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Dietary Supplementation of Calcium Propionate and Calcium Butyrate Improves Eggshell Quality of Laying Hens in the Late Phase of Production

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The aim of this study was to evaluate the effects of dietary supplementation of calcium propionate and calcium butyrate on the laying performance, eggshell quality, and expression of genes related to calcium and phosphorus metabolism in the tibia. One hundred and twenty 70-week-old Isa Brown hens were randomly assigned to three treatments, and each treatment had four replicates of 10 birds fed a basal diet (control) or a basal diet supplemented with 0.5% calcium propionate (CP) or 0.5% calcium butyrate (CB) for 8 weeks. The CB and CP treatments had no significant effect ($P>0.05$) on the laying rate, egg production, egg weight, and feed efficiency. The eggshell percentage was increased from week 2 ($P<0.05$) and eggshell thickness was elevated at week 8 ($P<0.01$) by both CP and CB treatments. Compared to the control treatment, the CB treatment increased serum calcium and phosphorus levels at week 4 ($P<0.05$), whereas the CP and CB treatments decreased serum phosphorus at weeks 6 and 8, respectively ($P<0.05$). Dietary supplementation had no effect on the bone index and bending strength of the tibia ($P>0.05$). The calcium and phosphorus content of the tibia was decreased by the CB treatment ($P<0.05$). In the spleen, *NF-κB* and *IL-6* transcript levels were not influenced ($P>0.05$) but *TNF-α* transcript levels were decreased by the CP treatment ($P<0.05$). In the tibia, the expression levels of *NF-κB*, *TNF-α*, and *IL-17* were not affected by the CP or CB treatment ($P>0.05$). The CP and CB treatments had no significant effect on the transcript levels of *RANKL*, *OPG*, *RNUX2*, *OPN*, *α-Clotho*, and *VDR* ($P>0.05$). In contrast, *PHEX* transcript levels were increased by the CP treatment ($P<0.05$). The expression levels of *osteocalcin* ($P=0.094$) and *FGF23* ($P=0.087$) tended to decrease under the CB treatment. In conclusion, dietary supplementation of 0.5% calcium butyrate or 0.5% calcium propionate improved the eggshell quality of aged laying hens, possibly as a result of decreased deposition or enhanced mobilization of bone calcium and phosphorus.

Key words: bone, calcium butyrate, calcium propionate, eggshell quality, laying hens

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Introduction

Extending the laying period of hens can increase their productive performance. The gradual deterioration of eggshell quality with age, however, has an undesirable influence on the laying performance of hens during the late phase of egg production. Effective calcium supply is a possible way to maintain the quality of hen eggs in the later stages of production (Whitehead and Fleming, 2000).

During the diurnal cycle of eggshell formation, large numbers of active osteoclasts are recruited to resorb and mobilize calcium from the medullary bone to meet the requirement of eggshell formation. Activated osteoclasts lead

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to the absorption of the bone matrix, which in turn increases serum calcium and phosphate levels (Mueller *et al*., 1964). The receptor activator of NF-κB (RANK) ligand (RANKL) is a type II transmembrane protein comprising 371 amino acids (Anderson *et al*., 1997). The binding of RANK and RANKL plays crucial roles in osteoclast survival, differentiation, and activation, resulting in bone resorption (Malliga *et al*., 2011; Huang *et al*., 2013). Osteoprotegerin (OPG) is a decoy receptor for RANKL, and the OPG–RANKL–RANK axis is involved in the regulation of bone metabolic balance (Klejna *et al*., 2009; Zhang *et al*., 2009). The functions of avian RANKL and its receptors (RANK and OPG) are evolutionarily conserved (Sutton *et al*., 2015).

Organic acids and their salts are generally considered safe and have been approved for use as feed additives in animal production by most European Union member states (Additives and Feed, 2011). Organic acids are used to protect feed from microbial and fungal contamination. In the hindgut, short-chain fatty acids, mainly acetate, propionate, and butyrate, are produced through the microbial fermentation of dietary fibers, and these fatty acids play important roles in the metabolic regulation of hosts. Calcium propionate and calcium butyrate serve as sources of organic acids and regulate metabolism. Addition of butyric acid to the diet of chicks significantly reduced *Salmonella* colonization in the cecum (Cox *et al*., 1994). Under acidic conditions, calcium propionate produces free propionic acid, exerting an antibacterial effect (Bintvihok and Kositcharoenkul, 2006). At low concentrations, butyrate decreased fat accumulation, with subsequent downregulation of lipogenic genes mediated by free fatty acid receptors, in broilers (Zhao *et al*., 2020). In contrast, propionate inhibited fat deposition by reducing feed intake, albeit not via the direct regulation of hepatic fat synthesis or adipocytic fat deposition, suggesting that propionate regulates fat deposition in a different manner from butyrate (Li *et al*., 2020). In laying hens, the effects of short-chain fatty acids on production performance may be more directly derived from their effects on mineral absorption. The low-pH environment caused by short-chain fatty acids is more conducive to the absorption of minerals. The supplementation of organic acid salts significantly increased egg production performance (Yesilbag and Colpan, 2006; Youssef *et al*., 2013). Hens fed diets supplemented with organic acid salts showed significantly higher egg production (Soltan, 2008; Dahiya *et al*., 2016). Hence, we hypothesized that calcium propionate and calcium butyrate have a beneficial effect on calcium metabolism.

In the present study, the effects of dietary supplementation of calcium propionate (0.5%) and calcium butyrate (0.5%) on eggshell quality and expression of genes related to osteoclast activation were investigated. Laying performance, egg quality, blood calcium and phosphorus, and the OPG–RANKL–RANK signaling pathway were also evaluated.

Materials and Methods

Ethics

All procedures used in this study were approved by the Animal Care Committee of Shandong Agricultural University (P. R. China) and were performed in accordance with the guidelines for experimental animals of the Ministry of Science and Technology (Beijing, P. R. China).

Experimental Design

One hundred and twenty 70-week-old Isa Brown laying hens were reared in a single cage (60 cm×45 cm×45 cm; length×width×height). The hens were divided into three groups of 40 hens and randomly assigned to one of the following three treatments: a basal diet (control), a basal diet (Shandong Zhongcheng Feed Technology Co., Ltd., China) supplemented with 0.5% calcium propionate (CP), and a basal diet supplemented with 0.5% calcium butyrate (CB). The calcium content in calcium propionate and calcium butyrate was 21.48% and 18.69%, respectively. The calcium content per kilogram of feed was 35.00 g for control, 35.90 g for CP (34.83 g basal+1.07 g added), and 35.76 g for CB (34.83 g basal+0.93 g added). In each treatment, there were 4 replicates of 10 hens each. The basal diet was formulated according to the recommendations of the National Research Council (NRC, 1994; Table 1). The experiment lasted 8 weeks. Housing temperature and relative humidity were maintained at 23±2℃ and 65%±3%, respectively. The photoperiod was 16 h of light and 8 h of dark. Each cage was equipped with a nipple drinker and feeder. All hens were provided *ad libitum* feed and water throughout the experimental period. Egg production and feed intake were recorded daily, and feed efficiency was calculated.

At weeks 4, 6, and 8, two hens were randomly selected from each replicate, and each treatment had 8 hens in total. After overnight feed withdrawal, 3 mL blood sample was

Table 1. **Composition of the experimental diet**

Items	Composition (%)
Ingredients	
Corn	63
Soybean meal	24
Limestone	8
NaCl	0.3
Choline chloride (50%)	0.1
Premix[a)]	4.6%
Total	100
Nutrient levels[b)]	
Metabolizable energy (Kcal/kg)	2700
Crude protein (%)	16.1
Lysine (%)	0.79
Methionine (%)	0.34
Calcium (%)	3.50
Available phosphorus (%)	0.45

[a)] The vitamin and mineral premix contains the following per kilogram of diet: vitamin A, 8800 IU; vitamin D3, 3,300 IU; vitamin K, 2.2 mg; vitamin E, 16.5 IU; cholecalciferol, 2,800 IU; riboflavin, 18 mg; niacin, 50 mg; pantothenic acid, 28 mg; biotin, 0.1 mg; folic acid, 0.6 mg; iron, 55 mg; selenium, 0.3 mg; copper, 5.5 mg; zinc, 88 mg; iodine, 1.7 mg; manganese, 88 mg; calcium, 5.7 g; and phosphorus, 3.3 g.

[b)] All nutrient levels were calculated values.

collected from a wing vein, and serum was separated by centrifugation at 1,500×*g* for 10 min at 4℃ and stored at −20℃ for further measurements.

At the end of the experiment, eight hens were randomly selected from each treatment. After overnight feed withdrawal, blood samples were collected from a wing vein at 08:00 a.m., and the chickens were euthanized by exsanguination after cervical dislocation (Close *et al*., 1997; Huang *et al*., 2015). After dissection, the spleen and right tibia were weighed and sampled. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80℃ for further analysis. Serum was separated by centrifugation at 1,500×*g* for 15 min and stored at −20℃ to measure the serum activity of alkaline phosphatase (ALP) and concentration of calcium and phosphorus.

Egg Quality

At weeks 2, 4, 6, and 8, 40 eggs were randomly collected for three consecutive days from each treatment and analyzed for egg quality. Eggshell thickness was determined by averaging measurements at three locations on the egg (air cell, equator, and sharp end) using an eggshell thickness tester (EFG-0503, ROBOTMATION, Tokyo, Japan). Eggshell strength was measured using an eggshell strength tester (EFG-0503, ROBOTMATION). Eggshells were weighed after drying for 12 h at 25℃ and expressed as the percentage of egg weight.

Serum Calcium and Phosphorus

Serum concentrations of ALP, calcium, and phosphorus were measured using commercial kits (Jian Cheng Bioengineering Institute, Nanjing, China). A fully automated biochemical analyzer (7160A, Hitachi, Tokyo, Japan) was used to determine serum calcium and phosphorus content and ALP activity. All procedures were performed according to the manufacturer's instructions.

Tibial Calcium and Phosphorus Content

The right tibial bone sample was treated with a mixture of alcohol and benzene (2:1) for 96 h for degreasing and then dried at 105℃ (Qixin, DHG-9070A, China) to a constant weight. The degreased bone sample was used to measure the calcium and phosphorus content. Calcium content was determined using the potassium permanganate method, and phosphorus content was determined using a spectrophotometric method.

The bones were incinerated in a muffle furnace (Xianke, KSJ, China) at 550℃ for 6 h. To avoid the formation of flames and smoke during incineration in the furnace, the bones were first burned in a crucible heated by an electric ceramic furnace.

Calcium analysis: Hydrochloric acid solution (10 mL) and a few drops of concentrated nitric acid were added to the crucible containing the ash. This solution was transferred to a 100 mL volumetric flask, cooled to room temperature, diluted to the mark with distilled water, and shaken well to form a sample decomposition solution. Next, 10−20 mL of the sample solution (calcium content, approximately 20 mg) was accurately pipetted into a 200 mL beaker, and 100 mL of distilled water and 2 drops of methyl red indicator were added; ammonia solution was added to the obtained solution until it turned orange. In-excess, hydrochloric acid solution was added to adjust to orange, and 2 more drops were added to turn the solution pink (pH 2.5−3.0). The sample was gradually boiled while slowly adding 10 mL of ammonium oxalate solution in a dropwise manner with constant stirring. When the solution turned orange, hydrochloric acid solution was added to make it red; the solution was boiled for several minutes and allowed to stand overnight to age the precipitate. The solution was filtered using a quantitative filter paper, and the precipitate was washed with 1+50 ammonia solution 6−8 times until there were no oxalate ions. After transferring the precipitate and filter paper to the original beaker, 10 mL of sulfuric acid solution and 50 mL of distilled water were added. The sample was heated to 75−80℃ and titrated with potassium permanganate standard until the solution turned pink and did not fade in half a minute as the end point. Simultaneously, the blank solution was analyzed.

$$X(\%)=\frac{(V-V_0)\times c\times 0.02}{m\times\frac{V'}{100}}\times 100=\frac{(V-V_0)\times c\times 200}{m\times V'}$$

X: Calcium content expressed as a mass fraction (%)
V: Volume of potassium permanganate standard solution consumed by the sample (mL)
V_0: Volume of potassium permanganate standard solution consumed by the blank solution (mL)
c: concentration of potassium permanganate standard solution (mol/L)
V' : Pipetted volume of the sample decomposition solution during titration (mL)
m: sample mass (g)
0.02: Calcium expressed in grams equivalent to 1.00 mL of potassium permanganate standard solution $\left[c\times\left(\frac{1}{5}KMnO_4\right)=1.000\,mol/L\right]$.

Two parallel samples were obtained from each sample for measurement, and arithmetic mean was used as the result.

Phosphorous analysis: In brief, 0.0, 1.0, 2.0, 4.0, 8.0, or 16.0 mL of phosphorus standard solution was accurately pipetted into a 50 mL volumetric flask, and 10 mL of ammonium vanadyl aluminate color reagent was add to each flask; the solution was diluted to the mark with water, shaken well, and allowed to stand at room temperature for 10 minutes Using the 0 mL solution as a reference and a 1 cm cuvette, the absorbance of each solution was measured with a spectrophotometer (BioTek, Winooski, VT, USA) at 400 nm. Phosphorus content was used as the abscissa and absorbance as the ordinate to draw a working curve.

Next, 1.0−10.0 mL of sample decomposition solution (phosphorus content, 50−750 μg) was accurately pipetted into a 50 mL volumetric flask, and 10 mL of ammonium molybdate vanadate color reagent was added. The sample was diluted with water to the mark, shaken well, and allowed to stand at room temperature for over 10 min. Absorbance of the sample decomposition solution was measured at 400 nm. Phosphorus content of the sample decomposition solution was

determined using the working curve.

$$X=\frac{m_1\times V}{m\times V_1\times 10^6}\times 100=\frac{m_1\times V}{m\times V_1\times 10^4}$$

X: Phosphorus content expressed as a mass fraction (%)
m_1: Phosphorus content of the sample decomposition solution determined based on the working curve (g)
V: Total volume of the sample decomposition solution (mL)
m: Mass of the sample (μg)
V_1: Measured volume of the sample decomposing solution (mL)

Ammonium vanadate molybdate color reagent: Briefly, 1.25 g of ammonium metavanadate was weighed, and 200 mL of water was added; the sample was heated to dissolve ammonium metavanadate, and 250 mL of nitric acid was added after cooling. Then, 25 g of ammonium molybdate was dissolved in 400 mL of water under cool conditions. The two solutions were mixed, diluted to 1,000 mL with water, and stored in the dark. If precipitation occurred, the sample could not be used.

Phosphorus standard solution: Potassium dihydrogen phosphate was dried at 105℃ for 1 h and cooled in a desiccator. Then, 0.2195 g of dried potassium dihydrogen phosphate was weighed and dissolved in water. The solution was quantitatively transferred to a 1,000 mL volumetric flask, and 3 mL of nitric acid was added. The solution was diluted to the mark with water and shaken well to obtain 50 μg/mL phosphorus standard solution.

Two parallel samples were obtained from each sample for measurement, and arithmetic mean was used as the result.

Bone Index and Bending Strength

After the tibia was obtained, all soft tissues and cartilaginous caps were removed and weighed. The tibial index was calculated as the ratio of tibial weight to body weight.

The mechanical (structural strength and stiffness) and material (flexural strength and modulus) properties of the tibia were quantified using a three-point bending test (Fleming *et al*., 1998). The tibia was centered over two supports (4 cm apart) with a 1 N preload before failure at a loading rate of 2 mm/min, with the anterior surface in tension. The three-point bending test was performed using a microcomputer-controlled electronic universal testing machine (Jinan Shi Jin Neng, China). When the bone fracture occurred, the maximum bending force F was measured, according to the following formula:

$$aw=(8\times F\times L)/\pi/d^3$$

Table 2. **Primers used for gene expression analysis using real-time polymerase chain reaction**

Gene name	GenBank accession number	Primers position	Primers sequence (5′ → 3′)
IL-6	HM179640.1	Forward	CGCCCAGAAATCCCTCCTC
		Reverse	AGGCACTGAAACTCCTGGTC
IL-17	AY744450.1	Forward	CTCCTCTGTTCAGACCACTGC
		Reverse	ATCCAGCATCTGCTTTCTTGA
NF-κB	NM.205129	Forward	CAGCCCATCTATGACAACCG
		Reverse	TCCCTGCGTCTCCTCTGTGA
RANKL	EF379383.1	Forward	TGTTGGCTCTGATGCTTGTC
		Reverse	TCCTGCTTCTGGCTCTCAAT
OPG	DQ098013.1	Forward	CGCTTGTGCTCTTGGACATT
		Reverse	GCTGCTTTACGTAGCTCCCA
β-actin	L08165	Forward	GAGAAATTGTGCGTGACATCAAGG
		Reverse	CACCTGAACCTCTCATTGCCA
TNF-α	HQ739087.1	Forward	GAGCGTTGACTTGGCTGTC
		Reverse	AAGCAACAACCAGCTATGCAC
FGF23	XM_425663.2	Forward	ATGCTGCTTGTGCTCTGTATC
		Reverse	CACTGTAAATGGTTTGGTGAGG
α-Klotho	XM_417105.5	Forward	ACCCGTCAATCCTGTTGG
		Reverse	TCAGCGTAGTCGTGGAAGAG
RUNX2	NM_204128.1	Forward	CACGCTGCTAAACCCAAACT
		Reverse	CAAACGGACTCATCCATCCT
OPN	NM_204535.4	Forward	GGCATTTCTTTGCTTGTGCT
		Reverse	AGCCAGGTCATTCTGTGTCTG
VDR	XM_015272607	Forward	AGAGGAAAGCGATGTTCACC
		Reverse	CCTTCATCATCCCAATGTCC
Osteocalcin	NM_205387.2	Forward	CCCAACGAGAGGTGTGTGA
		Reverse	TGCCTTTATTTCTGTCCATCCT
PHEX	XM_015302343	Forward	TGGTCAGAGAGGAGGTTTAGC
		Reverse	TTTGTCATCAGCAGCCACA

where L is the distance between two support points and d is the diameter of bone.

Real-time PCR

Total RNA was extracted from the spleen and tibia using TransZol Up (TransGen Biotech, Beijing, China). The RNA concentration was measured by spectrophotometry (Eppendorf, Hamburg, Germany), and RNA purity was verified by calculating the ratio of absorbance at 260 and 280 nm (A260/280 ≈ 1.75−2.01). Next, reverse transcription was performed using total RNA (1 μg) for first-strand cDNA synthesis using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). The cDNA was amplified in a 20 μL PCR reaction system containing 0.2 μmol/L of each specific primer (Sangon, Shanghai, China) and SYBR Green PCR Master Mix (Roche) according to the manufacturer's instructions. Real-time PCR was performed using the ABI Quant Studio 5 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Waltham, MA, USA). Primer sequences are listed in Table 2. The expression of *IL-6*, *IL-17*, *TNF-α*, nuclear factor kappa B (*NF-κB*), Runt-related transcription factor-2 (*RUNX2*), osteopontin (*OPN*), *osteocalcin*, phosphate-regulating gene with homology to endopeptidases on the X-chromosome (*PHEX*), *FGF23*, *α-Klotho*, *VDR*, *RANKL*, and *OPG* transcripts was measured. For each treatment, six to eight samples were analyzed, and each sample was measured twice.

Statistical Analysis

Data were analyzed using one-way ANOVA to estimate the main effects of dietary treatment (SAS version 8.1; SAS Institute Inc., Cary, NC, USA). When the main effect of the treatment was significant, the differences between the means were compared using Tukey's multiple comparison test. Statistical significance was set at $P<0.05$.

Results

Dietary CP and CB supplementation had no significant effect ($P>0.05$) on the laying rate, egg production, egg weight, and feed efficiency (Table 3). However, both CP and CB treatments decreased feed intake compared with the control treatment ($P<0.05$). Compared with the control value, the eggshell percentage was increased by the CP ($P<0.01$) and CB ($P<0.001$) treatments at week 2, 6 ($P<0.05$), and 8 ($P<0.05$) and by the CB treatment alone at week 4 ($P<0.01$) (Fig. 1A). Eggshell thickness was increased by the CB treatment at weeks 2 and 8 ($P<0.01$) and by the CP treatment at week 8 ($P<0.001$) (Fig. 1B). Compared with the control value, eggshell strength was increased by both CP and CB treatments at week 2 ($P<0.05$) and by the CP treatment alone at week 8 ($P<0.05$) (Fig. 1C).

Serum ALP level was not affected ($P>0.05$) by dietary supplementation. Compared with the control treatment, the CB treatment increased serum calcium level at week 4 ($P<0.05$), but neither CB nor CB treatment affected serum calcium level at weeks 6 and 8 ($P>0.05$, Fig. 2B). Compared with the control treatment, the CB treatment increased serum phosphorus level at week 4 ($P<0.05$), whereas the CP and CB treatments decreased serum phosphorus level at weeks 6 and 8, respectively ($P<0.01$, Fig. 2C).

Dietary supplementation had no significant effect on the tibial bone index and bending strength ($P>0.05$, Fig. 3A, B). However, tibial calcium and phosphorus levels were lower in hens received the CB diet than in those that received the control diet. In contrast, the tibial phosphorus content showed a decreasing trend ($P=0.070$) in hens that received the CP diet compared with that in hens that received the control diet (Fig. 3C, D).

Neither CP nor CB treatment had a significant effect ($P>0.05$) on the transcript levels of *RANKL*, *OPG*, *RNUX2*, *OPN*, *α-clotho*, and *VDR* in the tibia (Fig. 4), but *PHEX* transcript levels were increased by the CP treatment ($P<0.05$, Fig. 4F). The CB treatment tended to decrease the expression levels of *osteocalcin* ($P=0.094$) and *FGF23* ($P=0.087$) (Fig. 4E, G).

Furthermore, neither CP nor CB treatment had a significant effect on the expression levels of *NF-κB*, *TNF-α*, and *IL-17* in the tibia (Fig. 5A, B, C). Similarly, in the spleen, neither treatment affected *NF-κB* and *IL-6* transcript levels ($P>0.05$, Fig. 5D, F), but the CP treatment decreased *TNF-α* transcript levels ($P<0.05$, Fig. 5E).

Discussion

In the present study, the effect of dietary supplementation of CP and CB on bone health and laying performance of hens during the late phase of the first laying cycle was investigated. The results suggest that dietary supplementation of CP and CB can improve eggshell quality and stimulate bone mobilization.

In this study, feed intake was reduced by the CP and CB

Table 3. **Effect of dietary supplementation of calcium propionate (0.5%, CP) and calcium butyrate (0.5%, CB) on the laying performance of hens from 70 to 78 weeks of age**

	Control	CP	CB	*P*-value	F-value
Feed intake, g/d	131.9±3.3[a]	119.5±3.2[b]	118.2±2.4[b]	0.020	$F_{(2,9)}=6.26$
Laying rate, %	74.42±1.9	73.20±2.6	69.71±4.6	0.608	$F_{(2,9)}=0.53$
Egg weight, g	66.7±0.92	66.1±0.48	66.8±0.71	0.811	$F_{(2,9)}=0.21$
Egg production, g/h/d	44.1±1.2	41.4±2.4	39.7±2.8	0.413	$F_{(2,9)}=0.98$
Feed efficiency	3.32±0.15	2.99±0.21	3.13±0.16	0.510	$F_{(2,9)}=0.73$

Data are presented as mean±SD ($n=4$).
[a, b] Different superscript letters indicate significant differences ($P<0.05$).

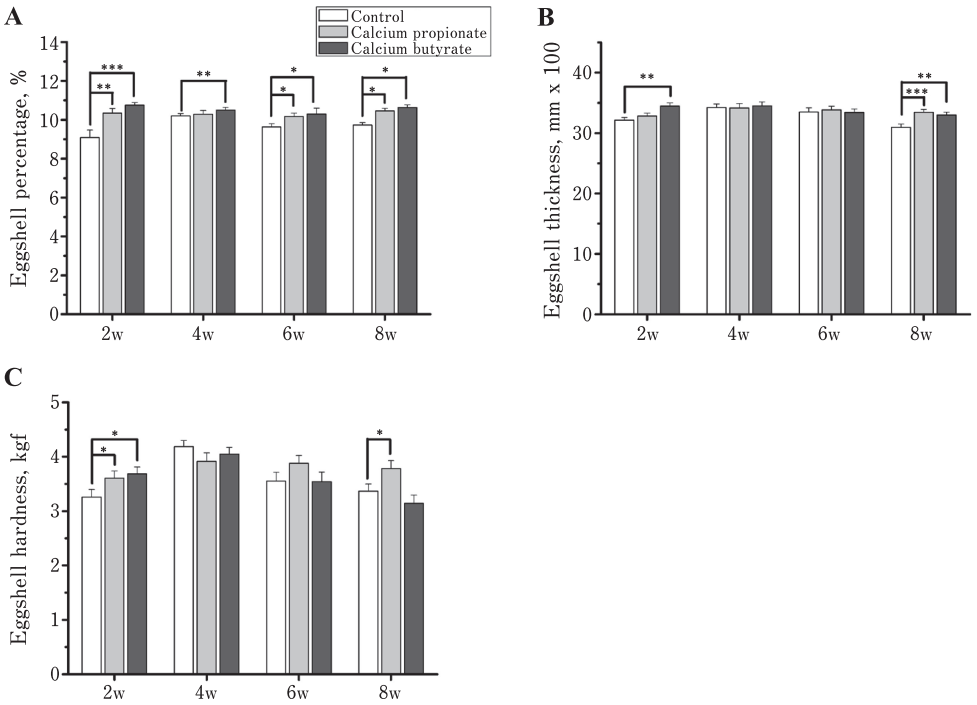

Fig. 1. **Effect of dietary supplementation of calcium propionate (0.5%) and calcium butyrate (0.5%) on eggshell percentage (A), eggshell thickness (B) and eggshell hardness (C) in 70-week-old laying hens.** Data are presented as mean±SD (n=38–40). * P <0.05; ** P<0.01; *** P<0.001.

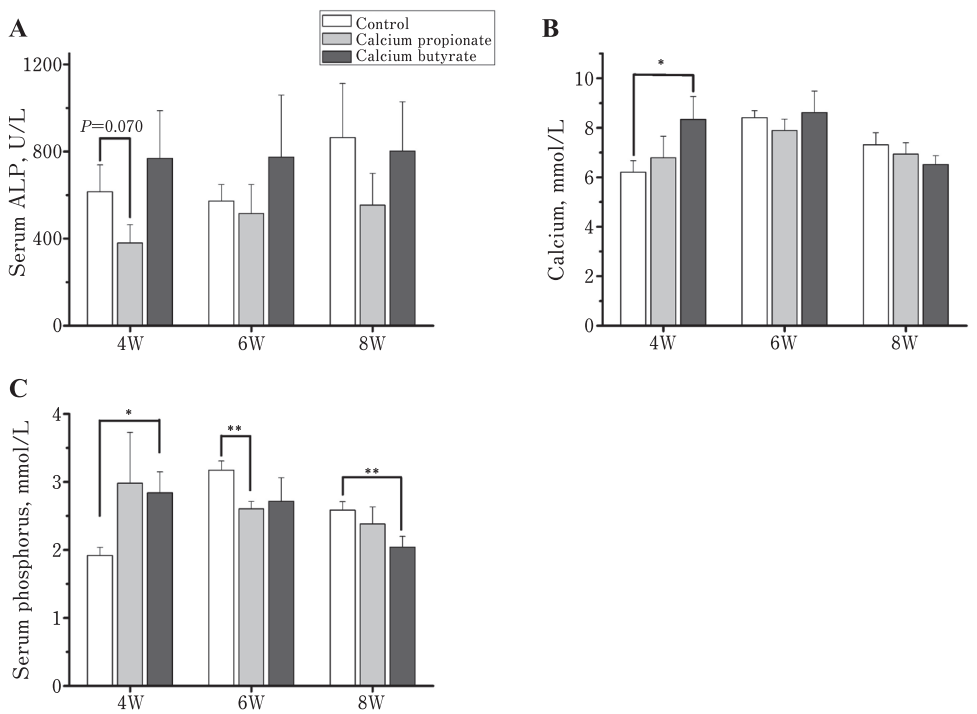

Fig. 2. **Effect of dietary supplementation of calcium propionate (0.5%) and calcium butyrate (0.5%) on serum alkaline phosphatase (ALP) (A), calcium (B), and phosphorus levels (C) in 70-weeks-age laying hens.** Data were presented as mean±SD (n= 8). *, P<0.05; **, P<0.01.

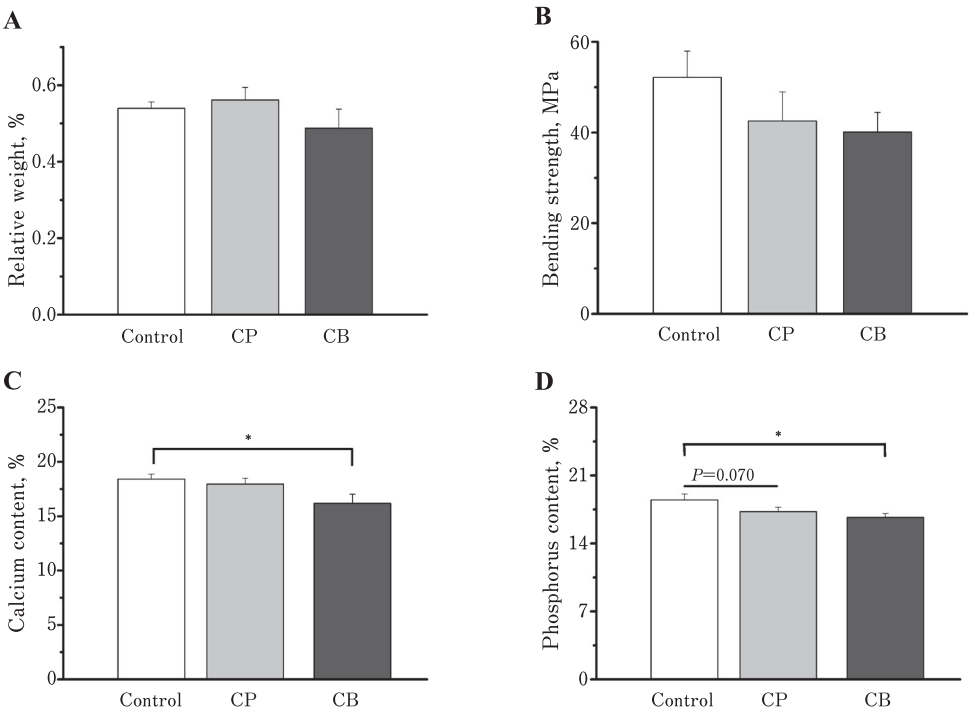

Fig. 3. **Effect of dietary supplementation of calcium propionate (0.5%, CP) and calcium butyrate (0.5%, CB) on tibia index (A), bending strength (B), calcium content (C), and phosphorus content (D) in tibia of 70-week-old laying hens.** Data are presented as mean±SD (n=8–10). *P<0.05.

treatments. This result was consistent with the report by Pinchasov and Elmaliah (1995) that voluntary feed and energy intake decreased significantly with the addition of acetic or propionic acid to the diet of broilers. In contrast, the laying rate, hen-day egg production, and feed efficiency were not influenced by dietary supplementation of CP and CB, indicating that laying performance is deteriorated by reduced feed intake. Similarly, Vieira *et al*. (2011) reported that sodium butyrate supplementation (20 mEq/kg) had no detectable influence on the laying performance of hens from 40 to 44 weeks of age. Arnouts *et al*. (2002) reported that supplementation of sodium butyrate (500 mg/kg) had no effect on egg weight. In contrast, dietary supplementation of 0.5%–1.5% sodium butyrate or calcium propionate improved the laying performance of hens from 24 to 40 weeks of age (Dahiya *et al*., 2016). Collectively, these results suggest that dietary CP or CB supplementation has no negative effect on the laying performance of hens.

Eggshell strength is an important indicator of egg quality. In laying hens, calcium and phosphorus play important roles in eggshell formation and quality. As laying hens age, the unsynchronous rise of calcium content with increase in eggshell weight is associated with the deterioration of eggshell quality (Bustany and Elwinger, 1987; Bolukbasi *et al*., 2005). Our results indicate that CP and CB increased eggshell percentage and thickness, while CP further elevated the break strength. These findings are in line with the results of a previous study by Arnouts *et al*. (2002), who reported that dietary supplementation of sodium butyrate positively affected eggshell quality by reducing the number of eggs with weak shells. Similarly, calcium balance was not influenced by the addition of sodium butyrate (Vieira *et al*., 2011). In this study, feed intake was decreased by the dietary supplementation of CP or CB, indicating that calcium and phosphorus intake was reduced in hens that received the CP and CB diets. In mice, lotus seed resistant starch enhanced the intestinal absorption of calcium, magnesium, and iron, and this effect was dependent on the type and concentration of short-chain fatty acids, specifically butyrate (Zeng *et al*., 2017). Blood calcium and phosphorus levels depend on absorption by the intestine, reabsorption by the kidney, and excretion by the eggshell gland. Our results showed that blood calcium and phosphorus levels fluctuated over time, and phosphorus showed a decreasing trend under the CB and CP treatments. In laying hens, the medullary bone (**MB**), a non-structural bone, is formed in the long bones when pullets reach sexual maturity (Whitehead and Fleming, 2000; Fleming, 2008). MB is resorbed during eggshell formation along with the structural bone, increasing the fracture risk in hens (Schweitzer *et al*., 2007; Shipov *et al*., 2010). The tibial bone index and bending strength were not significantly influenced by either CB or CP treatment, suggesting that no detectable change occurred in the tibia during the 8-week experimental period. However, the decreased

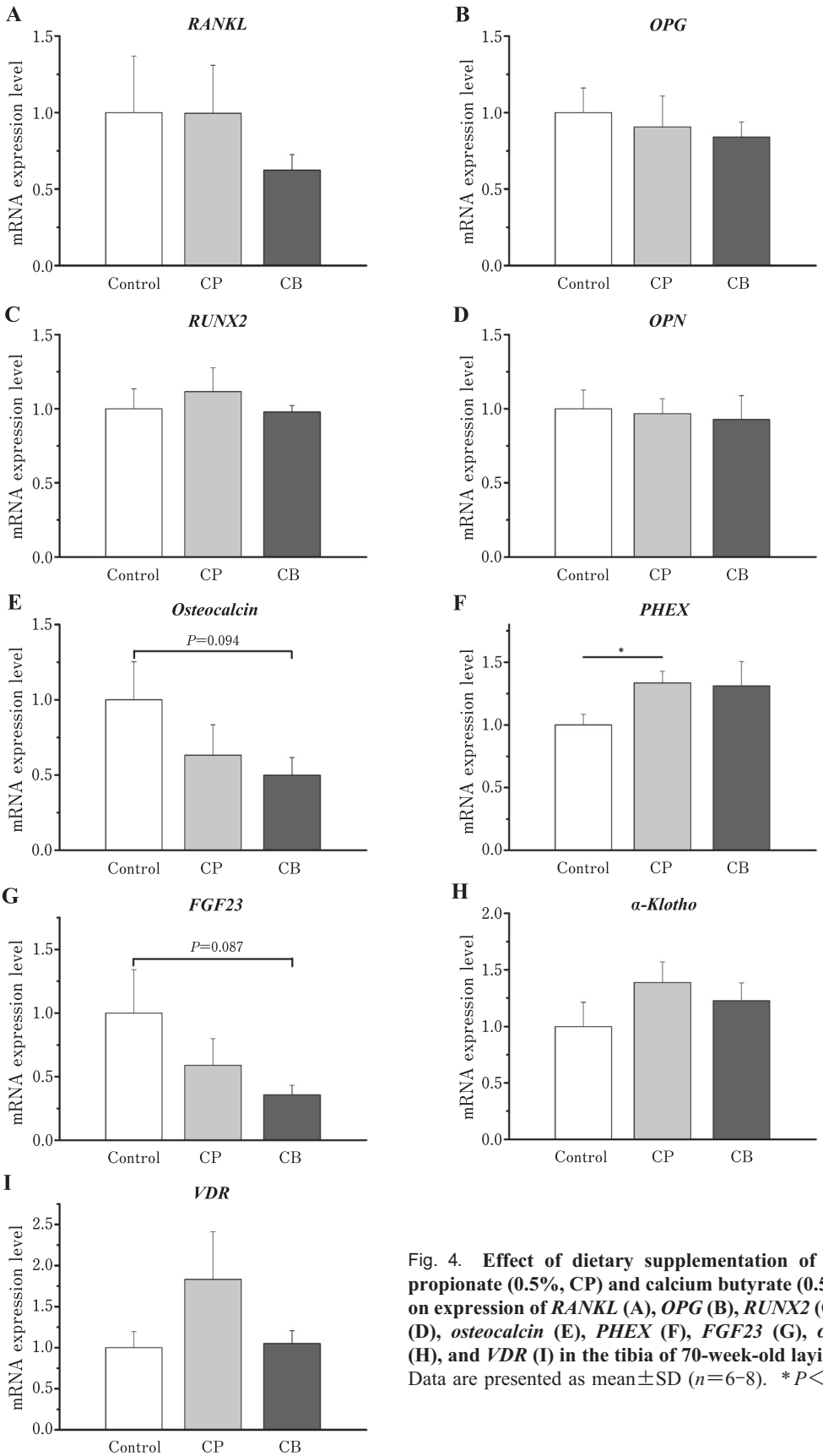

Fig. 4. **Effect of dietary supplementation of calcium propionate (0.5%, CP) and calcium butyrate (0.5%, CB) on expression of *RANKL* (A), *OPG* (B), *RUNX2* (C), *OPN* (D), *osteocalcin* (E), *PHEX* (F), *FGF23* (G), *α-Klotho* (H), and *VDR* (I) in the tibia of 70-week-old laying hens.** Data are presented as mean±SD (n=6–8). *$P<0.05$.

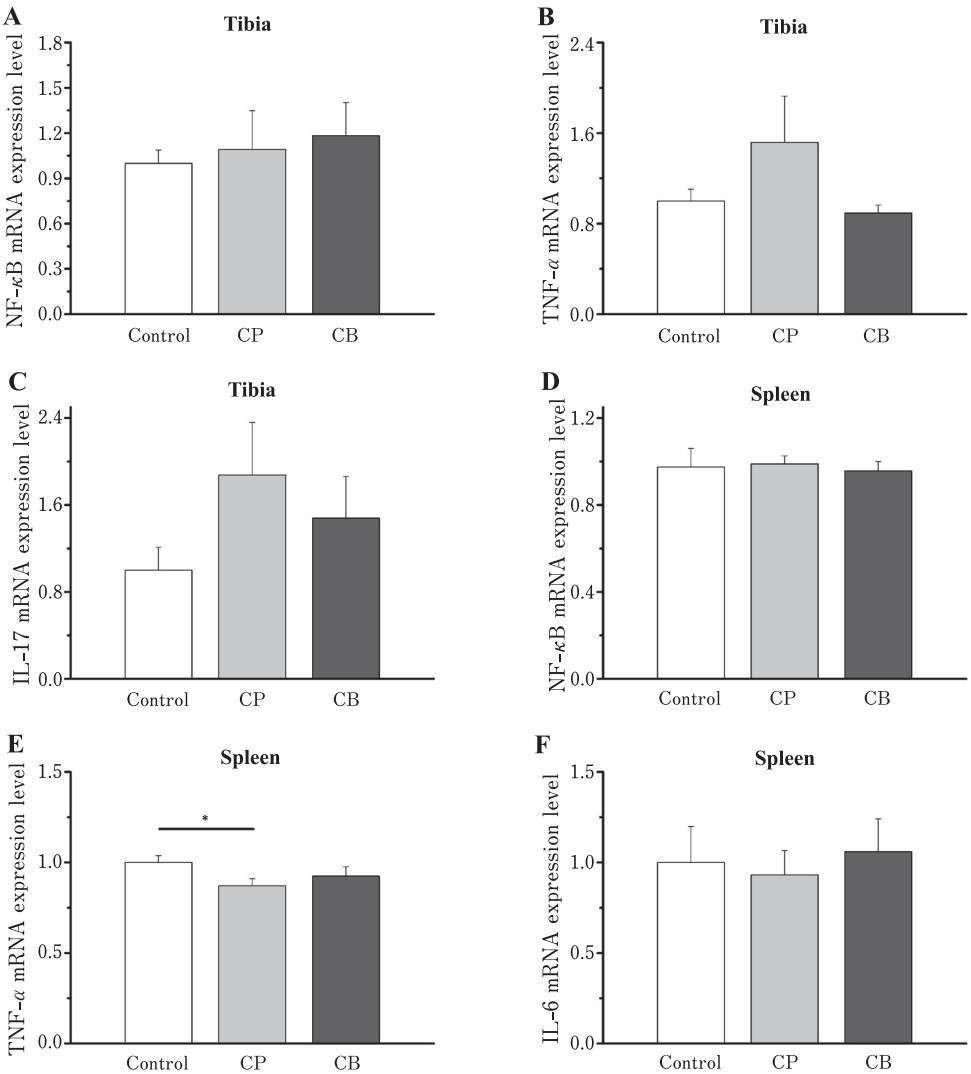

Fig. 5. **Effect of dietary supplementation of calcium propionate (0.5%, CP) and calcium butyrate (0.5%, CB) on the expression (A) *NF-κB*, (B) *TNF-α*, and (C) *IL-17* in the tibia and (D) *NF-κB*, (E) *TNF-α*, and (F) *IL-6* in the spleen of 70-week-old laying hens.** Data are presented as mean±SD (n=6–8). * $P<0.05$.

tibial calcium and phosphorus content by the CB treatment indicated enhanced bone resorption. A recent study reported that dietary supplementation of 0.2% butyric acid increased tibial ash content and decreased serum ALP levels (Nari and Ghasemi, 2020). Moreover, metabolic acids stimulate bone resorption, and the extracellular acidification of osteoclasts decreases intracellular pH and calcium levels (Teti *et al*., 1989). As positive controls supplemented with butyrate and propionate or calcium carbonate were not included in the present study, the effects of CP and CB supplementation on bone mineral deposition and the underlying mechanisms should be investigated in future studies.

Short-chain fatty acids promote immune function. The anti-inflammatory effects of butyrate are achieved by decreasing the expression of pro-inflammatory cytokines and reducing the proliferation and activation of lymphocytes via inhibition of the NF-κB pathway (Kyner *et al*., 1976; Segain *et al*., 2000; Meijer *et al*., 2010; Vinolo *et al*., 2011). In the present study, the expression of *NF-κB*, *TNF-α*, *IL-17*, and *IL-6* in the tibia and/or spleen was not altered by dietary supplementation, suggesting that neither treatment affected the immune function. However, the CP treatment decreased the expression of *TNF-α* in the spleen.

RANKL, a member of the TNFR ligand family (Anderson *et al*., 1997), is a type II transmembrane protein expressed in osteoblasts and chondrocytes. RANK and RANKL bind to each other and enhance bone resorption by promoting osteoclast maturation and differentiation (Malliga *et al*., 2011; Huang *et al*., 2013). OPG, a secreted glycoprotein that can suppress the binding of RANKL and RANK, inhibits osteo-

clast activity, reduces osteoclast differentiation, and increases bone density (Simonet *et al*., 1997; Harada and Takahashi, 2011). OPG, RANKL, and RANK interact to form the OPG–RANKL–RANK axis that regulates bone metabolic balance (Klejna *et al*., 2009). In this study, the expression of RANKL and OPG in the tibia was investigated. The unchanged expression of RANKL and OPG in the tibia implies that the CP and CB treatments did not activate the OPG–RANKL–RANK signaling pathway.

RUNX2 is a vital transcription factor for chondrocyte maturation and osteoblast differentiation (Arumugam *et al*., 2019; Liao *et al*., 2019). The non-collagenous proteins OPN and osteocalcin play important roles in bone formation (McKee *et al*., 1992). The expression levels of *RUNX2*, *OPN*, and *osteocalcin* were not significantly changed by the CP or CB treatment, suggesting that bone formation was not influenced by dietary supplementation.

Furthermore, expression levels of the phosphate-regulating genes *PHEX*, *FGF23* and its co-receptor *α-Klotho*, and *VDR* were further measured. *PHEX* regulates *FGF23* expression or stability, and loss of *PHEX* activity increases *FGF23* expression (Kiela and Ghishan, 2009). In this study, *PHEX* expression showed an increasing trend, while *FGF23* expression showed a decreasing trend in hens that received the CB diet. This result was in line with the decrease in phosphorus levels in the blood and tibia, suggesting that CB supplementation enhanced phosphorus mobilization or suppressed phosphorus deposition in the tibia.

In summary, dietary supplementation of 0.5% CP or 0.5% CP improved the eggshell quality of laying hens during the late phase of the first laying cycle, possibly as a result of decreased calcium and phosphorus deposition or enhanced mobilization in the bone.

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Conflicts of Interest

The authors declare no conflict of interest.

Availability of Data and Materials

All data generated or analysed during this study are included in this published article.

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