Effects of dietary *Bacillus subtilis* supplementation and calcium levels on performance and eggshell quality of laying hens in the late phase of production

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ABSTRACT The objective of this study was to evaluate the effects of dietary *Bacillus subtilis* supplementation and calcium (**Ca**) levels on performance, eggshell quality, intestinal morphology, and relative calbindin-D28k (**CALB1**) mRNA level of laying hens in the late phase of production. An experiment employing a 2 × 3 factorial arrangement of 3 levels of Ca (3.5, 4.0, and 4.5%) and the absence or presence of *B. subtilis* was carried out with a total of 576 Hy-Line Brown laying hens aged 72 to 79 wk. Every group had 8 replicates of 12 birds each. The results showed that 4.0 and 4.5% Ca levels improved ($P < 0.05$) apparent retention and serum Ca content of aged laying hens. Compared with the 3.5% Ca level, the 4.0% Ca level in diets increased ($P < 0.05$) thickness, eggshell weight, shell ratio, and eggshell Ca content of aged laying hens. Moreover, breaking strength, thickness, eggshell weight, shell ratio, eggshell Ca content, apparent retention of Ca in g/day, apparent retention of Ca in percent, villus height, villus height/crypt depth, serum Ca level, and relative CALB1 mRNA level of aged laying hens were all increased ($P < 0.05$) by *B. subtilis* supplementation in diets. The supplemental *B. subtilis* decreased feed conversion ratio ($P = 0.001$) significantly. In addition, there was an interaction effect between increased Ca levels from 3.5 to 4.5% and *B. subtilis* supplementation on crypt depth in the duodenum ($P < 0.05$). In conclusion, we found that both the increase in dietary Ca level from 3.5 to 4.5% and *B. subtilis* supplementation could enhance intestinal Ca absorption and improve eggshell quality of laying hens in the late phase of production (72–79 wk of age). Dietary supplementation of *B. subtilis* accompanying the 4.0% Ca level was appropriate in enhancement of eggshell quality.

Key words: *Bacillus subtilis*, calcium retention, eggshell quality, laying hen, late phase of production

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INTRODUCTION

Eggshell formation of laying hens is a classical and rapid biomineralization process. About 2 g of calcium (**Ca**) per egg deposits as the calcium carbonate in the eggshell gland in a span of less than 20 h. The decline in intestinal Ca absorption, leading to a decrease in Ca deposition and utilization, was reported to be partly responsible for the reduced eggshell quality in aged laying hens (Roland, 1988; Al-Batshan et al., 1994; Grobas et al., 1999).

A large body of research exists on dietary Ca manipulation as a primary means to improve eggshell quality, but the effects of increased dietary Ca level above 3.5% on intestinal Ca absorption and eggshell quality were variable. Despite decreased Ca availability, increased Ca level in diets could obtain better eggshell quality of aged laying hens through the improved Ca retention in some studies (Chowdhury and Smith, 2002; Lichovnikova, 2007; Saffa et al., 2008; An et al., 2016), while others could not (Lesson et al., 1993; Keshavarz and Nakajima, 1993). The effects of dietary Ca level above 3.5% on intestinal Ca absorption and eggshell quality need reevaluation.

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Enhancing Ca availability and absorption in the gut is another alternative for improving eggshell quality. *Bacillus subtilis* could work as a competitive exclusion agent (Knap et al., 2011), promote gut microflora balance (Knarreborg et al., 2008), and improve intestinal integrity and nutrients absorption efficiency (Sen et al., 2012). In a study by Abdelqader et al. (2013), *B. subtilis* was reported to decrease the pH of intestinal digesta and increase the intestinal absorption surface of aged laying hens for improving Ca availability. Effects of *B. subtilis* supplementation on the performance and eggshell quality of aged laying hens were also previously investigated in our laboratory and better eggshell quality could be obtained when *B. subtilis* was added at 2×10^9 CFU/kg (Cui, 2018). If so, with improved Ca availability, effects of increased Ca levels on Ca retention and eggshell quality may be ameliorated when *B. subtilis* is supplemented. Likewise, with different Ca levels, the effect of *B. subtilis* addition on Ca retention and eggshell quality may be different.

Thus, the combined effects of dietary Ca levels from 3.5 to 4.5% and *B. subtilis* supplementation may have a positive effect on intestinal Ca absorption and eggshell quality of aged laying hens. To test this hypothesis, this study was carried out to examine the effects of dietary Ca levels from 3.5 to 4.5% and *B. subtilis* addition on the performance and eggshell quality of aged laying hens. Besides, intestinal Ca absorption of laying hens is mainly mediated by transcellular and paracellular pathways in laying hens after sexual maturity (Gloux et al., 2019). To explore the patterns of Ca absorbed in the current study, the major player of transcellular Ca transport, namely calbindin-D28k (**CALB1**), was also examined.

MATERIALS AND METHODS

Experimental Design and Diets

This study was approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences. The prefeeding period lasted for 1 wk (71–72 wk of age) with corn-soybean meal basal diets containing 3.5% Ca. An experiment employing a 2×3 factorial arrangement of 3 levels of Ca (3.5, 4.0, and 4.5%) and the absence or presence of *B. subtilis* was carried out with a total of 576 Hy-Line Brown laying hens aged 72 to 79 wk. The 3 diets that met the nutrient specifications of NRC (1994) with varying Ca levels are shown in Table 1. Each group was replicated 8 times with 12 birds per replicate. Three birds were allotted to 1 cage (47 cm × 47 cm × 37 cm). The *B. subtilis* used was a naturally occurring strain of *B. subtilis* C-3102 (Calsporin; provided by Shanghai Muguan Enterprise Development Co., Ltd., Shanghai, China). The probiotic contained 1×10^{10} CFU/g of *B. subtilis* spores and its supplemental level was 2×10^9 CFU/kg. The actual count (9.3×10^9 CFU/g) of *B. subtilis* in this supplement was determined using the spread plate method. Hens were housed in 3-tier cages with ad libitum access to feed and water. Lighting was controlled automatically 16 h (4:30–20:30) per day.

Table 1. Composition and nutrient levels of the diets (air-dry basis) %.

Ingredients	3.5% Ca	4.0% Ca	4.5% Ca
Corn (79 g/kg CP)	64.020	63.400	63.090
Soybean meal (470 g/kg CP)	20.440	21.310	22.200
Limestone	8.620	10.013	11.370
Soybean oil	0.180	0.540	0.830
Wheat bran	4.418	2.413	0.181
Choline chloride (500 g/kg)	0.100	0.100	0.100
$CaHPO_4$	1.430	1.450	1.475
NaCl	0.300	0.300	0.300
Premix[1]	0.320	0.320	0.320
Met (DL-methionine, 980 g/kg)	0.100	0.099	0.097
Lys (L-lysine hydrochloride, 990 g/kg)	0.063	0.050	0.037
Thr (L-threonine, 990 g/kg)	0.009	0.005	0.000
Total	100.000	100.000	100.000
Nutrient levels[2]			
ME/(MJ/kg)	11.090	11.090	11.090
CP	15.500 (15.392)	15.500 (15.381)	15.500 (15.403)
Ca	3.510 (3.796)	4.010 (4.094)	4.500 (4.702)
TP	0.550 (0.548)	0.540 (0.533)	0.530 (0.537)
AP	0.346	0.346	0.346
Lys	0.778	0.778	0.778
Met	0.343	0.343	0.343

Abbreviations: AP, available phosphorus; Lys, lysine; Met, methionine; TP, total phosphorus.

[1]Provided per kilogram of diet: vitamin A 12,500 IU; vitamin D_3 4,125 IU; vitamin E 15 IU; vitamin K_3 2 mg; thiamine 1 mg; riboflavin 8.5 mg; calcium pantothenate 50 mg; niacin 32.5 mg; pyridoxine 8 mg; biotin 2 mg; folic acid 5 mg; vitamin B_{12} 5 mg; Zn 66 mg; I 1 mg; Fe 60 mg; Cu 8 mg; Se 0.3 mg; montmorillonite 1 g.

[2]Nutrition levels were all calculated values except the numbers in parentheses. Experimental diets were formulated according to Chinese Feeding Standard of Chicken (Ministry of Agriculture of China, 2004) and NRC (1994).

Performance and Eggshell Quality

During the feeding trial, egg production and egg weight were recorded daily, and feed consumption was recorded every 2 wk.

Eighteen eggs (3 successive days, 6 eggs each day with the weight close to the replicate average) from each replication were used to determine eggshell quality at the end of 75 and 79 wk of age. Eggshell thickness was determined by the Egg Shell Thickness Gauge (Orka Food Technology Ltd., Ramat Hasharon, Israel) with the membrane. Breaking strength was determined by the Egg Force Reader (the same as above). Eggshell weight was measured after the eggshell dried at room temperature for 48 h. The shell ratio was calculated as eggshell weight/egg weight × 100.

Ca and P Contents in Eggshell and Tibia

At the end of the trial, 6 eggshells (selected randomly from the 18 eggshells collected in 79 wk) from each replicate were collected as a sample to measure the Ca and P contents in eggshell. Firstly, the eggshells were washed with distilled water to obliterate dirt and eggshell membrane completely. After drying at room temperature for 48 h, the eggshells were weighed, grinded, and calcined. The Ca and P contents were analyzed by flame atomic absorption spectrophotometry (ZEEnit 700 P, Analytik Jena, Germany).

The left tibia from each laying hen was collected (1 bird of each replicate) to determine the Ca and P contents. After cleaning, the tibias were submerged in absolute alcohol and diethyl ether for 48 h, respectively. Subsequently, the Ca and P contents were analyzed by flame atomic absorption spectrophotometry (ZEEnit 700 P, Analytik Jena) after the tibia was dried, grinded, and calcined.

Apparent Retention of Ca and P

A metabolic experiment was carried out to measure the apparent retention of Ca and P. Three birds per replicate were placed in an individual cage and the daily feed intake and total excreta output were both recorded. A representative sample of excreta and feed from each pen was dried at 65°C for 3 d and subsequently analyzed for Ca and P using flame atomic absorption spectrophotometry (ZEEnit 700 P, Analytik Jena). Apparent retention was determined according to the procedure described by Chowdhury and Smith (2001) and Abdelqader et al. (2013).

$$\text{Apparent retention of Ca (g/d)} = \%\,\text{Ca in feed} \times \text{feed consumed} - \%\,\text{Ca in excreta} \times \text{excreta output}.$$

$$\text{Apparent retention of P (g/d)} = \%\,\text{P in feed} \times \text{feed consumed} - \%\,\text{P in excreta} \times \text{excreta output}.$$

$$\text{Apparent retention of Ca (\%)} = (\%\,\text{Ca in feed} \times \text{feed consumed} - \%\,\text{Ca in excreta} \times \text{excreta output}) \times /(\%\text{Ca in feed} \times \text{feed consumed}).$$

$$\text{Apparent retention of P (\%)} = (\%\,\text{P in feed} \times \text{feed consumed} - \%\,\text{P in excreta} \times \text{excreta output}) \times /(\%\,\text{P in feed} \times \text{feed consumed}).$$

Serum Biochemical Parameters

At the end of the feeding trial, 2 laying hens from each replication were selected randomly to collect blood from the wing vein and set at 37.5°C for 10 h to harvest serum. The serum was stored at −20°C until analysis. Serum levels of Ca, P, albumen, and alkaline phosphatase were determined by an automatic biochemical analyzer (model 7020, Hitachi, Tokyo, Japan).

Intestinal Morphology

At the end of the feeding trial, 2 laying hens from each replication were selected randomly and then euthanized with an overdose of pentobarbital (1.5 ml/kg). After cardiac arrest was confirmed, the small intestine was isolated immediately from the gastrointestinal tract and cut to obtain the duodenum (distal to the gizzard to 1 cm distal to the bile duct). The samples were washed in physiological saline solution, fixed in 10% buffered formalin overnight, processed into wax sections of 4 μm thickness, and stained with hematoxylin-eosin for histological analysis, according to the method of Choe et al. (2012). The morphometric variables included villus height (from the tip of the villi to the villus-crypt junction) and crypt depth (depth of the invagination between adjacent villi). The villus height and crypt depth were measured and recorded using an image analyzer (Image-Pro Plus 7.0, Media Cybernetics, Rockville, MD). A total of 3 intact, well-oriented crypt villus units were selected per bird for each intestinal cross-section, and the average of these values was used to express the mean values of villus height and crypt depth for each bird. The villus height/crypt depth (**V/C**) was calculated as the villus height relative to the crypt depth.

Total Quantitative Real-Time PCR Extraction, Reverse Transcription, and Quantitative Real-Time PCR

Samples from duodenum were frozen in liquid nitrogen and then stored at −80°C until further analysis. According to the instructions, total RNA was isolated from the duodenum with TRIzol reagent (Tiangen Biotech Co. Ltd., Beijing, China). Concentration and quality of the extracted RNA were assessed by spectrophotometry from 230 to 280 nm, using an UV-vis spectrophotometer (ND5000, Thermo Fisher Scientific, Waltham, MA). OD260/280 values ranged from 1.8 to 2.0 to ensure purity of total RNA. According to the instructions, reverse transcription was performed using 2 μg of total RNA with the FastQuant RT kit (KR106, Tiangen Biotech Co. Ltd.). Primers used in this study are shown in Table 2.

The CFX96 touch real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA) with SuperReal PreMix Plus (SYBR Green, FP205, Tiangen Biotech Co.) was used in accordance with manufacturer instructions to perform quantitative real-time PCR. The program was set at 95°C for 15 min, 40 cycles of 95°C for 10 s, 60°C for 30 s. Each sample was measured in duplicate. The size of all amplified products was confirmed by electrophoresis on a 1.5% (w/v) agarose gel with GelRed (SolarGelRed Nucleic Acid Gel Stain, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and visualized in Gel Doc XR+ System (Bio-Rad Laboratories Inc.). The relative mRNA expression levels were normalized to avian β-actin by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

All analyses were performed using SAS (SAS Institute Inc., Cary, NC). The replicate was considered to be an experimental unit for analysis. Main effects of Ca level and *B. subtilis* and their interactions were analyzed using general linear model procedures. For freeing some degrees of freedom and improving the power of this analysis, the interaction terms in the code were removed and data were reanalyzed when the interaction effects were not significant ($P > 0.05$). When interactions were significant ($P < 0.05$), differences between means were analyzed by Duncan's multiple range test. A P-value less than 0.05 was considered significant. Before analysis, egg production was subjected to an arcsine transformation.

RESULTS

Performance

The egg production, egg weight, ADFI, and feed conversion ratio (**FCR**) values are listed in Table 3. There was no interaction ($P > 0.05$) between *B. subtilis* supplementation and dietary Ca levels on the performance of laying hens. *B. subtilis* supplementation in diets decreased ($P < 0.05$) FCR significantly with no main effect of dietary Ca levels.

Eggshell Quality

Tables 4 and 5 show the breaking strength, thickness, weight, and shell ratio of eggshell. There were no interactions between *B. subtilis* supplementation and dietary Ca levels on eggshell quality parameters ($P > 0.05$). *B. subtilis* supplementation increased the breaking strength of the eggshell significantly at 79 wk of age ($P < 0.05$). Besides, eggshell thickness, eggshell weight, and shell ratio of laying hens fed with the 4.0% Ca level significantly increased ($P < 0.05$) compared with the 3.5% Ca level in diets.

Table 2. Nucleotide sequences of PCR primers used to assay gene expression by real-time quantitative PCR.

Gene names	Forward/reverse primer	Amplicon size (bp)	Accession
β-actin	GTGATGGACTCTGGTGATGGT TCGGCTGTGGTGGTGAAG	161	NM_205518
CALB1	GACATTAACAACCTTGCGACATAC CACAGAGAATGAGAGCCAGTTC	94	NM_205513.1

Abbreviation: CALB1, calbindin-D28k.

Table 3. Effects of dietary *Bacillus subtilis* supplementation and dietary Ca levels on performance of laying hens (72–79 wk of age).[1]

Items		Egg production (%)	Egg weight (g)	ADFI (g)	FCR[2]
Ca level (%)	*B. subtilis* level (CFU/kg)				
3.5	0	87.88	65.39	125.15	2.18
	2×10^9	89.86	65.83	122.89	2.05
4	0	88.82	66.08	124.40	2.13
	2×10^9	89.52	66.24	125.10	2.09
4.5	0	89.95	66.16	127.39	2.15
	2×10^9	89.12	66.22	124.92	2.09
SEM		0.37	0.11	0.40	0.01
Means of main effects					
Ca level (%)					
3.5		88.87	65.61	124.02	2.13
4		89.17	66.16	124.75	2.11
4.5		89.53	66.19	126.16	2.12
B. subtilis level (CFU/kg)					
0		88.88	65.85	125.65	2.15[b]
2×10^9		89.50	66.10	124.30	2.08[a]
P-value					
Ca level		0.718	0.051	0.081	0.849
B. subtilis level		0.356	0.275	0.091	0.001
Ca level × *B. subtilis* level		0.330	0.757	0.157	0.139

[a,b]Means assigned different superscripts within a factor of analysis (Ca level, *B. subtilis* level, and their interactions) differ, $P < 0.05$.
[1]Data are the mean of 8 replicates with 12 birds each.
[2]FCR, feed conversion ratio (feed:egg, g:g).

Tibia and Eggshell Ca and P Contents

As noted in Table 6, there were no interactions ($P > 0.05$) between Ca levels and *B. subtilis* supplementation on Ca and P contents in eggshell and tibia ash. The 4.0% Ca level had a significant increasing effect ($P < 0.05$) on the Ca content in eggshell compared with the 3.5% Ca level in the diet. In addition, supplemental *B. subtilis* in diets also increased ($P < 0.05$) the Ca content in eggshell of aged laying hens.

Table 4. Effects of *Bacillus subtilis* supplementation and dietary Ca levels on breaking strength and thickness of eggshell.[1]

		Breaking strength (N)		Thickness (× 0.01 mm)	
Items		75 wk	79 wk	75 wk	79 wk
Ca level (%)	*B. subtilis* level (CFU/kg)				
3.5	0	39.36	37.31	42.48	42.61
	2×10^9	38.84	38.22	42.27	43.25
4	0	38.92	37.93	42.67	43.43
	2×10^9	39.52	38.92	43.02	44.08
4.5	0	39.33	37.69	43.11	43.30
	2×10^9	39.46	38.24	42.82	43.56
SEM		0.34	0.18	0.13	0.11
Means of main effects					
Ca level (%)					
3.5		39.10	37.76	42.38	43.01[b]
4		39.22	38.42	42.85	43.70[a]
4.5		39.40	37.96	42.96	43.42[a,b]
B. subtilis level (CFU/kg)					
0		39.21	37.64[b]	42.75	43.17[b]
2×10^9		39.27	38.46[a]	42.70	43.57[a]
P-value					
Ca level		0.913	0.231	0.191	0.033
B. subtilis level		0.903	0.013	0.879	0.047
Ca level × *B. subtilis* level		0.821	0.862	0.530	0.632

[a,b]Means assigned different superscripts within a factor of analysis (Ca level, *B. subtilis* level, and their interactions) differ, $P < 0.05$.
[1]Data are the mean of 8 replicates with 18 eggs each.

Table 5. Effects of *Bacillus subtilis* supplementation and dietary Ca levels on shell ratio and eggshell weight.[1]

Items		Shell ratio (%)		Eggshell weight (g)	
		75 wk	79 wk	75 wk	79 wk
Ca level (%)	*B. subtilis* level (CFU/kg)				
3.5	0	9.56	9.61	6.23	6.25
	2×10^9	9.55	9.70	6.18	6.36
4	0	9.62	9.74	6.27	6.37
	2×10^9	9.65	9.90	6.39	6.49
4.5	0	9.69	9.73	6.30	6.38
	2×10^9	9.70	9.79	6.31	6.44
SEM		0.21	0.03	0.03	0.02
Means of main effects					
Ca level (%)					
3.5		9.56	9.66[b]	6.21	6.31[b]
4		9.64	9.81[a]	6.33	6.43[a]
4.5		9.70	9.75[a,b]	6.31	6.41[a,b]
B. subtilis level (CFU/kg)					
0		9.63	9.70[b]	6.27	6.33[b]
2×10^9		9.63	9.78[a]	6.31	6.43[a]
P-value					
Ca level		0.321	0.047	0.222	0.044
B. subtilis level		0.901	0.049	0.673	0.023
Ca level × *B. subtilis* level		0.982	0.634	0.470	0.862

[a,b]Means assigned different superscripts within a factor of analysis (Ca level, *B. subtilis* level, and their interactions) differ, $P < 0.05$.
[1]Data are the mean of 8 replicates with 18 eggs each.

Apparent Retention of Ca and P

Table 7 lists the apparent retention of Ca and P of laying hens. There was no interaction ($P > 0.05$) between Ca level and *B. subtilis* supplementation on the apparent retention of Ca. However, the 4.0 and 4.5% Ca levels improved ($P < 0.05$) the apparent Ca retention in g/day significantly and *B. subtilis*

Table 6. Effects of *Bacillus subtilis* supplementation and dietary Ca levels on Ca and P contents of tibia ash and eggshell (79 wk of age).

Items		Eggshell[1]		Tibia ash[2]	
		Ca (%)	P (%)	Ca (%)	P (%)
Ca level (%)	*B. subtilis* level (CFU/kg)				
3.5	0	30.87	0.13	39.76	17.75
	2×10^9	33.49	0.15	41.98	17.02
4	0	34.73	0.14	37.81	16.94
	2×10^9	36.71	0.14	39.00	15.99
4.5	0	31.44	0.14	38.55	15.76
	2×10^9	35.37	0.12	39.73	16.82
SEM		0.60	0.004	0.56	0.28
Means of main effects					
Ca level (%)					
3.5		32.17[b]	0.14	40.87	17.43
4		35.61[a]	0.14	38.47	16.46
4.5		33.01[a,b]	0.13	39.14	16.23
B. subtilis level (CFU/kg)					
0		32.49[b]	0.14	38.77	16.81
2×10^9		35.17[a]	0.14	40.24	16.61
P-value					
Ca level		0.041	0.176	0.169	0.202
B. subtilis level		0.025	0.770	0.729	0.196
Ca level × *B. subtilis* level		0.773	0.085	0.267	0.908

[a,b]Means assigned different superscripts within a factor of analysis (Ca level, *B. subtilis* level, and their interactions) differ, $P < 0.05$.
[1]Data are the mean of 8 replicates with 18 eggs each.
[2]Data are the mean of 8 replicates with 1 bird each.

Table 7. Effects of *Bacillus subtilis* supplementation and dietary Ca levels on apparent retention of Ca and P (79 wk of age).[1]

Items		Ca (g/day)	P (g/day)	Ca (%)	P (%)
Ca level (%)	*B. subtilis* level (CFU/kg)				
3.5	0	1.78	0.21	39.34	30.62
	2×10^9	2.92	0.19	62.15	28.21
4	0	2.26	0.21	50.22	31.67
	2×10^9	3.75	0.23	69.89	33.17
4.5	0	3.19	0.22	58.26	32.77
	2×10^9	3.83	0.21	71.80	31.90
SEM		0.15	0.01	2.83	1.12
Means of main effects					
Ca level (%)					
3.5		2.35[b]	0.20	50.74[b]	29.42
4		3.01[a]	0.22	57.37[a,b]	32.42
4.5		3.51[a]	0.22	63.67[a]	32.33
B. subtilis level (CFU/kg)					
0		2.41[b]	0.21	49.88[b]	31.69
2×10^9		3.50[a]	0.21	67.50[a]	31.09
P-value					
Ca level		0.012	0.703	0.033	0.170
B. subtilis level		<0.001	0.789	<0.001	0.262
Ca level × *B. subtilis* level		0.212	0.834	0.479	0.871

[a,b]Means assigned different superscripts within a factor of analysis (Ca level, *B. subtilis* level, and their interactions) differ, $P < 0.05$.
[1]Data are the mean of 8 replicates with 3 birds each.

supplementation increased ($P < 0.05$) the apparent Ca retention in g/day and apparent Ca retention in percent.

Intestinal Morphology and Relative CALB1 mRNA Level

The villus height, crypt depth, V/C, and relative CALB1 mRNA level in the duodenum are shown in Table 8. Dietary Ca levels did not interact with *B. subtilis* supplementation ($P > 0.05$) on the duodenal villus height and V/C, whereas the duodenal villus height and V/C were both increased ($P < 0.05$) by *B. subtilis* addition. An interaction ($P < 0.05$) was observed between dietary Ca levels and *B. subtilis* supplementation on the crypt depth in the duodenum, but there were no significant differences ($P > 0.05$) among treatments. *B. subtilis* addition strongly affected ($P < 0.05$) the duodenal relative CALB1 mRNA level and the 4.0% Ca level increased ($P < 0.05$) the relative CALB1 mRNA level compared with the 4.5% Ca level in diets.

Serum Biochemical Parameters

The serum biochemical parameters are shown in Table 9. No interaction ($P > 0.05$) was observed between Ca level and *B. subtilis* supplementation on Ca content in the serum. The dietary 4.0 and 4.5% Ca levels improved ($P < 0.05$) the serum Ca content significantly and the serum Ca content was also increased ($P < 0.05$) by *B. subtilis* supplementation.

DISCUSSION

From the results of the current study, we failed to observe any significant difference in the performance of laying hens when the Ca levels increased from 3.5 to 4.5%. In fact, several studies have demonstrated that increased Ca levels from 2.5 to 10% had no effect on laying hens' performance (Harms and Waldroup, 1971; Chowdhury and Smith, 2002; An et al., 2016). Conversely, the improvement in FCR was mainly attributed to the *B. subtilis* supplementation in our study, which was in accordance with other reports (Abdelqader et al., 2013; Guo et al., 2017). Increased nutrient availability may result in the improved FCR of birds caused by *B. subtilis* addition. It is evidenced by the increased villus height caused by supplemental *B. subtilis* in the current study, which could increase nutrient absorption of poultry (Sen et al., 2012; Abdelqader et al., 2013). In summary, laying hens in the late phase of production (72–79 wk of age) fed with a combination of Ca levels from 3.5 to 4.5% with *B. subtilis* supplementation could ameliorate performance.

Eggshell quality decrease in aged laying hens is well known in poultry production. *B. subtilis* supplementation in diets could ameliorate the eggshell quality of laying hens in the late phase of production, which was in accordance with other studies (Abdelqader et al., 2013; Sobczak and KozłOwski, 2015). The positive effect of 4.0% Ca level in diets on eggshell quality was in line with previous research reporting that Ca levels above 3.5% improved laying hens' eggshell quality (Chowdhury and Smith, 2002; Lichovnikova, 2007; Saffa et al., 2008; An et al., 2016).

Table 8. Effects of *Bacillus subtilis* addition and dietary Ca levels on intestinal morphology and relative CALB1 mRNA level (79 wk of age).[1]

Items		Villus height (μm)	Crypt depth (μm)	V/C	Relative CALB1 mRNA level
Ca level (%)	*B. subtilis* level (CFU/kg)				
3.5	0	1,234.90	267.20	4.59	1.22
	2×10^9	1,473.66	298.03	4.99	2.73
4	0	1,318.14	298.43	4.48	0.75
	2×10^9	1,339.39	276.10	4.85	4.77
4.5	0	1,362.08	294.00	4.64	0.40
	2×10^9	1,475.81	273.42	5.40	1.55
SEM		28.49	4.46	0.09	0.36
Means of main effects					
Ca level (%)					
3.5		1,354.28	282.62	4.79	2.04[a,b]
4		1,327.54	288.51	4.65	2.76[a]
4.5		1,422.29	283.11	5.04	1.03[b]
B. subtilis level (CFU/kg)					
0		1,303.41[b]	286.71	4.57[b]	0.79[b]
2×10^9		1,433.06[a]	282.76	5.09[a]	3.02[a]
P-value					
Ca level		0.389	0.840	0.234	0.047
B. subtilis level		0.021	0.663	0.005	0.001
Ca level × *B. subtilis* level		0.266	0.022	0.635	0.082

[a,b]Means assigned different superscripts within a factor of analysis (Ca level, *B. subtilis* level, and their interactions) differ, $P < 0.05$.
Abbreviations: CALB1, calcium binding protein calbindin-D28k; V/C, villus height/crypt depth.
[1]Data are the mean of 8 replicates with 2 birds each.

In addition to the beneficial effects of 4.0% Ca and *B. subtilis* supplementation on thickness, weight, and shell ratio of eggshell, supplemental *B. subtilis* also improved the eggshell breaking strength. The increased eggshell breaking strength in this study may result from the gut microflora balance caused by *B. subtilis* addition (Knarreborg et al., 2008). Changes in gut microflora may affect the oviduct microflora balance through cloaca (Camarda et al., 2000), which might alter the composition of organic matrix degrading microbial components, and then affect the eggshell breaking strength (Ahmed et al., 2005; Marie et al., 2015).

Table 9. Effects of *Bacillus subtilis* supplementation and dietary Ca levels on serum biochemical parameters (79 wk of age).[1]

Items		Ca (mmol/L)	P (mmol/L)	ALB (g/L)	ALP (U/L)
Ca level (%)	*B. subtilis* level (CFU/kg)				
3.5	0	3.50	3.07	21.92	360.25
	2×10^9	3.57	3.19	21.51	277.88
4	0	3.59	3.31	22.40	281.25
	2×10^9	3.64	3.17	22.05	226.00
4.5	0	3.63	3.31	22.81	297.00
	2×10^9	3.65	3.25	21.55	213.29
SEM		0.01	0.21	0.21	18.85
Means of main effects					
Ca level (%)					
3.5		3.54[b]	3.13	21.72	319.06
4		3.61[a]	3.24	22.23	253.63
4.5		3.64[a]	3.28	22.18	255.14
B. subtilis level (CFU/kg)					
0		3.58[b]	3.23	22.38	312.83
2×10^9		3.62[a]	3.20	21.71	239.05
P-value					
Ca level		<0.001	0.721	0.501	0.287
B. subtilis level		0.041	0.814	0.101	0.073
Ca level × *B. subtilis* level		0.385	0.772	0.592	0.944

[a,b]Means assigned different superscripts within a factor of analysis (Ca level, *B. subtilis* level, and their interactions) differ, $P < 0.05$.
Abbreviations: ALB, albumen; ALP, alkaline phosphatase.
[1]Data are the mean of 8 replicates with 2 birds each.

The decline in intestinal Ca absorption was reported to be partly responsible for the reduced eggshell quality in aged laying hens (Roland, 1988; Al-Batshan et al., 1994; Grobas et al., 1999). In accordance with other studies (Chowdhury and Smith, 2002; Saffa et al., 2008), the improved eggshell quality in our study may result from the increased intestinal Ca absorption, as indicated by the observed higher Ca retention and Ca levels in serum. Thus, *B. subtilis* supplementation and 4.0 or 4.5% Ca level in diets could increase intestinal Ca absorption. Moreover, *B. subtilis* supplementation and 4.0 or 4.5% Ca levels in diets both increased eggshell Ca contents without affecting tibia Ca contents, which indicated that the improved Ca absorption in our study primarily deposited in the shell gland. However, apart from 3.5 and 4.0% Ca levels in diets, the 4.5% Ca level in diets no longer increased eggshell Ca contents, which indicated that decreased Ca absorption was not the only factor declining the quality of eggshells. The quantity and composition of the organic matrix and the ions transport process during eggshell formation also could affect Ca deposition (Ahmed et al., 2005; Jonchère et al., 2012). In fact, a considerable amount of raw calcite appeared on the eggshells of laying hens fed a combination of 4.5% Ca level and *B. subtilis* supplementation in our research, indicating that this diet provided more Ca than the aged laying hens needed.

Better eggshell quality could be obtained by increased Ca absorption when laying hens were fed 4.0% Ca level and were provided *B. subtilis* supplementation in our study. Intestinal Ca absorption of laying hens was mainly mediated by transcellular and paracellular pathways after sexual maturity (Gloux et al., 2019). CALB1 is the major component of the transcellular active transport process in the absorption of Ca in the duodenum and upper jejunum. We further analyzed the relative CALB1 mRNA level and duodenal morphology of laying hens. The duodenal relative CALB1 mRNA level, increased by *B. subtilis* supplementation in our study, could increase the rate of intracellular diffusion and the total cytosolic Ca concentration (Bronner, 2003). The improved relative CALB1 mRNA level might be the reason that *B. subtilis* addition increased Ca absorption. Besides, in accordance with other studies (Abdelqader et al., 2013; Zhang et al., 2016), supplemental *B. subtilis* in diets could increase the villus height and V/C in our work. The increased absorptive area and improved intestinal morphology may be another reason for the increased Ca absorption caused by *B. subtilis* addition.

Apart from *B. subtilis* supplementation, the 4.0% Ca level in diets had no effect on the duodenal relative CALB1 mRNA level. Thus, the increased Ca retention of 4.0% Ca could not be obtained mainly through the transcellular active transport process. According to the research of Gloux et al. (2019), both transcellular active and paracellular passive pathways work cooperatively to support the high rate of Ca absorption, which suggested that the 4.0% Ca levels in diets may increase Ca absorption through the paracellular pathway. Besides, unlike the accordant increased Ca absorption by the intestine, the duodenal relative CALB1 mRNA level of 4.5% Ca levels decreased compared to the 4.0% Ca level in diets. Since intestinal Ca transport is mediated by the paracellular pathway when dietary Ca is high and by the transcellular pathway when dietary Ca is low (Christakos et al., 2014), the Ca absorption of the 4.5% Ca group might be mediated by the paracellular pathway, which needs to be further confirmed. In summary, the patterns of Ca absorbed by *B. subtilis* addition differed from that of increased Ca levels from 3.5 to 4.5% in diets, which may be one of the reasons attributing the above non-interaction results between *B. subtilis* and increased Ca levels. The single addition level of *B. subtilis* should also be considered. Moreover, significant interaction was observed between *B. subtilis* addition and Ca levels for crypt depth, but there were no significant differences ($P > 0.05$) among treatments. According to the study of Rosnow and Rosenthal (1989), group mean = interaction + factor A effect + factor B effect + grand mean. The interaction between dietary Ca levels and *B. subtilis* supplementation on the crypt depth may be negative, which needs further confirmation.

In conclusion, both the increases in dietary Ca level from 3.5 to 4.5% and *B. subtilis* supplementation could enhance intestinal Ca absorption and improve eggshell quality of laying hens in the late phase of production (72–79 wk of age). Dietary supplementation of *B. subtilis* accompanying the 4.0% Ca level was appropriate for the enhancement of eggshell quality. Nevertheless, the patterns to improve Ca absorption of laying hens may be different by dietary *B. subtilis* addition and increased Ca levels.

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DISCLOSURES

The authors declared that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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