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Non Ruminant Nutrition

Evaluation of optimal dietary calcium level by bone characteristics and calcium metabolism-related gene expression of broilers from 22 to 42 d of age

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Abstract

The current dietary Ca recommendation of broilers is primarily based on the previous studies carried out more than 30 yr ago. However, the modern commercial broilers are quite different from those more than 30 yr ago. The present experiment was conducted to evaluate an optimal dietary Ca level by bone characteristics and Ca metabolism-related gene expression of broilers fed a corn-soybean meal diet from 22 to 42 d of age. A total of 252 22-d-old Arbor Acres male broilers were randomly assigned to 1 of 7 treatments with 6 replicate cages of 6 birds per cage for each treatment. Broilers were fed the corn-soybean meal diets containing 0.50%, 0.60%, 0.70%, 0.80%, 0.90%, 1.00%, or 1.10% Ca for 21 d, and each diet contained 0.31% non-phytate P. The results showed that the mineral contents in tibia and middle toe bone, mineral density in tibia and middle toe bone, middle toe ash percentage, middle toe ash Ca percentage, and tibia alkaline phosphatase mRNA expression level of broilers were influenced ($P < 0.04$) by dietary Ca level and increased quadratically ($P < 0.05$) as dietary Ca level increased. The estimates of optimal dietary Ca levels were 0.55%, 0.60%, 0.70%, 0.72%, 0.63%, 0.66%, and 0.70%, respectively, based on the best fitted broken-line, quadratic, or asymptotic models ($P < 0.02$) of the above sensitive indices. These results indicate that the optimal dietary Ca level would be 0.72% to support all of the Ca metabolism and bone development of broilers fed the corn-soybean meal diet from 22 to 42 d of age.

Lay Summary

The present experiment was conducted to evaluate an optimal dietary Ca level by bone characteristics and Ca metabolism-related gene expression of broilers fed a corn-soybean meal diet from 22 to 42 d of age. A total of 252 22-d-old Arbor Acres male broilers were randomly assigned to 1 of 7 treatments with 6 replicate cages of 6 birds per cage for each treatment. Broilers were fed the corn-soybean meal diets containing 0.50%, 0.60%, 0.70%, 0.80%, 0.90%, 1.00%, or 1.10% Ca for 21 d, and each diet contained 0.31% non-phytate P. The results showed that the tibia and middle toe bone mineral contents, tibia and middle toe bone mineral density, middle toe ash percentage, middle toe ash Ca percentage, and tibia alkaline phosphatase mRNA expression level were sensitive criteria to estimate the optimal dietary Ca levels of broilers. The estimates of optimal dietary Ca levels were 0.55% to 0.72% based on the above sensitive criteria. These results indicate that the optimal dietary Ca level would be 0.72% to support all of the Ca metabolism and bone development of broilers fed the corn-soybean meal diet from 22 to 42 d of age.

Key words: bone characteristics, broiler, calcium, gene expression, requirement

Abbreviations: ADFI, average daily feed intake; ADG, average daily gain; ALP, alkaline phosphatase; BMC, bone mineral content; BMD, bone mineral density; BMP-2, bone morphogenetic protein-2; DMP-1, dentin mateix protein-1; FCR, feed:gain ratio; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IAS-CAAS, Institute of Animal Science, Chinese Academy of Agricultural Sciences; OC, osteocalcin; OPG, osteoprotegerin; SOST, sclerostion; TRACP, tartrate-resistant acid phosphatase

Introduction

Calcium, an essential mineral, plays an important role in growth, bone development, and metabolism of broilers (Rama Rao et al., 2006; Monika and Roselina, 2013; Li et al., 2020). The current Ca requirement of broilers recommended by NRC (1994) is based on previous literature published more than 30 yr ago (Edwards et al., 1963; Waldroup et al., 1974; Yoshida and Hoshii, 1982a, Yoshida and Hoshii, 1982b). However, modern broiler's growth potential has changed fundamentally over the past decades because of genetic improvements. In addition to these changes, increasing desire to optimize resource utilization validates the need to continuously review and revise the nutrient requirements of broilers (Fallah et al., 2019). Most studies on the Ca requirement of broilers have focused on the starter phase, but there are relatively few studies on the grower phase (Onyango et al., 2003; Valable et al., 2017; Ceylan et al., 2020). Besides, the lower P requirements for commercial broilers have been suggested by some researchers in recent years (Jiang et al., 2016; Liu et al., 2017). The Ca and P are co-existing and interdependent particularly in many biological functions of broilers (Qian et al., 1997; Hulan et al., 1985). Moreover, excessive Ca can

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interfere the digestion and absorption of microminerals and has the potential to suppress the growth of broilers (Hamdi et al., 2015). Therefore, it is necessary to reevaluate Ca requirements for modern commercial broilers from 22 to 42 d of age.

Growth performance is a commonly used indicator for evaluating Ca status or requirements of broilers in previous studies (Edwards et al., 1963; Waldroup et al., 1974). Bone ash and bone strength are often used to evaluate the Ca requirement of broilers by researchers (Bar et al., 2003; Rama Rao et al., 2003). Bone mineral content (BMC) and bone mineral density (BMD) are also sensitive indicators of bone ash percentage and Ca nutritional status in broilers (Onyango et al., 2003; Valable et al., 2017). A recent study from our laboratory indicated that the BMD of tibia and middle toe, alkaline phosphatase (ALP) activities in serum and tibia, and protein expression level of ALP in tibia could be used to evaluate the Ca requirement of broilers from 1 to 21 d of age, and the Ca requirements would be about 0.60% to obtain the best growth rate, and 1.00% to meet all of the Ca metabolisms and bone development of broilers (Bai et al., 2022). Jiang et al. (2013) found that the ALP activity in serum, and osteoprotegerin (OPG) and *ALP* mRNA levels and osteocalcin (OC) protein expression levels in bone could be used to evaluate the Ca status and requirements of hens. Zhang et al. (2019) reported that the tartrate-resistant acid phosphatase (TRACP) activity in serum and the *OPG* mRNA expression level in bone increased quadratically as dietary Ca level increased in meat ducks. In addition, sclerostion (SOST), dentin mateix protein-1 (DMP-1), and bone morphogenetic protein-2 (BMP-2) have been considered to be new biochemical markers of bone turnover and Ca metabolism utilization (Chapurlat et al., 2016). However, BMC, BMD, enzyme activities, and mRNA and protein expression levels of the above Ca metabolism-related enzymes and proteins have never been used to estimate dietary Ca requirements of chickens from 22 to 42 d of age.

Therefore, we hypothesized that the BMC, BMD, and mRNA or protein expression levels of ALP, OPG, OC, DMP1, BMP-2, SOST, or TRACP in bone might be new sensitive indices to evaluate dietary Ca requirements of broilers fed a practical corn-soybean meal diet from 22 to 42 d of age, and the Ca requirements of broilers from 22 to 42 d of age might be different from the current NRC Ca requirement (0.90%). The objective of the present study was to determine the effect of dietary Ca level on the growth performance, serum parameters, bone characteristics, and Ca metabolism-related gene expressions, so as to select sensitive indicators to estimate the optimal dietary Ca level of broilers fed a practical corn-soybean meal diet from 22 to 42 d of age.

Materials and Methods

All experimental procedures were approved by the Animal Management Committee (in charge of animal welfare issue) of the Institute of Animal Science, Chinese Academy of Agricultural Sciences (IAS-CAAS, Beijing, China) and performed in accordance with the guidelines. We have followed the ARRIVE guidelines for reporting animal research (Kilkenny et al., 2012).

Animals, diets, and management

A total of 300 1-d-old Arbor Acres male commercial broilers (Huadu Broiler Breeding Co., Beijing, China) were housed in thermostatically controlled stainless steel cages equipped with watering system, and were allowed ad libitum access to the experimental diets and tap water. The temperature of the feeding house was maintained at 32 °C to 35 °C for the first week, and then gradually decreased by 2 °C to 3 °C per week, and finally maintained at 24 °C after day 29. The relative humidity was maintained at 50% to 60%. Broilers were vaccinated with Newcastle disease vaccine at 7 and 21 d of age, and infectious bursal disease virus vaccine and bronchitis virus vaccine at 14 and 28 d of age, respectively. During 1 to 21 d of age, the broilers were fed with a corn-soybean meal basal diet containing 1.00% Ca (NRC, 1994; Bai et al., 2022) and 0.39% non-phytate phosphorus (NPP) (Liu et al., 2017) on an as-fed basis, and the diet was formulated to meet or exceed the requirements (NRC, 1994; Ministry of Agriculture of P. R. China, 2004) of starter broilers for all nutrients (Table 1). At 22 d of age, a total of 252 broilers with similar body weights were randomly allotted to 1 of 7 treatments with 6 replicate cages (6 chickens per cage) for each treatment in a completely

Table 1. Ingredients and nutrient levels of diets for broilers (%, as-fed basis)

Items	Days 1 to 21	Days 22 to 42
Ingredients		
Corn	53.38	58.62
Soybean meal	37.45	32.36
Soybean oil	5.00	5.00
$CaHPO_4$	1.80	1.35
Limestone	1.43	0.00
NaCl	0.30	0.30
DL-Methionine	0.31	0.16
Premix[1,2]	0.33	0.21
Sand[3]	0.00	2.00
Total	100	100
Nutrient composition		
Metabolizable energy, Kcal/kg	3024	3079
Crude protein[4]	21.89	19.92
Lys	1.12	1.00
Met	0.59	0.42
Met + cys	1.00	0.72
Non-phytate P	0.39	0.31
P[4]	0.66	0.57
Ca[4]	1.00	0.37

[1]Provided per kilogram of diet for d 1 to 21: vitamin A (all-trans retinol acetate), 15000 IU; cholecalciferol, 4500IU; vitamin E (all-rac-α-tocopherol acetate), 24 IU; vitamin K_3 (menadione sodium bisulfate), 3 mg; thiamin (thiamin mononitrate), 3 mg; riboflavin, 9.6 mg; pyridoxine, 3 mg; vitamin B_{12}, 0.018 mg; calcium pantothenate, 15 mg; niacin, 39 mg; folic acid, 1.5 mg; biotin, 0.15 mg; choline, 700 mg; Zn ($ZnSO_4 \bullet 7H_2O$), 60 mg; Cu ($CuSO_4 \bullet 5H_2O$), 8 mg; Mn ($MnSO_4 \bullet H_2O$), 110 mg; Fe ($FeSO_4 \bullet 7H_2O$), 40 mg; Se (Na_2SeO_3) 0.35 mg; I (KI), 0.35 mg; chlortetracycline, 50 mg.
[2]Provided per kilogram of diet for d 22 to 42: vitamin A (all-trans retinol acetate), 10000 IU; cholecalciferol, 3000 IU; vitamin E (all-rac-α-tocopherol acetate), 16 IU; vitamin K_3 (menadione sodium bisulfate), 2 mg; thiamin (thiamin mononitrate), 2 mg; riboflavin, 6.4 mg; pyridoxine, 2 mg; vitamin B_{12}, 0.012 mg; calcium pantothenate, 10 mg; niacin, 26 mg; folic acid, 1 mg; biotin, 0.1 mg; choline, 500 mg; Zn ($ZnSO_4 \bullet 7H_2O$), 40 mg; Cu ($CuSO_4 \bullet 5H_2O$), 8 mg; Mn ($MnSO_4 \bullet H_2O$), 80 mg; Fe ($FeSO_4 \bullet 7H_2O$), 30 mg; Se(Na_2SeO_3), 0.39 mg; I (KI), 0.35 mg.
[3]Sand was washed by deionized water, and contained no detectable Ca and P.
[4]Values determined by analysis. Each value based on triplicate determinations.

randomized design. The dietary Ca levels were calculated to be 0.50%, 0.60%, 0.70%, 0.80%, 0.90%, 1.00%, and 1.10%, respectively. The dietary Ca concentrations by analysis on an as-fed basis were 0.50%, 0.60%, 0.70%, 0.80%, 0.90%, 1.00%, and 1.07%, respectively. The basal diet (Table 1) was formulated to meet or exceed the requirements (NRC, 1994; Ministry of Agriculture of P. R. China, 2004) of grower broilers for all other nutrients except for Ca, and the concentration of NPP in the diet of each treatment was fixed at 0.31% (Jiang et al., 2016). Different treatment diets with different Ca levels were obtained by adjusting the ratio of sand and limestone from the basal diet as described before (Bai et al., 2022). Body weight, feed intake, and leg abnormality were recorded to calculate average daily weight gain (ADG), average daily feed intake (ADFI), feed:gain ratio (FCR), and incidence of leg abnormality during 22 to 42 d of age.

Sample collections and preparations

The feed ingredients and diets from the seven treatments were taken and submitted for Ca, CP, and P analyses before the initiation of the experiment to confirm their contents in diets. At the end of the experiment, 12 broilers (2 broilers per cage) from each treatment group were selected according to average body weight of each cage after a 12-h fast. Blood samples were taken from each broiler via vein, and then centrifuged to harvest serum for analyses of Ca, inorganic P and OC concentrations, and ALP and TRACP activities. The birds were then killed by cervical dislocation. The left tibia and middle toe were collected, and then stored at −20 °C for determinations of bone characteristics and the activities of ALP and TRACP. The right tibia was excised and immediately frozen in liquid nitrogen and then stored at −80 °C for assays of *ALP*, *OPG*, *OC*, *DMP-1*, *BMP-2*, *SOST*, or *TRACP* mRNA and protein expression levels.

Calcium, P, and CP concentrations

The CP level in basal diet was analyzed according to the method of Thiex et al. (2002) and Jiang et al. (2016). The concentrations of Ca in diets and bone ash were determined by inductively coupled plasma spectroscopy (Model IRIS Intrepid II, Thermo Jarrell Ash, Waltham, MA). Total P concentrations in diets and bone ash were determined with a spectrophotometer (procedure 3.4.11, AOAC, 2000). The soybean powder (GBW10013, National Institute of Standards and Technology, Beijing, China) was used as a standard reference material for validation of the Ca and P analysis. The concentrations of Ca and inorganic P in serum were measured by a colorimetric method with specific commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Bone Characteristics

Frozen tibia and middle toe bones were thawed at room temperature, and then the tibia bone was defleshed, and patella and cuticles of toe were removed. The BMC and BMD of tibia and middle toe bones were determined by dual-energy X-ray absorptiometry (DEXA) using the case of small animal model (DCS-600; Aloka, Tokyo, Japan) (Schallier et al., 2019). After the scan, tibia and middle toe bones were frozen immediately and stored at −20 °C until analysis of bone strength (Onyango et al., 2003). The tibia bone strength was determined by a texture analyzer (Stable Micro System, Goalming, UK). The ash percentage of tibia and middle toe bones was determined as described by Jiang et al. (2016).

Enzyme Activities and OC Contents

The ALP and TRACP activities as well as OC contents were measured by a colorimetric method using specific commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), respectively. All procedures were carried out according to the manufacturers' instructions.

RNA Extraction and Quantitative RT-PCR

Total RNA in tibia bone was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), and single-strand cDNA was synthesized using the PrimeScript RT Master Mix kit (Takara, Otsu, Japan) according to the manufacture's protocol. The concentration and integrity of the RNA were determined by using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA), respectively. Real-time quantitative PCR was performed on an Applied Biosystems 7500 Real-Time PCR System using SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA). Primer sequences for *ALP*, *OPG*, *OC*, *DMP-1*, *BMP-2*, *SOST*, *TRACP*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and β-actin (Table 2) were used for amplification reactions according to their gene sequences published in GenBank, respectively. Each gene was amplified independently in triplicate within a single instrument run. The mRNA levels of the target gene were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak et al., 2001), and the geometric mean of internal references (*GAPDH* and β-actin) was used to normalize the expression of the target gene (Sun et al., 2018).

Western Blotting Assay

Frozen tibia bone samples were homogenized in ice-cold RIPA lysis buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with protease inhibitor (Biotool, Houston, TX). The homogenate was centrifuged, and the supernatant was collected for total protein determination using a BCA protein assay kit (Pierce, Rockford, IL). Total proteins (30 μg) were separated on a 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Merck-Millipore, Munich, Germany). After blocked in 5% BSA blocking solution for 1 h at room temperature, the membrane was incubated overnight at 4 °C with the following primary antibodies: ALP (ABclonal, Wuhan, China), OPG (ABclonal, Wuhan, China), OC (ABclonal, Wuhan, China), BMP-2 (ABclonal, Wuhan, China), DMP-1 (Abcam, Cambridge, MA), β-actin (Huaxingbio, Beijing, China), and β-tubulin (Huaxingbio, Beijing, China), respectively. After 5 washes for 5 min in Tris-buffered saline with Tween, the strips were incubated with rabbit anti-goat or goat anti-mouse horseradish peroxidase-conjugated antibody (Huaxingbio, Beijing, China). The β-actin and β-tubulin proteins were used to normalize the expression levels of target proteins.

Statistical Analysis

The effect of dietary Ca treatment was analyzed by one-way ANOVA using the general liner model procedure of SAS (version 9.4, SAS Institute Inc.). The least significant difference

Table 2. Primer sequences for real-time PCR amplification

Genes	GenBank identity	Product length, bp	Primer sequences
β-actin	NM205518.1	95	Forward:5′-ACCTGAGCGCAAGTACTCTGTCT-3′ Reverse:5′-CATCGTACTCCTGCTTGCTGAT-3′
GAPDH	NM204305.1	128	Forward:5′-CTTTGGCATTGTGGAGGGTC-3′ Reverse:5′-ACGCTGGGATGTGTTCTGG-3′
ALP	NM205360.1	300	Forward:5′-GGAGAAGGACCCCGAATACTG-3′ Reverse:5′-TTGACGCCGCAGAGGTAAG-3′
OPG	XM015283019.2	186	Forward:5′-ATCTCAGTCAAGTGGAGCATC-3′ Reverse:5′-GTTCCAGTCTTCAGCGTAGTA-3′
OC	NM205387.3	143	Forward:5′-TGCTCGCAGTGCTAAAGCCTTCAT-3′ Reverse:5′-TCAGCTCACACACCTCTCGTT-3′
DMP-1	NM206993.1	226	Forward:5′-ACGCTTCTACACCTCTGCTG-3′ Reverse:5′-CTACGTCCGCATCACCAGT-3′
BMP-2	NM204358.1	136	Forward:5′-CCAACACCGTGTGCAGCTT-3′ Reverse:5′-TGGAGTTCAGCTGAGGTGACAGA-3′
SOST	XM025144077.1	269	Forward:5′-TGGGAACTGAGGCTGTCAGG-3′ Reverse:5′-AAGGGTGTGAGTGTGTGGTGTA-3′
TRACP	XM015302697.2	303	Forward:5′-TTTGGCCGTGGGTGATTGG-3′ Reverse:5′-CGCTCGGAGTGTCGGCTGTAT-3′

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *ALP*, alkaline phosphatase; *OPG*, osteoprotegerin; *OC*, osteocalcin; *DMP-1*, dentin matrix protein-1; *BMP-2*, bone morphogenetic protein-2; *SOST*, sclerostion; *TRACP*, tartrate-resistant acid phosphatase.

method was used to test the differences among means. The replicate cage served as the experimental unit. Orthogonal comparisons were applied for linear and quadratic responses of dependent variables to independent variables. Regression analyses of broken-line, quadratic, and asymptotic models were performed, respectively, and the best fitted model between responsive criteria and dietary Ca level was used to estimate the optimal dietary Ca level (the break point from the broken-line model, the maximum response from the quadratic model, or 95% of the maximum response from the asymptotic model) for broilers (Corzo et al., 2006; Robbins et al., 2007; Ma et al., 2016; Wang et al., 2022). The level of statistical significance was set at $P \leq 0.05$.

Results

Growth performance and incidence of leg abnormality

Dietary Ca level did not affect ($P > 0.31$) ADFI, ADG, FCR, and incidence of leg abnormality of broilers from 22 to 42 d of age as described in our previous study (Wang et al., 2021).

Calcium, P, OC concentration, and enzyme activities

Dietary Ca level did not affect ($P > 0.21$) Ca, P, and OC concentrations in serum, ALP activities in serum and tibia, and TRACP activity in tibia, but affected ($P = 0.048$) TRACP activity in serum (Table 3). The TRACP activity in serum increased linearly ($P = 0.006$) as dietary Ca level increased.

Middle toe bone parameters

Dietary Ca level did not affect ($P = 0.25$) ash P percentage of middle toe bone, but affected ($P < 0.04$) ash percentage, ash Ca percentage, and BMC and BMD of middle toe bone (Table 4). Ash percentage and BMC of middle toe bone increased quadratically ($P < 0.03$) as dietary Ca level increased; ash Ca percentage and BMD of middle toe bone increased linearly ($P < 0.03$) and quadratically ($P < 0.03$) as dietary Ca level increased. Ash percentage, ash Ca percentage and BMC of middle toe bone reached their highest points at a dietary Ca level of 0.70%; BMD of middle toe bone reached a plateau at 0.60% Ca.

Tibia bone parameters

Dietary Ca level did not affect ($P > 0.06$) ash percentage and strength of tibia bone; however, it affected ($P < 0.02$) ash Ca and P percentage, BMC, and BMD of tibia bone (Table 5). Ash Ca and P percentage of tibia bone increased linearly ($P < 0.04$) as dietary Ca level increased; BMC and BMD of tibia bone increased linearly ($P < 0.003$) and quadratically ($P < 0.003$) as dietary Ca level increased. BMC and BMD of tibia bone reached plateaus at 0.60% Ca.

mRNA expression levels of Ca metabolism-related enzymes and proteins in tibia bone

Dietary Ca level did not affect ($P > 0.17$) the mRNA expression levels of *SOST* and *TRACP* in tibia bone; however, it affected ($P < 0.04$) the mRNA expression levels of *ALP*, *OPG*, *OC*, *DMP-1*, and *BMP-2* in tibia bone (Table 6). The mRNA expression levels of *OPG*, *OC*, *DMP-1*, and *BMP-2* in tibia bone decreased linearly ($P < 0.002$) as dietary Ca level increased; the mRNA expression levels of *ALP* decreased quadratically ($P = 0.045$) as dietary Ca level increased. The mRNA expression levels of *ALP* reached its highest point at 0.70% Ca.

Protein expression levels of Ca metabolism-related enzymes and proteins in tibia bone

Dietary Ca level did not affect ($P > 0.46$) the protein expression levels of ALP, OPG, OC, DMP-1, BMP-2, SOST, and TRACP in tibia bone (Table 7).

Optimal dietary Ca levels

Results of the optimal dietary Ca levels of broilers from 22 to 42 d of age as estimated by the nonlinear regression analyses

Table 3. Effect of dietary Ca level on Ca, P, and OC contents in serum and ALP and TRACP activities in serum and tibia of 42-d-old broilers[1]

Dietary Ca, %	Serum					Tibia	
	Ca content, mmol/L	P content, mmol/L	OC content, ng/L	ALP, King unit/100 mL	TRACP, King unit/100 mL	ALP, King unit/g prot	TRACP, King unit/g prot
0.50	2.24	2.48	14.0	301	14.9[b]	0.386	3.12
0.60	2.34	2.55	14.6	290	15.8[b]	0.407	1.13
0.70	2.39	2.63	14.0	266	15.4[b]	0.498	3.21
0.80	2.28	2.41	14.9	212	17.7[ab]	0.310	2.98
0.90	2.43	2.50	14.0	211	18.9[a]	0.292	3.69
1.00	2.47	2.24	13.9	211	17.1[ab]	0.487	1.67
1.10	2.37	2.17	13.9	138	16.2[b]	0.300	2.24
SEM	0.172	0.145	0.69	53.9	0.85	0.1419	0.860
P-value							
Ca level	0.22	0.28	0.93	0.32	0.048	0.86	0.68
Linear	—	—	—	—	0.006	—	—
Quadratic	—	—	—	—	0.17	—	—

[1]Data represent the means of 6 replicates (n = 6).
[a,b]Values with different superscript letters within a column differ (P < 0.05).
ALP, alkaline phosphatase; OC, osteocalcin; TRACP, tartrate-resistant acid phosphatase.

Table 4. Effect of dietary Ca level on middle toe bone parameters of 42-d-old broilers[1]

Dietary Ca, %	Ash percentage, %	Ash Ca percentage, %	Ash P percentage, %	BMC, g	BMD, g/cm^2
0.50	45.4[b]	36.1[ab]	17.2	0.400[b]	0.113[b]
0.60	46.6[ab]	36.2[a]	17.4	0.447[ab]	0.122[a]
0.70	47.6[a]	36.4[a]	17.5	0.468[a]	0.124[a]
0.80	46.4[ab]	36.2[a]	17.4	0.468[a]	0.125[a]
0.90	46.2[b]	36.0[ab]	17.3	0.428[ab]	0.118[ab]
1.00	46.1[b]	35.9[ab]	17.4	0.433[ab]	0.121[a]
1.10	46.1[b]	35.7[b]	16.9	0.398[b]	0.123[a]
SEM	4.01	0.15	0.17	0.0152	0.0026
P-value					
Ca level	0.026	0.037	0.25	0.009	0.029
Linear	0.55	0.008	—	0.45	0.021
Quadratic	0.026	0.014	—	0.0003	0.020

[1]Data represent the means of 6 replicates (n = 6).
[a,b]Values with different superscript letters within a column differ (P < 0.05).
BMC, bone mineral content; BMD, bone mineral density.

are shown in Table 8. Based on the best fitted broken-line, quadratic, or asymptotic models (P < 0.02) of tibia BMC, BMD, and *ALP* mRNA level, middle toe BMC, BMD, ash percentage, and ash Ca percentage, the optimal dietary Ca levels were estimated to be 0.55% to 0.72% for broilers fed a corn-soybean meal diet from 22 to 42 d of age.

Discussion

Our hypotheses that the BMC, BMD, and mRNA or protein expression levels of ALP, OPG, OC, DMP1, BMP-2, SOST, or TRACP in bone might be new sensitive indices to evaluate dietary Ca requirements of broilers fed a practical corn-soybean meal diet from 22 to 42 d of age, and the Ca requirements of broilers from 22 to 42 d of age might be different from the current NRC Ca requirement (0.9%) have been supported by the results of the present study. The present study demonstrated that the BMC and BMD of tibia and middle toe, middle toe ash Ca percentage, and tibia ALP mRNA level were new sensitive indicators to evaluate dietary Ca requirement of broilers fed a practical corn-soybean meal diet from 22 to 42 d of age, and the optimal dietary Ca level would be 0.72% to meet all of the Ca metabolism and bone development of broilers fed a corn-soybean meal diet from 22 to 42 d of age, which is 20% lower than the current NRC Ca requirement. Our findings are of great significance to ensure the healthy growth of broilers and reduce the interference of Ca on the palatability to broiler diets and absorption and utilization of other nutrients, as well as save Ca resources and reduce feed costs.

The Ca requirements for broilers have been studied extensively over almost a century. Growth performance is the commonly used response criterion to evaluate Ca requirements of broilers from 1 to 21 d of age in some previous

Table 5. Effect of dietary Ca level on tibia bone parameters of 42-d-old broilers[1]

Dietary Ca, %	Ash percentage, %	Ash Ca percentage, %	Ash P percentage, %	BMC, g	BMD, g/cm^2	Bone strength, N
0.50	52.0	35.6^{b}	17.8^{a}	3.01^{b}	0.261^{b}	241
0.60	52.3	36.2^{ab}	17.9^{a}	3.65^{a}	0.283^{a}	303
0.70	52.4	36.4^{a}	17.8^{a}	3.76^{a}	0.292^{a}	322
0.80	52.8	36.1^{a}	17.9^{a}	3.70^{a}	0.295^{a}	321
0.90	52.8	35.8^{ab}	16.7^{b}	3.30^{a}	0.287^{a}	278
1.00	53.3	36.2^{a}	17.7^{ab}	3.66^{a}	0.289^{a}	326
1.10	52.1	36.3^{a}	17.0^{bc}	3.69^{a}	0.292^{a}	345
SEM	0.15	0.16	0.02	0.127	0.0058	17.7
P-value						
Ca level	0.069	0.011	0.0006	0.0004	0.005	0.14
Linear	—	0.032	0.002	0.006	0.002	—
Quadratic	—	0.20	0.78	0.002	0.007	—

[1]Data represent the means of 6 replicates (n = 6).
[a,b]Values with different superscript letter within a column differ (P < 0.05).
BMC, bone mineral content; BMD, bone mineral density.

Table 6. Effect of dietary Ca level on mRNA expression levels of Ca metabolism-related enzymes and proteins in tibia bone of 42-d-old broilers[1]

Dietary Ca, %	*ALP*, RQ	*OPG*, RQ	*OC*, RQ	*DMP-1*,RQ	*BMP-2*, RQ	*SOST*,RQ	*TRACP*,RQ
0.50	1.045^{ab}	1.028^{a}	1.039^{a}	1.007^{a}	0.999^{ab}	1.070	0.911
0.60	0.954^{abc}	0.745^{bc}	0.996^{ab}	0.959^{a}	0.916^{abc}	0.953	1.376
0.70	1.327^{a}	0.895^{ab}	1.953^{ab}	1.002^{a}	1.161^{a}	0.933	1.352
0.80	1.044^{ab}	0.673^{bc}	0.687^{abc}	0.763^{ab}	0.790^{bc}	0.829	1.860
0.90	1.087^{ab}	0.716^{bc}	0.762^{abc}	0.660^{b}	0.851^{abc}	0.877	0.970
1.00	0.648^{c}	0.622^{c}	0.579^{c}	0.512^{b}	0.616^{c}	0.871	0.523
1.10	0.768^{bc}	0.673^{bc}	0.642^{bc}	0.615^{b}	0.795^{bc}	0.931	0.598
SEM	0.1209	0.0810	0.1140	0.0980	0.1057	0.1390	0.2621
P-value							
Ca level	0.012	0.015	0.029	0.003	0.03	0.93	0.18
Linear	0.13	0.001	0.0008	< 0.0001	< 0.0001	—	—
Quadratic	0.045	0.27	0.81	0.73	0.73	—	—

The mRNA levels were calculated as the relative quantity (RQ) of the target gene mRNA to the geometric mean of β-actin mRNA and GAPDH mRNA, RQ = $2^{-\Delta\Delta Ct}$ (Ct, threshold cycle).
[1]Data represent the means of 6 replicates (n = 6).
[a, b, c]Values with different superscript letter within a column differ (P < 0.05).
ALP, alkaline phosphatase; *OPG*, osteoprotegerin; *OC*, osteocalcin; *DMP-1*, dentin matrix protein-1; *BMP-2*, bone morphogenetic protein-2; *SOST*, sclerostion; *TRACP*, tartrate resistant acid phosphatase.

studies (Bar et al., 2003; Gautier et al., 2017). It has been demonstrated that the body weight of broilers from 1 to 21 d of age decreased linearly as the increase of dietary Ca level (Guatier et al., 2017; Bai et al., 2022). Similar results were observed in broilers from 1 to 14, 28 or 42 d of age fed a low-NPP (0.3% or 0.35%) diet (Rama Rao et al., 2006). However, Driver et al. (2005) found that there were no significant linear or quadratic responses on body weight gain and feed intake of broiler chickens from 19 to 42 d of age with the increase of dietary Ca level. The results from the present study are in line with the results of Driver et al. (2005), indicating that the growth performance was unsuitable for evaluating dietary Ca requirements of broilers from 22 to 42 d of age.

The concentrations of Ca and P in serum and bone are important indicators to evaluate Ca absorption and utilization in the body. The Ca and P contents in both serum and bone ash of broilers responded sensitively to dietary Ca level in a previous study (Hulan et al., 1985). The results from the present study showed that the Ca content in middle toe bone ash increased quadratically with the increase of dietary Ca level and can be used to evaluate the dietary Ca requirement of broilers from 22 to 42 d of age. However, our previous study showed that the Ca content in middle toe ash of broilers on day 21 increased linearly with increasing dietary Ca levels and was not suitable to estimate dietary Ca requirement of broilers from 1 to 21 d of age (Bai et al., 2022). Different ages of the birds in the two studies might account for the discrepancy. In addition, in the present study, we also found that the Ca and P contents in tibia ash changed linearly as the increase of dietary Ca level, illustrating that Ca and P contents in tibia ash are sensitive indices to assess Ca nutritional status, but

Table 7. Effect of dietary Ca level on protein expression levels of Ca metabolism-related enzymes and proteins in tibia bone of 42-d-old broilers [1]

Dietary Ca, %	ALP, RQ	OPG, RQ	BMP-2, RQ	OC, RQ	SOST, RQ	DMP-1, RQ	TRACP, RQ
0.5	0.781	0.614	0.774	0.739	0.520	0.618	0.588
0.6	0.810	0.795	0.980	0.704	0.643	0.617	0.499
0.7	1.002	0.931	0.978	0.723	0.657	0.958	0.899
0.8	0.865	0.950	1.002	0.930	0.682	0.716	0.626
0.9	1.003	0.815	1.011	0.647	0.922	0.749	0.644
1.0	1.043	0.955	1.010	0.922	0.730	0.765	0.977
1.10	0.707	0.808	0.857	1.010	0.987	1.000	1.052
SEM	0.1470	0.1719	0.1644	0.2188	0.2403	0.2103	0.2415
P-value	0.61	0.82	0.92	0.86	0.85	0.80	0.47

The protein levels were calculated as the relative quantity (RQ) of the target gene protein to the β-actin or β-tubulin protein.
[1]Data represent the means of 6 replicates (n = 6).
ALP, alkaline phosphatase; OPG, osteoprotegerin; OC, osteocalcin; DMP-1, dentin matrix protein-1; BMP-2, bone morphogenetic protein-2; SOST, sclerostion; TRACP, tartrate-resistant acid phosphatase.

Table 8. The optimal dietary Ca levels of broilers from 22 to 42 d of age as estimated based on the best fitted models

Dependent variable	Regression equation[1]	*P*-value	Optimal dietary Ca levels, %
Tibia BMC	$Y = 0.29.9 - 62.5176 \times e^{-15.2703X}$	0.0001	0.55
Tibia BMD	$Y = -71.6305 + 125.4146X$ ($0.50 \le X \le 0.70$); $Y = 3.6314 - 0.0219X$ ($0.60 \le X \le 1.07$)	0.001	0.60
Tibia ALP mRNA expression level	$Y = -0.5268 + 2.5129X$ ($0.50 \le X \le 0.70$); $Y = 2.3213 - 1.5320X$ ($0.70 \le X \le 1.07$)	0.010	0.70
Middle toe BMC	$Y = 0.0522 + 0.5909X$ ($0.50 \le X \le 0.72$); $Y = 0.6396 - 0.2211X$ ($0.72 \le X \le 1.07$)	0.002	0.72
Middle toe BMD	$Y = -0.1119 + 0.3765X$ ($0.50 \le X \le 0.63$); $Y = 0.1305 - 0.0092X$ ($0.63 \le X \le 1.07$)	0.012	0.63
Middle toe ash percentage	$Y = 0.2727 + 0.3045X$ ($0.50 \le X \le 0.66$); $Y = 0.4987 - 0.0377X$ ($0.66 \le X \le 1.07$)	0.006	0.66
Middle toe ash Ca percentage	$Y = 0.3767 + 0.2370X - 0.1517X^2$	0.002	0.70

[1]Regression equations based on analyzed dietary Ca level (%) in the diet.
BMC, bone mineral content; BMD, bone mineral density; ALP, alkaline phosphatase.

not useful markers for the assessment of Ca requirement of broilers from 22 to 42 d of age.

Bone characteristics are commonly used to access dietary Ca requirement or deficiency of broilers. Bone strength is a direct responsive criterion for the risk of bone fracture. Rowland et al. (1967) reported that tibia bone strength was a sensitive indicator for evaluating Ca requirement of broilers from 1 to 28 d of age. However, the results from the current study indicated that tibia bone strength was not sensitive for estimating Ca requirements of broilers from 22 to 42 d of age, and the similar results have been found in other studies (Xia et al., 2015; Valable et al., 2017). In addition, Bone ash has been used to evaluate bone mineralization in poultry (Rama Rao et al., 2003). Edwards et al. (1963) and Bar et al. (2003) found that bone ash percentage of broilers on day 21 increased quadratically as dietary Ca level increased. There is a positive correlation between BMC or BMD measured by DEXA and bone ash percentage (Onyango et al., 2003). The BMC and BMD of broilers showed quadratic increases as the dietary Ca increased (Onyango et al., 2003; Valable et al., 2017). Similarly, in the present study, the ash percentage of middle toe, and BMC and BMD of middle toe and tibia showed quadratic responses to dietary Ca concentrations, and were sensitive indicators to evaluate Ca requirement of broilers. A recent study from our laboratory also indicated that the tibia BMD of broilers could be used to evaluate the Ca requirements of broilers from 1 to 21 d of age (Bai et al., 2022).

In recent years, bone Ca metabolism-related enzymes and proteins have been used as criteria for estimation of bone development in poultry (Jiang et al., 2013; Xia et al., 2015; Zhang et al., 2019). Alkaline phosphatase is an active protein which is mainly synthesized and secreted by osteoblasts and can promote bone formation (Ross et al., 2000). Xia et al. (2015) found that ALP activity in serum showed a quadratic response to dietary Ca level and could be used to evaluate dietary Ca requirements of laying Longyan shelducks. A recent study from our laboratory also found that ALP activity in serum and tibia and the protein expression level of ALP in tibia on day 21 could be used as sensitive criteria for evaluating Ca requirements of broilers from 1 to 21 d of age (Bai et al., 2022). In addition, the current study showed that the mRNA expression of ALP in tibia on day 42 decreased quadratically as dietary Ca level increased, and could act as a biomarker for the assessment of dietary Ca requirement of broilers from 22 to 42 d of age. Osteocalcin is essential for bone mineralization and widely

accepted as a marker of bone formation (Cepelak and Cvoriscec, 2009). Tartrate-resistant acid phosphatase and OPG have been used as markers of bone resorption (Shidara et al., 2008). The TRACP activity is increased in patients with osteoporosis and had a significant negative correlation with BMD (Shidara et al., 2008). An increase in OPG expression will suppress osteoclast differentiation and bone resorption (Yamamoto et al., 2006; Zhang et al., 2019). Jiang et al. (2013) found that high dietary Ca increased the *OPG* mRNA expression and OC protein expression in keel of hens. Zhang et al. (2019) reported that TRACP activity in serum decreased, and the mRNA expression of OPG in sternum increased quadratically as the dietary Ca level increased in meat ducks. Dentin mateix protein-1(DMP-1), BMP-2, and SOST have also been reported to be novel biological markers to reflect bone turnover (Chapurlat et al., 2016). However, in the current study, dietary Ca level did not affect the protein expression levels of the above enzymes and proteins in tibia, whereas the mRNA expression levels of *OPG*, *OC*, *DMP-1* and *BMP-2* in tibia decreased linearly as dietary Ca level increased, indicating that all these criteria are not suitable for estimating Ca requirements of broilers from 22 to 42 d of age. Similar results were observed in our recent study with starter broilers (Bai et al., 2022), indicating that the tibia *OC* and *BMP-2* mRNA levels decreased linearly as dietary Ca levels increased.

The current NRC Ca requirement of broilers from 22 to 42 d of age based on previous studies (Edwards et al., 1963; Waldroup et al., 1974; Yoshida and Hoshii, 1982a, b) is 0.9%. However, Driver et al. (2005) suggested that 0.9% of the Ca requirements for grower birds may be excessive for optimum body weight gain, FCR, and tibia ash of grower broilers. Recently, Ceylan et al. (2020) reported that 0.75% dietary Ca was enough for skeletal development and maintain bone health of grower broilers. The current study also showed that the optimal Ca level for all of the Ca metabolism and bone development of broilers from 22 to 42 d of age was 0.72%, which is in line with the result of Ceylan et al. (2020). Previous studies have shown that Ca supplied in excess of requirement may have a negative effect on the absorption and utilization of other nutrients (Sebastian et al., 1996; Hamdi et al.,). Therefore, our current results would be of great significance for reducing the feed cost and improving nutrient availability of broilers.

In conclusion, the results from the current study indicate that the optimal dietary Ca level would be 0.72% to support all of the bone development and Ca metabolism requirements of broilers fed a practical corn-soybean meal diet from 22 to 42 d of age, which is 20% lower than the current NRC Ca requirement (0.90%).

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Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

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