Effect of zinc and phytase supplementation on performance, immune response, digestibility and intestinal features in broilers fed a wheat-soybean meal diet

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ABSTRACT
The aim of this study was to evaluate the effects of dietary zinc (Zn) supplementation on performance, immune responses and gastrointestinal tract features of broilers fed wheat-soybean meal diet. In addition, optimised dietary Zn level was estimated based on dose-response data. A total of 500 one-day-old male Ross-308 broiler chicks were randomly assigned to 10 dietary treatments with five replicates and ten birds per replicate in a 5\times2 factorial arrangement of five graded (+40 mg/kg) dietary Zn levels with or without phytase (500 FTU/kg of diet) supplementation. By increasing dietary Zn concentration, final live body weight (LBW), daily weight gain (WG), feed intake (FI), and European Production Efficiency Factor (EPEF), antibody titres against the sheep red blood cell inoculation, cutaneous basophils hypersensitivity to PHA-P injection, jejunum villus height, and apparent dry matter digestibility (ADMD) increased with a significant linear trend. Phytase supplementation improved WG and ADMD and decreased pancreas and intestine relative weights. The interaction effects of dietary Zn concentration and phytase supplementation on the whole of evaluated traits were not significant. Optimum dietary Zn levels were estimated by the regression models at 70, 82, 98, and 110 mg/kg of diet for optimised EPEF and immunity, LBW and WG, appetites, and ADMD, respectively. It is concluded, in the broiler chickens fed wheat-soybean meal diet, Zn and phytase supplementation can improve growth performance, immune response, digestibility, and intestinal features. The Zn requirement is higher than the NRC (1994) recommendation. The efficacy of dietary Zn was not affected by dietary phytase supplementation.

HIGHLIGHTS
In the broilers fed wheat-soy diet:
- Dietary Zn and phytase supplementation improve growth performance.
- The Zn requirement is higher than the NRC recommendation.

Introduction

Zinc (Zn) is an essential trace element in the living system and has a significant role in various biological processes (Zakaria et al. 2017). It is an essential component of many metalloenzymes which significantly affects their structural and functional properties. More than 300 enzyme activity depends on Zn (Akbari Moghaddam Kakhki et al. 2017). Because of its outstanding contribution to enzymatic functions, Zn plays an essential role in complex biochemical functions such as energy metabolism, proteins turnover, nucleic acid synthesis, and cellular proliferation (Dibaiee-Nia et al. 2017). Therefore, it is necessary for growth (Huang et al. 2007; Shao et al. 2014; Dibaiee-Nia et al. 2017), immunity (Prasad 2008; Sunder et al. 2008; Roy et al. 2014), hormone secretion (Abedini et al. 2018), antioxidant protection system (Vakili and Rashidi 2011), and many other biochemical processes. Various investigators have added Zn in inorganic (Edwards and Baker 2000; Hudson et al. 2005; Liu et al. 2011; Mwangi et al. 2017) or organic (Hudson et al. 2005; Ao et al. 2006, 2009) form and reported positive effects on broilers growth performance.

However, the national research council had recommended a dietary Zn concentration of 40 mg/kg for broiler chickens (NRC 1994). Since 1994, the broiler
growth rate has considerably increased and, therefore, the Zn requirement for broiler chickens would need to be reassessed. Moreover, the higher dietary Zn levels have been used in the broiler industry for the purpose of growth improvement (Roy et al. 2014; Akter et al. 2017; Aviagen 2019). On the contrary, some researchers did not find any distinctive effects of dietary Zn supplementation in both forms; inorganic (Wang et al. 2002; Akbari Moghaddam Kakhi et al. 2017) and organic (Kidd et al. 1992, 1993; Salim et al. 2012). Since high levels of mineral salts in the diet cause high excretion of minerals, not only is it not productive, but it also pollutes the environment (Bao et al. 2007). Burrell et al. (2004) reported that excessive dietary Zn is ultimately excreted through the droppings and can pose a significant environmental threat.

On the contrary, anti-nutrient agents such as phytate in feedstuff can reduce the bioavailability of the nutrients (Zaghari et al. 2015; Morgan et al. 2017). Phytic acid has a high chelating capacity for two valent elements (Ca, P, Zn, Fe and Cu) and reduces their bioavailability (Ao et al. 2007; Akter et al. 2017). Zinc is more susceptible to trapping than other minerals, which creates an insoluble solid Zn-phytate compound and impairs Zn availability (Akter et al. 2017). Edwards and Baker (2000) reported Zn-antagonizing components, primarily phytate, reduce the utilisation of inorganic Zn added to diets. Therefore, the strategy emphasised improving the absorption of Zn without exceeding Zn intake by using microbial phytase enzyme that hydrolysates phytic acid to inositol and improves the availability of Zn (Roy et al. 2014). Certain feed ingredients, particularly wheat, possess intrinsic phytase activity. However, the importance of plant phytase in standard diets is questionable because it is less effective than microbial phytases at gastrointestinal pH and may be inactivated by acidic pH levels in the gut (Bedford 2000). Plant phytases are effective only at pH 5, while exogenous microbial origin phytases are effective at pH 3 to 7 (Leeson and Summers 2005). Moreover, since the activity of plant phytase can be reduced by the process of pelleting of poultry diets. Plant phytases are heat-labile and, in purified form, most are destroyed at temperatures above 70°C within minutes (Konietzny and Greiner 2002).

Given this background, this study was undertaken to evaluate the effects of different levels of Zn on growth performance, carcase yields, humoral and cellular immune responses, and gastrointestinal tract (GIT) features of broilers fed a wheat-soybean meal diet with or without phytase supplementation. In addition, it was hypothesised that it is possible to find an optimal inclusion level of zinc for broiler chickens fed a wheat-soybean meal diet to reach optimum growth performance.

**Materials and methods**

The experiment was conducted with the approval of the Animal Care Committee of the Ferdowsi University of Mashhad, Mashhad, Iran (Approval no: 271/329/2013).

**Animals, housing, design and diets**

A total of 500 one-day-old male Ross-308 broiler chicks from a local hatchery were purchased. The chicks were randomly distributed in 50 pens (10 birds each; 0.1 m²/b), each was equipped with one pan feeder and nipple waterer. The pen floor was covered with wood shavings. During the experimental period the lighting program and relative humidity were 21 L: 3 D and 50–60%, respectively. The temperature was set at 33 ± 1°C for the first three days of the experiment, then reduced by 0.5 °C/d to reach 20–22°C and remained constant after that. Animals and housing facilities were inspected three times daily. A completely randomised design experiment was run as a 5 × 2 factorial arrangement comprised of five graded (+40 mg/kg) dietary Zn concentrations with/without phytase supplemental. Before the trial, samples of the main ingredients (soybean meal: DM, 893; CP, 456; EE, 21; and ash, 50 g/kg; and wheat: DM, 921; CP, 132; EE, 26; and ash, 24 g/kg) were analysed for proximate composition. These values were used in a least-cost equation to formulate a basal diet. Starter (d1–10), grower (d10–25), and finisher (d25–35) diets were formulated to meet or exceed Ross-308 nutrients recommendations (Aviagen 2019) except zinc. Starter, grower, and finisher diets contained 30.1, 29.2, and 28.9 mg/kg of Zn from raw materials, respectively (measured by Atomic Absorption Spectrometry analysis). Ingredient and diet compositions are presented in Table 1. All experimental diets were provided in mash form, a way that batch of basal diets for each period was made and then divided into five equal portions. The Zn supplement (reagent-grade ZnSO₄·7H₂O, 22.60% Zn, Sigma-Aldrich Chemical Co., St. Louis, MO, USA) at the rate of 0, 177, 354, 531, and 708 mg/kg were added on top of each portion and mixed to provide five diets contained 30 (basal diet), 70, 110, 150,
and 190 mg Zn/kg of diet (Table 2). Then each one of those, divided into two equal portions, phytase enzyme (1000 U/g phytase activity, Phileo Lesaffre Animal Care, Co. Marcq-en-Baroeul, France) were added at the rate of 0 or 0.5 g/kg of diet on top of each one of them and mixed to provide ten experimental diets with or without phytase (500 FTU/kg of diet).

### Table 1. Ingredients and nutrients composition of basal diets.a

| Items | Starter (d1–10) | Grower (d11–24) | Finisher (d25–35) |
|-------|----------------|-----------------|-------------------|
| Ingredient, g/kg as fed basis | | | |
| Wheat (CP = 13.15%, ME = 3100 kcal/kg) | 579.2 | 613.3 | 670.8 |
| Soybean meal (CP = 45.55%, ME = 2200 kcal/kg) | 320.4 | 280.2 | 217.9 |
| Soybean oil (ME = 8820 kcal/kg) | 52.3 | 62.8 | 70.3 |
| Limestone (Ca = 38%) | 9.9 | 9.20 | 8.5 |
| Dicalcium phosphate (P = 18.5%, Ca = 22%) | 20.0 | 17.8 | 16.0 |
| Common salt | 1.6 | 1.6 | 1.4 |
| Sodium bicarbonate | 1.5 | 1.5 | 1.5 |
| Vitamin premixb | 2.5 | 2.5 | 2.5 |
| Mineral premixc | 2.5 | 2.5 | 2.5 |
| DL-methionine | 3.9 | 3.4 | 3.1 |
| L-Threonine | 1.9 | 1.6 | 1.5 |
| L-Lysine-HCL | 4.3 | 3.6 | 4.0 |
| Determined nutrient compositiond, as-fed basis | | | |
| Metabolisable energy, kcal/kg | 3000 | 3100 | 3200 |
| Crude protein, g/kg | 230.0 | 215.0 | 195.0 |
| Calcium, g/kg | 9.3 | 8.4 | 7.6 |
| Available phosphorus, g/kg | 5.0 | 4.6 | 4.2 |
| Sodium, g/kg | 1.6 | 1.6 | 1.6 |
| Arginine, g/kg | 14.2 | 13.1 | 11.4 |
| Lysine, g/kg | 14.4 | 12.9 | 11.6 |
| Methionine, g/kg | 6.9 | 6.2 | 5.7 |
| Methionine + Cystine, g/kg | 10.6 | 9.8 | 9.0 |
| Threonine, g/kg | 9.7 | 8.9 | 7.9 |
| Valine, g/kg | 10.1 | 9.4 | 8.4 |
| Zinc, mg/kg | 30.0 | 29.2 | 28.9 |

**ME**: metabolisable energy; **CP**: crude protein; **Ca**: calcium; **P**: phosphorus

*aThe diets were provided in a way that a batch of basal diet (without Zn supplementation) for each period was made and then divided into five equal portions, the Zinc sulphate supplement “as a reagent-grade ZnSO_4·7H_2O, 22.60% Zn, Sigma-Aldrich Chemical Co., St. Louis, MO”, at the rate of 0, 177, 354, 531 and 708 mg/kg were added on top of each portion and mixed to provide five diets contained 30 (non-supplemented basal diet), 70, 110, 150, and 190 mg Zn/kg and then each one of those divided into two equal portion.

**b**Phytase enzyme “1000 U/g phytase activity, Phileo Lesaffre Animal Care, Co. Marcq-en-Baroeul, France”, were added at the rate of 0 and 500 mg/kg on top of each portion and mixed to provide 10 experimental diets.

*bVitamin premix supplied the followings per kilogram of diet: vitamin A (all-trans-retinol), 12000 IU; vitamin D3 (cholecalciferol), 5000 IU; vitamin E (α-tocopherol), 18 IU; vitamin K3 (menadione), 2.65 mg; vitamin B1 (thiamin), 2.97 mg; vitamin B2 (riboflavin), 8.0 mg; vitamin B3 (niacin), 57.42 mg; vitamin B5 (pantothenic acid), 17.86 mg; vitamin B6 (pyridoxine), 4.45 mg; vitamin B9 (folic acid), 1.9 mg; vitamin B12 (cyanocobalamin), 0.02 mg; vitamin B1 (biotin), 0.18 mg; choline chloride, 487.5 mg, and antioxidant 1.0 mg.

**d**The determined ingredient analysis was used to calculate nutrient composition (crude protein, calcium, sodium and zinc were measured by the AOAC (2002) methods; metabolisable energy, digestible amino acids and available phosphorus were measured by NIR analysis.

### Table 2. Zinc sulphate supplement level and diets analysed Zn concentration.

| Zinc sulphate supplement levela, mg/kg | Starter | Grower | Finisher |
|--------------------------------------|--------|--------|---------|
| 0 | 30.05 | 29.16 | 28.91 |
| 177 | 70.05 | 69.16 | 67.91 |
| 354 | 110.05 | 109.16 | 107.91 |
| 531 | 150.05 | 149.16 | 147.91 |
| 708 | 190.05 | 189.16 | 187.91 |

*aZinc sulphate supplement was as reagent-grade, ZnSO_4·7H_2O, 22.6% Zn, Sigma-Aldrich Chemical Co., St. Louis, MO.

#### Growth performance

Live body weight (LBW) was determined through weighing all the birds in a pen on d 1 and 35. Feed intake was calculated by weighing the residual feed in feeders for the respective period. The growth performance as mean live body weight (LBW), daily weight
gain (WG), and daily feed intake (FI) was calculated. Died chicks were weighed, recorded, and used to correct the growth performance. The feed conversion ratio (FCR), adjusted for mortality and calculated as; total feed intake divided by total gain, including the weight of lost birds. The European Production Efficiency Factor (EPEF) was calculated as follows (Zarghi et al. 2020):

\[
\text{EPEF} = \frac{\text{Viability d0–35} \times \text{LBW d35} \times \text{FCR d0–35}}{\text{age (d35)} \times \text{FCR d0–35}} \times 100
\]

Nutrient digestibility

Chromic oxide (Cr₂O₃) was used at the rate of 3 g/kg of diet as an inert marker during 24-30 days of age. Excreta samples were collected three times daily for 72 h from a plastic sheet that was placed on the litter of each pen after four days adaptation period (27-30d). Collected excreta samples for each pen were pooled, thoroughly mixed, and about 100 g of the uniform mix was immediately stored at -20°C. A 100 g excreta sample from each pen was later dried in a forced-air oven at 60°C for 72 h. The feed and dried excreta samples were ground to pass through a 40-mesh screen and mixed thoroughly before analysis. Apparent digestibility coefficients of dry matter (DM), organic dry matter (ODM), and crude protein (CP) was calculated according to the formula using the marker ratio in the diet and excreta (Tiemouri et al. 2019):

\[
\text{Apparent nutrient digestibility (%) =} \frac{(N/M) \cdot d - (N/M) \cdot e}{(N/M) \cdot d} \times 100,
\]

where (N/M)₀ is the ratio of nutrient to marker in diet and (N/M)ₑ is the ratio of nutrient to marker in excreta.

Chemical analysis

The Cr₂O₃ in feed and excreta samples was determined by a developed spectrophotometric procedure (Fenton and Fenton 1979). Samples of feed or faeces were weighed into 50-ml pyrex beakers and ashed overnight in a muffle furnace at 450°C. After cooling, 15.0 ml of a digestion mixture (10 g of sodium molybdate dihydrate dissolved in 500 ml of a 150:150:200 mixture of distilled water-concentrated H₂SO₄-70% HClO₄) was added to each sample and heated on a hot plate (surface temperature up to 300°C) until a yellowish or reddish colour developed. The samples were heated for a further 10–15 min, removed from the hot plate and allowed to cool. The digests were then quantitatively transferred to 200-ml volumetric flasks with distilled water and made to volume. Approximately 10 ml of the diluted digest was poured into a tube (disposable polystyrene culture tube (17 × 100 mm) with polyethylene cap) and centrifuged for 5 min at 700 g. The optical density was measured in an 1-cm cuvette vs. distilled water at 440 nm on a UV spectrophotometer (UNICO- 2100-UV Spectrophotometer, Princeton, New Jersey, USA). The Zn in feed sample was determined in an Air-Acetylene flame on an atomic absorption spectrophotometer (Perkin Elmer A Analyst 100, Massachusetts, Wellesley, USA) after digestion with a tri-acid mixture; HNO₃: H₂SO₄: HClO₄ in the ratio of 15:2:4 (Jahanian and Rasouli 2015). The oven drying and Kjeldahl methods were performed according to methods 926.12 and 981.10 of the AOAC International, respectively, for determining DM and CP contents in feed and excreta samples (Latimer 2016).

Humoral immune response

Sheep red blood cells (SRBC), as a non-pathogenic antigen, were used to evaluate the humoral immune response in broiler chickens. Ten birds from each treatment (two bird/pen close to the average pen weight) were marked with dye and injected with 0.1 ml of 5% SRBC suspension into the brachial vein on 21 days of age. Seven days after the first challenge, the birds were given a booster injection of the 5% SRBC for secondary antibody production. This procedure was followed because the antibody produced during a secondary response is higher than during the primary response (Allahdo et al. 2018). The blood samples were taken on the seventh-day post each inoculation. The blood samples were left at room temperature for two hours for clot creating, then wrung with a wooden applicator stick, and placed in a 4°C refrigerator overnight for maximum sera yield, then were centrifuged at 1900 x g for 10 min at 4°C to extract serum (Bartlett and Smith 2003).

Serum samples were tested for total antibody (IgT) response, then specifically for IgM and IgY using the 2-mercaptoethanol sensitive (ME- sensitive) technique (Qureshi and Havenstein 1994; Lepage et al. 1996). Briefly, serum pipetted into microcentrifuge tubes and inactivated by heat in a 56°C water bath for 30 min. Then, 50 μL of PBS was placed in the first row of wells in a 96-well V-bottom micro-titration plate. To the same wells, 50 μL of serum was added, and plates
were sealed and incubated at 37°C for 30 min. Plates were removed from the incubator, and 50 μL PBS was added to the 11 remaining wells in each row. A two-fold serial dilution of the samples was made on successive rows, 50 μL of a 2.5% SRBC suspension added to each well, and plates were again sealed and incubated for 30 min. The ME-sensitive (IgM) and ME-resistant (IgY) antibody titres were assessed using the same procedure as total titres, except that 50 μL of 2-ME was added to the first row of wells. Titres were read by holding plates over a lighted mirror to observe agglutination wells. All antibody titres were reported as log2 of the reciprocal of the last dilution in which agglutination was observed (Bartlett and Smith 2003; Allahdoo et al. 2018).

**Cellular immune response**

The cell-mediated immune (CMI) response was assessed by measuring the response of cutaneous basophils hypersensitivity (CBH) to phytohemagglutinin-P (PHA-P) by intra-dermally injection at 35d of age. The thickness of the web between the third and fourth inter-digital space on the left and right feet was measured with a micrometre. Ten broilers from each treatment (two bird/pen close to the average pen weight) were injected with 100 μg of PHA-P suspended in 0.1 ml of phosphate-buffered saline (PBS) into the web of the right foot, while in each bird, the left web (Control) was injected with 0.1 ml of PBS. The web swelling of both feet was measured 24 hours after injection. The response was determined by subtracting the skin thickness of the first measurement from the second and the values of the left foot (control) from the right foot (Allahdoo et al. 2018).

**Carcase and visceral organs relative weight**

Ten birds from each treatment (two bird/pen close to the average pen weight) were selected, and after 4 hours of feed withdrawal but with free access to drinking water, weighed, slaughtered, plucked, and gastrointestinal tract and other inner organs excised to determine the carcase and visceral organs relative weight on 35d of age. Carcase is obtained by removing the head, feathers, feet, and visceral organs. After chilling for 24 hours at 4°C, carcases were weighed and were cut according to a standardised procedure to determine the carcase yield (Tabatabaie et al. 2017). The carcase, breast, legs, abdominal fat, and visceral organ weights were determined by a weighing scale (0.001 g, model GF 400, A&D Weighing, San Jose, CA, USA) to calculate relative organ weight (g/100 live body weight).

**Jejunum morphology**

The middle part of the jejunum was excised for morphological study. Tissue samples (0.5 cm2) were taken from the jejunum midpoint and then immersed in a 10% buffered formalin solution for 72 hours. The samples were treated in a tissue processor apparatus and embedded in paraffin wax. Transverse sections were cut (6 μm) using a rotary microtome (Leica RM 2145), placed on a glass slide, and stained with Haematoxylin and Eosin (H&E). The histological slides were evaluated under a light microscope to determine morphological indices (all chemicals purchased from Sigma chemical company). Micrographs taken with an Olympus BX41 microscope (Olympus Corporation, Tokyo, Japan) fitted with a digital video camera. The images were analysed using image software (Image-Pro Plus v 4.5) on nine villi chosen from each slide, and only vertically oriented villus was selected for measuring (Teymouri et al. 2018). The measured morphological traits were as follows: (1) villus height, (2) villus width, (3) crypt depth, (4) intestinal muscular thickness, and (5) the apparent villus surface area (AVSA) is calculated as follows:

\[
\text{Apparent villus surface area} = \frac{1}{2} \times \text{villus width} \times \text{Villus height} \times 2 \times 3.14
\]

**Statistical analysis**

All data were analysed for normality using SAS software through the Univariate plot normal procedure. Data were analysed by the GLM procedure of SAS (SAS 9.1.3 2009). Differences among means were separated using the LS-MEANS option of SAS adjusted for Tukey’s test at p<.05. The dietary Zn level for maximum response in performance variables, that’s R² was significant, were predicted using the nonlinear modelling option in SAS with the dietary Zn concentration as an independent variable. The iterative procedure makes repeated estimates for coefficients and minimises residual error until the best-fit lines are achieved (Robbins et al. 2006; Pesti et al. 2009). The two lines are fitted to the values using the following equations:

- **For linear broken line**: \[ Y = L + U \times (R - X) \times I \]
- **For Quadratic broken line**: \[ Y = L + U \times (R - X)^2 \times I \]

where \( Y \) = dependent variable, \( L \) = theoretical
maximum, \( R = \text{requirement}, X = \text{independent variable}, \ i = 1 \) \( (X < R) \) or \( i = 0 \) \( (X > R) \), and \( U = \text{rate constant} \).

To assist in choosing an appropriate model, coefficient of determination \( (R^2) \), adjusted coefficient of determination \( (\text{adj. } R^2) \), root means square error \( (\text{RMSE}) \), and Akaike’s information criterion \( (\text{AIC}) \) values were calculated using the following formulas:

\[
R^2 = \frac{\text{corrected total sum of squares} - \text{sums of squares for error}}{\text{corrected total sum of squares}}
\]

\[
\text{Adjusted } R^2 = 1 - \left[ \frac{\text{sums of squares for error}}{(N - 1)} \right] \div \left[ \frac{\text{corrected total sum of squares}}{(N - 1)} \right]
\]

\[
\text{RMSE} = \sqrt{\frac{\sum_{t=1}^{n} (yt - \hat{yt})^2}{N}}
\]

\[
\text{AIC} = N \times \ln\left(\frac{\text{sums of squares for error}}{N}\right) + 2P
\]

where \( yt = \text{observed and predicted values}, \hat{yt} = \text{predicted values}, N = \text{number of observations}, P = k + 1, \) and \( k \) is the number of parameters.

The exploration of the models according to the fit (quality of prediction) was done using the adj. \( R^2 \), RMSE, and AIC criterion, a smaller numerical value of RMSE, AIC, and higher value of adj. \( R^2 \) indicates a better fit when comparing models.

### Results

The interaction effects of Zn levels and enzyme supplementation on the evaluated traits were insignificant.

### Performance

This study demonstrated that Zn and phytase supplementation had a significant effect on growth performance traits (Table 3). Live body weight, FL, and WG improved as dietary Zn level increased (linear effect; \( p < 0.001 \)). Birds fed diet contain over 70 mg/kg Zn, performed higher LBW, FL, and WG than those fed non-Zn supplemented diet (Zn level = 30 mg/kg). The feed conversion ratio was not affected by dietary Zn level (\( p > 0.05 \)). European Production Efficiency Factor (EPEF) was improved in a linear manner (\( p < 0.005 \)) as an effect of increased levels of Zn supplementation. The results showed that broilers fed the diet containing 70 mg/kg Zn performed the highest EPEF. Dietary phytase supplementation significantly increased LBW d35, WG d1-35 (\( p < 0.001 \)), and EPEF (\( p < 0.008 \)).

### Nutrient digestibility

The effects of dietary Zn and phytase supplementation on apparent nutrient digestibility are shown in Table 4. There was a significant linear response in nutrient digestibility to increasing dietary Zn levels. By
increasing dietary Zn concentration, linearly increased apparent dry matter (ADMD) and crude protein (CPD) digestibility (p<0.05). Supplemental phytase significantly (p<0.01) increased CP apparent digestibility.

**Table 4.** Effect of dietary Zn and phytase supplementation on apparent nutrient digestibility in male broilers fed wheat-soybean meal diets.

| Items             | Dry matter | Ash | Organic dry matter | Crude protein |
|-------------------|------------|-----|--------------------|---------------|
| Phytaseb, mg/kg   |            |     |                    |               |
| 0                 | 66.83      | 34.13 | 69.18              | 62.22ab       |
| 500               | 67.21      | 36.40 | 69.42              | 64.37a        |
| SEM               | 0.34       | 0.89 | 0.38               | 0.49          |
| Zn levels, mg/kg  |            |     |                    |               |
| 30                | 66.03ab    | 34.51 | 67.85              | 60.26b        |
| 70                | 66.36ab    | 36.03 | 68.53              | 62.00ab       |
| 110               | 67.63ab    | 34.46 | 69.59b             | 62.81ab       |
| 150               | 67.66b     | 36.21 | 69.92a             | 63.96b        |
| 190               | 67.31ab    | 35.12 | 69.63a             | 64.44a        |
| SEM               | 0.34       | 1.41 | 0.60               | 0.77          |

Source of variation, p-value
- Phytase: 0.07 0.07 0.14 0.01
- Zn: 0.03 0.84 0.03 0.04
- Zn × Phytase: 0.80 0.96 0.81 0.59

Zn dose response, p-value
- Linear: 0.97 0.74 0.02 0.04
- Quadratic: 0.05 0.88 0.27 0.12

*The values are means of 25, 10, and 5 replicates for Phytase, Zn, and interaction effects, respectively.

**Immune response**

The results for immunity responses are shown in Table 5. A significantly linear response to increasing Zn concentration was observed for total antibody titre in response to sheep red blood cell inoculation at day seven post-inoculation. However, this effect was not significant when measured at the second injection. Increasing dietary Zn level linearly enhanced primary antibody titres’ response to SRBC inoculation and decreased the CBH reaction to PHA-P injection (p<0.05). In the birds fed a diet containing over 110 mg/kg Zn, the total antibody titres against SRBC inoculation after 7d were significantly higher, and the thickness of the web between the third and fourth interdigital space at 24 hours post-injection were significantly lower than those fed non-Zn supplemented diet (Zn level = 30 mg/kg). The enzyme supplemental did not affect the humoral and cellular immune response (p > 0.05). The relative weight of lymphoid organs was not affected by dietary Zn levels and phytase supplementation (p > 0.05).

**Carcase yield and visceral organs relative weight**

The results for meat yield and visceral organs relative weight are shown in Table 6. The effects of dietary Zn supplementation on carcase yield and visceral organs relative weight (g/100g of live body weight) were not significant (p > 0.05). By dietary phytase

**Table 5.** Effect of dietary Zn and phytase supplementation on immune responses (humoral immune response to sheep red blood cell inoculation, cell-mediated response to PHA-P injection, and lymphoid organs weight) in male broilers fed wheat-soybean meal diets.

| Items                      | Antibody titres response to SRBC inoculation (log2)b | PHA-P response | Lymphoid organs weight (g/100g of LBW) |
|----------------------------|------------------------------------------------------|----------------|---------------------------------------|
|                            | d7 After injection                                  | d14 After injection | Hours after injection | Bursa | Spleen |
| Phytasec, mg/kg            | Ig T  Ig Y  Ig M                                    | Ig T  Ig Y  Ig M | 8h  16h  24h |           |
| 0                          | 7.10  5.20  1.90                                   | 8.83  6.93  1.90 | 0.45  0.48  0.42 | 0.11  0.09 |
| 500                        | 7.50  5.30  2.20                                   | 8.70  6.77  1.93 | 0.51  0.55  0.41 | 0.09  0.08 |
| SEM                        | 0.15  0.16  0.18                                   | 0.15  0.18  0.16 | 0.05  0.06  0.06 | 0.01  0.01 |
| Zn levels, mg/kg           | 6.55b  5.25  1.50                                 | 8.33  6.67  1.67 | 0.55  0.70  0.60b | 0.10  0.09 |
| 70                         | 6.67ab  5.17  2.50                                | 8.92  7.00  1.92 | 0.42  0.39  0.42b | 0.10  0.08 |
| 110                        | 7.08b  5.17  1.92                                | 8.67  6.92  1.75 | 0.52  0.62  0.30b | 0.13  0.11 |
| 150                        | 7.50a  5.25  2.25                                | 9.17  7.00  2.17 | 0.33  0.33  0.36b | 0.09  0.08 |
| 190                        | 7.50b  5.42  2.08                               | 8.75  6.67  2.08 | 0.61  0.55  0.39b | 0.12  0.09 |
| SEM                        | 0.25  0.24  0.28                                 | 0.23  0.28  0.25 | 0.09  0.09  0.10 | 0.01  0.01 |
| Source of variation, p-Value| Phytase 0.08  0.65  0.24  0.52  0.51  0.88 | 0.54  0.38  0.94 | 0.11  0.62 |
| Zn 0.04  0.95  0.15  0.14  0.83  0.59 | 0.16  0.06  0.03 | 0.45  0.11 | 0.74  0.63 |
| Zn × Phytase 0.09  0.14  0.91  0.56  0.78  0.73 | 0.53  0.46  0.52 | 0.84  0.81 |

*The values are means of 25, 10, and 5 replicates for Phytase, Zn, and interaction effects, respectively.

bData represent means of log2 of the reciprocal of the last dilution exhibiting agglutination. Ig T; total, IgM (2-mercaptoethanol sensitive), and IgY (2-mercaptoethanol-resistant) antibody titres.

bData contain 1000 U/g phytase activity.

bData contain 1000 U/g phytase activity.

bMean values within the same variable for each effect with unlike superscript letters were significantly different (p<0.05).
supplementation, carcase yield significantly increased, and the small intestine and pancreas relative weights significantly decreased ($p < .01$).

### Gastrointestinal tract features

The result for the small intestine containing viscosity and jejunum histology are shown in Table 7. The villus height (VH) and villus height to crypt depth ratio (VH/CD) were linearly enhanced by increasing dietary Zn levels ($p < .01$). Dietary phytase supplementation effects were insignificant on intestinal morphological observations ($p > .05$). Dietary phytase supplementation significantly decreased ileal viscosity ($p < .01$).

### Estimated Zn requirement

A critical goal of this study was to estimate the Zn requirement of broilers. The optimisation models were
Table 8. Estimated Zn requirements (mg/kg of diet) for optimisation growth performance, immune and digestibility in broiler chickens by NLIN models.

| Parameters                  | Requirement | Lower 95% | Upper 95% | p-Value | R²     | Adj. R² | Predicted value | RMSE | AIC | Equation
|-----------------------------|-------------|-----------|-----------|---------|--------|---------|-----------------|------|-----|---------|
| Live body weight d35        | 81.57       | 50.51     | 112.63    | 0.0001  | 0.33   | 0.32    | 1917           | 27.96 | 386 | $Y = 1916 - 0.032(81.57 - X)^2$ × I |
| Daily weight gain           | 81.49       | 55.53     | 107.45    | 0.0001  | 0.42   | 0.40    | 53.50           | 1.15  | 84  | $Y = 53.5 - 0.001(81.49 - X)^2$ × I |
| Daily feed intake           | 97.84       | 70.59     | 125.09    | 0.0006  | 0.27   | 0.26    | 92.98           | 2.40  | 114 | $Y = 93.0 - 0.055(97.84 - X)^2$ × I |
| EPEF                       | 69.99       | 45.44     | 95.43     | 0.0001  | 0.34   | 0.32    | 304             | 3.53  | 225 | $Y = 304 - 0.011(69.99 - X)^2$ × I |
| Immune responses            | 70.00       | 46.22     | 94.23     | 0.0001  | 0.36   | 0.35    | 7.60            | 0.81  | 90  | $Y = 7.6 - 0.001(70 - X)^2$ × I |
| Dry matter digestibility    | 111.50      | 79.03     | 143.97    | 0.0408  | 0.31   | 0.29    | 67.49           | 1.59  | 93  | $Y = 67.49 - 0.019(111.5 - X)^2$ × I |

R²: coefficient of determination; Adj. R²: adjusted coefficient of determination; RMSE: root means square error; AIC: Akaike’s information criterion; EPEF: European Production Efficiency Factor calculated as: [Viability d0-35 (%) × LBW d35 (kg) × 100/(Age d35) × FCR d0-35].

*Responses did not fit in the models to estimate the digestible threonine requirements for feed conversion ratio

*Expressed as mg/kg of diet.

*Predicted values by models to obtain optimum growth performance, immune and digestibility.

$^d$: 1 if X < Estimated Zn requirements or 0 if X > Estimated Zn requirements.

Discussion

It was hypothesised that the dietary Zn concentration effects might depend on the presence of phytate in wheat-based diets. Surprisingly, no interactions were found between dietary Zn levels and phytase supplementation.

Performance

This study achievements on performance traits have an agreement with other research, which reported dietary zinc supplementation improved growth performance in broilers (Collins and Moran 1999; Hegazy and Adachi 2000; Huang et al. 2007). In this study, birds given the non-Zn-supplemented diet (30 mg/kg of diet Zn concentration) showed reduced feed intake and body weight gain at 35 days of age. Zinc is an essential component of many metalloenzymes which significantly affects their structural and functional properties. However, the exact mechanism by which dietary Zn influences daily FI is still unclear but can be related to satiety regulation. It has been reported that Zn-deficient diets could increase the gene expression of mRNA for cholecystokinin (CCK) production in the intestine, which negatively affects the appetite of animals (Blanchard and Cousins 2000). Also, increasing daily FI in birds fed Zn supplemented diet may be linked with higher production of digestive enzymes.
The poor weight gain can be attributed to increasing feed intake, associated with a Zinc deficiency. Moreover, the weight gain of birds depends on the effective utilisation of consumed feed and its subsequent use in cell proliferation, mainly in the muscle. Because of its outstanding contribution to enzymatic functions, Zn plays a role in complex biochemical functions such as energy metabolism, protein turnover, and nucleic acid synthesis (Dibaiee-Nia et al. 2017). Therefore, Zn deficiency may cause retarded growth and poor weight gain due to its role in DNA/RNA synthesis and carbohydrate, fat, or protein metabolism (Akter et al. 2017). Previous studies (Bao et al. 1994; Bartlett and Smith 2003; Burrell et al. 2004; Hudson et al. 2005; Sunder et al. 2008; Liao et al. 2013) have reported the same trend in birds fed a low or deficient Zn diet. Moreover, Zn has an essential role in the metabolism of energy and protein (Bao Y et al. 2009). As a result, the improvement in weight gain may be due to the role of Zn in many biochemical processes and physiological functions. Disagreement with the present results in this experiment and above reports, some studies found that dietary Zn supplementation did not affect the growth performance of broiler chickens (Kidd et al. 1994; Bartlett and Smith 2003; Burrell et al. 2004; Hudson et al. 2005; Sunder et al. 2008; Liao et al. 2013). The lack of a significant effect of diet Zn levels on FCR is following the reports of other researchers (Bartlett and Smith 2003; Huang et al. 2007; Liao et al. 2013). Increased dietary Zn level through organic (Rossi et al. 2007), inorganic and their mixtures (Burrell et al. 2004; Attia et al. 2019) did not show any significant effect on the FCR of broilers. In contrast, the beneficial effect of supplemental Zn on FCR has been demonstrated in another study (Hess et al. 2001). They showed increased dietary Zn up to 95 mg/kg improved FCR in broilers. The discrepancy may be due to the use of different amounts and sources of Zn in these experiments.
Phytase supplementation did not affect FI but improved the daily WG of birds, irrespective of Zn level. It as been reported that dietary phytase supplementation improves growth performance when the first critical nutrient in a diet is P (Cowieson and Adeola, 2005). As feed intake of birds was unaffected by phytase supplementation, the improved daily WG of the same group of birds could be interpreted as being a consequence of phytate-bound minerals and other nutrients by phytase (Akter et al. 2017). This result also suggests that Zn levels used in this study did not reduce the ability of the phytase to degrade phytate. The beneficial effect of phytase on WG has also been reported in previous studies (Bedford 2000; Thammarutwasik et al. 2009; Akter et al. 2017). The lack of response to phytase on FCR is in agreement with Roy et al. (2014).

**Nutrient digestibility**

The results of this study revealed that a significant linear increase in apparent digestibility of dry matter and crude protein by the increase in dietary Zn concentration (Table 4). Zinc serves as a cofactor for many digestive enzymes, such as Metallo-proteinase and Metallo-carboxypeptidases (Sahin et al. 2009; Attia et al. 2019). It is reported that Zn can be found in high concentrations in various glandular organs, especially digestive tract secretions organs (Hedemann et al. 2006), and the pancreatic enzyme activity was increased by dietary Zn supplementation (Szabo et al. 2004). Dry matter digestibility was not affected by the phytase at all. The diet supplemental with phytase showed a higher 3.45 crude protein digestibility than the non-supplemented diet. This increased digestibility may be explained by the fact that phytate-protein bonds were, to some extent, cleaved by phytase (Namkung and Leeson 1999). Reported a 1–2% improvement in amino acid digestibilities when 750 FTU/kg phytase was added to mash diet for turkey poults (Yi et al. 1996). In an experiment that involving cecectomized roosters, adding 1,200 FTU/kg increased true amino acid digestibility by approximately 2% (Biehl and Baker 1997).

**Immune response**

This study achievement on immune responses is consistent with the previous study that showed diets supplemented with zinc tend to improve immune status in broiler chickens (Kidd et al. 1996; Bartlett and Smith 2003; Sunder et al. 2008). According to Sunder et al. (2008), humoral immune responses were significantly higher in broilers fed a diet supplemented with 80 mg/kg or a greater quantity of Zn than those fed diets with lower Zn concentration. Zinc is an essential micronutrient that affects multiple aspects of the immune system (Prasad 2008). In this study, increasing dietary Zn concentration linearly decreased CBH response, the thickness of the web between the third and fourth interdigital space decreased linearly with the increasing levels of dietary Zn at 24 hours post-injection. Disagreement with the current result, Sajadifar et al. (2013) reported that supplementation of broiler diets with 200 mg/kg Zn led to a higher CBH response. Sunder et al. (2008) reported that the cell-mediated immune responses were significantly higher in broilers supplemented with 80 mg/kg or greater quantity of Zn than those supplemented with less than 80 mg/kg of Zn. Hudson et al. (2005) observed a higher cellular immune response to PHA and antibody titres against the Newcastle disease in broiler breeders fed diets supplemented with organic sources of Zn, as compared to inorganic sources.

Overall, the results of this study indicated that Zn supplementation improves immunity, as compared to the control; however, it was not affected lymphoid organs (Table 5). In agreement with the current results, Bartlett and Smith (2003) reported that none of the lymphoid organs (thymus, bursa, spleen, or liver) of broiler chickens was significantly affected by the diet Zn levels. Also, Sunder et al. (2008) reported that the weights of bursa and spleen were significantly higher in broilers fed a diet supplemented with 40 mg/kg compared to those fed non-supplemented diets. The immune system is dependent on the functions of cellular metabolism. Zn is ubiquitous in cellular metabolism and functions structurally and catalytically in metalloenzymes (Abedini et al. 2018). Zinc deficiency causes a decrease in cellular immunity and adversely affects thymus, spleen, and interleukin production (Sahin et al. 2009). Zinc is essential for thymulin, a thymic hormone regulating T lymphocyte maturation. Thus, birds fed diets supplemented with a more available Zn source might have more thymulin activity and, therefore, promote immune responses through the increased maturation of T-lymphocytes and activation of B-lymphocytes by T-helper cells (Abedini et al. 2018). Zinc is an essential element for all aspects of immunity and is critical for the integrity of the cells involved in the immune response (Bartlett and Smith 2003). Following the results of this study, Roy et al. (2014) reported that the effect of phytase supplementation on immunity in broilers is scanty.
However, Liu et al. (2008) reported, antibodies against the Newcastle disease virus vaccine were enhanced by dietary phytase supplementation.

**Carcase yield and visceral organs relative weight**

Consistent with the current result, Shyam Sunder et al. (2008) reported no effect on carcase weight of broiler chickens with dietary Zn supplementation. Sunder et al. (2008) showed no effect on carcase weight of broiler chickens with dietary Zn supplementation. In contrast, other researchers showed an increase in carcase yield of broiler chickens when the diet was supplemented with Zn (Ao et al. 2007; 2011). This study demonstrated that dietary Phytase supplementation improved carcase yield and decreased relative small intestine and pancreas weight (Table 6).

**Gastrointestinal tract competence**

In this study, Zn supplementation of diet led to an increase in VH and VH/CD ratio in the jejunum. In agreement with this study, Olkowski et al. (2005) reported positive effects of supplemental zinc in the wheat-soy diet on intestinal mucosa (Olkowski et al. 2005), villus width, crypt depth, the number of goblet cells, and mucin production (Piel et al. 2005). Zinc has positive effects on the broiler chicken’s intestine through increased cell proliferation and enhanced protein synthesis (Shao et al. 2014). It also improves intestinal health through enhanced crypt cell production (Duff and Ettarh 2002), enterocytes integrity, and barrier structures and functions (Lambert et al. 2004). Villus height and crypt depth of the small intestine directly represent intestinal function and health. The current result in this experiment showed that morphometric evaluation of the small intestine revealed increased VH in all supplemented groups. The increased VH and VH/CD in Zn supplemented groups can be linked to a rapid proliferation of crypt cells due to Zn availability (Tako et al. 2005).

**Estimated Zn requirement**

The estimated Zn requirements in the current experiment were higher than those (40 mg/kg of diet) recommended by the National Research Council (NRC 1994). Nevertheless, they were lower than those (added 110 mg/kg of diet Zn) recommended by Ross-308 and Cobb-500 manuals (Cobb-Vantress 2021; Aviagen 2019). Zn is required for the normal function of many structural proteins, enzymes, hormones and is necessary for growth and development. Moreover, Zn has an essential role in the metabolism of energy and protein (Bao Y et al. 2009). As a result, the improvement in WG may be due to the role of Zn in many biochemical processes and physiological functions.

In various studies, the different dietary Zn concentration was reported to improve the performance of broiler chickens; including 181 mg/kg (Bartlett and Smith 2003), 110 mg/kg (Dozier et al. 2003; Burrell et al. 2004; Sunder et al. 2008), 95 mg/kg (Wedekind et al. 1992; Hess et al. 2001), 85 mg/kg (Rossi et al. 2007), 48 mg/kg (Huang et al. 2007), and 45 mg/kg (Mohanna and Nys 1999; Salim et al. 2008). The differences in Zn requirement in various reports may be due to genetic, bird age, diets, and rearing conditions used in different experiments (Zakaria et al. 2017; Attia et al. 2019).

This study showed that the optimum dietary Zn requirement for broilers fed on wheat-soybean meal diets was higher than the NRCS recommended level. This consequence is agreed with Chen et al. (2017). Liu et al. (2015) reported that dietary supplementation of 60 mg Zn/kg in the corn-soy meal basal diet (a total dietary Zn of about 90 mg/kg) was adequate for improving the performance of broilers. They showed that the amount (29 mg/kg) of Zn from the basal diet source was not adequate to support the optimum growth performance of broilers, and it is necessary to increase dietary Zn levels. Broiler chicken’s Zn requirements have been estimated to be 37 mg/kg from organic sources (Ao et al., 2007) and 84 mg/kg from inorganic sources (Huang et al. 2007). In contrast, some studies showed that the corn-soy meal contains 30 mg/kg of Zn, and this amount is adequate to support optimum performance during the first 3wk (Burrell et al. 2004; Sunder et al. 2008). Bartlett and Smith (2003) reported that a diet containing 34, 68, and 181 mg Zn/kg of corn-based diet did not affect the growth performance of broiler chickens.

**Conclusion**

Under the conditions of this study, Zn supplementation to a wheat-based diet had some beneficial effects on growth performance, nutrient utilisation, and immune response in broiler chickens. Phytase supplementation improved live body weight and nutrient
utilisation. Using regression models, optimum dietary Zn levels were determined to be 70, 82, 98, and 110 mg/kg for optimised European Production Efficiency Factor and immunity, weight gain, appetites, and nutrient digestibility, respectively. These values are higher than the NRC (1994) recommendation (40 mg/kg of diet) and close to commercial broilers strain recommendation (80-120 mg/kg of diet), which is used to enhance performance. Overall, dietary Zn supplementation could improve broiler chicken’s growth performance via regulating some aspects of intestinal features and health. The dietary phytase supplementation did not affect the optimum dietary Zn levels found in this study.

**Ethical approval**

The experiment was conducted with the approval of the Animal Care Committee of the Ferdowsi University of Mashhad (Mashhad, Iran).

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**Disclosure statement**

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