Dietary *Bacillus subtilis* B2A strain in laying hens challenged with *Salmonella gallinarum*: effects on egg production, egg quality, blood haptoglobin and targeted intestinal *Salmonella* shedding

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**ABSTRACT**

A total of 240 Hy-Line 40-week-old *Salmonella*-free layers were assigned to 5 dietary treatments composed of 12 replications; with 4 laying hens per replications in a 5-week experiment trial on laying hens challenged with *Salmonella gallinarum*. Dietary treatment comprised: (1) NC, basal diet; (2) PC, NC + oral *S. gallinarum* administration; (3) B8, PC + 0.1% *Bacillus subtilis* (10^6 cfu/g); (4) B9, PC + 0.1% *B. subtilis* (10^7 cfu/g) and (5) B10, PC + 0.1% *B. subtilis* (10^8 cfu/g). *B. subtilis* groups reduced the population of *S. gallinarum* in both large intestine and excreta compared with PC laying hens. The population of *S. gallinarum* in challenged laying hens fed *B. subtilis* was significantly lower in small intestine, large intestine and excreta. In the small intestine, *Lactobacillus* population was higher in B10 compared with PC. Addition of *B. subtilis* improved eggshell thickness. Egg shell strength showed an improvement in week 5 and it was higher in B9 and B10 compared with PC. Haptoglobin concentration was higher in the PC group compared with other groups. In conclusion, diets supplemented with *B. subtilis* as a novel anti-salmonella bacteria show potential for decreasing *S. gallinarum* in the intestinal tract and improving egg gravity, eggshell strength and eggshell quality during the laying period.

**Introduction**

Due to the focus on the protection of human health, there are increasing concerns about food safety and reduction of food-borne pathogens in food animals and their products. *Salmonella* remains the leading cause of costly bird bacterial food-borne disease due to its extended prevalence in poultry farms (Mead et al. 1999). Due to recently increased emphasis on production of animal products without the use of drugs, many antibiotic growth promoters have been banned worldwide and the products from chickens treated with antibiotics growth promoters can no longer be marketed.

The recent international legislation to ban antibiotics in most of the countries has been accompanied by increased incidence of poultry salmonellosis. This has provided impetus to the application of biological substances such as probiotics, which have been reported to be beneficial for food animal production. Several investigators have examined the positive effect of probiotics on *Salmonella* proliferation in poultry (Knap et al. 2011; Park & Kim 2014; Upadhyaya et al. 2016) and pig (Kim et al. 2014; Zhao & Kim 2015). *Bacillus subtilis* is a spore-forming anaerobic bacterium (Wu et al. 2009), which can be used as a single bacterial strain or a mixture of different species to prevent the colonization of pathogens in the gastrointestinal tract of poultry and to improve some aspects of their health.

There is extensive literature describing the competitive effects of *B. subtilis* spores in poultry; most of the studies report an increase in modulation of intestinal microbiota in favour of lactic acid bacteria (Wu et al. 2009; Park & Kim 2014). The ability of *B. subtilis* spores to protect against the *Salmonella* Heidelberg challenge with 58% reduction in *Salmonella*-positive drag swabs compared with control chickens may be due to the acidification of the intestinal environment which inhibits the growth of detrimental bacteria in chickens (Knap et al. 2011). In a study assessing the effects of *B. subtilis* in laying hens challenged with *Salmonella gallinarum*, Upadhyaya et al. (2016) showed that *B. subtilis* spores reduced colonization of the *Salmonella* spp. They reported that only 38% of laying hens from the *B. subtilis*-treated group were *Salmonella*-positive, whereas 62% of laying hens were still *Salmonella*-negative in the untreated control treatment; however, no significant differences were detected in performance variables in Leghorn chickens. Park and Kim (2014) also reviewed the activity of *B. subtilis* spores and indicated that they inhibit *Salmonella* proliferation in the large and small intestine, decrease ammonia emission from excreta and decrease haptoglobin level in plasma.

The objective of this study was to determine the effects of dietary supplementation of a *B. subtilis* B2A strain on egg production, blood parameters, and excreta and intestinal microbiota of laying hens with or without *Salmonella enterica* serovar Gallinarum KVCC BA 0700722 (S. gallinarum) challenge. The aim of this study was to find a novel probiotic strain which can inhibit the infectious activity of experimentally induced *S. gallinarum* infection in laying hens.

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Material and methods

Isolation of bacterial strain

The isolates were obtained by serial dilutions of soil samples, plating on nutrient agar (Difco Laboratories, Detroit, MI, USA), incubating for 24 h at 37°C and picking 5–6 colonies. Among them, we selected two bacterial strains that positively showed inhibitory activity against *S. gallinarum* KVCC BA 0700722. The strain species were identified as *B. subtilis* by methods that included bacteria morphology, 16S rRNA gene sequencing and the NCBI (National Center for Biotechnology Information) database, using the BLAST search program. We therefore named this strain *B. subtilis* B2A, which was produced by mixing of excipients with lyophilized *B. subtilis* B2A.

Experimental laying hens and design

A total of 240 Hy-Line Brown 40-week-old *Salmonella*-free layers were raised in a windowless and environmentally controlled room that was maintained at 23°C for 5 weeks. Sixteen hours (0500 to 2100 h) of artificial lighting with a light intensity of 5.2 lx at bird level was provided daily. Layers were kept individually, and 12 pens (20 cm width × 50 cm length × 40 cm height) were regarded as a replication. Hens were assigned to 5 dietary treatments with 12 replications per treatment. Experimental diets were formulated to meet the NRC (1994) recommendations. The experimental diet was typically of corn–soybean mash meal and contained 12,154 mJ/kg ME and 15.02% CP (Table 1). *S. gallinarum* (KVCC BA00722) was purchased from Korea Veterinary Culture Collection (KVCC, Korea) and was used in salmonella-challenged laying hens. Dietary treatment groups were: NC, basal diet; PC, NC + oral *S. gallinarum* (10⁶ cfu/g); B8, PC + 0.1% *B. subtilis* B2A (10⁶ cfu/g); B9, PC + 0.1% *B. subtilis* B2A (10⁹ cfu/g) and B10, PC + 0.1% *B. subtilis* B2A (10¹⁰ cfu/g). All laying hens except NC (n = 192) were orally challenged with 1 mL suspension of 10⁸ cfu/mL *S. gallinarum* at days 14 and 28 after the initiation of experiment by injection into the crop using a syringe with an attached flexible tube to inject each bird.

Chemical analysis

Feed samples were ground to pass through a 1-mm screen, after which they were 2000), N (method 968.06; AOAC 2000), ether extract (method 920.39; AOAC 2000), Ca (method 984.01; AOAC 1995) and P (method 965.17; AOAC 1995). Individual AA composition was measured using an AA reactor. Performic acid is an oxidizing reagent that converts Cys quantitatively to cysteic acid and Met to Met-sulfone. Nitrogen was determined (Kjeldahl 2300 Nitrogen Analyser; Foss Tecator AB, Hoeganaes, Sweden), and CP was calculated as N × 6.25.

Excreta and intestinal microbiota analysis

At the end of the experiment, excreta samples were collected from 10 layers per treatment, then pooled and placed on ice for transportation to the laboratory, where analysis was immediately performed by the method of Wang and Kim (2011). After collection of excreta samples, the same birds were slaughtered, and the large and small intestines were sampled for the enumeration of *Salmonella*, *E. coli* and *Lactobacillus*. The small intestinal digesta were collected from a 4–5 cm segment between the front and rear parts of the Meckel’s diverticulum, and the large intestinal digesta were collected from a 2–3 cm front part of the cloaca. One gram of the composite excreta and small and large intestine digesta samples from each replication were diluted with 9 mL of 1% peptone broth (Becton, Dickinson and Co., Rutherford, NJ) and homogenized. Viable counts of bacteria in the excreta samples were then determined by plating serial 10-fold dilutions (in 10 g/L peptone solution) onto Mac Conkey agar, Lactobacilli MRS agar and *Salmonella Shigella* agar plates to verify *Escherichia coli*, *Lactobacillus* and *Salmonella*, respectively. The Lactobacilli MRS agar plates were incubated for 48 h at 39°C, and the Mac Conkey agar and *Salmonella Shigella* agar plates were incubated for 24 h at 37°C under anaerobic conditions. The bacterial colonies were counted immediately after removal from the incubator. A single colony was obtained from selective media plates and cultivated in peptone yeast glucose broth. Subsequently, the bacteria were characterized to the genus level on the basis of colonial appearance, gram reaction, spore production, cell morphology and fermentation end product formation.

Egg production

Hens were allowed *ad libitum* access to feed and water throughout the experimental period. Eggs were collected on a daily
basis, and egg production was calculated as an average hen-day production.

**Egg quality**

A total of 30 eggs with the exception of soft and broken eggs were randomly collected at 1700 h from each treatment on a weekly basis and were used to determine the egg quality at 2000 h the same day. Eggshell-breaking strength (kg/cm²) was evaluated (Eggshell Force Gauge Model II; Robotmation Co., Tokyo, Japan). Eggshell thickness was measured at the large end, the equatorial region and the small end (Dial Pipe Gauge; Ozaki MFG. Co., Tokyo, Japan). Finally, the egg weight, yolk colour and Haugh unit were evaluated (Egg Multi Tester; Touhoku Rhythm Co., Tokyo, Japan).

**Blood parameters and serum haptoglobin concentration**

At the end of the experiment, during the first week post the second *Salmonella* challenge, 10 layers per treatment were selected, and 5 mL of blood was collected from the left jugular vein using a sterilized needle into vacuum tubes containing no additive and tubes containing K₃EDTA (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) to obtain serum and whole blood, respectively. Blood samples were centrifuged at 3000×g for 20 min at 4°C, and serum samples were stored at −4°C. Serum haptoglobin was determined, using an enzyme-linked immunosorbent assay kit (TP801; Tri-Delta Diagnostics, Morris Plains, NJ, USA).

**Statistical analysis**

Data were analysed statistically via GLM procedure of the SAS/STAT9.2 software (SAS Institute 2008), for a completely randomized design. Differences among all treatments were assessed by Tukey’s range test. Variability in the data was expressed as the pooled SE and *P* values of <.05 were considered to indicate statistical significance.

### Results

**Excreta and intestinal microbiota**

The *Salmonella* population in the small intestine was lower (*P* < .05) in the B8, B9, B10 and NC groups than in the PC group (Table 2). *B. subtilis* also reduced the population of *Salmonella* in both large intestine and excreta compared with that in PC laying hens at the third week of experiment. In the small intestine, *Lactobacillus* population was higher in the B10 group compared with the PC group, whereas there were no significant effects on the number of lactobacilli in either the large intestine or excreta (Table 2). Diet treatment generally had no effect on *E. coli* population in the intestinal tract and excreta.

**Egg production**

Egg production and feed intake results are shown in Table 3. The production of eggs was not different (*P* < .05) during the weeks. The daily feed intake also did not differ among treatments during all weeks (Table 3).

**Egg quality**

The effects of *B. subtilis* strain on egg quality in layers challenged with *S. gallinarum* are presented in Table 4. Eggshell strength showed an improvement at week 5, and it was lower in the PC group compared with the B9 and B10 groups. Among the groups, the PC group exhibited the lowest (*P* < .05) eggshell thickness and egg gravity compared with the other groups during the fifth week.

**Haptoglobin**

Haptoglobin concentration was higher (*P* < .05) in the PC group compared with the other groups (Table 5).

**Discussion**

Since *Salmonella* is the most common bacterial cause *B. subtilis* (Wu et al. 2009; Lee et al. 2014), which is regarded as the
The effects of *B. subtilis* B2A on egg quality in laying hens.

| Items                   | NC (g)       | PC (g)       | B8 (g)       | B9 (g)       | B10 (g)       |
|-------------------------|--------------|--------------|--------------|--------------|--------------|
| Egg weight              | 61.5 ± 0.96  | 61.3 ± 0.55  | 61.8 ± 0.87  | 61.9 ± 0.94  | 61.8 ± 0.91  |
| Yolk height (mm)        | 7.98 ± 0.15  | 8.05 ± 0.10  | 8.14 ± 0.06  | 8.13 ± 0.22  | 8.04 ± 0.11  |
| Yolk colour             | 7.64 ± 0.17  | 7.31 ± 0.14  | 7.45 ± 0.19  | 7.40 ± 0.20  | 7.40 ± 0.18  |
| Haugh unit              | 89.9 ± 0.43  | 89.1 ± 0.61  | 89.9 ± 0.49  | 89.6 ± 1.28  | 89.3 ± 0.65  |
| Eggshell strength (kg/cm²) | 4.07 ± 0.13 | 4.09 ± 0.10  | 4.05 ± 0.11  | 4.10 ± 0.12  | 4.05 ± 0.08  |
| Eggshell thickness (mm²) | 38.7 ± 0.54 | 39.3 ± 0.46  | 39.1 ± 0.46  | 39.1 ± 0.59  | 39.1 ± 0.46  |
| Egg gravity             | 1096 ± 1.1   | 1098 ± 3.3   | 1095 ± 1.4   | 1095 ± 2.4   | 1097 ± 2.8   |
| Haugh unit              | 7.99 ± 0.11  | 8.11 ± 0.08  | 8.08 ± 0.08  | 8.06 ± 0.11  | 8.23 ± 0.15  |
| 2 weeks                 | 61.2 ± 0.69  | 62.2 ± 0.42  | 61.4 ± 0.56  | 61.5 ± 0.33  | 61.6 ± 0.52  |
| Yolk height (mm)        | 89.7 ± 0.70  | 89.0 ± 0.57  | 88.6 ± 0.82  | 90.7 ± 0.84  | 907.8 ± 0.04  |
| Yolk colour             | 7.33 ± 0.18  | 7.35 ± 0.17  | 7.35 ± 0.20  | 7.15 ± 0.21  | 7.15 ± 0.21  |
| 3 weeks                 | 60.9 ± 0.80  | 60.6 ± 0.33  | 61.4 ± 0.55  | 61.2 ± 0.33  | 61.7 ± 0.52  |
| Yolk height (mm)        | 7.95 ± 0.09  | 7.89 ± 0.07  | 7.97 ± 0.08  | 7.95 ± 0.11  | 8.13 ± 0.12  |
| Yolk colour             | 7.2 ± 0.25   | 7.53 ± 0.20  | 7.5 ± 0.28   | 7.55 ± 0.27  | 7.25 ± 0.29  |
| 4 weeks                 | 61 ± 0.98    | 60.1 ± 0.47  | 61.1 ± 0.78  | 61.2 ± 0.73  | 60.9 ± 0.58  |
| Egg weight              | 8.28 ± 0.08  | 8.01 ± 0.04  | 8.28 ± 0.06  | 8.22 ± 0.22  | 8.14 ± 0.11  |
| Yolk height (mm)        | 7.47 ± 0.22  | 7.51 ± 0.14  | 7.50 ± 0.16  | 7.30 ± 0.20  | 7.50 ± 0.17  |
| Yolk colour             | 9.11 ± 0.43  | 9.02 ± 0.44  | 9.17 ± 0.50  | 91.1 ± 0.68  | 91.1 ± 0.68  |
| Eggshell strength (kg/cm²) | 4.00 ± 0.10 | 4.05 ± 0.10  | 4.00 ± 0.11  | 4.16 ± 0.12  | 4.16 ± 0.08  |
| 5 weeks                 | 60.9 ± 0.72  | 60.6 ± 0.61  | 61.2 ± 0.89  | 61.2 ± 0.81  | 61 ± 0.62    |
| Yolk height (mm)        | 7.78 ± 0.17  | 7.43 ± 0.15  | 7.55 ± 0.22  | 7.35 ± 0.21  | 7.49 ± 0.18  |
| Egg gravity             | 1097 ± 1.1   | 1091 ± 1.5   | 1096 ± 1.4   | 1095 ± 1.5   | 1096 ± 1.1   |

Note: Means in the same row with different superscripts differ (*P < .05*). Mean ± SE.

Abbreviation: NC, basal diet; PC, NC + *Salmonella* challenge; B8, PC + 0.1% *B. subtilis* B2A (10⁸ cfu/g); B9, PC + 0.1% *B. subtilis* B2A (10⁹ cfu/g); B10, PC + 0.1% *B. subtilis* B2A (10¹⁰ cfu/g).
have a lower incidence of *Salmonella* than those fed control diets (Wu et al. 2009; Knap et al. 2011). A recent study reported by Park and Kim (2014) showed that when 21-day-old chicks were dosed with *B. subtilis* B2A or *B. subtilis* RX7 and later challenged with *S. gallinarum*, *B. subtilis* showed significant differences in *Salmonella* population in the intestine, it has been suggested that this might be the metabolic effects of obligate spore-forming aerobes on pathogens. In this study, the presence of *B. subtilis* reduced the release of *S. gallinarum* into the *Salmonella* reduction has been associated with an increased concentration of lactic acid in the caeca (Knarreborg et al. 2008), which is a primary place of *Salmonella* multiplication. Germination of *B. subtilis* strain spores causes a lower pH in the intestine due to *Lactobacillus* proliferation and more lactic acids, thus promoting the inhibition of *Salmonella* growth. Apart from the germinating or vegetative characteristics of *B. subtilis* spores, another prominent mechanism which is believed to play a crucial role is the biofilm and peptides released in the gut lumen which may cause inhibition of adhesion or colonization of pathogens (Stanley & Lazazzera 2004). Biofilms are microbial communities that are able to adhere to epithelial cells to form pathogenic ecosystems (Stanley & Lazazzera 2004). Sporulating *B. subtilis* cells produce and export the antibiotic-like killing factor and also surfactin (Gonzalez-pastor et al. 2003) in a biofilm and, interestingly, surfactin is also able to inhibit biofilm formation in the human pathogen *Salmonella* (Mireles et al. 2001).

In this study, the influence of dietary *B. subtilis* supplementation on laying hens with and without *Salmonella* challenge was investigated. In terms of performance, the results of the current study showed that laying hens consuming the *B. subtilis* diets did not change egg production during the experiment. In line with our findings, several studies have noted no significant effects of *B. subtilis* on egg production, suggesting that *B. subtilis* does not negatively affect performance in a non-challenged (Mahdavi et al. 2005) or a challenged (Knap et al. 2011; Park & Kim 2014) setting. In contrast, Abdelqader et al. (2013) found that $2.3 \times 10^9$ cfu/g of *B. subtilis* spores isolated from the intestinal tract of chicken significantly increased egg production and eggshell quality of laying hens. In a comparative study, Khan et al. (2011) observed enhanced BW in broiler chickens during the 39-day experiment when they used probiotic including $1.5 \times 10^9$ cfu *B. subtilis*. The effect of *B. subtilis* on microbiota pathogens is distinctive, but its effects on production factors vary. Contradictory results of those studies which are found regularly in the probiotic or gut microbiology literature could be due to a variety of reasons such as differences in the species, their concentrations in the diet, viability in the gastrointestinal tract and type of challenge performed.

Eggshell-breaking strength and eggshell thickness were increased significantly as a result of *B. subtilis* supplementation in the last week of study. A better eggshell quality was seen in the study by Abdelqader et al. (2013), who found that *B. subtilis* and inulin supplementation of layers increased the colonization of beneficial bacteria such as *Lactobacillus* and *Bifidobacterium* in the intestinal environment and this indicated induction of lower pH in the excreta, intestinal digesta and caecal content. Conversely, Ribeiro et al. (2014) reported no significant influence on eggshell quality after *B. subtilis* supplementation in laying hens. The transport of calcium through the enterocyte is attributed to calcium-binding proteins which show a higher efficiency at a lower pH (Allen 1982). Thus, lowering the pH may increase the solubility and absorption of minerals such as calcium and phosphorus salts in the intestinal environment.

Haptoglobin is an anti-inflammatory protein that modulates prostaglandin synthesis and its plasma level increases during any inflammatory process such as infection and injury (Ceron et al. 2005). Groups of commercial layers in a *S. gallinarum* challenge experiment showed an increase in the serum levels of haptoglobin, which is an acute-phase protein, as compared to the control group (Garcia et al. 2009). Reduced concentration of haptoglobin due to *B. subtilis* feeding in these *Salmonella*-challenged laying hens could be attributed to reduction in infection-induced stress and the related injuries.

### Table 5. The effect of *B. subtilis* B2A on blood parameters in laying hens.

| Items, log10 cfu/g          | NC  | PC  | B8  | B9  | B10 |
|----------------------------|-----|-----|-----|-----|-----|
| Haptoglobin (mg/dL)        | 0.56 ± 0.10$^a$ | 0.76 ± 0.02$^b$ | 0.53 ± 0.08$^b$ | 0.61 ± 0.03$^b$ | 0.63 ± 0.02$^b$ |

Note: Means in the same row with different superscripts differ ($P<.05$). Mean ± SE. Abbreviation: NC, basal diet; PC, NC + *Salmonella* challenge; B8, PC + 0.1% *B. subtilis* B2A (10$^8$ cfu/g); B9, PC + 0.1% *B. subtilis* B2A (10$^9$ cfu/g); B10, PC + 0.1% *B. subtilis* B2A (10$^{10}$ cfu/g).

#### Conclusion

The results of the present study support our hypothesis that dietary *B. subtilis* plays a significant role in decreasing *S. gallinarum* populations in the intestinal tract of laying hens. Diets supplemented with *B. subtilis* B2A probiotic show potential for improving egg gravity, eggshell strength and eggshell quality during the laying period. Therefore, *B. subtilis* B2A strain may have beneficial effects in improving the host response to *S. gallinarum* infection in laying hens.

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#### Disclosure statement

No potential conflict of interest was reported by the authors.

#### References

Abdelqader A, Al-Fatafth AR, Daş G. 2013. Effects of dietary *Bacillus subtilis* and inulin supplementation on performance, eggshell quality, intestinal morphology and microflora composition of laying hens in the late phase of production. *Anim Feed Sci Technol.* 179:103–111.

Allen LH. 1982. Calcium bioavailability and absorption: a review. *Am J Clin Nutr.* 35:783–808.

AOAC. 1995. *Official methods of analysis.* 16th ed. Arlington, VA: Association of Official Analytical Chemists.
Ceron JJ, Eckersall PD, Martínez-subiela S. 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. Vet. Clin. Path. 34:85–99.

Garcia KO, Berchieri-júnior A, Santana AM, Freitas-neto OC, Fagliari JJ. 2009. Experimental infection of commercial layers using a Salmonella enterica serovar Gallinarum strain: Leukogram and serum acute-phase protein concentrations. Braz J Poult Sci. 4:263–270.

Gonzalez-pastor JE, Hobbsand EC, Losick R. 2003. Cannibalism by sporulating bacteria. Science. 301:510–513.

Khan SH, Yousaf B, Mian AA, Rehman A, Farooq MS. 2011. Assessing the effect of administering different probiotics in drinking water supplement on broiler performance, blood biochemistry and immune response. J Appl Anim Res. 39:418–428.

Kim KH, Ingale SL, Kim JS, Lee SH, Lee JH, Kwon IK, Chae BJ. 2014. Bacteriophage and probiotics both enhance the performance of growing pigs but bacteriophage are more effective. Anim Feed Sci Technol. 196:88–95.

Mead PS, Slutsker L, Dietz V, Mccaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. 1999. Food-related illness and death in the United States. Emerg Infect Dis. 5:607–625.

Mireles JR, Toguchi A, Harshey RM. 2001. Salmonella enterica serovar Typhimurium swarming mutants with altered biofilm-forming abilities: surfactin inhibits biofilm formation. J Bacteriol. 183:5848–5854.

NRC. 1994. Nutrient requirements of poultry. 9th rev. ed. Washington (DC): Natl. Acad. Press.