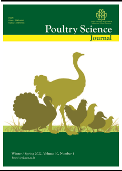

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Effect of Different Dietary Levels of Calcium and Non-Phytate Phosphorus, with a Constant Ratio of 2:1, in Starter and Grower Periods on Performance of Broiler Chickens

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Abstract

This experiment was conducted to determine the influence of different concentration of calcium (Ca) and non-phytate phosphorus (NPP), with a constant ratio of 2:1(Ca:NPP), on young broiler chickens. A total of 900 Ross 308 d-old male broiler chickens were randomly allocated to 60 pens (15 birds per pen). Four dietary treatments including high concentration of Ca and NPP (H), moderate concentration of Ca and NPP (M), low concentration of Ca and NPP (L), and very low concentration of Ca and NPP (VL) were given to the birds. The concentration of Ca was 9.6, 7.6, 5.6, and 3.6 g/kg of diet, respectively. In starter period, broiler chickens fed the M diet showed the lowest feed conversion ratio (FCR) in comparison to those received the H, L and VL diets. In grower periods, broiler chickens fed the H diet had the highest average daily feed intake (ADFI); M and L diets showed the highest average daily weight gain (ADG) compared to H and VL diets. In whole period, broiler chickens fed the H and M diets showed the highest ADFI in comparison to those received the L and VL diets and FCR was higher in broiler chickens received H diet in comparison to those fed M, L and VL diets. Decreasing the dietary Ca and NPP level elicited linear reductions in tibia Ca. The count of lactic acid bacteria in the duodenum improved with increasing levels of Ca and NPP. In conclusion, M treatment could support maximal ADG and body weight, while lowest FCR was obtained from birds received L diets. The use of L treatment resulted in comparable Ca and phosphorus content of tibia bone compared to those of M treatment.

Introduction

The management of calcium (Ca) and phosphorus (P) availability in poultry nutrition is a key issue in achieving an optimal level of integral functions in metabolism and skeletal health (Rath *et al*., 2000; Sharpley *et al*., 2007). Diets with inadequate Ca and non-phytate phosphorus (NPP) concentrations or imbalanced in Ca:NPP ratio can lead to skeletal disorders and impaired growth performance (Applegate *et al*., 2003; Gautier *et al*., 2017). High Ca and P contents in the diet reduce the energy value of the diet and interfere with the availability of other minerals, which result in growth depression and retarded skeletal mineralization (Shafey, 1993; Sebastian *et al*., 1997; Selle *et al*., 2009; Bradbury *et al*., 2014). Moreover, excess dietary Ca and P build complexes with proteins and amino acids (AA), resulting in reduced AA digestibility and increased endogenous AA losses (Shafey and McDonald, 1991; Cowieson *et al*., 2004). On the other hand, feeding Ca and P closer to the nutritional requirement of the broiler chickens could increase the digestibility of P and Ca, which is the main approach to reduce the high concentration of Ca and P in broiler chickens' manure (Moore, 1998; McGrath *et al*., 2005). Therefore, to minimize overfeeding of these macro minerals, application of decent nutritional strategies for effective use of Ca and P is necessary.

One of Ca and P feeding strategies involves formulating diets with a constant ratio of Ca and

NPP. Studies have indicated that dietary needs for Ca and NPP are interdependent (Applegate *et al*., 2003; Yan *et al*., 2005), and high Ca and NPP concentrations or a wide dietary Ca:NPP ratio causes lower utilization of NPP (Driver *et al*., 2005b; Gautier *et al*., 2017). It has been shown that different dietary concentrations of Ca and NPP compared to a constant ratio of 2:1 have a comparable effect on broiler chickens performance during the starter period (Gautier *et al*., 2017). More noticeable, some researchers indicated that Ca:NPP ratio might be more significant than absolute individual dietary concentrations of Ca and NPP for mineral digestibility (Wilkinson *et al*., 2014). Also, Díaz-Alonso *et al*. (2019) suggested that by considerng a constant ratio of 2:1, highest body weight gain, highest Ca and P level in the tibia ash and weight of ash can be reached in broiler chickens fed diets with an aP level of 5.3 g/kg. A narrow Ca:NPP ratio may decrease the development of insoluble phytate complexes and the formation of Ca orthophosphate and consequently increases the availability of Ca and NPP for absorption (Gibson and Ullah, 1990; Gautier *et al*., 2017).

Another strategy of Ca and P feeding is applying early dietary Ca and NPP restriction to improve the absorption efficiency of Ca and NPP in broiler chicken. A few studies have evaluated the capacity of chickens to adapt to low Ca and NPP concentrations (Yan *et al*., 2005; Rousseau *et al*., 2016). With maintaining a similar Ca:NPP ratio of 2.1:1, after an 11-d period of NPP depletion, sub-deficient chicks were able to compensate for NPP and Ca deficiency (e.g. 0.28% NPP and 0.6% Ca) and improve growth performance and bone characteristics to a level not significantly different from the positive control (e.g. 0.39% NPP and 0.69% Ca) (Letourneau-Montminy *et al*., 2008). Therefore, the inclusion of the lowest possible Ca and NPP concentration in the early stages can help maximize growth performance (Yan *et al*., 2005; Letourneau-Montminy *et al*., 2008; Rousseau *et al*., 2016).

The microbial population and composition in the gastrointestinal tract (GIT) of broiler chickens is important for feed digestion, pathogen exclusion, and immune system stimulation (Zhu *et al*., 2002). Several studies have shown that changes in dietary Ca and P supplementation have an effect on the activity, population and composition of the microbial community colonizing in GIT of broiler chickens (Ptak *et al*., 2015; Borda-Molina *et al* , 2016). Also, several studies in pigs and poultry revealed that dietary P and Ca have some impact on immune system through changing the bacterial microbiota and pH in gastrointestinal tracts (Metzler *et al*., 2010; Walk *et al*., 2012). Because gut microbiota is engaged in the enzymatic hydrolysis of nutrient components in the GIT, it's important to understand the role of the gut microbiota in improving the use of minerals like Ca and P by birds.

The objective of this experiment was to determine the influence of the constant Ca: NPP ratio of 2:1 over a range of Ca and NPP concentrations in corn-soybean meal diets fed to broiler chickens from 1 to 10 and 11 to 24 days of age. In addition, bone parameters, carcass characteristics, antibody titer against Newcastle disease virus and duodenal microbiota were measured in this study.

Materials and methods

Birds housing and dietary treatments

All procedures were approved by the University of Zanjan's Animal Care and Use Committee. This study was conducted at the research farm of Zarbal Company (Mazandaran, Iran). The nine-hundred one-day-old male broiler chickens (Ross 308) were randomly assigned to 60 pens (15 chicks per pen, 1.5×1.5 m). Initial mean and range of BW were similar (38 ± 0.5 g) for all pens. Water and mash diets were provided *ad libitum* for birds throughout the experiment. Wood shaving was used as bedding material in floor pens. Room temperature was kept at 32 ºC during the first 3 d of age, and then it was reduced gradually until reaching 21ºC. Birds were reared under 24 h light on day 1, 23 h on day 2 and 18 h thereafter.

This experiment involved four dietary treatments over starter (1 to 10 days) and grower (11 to 24 days) periods (Table 1). Diets were based on corn and soybean meal and formulated based on the nutrient requirements catalogue of the Ross 308 (2014). Diets were iso-energetic and iso-nitrogenous, except for Ca and NPP. Broiler chickens were received one of the following diets: H) high concentration of calcium (9.6 g/kg of diet) and NPP (4.8 g/kg of diet), M) medium concentration of calcium (7.6 g/kg of diet) and NPP (3.8 g/kg of diet), L) low concentration of calcium (5.6 g/kg of diet) and NPP (2.8 g/kg of diet), and VL) very low concentration of calcium (3.6 g/kg of diet) and NPP (1.8 g/kg of diet). The number of replications per treatment followed as 24, 18, 12 and 6, respectively, for H, M, L and VL treatments.

Chemical analysis

The experimental diets were sampled and analyzed for Ca (AOAC, 1995; method 935.13), total P (AOAC, 1995; method 965.17) and crude protein of diets was also measured (AOAC, 1995; method 984.13). The protocol of Van Soest *et al*. (1991) was applied to measure aNDFom (ash-free neutral detergent fiber, without sodium sulfite) and ADFom (ash-free acid detergent fiber) of diets.

Table 1. Ingredient composition and nutrient content of the diets from 1 to 24 d of age (%, as-fed basis, unless otherwise indicated).

	H[6]		M[7]		L[8]		VL[9]	
	1-10 d	11-24 d	1-10 d	11-24 d	1-10 d	11-24 d	1-10 d	11-24 d
Ingredient								
Corn	46.35	51.51	47.92	53.10	49.49	54.64	51.07	56.22
Soybean meal	44.18	38.36	43.88	38.05	43.58	37.75	43.27	37.45
Soybean oil	4.90	5.55	4.39	5.04	3.87	4.53	3.36	4.02
Calcium carbonate	1.12	1.13	0.92	0.92	0.72	0.72	0.52	0.52
Dicalcium phosphate	1.80	1.86	1.24	1.30	0.68	0.75	0.12	0.19
Common Salt	0.31	0.31	0.31	0.31	0.31	0.31	0.30	0.31
Sodium bicarbonate	0.24	0.23	0.24	0.23	0.24	0.24	0.25	0.24
DL-Methionine	0.35	0.32	0.35	0.32	0.35	0.32	0.35	0.31
L-Lysine HCl	0.17	0.16	0.17	0.16	0.18	0.17	0.18	0.17
L-Threonine	0.08	0.07	0.08	0.07	0.08	0.07	0.08	0.07
Vitamin premix[1]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Mineral premix[2]	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Calculated analysis[3]								
AME_n[4] (kcal/kg)	3000	3100	3000	3100	3000	3100	3000	3100
CP	23.80	21.64	23.80	21.64	23.80	21.65	23.80	21.65
Lysine	1.44	1.29	1.44	1.29	1.44	1.29	1.44	1.29
Methionine + Cystine	1.08	0.99	1.08	0.99	1.08	0.99	1.08	0.99
Threonine	0.97	0.88	0.97	0.88	0.97	0.88	0.97	0.88
Valine	1.10	1.00	1.10	1.00	1.10	1.00	1.10	1.00
Isoleucine	1.00	0.90	1.00	0.90	1.00	0.90	1.00	0.90
Calcium	0.96	0.96	0.76	0.76	0.56	0.56	0.36	0.36
Non-phytate phosphorus	0.48	0.48	0.38	0.38	0.28	0.28	0.18	0.18
Total phosphorus	0.74	0.73	0.64	0.63	0.54	0.53	0.45	0.43
Sodium	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Chlorine	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26
Potassium	1.03	0.93	1.02	0.92	1.02	0.92	1.02	0.92
DCAD[5] (mEq/kg)	277	251	276	250	275	249	275	249
Determined analysis								
Dry matter	91.80	92.00	91.90	91.80	91.90	91.90	92.00	91.90
CP	23.70	21.90	23.70	21.60	23.60	21.80	23.50	22.00
Calcium	0.99	1.04	0.79	0.80	0.55	0.51	0.40	0.40
Total phosphorus	0.69	0.70	0.60	0.61	0.54	0.53	0.47	0.50
Ash	7.4	6.8	7.8	6.7	6.8	6.5	6.6	6.4
aNDFom	12.8	12.5	12.2	12.1	12.3	12.1	12.0	11.9
ADFom	6.2	6.1	6.4	6.0	6.2	6.0	6.0	5.9

[1]Provided the following per kilogram of diets: vitamin A, 9,000 IU (retinyl acetate); cholecalciferol, 2,000 IU; vitamin E, 36 IU (dl-α-tocopheryl acetate); vitamin B_{12}, 0.015 mg; menadione, 2 mg; riboflavin, 6.6 mg; thiamine, 1.8 mg; pantothenic calcium, 10 mg; niacin, 30 mg; folic acid, 1 mg; biotin, 0.1 mg; pyridoxine, 3 mg.
[2]Provided the following per kilogram of diets: manganese ($MnSO_4 \cdot H_2O$), 100 mg; zinc (ZnO), 85 mg; iron ($FeSO_4 \cdot 7H_2O$), 50 mg; copper ($CuSO_4 \cdot 5H_2O$), 10 mg; selenium (Na_2SeO_3), 0.2 mg; iodine (calcium iodate), 1 mg; choline (choline chloride), 250 mg.
[3]The information from National Research Council (1994) was used for calculation of nutrients' content.
[4]AME_n = apparent metabolizable energy corrected for nitrogen.
[5]DCAD = dietary cation-anion difference (Na+K-Cl).
[6]H = high concentration of calcium and phosphorus in the starter and grower diets.
[7]M = medium concentration of calcium and phosphorus in the starter and grower diets.
[8]L = low concentration of calcium and phosphorus in the starter and grower diets.
[9]VL = very low concentration of calcium and phosphorus in the starter and grower diets.

Growth performance and carcass characteristics

Feed intake and body weight (BW) were measured on days 1, 10 and 24 and ADFI, ADG and FCR were calculated for rearing periods (1 to 10, 11 to 24 and 1 to 24 days of age). Feed intake was adjusted for mortality, and the relevant ADG was included in the calculation of adjusted FCR. Subsequent to the weighing of broiler chickens at the end of the experiment (24 days), 3 birds per replicate (6 replicates per treatment, 18 birds per treatment) were randomly selected and slaughtered by cervical dislocation to determine some carcass characteristics (hot carcass, breast and thigh + drumstick) and relative weight of internal organs (liver, heart, abdominal fat, and gizzard) as g/100 g BW.

Bone characteristics

At the end of day 24 (after blood sampling and measuring BW of birds), 3 birds per replicate (6 replicates per treatment) were slaughtered to have tibia samples. After measuring the length and width of tibia samples, the concentration of Ca and P of tibia were determined. The concentration of Ca and P was determined using the following method: the tissue was stripped off from bones, and tibia was dried overnight at 100 °C and ashed in a muffle furnace at 540 °C for 6 h, then ash was solubilized with Ultra-pure HNO3 (16 M) and hydrogen peroxide (30%) and left on a digestion block until it was completely dissolved in nitric acid (0.4 M). Afterwards, the dilution was performed in 0.1 g/L lanthanum oxide solution for determination of Ca. The concentration of calcium in the prepared solution was measured by atomic absorption spectrophotometer. Acid molybdate reducer solutions were used for analysis of P concentration (through the formation of a phosphomolybdenum complex) by spectrophotometer (Perkin Elmer Optima 2100 DV) at the wavelength of 400 nm (AOAC, 2006).

Antibody titer against Newcastle disease virus

On day 24, 3 birds per replicate (6 replicates per treatment) were randomly selected, and blood sampled via brachial vein. After centrifugation (4000 *g*, 10 minutes), serum samples were tested for antibodies titer against NDV using IDEXX ELISA Kit.

Duodenal microbiota

At the end of day 24 of experiment, 3 birds per replicate (6 replicates per treatment) were chosen, slaughtered and the digesta of the duodenum was gently flushed out using distilled water and pooled for all birds of one pen separately. The plate culture method was used to obtain the count of lactic acid bacteria and *Escherichia coli* (*E. coli*). Briefly, one g of duodenal digesta was sampled, serially diluted and plated on duplicate using Eosin Methylene-Blue agar media (Merck, Germany) to enumerate *E. coli*, Rogosa agar media (Merck, Germany) to enumerate lactic acid bacteria, and plate count agar (Merck, Germany) to enumerate total aerobic bacteria. Plates were incubated at 37 °C for 24 h aerobically (for the count of total aerobic bacteria and *E. coli*) and 41°C for 72 h anaerobically (for the count of lactic acid bacteria). After the incubation period, the number of colonies on each pellet was counted. Dilutions with 30 to 300 colonies were counted. Manual method was used for colony counting. In this method, the plate is placed upside down on white paper and a checkered counting glass is placed between the plate and the paper, and the number of colonies is obtained by marking the cells. The average count in two plates was calculated and the number of colonies was determined by taking into account the dilution coefficient.

Statistical analysis

Data were analyzed as a completely randomized design with four treatments (diets) using the GLM procedure of SAS (SAS, 2003). Pen was considered as the experimental unit for all parameters. Some contrasts were used to determine the linear (Lin) and quadratic (Q) effect of H vs. M vs. L vs. VL in the whole period. All statements of significance are based on $P \leq 0.05$ and tendency was based on $0.05 < P \leq 0.10$.

Results

Growth performance

The effect of dietary treatments on growth performance from 1 to 10 and 11 to 24 days of age (starter and grower periods) is presented in Table 2. The results of the present study showed that in starter period, broiler chickens fed the H and M diets showed the highest ADFI in compare to those received L diet ($P \leq 0.001$; Q, $P \leq 0.05$). The birds under M treatment showed the highest ADG during the starter period ($P \leq 0.05$). Broiler chickens received the M and L diets showed the lowest FCR in compare to those received the H and VL diets (Q, $P \leq 0.05$).

In the grower period, broiler chickens fed the H and M diets had higher ADFI than L and VL groups (Lin, $P \leq 0.001$; Q, $P \leq 0.05$). Broiler chickens fed M and L diets showed the highest ADG in comparison to the H and VL diets (Lin, Q, $P \leq 0.001$). Broiler chickens fed the H diet had higher FCR in comparison to those fed M, L and VL diets. Moreover, the use of M diet improved the FCR compared to the L diet (Lin, Q, $P \leq 0.001$).

In terms of growth performance for the whole period (1 to 24 days), H and M diets showed the highest ADFI compared to L and VL diets (Lin, $P \leq 0.001$; Q, $P \leq 0.05$). Broiler chickens fed M and L diets showed the highest ADG compared to those received H and VL diets (Q, $P \leq 0.001$). Broiler chickens received the M and L diets had the highest BW than those received the H and VL diets (Q, $P \leq 0.0001$). Broiler chickens fed H diet showed higher FCR than those fed M, L and VL diets. Also, the M treatment improved FCR when compared to the L treatment (Lin, Q, $P \leq 0.001$). The highest percentage of mortality was observed in broiler chickens fed VL diet (Lin, $P \leq 0.01$; Q, $P \leq 0.05$).

Carcass characteristics

The effect of dietary treatments on carcass characteristics, liver, heart, abdominal fat, and gizzard are represented in Table 3. There was no significant difference in carcass characteristics, the relative weight of liver, heart and abdominal fat among dietary treatments. However, the M diet increased the relative weight of the gizzard compared to that of the H, L and VL diet (Lin, $P \leq 0.05$).

Table 2. Growth performance and mortality of broiler chickens fed with different diets.

	1-10 d				11-24 d			1-24 d				
Diets	ADFI[6] (g)	ADG[7] (g)	BW[8] (g)	FCR[9]	ADFI (g)	ADG (g)	FCR	ADFI (g)	ADG (g)	BW (g)	FCR	Mortality (%)
H[1] (n = 24)	29.4^{a}	19.1^{b}	237	1.54^{a}	88.2^{a}	52.0^{b}	1.69^{a}	63.7^{a}	38.3^{b}	965^{b}	1.66^{a}	0.83^{b}
M[2] (n = 18)	29.6^{a}	19.8^{a}	244	1.49^{b}	87.6^{a}	57.3^{a}	1.53^{b}	63.4^{a}	41.7^{a}	1045^{a}	1.52^{b}	0.00^{b}
L[3] (n = 12)	28.1^{b}	18.7^{b}	233	1.50^{b}	83.4^{b}	56.7^{a}	1.47^{c}	60.4^{b}	40.9^{a}	1026^{a}	1.48^{c}	1.67^{b}
VL[4] (n = 6)	29.0^{ab}	18.7^{b}	232	1.55^{a}	78.6^{c}	52.8^{b}	1.49^{bc}	58.0^{c}	38.6^{b}	971^{b}	1.50^{bc}	4.44^{a}
SEM[5]	0.29	0.26	2.9	0.013	0.65	0.45	0.013	0.45	0.33	8.0	0.011	1.07
P-value												
Diets	0.005	0.02	0.01	0.01	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.01
Linear	0.10	0.11	0.10	0.48	<0.0001	0.51	<0.0001	<0.0001	0.99	0.99	<0.0001	0.003
Quadratic	0.25	0.23	0.25	0.002	0.007	<0.0001	<0.0001	0.04	<0.0001	<0.0001	<0.0001	0.02

[1]H = high concentration of calcium and phosphorus in the starter and grower diets (n = 24).
[2]M = medium concentration of calcium and phosphorus in the starter and grower diets (n = 18).
[3]L = low concentration of calcium and phosphorus in the starter and grower diets (n = 12).
[4]VL = very low concentration of calcium and phosphorus in the starter and grower diets (n = 6).
[5] *SEM* = standard error of the mean.
[6]ADFI = average daily feed intake.
[7]ADG = average daily gain.
[8]BW = body weight.
[9]FCR = feed conversion ratio.

Bone characteristics

The effect of experimental diets on bone characteristics at 24 day is presented in Table 4. Tibia Ca decreased linearly ($P \leq 0.001$) when dietary Ca and NPP level decreased. The tibia P was affected quadratically ($P \leq 0.05$) by dietary treatments; the H and L diets had the lowest and highest values, respectively. The H diet had the highest tibia width (Lin, Q, $P \leq 0.05$) in comparison to those of the M, L and VL diets.

Table 3. Carcass characteristics (% of body weight) of broiler chickens fed with different diets.

Diets	Hot carcass	Breast	Thigh+Drumstick	Abdominal fat	Liver	Heart	Gizzard
H[1]	65.0	20.6	19.3	0.145	1.13	0.52	2.29[ab]
M[2]	66.0	22.0	20.7	0.140	1.13	0.53	2.39[a]
L[3]	65.6	20.3	19.0	0.145	1.11	0.51	2.24[b]
VL[4]	65.6	20.9	19.6	0.143	1.11	0.52	2.22[b]
SEM[5]	0.70	0.54	0.53	0.002	0.013	0.006	0.037
P-value							
Diets	0.79	0.16	0.15	0.59	0.30	0.28	0.03
Linear	0.61	0.79	0.75	1.00	0.12	0.53	0.05
Quadratic	0.50	0.50	0.49	0.57	0.72	0.81	0.12

[1]H = high concentration of calcium and phosphorus in the starter and grower diets.
[2]M = medium concentration of calcium and phosphorus in the starter and grower diets.
[3]L = low concentration of calcium and phosphorus in the starter and grower diets.
[4]VL = very low concentration of calcium and phosphorus in the starter and grower diets.
[5] *SEM* = standard error of the mean.

Table 4. Bone parameters of broiler chickens fed with different diets.

Diets	Tibia			
	Calcium (% of ash)	Phosphorus (% of ash)	Length (mm/ 100 g BW)	Width (mm/ 100 g BW)
H[1]	39.4[a]	18.7	5.93	0.75[a]
M[2]	39.1[a]	19.5	5.62	0.69[b]
L[3]	38.8[ab]	19.8	5.54	0.69[b]
VL[4]	38.2[b]	19.3	5.71	0.70[b]
SEM[5]	0.24	0.32	0.168	0.019
P-value				
Diets	0.009	0.12	0.41	0.05
Linear	0.001	0.17	0.33	0.05
Quadratic	0.51	0.04	0.17	0.05

[1]H = high concentration of calcium and phosphorus in the starter and grower diets.
[2]M = medium concentration of calcium and phosphorus in the starter and grower diets.
[3]L = low concentration of calcium and phosphorus in the starter and grower diets.
[4]VL = very low concentration of calcium and phosphorus in the starter and grower diets.
[5] *SEM* = standard error of the mean.

Table 5. Antibody titer against Newcastle disease virus and duodenal microbial population ($\log_{10}$ CFU/g of fresh digesta) of broiler chickens fed with different diets.

Diets	Antibody titer (%)	Microbial population		
		Total bacteria	*E. coli*	Lactic acid bacteria
H[1]	5.61	6.17[a]	4.82	5.40[a]
M[2]	5.16	5.83[ab]	4.84	5.06[ab]
L[3]	5.11	5.34[b]	4.40	4.61[b]
VL[4]	5.66	5.23[b]	4.40	4.55[b]
SEM[5]	0.351	0.218	0.224	0.220
P-value				
Diets	0.57	0.02	0.38	0.04
Linear	0.94	0.002	0.12	0.006
Quadratic	0.17	0.59	0.96	0.54

[1]H = high concentration of calcium and phosphorus in the starter and grower diets.
[2]M = medium concentration of calcium and phosphorus in the starter and grower diets.
[3]L = low concentration of calcium and phosphorus in the starter and grower diets.
[4]VL = very low concentration of calcium and phosphorus in the starter and grower diets.
[5] *SEM* = standard error of the mean.

Antibody titer against Newcastle disease virus and duodenal microbiota

The effect of dietary treatments on antibody titer against Newcastle disease virus and duodenal microflora population are presented in Table 5. Changing dietary Ca and NPP levels had no impact on antibody titer against Newcastle disease virus at 24 d. The count of total aerobic bacteria in duodenal microbiota increased with increasing levels of Ca and NPP, and broiler chickens fed the H diet had the highest intestinal total aerobic bacteria in compare to the M, L and VL diets (Lin, $P \leq 0.05$). Also, the count of lactic acid bacteria in duodenal microbiota population increased with increasing levels of Ca and NPP, and broiler chickens fed the H diet had the highest intestinal lactic acid bacteria count in compare to M, L and VL diets (Lin, $P \leq 0.05$).

Discussion

The majority of research is based on inconstant ratio of Ca: NPP at different levels for evaluation of growth performance and bone characteristics and adaptive response. Therefore, the objective of this experiment was to determine the influence of the constant Ca:NPP ratio of 2:1 over a range of Ca and NPP concentrations. Findings of previous research on the influence of Ca: NPP ratio in different concentrations of Ca and NPP provide a clear indication that the inclusion of Ca and NPP beyond bird requirements negatively affect the homeostasis of these minerals (Rao *et al*., 2006; Hamdi *et al*., 2015; Gautier *et al*., 2017, Xu *et al*., 2021). Hence, broiler chicken growth performance and nutrient retention can be deteriorated due to the ability of excess Ca to chelate with both P and phytate (Selle *et al*., 2009) and the ability of excess Ca and P to interfere with the availability of other minerals (Bradbury *et al*., 2014; Wilkinson *et al*., 2014; Gautier *et al*., 2017); therefore, the absolute concentrations of Ca and NPP while Ca:NPP ratio is maintained at constant ratio of 2:1, is also important.

In the current study, over starter/grower periods, dietary treatments that contained 0.76% Ca and 0.38% NPP inclusion were able to support maximal ADG and BW, while lowest FCR was obtained from birds fed diets containing 0.56% Ca and 0.28% NPP. Overall, in starter/grower periods, the Ca:NPP ratio at 2:1 with dietary Ca and P inclusion of 0.76% Ca and 0.38% NPP maximized growth performance parameters. This finding coincides with data from Gautier *et al.* (2017) who conducted an experiment with three concentrations of Ca (0.4, 1.0, or 1.6%) with constant NPP concentrations either at 0.45% or adjusted to maintain a dietary Ca:NPP ratio of 2:1. These researchers reported that responses in growth performance were greatest in birds that received diets containing 0.6% Ca while NPP concentration was maintained at 0.3%. Furthermore, Mello *et al*. (2012) revealed that while keeping the Ca:aP ratio equal to 2:1, ADG increased until reaching a plateau level at 0.33% aP. Also, in accordance with our findings, David *et al*. (2021) indicated that the estimated Ca and P requirement for weight gain is lower than the current Ca recommendation by commercial strains (0.96% Ca and 0.48% aP) for broiler chicken starter diets.

Dietary P restriction lead to hypercalcaemia and hypophosphataemia while dietary Ca restriction can cause mild hypocalcaemia (Bar *et al*., 2003). Also, calcium appetite can be inhibited by increased concentrations of ionic calcium in the blood, and the change in behavior is sufficiently fast to adjust the calcium homeostasis of birds (Lobaugh *et al*., 1981). On the other hand, it has been shown that excess dietary Ca can reduce digesta transit time, restrict the availability of other minerals, and impair absorption (Shafey and McDonald, 1991; Yan *et al*., 2005; Wilkinson *et al*., 2014).

In the present study, Ca and NPP levels did not affect carcass, breast, thigh + drumstick and abdominal fat percentage. Also, relative weight of liver and heart were not affected by dietary treatments. However, birds fed M diet had the highest gizzard weight in comparison to other levels. This result is in line with the result of Abdulla *et al*., (2017) who reported no significant difference in the carcass percentage of broiler chickens fed different levels of Ca. Our results are in contrast to the study of Talpur *et al*. (2012) who observed that dressing percentage was higher in broiler chicken fed 1% of the calcium in comparison to those fed 2% and 3%. Also, Ghobadi *et al*., (2010) reported that a reduction in dietary aP reduced the carcass weight. These contradictory results could be due to different BW observed in birds fed different levels of Ca and P compared with other levels in various studies. Poultry can compensate for minor Ca and P deficiency by displacing bone reserves, increasing renal reabsorption, and other physiological mechanisms, but if the deficiency of Ca and P is more severe than the tolerance threshold of birds, it will mainly affect feed efficiency and body weight (Walk *et al*., 2012).

It has been demonstrated that maintaining Ca: NPP ratio of 2:1 make a possibility of reducing Ca and NPP inclusion in diets without a negative impact on growth performance (Mello *et al*., 2012; Rousseau *et al*., 2016). Moreover, Yan *et al*. (2005) indicated that broiler chickens received low Ca and NPP diets had higher apparent retention of total Ca and P, which confirms the ability of birds to adapt to moderate deficiency. Also, previous research demonstrated that chickens fed a diet moderately deficient in P and Ca in the earlier rearing phase can partially adapt to the deficiency through the improvement of digestive efficiency, the increased ileal P disappearance, increased ileal absorption of P and Ca, compensatory growth performance, and compensatory improvement in bone parameters in a later growth phase (Yan *et al*., 2005; Rousseau *et al*., 2016). Furthermore, because of carry-over effect of feeding diets with sufficient level of NPP in the previous phase, performance parameters may not be deteriorated by deficient dietary NPP in birds in later phase (Nelson *et al*., 1990). When inclusion of NPP in the diet is below requirement, chickens can provide P by immobilization form the bones for the physiological and metabolic needs; therefore in range of certain deficiency, performance is not affected when broiler chickens receive NPP-deficient diets in later phases, particularly after 32 d of age (Skinner *et al*., 1992). However, the practical application of this adaptation process to further fine-tune the dietary levels and the chronology of dietary P and Ca provision are not defined. Considering findings of current and previous research, it can be suggested that applying the adaptation principle in broiler chicken combined with maintaining Ca:NPP ratio a 2:1 over dietary phases may allow for reducing the level of Ca and NPP without compromising performance.

According to most recent experiments with a constant Ca:NPP ratio of 2:1 there is a possibility of reducing Ca and NPP inclusion in diets with maintaining a ratio of 2:1, but inclusion of Ca and NPP under certain amount could not support the optimum growth. For example, Mello *et al*. (2012) recommended 0.36% and 0.72% of aP and Ca for 22 to 33 d and 0.26% and 0.51% of aP and Ca for 34 to 46 d for optimum growth performance in female broiler chickens. Also, in experiments by Yan *et al*. (2005) and Rousseau *et al*. (2016), inclusion of Ca and NPP levels less than 0.6% Ca, 0.3% NPP were not investigated. According to Gautier *et al*. (2017), very low level of Ca and NPP (0.4% Ca, 0.2% NPP) could not catch up to the birds fed higher levels of Ca and NPP with the same constant ratio of 2:1 over 1 to 21 days. Moreover, some researchers concluded that performance parameters are not the best indicator of NPP requirement and NPP requirement when performance is the sole criterion, would be less than 0.15% (Yan *et al*., 2001; Dhandu and Angel, 2003). Therefore, considering different findings in the literature, it can be concluded that the requirement of Ca and NPP in broiler chickens can be different depending on whether the interdependent relation of Ca and NPP ratio is considered in the assessment method or not.

Ca content of tibia was influenced by dietary treatments in starter/grower periods. Unlike growth performance, highest Ca content and width of tibia bones were obtained from birds fed high levels of Ca and NPP. These findings are consistent with other studies that show growth performance and bone mineralization respond in different direction to dietary manipulations of Ca (Letourneau-Montminy *et al*., 2008; Gautier *et al*., 2017). Gautier *et al*. (2017) showed that tibia height, length, and width were greater in birds fed diets that contained a 0.4% or 0.6% Ca compared with birds fed higher Ca inclusions. However, these researchers indicated that tibia break force and ash were reduced at the lowest Ca (0.4%) and NPP (0.2%) concentrations even ratio of Ca:NPP was maintained constant at 2:1 from 2 to 23 days. Difference in observed effects reported in different studies on using low Ca diets in broiler chicken can be as a result of a narrow ratio of Ca:P balance in diets rather than a Ca deficiency (Delezie *et al*., 2012). Also, it has been shown that high Ca levels can aggravate P deficiency, resulting in appetite loss and decreased growth of bone tissues (Driver *et al*., 2005a). Moreover, although it has been indicated that concomitant and coordinated reduction of dietary Ca and P levels had no negative effects on bone mineralization, it is recommended that maximal performance and retention results can be obtained by reduce the minerals in a balanced way (Delezie *et al*., 2012).

In the current study, the addition of Ca and NPP increased the total number of bacteria, as well as lactic acid bacteria. In line with our finding, Ptak *et al*. (2015) showed that with decreasing Ca and P levels in the diet, the total microbial population in the intestine decreased. These researchers also reported that the count of *Clostridium perfringens* and *Enterobacteriaceae* decreased in chickens that fed low Ca and aP diets, but different levels of available Ca and P did not affect the count of *Streptococcus* and *Lactococcus* bacteria. Some other studies in broiler chickens and pigs have shown that supplementing diets with Ca and P increases Lactobacillus abundance (Metzler-Zebeli *et al*., 2010; Borda-Molina *et al*., 2016). Ca and P supply can play a controlling role in the total numbers of bacteria; it has been indicated that decreasing Ca, and P in the diet leads to a decrease in the levels of short-chain fatty acids, lactate and acetic acid in the ileum of broiler chickens (Ptak *et al*., 2015). These findings suggest that Ca and P may be limiting factors for fermentation in the ileum and moderating microflora in the intestine.

The antibody titer against the Newcastle disease virus was not affected by dietary Ca and NPP levels. Our results contrast with the study of Liu *et al*. (2008) and Nie *et al*. (2018) who reported that increasing the NPP level can lead to changes in the rate of efficiency and function of lymphocytes in birds and consequently improve the function of the immune system. Also, Emami *et al*. (2013) showed that reducing aP levels in the diet compared to the high levels of aP in diet decreased the antibody titer against sheep red blood cells (SRBC). Emami *et al*. (2013) indicated that the reduction of aP in the diet of broiler chickens led to a decrease in the levels of immunoglobulin G and M (IgM and IgG) in the blood.

Mortality rate in the current study was significantly affected by Ca and NPP level in diets. This effect was more evident at the lowest levels of Ca and NPP (VLVL). It was expected that the dietary treatment used in this study would affect mortality because previous research showed that 0.2% NPP is necessary to minimize problems with mortality. Waldroup *et al*. (2000) concluded that NPP requirements for maximum growth performance are higher in comparison to the NPP requirements for minimum mortality rate.

Conclusion

According to the current study, over starter/grower periods, dietary treatments that contained 0.76% Ca and 0.38% NPP were able to support maximal ADG and BW, while lowest FCR was obtained from birds fed diets containing 0.56% Ca and 0.28% NPP. Use of 0.56% Ca and 0.28% NPP during the starter and grower period showed a comparable bone Ca and P in comparison to those of 0.76% Ca and 0.38% NPP. Furthermore, the findings of the current study are not in line with the current recommendation of broiler chicken's nutrient guidelines, where they suggest a slightly decreasing level of Ca and NPP from starter period to grower period. Reconsideration of current recommendation by a different provider of commercial strains of broiler chicken in this respect would be of worth. Also, further studies on different levels of Ca and NPP in starter, grower and finisher periods and their carry-over effects on the following periods are required.

Conflict of interest

None.

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