

Probiotic *Bacillus subtilis* C-3102 Improves Eggshell Quality after Forced Molting in Aged Laying Hens

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This study was carried out to evaluate the effects of probiotic *Bacillus subtilis* C-3102 feed additive on quality characteristics including strength, thickness, and weight of eggshells of Boris Brown laying hens. The control group ($n=64$) was fed a basal diet comprised of maize and feed rice, whereas the experimental group ($n=64$) was fed a basal diet supplemented with *B. subtilis* C-3102 (3×10^5 CFU/g) starting at 49 weeks of age. From 67 to 69 weeks, all hens were induced to molt using an anorexic program; then, the birds in both groups returned to their respective diets (from 69 to 82 weeks). Eggshell strength, measured six times with 60 eggs selected before the molting treatment, was significantly greater in the C-3102 group than in the control group at 51, 59, 63, and 66 weeks (3.45, 3.44, 3.28, and 3.13 kg/cm²; $P < 0.05$, 0.05, 0.01, and 0.01, respectively). Moreover, eggshell strength—measured three times after the molting treatment—was significantly greater in the C-3102 group than in the control group at 73 and 77 weeks (3.79 and 3.65 kg/cm²; $P < 0.01$ and 0.01, respectively). Eggshell thickness was also significantly greater in the C-3102 group than in the control group at 73 and 77 weeks (0.400 and 0.390 mm; $P < 0.01$ and 0.01, respectively). Fecal samples collected from eight hens of each group at 70 weeks of age after forced molting, showed a significantly higher proportion of *Lactobacillus* spp. in the C-3102 group (8.94 log CFU/g) ($P < 0.05$) than in the control group (8.63 log CFU/g). *Clostridium* spp. abundance was significantly lower in the C-3102 group (2.92 log CFU/g) than in the control group (4.3 log CFU/g). These results suggest that C-3102 supplementation improves eggshell quality in aged laying hens, particularly after forced molting.

Key words: *Bacillus subtilis* C-3102, Boris Brown laying hen, eggshell quality, eggshell strength, intestinal microflora
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Introduction

Controlling egg quality, productivity, and eggshell quality is important in the egg industry. Egg loss is usually related to poor eggshell quality in aged laying hens (Karcher *et al.*, 2015), and the average daily egg loss due to substandard eggshell quality is estimated to be 13%–20% (Roland, 1988). The reported frequency of broken eggs in brown egg-laying hens increases from 0.43% at 22 weeks of age to 1.81% at 74 weeks of age (Grobias *et al.*, 1999). The decrease in eggshell

quality with age is most likely associated with lower Ca uptake from the intestine and the larger egg size of older laying hens (Al-Batshan *et al.*, 1994). As a response to the formation of larger eggs with reduced Ca levels in aged laying hens, eggshell thickness decreases (Roland, 1980). Calcium is a key nutrient for optimal eggshell quality in egg production (Ahmed *et al.*, 2013), and increased Ca levels in the basal diet of laying hens partially improves eggshell quality (Park and Sohn, 2018). Therefore, most approaches previously used to control eggshell quality in aged laying hens involved increasing the Ca level in the basal diet. However, excessive Ca in the basal diet is not an effective means to improve Ca uptake and eggshell quality in aged laying hens (An *et al.*, 2016).

In broiler chickens, probiotics exert beneficial effects on host health, such as immune stimulation against pathogenic bacterial infections (Wang *et al.*, 2015; Wang *et al.*, 2017), improved egg quality (Forte *et al.*, 2016), weight gain, reduced mortality rate (Huff *et al.*, 2015), and heat stress relief

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(Lan *et al.*, 2004). Most of these beneficial effects are known to occur via the establishment of a balance between beneficial and pathogenic bacterial populations in the intestine (Ziprin and Deloach, 1993; Jin *et al.*, 1997). A recent study suggested that diet supplementation with probiotic *Pediococcus acidilactici* has the potential to improve hen performance and eggshell quality during the early laying period (Mikulski *et al.*, 2012).

Bacillus spp. are advantageous in maintaining the stability of feed additive products because of their robust, spore-forming characteristics, which can improve weight gain and feed efficiency (Xu *et al.*, 2006). A unique approach reported by Lei *et al.* (2013) showed the potential of probiotic *Bacillus licheniformis* to improve egg quality and intestinal barrier function in laying hens. Among the reported probiotic *Bacillus* strains, *B. subtilis* C-3102 is authorized by the European Food Safety Authority (EFSA) to provide nutrition to chickens, weaned piglets, laying hens, turkeys, and other avian species (EFSA, 2015).

To counter the decline in egg production in aged hens, a forced molt induction approach has been traditionally used by means of 1–2 weeks of feed withdrawal, accompanied by withdrawal of water (Dickey *et al.*, 2012). Unfortunately, the stress associated with feed withdrawal results in increased susceptibility to *Salmonella* infection, marked by increased intestinal shedding and dissemination of *Salmonella* to internal organs such as the liver, spleen, and ovaries (Kubena *et al.*, 2005). Consequently, molting treatment is no longer permitted in Europe and is less frequently applied in the USA and Canada. However, *B. subtilis* C-3102 has the potential to improve microbiological status and reduce pathogenic bacteria in broiler chickens (Maruta *et al.*, 1995; Fritts *et al.*, 2000).

Therefore, in the present study, the effects of supplementing probiotic *B. subtilis* C-3102 in the diet of aged laying hens on eggshell quality were investigated. Furthermore, the effects were compared before and after stress-induced forced molting.

Materials and Methods

Birds, Management, and Diet

One hundred and twenty-eight 49-week-old Boris Brown laying hens from the same flock of equal quality were randomly divided into control and C-3102 groups of 64 hens each. All hens were housed under the same management conditions (750 cm²/hen, 10–30°C) in a ventilated poultry house in Santoku Farm (Chiba, Japan). Trials with hens lasted from 49 to 67 weeks of age and after 2 weeks of forced molting, from 69 to 82 weeks of age, as shown in Fig. 1. Except during the forced molting program, the laying hens were fed a basal diet or a *B. subtilis* C-3102-containing diet and provided water *ad libitum* throughout the experimental period. During molting, the hens had *ad libitum* access to water under anorexic conditions; this treatment lasted until the weights of the hens decreased by 25% relative to that before forced molting. All hens were housed in stainless steel cages (2 hens per cage) and exposed to 16 h of light and

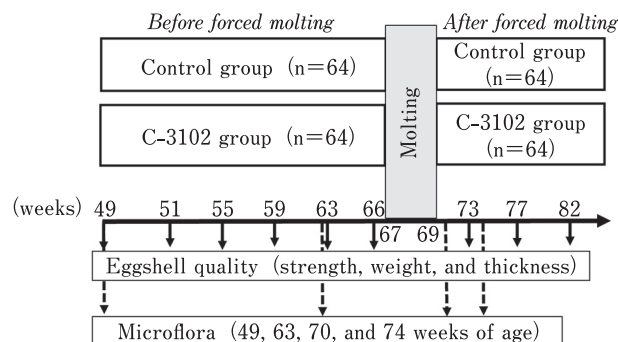


Fig. 1. **Outline of treatment schedule and analytical parameters before and after forced molting.** Forced molting started on week 67 and ended on week 69.

8 h of dark periods per day. The experiments were conducted in Santoku Farm according to the “Breeding management guidelines for hens that correspond to the idea of animal welfare” (Japan Livestock Technology Association). The guidelines can be found through the following link to the Terrestrial Animal Health Code, published by the World Organisation for Animal Health (OIE) (<https://www.oie.int/international-standard-setting/terrestrial-code/access-online/>).

The hens in the control group were fed a basal diet (the composition is listed in Table 1) and those in the C-3102 group received 0.003% *B. subtilis* C-3102 along with the basal diet. *Bacillus subtilis* C-3102 was supplied at 3×10^5 CFU/g feed. The composition of the basal diet was primarily maize, feed rice, and soybean meal.

Sampling

At 49, 51, 55, 59, 63, and 66 weeks of age, before forced molting; and at 73, 77, and 82 weeks of age, after forced molting; 60 eggs were collected and analyzed within 48 h of sampling and stored at room temperature (20–25°C) until analysis (Fig. 1). Fresh fecal samples were collected from eight randomly selected hens from each group before forced molting at 49 and 63 weeks of age and after forced molting at 70 and 74 weeks of age (Fig. 1).

Eggshell Quality Assessment

Productive performance metrics—including hen-day egg production, eggshell strength, eggshell weight, egg weight, eggshell thickness, and eggshell membrane weight—were measured according to methods described by Tsushima *et al.* (2015). A total of 60 eggs per group were collected to analyze egg weight and eggshell strength, while 30 eggs per group were randomly selected to evaluate the other eggshell quality indices. Egg weight was measured using an electronic balance (Shimadzu Corporation, Kyoto, Japan). Eggshell strength along the minor axis was determined using an eggshell strength tester (Fujihira Industry, Tokyo, Japan). After measuring the egg weight and eggshell strength, each eggshell was cracked and washed with water and then dried to analyze eggshell thickness and eggshell weight. A piece of cracked eggshell without the membrane was used to measure eggshell thickness using a PEACOCK dial pipe

Table 1. Nutrient composition of the basal diet used in the study

Ingredients	Composition	Nutrients	
Corn (%)	60.0	Crude protein (%)	17.0
Soybean meal (%)	22.0	Crude ash (%)	14.5
Fish meal (%)	2.0	Crude fiber (%)	6.0
Rice bran (%)	2.0	Calcium (%)	3.6
Other ¹ (%)	14.0	Crude fat (%)	2.0
		Phosphorus, available (%)	0.4
Total (%)	100.0	Metabolisable energy (MJ/kg)	12.0

Detailed composition of other ingredients are not disclosed due to commercial diet.

¹Other ingredients: Lime stone, Alfalfa meal, Fish oil, Zeolite, Seaweed, Wood vinegar, Mulberry leaves, Japanese mugwort, Salt, Paprika powder (Extracted), Silicic anhydride, Vegetable fermented powder, Grape bran and Vitamin-Mineral premix².

²Vitamin-Mineral Premix: vitamin A, vitamin D3, vitamin E, vitamin K3, vitamin B1, vitamin B2, vitamin B6, vitamin B12, pantothenic acid, nicotinic acid, folic acid, biotin, manganese sulphate, zinc carbonate, ferrous sulphate, copper sulphate, Cobalt sulphate, calcium iodate, methionine, lysine, ethoxyne, phytase, threonine, choline and calcium propionate.

gauge (Fujihira Industry), and then the dried whole eggshell weight was measured using an electronic balance. Whole eggshells were subsequently dissolved in 35 mL of diluted solvent (methanol: concentrated HCl=3:4). The solution was then centrifuged at 20,000×g for 30 min at 4°C. The precipitate was washed with water and dried, and the weight of the remaining eggshell membrane was measured. Eggshell weight without the membrane was calculated as follows: (whole eggshell weight) - (eggshell membrane weight).

Fecal Sample Analysis

To understand the effect of *B. subtilis* C-3102 on intestinal microflora, a microfloral analysis of the fecal samples was conducted using the fermented methods described by Mitsuoka *et al.* (1976). The fecal samples were serially diluted (10^{-1} to 10^{-8}), and 0.05-mL aliquots were cultured on various nonselective and selective agar plates. The cell counts for the serially diluted samples were obtained from the agar plates. The selective agar plates used were glucose blood liver (BL), trypticase soy (TS), neomycin Nagler (NN), modified *Lactobacillus* selective (LBS) medium, and deoxycholate hydrogen sulfide lactose (DHL) plates. The BL, NN, and LBS agar plates were incubated at 37°C in jars with an AneropackTM oxygen generator/carbon dioxide absorbent package (Mitsubishi Gas Chemical Company, Tokyo, Japan) for 2 d. The TS and DHL agar plates were incubated aerobically at 37°C for 1 d. The limit of detection for microbes on these plates was 2×10^2 CFU/g feces.

Statistical Analysis

In this study, a two-sample independent sample *t*-test was performed to analyze the effects of probiotic *B. subtilis* C-3102 on the parameters measured. An alpha (α) level of 0.05 was used as the threshold to determine statistical significance, and a *P*-value of 0.10 represented a trend. All analyses were conducted using the statistical software Statistix 10 (Analytical Software, FL, USA).

Results

Productive Performance and General Egg Quality

Throughout the experiment, none of the diets (basal diet and experimental diet supplemented with C-3102) exerted adverse effects on the hens. Moreover, there were no significant differences in egg production, egg mass, body weight, and feed intake between the C-3102 and basal diet groups of these Boris Brown laying hens (data not shown).

Eggshell Strength, Weight, and Thickness

Before the forced molting treatment, eggshell strength, weight and thickness and eggshell weight/egg weight in the control group marginally decreased with time (Table 2). However, eggshell strength and eggshell weight (with and without the egg membrane) in the control group were strongly associated with eggshell quality, and they improved after the forced molting treatment (Figs 2 and 3; Tables 2 and 3). In contrast, the eggshell strength of hens in the group fed the experimental diet supplemented with C-3102 was significantly improved compared with the control group, both before (at 51, 59, 63, and 66 weeks of age) and after (at 73 and 77 weeks of age) the forced molting treatment ($P < 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.01$, $P < 0.01$, and $P < 0.01$, respectively) (Tables 2 and 3). After the start of diet supplementation, the eggshell strength in the control group at 49 weeks of age was 3.46 kg/cm², which decreased to 2.81 kg/cm² (81.2%) at 66 weeks of age, before forced molting. However, in the C-3102 group, the eggshell strength at 49 weeks of age was 3.49 kg/cm² and 3.13 kg/cm² (89.7%) at 66 weeks of age (Fig. 2A). These trends were obvious in the analysis using the linear approximate equations for the control ($Y = -0.029 X + 4.786$) and C-3102 ($Y = -0.017 X + 4.295$) groups. The gap between both values increased during the treatment period ($P < 0.1$). These findings revealed that experimental diet supplementation with C-3102 for 17 weeks improved eggshell strength by approximately 8.5%. This difference corresponded to a delay in the decrease in the eggshell strength by approximately 36 d in the C-3102 group

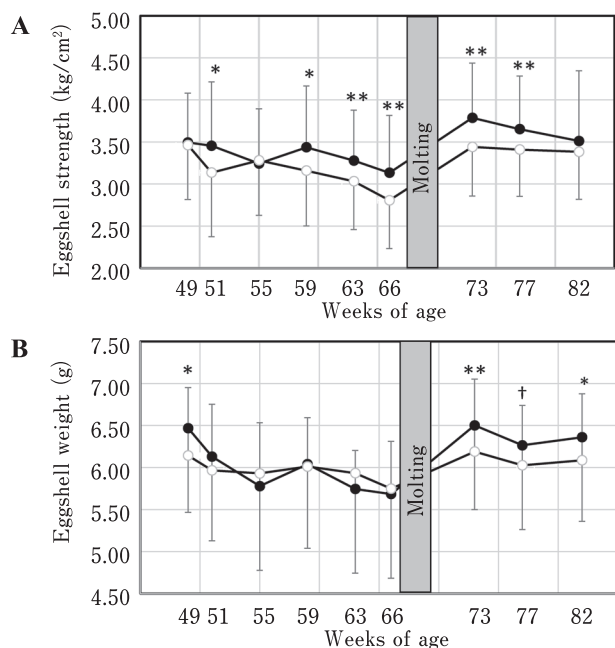


Fig. 2. Changes in eggshell strength and weight of laying hens before and after the forced molting treatment. Changes in eggshell strength (A) of laying hens ($n=60$ /group) and eggshell weight (B) of laying hens ($n=30$ /group). Different symbols indicate hens fed a basal diet (○) or an experimental diet containing C-3102 (●). Significant differences between the control and C-3102 groups are indicated as follows: † $P<0.10$, * $P<0.05$, and ** $P<0.01$.

compared with the control group.

Eggshell weight in the C-3102 group was significantly higher than that in the control group at 73 and 82 weeks of age ($P<0.01$ and $P<0.05$, respectively) (Fig. 2B). The eggshell strength or eggshell weight in the control group decreased throughout the experimental period (Fig. 3B), whereas it improved after forced molting in the C-3102 group compared with the control group at 73, 77, and 82 weeks of age ($P<0.05$, $P<0.001$, and $P<0.01$, respectively). Interestingly, there was no significant difference in eggshell thickness before the forced molting treatment between the two groups, but eggshell thickness was significantly improved in the C-3102 group compared with the control group after the forced molting treatment at 73 and 77 weeks of age ($P<0.01$ and $P<0.01$, respectively).

Microfloral and Fecal Sample Analyses

As shown in Table 4, the number of *Bacillus* spp. on the TS agar plates, including aerobic bacteria, from the control group showed an increasing trend with the supplementation of experimental diet with C-3102 in C-3102 group. The clostridial count among the eight hens analyzed in both groups increased gradually during the experimental period. In particular, the count of *Clostridium perfringens* increased after forced molting, although it was significantly lower in

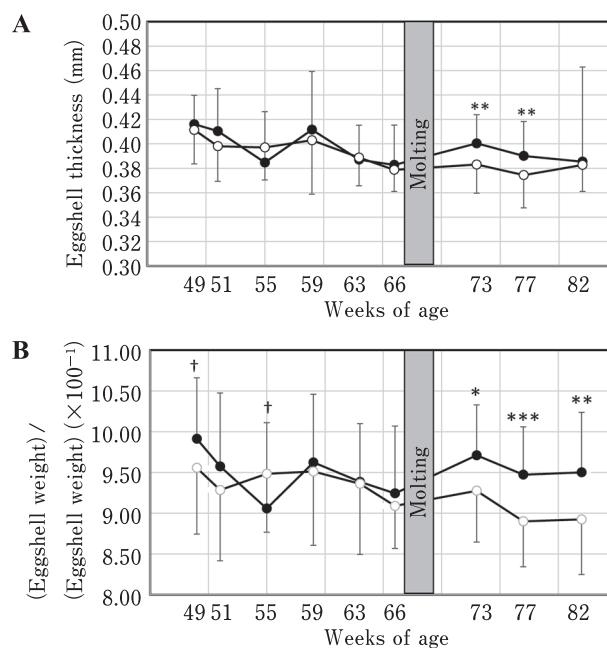


Fig. 3. Changes in eggshell thickness and eggshell weight/egg weight of laying hens before and after the forced molting treatment. Changes in eggshell thickness (A) and eggshell weight/egg weight (B) of laying hens ($n=30$ /group). Different symbols indicate hens fed a basal diet (○) or an experimental diet containing C-3102 (●). Significant differences between the control and C-3102 groups are indicated as follows: † $P<0.10$, * $P<0.05$, ** $P<0.01$, and *** $P<0.001$. Statistically different trends between the two groups is shown by † $P<0.10$.

the C-3102 group than in the control group at 74 weeks of age (2.92 and 4.31 log CFU/g, respectively) ($P<0.05$) (Table 4). After forced molting, the count of *Lactobacilli* on the LBS agar plates was significantly higher in the C-3102 group (8.94 log CFU/g) than in the control group (8.63 log CFU/g) ($P<0.05$) at 70 weeks of age (Table 4). Moreover, the total number of anaerobic bacteria was significantly higher in the C-3102 group (9.22 log CFU/g) than in the control group (8.98 log CFU/g) ($P<0.05$) at 70 weeks of age. These results suggest that the supplementation of the experimental diet with C-3102 elevates the proportion of *Lactobacilli* and decreases the proportion of pathogenic bacteria such as *Clostridium* in aged laying hens.

Discussion

As egg production typically declines with the age of hens, management of egg productivity and shell quality is the most important aspect in egg production (Karcher *et al.*, 2015). The decreased uptake of Ca by aged laying hens indicates that they require a higher Ca supply (uptake) than younger hens to produce larger eggs. Calcium supplementation in the basal diet might be effective, but it is still insufficient to ensure efficient Ca uptake by aged hens, which typically experience reduced mineral uptake with age (An *et al.*,

Table 2. Impact of *Bacillus subtilis* C-3102 feeding on eggshell quality of Boris Brown hens before the forced molting period

Week age	49 weeks		51 weeks		55 weeks	
	Control	C-3102	Control	C-3102	Control	C-3102
Eggshell strength (kg/cm ²)	3.46±0.65	3.49±0.59	3.13±0.77	3.45±0.77*	3.28±0.66	3.24±0.66
Egg weight (g)	64.5±5.6	65.8±4.5	64.8±4.6	64.0±4.4	63.6±5.2	63.5±4.6
Eggshell weight (with membrane) (g)	6.31±0.66	6.65±0.50*	6.15±0.59	6.31±0.63	6.10±0.61	5.96±0.78
Eggshell weight (without membrane) (g)	6.14±0.65	6.47±0.49*	5.97±0.59	6.13±0.63	5.93±0.60	5.78±0.77
Eggshell weight/Egg weight	9.56±0.83	9.91±0.76 †	9.28±0.88	9.57±0.92	9.48±0.73	9.06±1.07 †
Eggshell thickness (mm)	0.411±0.028	0.416±0.024	0.398±0.029	0.410±0.035	0.397±0.027	0.385±0.042
Eggshell membrane weight (g)	0.165±0.033	0.181±0.032 †	0.185±0.036	0.184±0.029	0.168±0.025	0.195±0.057*

Week age	59 weeks		63 weeks		66 weeks	
	Control	C-3102	Control	C-3102	Control	C-3102
Eggshell strength (kg/cm ²)	3.16±0.40	3.44±0.74*	3.03±0.58	3.28±0.61**	2.81±0.58	3.13±0.69**
Egg weight (g)	63.7±5.2	63.1±4.9	63.9±5.9	61.2±4.5**	64.2±5.7	62.1±4.6**
Eggshell weight (with membrane) (g)	6.19±0.61	6.21±0.57	6.10±0.43	5.91±0.46	5.91±0.41	5.85±0.64
Eggshell weight (without membrane) (g)	6.01±0.61	6.04±0.56	5.93±0.43	5.74±0.47	5.75±0.40	5.68±0.64
Eggshell weight/Egg weight	9.51±0.92	9.62±0.85	9.36±0.88	9.38±0.73	9.09±0.53	9.24±0.84
Eggshell thickness (mm)	0.403±0.045	0.412±0.049	0.389±0.023	0.387±0.029	0.379±0.018	0.383±0.033
Eggshell membrane weight (g)	0.178±0.031	0.169±0.027	0.168±0.027	0.166±0.026	0.161±0.019	0.163±0.030

Significant differences between two groups are indicated as follows: * $P < 0.05$ and ** $P < 0.01$.
 † shows statistically different trend between the two group († $P < 0.10$).

Table 3. Impact of *Bacillus subtilis* C-3102 feeding on eggshell quality of Boris Brown hens after forced molting period

Week age	73 weeks (4 weeks after molting)		77 weeks (8 weeks after molting)		82 weeks (13 weeks after molting)	
	Control	C-3102	Control	C-3102	Control	C-3102
Eggshell strength (kg/cm ²)	3.44±0.59	3.79±0.66**	3.41±0.56	3.65±0.64**	3.39±0.57	3.51±0.84
Egg weight (g)	66.6±5.0	67.9±5.0	67.7±5.1	67.1±4.5	68.1±5.2	68.1±5.4
Eggshell weight (with membrane) (g)	6.35±0.51	6.67±0.57**	6.20±0.47	6.44±0.49 †	6.25±0.46	6.53±0.54*
Eggshell weight (without membrane) (g)	6.19±0.51	6.50±0.56**	6.03±0.45	6.26±0.48 †	6.09±0.45	6.36±0.53*
Eggshell weight/Egg weight	9.27±0.64	9.71±0.63*	8.90±0.57	9.47±0.60***	8.92±0.69	9.50±0.75**
Eggshell thickness (mm)	0.383±0.024	0.400±0.024**	0.374±0.027	0.390±0.029**	0.383±0.022	0.385±0.079
Eggshell membrane weight (g)	0.164±0.020	0.172±0.024	0.177±0.028	0.176±0.028	0.167±0.021	0.167±0.027

Significant differences between two groups are indicated as follows: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.
 † shows statistically different trend between the two group († $P < 0.10$).

Table 4. Change of microflora by supplementation of *Bacillus subtilis* C-3102 during the test period

Week age	Before the forced molting				After the forced molting			
	49 weeks		63 weeks		70 weeks		74 weeks	
	Control	C-3102	Control	C-3102	Control	C-3102	Control	C-3102
Enterobacteriaceae, Log CFU/g	3.84±0.71	4.37±0.77	6.28±0.42	6.43±0.82	6.24±0.71	6.67±1.17	4.87±0.70	5.63±0.58*
<i>Clostridium perfringens</i> , Log CFU/g	3.48±0.89	2.33	2.74±0.20	2.81	3.12±0.82	3.89±0.98	4.31±1.50	2.92±0.41*
Detection rate (positive sample/all sample)	(6/8)	(1/8)	(4/8)	(3/8)	(8/8)	(8/8)	(8/8)	(8/8)
<i>Lactobacillus</i> , Log CFU/g	8.29±0.22	8.32±0.28	8.67±0.14	8.74±0.23	8.63±0.27	8.94±0.21*	8.76±0.23	8.87±0.24
Total anaerobic bacteriaceae, Log CFU/g	8.52±0.14	8.56±0.16	8.88±0.20	8.95±0.33	8.98±0.26	9.22±0.15*	8.97±0.26	9.04±0.24
<i>Bacillus subtilis</i> , Log CFU/g	3.85±0.19	4.18±0.21	3.99±0.07	5.11±0.10	4.05±0.03	5.34±0.04	3.64±0.43	5.32±0.03

Significant differences between two groups are indicated as follows: * $P < 0.05$.

2016). In the present study, we observed a promising effect of *B. subtilis* C-3102 on the eggshell quality of aged laying hens (Fig. 2 and Table 2). Specifically, C-3102 supplementation in the diet of aged laying hens from 343 to 466 d (17 weeks) before the forced molting period resulted in a decline in eggshell strength to 89.7% (2.81/3.46), while the eggshell strength of the control group dropped to 81.2% (2.81/3.46). This indicates an 8.5% (89.7%–81.2%) improvement in eggshell strength after 17 weeks of C-3102 treatment. Several eggshell qualities have been reported to strongly affect the risk of egg breakage (Strong, 1989). When the ratio of the eggshell weight/egg weight was over 9.4%, the breaking rate was only 0%–1%, whereas when it was less than 9.0%, the breaking rate was as high as 3%–4% (Fukuhara *et al.*, 1999). In this study, the eggshell weight/egg weight ratio of the control group was less than 9% but was more than 9.5% for the C-3102 group at 77 and 82 weeks of age. Thus, the potential breaking rate was likely improved from 3%–4% to 0%–1% by C-3102 supplementation.

Forced molting is a general approach used to improve the egg-laying performance of old broiler breeds (Tona *et al.*, 2002). The relative hatchling weight from the eggs of young broilers was reportedly higher than that of more mature broilers after treatment (Tona *et al.*, 2002). Furthermore, the eggs of hens after molting lost weight during incubation, possibly due to better eggshell formation (Christensen and McCorkle, 1982). In the present study, eggshell parameters that decreased over time in the control group before the forced molting treatment (Table 2) were recovered after forced molting (Table 3). However, also after the forced molting period, various parameters associated with eggshell quality in the C-3102 group were substantially improved relative to those in the control group. Therefore, the probiotic effects of C-3102 on laying hens were considerably higher after molting than before molting. Our preliminary studies suggested that the probiotic effects of C-3102 were more effective in broiler breeds housed under more stressful conditions, with frequent introduction of pathogenic bacteria (data not shown). This may be because C-3102 had a greater effect on highly damaged tight junctions, dysfunctional systemic immunity, and hormonal imbalance that resulted after the forced molting period. In this study, the forced molting procedure improved eggshell quality, especially in aged laying hens. The improved eggshell quality and gut health will enable birds to have a prolonged egg production cycle with good egg production and shell quality, as observed in a previous study (Zhang *et al.*, 2017). However, the forced molting procedure has raised concerns within the society and animal welfare groups for the well-being of laying hens. Therefore, C-3102 supplementation could overcome these challenges by allowing shorter molting periods and less diet restriction during forced molting, including watering.

The supplementation of C-3102 improved eggshell strength both before and after the forced molting treatment; however, the mechanism of action for these improvements may be slightly different between the two periods. Briefly, eggshell strength, which declines with age, was significantly im-

proved by supplementation with C-3102 compared with the basal diet in the pre-molting period, but the other parameters associated with eggshell quality did not differ between the two groups (Table 2). In contrast, after molting induction, various parameters associated with eggshell quality, such as eggshell strength, eggshell weight/egg weight, and microflora, were significantly improved in the C-3102 group compared with the control group (Tables 3 and 4). Before the forced molting period, the eggshell strength was improved in the C-3102 group without a concurrent increase in eggshell weight and thickness, with no change in Ca accumulation during this period (Fig. 2). More than 500 proteins have been identified to date in the acid-soluble part of the eggshell matrix (Mann *et al.*, 2006). Of these, claudins might play crucial roles not only in barrier formation at tight junctions but also in the development of the oviductal mucosal epithelium (Turksen and Troy, 2004; Ariyadi *et al.*, 2013), which is also important for egg formation. Moreover, changes in the expression of shell layer-related genes, which are related to shell strength, have been studied using microarray analysis (Liu *et al.*, 2013), and such genes and their products may be affected by C-3102 supplementation. The results of the present study suggest that the eggshell structure of hens supplemented with C-3102 may have been affected by changes in the expression of some eggshell-specific proteins involved in eggshell formation before the forced molting period. Proteomic analysis of the eggshell proteins in both groups should be carried out to confirm our findings.

After the forced molting treatment, the count of *Clostridium* was significantly decreased and that of Enterobacteriaceae members was higher in the C-3102 group than in the control group at 79 weeks of age. After the forced molting treatment, an increase in lactic acid bacteria was observed in fecal samples from the C-3102 group (Table 4). In broilers, an increase in *Lactobacillus* spp. by C-3102 supplementation has been reported to be due to the creation of anaerobic conditions by C-3102 germination, providing better growth conditions for lactobacilli (Maruta *et al.*, 1995). Generally, the pH of the fecal samples decreased because of lactic acid released by the increased numbers of *Lactobacillus* spp. and acetic acid released by *Bifidobacterium* spp. in the intestine. If powdered calcium carbonate supplied in the basal diet could be easily solubilized under low pH conditions in the intestine, Ca absorption may be improved. Therefore, the relationship between pH and Ca level in the fecal samples from the C-3102 and control groups must be compared in future studies to understand the probiotic effects of C-3102.

In conclusion, supplementing the probiotic C-3102 feed additive before forced molting was effective in improving eggshell strength, which declines with age in hens. It increased the eggshell strength, weight, and thickness compared with the basal diet under stress conditions introduced by forced molting.

Conflicts of Interest

All authors declare no conflict of interest relevant to this

study.

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