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# Effects of Feeding Dried Culture of *Bacillus subtilis* Transformed with Ferritin Gene on Productivity and Egg Quality of Laying Hens After 90 Weeks of Age

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## ABSTRACT

**Background:** This study evaluated the effect of adding dried Bacillus subtilisculture (BSC) with Periserrula leucophryna-derived ferritin to the feed of laying hens after 90 weeks of age, focusing on productivity and egg quality.

**Methods:** An experiment was conducted using 36,000 90-week-old ISA Brown hens, fed either a control diet or a diet supplemented with 0.1% ferritin-transferred BSC for 10 weeks. Two treatments with three replicates were tested. Productivity (mortality, egg production rate, egg weight) and egg quality (eggshell thickness, strength, color; yolk color, index; Haugh unit) were measured.

**Result:** Mortality did not differ before and after BSC feeding. Egg production remained stable until week 6, then declined. Egg weight increased initially ($p<0.05$) but later decreased due to a higher proportion of smaller eggs. Eggshell strength and thickness were maintained and shell color showed a slight, non-significant improvement. Yolk color increased slightly at weeks 6-8, while the yolk index improved significantly ($p<0.05$). Haugh units remained stable, indicating preserved freshness. These findings suggest BSC supplementation helped maintain productivity and egg quality in aging hens, with potential economic benefits.

**Key words:** *Bacillus subtilis* culture (BSC), Egg yolk index, Ferritin gene, Haugh unit, Laying hens.

## INTRODUCTION

In Korea, laying hen feed relies heavily on imports and recent increases in grain prices, exchange rates and logistics costs have raised feed expenses sharply. Under free trade agreements, reducing production costs and maintaining egg quality-especially during the late laying period-is critical for farm sustainability. Brown commercial hens peak at 95–96% laying rate at 27 weeks, declining to about 80% by 70 weeks, with reduced egg quality (Hy-Line Brown Management Guide, 2022).

To counter these declines, feed additives such as enzymes, minerals, vitamins, plant extracts and probiotics have been studied (Chung *et al*., 2021; Shin *et al*., 2008; Lim *et al*., 2018; Li *et al*., 2017). Probiotics enhance microbial balance, nutrient absorption and egg production while inhibiting pathogens like *Escherichia coli* and *Salmonella* (Parker, 1974; Fuller, 1989; Reuter, 2001; Kwon *et al*., 2002; Sheoran *et al*., 2017). Among these, *Bacillus subtilis* (BS) is widely used to improve productivity, egg quality and feed efficiency (Santoso *et al*., 1995; Ryu *et al*., 1999; Junbo *et al*., 2009). Recombinant probiotics, such as Lactobacillus reuteriex- pressing cellulase genes, have further improved nutrient digestibility and gut adhesion in poultry (Wang *et al*., 2020).

Ferritin, an intracellular iron-storage protein, is vital for iron homeostasis (Harrison and Arosio, 1996). The ferritin gene from Periserrula leucophryma, a marine species native to Korea (GenBank DQ207752), stores up to 4,500 $Fe^{3+}$ atoms and is more effective with fewer side effects than inorganic iron (Jeong *et al*., 2006). Trace minerals and antioxidants, including selenium, also improve egg quality and bird health (Zia *et al*., 2016). A novel additive combining ferritin with BS offers both probiotic and iron supplementation benefits (Choi, 2016) and has been reported to enhance egg weight, shell color, freshness and overall quality (Lee *et al*., 2019).

Therefore, this study aimed to evaluate the effects of feeding dried Bacillus subtilisculture (BSC) containing the P. leucophryma-derived ferritin gene to late-laying hens, focusing on productivity, eggshell quality, yolk quality and egg freshness.

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## MATERIALS AND METHODS

### Experimental design

A basal commercial-type layer feed was supplemented with 0.1% dried Bacillus subtilisculture (BSC) expressing the Periserrula leucophrymaferritin gene (FerriProÇ$, Turtl Bio, Korea) from 90 to 100 weeks of age. The control group (CON) received no additive. Productivity data and egg samples were collected biweekly.

### Experimental animals

A total of 36,000 ISA Brown hens (90 weeks old) were allocated to two treatments with three replicates each. The trial was conducted from December 17, 2020, to February 24, 2021, at a commercial farm in Cheongsong-gun, Korea, following Daegu University Animal Ethics guidelines (DUIACC-2020-14-0901-001).

The corn-soybean diet provided 2,800 kcal/kg ME, 17.5% crude protein and 3.8% calcium, meeting or exceeding NRC (1994) requirements (Bang, 2020). Birds were housed in cages with nipple drinkers, fed and watered ad libitum and maintained under 23 h light/1 h dark. Environmental control followed automatic poultry management standards.

### Measurements

$$\bullet\text{Mortality rate (\%)} = \frac{\text{Number of deaths}}{\text{Total birds}} \times 100$$

$$\bullet\text{Laying rate (\%)} = \frac{\text{Number of eggs laid}}{\text{Total birds}} \times 100$$

•**Egg weight (g):** Weekly average of normal eggs.

•**Egg size distribution (%):** Extra-large (≥ 68 g), Large (60-68 g), Medium (52-60 g), Small (44–52 g), Peewee (<44 g) per Livestock Products Quality Evaluation Institute standards.

$$\bullet\text{Blue egg rate (\%)} = \frac{\text{Blue/cracked eggs}}{\text{Total eggs}} \times 100$$

•**Eggshell quality:** Strength (IMADA gauge), thickness (Mitutoyo caliper; average of three shell points) and color (Roche/DSM color fan).

•**Egg yolk quality:** Yolk color (color fan) and yolk index (height ÷ diameter, measured with Mitutoyo instruments).

•**Haugh unit (HU):** 100 log (H + 7.57 - 1.7 W^0.37)
Where,
H = Albumen height (mm).
W = Egg weight (g).

Thirty eggs per group were sampled weekly for quality analysis.

### Statistical analysis

Data were analyzed using ANOVA in SAS (SAS Institute, 2002) under a completely randomized design. Means were compared by duncan's multiple range test at p<0.05.

## RESULTS AND DISCUSSION

### Productivity evaluation

### Mortality rate

Before BSC supplementation, the daily mortality rate in the control group was 0.019%. After supplementation, it ranged from 0.0187% to 0.0244% during weeks 2–10, with no significant difference. According to ISA Brown (2022) and Hy-Line Brown (2022) guidelines, the average mortality rate at 90 weeks is 6% and 6.5%, respectively. In this study, it was 7.8% at 90 weeks. At 100 weeks, the guideline rate is 9%, while this study recorded 9.15%. The gap from the standard decreased from 1.3% at the start to 0.15% at the end of the 10-week trial, indicating potential economic benefit.

### Laying rate

The pre-supplementation laying rate was 88.54%. From weeks 2-6, rates ranged between 87.07% and 88.49%, with no significant difference. At weeks 8 and 10, rates dropped significantly to 84.91% and 84.62%, respectively (p<0.05).

### Egg weight

Egg weights were measured to examine the impact of BSC feeding on the productivity of laying hens in the late laying period. The results are shown in Table 1. The average egg weight was 62.90 g in the control group before BSC feeding, however, an increase in the egg weight was observed after BSC feeding. The highest egg weight was 63.00 g in the second week after feeding (p<0.05) but showed a gradual decrease to 62.80-62.50 g in weeks 6-10, significantly lower than before feeding (p<0.05).

### Percentage distribution of egg weight categories (%)

Egg weight categories (jumbo, extra-large, large, medium, small) were analyzed to assess the effect of BSC feeding (Table 2). Jumbo eggs increased from 13.55% pre-feeding to 14.06% at week 2 (p<0.05), then gradually declined to ~12% by weeks 8–10 (p<0.05). Extra-large eggs peaked at ~58% in week 2 but decreased steadily to ~55% by weeks 8–10 (p<0.05). In contrast, large and medium eggs rose from ~25% and 0.65% at baseline to ~29% and 0.9% by

**Table 1:** Egg weight from laying hens fed diets with 0.1% BSC during a period of 10 weeks [1,2,3].

| Weeks | Egg weight (g) |
|---|---|
| 0 | 62.90.01b |
| 2 | 63.00.03a |
| 4 | 62.90.01b |
| 6 | 62.80.01c |
| 8 | 62.62.05e |
| 10 | 62.50.05d |

[1] BSC; Dried *Bacillus subtilis* Culture.
[2] Values are expressed as means±standard errors.
[3] Means in the same column with no common superscript are significantly different (p<0.05).

**Table 2:** Egg size distribution from laying hens fed diets with 0.1% BSC during a period of 10 weeks [1,2,3].

| Weeks | Extra large | Very large | Large | Medium | Small+off-grade | Broken |
|---|---|---|---|---|---|---|
| 0 | 13.55b | 58.25ab | 26.00b | 0.66c | 0.02 | 1.53c |
| 2 | 14.06a | 58.36a | 25.23c | 0.67c | 0.02 | 1.66c |
| 4 | 13.73b | 58.03b | 25.70b | 0.65c | 0.02 | 1.87b |
| 6 | 13.71b | 57.46c | 26.10b | 0.78b | 0.02 | 1.92b |
| 8 | 12.07c | 55.71d | 28.98a | 0.95a | 0.01 | 2.27a |
| 10 | 12.33c | 55.83d | 28.57a | 0.88a | 0.02 | 2.36a |
| SEM | 0.27 | 0.25 | 0.38 | 0.06 | 0.0077 | 0.16 |

[1] BSC; Dried *Bacillus subtilis* Culture.

[2] SEM: Standard error of the mean.

[3] Means in the same column with no common superscript are significantly different (p<0.05).

weeks 8–10 (p<0.05). Small eggs also increased, reaching ~2.3% in weeks 8-10 (p<0.05).

### Evaluation of egg quality

### Shell quality of eggs

### Shell strength

Shell strength before BSC feeding was 4.07 kg/cm. During weeks 2-10, it ranged from 3.80 to 4.08 kg/cm, peaking at week 6 (4.08 kg/cm, p<0.05). No significant difference was found compared to the control, but a gradual decline occurred after week 6, reaching 3.87 kg/cm at week 10 (p<0.05).

### Shell thickness

As shown in Table 3, shell thickness ranged from 0.337 mm pre-BSC feeding to 0.334–0.346 mm during weeks 2-10. Thickness increased gradually, peaking at 0.346 mm in week 4 (p<0.05), then declined to 0.335 mm by week 10 (p<0.05 vs. week 4).

### Shell color

Table 4 shows that shell color averaged 11.50 pre-feeding, dropping to 11.37 at week 2 (p<0.05), then increasing to a peak of 11.97 in week 6 (p<0.05). Slight declines followed, with values of 11.93 at week 8 and 11.87 at week 10, with no significant final difference from baseline.

### Quality of egg yolk

### Egg yolk color

As shown in Table 5, yolk color decreased from 6.59 pre-feeding to 6.39 at week 2, then increased to 6.55 by week 4 and peaked at 6.83 in week 6. It slightly decreased to 6.70 at week 8 and 6.36 by week 10, with no significant differences after week 6.

### Yolk index of eggs

As shown in Table 6, yolk weight was lowest pre-BSC at 16.77 g (p<0.05), increased to 17.83 g at week 6 (p<0.05) and declined to 17.12 g and 17.05 g at weeks 8 and 10 (p<0.05). Yolk height followed a similar trend, starting lowest pre-feeding, peaking at 16.91 mm in week 6 and decreasing to 16.60 mm by week 10 (p<0.05). Yolk diameter

**Table 3:** Eggshell thickness from laying hens fed diets with 0.1% BSC during a period of 10 weeks [1,2,3].

| Weeks | Eggshell thickness (mm) |
|---|---|
| 0 | 0.337.002bcd |
| 2 | 0.334.002d |
| 4 | 0.346.002a |
| 6 | 0.342.003abc |
| 8 | 0.343.003ab |
| 10 | 0.335.002cd |

[1] BSC; Dried *Bacillus subtilis* Culture.

[2] Values are expressed as means±standard errors.

[3] Means in the same column with no common superscript are significantly different (*p*<0.05).

**Table 4:** Eggshell color from laying hens fed diets with 0.1% BSC during a period of 10 weeks [1,2,3].

| Weeks | Eggshell color (No.) |
|---|---|
| 0 | 11.50.17ab |
| 2 | 11.37.16b |
| 4 | 11.63.19ab |
| 6 | 11.97.15a |
| 8 | 11.93.14a |
| 10 | 11.87.12a |

[1] BSC; Dried *Bacillus subtilis* Culture.

[2] Values are expressed as means±standard errors.

[3] Means in the same column with no common superscript are significantly different (*p*<0.05).

**Table 5:** Egg yolk color from laying hens fed diets with 0.1% BSC during a period of 10 weeks [1,2].

| Weeks | Egg yolk color (No.) |
|---|---|
| 0 | 6.59.20 |
| 2 | 6.39.14 |
| 4 | 6.55.14 |
| 6 | 6.83.14 |
| 8 | 6.79.14 |
| 10 | 6.36.14 |

[1] BSC; Dried *Bacillus subtilis* Culture.

[2] Values are expressed as means±standard errors.

**Table 6:** Egg yolk index from laying hens fed diets with 0.1% BSC during a period of 10 weeks [1,2,3].

| Weeks | Yolk weight (g) | Yolk height(mm) | Yolk diameter(mm) | Egg yolk index (mm) |
|---|---|---|---|---|
| 0 | 16.77b | 15.65d | 42.68a | 0.37e |
| 2 | 16.81b | 16.21c | 39.57c | 0.41b |
| 4 | 16.95b | 16.90a | 39.62c | 0.43a |
| 6 | 17.83a | 16.91a | 42.21b | 0.40c |
| 8 | 17.12b | 16.84ab | 42.12b | 0.40c |
| 10 | 17.05b | 16.60b | 42.09b | 0.39d |
| SEM | 0.54 | 0.43 | 0.54 | 0.01 |

1) BSC; Dried *Bacillus subtilis* Culture.

2) SEM: Standard error of the mean.

3) Means in the same column with no common superscript are significantly different ($p$<0.05).

**Table 7:** Haugh unit from laying hens fed diets with 0.1% BSC during a period of 10 weeks [1,2].

| Weeks | Haugh unit (HU) |
|---|---|
| 0 | 60.00.68 |
| 2 | 60.18.71 |
| 4 | 60.52.69 |
| 6 | 60.58.69 |
| 8 | 60.51.69 |
| 10 | 59.80.73 |

1) BSC; Dried *Bacillus subtilis* Culture.

2) Values are expressed as means ±standard errors.

was highest pre-feeding at 42.68 mm and ranged 39.75-42.21 mm during the trial, with no significant increase over time. The yolk index rose from 0.37 pre-feeding to 0.43 in week 4, then declined to 0.39 by week 10 (p<0.05).

**Haugh unit**

Table 7 shows that HU started at 60.00 pre-feeding, increased to 60.58 at week 6, then decreased to 59.80 by week 10. Although no significant differences were detected, values indicated improved freshness during the early feeding period, followed by a slight decline after week 8.

Global increases in grain and oil prices have heightened the need for improving egg quality while reducing production costs in poultry farming, as feed expenses account for a substantial proportion of total operating costs. Under such economic pressures, dietary strategies such as probiotic supplementation have emerged as alternatives to antibiotic growth promoters, offering benefits in feed efficiency (Leitner *et al*., 2001) and overall livestock performance (Yeo and Kim, 1997). *Bacillus subtilis*, in particular, has been widely documented to improve laying rate, egg quality and reduce mortality in laying hens (Ryu *et al*., 1999; Santoso *et al*., 1995; Junbo *et al*., 2009). Supplementation with *B. subtilis* engineered to express ferritin-a cellular iron-storage protein-has been reported to enhance productivity and egg quality in both broilers (Choi, 2016) and laying hens (Lee *et al*., 2019).

The present study aimed to evaluate the effect of dried B. subtilisculture (BSC) containing a ferritin gene derived from Periserrula leucophrymaon the productivity and egg quality of late-phase laying hens. Using a large commercial flock of 3,600 ISA Brown hens at 90 weeks of age, hens were fed a basal diet supplemented with 0.1% ferritin-expressing BSC for 10 weeks. Mortality rates in late-phase Hy-Line Brown hens typically rise toward 100 weeks of age (Hy-Line Brown Management Guide, 2022). In contrast, mortality in the present study showed no significant increase over the trial period, suggesting that ferritin-enriched *B. subtilis* supplementation contributed to suppressing mortality trends during the late laying period.

In terms of laying rate, no significant differences were observed between the pre-supplementation rate (88.54%) and the average rate from weeks 2 to 6 (87.88%). A decline in laying rate became apparent from week 6 (96 weeks of age) onward. Considering that a natural decline is expected from 90 to 96 weeks in Hy-Line Browns (Hy-Line Brown Management Guide, 2022), our results suggest that BSC supplementation partially mitigated the decline.

Eggshell quality is known to deteriorate with advancing age due to physiological factors and external influences such as storage temperature (Samli *et al*., 2005), nutrition and stress (Roland, 1988; McLoughlin and Soares, 1976). In this study, eggshell weight increased significantly at the initial stage of BSC feeding, consistent with findings in ISA Browns supplemented with ferritin protein *via* drinking water at 80 weeks (Lee *et al*., 2019). The subsequent decrease in eggshell weight may be explained by the age-related decline in production rate and the shift in egg size distribution toward lighter categories. Despite this, eggshell strength and thickness were maintained up to week 8 and showed no significant reduction by week 10 compared to baseline. This aligns with reports that organic iron or iron-protein supplementation can enhance eggshell thickness and strength in laying hens (Yang *et al*., 2004).

Shell color, an important commercial trait influencing consumer preference (Lee *et al*., 2003), showed no significant differences between control and treatment groups during weeks 4 to 10, but a numerical increase was observed, suggesting a trend toward darker shell coloration. This is in agreement with Lee *et al*. (2019), who reported darker brown shells with ferritin supplementation.

Regarding yolk color, no significant differences were observed before and after supplementation; however, a slight increase was noted during weeks 6 to 8, coinciding with an overall improvement in yolk index throughout the trial. Previous studies have reported mixed effects of probiotics on yolk pigmentation, with some showing no effect (Na *et al*., 2003) and others reporting improvements (Kwon *et al*., 2002). The addition of ferritin protein has also been associated with enhanced yolk color (Lee *et al*., 2019). Therefore, the combination of *B. subtilis* and ferritin may positively influence yolk quality in late-phase layers.

Overall, our findings indicate that dietary supplementation with ferritin-expressing B. subtiliscan contribute to maintaining productivity, eggshell quality and certain aspects of egg quality during the late laying period under commercial conditions. These results support the potential for integrating genetically enhanced probiotics into feeding strategies for extending the productive life of laying hens while maintaining product quality.

## CONCLUSION

The findings of this study demonstrated that adding dried Bacillus subtilis culture (BSC) containing *Periserrula leucophryna*-derived ferritin gene transfection to the feed of laying hens over a period of 90 weeks of age prevented a decline in productivity and egg quality in the late laying period and resulted in improved reproductive performance. Conduct of future study is planned to assess productivity and for performance of blood analysis of laying hens at various ages after BSC supplementation, to examine the productivity and blood analysis of laying hens based on changes in BSC concentration following BSC supplementation and for conduct of feeding level testing in a variety of livestock species after BSC supplementation. Based on our findings, it is expected that supplementation with ferritin gene Bacillus subtilis will contribute to the effort to increase income for livestock farms and fostering development within the livestock industry.

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### Disclaimers

The views and conclusions expressed in this paper is solely those of the authors and do not necessarily express the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

### Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

### Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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