Evaluation of interactive effects of phytase and benzoic acid supplementation on performance, nutrients digestibility, tibia mineralisation, gut morphology and serum traits in male broiler chickens

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ABSTRACT
A total of 720 one-day-old male broiler chickens were randomly allotted into nine treatments to evaluate the effects of different levels of phytase (Phy, 0.0, 500, and 1000 FTU/kg) and benzoic acid (BA, 0.0, 0.5, and 1.0 g/kg) supplementation on performance, nutrients digestibility, tibia mineralisation, intestinal morphology and serum traits in broiler chickens fed nutrient deficient diets (NC). The experiment was done in a 3\(^3\)X3 factorial arrangements with a completely randomised design until 42 days of age. Body weight (BW), body weight gain (BWG), and feed conversion ratio (FCR) improved when Phy (at both levels) was added to NC diets (\(p<0.05\)). Phy at 1X and 2X increased serum P, decreased serum Ca and litter moisture content (1000 FTU/kg) at d 28 (\(p<0.05\)). Furthermore, Phy supplementation increased tibia length and ash content as well as apparent ileal digestibility of P and ileal villus height (VH) (\(p<0.05\)). Addition of BA at 0.5 g/kg to the diets increased litter moisture, serum Ca content, jejunal villus height and ileal villus width (VW) (\(p<0.05\)). However, the combination of Phy and BA supplementation decreased apparent ileal digestibility of Ca (at 2X), and increased VH/CD ratio of the jejunum (at 1X). In conclusion, Phy was effective in improving performance traits, bone mineralisation, apparent ileal nutrients digestibility, and intestinal morphology. BA had no impact on performance traits, bone mineralisation, and ileal nutrients digestibility but at recommended level (0.5 g/kg) increased VH and VW in the ileum and jejunum.

HIGHLIGHTS
- Supplementation of nutrient deficient diet with phytase significantly improved body weight and body weight gain in male broiler chickens.
- Phytase supplementation increased serum phosphorus content and decreased serum calcium and litter moisture contents.
- Phytase supplementation increased tibia length and ash content, apparent ileal digestibility of phosphorus and ileal villus height.
- Addition of benzoic acid to nutrient deficient diet increased litter moisture, serum calcium content, jejunal villus height and ileal villus width.
- Combination of Phytase and benzoic acid supplementations decreased apparent ileal digestibility of calcium, crypt depth of the ileum and increased villus width and villus height/crypt depth ratio of the jejunum and ileum, respectively.

Introduction
The poultry industry is highly concerned by the environmental impacts of unsustainable poultry operations, especially excretion of phosphorus (P) and nitrogen (N). Unlike official restrictions on inclusion of antibiotic growth promoters in poultry feed in increasing number of countries (Giannenas et al. 2014; Olukosi and Dono 2014), there are no official mandates rather than voluntarily efforts to reduce environmental pollutants from poultry production operations. Both P and N are among the most expensive nutrients, which make any policy to diminish their excretion in the excreta, economically justified. Approximately two-thirds of the total P in plants is in the form of phytate and is...
unavailable or poorly utilised by monogastric animals. This unavailability is due to the very low phytase (Phy) activity found in the digestive tract. (Aydin et al. 2010; Karimi et al. 2013). Exogenous Phy has been supplemented to commercial poultry and pig diets for many years, with the aim to improve growth performance, releasing P and other nutrients (e.g., Ca, Zn, Fe, Cu) as well as reducing environmental P pollution (Beeson et al. 2017). The liberation of P from phytate by Phy is, however, far from complete and averaged only 29% of phytate-P present in poultry diets (Woyengo et al. 2010). The most common dosage of Phy in broiler nutrition is 500 FTU/kg of feed (Beeson et al. 2017). Several methods including dietary supplementation with higher dosage of Phy (1000–12,000 FTU/kg) have been investigated so far, aiming to improve the release of P from phytate (Walk et al. 2014). Increased phytate hydrolysis with using higher dosage of Phy may improve overall performance and efficiency of nutrient utilisation. These improvements have been speculated to be partly results of an increase in availability of the nutrients that had previously been bound with phytate, including protein and minerals (Karimi et al. 2013; Pieniazek et al. 2017). Previous researches have shown that poultry digestive tract acidity is not desirable to complete hydrolyse or establish phytate by Phy (Pieniazek et al. 2017). Optimisation of the pH of GIT by inclusion of organic acids (OA) in the diet has been also proposed as another approach to maximise Phy activity in the gastrointestinal tract (Adil et al. 2010). Benzoic acid (BA) as an OA can reduce the pH of digesta, which can then result in increased dissociation between phytate and minerals and increased activity of most Phys, which express their optimal activity at low pH, because the Phy efficiency is correlated with both acidity and concentration of other free cations (Woyengo et al. 2010; Nourmohammadi et al. 2011). Amaechi and Anueyiagu (2012) stated that the 1.2% inclusion level of BA in the broiler diet helped improve the growth performance and gut health of the broilers, however, Józefiak et al. (2007) reported that dosages of this acid higher than 0.25% caused growth depression and poor feed conversion. Addition of BA to diet have ability to reduce the pH of digesta, which accelerates the conversion of pepsinogen to pepsin and decrease the formation of insoluble mineral–phytate complexes, thus improving the absorption rate of proteins, amino acids, and minerals and improve the digestion of protein (Emami et al. 2013; Sohail et al. 2015). Thus, it was hypothesised that the concurrent supplementation of Phy and BA may have synergistic effects on growth performance and nutrient utilisation by broiler chickens, though less information available dealing the in vivo interaction between Phy and BA in broiler chickens, especially at levels higher than current recommendations. Therefore, the objective of the current study was to determine the effect of supplementing a negative control diet (NC) diet with Phy (at 1X and 2X of recommendation) alone or in combination with BA at 1X and 2X of current recommendation on growth performance, bone mineralisation, nutrient digestibility and gut morphology in broiler chickens.

Materials and methods

All procedures used in this experiment were approved by the Animal Ethics Committee of Kurdistan University (IR. UOK. REC. 1398.033) and complied with the guidelines for the care and use of animals in research (Federation of Animal Science Societies Writing Committee 2010).

Birds and managements

A total of 720 one-day-old male broiler chickens (Ross 308) was purchased from a commercial hatchery. On arrival, all chickens were randomly assigned to nine experimental diets, as a completely randomised design in a 3 × 3 factorial arrangement. Each treatment was replicated five times, with 16 chickens per replication. The lighting program in the first two days of arrival was 24 h and then kept on 23 h light and 1 h dark schedule thereafter. The house temperature was set at 32°C in the first week, then gradually decreased to 22°C and kept at this temperature during the rest of the experiment. The rearing period was divided into 3 phases of 1–11 days, 12–24 days and 25–42 days of ages. Broilers chickens had ad-libitum access to freshwater and experimental diets in a mash form throughout the experiment.

Dietary treatments

A negative control maize-soybean meal based diet (NC, Table 1), without anticoccidial or antibiotic growth-promoting agents, was formulated with lower crude protein (−5.01 g/kg), AME (−0.20934 MJ/kg), available P (−1.5 g/kg), Ca (−1.5 g/kg), sodium (−0.42 g/kg), lysine (−0.21 g/kg), methionine (−0.09 g/kg), cysteine (−0.15 g/kg) and TSAA (−0.24 g/kg) contents in three phases of growth to reflect suggested nutrient matrix value of applied Phy (500 FTU/kg,
CIBENZA® PHYTAVERSE® G10, Novus International, Inc., St. Charles, MO, USA) in the present study. The NC diet was supplemented with 0.0, 500 or 1000 FTU/kg of Phy or 0.0, 0.5 or 1.0 g/kg of BA (Avimatrix, Novus International Inc., St. Charles, MO, USA) or their combination. In total, nine experimental diets were produced as follows: 1) NC diet without additive, 2) NC diet plus 0.5 g/kg of BA, 3) NC diet plus 1.0 g/kg of BA, 4) NC diet plus 500 FTU/kg of Phy, 5) NC diet plus 1000 FTU/kg of Phy, 5) NC diet supplemented with 0.5 g/kg of BA plus 500 FTU/kg of Phy, 6) NC diet supplemented with 1.0 g/kg of BA plus 1000 FTU/kg of Phy, 7) NC diet supplemented with 0.5 g/kg of BA plus 1000 FTU/kg of Phy, 8) NC diet supplemented with 0.5 g/kg of BA plus 1000 FTU/kg of Phy, 9) NC diet supplemented with 1.0 g/kg of BA plus 1000 FTU/kg of Phy. To achieve optimum homogeneity of the added BA and Phy, at first, a small quantity of fine ground maize was mixed with the BA and/or Phy, then mixed with the whole of the diet to get the final concentration. It is noteworthy that BA of Avimatrix (containing 45% BA) is encapsulated in a vegetable oil matrix. The hypothesis behind the encapsulation of BA in a vegetable oil matrix is to slow the release of BA aiming to increase its concentration and thus activity in the lower parts of the gut.

### Experimental procedure

Body weight (BW, g) and feed intake (FI, g) were measured at the end of 0–11 days, 12–24 days and 25–42 days growth periods. The average daily FI, body weight gain (BWG, g) and feed conversion ratio (FCR, g g\(^{-1}\)) were calculated for each growing period and the entire rearing period (1–42 days). The FI, BWG, and FCR values were adjusted according to the weight of dead broiler chickens. At d 28, and after 72 h of feeding of the experimental diets supplemented with 3 g/kg of titanium dioxide (TiO2) (as an indigestible marker), a total of 90 broiler chickens (two birds per pen; 10 birds per treatment) with BW closest to the pen average, were selected and taken to the animal house slaughter facility. Blood samples were taken from the jugular vein and centrifuged at 865 \(\times\) g for 10 min and sera were separated and stored at \(-20^\circ\)C until further analysis. As described in Karami et al. (2020), immediately after blood sampling, the selected birds weighed, and then killed by stunning followed by cervical dislocation. Then the legs of the 2 samples birds were dissected, and the left tibia bones were cleaned of attached tissues and debris without boiling and then stored at \(-20^\circ\)C until further analysis (ash, Ca and P). The right and left toes were cut and cleaned of as litter and excrement and were kept intact in plastic bags and stored at \(-20^\circ\)C until further analysis.

The ileum contents of sacrificed birds (between Meckel’s diverticulum and 2 cm above the ileal-cecal junction) were collected by smooth squeezing. Then the collected ileum samples of broiler chickens from each replicate were pooled and stored at \(-20^\circ\)C. The toes were dried at 105 °C for 24 h and placed in a desiccator, and the weights were recorded, and then ashed at 600 °C overnight in porcelain crucibles. Toe ash was expressed as the percentages of toe ash relative to toe dry weights. The left bones were dehydrated in ethanol for 72 h, defatted for 72 h in diethyl ether: methanol (9:1), drying at 105 °C for at least 24 h to reach to a constant weight, then ashed at 600 °C for 6 h. Tibia ash was determined on a fat-free dry matter basis (Method 942.05 AOAC). The Ca level in tibia ash contents, excreta and diet samples were

### Table 1. Composition and calculated nutrient content of experimental diets 1.

| Item, g/kg (unless indicated) | 1–11 days | 12–24 days | 25–42 days |
|-------------------------------|-----------|------------|------------|
| Com                           | 566.6     | 579.7      | 635.7      |
| Soybean meal (465 g/kg CP)    | 379.1     | 356.9      | 312.8      |
| Soy oil                       | 20.9      | 33.5       | 23.5       |
| Salt                          | 2.9       | 2.6        | 2.6        |
| DL Methionine                 | 2.1       | 2.2        | 2.5        |
| Lysine                        | 3.2       | 2.0        | 1.6        |
| Calcium Carbonate            | 11.3      | 10.7       | 10.2       |
| Dicalcium phosphate           | 8.9       | 7.4        | 6.1        |
| Vitamin and mineral premix    | 5.0       | 5.0        | 5.0        |
| Calculated analysis           |           |            |            |
| Crude protein                 | 220.0     | 210.0      | 195.0      |
| Metabolisable energy, MJ/kg   | 12.35     | 12.77      | 12.77      |
| Calcium                       | 7.5       | 6.9        | 6.3        |
| Total phosphorus              | 5.7       | 5.3        | 4.9        |
| Avail phosphorus              | 3.0       | 2.7        | 2.4        |
| Lysine                        | 14.2      | 12.7       | 11.3       |
| Methionine                    | 5.4       | 5.5        | 5.6        |
| Total sulphur amino acids     | 8.9       | 8.9        | 8.8        |
| Sodium                        | 1.3       | 1.2        | 1.2        |
| Analyzed nutrients            |           |            |            |
| Dry matter                    | 905.7     | 905.5      | 891.2      |
| Crude protein                 | 222.0     | 212.5      | 201.0      |
| Crude ash                     | 52.1      | 47.5       | 49.9       |
| Calcium                       | 7.7       | 7.3        | 6.7        |
| Total phosphorus              | 5.1       | 4.8        | 4.6        |

1Negative control (NC), maize-soybean meal based diet with nutrient deficiency equal to matrix value of 500 FTU/kg of Phytase (with lower crude protein (\(-5.01 g/kg\)), AME (\(-0.20934 MJ/kg\)), available P (\(-1.5 g/kg\)), Ca (\(-1.5 g/kg\)), sodium (\(-0.42 g/kg\)), digestible lysine (\(-0.21 g/kg\)), digestible methionine (\(-0.09 g/kg\)), digestible cysteine (\(-0.15 g/kg\)), and digestible TSAA (\(-0.24 g/kg\)) contents

2For analysed composition, g/kg dry matter and for rest parts, g/kg as fed.

380 g/kg Ca

4190 g/kgP and 210 g/kg Ca

5The vitamin premix supplied the following per kilogram of diet: vitamin A, 9000 IU; vitamin D3, 2000 IU; vitamin E, 18 IU; vitamin K3, 2 mg; thiamine, 1.8 mg; riboflavin, 6.6 mg; vitamin B6, 3 mg; vitamin B12, 0.015 mg; niacin, 9000 U; vitamin D3, 2000 U; vitamin E, 18 U; vitamin K3, 2 mg; thiamine, 1.8 mg; riboflavin, 6.6 mg; vitamin B6, 3 mg; vitamin B12, 0.015 mg; niacin, 9000 U; vitamin D3, 2000 U; vitamin E, 18 U; vitamin K3, 2 mg; thiamine, 1.8 mg; riboflavin, 6.6 mg; vitamin B6, 3 mg; vitamin B12, 0.015 mg; niacin, 84.7 mg; Cu, 10 mg; I, 1 mg. Se, .15 mg.

6The chemical composition of experimental diets were calculated based on NRC (1994) feed ingredient database.
determined by standard procedure following titration with 0.1% N potassium permanganate (method 972.02; AOAC, 1990). The CP (Method 990.03 AOAC) in collected excreta and diet samples were measured according to the standard laboratory procedures (AOAC, 1990). The total P in tibia ash, collected excreta and diet samples were measured using standard procedure following the colorimetric method of ammonium vanadate/molybdate (method 965.17; AOAC, 1990). The diets and ileum digesta samples were analysed for TiO₂ concentrations as described by Short et al. (1996). Each sample was analysed in duplicate and the absorbance measured using the spectrophotometer (Jasco V570, Tokyo, Japan) at the absorbance of 410 nm. Intestinal tissue samples of jejunum and ileum were obtained from the slaughtered broiler chickens and approximately 2 cm of the middle portions of the jejunum and ileum were excised and fixed in 10% buffered formalin for one week and used to measure villus height (VH), villus width (VW) and the depth of the crypts (CD). Tissues were dehydrated by immersing through a series of alcohols with increasing percentage.

**Calculations and statistical analysis**

Nutrient digestibility was calculated using the following equation:

\[
ND (%) = 1 - \left( \frac{Ti \, in \, diet}{Ti \, in \, sample} \times \frac{nutrient \, in \, sample}{Nutrient \, in \, diet} \right) \times 100
\]

All data were collected and pen means were considered as the experimental unit. Firstly, data were analysed as a completely randomised design using the GLM procedure of SAS (2001). Then the data were analysed as a 3×3 factorial arrangement of treatments including three levels of Phy (0X, 1X, and 2X; X = 500 FTU/kg) and three-levels of BA (0X, 1X, and 2X; X = 0.5 g.kg⁻¹). The following statistical model was chosen: \(Y_{ijk} = \mu + P_j + B_i + PB_{ij} + e_{ijk}\), where, \(Y_{ijk}\) is the response measured, \(\mu\) is the overall mean, \(P_j\) is the effect level of Phy, \(B_i\) is effect level of BA, \(PB_{ij}\) is the interaction between Phy and BA, and \(e_{ijk}\) is the residual error. Significant differences among all dietary treatments means were determined at \(p \leq 0.05\) by Tukey tests. Where the interaction effect was significant, the effects of the main factors were not discussed.

**Results**

**Growth performance**

The results of the present experiments are summarised in Tables 2 and 3. Addition of Phy to NC diets at 1X and 2X of the most common recommended dosage (500 FTU/kg) increased \((p \leq 0.05)\) BW at d 11 and 24, BWG (1–11 days) and decreased FCR (1–11, 12–24 and 1–42 days), however, FI was not influenced by Phy supplementation \((p > 0.05)\). Addition of BA at 1X and 2X of the current recommendation (0.5 g/kg) to the NC diets had no \((p > 0.05)\) effect on growth performance traits of broiler chickens at the end of the different experimental periods, though broiler chickens fed NC diets with BA tended to have higher BW at d 42 \((p < 0.093)\) and higher FI during the finisher period \((p < 0.64)\) and entire experiment \((p < 0.67)\). In this study, the performance traits were not influenced by BA × Phy interaction \((p > 0.05)\).

**Bone mineralisation and litter moisture**

As shown in Table 4, tibia length and tibia ash content were increased \((p \leq 0.05)\) when Phy was added at 1X and 2X of the recommended dosage but toe ash and tibia minerals (Ca and P) contents were not affected by adding Phy to the diets \((p > 0.05)\). Tibia length, toe and tibia ash as well as Ca and P contents were not \((p > 0.05)\) changed by the addition of BA to the NC diet \((p > 0.05)\). Furthermore, no tibia and toe mineralisation traits were influenced by Phy × BA interaction at d 28 \((p > 0.05)\). Results for the litter moisture are shown in Table 4. The inclusion of Phy in the NC diets, especially at 2X level, reduced litter moisture content at d 30 \((p \leq 0.05)\). Adding BA to the NC diets at 1X level increased litter moisture content at d 30 \((p \leq 0.05)\). The litter moisture content was not influenced by the interaction between Phy × BA \((p > 0.05)\).

**Apparent ileal digestibility of nutrients**

The present results showed that Phy supplementation significantly improved apparent ileal digestibility of P
regardless of Phy dosage \((p \leq 0.05)\), but Phy supplementation did not affect ileal DM and Ash digestibility \((p \geq 0.05)\). As shown in Table 5, BA supplementation of the NC diets had no effect on ileal Ash and P digestibility \((p > 0.05)\). However, ileal Ca digestibility was affected by Phy \(\times\) BA interaction \((p > 0.05)\). As shown in Table 5, the broiler chickens fed with the NC diet containing BA in 2X level and NC diet containing Phy in 1X level had higher Ca digestibility compared with broiler chickens fed with the NC diet, on the other hand, NC diet containing 2X level of BA and Phy, showed lower ileal Ca digestibility compared with other treatments. Other groups demonstrated identical ileal Ca digestibility.

**Table 2.** Effects of phytase (Phy) and benzoic acid (BA) supplementation on body weight (g) and weight gain (g) in broiler chicks from 1 to 42 days of age.

| Treatment | Dietary treatments | Body weight, g | Weight gain, g |
|-----------|--------------------|----------------|----------------|
|           | Phy, U. kg\(^{-1}\) | BA, g. kg\(^{-1}\) | 11 days | 24 days | 42 days | 11 days | 24 days | 42 days | 11–42 days |
| 1         | 0.0 | 0.0 | 235 | 788 | 2158 | 192 | 553 | 1370 | 2116 |
| 2         | 0.0 | 0.5 | 246 | 828 | 2227 | 204 | 582 | 1399 | 2185 |
| 3         | 0.0 | 1.0 | 250 | 876 | 2312 | 216 | 625 | 1437 | 2270 |
| 4         | 500 | 0.0 | 260 | 863 | 2221 | 218 | 603 | 1358 | 2178 |
| 5         | 500 | 0.5 | 255 | 901 | 2451 | 213 | 645 | 1550 | 2408 |
| 6         | 500 | 1.0 | 256 | 880 | 2405 | 214 | 624 | 1525 | 2363 |
| 7         | 1000 | 0.0 | 260 | 899 | 2376 | 217 | 639 | 1477 | 2333 |
| 8         | 1000 | 0.5 | 266 | 913 | 2400 | 222 | 648 | 1487 | 2357 |

**Main effects**

| Source of variation | Probability |
|---------------------|-------------|
| Phy                | 0.001       |
| BA                 | 0.547       |
| Phy \(\times\) BA  | 0.401       |
| Pooled SEM         | 2.1         |

**SEM:** Pooled Standard Error of Means.

**Table 3.** Effects of phytase (Phy) and benzoic acid (BA) supplementation on feed intake (g) and feed conversion ratio (g.g\(^{-1}\)) in broiler chicks from 1 to 42 days of age.

| Treatment | Dietary treatments | Feed intake, g | Feed conversion ratio, g.g\(^{-1}\) |
|-----------|--------------------|----------------|--------------------------------------|
|           | Phy, U. kg\(^{-1}\) | BA, g. kg\(^{-1}\) | 0–11 days | 12–24 days | 25–42 days | 1–42 days | 0–11 days | 12–24 days | 25–42 days | 1–42 days |
| 1         | 0.0 | 0.0 | 284 | 864 | 2303 | 3444 | 1.48 | 1.56 | 1.68 | 1.63 |
| 2         | 0.0 | 0.5 | 290 | 897 | 2376 | 3553 | 1.42 | 1.54 | 1.70 | 1.63 |
| 3         | 0.0 | 1.0 | 286 | 923 | 2452 | 3635 | 1.42 | 1.48 | 1.69 | 1.60 |
| 4         | 500 | 0.0 | 287 | 903 | 2288 | 3466 | 1.32 | 1.50 | 1.69 | 1.59 |
| 5         | 500 | 0.5 | 280 | 944 | 2553 | 3765 | 1.32 | 1.47 | 1.65 | 1.57 |
| 6         | 500 | 1.0 | 285 | 935 | 2475 | 3683 | 1.34 | 1.50 | 1.62 | 1.56 |
| 7         | 1000 | 0.0 | 282 | 927 | 2385 | 3587 | 1.29 | 1.45 | 1.63 | 1.53 |
| 8         | 1000 | 0.5 | 283 | 950 | 2515 | 3728 | 1.30 | 1.49 | 1.70 | 1.60 |
| 9         | 1000 | 1.0 | 287 | 935 | 2532 | 3754 | 1.29 | 1.44 | 1.70 | 1.59 |

**Main effects**

| Source of variation | Probability |
|---------------------|-------------|
| Phy                 | 0.825       |
| BA                  | 0.911       |
| Phy \(\times\) BA   | 0.836       |
| Pooled SEM          | 1.8         |

**SEM:** Pooled Standard Error of Means.
Broiler chickens fed NC diets with Phy, especially at 2X level (1000 FTU/kg), had \((p \leq .05)\) higher serum P content at d 28 (Table 5), while it decreased serum Ca content at d 28 \((p \leq .05)\). Inclusion of BA had no impact on serum P and ALP contents \((p \geq .05)\); however, serum Ca content increased by BA supplementation at 1X (0.5 g/kg, \(p \leq .05)\). Serum traits were not influenced by Phy \(\times\) BA interaction \((p \geq .05)\).

### Table 4. Effects of phytase (Phy) and benzoic acid (BA) supplementation on bone mineralisation and litter moisture content (%) in broiler chicks at 28 days of age.

| Treatment | Phy, U.kg\(^{-1}\) | BA, g.kg\(^{-1}\) | Tibia length, mm | Toe ash | Tibia ash | Tibia P | Tibia Ca | Litter moisture, % |
|-----------|------------------|------------------|------------------|---------|-----------|---------|---------|-------------------|
| 1         | 0.0              | 0.0              | 80.8             | 13.45   | 40.83     | 15.51   | 34.01   | 19.81             |
| 2         | 0.0              | 0.5              | 83.0             | 12.75   | 42.36     | 15.37   | 35.14   | 25.45             |
| 3         | 0.0              | 1.0              | 81.0             | 12.83   | 42.57     | 15.50   | 34.61   | 21.49             |
| 4         | 500              | 0.0              | 84.5             | 13.15   | 43.35     | 15.98   | 38.42   | 16.80             |
| 5         | 500              | 0.5              | 83.5             | 13.29   | 44.24     | 15.50   | 36.61   | 21.45             |
| 6         | 500              | 1.0              | 83.4             | 13.67   | 43.66     | 15.68   | 34.37   | 21.66             |
| 7         | 1000             | 0.0              | 83.4             | 13.13   | 43.53     | 15.88   | 34.49   | 22.56             |
| 8         | 1000             | 0.5              | 84.1             | 14.01   | 42.98     | 15.52   | 34.63   | 25.66             |
| 9         | 1000             | 1.0              | 83.6             | 13.79   | 43.99     | 15.46   | 34.71   | 25.01             |

**Main effects**

| Phy, U.kg\(^{-1}\) | Bone Mineralisation, % |
|------------------|-------------------------|
| 0.0              | 81.6 b                  |
| 500              | 83.8 a                  |
| 1000             | 83.7 a                  |
| BA, g.kg\(^{-1}\) |                        |
| 0.0              | 82.9                    |
| 0.5              | 83.5                    |
| 1.0              | 82.7                    |

**Source of variation**

| Probability |
|-------------|
| Phy 0.015   |
| BA 0.575    |
| Phy \(\times\) BA 0.610 |

**Pooled SEM**

| Probability |
|-------------|
| Phy 0.350   |
| BA 0.145    |
| Pooled SEM 0.585 |

*Means within a column and under each main effects with common superscript do not differ significantly \((p \leq .05)\); SEM: Pooled Standard Error of Means.

### Table 5. Effects of phytase (Phy) and benzoic acid (BA) supplementation on ileum nutrient digestibility (%), and serum metabolites (mg. dL\(^{-1}\)) in broiler chickens at 28 days of age.

| Treatment | Phy, U.kg\(^{-1}\) | BA, g.kg\(^{-1}\) | DM | Ash | P | Ca | P, mg. dL\(^{-1}\) | Ca, mg. dL\(^{-1}\) | ALP, U.L |
|-----------|------------------|------------------|----|-----|---|----|-----------------|-----------------|---------|
| 1         | 0.0              | 0.0              | 93.7 | 43.10 | 42.50 | 42.81\(^{cd}\) | 3.22           | 10.12   | 1519   |
| 2         | 0.0              | 0.5              | 93.7 | 46.03 | 49.25 | 45.28\(^{ab}\) | 2.83           | 10.33   | 1494   |
| 3         | 0.0              | 1.0              | 93.7 | 49.82 | 51.07 | 58.97\(^{a}\)  | 3.12           | 9.99    | 1362   |
| 4         | 500              | 0.0              | 94.17| 56.69 | 67.70 | 56.72\(^{ab}\) | 3.82           | 8.36    | 1218   |
| 5         | 500              | 0.5              | 93.57| 46.71 | 63.05 | 47.93\(^{ab}\) | 3.79           | 10.53   | 1522   |
| 6         | 500              | 1.0              | 94.20| 50.59 | 68.05 | 52.34\(^{ab}\) | 3.83           | 9.27    | 1538   |
| 7         | 1000             | 0.0              | 94.52| 50.02 | 68.41 | 47.28\(^{ab}\) | 3.66           | 8.91    | 1268   |
| 8         | 1000             | 0.5              | 93.57| 45.29 | 65.11 | 44.21\(^{c}\)  | 3.92           | 9.36    | 1436   |
| 9         | 1000             | 1.0              | 92.40| 45.68 | 64.59 | 32.91\(^{d}\)  | 4.25           | 8.79    | 1578   |

**Main effects**

| Phy, U.kg\(^{-1}\) | Ileum nutrient digestibility, % |
|------------------|---------------------------------|
| 0.0              | 93.7 46.3 47.6\(^{b}\) 49.0\(^{ab}\) |
| 500              | 94.0 51.3 65.9\(^{a}\) 52.3\(^{a}\) |
| 1000             | 93.5 47.0 66.0\(^{a}\) 41.5\(^{b}\) |
| BA, g.kg\(^{-1}\) |                                |
| 0.0              | 94.2 49.9 59.2 48.9            |
| 0.5              | 93.6 46.0 59.1 45.8            |
| 1.0              | 93.4 48.7 61.2 48.1            |

**Source of variation**

| Probability |
|-------------|
| Phy 0.508   |
| BA 0.218    |
| Phy \(\times\) BA 0.184 |

**Pooled SEM**

| Probability |
|-------------|
| Phy 0.239   |
| BA 0.454    |
| Pooled SEM 0.158 |

*Means within a column and under each main effects with common superscript do not differ significantly \((p \leq .05)\); SEM: Pooled Standard Error of Means.

**Serum traits**

Broiler chickens fed NC diets with Phy, especially at 2X level (1000 FTU/kg), had \((p \leq .05)\) higher serum P content at d 28 (Table 5), while it decreased serum Ca content at d 28 \((p \leq .05)\). Inclusion of BA had no impact on serum P and ALP contents \((p \geq .05)\); however, serum Ca content increased by BA supplementation at 1X (0.5 g/kg, \(p \leq .05)\). Serum traits were not influenced by Phy \(\times\) BA interaction \((p \geq .05)\).
Intestinal morphology

Results for the intestinal morphology are shown in Table 6. Results showed that Phy supplementation had no effect \((p > 0.05)\) on the jejunal morphometric traits, but increased ileal VH \((p < 0.05)\), while at level 2X decreased ileal VW \((p < 0.05)\) at d 28. BA supplementation of NC diets at 1X level increased VH in the jejunum, but decreased ileal VW \((p < 0.05)\). The jejunal VW, ileal CD, and VH/CD ratio were affected \((p < 0.005)\) by Phy \(\times\) BA interaction. As shown in Table 6, the addition of BA and Phy at 1X level to the NC diets significantly increased the jejunal VW compared with other experimental treatments \((p < 0.05)\), however, compared with other experimental treatments, adding Phy at 1X level and BA at 2X level to the NC diets significantly reduced the jejunal VW \((p < 0.05)\). In the NC diet containing 1X of current BA, the ileal CD decreased, but with addition of 2X BA to the NC diet the ileum CD increased compared with other experimental treatments \((p < 0.05)\). The ileum VH/CD ratio in the broiler chickens fed diet containing 2X of current Phy plus 1X of current BA, was increased but in broiler chickens fed diet containing 2X of current BA decreased, that this effect is consistent with an increase in CD and decrease ileum VH induced by this supplement.

### Discussion

The aim of the present study was to evaluate the interactions between Phy and BA inclusion in broiler chickens diets with reducing levels of nutrients on performance, nutrients digestibility, tibia mineralisation, gut morphology and serum traits.

The results of the present experiment showed that supplementation of NC diet with Phy (500 and 1000 FTU/kg) was highly effective to improve BW, BWG, and FCR of broiler chickens at the early life stages \((p < 0.05)\). Inclusion of Phy in NC diets increased ileal P digestibility, tibia ash, tibia length, serum P, ileal VH and VW \((p < 0.05)\). Though, it has been argued that higher levels of Phy, i.e. <1000 FTU/kg, could further improve performance and bone mineralsation (Walk et al. 2014; Manobhavan et al. 2016), however, in the present study, inclusion of high doses of Phy had no further significant effect on broiler performance, despite the numerically greater BW, BWG, and reduce FCR compared to the broiler chicken fed a diet without Phy. In general, the specific benefits of Phy supplementation in nutrient-deficient (NC) diets may vary between studies, due to the deficiency of available P and other nutrients, different responses to Phy and the effects of the dose used by Phy are given (Beeson et al. 2017). There are many reports on the nutritional advantages

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**Table 6. Effects of dietary nutrient level (DNL), phytase (Phy) and benzoic acid (BA) supplementation on histomorphology of jejunum and ileum in broiler chicks at 28 days of age.**

| Treatment | Dietary treatments | Jejunum, \(\mu m\) | Ileum, \(\mu m\) |
|-----------|--------------------|---------------------|-----------------|
|           | Phy, U/kg\(^{-1}\) | BA, g/kg\(^{-1}\) | Villus height | Villus width | Crypt depth | Villus height | Villus width | Crypt depth | Villus height/Crept depth ratio |
| 1         | 0.0                | 0.0                | 561           | 140\(^{bc}d\) | 164         | 3.46          | 556           | 136         | 231\(^{ab}\) | 2.81\(^{cd}\) |
| 2         | 0.0                | 0.5                | 836           | 167\(^{b}\)   | 210         | 4.03          | 611           | 141         | 166\(^{a}\) | 3.54\(^{bc}\) |
| 3         | 0.0                | 1.0                | 584           | 150\(^{cd}\)   | 190         | 3.08          | 539           | 142         | 236\(^{b}\) | 2.27\(^{d}\) |
| 4         | 500                | 0.0                | 714           | 118\(^{a}\)    | 214         | 3.29          | 706           | 193         | 199\(^{abcd}\) | 3.70\(^{b}\) |
| 5         | 500                | 0.5                | 841           | 213\(^{a}\)    | 235         | 3.65          | 634           | 145         | 213\(^{abc}\) | 3.04\(^{abc}\) |
| 6         | 500                | 1.0                | 596           | 104\(^{b}\)    | 188         | 3.26          | 639           | 178         | 173\(^{cd}\) | 3.61\(^{abc}\) |
| 7         | 1000               | 0.0                | 753           | 148\(^{b}\)    | 192         | 3.88          | 671           | 132         | 191\(^{bcd}\) | 3.67\(^{ab}\) |
| 8         | 1000               | 0.5                | 798           | 174\(^{b}\)    | 195         | 3.52          | 655           | 118         | 177\(^{cd}\) | 4.05\(^{a}\) |
| 9         | 1000               | 1.0                | 692           | 169\(^{b}\)    | 192         | 3.70          | 633           | 139         | 176\(^{cd}\) | 3.30\(^{abc}\) |

Main effects

| Phy, U/kg\(^{-1}\) | BA, g/kg\(^{-1}\) | Villus height | Villus width | Crypt depth | Villus height | Villus width | Crypt depth | Villus height/Crept depth ratio |
|---------------------|---------------------|---------------|--------------|-------------|---------------|--------------|-------------|--------------------------------|
| 0.0                 | 0.0                 | 660           | 152         | 188         | 3.52          | 569\(^{b}\) | 139\(^{b}\) | 211 | 2.87\(^{b}\) |
| 500                 | 0.0                 | 717           | 145         | 212         | 3.40          | 660\(^{a}\) | 172\(^{a}\) | 197 | 3.45\(^{a}\) |
| 1000                | 0.0                 | 742           | 164         | 193         | 3.70          | 653\(^{a}\) | 130\(^{b}\) | 181 | 3.67\(^{a}\) |
| 0.0                 | 0.5                 | 670\(^{b}\)    | 135\(^{b}\) | 190         | 3.55          | 644         | 154\(^{a}\) | 207 | 3.39 |
| 0.5                 | 825\(^{a}\)         | 185\(^{a}\)    | 213         | 3.73          | 633         | 134\(^{a}\) | 185 | 3.54 |
| 1.0                 | 624\(^{b}\)         | 141\(^{b}\)    | 190         | 3.35          | 604         | 153\(^{a}\) | 196 | 3.06 |

Source of variation Probability

| Phy                  | BA                  |
|----------------------|---------------------|
| 0.139                | 0.242               |
| <0.0001              | <0.0001             |
| 0.015                | 0.245               |
| 0.133                | 0.242               |

Pooled SEM 21.9 6.19 4.84 0.109 13.60 4.60 5.65 0.116

\(^{a}\)–\(^{e}\)Means within a column and under each main effects with common superscript do not differ significantly \((p > 0.05)\); SEM: Pooled Standard Error of Means.
of the addition of Phy to broiler chickens diets such as improvement in growth performance and bone mineralisation, especially in P deficient diets. Rutherfurd et al. (2012), Pieniazek et al. (2017), and Beeson et al. (2017) reported that Phy (250–2000 FTU/kg) improved BWG and FI of broiler chickens fed the NC diet but had no effect on FCR. However, Walk et al. (2012) observed that Phy (500 and 5000 FTU/kg) improved FCR, but no effects on BWG and FI. Several mechanisms have been proposed for the effectiveness of Phy in broiler chickens, in which the partial breakdown of phytate complex and releasing of bounded nutrients (Dos Santos et al. 2013), especially P is suggested as the main reason for observed benefits of Phy.

Though the positive influence of Phy in broiler chicken nutrition has been widely anticipated, however, the extent of Phy ability to release bounded nutrients as it is called the nutrients equivalency of Phy or ‘Phy nutrient matrix values’ is a highly arguable subject and depend upon factors such as the Phy origin, Phy dosage, birds age, dietary nutrient level, etc., which are all practically important when diet nutrient specification should be adjusted based on suggested matrix value. It has been argued that setting a single nutrient matrix value for a Phy product is not a suitable option (Karimi et al. 2013) and probably nutrient matrix value of Phy would need to be adjusted based on birds age, the diet formulation and nutrient composition of the ingredients used (Viveros et al. 2002; Farhadi et al. 2017).

These results demonstrate that meeting P and other nutrients requirements of broiler chickens are essential for proper development of the skeletal system. In fact, several studies showed that tibia ash percentage is a reliable index to monitor the P and Ca sufficiency in monogastric animal diets. In the present study, higher level of tibia ash contents of broiler chickens fed NC supplemented with Phy (1X and 2X dosage) clearly demonstrate that Phy applied in the present experiment were release bounded P, which is in agreements with other reports (Viveros et al. 2002; Pieniazek et al. 2017). Pieniazek et al. (2017) observed that, the inclusion of Phy at both 500 and 2000 U/kg was increased bone ash weight and percentage in broiler chickens fed P-deficient diet. Zeller et al. (2015) also reported that in broiler chickens fed with 500 and 12,500 FTU/kg Phy supplemented diets, tibia dry weight, tibia ash weight, bone-breaking strength, and cortical content were increased. Rutherfurd et al. (2012) state that in broiler chickens fed the low-P diet supplemented with either 1000 or 2000 U/kg of Phy, higher ileal P digestibility and lower litter moister contents of broiler chickens fed Phy supplemented diets in the present experiment is in agreements with our previous findings (Farhadi et al. 2017), which may be related to potential of Phy in improving P utilisation and dietary electrolyte balances, and preventing of the formation of insoluble Ca-Phytate complexes in poultry diets, which has been reflected in higher serum P content but lower serum Ca content in the present experiment. These observations are in agreement with published studies (Nourmohammadi et al. 2011; Rutherfurd et al. 2012; Farhadi et al. 2017). In the present study, observed increase in serum P levels due to Phy supplementation supports this result. Amerah et al. (2014) also reported that adding 1000 FTU/kg of Phy to broiler chickens diet with different Ca to P ratio (1.43, 2.14, 2.86, 3.57), significantly improved P digestibility without influencing Ca digestibility.

In accordance with the results of the present experiment, a number of studies reported no effect of BA supplementation on growth performance parameters of broiler chickens (Olukosi and Dono 2014; Yousaf et al. 2017). Hassan and Raheem (2016) reported that in broiler chickens fed diets containing 4 g/kg and 8 g/kg free form of BA, performance traits were not influenced by dietary treatments. On the other hand, Józefiak et al. (2007) reported that inclusion of 5 and 7.5 g/kg of free form of BA in the broiler chickens diet, decreased BWG and increased FCR. These conflicting results from the effects of BA in broiler chickens performance parameters may derive from factors such as level and type of organic acid, buffering capacity of dietary ingredients, antimicrobial compounds in the diet, the sanitation conditions in the production facility as well as the profile of the gut microbiota (Broom 2015; Yousaf et al. 2017). In the current experiment, the addition of BA to the experimental diets generally had no impact on tibia mineralisation, but increased blood serum Ca content (at 0.5 g/kg) as the main factor, which need more studies to dermine why it is not reflected in tibia mineral contents. In agreement with the present data, 5 g/kg BA supplementation did not affect the bone parameters such as breaking strength and ash, Ca, P and Mg content in pigs weaned (Gutzwiller et al. 2014). While adding 10 and 20 g/kg BA to the growing pigs diets linearly increased bone ash P concentration.
and linearly decreased bone Ca content, but plasma concentrations of Ca and P increased linearly on days 10 and 21 of the experiment (Sauer et al. 2009). The increase in Ca and P content of blood serum produced by the addition of OAs may be attributed to the lowering of GI-tract pH by using acids which increases the absorption of such minerals from the gut into the blood stream (Nourmohammadi et al. 2011). Bühler et al. (2010a) reported that the interaction between Phy and BA inclusions was significant for Ca ileal digestibility during finisher period. Broiler chickens fed diets containing 2X level of BA and Phy had lower Ca ileal digestibility compared with those fed diets containing only Phy and BA. Consistent with this result, Bühler et al. (2010a) reported that the interaction between BA and Phy significantly decreased Ca digestibility in growing pigs. There are several hypotheses to explain the interaction between Phy and BA on the digestibility of Ca. Phy activity may be inhibited due to acidification of the digestive tract (decrease pH) using BA, because the optimum pH for Phy activity the range is 4.5–5. On the other hand, it has been shown that high amounts of acid may reduce Phy activity in the stomach without affecting its efficiency. BA may affect Phy activity as well as its efficiency in the stomach. Another explanation is that the aromatic structure of BA may inhibit the enzyme (Bühler et al. 2010a). The addition of 2 g/kg BA to broiler chickens diets had no impact on the ileal digestibility of DM and CP. However, it improved the total tract digestibility of dry matter, apparent metabolisable energy and nitrogen-corrected apparent metabolisable energy (Olukosi and Dono 2014). Inclusion of 0.5 and 1% BA into heavy pigs’ diet had no impact on total tract digestibility of CP and P (Galassi et al. 2011). On the other hand, Klug et al. (2010) reported that adding 2% BA into lactating pigs’ diets significantly increased CP digestibility compared to the control group, they state that the effect of BA may be due to its beneficial effects on microbial flora.

In general, in the present study, when the NC diet was supplemented with Phy, BA, or both, the jejunal VW, the ileal CD, and the VH/CD ratio were restored and had significant differences with the same parameters obtained with the NC diet. Mohammadagheri et al. (2016), reported that Phy increased the VW and decreased the CD and in combination with citric acid decreased the CD and increased VH/CD ratio significantly in broiler chicks. Increases in VH (greater surface area and absorptive capacity) and decreases in CD result in high VH/CD ratio, which is an indicator that the broiler chickens has mature enterocytes at the villus tips, a balanced enterocyte migration, and sloughing, because VH/CD ratio can be directly correlated with the balance of VH and CD (Mohammadagheri et al. 2016). In another study with the simultaneous addition of Phy, BA, and butyric acid to diets of weaned pigs, there was no interaction between Phy, BA and butyric acid on CD, but significantly increased VH and VH/CD ratio of the duodenum (Rufino et al. 2017). As mentioned before, OAs, reduce gram-negative bacteria and decreasing the intestinal colonisation and infectious processes and higher levels of lactobacillus, ultimately decreasing the inflammatory reactions at the intestinal mucosa, which increases the VH and functions of secretion, digestion, and absorption of nutrients by the mucosa (Emami et al. 2013; Mohammadagheri et al. 2016). Diao et al. (2016) reported that inclusion 5000 mg/kg BA supplementation to diet significantly increased the VH/CD ratio, and decreased CD in the jejunum of pigs. In agreement with the present data, 0.6, 1.2, 1.8, and 2.4% inclusion of BA supplementation into broiler chickens diets, increased the length of duodenal and jejunal villus (Amaechi and Anueyiagu 2012). Moreover, adding 1 and 2 g/kg BA to broiler chickens diets significantly increased VH and VW, as well as CD of jejunum compared with the control but there were no treatment effects on VH/CD (Olukosi and Dono 2014). The improved morphology in digestive tract was related to a more acidic environment and higher concentration of volatile fatty acids and higher sucrase and lactase activities in the striated border, which could give rise to promoting cell growth and division through stabilising DNA and repair damage, and these factors may contribute to the better morphology in the jejunum brought about by BA (Diao et al. 2016).

Conclusions
In conclusion, based on the results of this research, addition of both levels of Phy to the diet can
beneficially influence growth performance, bone mineralisation and intestinal histomorphology in nutrients deficient corn-soybean meal based diets. However, the addition of Phy to the BA supplemented broiler diets cannot result in improved growth performance and nutrient digestibility. As well, under the condition of this study, no further benefits were achieved because of increasing the dietary BA and Phy levels beyond current recommendations, though 1000 FTU/kg of Phy was more effective to reduce litter moisture content. The reason of these results may be due to type and dose of BA and Phy in these studies. Further studies are needed to throw more light on developmental effects of those BA on the performance and intestinal histomorphology of broiler chickens.

Acknowledgements
The Authors thanks Novus International, Inc. for providing phytase and benzoic acid samples.

Ethical approval
All experimental procedures were conducted according to the international protocols and approved by Research Committe of University of Kurdistan, IRAN.

Disclosure statement
No potential conflict of interest was reported by the authors.

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