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# Estimation of the standardized ileal digestible calcium and phosphorus requirements of broiler chickens from 10 to 21 days of age

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## Abstract

The current study aimed to estimate the standardized ileal digestible (SID) calcium (Ca) and phosphorus (P) requirements of broiler chickens based on response surface methodology (RSM). Nine experimental diets were formulated with different SID Ca and P concentrations (2.80 and 5.50, 3.44 and 4.44, 3.44 and 6.56, 5.00 and 7.00, 5.00 and 5.50, 5.00 and 4.00, 6.56 and 4.44, 6.56 and 6.56, and 7.20 and 5.50 g/kg, respectively). A total of 480 10-day-old Ross 308 male broilers were weighed and randomly assigned to nine treatments based on body weight. Each treatment had five replicates, except for the central treatment (5.00 g/kg Ca and 5.50 g/kg P), which had eight replicates with 10 birds per pen. On day 21, body weight and feed leftovers were recorded to calculate body weight gain (BWG) and the gain-to-feed ratio (G:F). Left tibia bones were also collected for compositional analysis and bone mineral density (BMD) assessment. Response surface analysis revealed significant quadratic models for all criteria. The maximum BWG was estimated at 5.13 and 5.74 g/kg for SID Ca and P, respectively. The maximum G:F was observed when 6.41 and 7.00 g/kg of SID Ca and P were used, respectively. Multi-objective optimization analysis demonstrated that 6.02 g/kg of SID Ca and 6.61 g/kg of SID P were required to achieve both optimal BWG and G:F. Furthermore, the ideal SID Ca and P concentrations for optimal tibia ash, tibia Ca, tibia P, and BMD were estimated at 7.20 and 7.00 g/kg, 5.75 and 5.87 g/kg, 7.20 and 7.00 g/kg, and 7.20 and 6.96 g/kg, respectively. Multi-objective optimization indicated that 6.50 and 6.83 g/kg of SID Ca and P, respectively, are required to achieve optimal growth performance and bone mineralization. This study's findings suggest that RSM is a feasible and effective approach to determining the optimal SID Ca and P requirements of broiler chickens, as it efficiently evaluates multiple factors while considering several response criteria.

**Keywords:** Broiler, Calcium, Phosphorus, Requirement, Standardized ileal digestible, Response surface methodology

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**Availability of data and material**
Upon reasonable request, the datasets of this study can be available from the corresponding author.

**Authors' contributions**
Conceptualization: Kong C.
Data curation: Kong C.
Formal analysis: Lee CW, Kong C.
Methodology: Kong C.
Software: Lee CW, Kong C.
Validation: Lee CW, Kong C.
Investigation: Lee CW, Kong C.
Writing - original draft: Lee CW, Kong C.
Writing - review & editing: Lee CW, Kong C.

**Ethics approval and consent to participate**
The protocols for the present study were reviewed and approved by the Institutional Animal Care and Use Committee of Kyungpook National University (KNU 2023-0226).

# INTRODUCTION

Calcium (Ca) and phosphorus (P) are the most abundant macro-minerals deposited in bones, playing important roles in nutrient metabolism and bone mineralization [1, 2]. Ca, which acts as a cation in the digestive tract, increases intestinal pH. Additionally, it reduces the digestibility of both Ca and P by binding to phytate and forming a calcium–phytate complex [3–5]. Since Ca and P concentrations influence each other in terms of digestion and absorption, their interaction should be considered when seeking to enhance growth performance and promote the efficient utilization of minerals. Recent studies have investigated the interactive effects of Ca and P on their availability in broilers by simultaneously adjusting their dietary levels in experimental diets [6–8].

In general, poultry diet is formulated based on the total Ca and available or non-phytate P (NPP) concentrations. "Available nutrients" refer to the actual amount of dietary nutrients that are digested and absorbed by an animal, with the commonly used NPP content representing the available P content in diet [9]. However, this does not account for the fact that NPP is not fully utilized, and some of the phytate P is also utilized [10]. Therefore, as digestibility has become a common criterion for evaluating the nutrient availability of poultry feed ingredients, there has been a shift toward using digestible Ca and P bases for diet formulation, rather than total Ca and available P or NPP bases [11, 12]. Several studies have measured the standardized ileal digestibility values of Ca and P, which have been corrected for endogenous losses from apparent ileal digestibility [13–15].

Response surface methodology (RSM) is a mathematical and statistical method used to analyze the interactive effects of multiple factors on response variables and determine the optimal factor settings for the best response [16, 17]. RSM helps evaluate multiple parameters and their interactions using quantitative data and has been used in various experiments, including studies on broilers, to determine nutrient requirements in animal nutrition [18–20]. Growth performance and bone mineralization have been evaluated as response variables owing to their sensitivity to dietary Ca and P concentrations [21, 22]. While certain studies have assessed dietary Ca and P requirements using RSM based on total Ca and NPP levels, to the best of our knowledge, no study has simultaneously estimated standardized ileal digestible (SID) Ca and P requirements. Therefore, this study aimed to estimate SID Ca and P requirements using RSM by measuring growth performance and bone mineralization in broiler chickens fed diets with varying SID Ca and P levels.

# MATERIALS AND METHODS

## Animal ethics

The Institutional Animal Care and Use Committee of Kyungpook National University, Korea, reviewed and approved all experimental procedures (approval number: KNU 2023-0226).

## Animals, management, and experimental design

A total of 480 10-day-old Ross 308 male broiler chickens were randomly allocated to nine dietary treatments using a five-level, two-factor central composite design (CCD). Each treatment was replicated five times, except for the central treatment (run no. 5), which had eight replicates), and each replicate comprised 10 birds. From d 10 to 21, birds were allowed *ad libitum* access to water and experimental diets for 11 days. House temperature was maintained at 34℃ on the first day and gradually reduced to 25℃ by 21 days of age at a rate of 3℃ per wk. All birds were housed in 1 × 1 m floor pens under a continuous 24-hour lighting program. Lighting intensity was maintained at 30 lux throughout the experiment.

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## Dietary treatments

SID Ca and P concentrations in feed ingredients were obtained from previous research in the same laboratory [23]. A corn–soybean meal basal diet was formulated to meet or exceed nutrient specifications of broilers [24], except those for Ca and P (Table 1). All experimental diets were isoenergetic and isonitrogenous. The basal diet was adjusted with appropriate amounts of cornstarch, soybean oil, limestone, monocalcium phosphate, and monosodium phosphate to formulate nine experimental diets containing varying SID Ca and SID P concentrations, as indicated in Table 1: Diet 1: 2.80 and 5.50 g/kg, Diet 2: 3.44 and 4.44 g/kg, Diet 3: 3.44 and 6.56 g/kg, Diet 4: 5.00 and 7.00 g/kg, Diet 5 (central treatment): 5.00 and 5.50 g/kg, Diet 6: 5.00 and 4.00 g/kg, Diet 7: 6.56

**Table 1. Ingredient and chemical compositions of experimental diets on an as-fed basis**

| Ingredient, g/kg | Dietary treatments | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| Corn | 560.0 | 560.0 | 560.0 | 560.0 | 560.0 | 560.0 | 560.0 | 560.0 | 560.0 |
| Soybean meal | 330.0 | 330.0 | 330.0 | 330.0 | 330.0 | 330.0 | 330.0 | 330.0 | 330.0 |
| Cornstarch | 61.0 | 60.4 | 51.0 | 40.5 | 43.0 | 45.5 | 29.3 | 25.8 | 21.0 |
| Soybean oil | 14.9 | 15.2 | 19.5 | 24.4 | 23.2 | 22.1 | 29.6 | 31.2 | 33.4 |
| Sodium bicarbonate | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| Limestone | - | 5.5 | - | 2.8 | 9.3 | 15.8 | 22.2 | 13.0 | 21.1 |
| Monocalcium phosphate | 11.1 | 8.6 | 15.3 | 22.0 | 14.2 | 6.3 | 8.6 | 19.7 | 14.2 |
| Monosodium phosphate | 2.7 | - | 3.9 | - | - | - | - | - | |
| Sodium chloride | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| Vitamin premix[1)] | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Mineral premix[2)] | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 |
| Choline chloride | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| L-Arg | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| L-Ile | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| L-Lys-HCl | 2.4 | 2.4 | 2.4 | 2.4 | 2.4 | 2.4 | 2.4 | 2.4 | 2.4 |
| L-Met | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 |
| L-Cys | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 | 1.6 |
| L-Thr | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 |
| L-Val | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 | 1.1 |
| Calculated value | | | | | | | | | |
| AME, kcal/kg | 3000.0 | 3000.0 | 3000.0 | 3000.0 | 3000.0 | 3000.0 | 3000.0 | 3000.0 | 3000.0 |
| Crude protein | 203.3 | 203.3 | 203.3 | 203.3 | 203.3 | 203.3 | 203.3 | 203.3 | 203.3 |
| Total Ca | 3.3 | 4.8 | 4.1 | 6.3 | 7.2 | 8.1 | 10.7 | 9.5 | 11.4 |
| Total P | 6.3 | 5.2 | 7.5 | 8.0 | 6.4 | 4.7 | 5.2 | 7.5 | 6.4 |
| Non-phytate P | 3.8 | 2.7 | 5.0 | 5.5 | 3.8 | 2.2 | 2.7 | 5.0 | 3.8 |
| SID Ca[3)] | 2.80 | 3.44 | 3.44 | 5.00 | 5.00 | 5.00 | 6.55 | 6.56 | 7.20 |
| SID P[3)] | 5.50 | 4.44 | 6.56 | 7.00 | 5.50 | 4.00 | 4.44 | 6.56 | 5.50 |
| SID Ca: SID P ratio | 0.51 | 0.77 | 0.52 | 0.71 | 0.91 | 1.25 | 1.48 | 1.00 | 1.31 |
| Analyzed value | | | | | | | | | |
| Total Ca | 3.6 | 5.5 | 4.4 | 6.4 | 7.5 | 7.2 | 11.4 | 9.8 | 11.7 |
| Total P | 6.5 | 5.5 | 7.6 | 8.0 | 6.7 | 4.7 | 5.7 | 8.0 | 6.7 |

[1)] Supplies the following quantities per kilogram of diet: vitamin A, 18,000 IU; vitamin $D_3$, 6,000 IU; vitamin E, 75 mg; Vitamin $K_3$, 5 mg; Thiamin, 5 mg; Riboflavin, 13 mg; Nicotinic acid, 90 mg; Pantothenic acid, 30 mg; Pyridoxine, 6.8 mg; cobalamin, 0.03 mg; Folacin, 3.3 mg; Biotin 0.33 mg.

[2)] Supplies the following quantities per kilogram of diet: Mn, 180 mg; Zn, 165 mg; Fe, 75 mg; Co, 24 mg; I, 1.9 mg; Se, 0.5 mg.

[3)] Based on SID values determined in a previous digestibility study conducted in the same laboratory [23].

AME, apparent metabolizable energy; Ca, calcium; P, phosphorus; SID, standardized ileal digestible.

and 4.44 g/kg, Diet 8: 6.56 and 6.56 g/kg, Diet 9: 7.20 and 5.50 g/kg.

### Measurement and chemical analyses

On day 10 and 21, all chickens were individually weighed, and feed intake was recorded to determine the mean body weight, body weight gain (BWG), and the gain-to-feed ratio (G:F). Mortality was monitored daily and used to adjust the feed intake values. At the end of the experiment (21 d of age), two birds with body weights representing the median from each pen euthanized by $CO_2$ asphyxiation for left tibias collection. The tibias were placed in labeled plastic bags and stored in a freezer at −20℃ until further analysis. After separating the meat and soft tissues from each tibia, the tibias were defatted by soaking in ethyl ether for 48 h and subsequently dried to a constant weight using a drying oven at 105℃ for 24 h. Bone mineral density (BMD) was determined using dual-energy X-ray absorptiometry (InAlyzer; Medikors). Thereafter, the samples were ashed in a muffle furnace at 550℃ for 12 h to determine tibial ash, total Ca, and total P contents. All experimental diets were ground using a mill grinder (CT 293 Cyclotec™, Foss) through a 1.0-mm screen. The total Ca and P concentrations in the experimental diets and tibia were analyzed using inductively coupled plasma-optical emission spectrometry (Optima 8300, PerkinElmer) [25].

### Statistical Analyses

Experimental data were analyzed using the MIXED procedure of SAS 9.4 (SAS). The statistical model included dietary treatment as the fixed variable and the replications as random variables. Statistical significance was set at $p < 0.05$. Where dietary effects were significant, the Tukey–Kramer method was used to compare mean values among treatments.

In this experiment, a two-factor, five-level CCD for SID Ca (2.8, 3.4, 5.0, 6.6, and 7.2 g/kg) and SID P (4.0, 4.4, 5.5, 6.6, and 7.0 g/kg) was used. Each pen was set as an experimental unit. Experimental data were fitted to the following second-order polynomial regression equation:

$$Y = \beta_0 + \beta_i x_i + \beta_j x_j + \beta_{ii} x_i^2 + \beta_{ij} x_j^2 + \beta_{ij} x_i x_j + \varepsilon$$

where Y is the response of interest; $\beta_0$ denotes the intercept; and $\beta_i$, $\beta_j$, $\beta_{ii}$, $\beta_{jj}$, and $\beta_{ij}$ are the coefficients estimated by the model. Furthermore, $x_i$ and $x_j$ represent SID Ca and SID P, respectively, and ε is the residual associated with the experiment. The experimental data were analyzed using Design-Expert® software (version 13; Stat-Ease) to optimize requirements. $R^2$ indicates the extent to which each independent variable is interpreted concerning changes in the dependent variable and is used to assess how closely the predicted values align with the actual values [26]. The discrepancy between the predicted $R^2$ and the adjusted $R^2$ values was examined to confirm the regression fit, ensuring it was < 0.2. The desirability function and importance were used to evaluate the effectiveness of each factor in determining the SID Ca and P requirements of chickens. Desirability scores range from 0 to 1, and the value closest to 1 was selected as the optimal point. According to Design-Expert software, importance values range from 1 to 5, with a score of 5 assigned to growth performance and that of 3 to bone mineralization. However, while tibial ash and mineral contents exhibited a quadratic response, the "lack of fit" was significant. Therefore, an importance level of 1 was assigned to tibial ash and mineral content for multi-objective optimization.

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## RESULTS

During the experimental period, the mortality rate was 0.62%, and it was not influenced by the experimental diets. The total Ca and P contents of the experimental diets were analyzed on an as-fed basis, as expected (Table 1). Increasing supplementation of SID Ca and P exerted a quadratic effect ($p < 0.05$) on growth performance (Table 2) and bone mineralization (Table 3), indicating that the regression model was adequate to determine requirements. The lack-of-fit test of the RSM model was not significant for BWG, G:F, and BMD. The difference between the predicted $R^2$ and the adjusted $R^2$ values was < 0.2, indicating that the quadratic model adequately fit the data for all response criteria.

Birds fed Diet 5 exhibited the greatest BWG, and Diets 2, 4, 5, and 8 yielded the highest G:F values (Table 2). As shown in Table 3, bone mineralization also displayed differences among treatments, although the values were comparable. The tibia ash, Ca, and P contents were highest in Diets 5 and 9, Diets 5 and 9, and Diets 5 and 8, respectively, while BMD peaked with Diet 8 (Table 3). Single-objective optimization indicated that the estimated optimal values of SID Ca and P for maximal BWG and G:F were 5.13 and 5.74 g/kg and 6.41 and 7.00 g/kg, respectively (Table 4). The ideal SID Ca and P concentrations for tibia ash, tibia Ca, tibia P, and BMD were 7.20 and 7.00 g/kg, 5.75 and 5.87 g/kg, 7.20 and 7.00 g/kg, and 7.20 and 6.96 g/kg, respectively (Table 4). Multi-objective optimization demonstrated that 6.02 g/kg of SID Ca and 6.61 g/kg of SID P were required to optimize growth performance, while 7.13 g/kg of SID Ca and 6.80 g/kg of SID P were required to maximize bone mineralization (Table 4). The most desirable concentrations of SID Ca and P for all responses were 6.50 and 6.83 g/kg, respectively (Table 4).

**Table 2. Growth performance of broiler chickens fed diets varying in concentrations of standardized ileal digestible (SID) calcium (Ca) and phosphorous (P) from d 10 to 21**

| | Input variables, g/kg | | Output variables | | | |
|---|---|---|---|---|---|---|
| Run no. (n[1)]) | SID Ca | SID P | BWG (g) | SEM | G:F (g/g) | SEM |
| 1 (5) | 2.80 | 5.50 | 495.8[bcd] | 11.40 | 0.72[ab] | 0.008 |
| 2 (5) | 3.44 | 4.44 | 542.2[ab] | 11.40 | 0.75[a] | 0.008 |
| 3 (5) | 3.44 | 6.56 | 483.0[cd] | 11.40 | 0.72[ab] | 0.008 |
| 4 (5) | 5.00 | 7.00 | 527.4[abc] | 11.40 | 0.75[a] | 0.008 |
| 5 (8) | 5.00 | 4.00 | 545.3[a] | 9.01 | 0.75[a] | 0.006 |
| 6 (5) | 5.00 | 5.50 | 503.0[abcd] | 11.40 | 0.73[ab] | 0.008 |
| 7 (5) | 6.55 | 4.44 | 456.0[d] | 11.40 | 0.70[b] | 0.008 |
| 8 (5) | 6.56 | 6.56 | 521.4[abc] | 11.40 | 0.75[a] | 0.008 |
| 9 (4) | 7.20 | 5.50 | 516.5[abc] | 12.74 | 0.73[ab] | 0.009 |
| Adjusted $R^2$ | | | 0.5202 | | 0.5117 | |
| Predicted $R^2$ | | | 0.3679 | | 0.3520 | |
| *p*-values | | | | | | |
| Diet | | | < 0.0001 | | 0.0003 | |
| Quadratic source | | | | | | |
| Sequential | | | < 0.001 | | < 0.001 | |
| Lack of fit | | | 0.078 | | 0.718 | |

The average initial body weight was 301 ± 31.3 g.
[1)]Number of observations (pens) per treatment after excluding outliers.
[a–d]Values with a different superscript within the column differ significantly ($p < 0.05$).
BWG, body weight gain; G:F, gain-to-feed ratio; SEM, standard error of the mean.

**Table 3. Bone mineralization of broiler chickens fed diets varying in concentrations of standardized ileal digestible (SID) calcium (Ca) and phosphorous (P) from d 10 to 21**

| | Input variables, g/kg | | Output variables | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Run no. (n[1)]) | SID Ca | SID P | Tibia ash (%) | SEM | Tibia Ca (%) | SEM | Tibia P (%) | SEM | BMD (g/cm$^2$) | SEM |
| 1 (5) | 2.8 | 5.5 | 45.26$^{d}$ | 0.635 | 16.40$^{c}$ | 0.426 | 9.27$^{cd}$ | 0.202 | 0.105$^{d}$ | 0.0025 |
| 2 (5) | 3.4 | 4.4 | 48.20$^{bc}$ | 0.635 | 17.95$^{abc}$ | 0.426 | 9.98$^{abc}$ | 0.202 | 0.114$^{cd}$ | 0.0025 |
| 3 (5) | 3.4 | 6.6 | 46.97$^{cd}$ | 0.635 | 17.47$^{abc}$ | 0.426 | 9.62$^{bcd}$ | 0.202 | 0.111$^{cd}$ | 0.0025 |
| 4 (5) | 5.0 | 4.0 | 50.76$^{ab}$ | 0.635 | 18.47$^{ab}$ | 0.426 | 10.31$^{ab}$ | 0.202 | 0.131$^{b}$ | 0.0025 |
| 5 (8) | 5.0 | 5.5 | 51.09$^{a}$ | 0.505 | 19.06$^{a}$ | 0.336 | 10.42$^{a}$ | 0.169 | 0.134$^{ab}$ | 0.0020 |
| 6 (5) | 5.0 | 7.0 | 45.77$^{cd}$ | 0.635 | 16.83$^{bc}$ | 0.426 | 9.00$^{d}$ | 0.202 | 0.115$^{cd}$ | 0.0025 |
| 7 (5) | 6.6 | 4.4 | 46.39$^{cd}$ | 0.635 | 17.63$^{abc}$ | 0.426 | 9.05$^{d}$ | 0.202 | 0.119$^{c}$ | 0.0025 |
| 8 (5) | 6.6 | 6.6 | 51.12$^{a}$ | 0.635 | 18.35$^{ab}$ | 0.426 | 10.54$^{a}$ | 0.202 | 0.144$^{a}$ | 0.0025 |
| 9 (5) | 7.2 | 5.5 | 51.16$^{a}$ | 0.635 | 18.94$^{a}$ | 0.426 | 10.35$^{ab}$ | 0.202 | 0.138$^{ab}$ | 0.0025 |
| Adjusted $R^2$ | | | 0.3803 | | 0.3475 | | 0.5424 | | 0.8302 | |
| Predicted $R^2$ | | | 0.3127 | | 0.1878 | | 0.4304 | | 0.7889 | |
| *p*-values | | | | | | | | | | |
| Diet | | | < 0.0001 | | 0.0001 | | < 0.0001 | | < 0.0001 | |
| Quadratic source | | | | | | | | | | |
| Sequential | | | < 0.001 | | 0.006 | | 0.004 | | < 0.001 | |
| Lack of fit | | | 0.003 | | 0.029 | | 0.038 | | 0.672 | |

[1)]Number of observations (pens) per treatment.

[a–d]Values with a different superscript within the column differ significantly ($p < 0.05$).

SEM, standard error of the mean; BMD, bone mineral density.

## DISCUSSION

Ca and P homeostasis is determined by the interaction between these minerals, and since their respective concentrations influence their utilization, their interrelationships serve an important role in defining mineral requirements [27]. Therefore, when estimating Ca or P requirements, formulating experimental diets that simultaneously consider both factors rather than setting only one nutrient as an independent variable is more accurate. Several factorial experiments have been conducted to examine the interaction between Ca and P by concurrently considering their levels [8, 28, 29]. Establishing SID Ca and P levels at five levels each, as in the current study, would typically require 25 treatments under a 5 × 5 full factorial arrangement. However, applying a CCD, a more efficient alternative, reduces the number of dietary treatments required in poultry research to only nine [17]. Specifically, the CCD is designed to generate maximal information regarding parameters from a minimal number of treatments by identifying three distinct points: a central point as well as factorial and axial points, which are equidistantly positioned from the central point [30, 31]. Moreover, as a design applied in RSM, the CCD incorporates five levels for independent factors, facilitating the statistical optimization of variables that influence responses [32].

In this study, the dietary SID Ca and P concentrations of the experimental diets were adjusted accordingly to ensure that the estimated requirements, based on a literature review, were centered within the CCD framework. Subsequently, single-objective optimization identified 5.13 and 5.74 g/kg as the estimated optimal SID Ca and P levels for maximal BWG, respectively. Since feed intake did not exhibit a significant difference among treatments, it was excluded from the multi-objective optimization analysis. Multi-objective optimization revealed that 6.02 g/kg of SID Ca and 6.61 g/kg of SID P were required to maximize BWG and G:F, while 7.13 g/kg of SID Ca and 6.80

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**Table 4. The single-objective and multi-objective optimization of performance of broilers fed experimental diets from day 10 to 21**

| Item | Input variables, g/kg | | Predicted output at optimal point | Desirability |
|---|---|---|---|---|
| | SID Ca | SID P | | |
| BWG | 5.13 | 5.74 | Maximum = 545.5 (g) | 0.78 |
| G:F | 6.41 | 7.00 | Maximum = 0.76 (g/g) | 0.78 |
| Tibia ash | 7.20 | 7.00 | Maximum = 52.52 (%) | 0.97 |
| Tibia Ca | 5.75 | 5.87 | Maximum =19.24 (%) | 0.69 |
| Tibia P | 7.20 | 7.00 | Maximum =10.85 (%) | 0.90 |
| BMD | 7.20 | 6.96 | Maximum = 0.147 (g/cm$^2$) | 0.95 |
| Growth performance[1)] | 6.02 | 6.61 | Maximum BWG = 541.1 (g)<br>Maximum G:F = 0.75 (g/g) | 0.75 |
| Bone mineralization[2)] | 7.13 | 6.80 | Maximum ash = 52.20 (%)<br>Maximum Ca = 18.47 (%)<br>Maximum P = 10.83 (%)<br>Maximum BMD = 0.147 (g/cm$^2$) | 0.86 |
| Total[3)] | 6.50 | 6.83 | Maximum BWG = 537.9 (g)<br>Maximum G:F = 0.76 (g/g)<br>Maximum ash = 51.70 (%)<br>Maximum Ca = 18.69 (%)<br>Maximum P = 10.79 (%)<br>Maximum BMD = 0.145 (g/cm$^2$) | 0.78 |

[1)]Importance in determining SID Ca and P requirements according to Design-Expert software: BWG = 5; G:F = 5.

[2)]Importance in determining SID Ca and P requirements according to Design-Expert software: Tibia ash = 1; Tibia Ca = 1; Tibia P = 1; BMD = 3.

[3)]Importance in determining SID Ca and P requirements according to Design-Expert software: BWG = 5; G:F = 5; Tibia ash = 1; Tibia Ca = 1; Tibia P = 1; BMD = 3.

Ca, calcium; P, phosphorus; SID, standardized ileal digestible; BWG, body weight gain; G:F, gain-to-feed ratio; BMD, bone mineral density.

g/kg of SID P were required to maximize tibia ash, tibia Ca, tibia P, and BMD. Meanwhile, the SID Ca requirement based on tibia ash, tibia P, and BMD was determined to be 7.2 g/kg, equating to the highest SID Ca concentration incorporated into the experimental diets. This finding was unanticipated, considering that the total dietary Ca and P levels exceeded the recommended Aviagen specifications. The comparatively high SID Ca and P requirements generated based on bone mineralization probably emanated from its sensitivity to Ca and P concentrations. Tibia bone development in broilers is commonly evaluated using parameters reflecting bone mineralization [8, 28]. Research has consistently demonstrated that broilers require higher levels of NPP when bone mineralization serves as the evaluation criterion compared to when growth performance is used [33]. Approximately 99% of Ca and 80% of P are stored in the bones, while the remaining 20% of P participates in various metabolic processes, playing essential roles in growth, cellular and membrane function, energy metabolism, and acid–base balance [34]. Low plasma Ca concentrations stimulate parathyroid hormone release, triggering Ca and P release from bones to maintain homeostasis [34]. Similarly, low plasma P concentrations cause bone resorption, providing the P required for bird maintenance and growth [35]. Considering this physiological regulation, the dietary Ca and P consumed by broilers primarily accumulate in the bones, and as bones serve as a storage reservoir for Ca and P, they may release these minerals when the amounts required for maintenance and growth become insufficient. In this study, broilers were fed a pre-starter diet that met nutrient recommendations up to d 10, ensuring the sufficient storage of Ca and P in the bones. Consequently, even when experimental diets with lower Ca and P levels than those recommended by Aviagen were administered, the birds' stored mineral reserves potentially compensated for the deficiency, allowing them to maintain essential physiological functions. Walk et al. [36] reported no effect of graded SID Ca levels (0.16%–0.56%) on feed intake or BWG, whereas tibial ash content responded quadratically to these levels. Additionally, Walk et al. [21], who observed no linear or quadratic effects on BWG in birds fed a diet with SID Ca levels of 0.2%–0.6%, found significant differences in tibial ash concentration depending on the SID Ca level. In broilers, Fallah et al. [7] also observed higher total Ca and NPP requirements for bone mineralization than for performance traits, emphasizing the role of Ca and P in regulating bone mineralization and growth performance. These findings suggest that tibial mineralization, as a response criterion, may be a more sensitive measure of Ca and P requirements than growth performance. Moreover, future studies should consider higher maximum dietary SID Ca and P levels to obtain more accurate estimations.

The current study found the optimal SID Ca-to-SID P ratio to be 0.95, based on all analyzed criteria. The diets with similar ratios, listed in order, were Diets 5 (5 g/kg Ca), 8 (6.56 g/kg Ca), and 2 (3.44 g/kg Ca). Diet 5 yielded the greatest BWG, while Diets 5 and 8 yielded the highest tibial ash content. Moreover, the tibia Ca and P contents were highest in Diets 5 and 9 and Diets 5 and 8, respectively, while BMD peaked with Diet 8. These results indicate that the experimental diets with SID Ca-to-SID P ratios approximating 0.95 might have contributed to improvements in growth performance and bone mineralization. David et al. [37] estimated the SID Ca requirement for maximum weight gain in broilers aged 24 days to be 3.05 g/kg, while that for maximum tibial ash content was 3.69 g/kg, with a recommended SID P concentration of 3.5 g/kg. These values indicated SID Ca-to-SID P ratios of 1.05 and 0.87, respectively. The relatively high SID Ca requirements observed in the current study possibly reflect differences in the evaluated SID Ca and P concentration ranges. While David et al. [37] examined SID Ca levels of 1.8–4.55 g/kg, the current study used wider ranges: 2.8–7.2 g/kg for SID Ca and 4.4–7.0 g/kg for SID P. However, the SID Ca-to-SID P ratio of 0.95 obtained in the current study remained within the range (1.05–0.87) suggested by David et al. [37], suggesting partial alignment between the findings of the two experiments. Furthermore, their study demonstrated that SID Ca requirements potentially increase with increasing dietary P levels, a trend consistent with the results of the current study [37].

In the current study, Diet 7, which had the highest SID Ca-to-SID P ratio, resulted in the lowest BWG and G:F values. Gautier et al. [38] demonstrated that when the NPP content was fixed and Ca content increased to amplify the Ca ratio, growth performance decreased with increasing Ca levels. However, when the Ca-to-NPP ratio was fixed at 2:1, and both dietary Ca and P levels had been elevated, growth performance was not affected by dietary treatments [38]. These results are consistent with those of Fallah et al. [7], who reported that increasing dietary Ca levels, particularly in low-NPP diets, negatively affected the performance of broiler chickens. High dietary Ca levels have been reported to decrease growth performance in broiler chickens, and this decrease can be alleviated by increasing the dietary NPP level [39, 40]. The reduction in growth performance observed with high dietary Ca levels possibly resulted from the effects of Ca circulation processes aimed at maintaining homeostasis in the body. Once Ca is digested, it is absorbed into the bloodstream and subsequently deposited in the bones [41]. High plasma Ca levels stimulate the secretion of calcitonin, which inhibits osteoclast activity and promotes the renal excretion of both Ca and P [42, 43]. In another study, the maintenance of a balanced Ca:NPP ratio in a low-P diet did not affect serum P levels, suggesting that an imbalanced Ca:NPP ratio potentially influences fluctuations in serum P levels [44]. Additionally, high Ca levels raise the pH of the gastrointestinal tract, reducing the soluble fraction of other nutrients and consequently decreasing their availability

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and absorption [45, 46]. Li et al. [47] reported that at an NPP level of 1.9 g/kg, increasing dietary Ca levels from 6.5 to 9.5 g/kg decreased apparent ileal P digestibility. Furthermore, phytate can bind proteins to form protein–phytate complexes, which depress protein utilization [3, 48]. These adverse effects suggest that an imbalance of dietary Ca and P may diminish growth performance. In a study by Noruzi et al. [49], reducing dietary Ca and available P by up to 30% while maintaining the same ratio did not affect performance. Moreover, since broilers can adapt to diets with low dietary P and Ca levels by increasing digestibility and absorption [50], considering both the absolute concentrations and Ca-to-P ratio may provide a more accurate estimation of Ca and P requirements.

The optimal conditions for the multi-objective optimization of growth performance and bone mineralization in broilers from day 10 to 21 were achieved at SID Ca and P levels of 6.50 and 6.83 g/kg, respectively, at an SID Ca-to-SID P ratio of 0.95. Based on growth performance, the optimal levels of SID Ca (6.02 g/kg) and SID P (6.61 g/kg) estimated in this study correspond to approximately 7.9–8.3 g/kg of total Ca and 3.7–4.2 g/kg of NPP. These values are lower than those recommended by NRC [51] for both Ca and NPP. Compared to the Aviagen guidelines, NPP levels were similar, but Ca levels were estimated to be slightly higher. Since optimal bone development requires higher levels of Ca and P than those needed for growth performance alone, nutrient requirement recommendations based solely on performance outcomes may not adequately reflect the levels necessary for proper skeletal health and development. Additionally, although the diets had identical SID values, differences in total Ca and NPP concentrations resulting from the variation in ingredient composition limit the direct comparison of the present results with existing feeding guidelines. The results also suggest that further research is required to establish the precise requirements by varying SID Ca and P levels while maintaining a fixed SID Ca-to-SID P ratio. The findings of the current study also suggest that using RSM to determine the optimal SID Ca and P requirements for broiler chickens is a feasible and effective approach, as it enables the simultaneous evaluation of multiple factors while considering response criteria to optimize nutrient requirements efficiently.

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