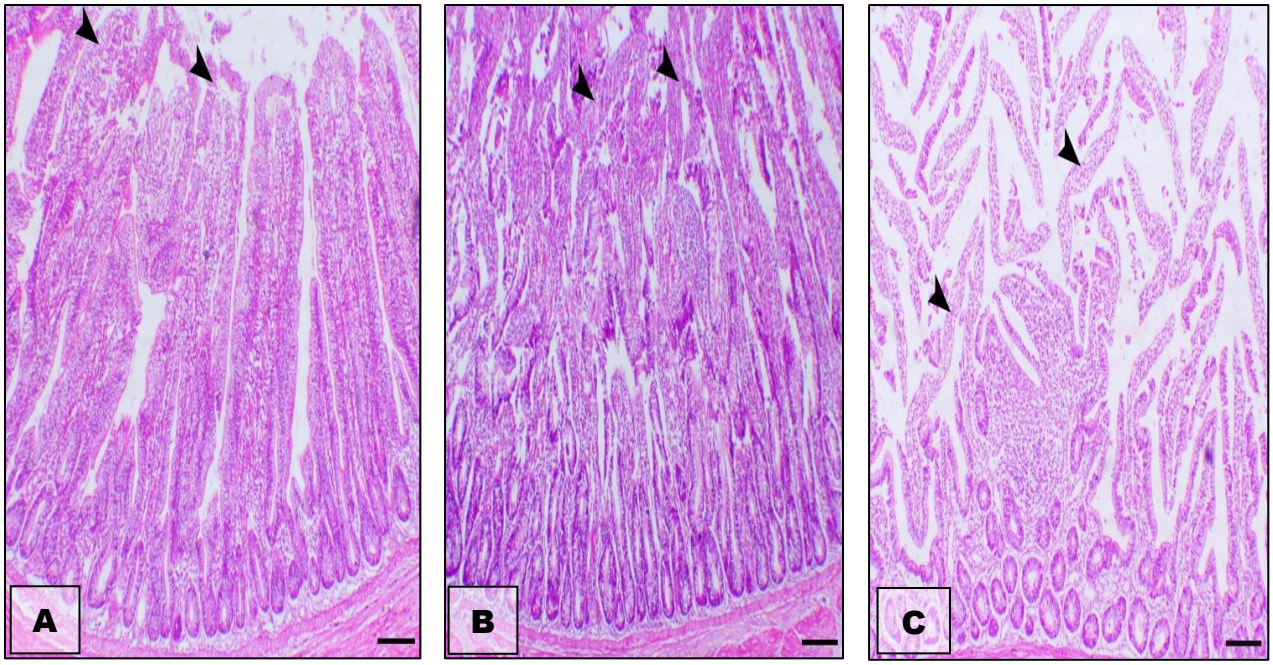

Figure 4. Ilium histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. Necrotic enteritis associated with necrosis of the villi and infiltration of inflammatory cells are shown in chickens fed on a diet containing inorganic zinc (A); while group fed on 100 mg/kg diet Nano zinc showing marked a decrease in the features of enteritis with normal villi (B), moreover group fed 50 mg/kg diet Nano zinc indicating a decrease in the features of enteritis (C). H&E, X200, bar= 50 µm.

Liver and spleen histopathology

Microscopically, liver tissues show normal hepatocytes forming hepatic plates around the central vein in all broiler chicken groups fed on different zinc sources and levels (Figure 5). On the other hand, it was observed that the broiler chickens group fed on inorganic zinc supplemented diet with SK challenge had marked hepatitis associated with severe degree heterophilic cells infiltration around the portal area that extended into hepatic tissues and multifocal necrosis and degenerative changes within hepatic cells (Figure, 6). However, replacement of ZnO by 100 or 50 mg of ZnONPs/kg diet with SK challenge indicated a marked decrease in hepatitis features representing a marked decrease in the hepatic degenerative changes and perivascular infiltration of inflammatory cells. Generally, the spleen showed normal lymphoid follicles in all broiler chicken groups fed on different zinc sources and levels (Figure 7). On the other hand, the broiler chickens group fed on inorganic zinc supplemented diet with SK challenge indicated congestion of the red pulp and marked degree of lymphoid depletion associated with lymphoid cells necrosis with the marked appearance of the reticular fibers (Figure 8). On the other hand, replacing ZnO by 100 or 50 mg of ZnONPs/kg diet with SK challenge presented a marked increase of the lymphoid content within the white pulp.

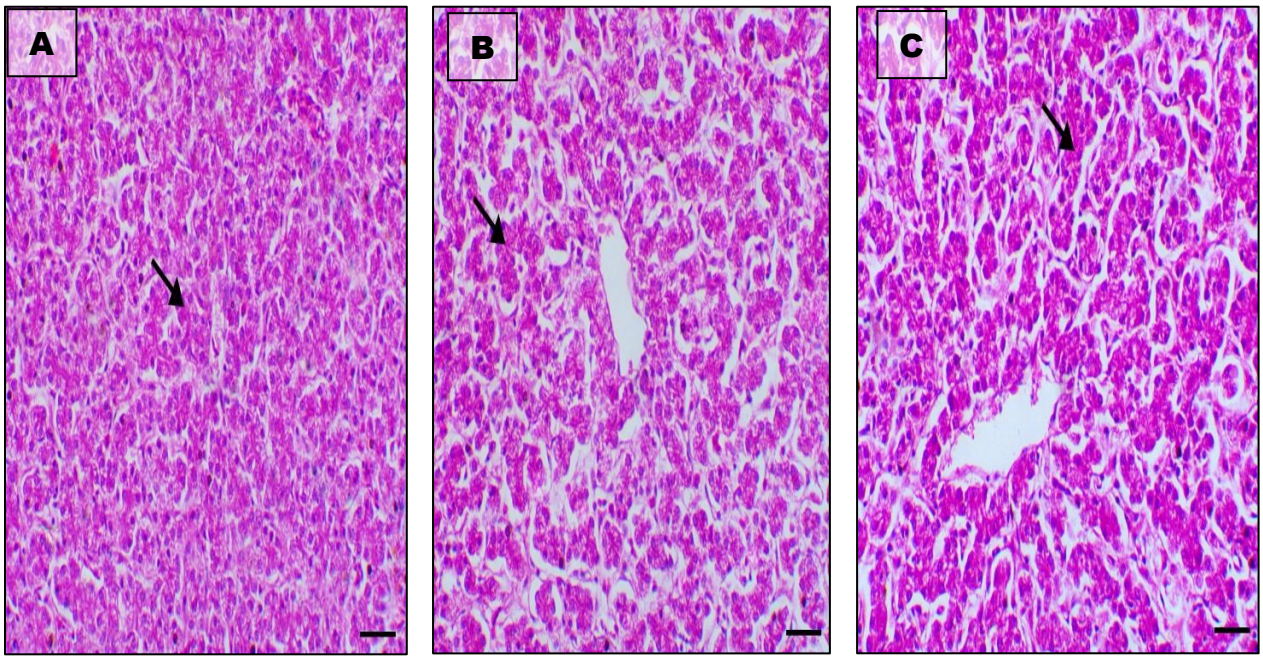

Figure 5. Liver histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. The normal hepatocytes forming hepatic plates around the central vein in chickens fed on a diet containing inorganic zinc (A); while group fed on 100 mg/kg diet Nano zinc showing normal hepatocytes forming hepatic plates around the central vein (B), moreover group fed 50 mg/kg diet Nano zinc indicating normal hepatic tissues (C, arrow indicates normal hepatocytes around the central vein). H&E, X200, bar = 50 µm.

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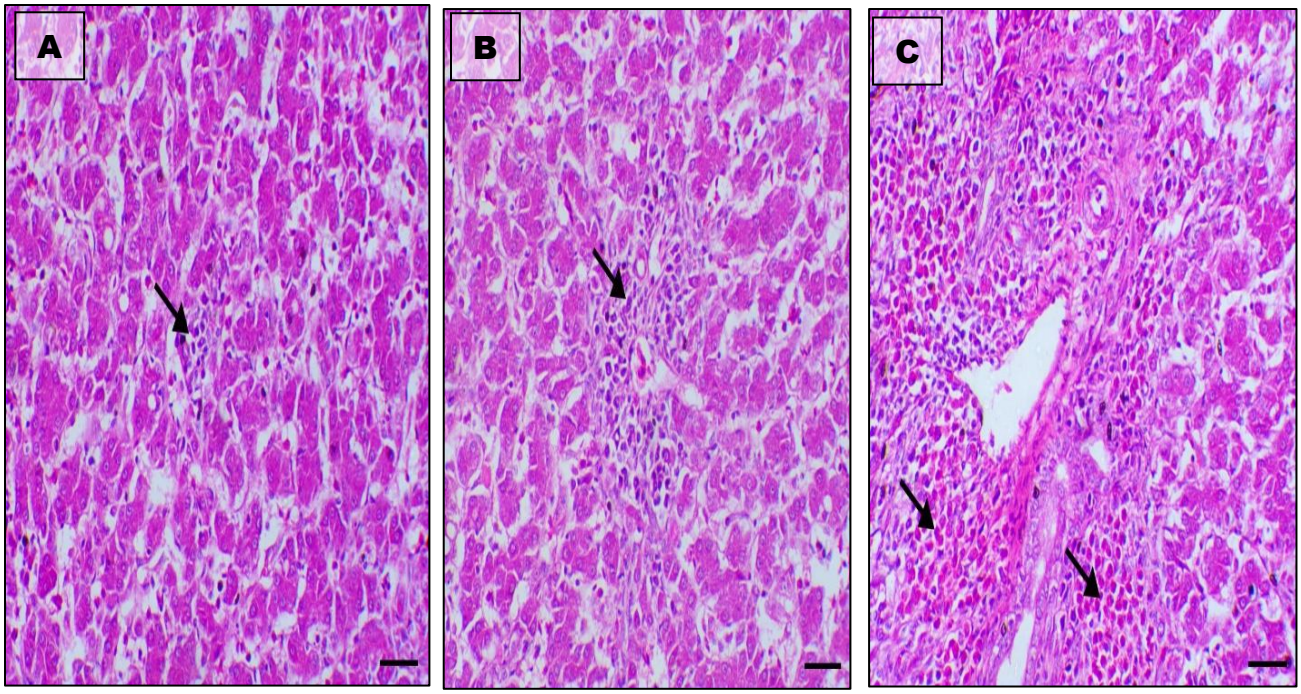

Figure 6. Liver histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. Marked hepatitis associated with severe degree heterophilic cells infiltration (arrows) around the portal area extending into hepatic tissues and multifocal necrosis and degenerative changes within hepatic cells are shown in chickens fed on a diet containing inorganic zinc (A), while group fed on 100 mg/kg diet Nano zinc showing marked decrease hepatitis features represented with marked decrease the hepatic degenerative changes and perivascular infiltration of inflammatory cells (B), moreover group fed 50 mg/kg diet Nano zinc indicating decreased periportal inflammatory cells and hepatic degenerative changes (C, arrow indicates inflammatory cells infiltration including lymphocytes, macrophages, and few heterophils). H&E, X200, bar= 50 µm.

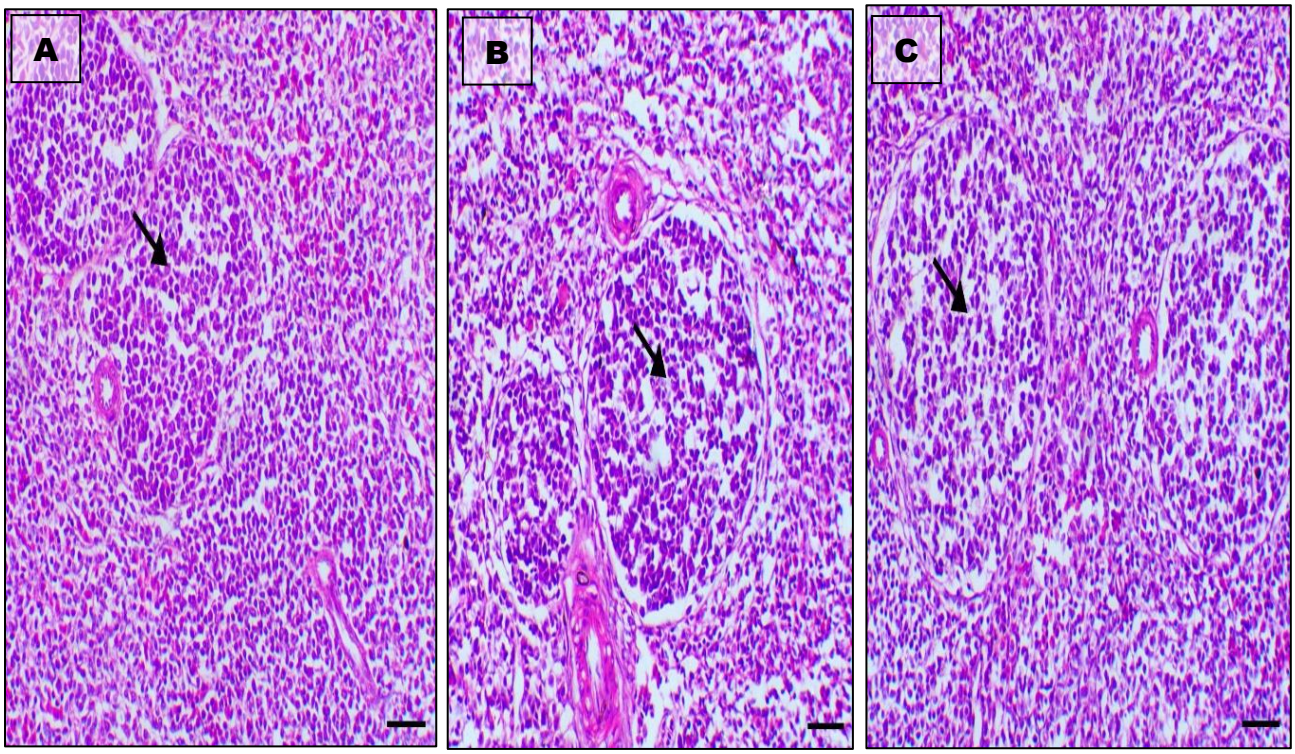

Figure 7. Spleen histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. Spleen histopathology of broiler chickens showing normal lymphoid follicles in chickens fed on a diet containing inorganic zinc (A); while group fed on 100 mg/kg diet Nano zinc showing normal lymphoid follicles (B), moreover group fed 50 mg/kg diet Nano zinc revealing normal lymphoid follicles (arrow, C), H&E, X200, bar= 50 µm.

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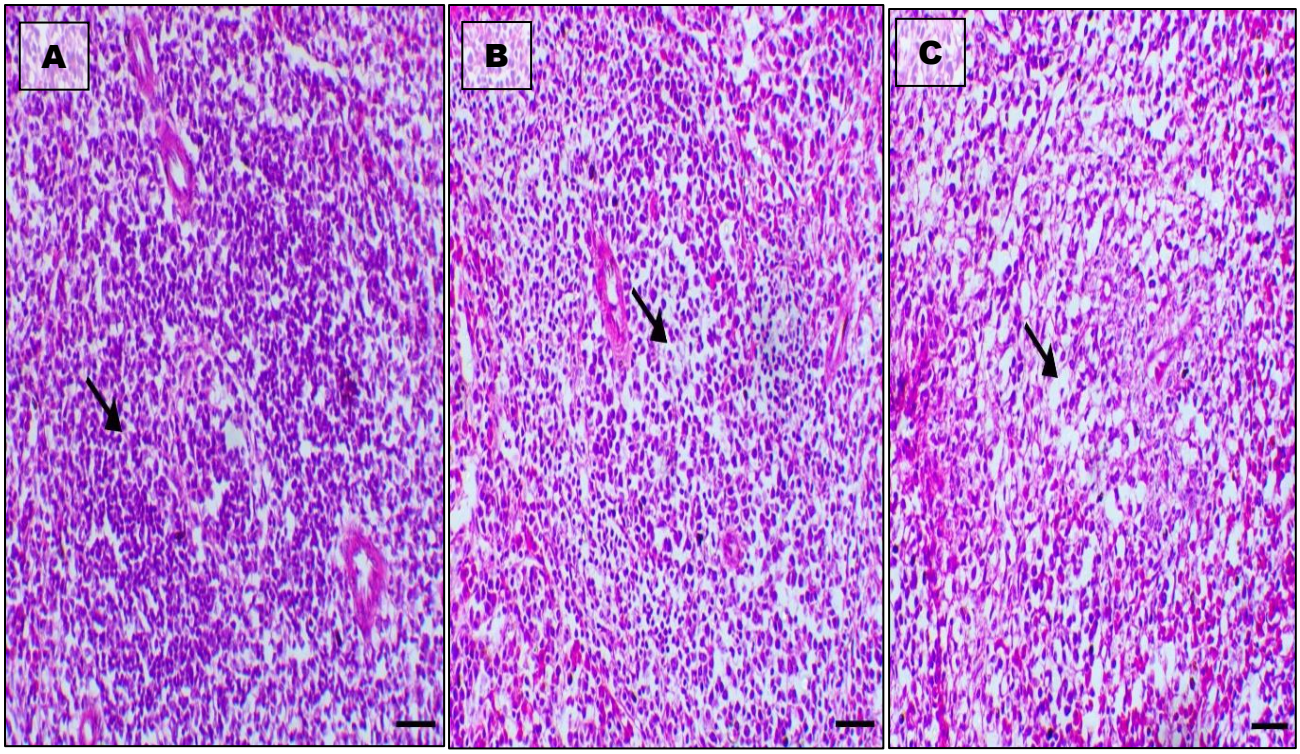

Figure 8. Spleen histopathological changes of challenged chicks with *Salmonella* Kentucky at 35 days of age. The congestion of the red pulp and marked degree of lymphoid depletion associated with lymphoid cells necrosis with the marked appearance of the reticular fibers (arrow) are shown in chickens fed on a diet containing inorganic zinc (A); while group fed on 100 mg/kg diet Nano zinc revealing a marked increase of the lymphoid content within the white pulp (B), moreover group fed 50 mg/kg diet Nano zinc showing an increase of the content of the lymphoid cells within the white pulp (arrow, C), H&E, X200, bar= 50 µm.

DISCUSSION

Salmonella is the most important and common pathogen of food-borne diseases, moreover, poultry products are considered the main source of *Salmonella* spp., which is associated with food-borne infections in humans (Setta et al., 2012). Overusing antibiotics for the treatment of bacterial outbreaks or improvement of growth performance in broiler chicken production leads to the development of bacterial resistance and subsequently reduces its efficacy (Du Pont and Steels, 1987). One way to prevent the spread of this infectious agent is to replace antibiotics with new alternative antibacterial, and consequently prevent antibiotic-bacterial resistance. The expected new antibacterial factors include metal nanoparticles (such as nano Zinc) which were used recently in broiler chickens fields not only as feed additives but also as antibacterials.

Growth performance parameters

It is well known that zinc deficiency in the broiler chickens' diet reduced their appetite and growth performance parameters (Zhao et al., 2014). *Salmonella* challenge in broiler chicken leads to general signs of illness, respiratory distress, diarrhea, and reduction of final body weight. In the present study, reduction in body weight gain and feed efficiency parameters occurred after oral SK challenge, whereas high dietary nano Zn (100 mg/kg diet) tended to alleviate the reduced body weight gain and decreased feed efficiency parameters resulting from the SK challenge. The available data which clarify the effects of nano Zinc on *Salmonella* infection of broiler chickens are very few. Hegazy and Adachi (2000) reported a significant improvement in growth performance, represented by relative body gain and feed efficiency, for the Zn-enriched diets fed to the *Salmonella*-challenged group. These data suggest that the higher nano Zn level in the broiler chicken diet might exert a protection role in controlling *Salmonella* infection. However, the actual mechanisms underlying the protective effects of Zn are still not fully understood. There is a growing interest in the application of nanotechnology to improve the feed utilization efficiency of nano trace elements in diets (Al-Beitawi et al., 2017). The application of ZnONPs can be considered a good alternative in poultry feeding to replace inorganic Zn sources. The present study indicated that the body weight gain of broiler chickens fed 100 mg ZnONPs/kg diet exhibited no statistical changes at the end of the experimental period. The improvements resulted from supplementing broilers' diets with 100mg ZnONPs/kg diet as the only source of Zn indicated that Zn nanoparticles were more available and effective to induce the positive effects on performance parameters. Moreover, this might be related to the role of Zn as an integral part of more than 300 enzyme systems involved in energy nucleic acids and protein metabolism (Tabatabaie et al., 2007).

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Some blood pictures, serum lipid profile, liver, and kidney functions

The hematological data of the present study indicated that there was no clear effect between treatments of broiler chicken, moreover, zinc sources had no effect on lipid profile, serum AST and ALT activities. The present results are supported by those obtained by Aliarabi et al. (2015), who indicated that hematological parameters decreased ($p < 0.05$) by a lower zinc supplemented diet including Hb concentration, total RBCs count, and PCV with the proportion being dose-dependent. However, SK challenge groups slightly reduced the hematological parameters, compared to the broiler chicks on the same diet without SK challenge. The data are supported by those obtained by Samia and Samia (2011) indicating that values of RBCs count, Hb concentration, and PCV significantly decreased in *Salmonella*-challenged broiler chicks. Moreover, SK challenged group exhibited higher serum AST and ALT activities which may be related to the effect of *Salmonella* endotoxin on the hepatic cells (Saif et al., 2003). The hepatotoxic effect with the SK challenge was confirmed by liver histopathological lesions (Figure 6).

Immune response

The present data revealed that broiler chickens fed on 100 or 50 mg ZnONPs/kg diet instead of 100 mg ZnO/kg diet exhibited higher lysosomal activity and phagocytic activity, compared to the broiler chicken group fed on the basal diet supplemented by inorganic zinc. The obtained data are supported by El-Katcha et al. (2017) who reported that broiler chicken fed on diets supplemented by different levels of organic or nano Zinc had improved phagocytic activity and index, compared to the broiler chicks fed on the basal diet with inorganic zinc supplementation. Moreover, Azza et al. (2020) found that ZnONPs addition instead of ZnO in broiler chicks' diet improved phagocytic activity and phagocytic index. The insignificant improvement of immune organs relative to weights of broiler chicken group fed on a diet supplemented with 100 mg of ZnONPs/kg, compared to other groups may be related to higher feed intake (Table 10). Thus, the replacement of inorganic zinc by 100 mg of ZnONPs/kg increased nutrients supply for the development of immune organs (Bartlett and Smith, 2003; Moghaddam and Jahanian, 2009; Sahoo et al., 2014). Therefore, the high bioavailability of ZnONPs might activate immune responses in broiler chickens through the improvement of thymulin activity, maturation of T lymphocytes, and the activation of B lymphocytes (Abedini et al., 2018).

On the other hand, it was observed that SK challenged group fed on inorganic Zinc supplemented diet had lower phagocytic activity, phagocytic index, and lysosomal activity, compared to the broiler chicks fed on the same diet without SK challenge. This indicates that *Salmonella* is an intracellular bacterium that survives and multiplies inside the parasitophorous vacuoles of macrophages of internal organs, such as the liver and spleen (systemic phase). This internal localization allows *Salmonella* to attack the host immune response (Beal and Smith, 2007). Macrophages, as a part of the innate immune system, are considered the main way of killing pathogenic bacteria through phagocytosis and the production of antimicrobial products (MacMicking et al., 1997). The obtained data showed SK challenge decreased macrophage, phagocytic cells, and immune response of broiler chickens. Moreover, replacement inorganic Zinc by both levels of ZnONPs with SK challenge improved immune stimulant parameters of broiler chickens, compared to the chickens fed on ZnO supplemented diet. The effect of the ZONPs on the relative weight of the immune organs was in harmony with the results of a study by Sagar et al. (2018) indicating that ZONPs supplementation of broilers diet improved relative weight of immune system organs (thymus, bursa, and spleen). This may be related to the antimicrobial properties of ZONPs that reduced the pathogenic microbes and improved gut health (Sahoo et al., 2014).

***Salmonella* shedding and cecal microbiota**

The SK shedding and lower decal pathogenic bacteria with high *lactobacillus* count of broiler chicken group fed on both levels ZnONPs supplemented diets without or with SK challenge indicated that ZnONPs can be considered as a treatment for Gram-positive and Gram-negative bacteria (Nakamura et al., 1993). The present data are supported by Arabi et al. (2012) and Mahmoud et al. (2020). The higher concentration of ZONPs (100 ppm) showed the highest antibacterial activity against the total bacterial count. Some previous studies suggested that increasing bacterial cell permeability increased bacterial death (Siddiqi et al., 2018). The present data are supported by Reda et al. (2021) as stated that ZnO in aqueous suspensions had high bactericidal activity against the Gram-negative bacterium (*Eschericha coli*) than against the Gram-positive bacterium (*Staphulococcus aureus*).

Histopathological changes

Generally, the small intestine plays an important role in nutrients absorption, and intestinal villi height with crypt depth ratio is considered as an essential indicator for intestinal health (Lei et al., 2014). The present data indicated that ZnONPs supplementation in broiler chicken diet instead of inorganic zinc increased villi length which reflects higher nutrient absorption (Awad et al., 2008; Ali et al., 2017). The improvement of the ileum wall may be related to higher ZnONPs availability which is responsible for intestinal epithelial barrier integrity health and functions (Ali et al., 2017; Shah et al., 2019). Deeper ileal villi crypts indicate a higher metabolism and allow rapid villi renewal and regeneration (Hamedi et al., 2011). On the other hand, reduction of villi height and crypt depth of the broiler intestine may be

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responsible for lowering nutrients absorption (Saeid et al., 2013). Moreover, SK challenge reduced ileal villi height and crypt depth and produce necrotic enteritis with infiltration of inflammatory cells, compared to broiler chickens fed on the same diet without SK challenge. Replacement of ZnO by different levels of ZnONPs with SK challenge improved ileal morphology and decreased enteritis suggesting that ZnONPs play an essential role in improving health and function of the gastrointestinal tract under different conditions, such as disease, stress, and pathogen challenge (Zhang et al., 2012).

Hepatic histopathology of SK challenged broiler chickens fed on a diet supplemented by inorganic zinc included marked hepatitis associated with a severe degree of heterophilic cells infiltration around the portal area and degenerative changes. Moreover, spleen morphology of the same challenged group included congestion of the red pulp and marked degree of lymphoid depletion associated with lymphoid cells necrosis with the marked appearance of the reticular fibers are supported by Freitas Neto et al. (2007) and Nazir et al. (2012). On the other hand, it was observed that inorganic zinc replacement by both levels of ZnONPs led to a marked decrease in hepatitis features represented by a significant decrease in the hepatic degenerative changes and perivascular infiltration of inflammatory cells and increased the lymphoid content within the white pulp indicating that nano zinc had hepato-protective role (Zhang et al., 2012).

CONCLUSION

In conclusion, the obtained data of the current study indicated that ZnONPs of the broiler chicken diet is a considerable zinc source with positive effects on growth performance, feed efficiency, and health status. Moreover, dietary ZnONPs supplementation appears to alleviate the adverse effect of *Salmonella* Kentucky on broiler chicken growth, immune response, intestinal and hepatic health. These results provide new information on the critical role played by dietary Nano zinc to control Salmonella infection of broiler chicken and require further studies to prove these results and provide information about its mode of action.

DECLARATIONS

Authors contribution

Abeer Mohamed El-Shenawy designed diet formulation, measured growth performance parameters, measured biochemistry parameters, and performed statistical analysis. Atef Abdelmageed Salim followed up on clinical signs and conducted a microbiological examination. Mofeed Youssef Gouda examined pathological changes. Moreover, all authors shared the interpretations of the results and confirmed the final draft of the manuscript.

Competing interests

The authors declared that there are no competing interests related to this work, which can negatively impact its publication

Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, double publication and/or submission, and redundancy) have been checked by the authors.

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