The effect of dietary *Bacillus subtilis* supplementation on the growth performance, blood profile, nutrient retention, and caecal microflora in broiler chickens

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**ABSTRACT**

The objective of this study was to evaluate the effects of the *B. subtilis* RX7 and *B. subtilis* C14 on the growth performance, blood profiles, nutrient retention, and caecal microflora of broiler chickens. A total of 288, 1-d-old Ross 308 male broilers were assigned to one of four dietary treatment groups for 35 days: the negative control (NC; basal diet with no antibiotic or *B. subtilis*), positive control (PC; NC + 40 ppm avilamycin), T1 (NC + 0.1% *B. subtilis* RX7 1.0×10⁹ cfu/g), and T2 (NC + 0.1% *B. subtilis* C14 1.0×10⁹ cfu/g). *B. subtilis* supplementation led to significantly higher weight gain than that in the NC. The serum haptoglobin concentration was lower in the Bacillus groups than in the NC and PC groups. The energy retention of broilers fed *B. subtilis* was significantly higher than that of the NC and PC broilers. The numbers of caecal Lactobacillus or Salmonella in the Bacillus groups were higher and lower, respectively, than those in the numbers in the NC and PC. The results showed that *B. subtilis* RX7 and C14 increased the weight gain, energy retention, and caecal Lactobacillus numbers and decreased serum haptoglobin levels and caecal Salmonella numbers in broiler chickens.

1. Introduction

Antibiotics have long been used as feed additives and have been shown to promote growth, stabilize the microbial flora in the intestinal tract, and prevent some pathologies (Dibner and Richards 2005). However, the overuse of antibiotics in farm animals can lead to antibiotic resistance in intestinal bacteria (Kolar et al. 2002). Luangtongkum et al. (2006) reported that the rates of resistant pathogenic bacteria in conventionally raised broilers and turkeys treated with antibiotics were higher than the rates in organic broilers and turkeys. In addition, the use of antibiotics results in the presence of antibiotic residue in animal products (Donoghue 2003). Therefore, to ensure the safety of animal products for consumers, the use of antibiotics as a growth promoter in animal diets has been strictly prohibited in many countries, including Korea.

Many alternatives to antibiotics have been proposed, such as probiotics, prebiotics, organic acids, phytotherapeutic extracts, and other functional materials (Dibner and Buttin 2002; Joerger 2003; Biggs et al. 2007; Park et al. 2014; Park and Kim 2014). Over the last decade, these additives have been shown to be potentially useful for pathogen control and performance enhancement, and have been successfully applied in poultry. Among the many feed additives used as substitutes for antibiotics, probiotics are expected to have the useful functions necessary to effectively replace antibiotics. The positive effects of *Bacillus* probiotic strains in poultry include growth performance, nutrient retention, and modulation of intestinal microflora, pathogen inhibition, favourable intestinal histological changes, immunomodulation, and improvement of certain serum biochemical parameters (Liu et al. 2012; Lee et al. 2013; Park and Kim 2015; Mahmoud et al. 2017; Oh et al. 2017).

This study was conducted to investigate the effects of two *Bacillus subtilis* strains as candidate probiotics in broiler chickens by examining their antibacterial activity against *Salmonella* and their effects on growth performance, blood profiles, nutrient retention, and intestinal microflora populations.

2. Materials and methods

All animal management and experimental procedures were approved by the Animal Care and Use Committee of Dankook University.

2.1. Isolation of bacterial strains as potential probiotics

To select bacterial strains for their probiotic potential, 30 isolates were obtained from soil samples collected randomly from several animal farms and pastures in Cheonan, Korea. These isolates were obtained by serially diluting soil samples, plating on Nutrient agar (Difco Laboratories, Detroit, MI, USA), incubating for 24 h at 37°C, and picking five to six colonies for analysis. Among these strains, we selected two that showed inhibitory activity against *S. gallinarum* ATCC 9184. The isolated strains were identified as *B. subtilis* based on bacterial morphology and 16S rRNA gene sequence analysis.

2.2. Experimental design

To assess the effects of the two isolates in broilers, 288 one-day-old male Ross 308 broilers were housed in 24 battery cages
(80 cm × 200 cm × 45 cm; w × l × h) equipped with feeders and nipple drinkers for 35 days. The birds were randomly assigned to 4 treatment groups with 72 birds per treatment and 6 replicates (12 birds/cage), with similar body weights (46.8 ± 0.9 g). The environmental temperature was maintained at 32°C for the first week and then gradually reduced to 22°C by the fifth week. Relative humidity was gradually increased from 60% (days 1–21) to 70% (days 22–35). Experimental diet and fresh water were offered ad libitum, and the animals were housed under a 23-h/1-h light/dark cycle throughout the experimental period.

The experimental diets consisted of corn-soybean mash meal, and contained 13.2 MJ/kg ME and 22% CP until 21 days of age and 13.4 MJ/kg ME and 20% CP until 35 days of age (Table 1). Dietary treatments in the experimental groups included the basal diet (negative control; NC), a basal diet supplemented with 40 ppm avilamycin (positive control; PC), and 2 Bacillus treatment groups (basal diet + 0.1% B. subtilis RX7 1.0 × 10⁹ cfu/g or 0.1% B. subtilis C14 1.0 × 10⁹ cfu/g).

2.3. Growth performance and blood collection

Body weight and feed intake per cage were measured at the end of the experiment, and feed conversion was calculated based on feed intake divided by body weight gain. At the end of the feeding period, blood samples were collected after a 12-h fast from the jugular vein of 12 broilers from each treatment to determine haematological parameters. For haematology, approximately 3 mL of blood was collected in tubes containing K₂EDTA (BD Vacutainer; Plymouth, PL6 7BP, UK), and was analysed immediately after collection. For haptoglobin determination, blood samples were placed in serum separator tubes (BD Vacutainer), centrifuged at 3000 rpm for 15 min, and stored at −80°C until use.

2.4. Haematological and haptoglobin analysis

Haematological parameters, such as the numbers of white blood cells, red blood cells, and lymphocytes, were estimated with an automatic blood analyser (ADVIA 120; Bayer, Tarrytown, NY, USA). Serum haptoglobin was determined using an enzyme-linked immunosorbent assay kit (TP801; Tri-Delta Diagnostics, Inc., Morris Plains, NJ, USA).

2.5. Total tract nutrient retention

To determine nutrient retention, 0.2% chromium oxide (Cr₂O₃) was added to the experimental diets as an indigestible marker four days prior to collection. Then, the excreta of the birds were collected daily for three days (the last three days of the experiment period) and dried in a 60°C oven for 72 h. After drying, they were pulverized, passed through a 1-mm screen, and analysed for total tract retention of dry matter (DM), nitrogen (N), and energy. DM content was assessed by oven drying at 105°C for 8 h according to AOAC guidelines (2000). The N contents of the feed and excreta samples were analysed using the Kjeldahl method (AOAC, 2000). The gross energy of feed and excreta samples was measured using a bomb calorimeter (Parr 6100; Parr instrument Co., Moline, IL, USA).

2.6. Caecal microbial analysis

Fresh caecal contents from broilers in each treatment group were obtained at the end of the experiment, and six birds per treatment were slaughtered. To measure caecal Lactobacillus, E. coli, and Salmonella, approximately 1 g of caecal samples was diluted 10-fold (1:9, w/v) in sterilized phosphate-buffered saline (PBS, 0.1 M, pH 7.0) and homogenized. Then, a 0.1-mL sample was serially diluted 10⁻³ to 10⁻⁹ and spread on Rogosa agar (Difco Laboratories, Detroit, MI, USA), and SS agar (Difco Laboratories, Detroit, MI, USA), for Lactobacillus, E. coli and Salmonella, respectively. Lactobacillus agar plates were incubated anaerobically at 37°C for 48 h, whereas E. coli and Salmonella agar plates were incubated under aerobic conditions at 37°C for 24 h. The colonies on each plate were counted using a colony counter, and the results are presented as log₁₀ colony-forming units (CFU) per gram.

2.7. Statistical analysis

The cage was the experimental unit for growth performance and nutrient retention, whereas each bird was the experimental unit for blood profiles and caecal microbial counts. All data were analysed using the General Linear Model (GLM) in SAS. Tukey’s test was performed to determine the significance of differences among groups. Variability in the data was expressed as the pooled SEM, and P values less than .05 were considered statistically significant.

### Table 1. Formulation and chemical composition of the experimental diets (on an as-fed basis).

| Ingredients, %              | Starter  | Grower  |
|------------------------------|----------|---------|
| Corn                         | 55.33    | 63.03   |
| Soybean meal (CP 48%)        | 28.34    | 24.56   |
| Corn gluten meal (CP 60%)    | 6.50     | 3.50    |
| Soybean oil                  | 5.50     | 4.89    |
| Dicalcium phosphate          | 2.46     | 2.29    |
| Limestone                    | 0.89     | 0.75    |
| Salt                         | 0.20     | 0.20    |
| DL-Methionine (98%)          | 0.17     | 0.17    |
| L-Lysine-HCl (78%)           | 0.21     | 0.21    |
| Vitamin premix               | 0.20     | 0.20    |
| Mineral premix               | 0.20     | 0.20    |
| Total                        | 100      | 100     |

**Calculated composition**

| ME, MJ/kg                  | 13.2     | 13.4    |
| Digestible lysine, %       | 0.93     | 0.90    |
| Digestible methionine, %   | 0.48     | 0.38    |

**Analysed composition**

| CP, %                      | 22.4     | 20.5    |
| Ca, %                      | 1.06     | 0.87    |
| Lysine, %                  | 1.05     | 0.98    |
| Methionine, %              | 0.54     | 0.41    |
| Total P, %                 | 0.83     | 0.75    |
| Crude fat, %               | 4.32     | 5.87    |
| Crude fibre, %             | 4.71     | 6.21    |

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*Starter diet was provided on days 1–21, and finisher diet was provided on days 22–35.*

*Provided per kg of complete diet: 15,000 IU of vitamin A, 3750 IU of vitamin D₃, 37.5 mg of vitamin E, 2.5 mg of vitamin K₃, 3 mg of thiamin, 7.5 mg of riboflavin, 4.5 mg of vitamin B₆, 24 µg of vitamin B₁₂, 51 mg of niacin, 1.5 mg of folic acid, 0.2 mg of biotin, and 13.5 mg of Ca-pantothenate.*

*Provided per kg of complete diet: 37.5 mg of Zn (as ZnSO₄), 37.5 mg of Mn (as MnO₂), 37.5 mg of Fe (as FeSO₄·7H₂O), 3.75 mg of Cu (as CuSO₄·5H₂O), 0.83 mg of I (as KI), and 0.23 mg of Se (as Na₂SeO₃·5H₂O).*
3. Results

3.1. Growth performance

During the 35-d trial, feed intake and feed conversion were not affected by supplementation with *B. subtilis* RX7 or C14 (Table 2). The weight gain of the broilers in the *B. subtilis* RX7 and C14 treatment groups was higher than those in the NC (P < .05); however, no significant differences in weight gain were observed between the *B. subtilis* treatment groups and the PC.

3.2. Blood profiles

*B. subtilis* RX7 and C14 treatment had no significant effects on the number of white blood cells, red blood cells, or lymphocytes (Table 3). However, the haptoglobin concentration was lower in birds in the *B. subtilis* RX7 and C14-supplemented groups than in NC and PC birds (P < .05).

3.3. Total tract nutrient retention

The energy retention of broilers fed *B. subtilis* RX7 and C14 was significantly higher (P < .05) than that in NC and PC broilers (Table 4). However, dietary *B. subtilis* supplementation did not affect the retention of DM or N.

3.4. Caecal microflora

*B. subtilis* supplementation led to significantly higher (P < .05) numbers of *Lactobacillus* in caecum than in the NC and PC (Table 5). In addition, lower numbers of *Salmonella* were observed in the caecum of birds in the *B. subtilis* treatment groups. However, no difference was observed between the *B. subtilis* treatment groups and the PC.

4. Discussion

The goal of this study was to examine the effects of two bacterial strains, *B. subtilis* RX7 and *B. subtilis* C14, that inhibit *Salmonella*, as potential antibiotic alternatives. First, we found that *B. subtilis* RX7 and *B. subtilis* C14 effectively inhibit the growth of *S. gallinarum*, exhibiting 20 mm and 22 mm of diameter of zone of inhibition, respectively, through an agar-well diffusion assay. Then, we performed an in vivo broiler study for 35 days. Based on the *in vitro* inhibitory effects, we hypothesized that supplementation of broiler diets with these *B. subtilis* strains would have inhibitory effects on intestinal *Salmonella*.

As expected, we also observed probiotic effects of *B. subtilis* RX7 and *B. subtilis* C14 on weight gain in the *in vivo* broiler experiment, as well as growth-promoting effects that were on par with those of the PC. Probiotics are live microorganisms that, when administered in adequate quantities, have beneficial effects on the host by improving the intestinal bacterial balance (Fuller 1989). Dietary probiotics have been shown to improve weight gain, reduce mortality, and enhance feed conversion, resulting in increased broiler productivity (Gerendai and Gippert 1988; Owings et al. 1990). *Bacillus* supplementation has also been shown to have beneficial effects on productivity, mortality, modulation of intestinal microflora, pathogen inhibition, and immune system stimulation in poultry (La Ragione et al. 2001; La Ragione and Woodward 2003; Lee et al. 2010). In our study, the increased weight gain following the addition of *B. subtilis* to the broiler diet was attributed to their probiotic effects, including maintenance of beneficial microbial populations and improved digestion, as shown in Tables 4 and 5. Therefore, the results of the current study suggest that inclusion of *B. subtilis* in broiler diets may improve performance by increasing body weight, although no significant differences were observed in feed intake and feed conversion between the *B. subtilis*-supplemented groups and the NC.

In this study, broilers fed a diet containing *B. subtilis* showed lower levels of haptoglobin than NC and PC broilers. Serum haptoglobin levels are increased by infection and by various physiological changes (Murata et al. 2004). Many previous studies have reported serum haptoglobin to be a clinically important element for measuring the occurrence and severity of inflammatory responses in pigs and cattle with various inflammatory diseases or experimental infection (Deignan et al. 2000; Petersen et al. 2002; Nazifi et al. 2009). Koutsos et al. (2006) reported that challenge with lipopolysaccharides derived from *S. typhimurium* in growing chicks promoted inflammatory immune

### Table 2. Effects of *B. subtilis* supplementation on growth performance in broilers.

| Parameter       | NC  | PC  | T1  | T2  | SEM  | P-value |
|-----------------|-----|-----|-----|-----|------|---------|
| Weight gain, g  | 1.602<sup>a</sup> | 1.655<sup>b</sup> | 1.669<sup>a</sup> | 1.677<sup>a</sup> | 12.44 | .035    |
| Feed intake, g  | 2.585 | 2.651 | 2.690 | 2.685 | 19.55 | .594    |
| Feed conversion | 1.614 | 1.602 | 1.612 | 1.601 | 0.02  | .826    |

<sup>1</sup>NC, basal diet with no antibiotics or *B. subtilis*; PC, basal diet + 40 ppm avilamycin; T1, basal diet + *B. subtilis* RX7; T2, basal diet + *B. subtilis* C14.

<sup>2</sup>Standard error of the mean.

<sup>3</sup>Means in the same row with different superscripts differ significantly (P < .05).

### Table 3. Effects of *B. subtilis* on blood profiles in broilers.

| Blood component | NC  | PC  | T1  | T2  | SEM  | P-value |
|-----------------|-----|-----|-----|-----|------|---------|
| White blood cells, 10<sup>3</sup>/µL | 29.9 | 30.1 | 31.0 | 29.2 | 1.72 | .861    |
| Red blood cells, 10<sup>9</sup>/µL | 2.24 | 2.18 | 2.22 | 2.17 | 0.08 | .459    |
| Lymphocyte, % | 85.6 | 84.6 | 84.4 | 85.3 | 1.30 | .588    |
| Haptoglobin, g/dL | 10.8<sup>a</sup> | 10.8<sup>b</sup> | 9.56<sup>b</sup> | 9.95<sup>b</sup> | 0.25 | .036    |

<sup>1</sup>NC, basal diet with no antibiotics or *B. subtilis*; PC, basal diet + 40 ppm avilamycin; T1, basal diet + *B. subtilis* RX7; T2, basal diet + *B. subtilis* C14.

<sup>2</sup>Standard error of the mean.

<sup>3</sup>Means in the same row with different superscripts differ significantly (P < .05).

### Table 4. Effects of *B. subtilis* on total tract nutrient retention in broilers.

| Digestibility, % | NC  | PC  | T1  | T2  | SEM  | P-value |
|------------------|-----|-----|-----|-----|------|---------|
| Dry matter       | 75.3 | 75.0 | 75.0 | 74.7 | 1.25 | .785    |
| Nitrogen         | 64.5 | 64.0 | 67.8 | 68.1 | 1.77 | .574    |
| Energy           | 76.3<sup>a</sup> | 76.3<sup>b</sup> | 80.0<sup>a</sup> | 79.1<sup>a</sup> | 1.09 | .042    |

<sup>1</sup>NC, basal diet with no antibiotics or *B. subtilis*; PC, basal diet + 40 ppm avilamycin; T1, basal diet + *B. subtilis* RX7; T2, basal diet + *B. subtilis* C14.

<sup>2</sup>Standard error of the mean.

<sup>3</sup>Means in the same row with different superscripts differ significantly (P < .05).

### Table 5. Effect of *B. subtilis* on caecal microflora in broilers.

| Bacteria, cfu/g | NC  | PC  | T1  | T2  | SEM  | P-value |
|-----------------|-----|-----|-----|-----|------|---------|
| Lactobacillus   | 7.56<sup>b</sup> | 7.37<sup>b</sup> | 7.78<sup>a</sup> | 7.70<sup>a</sup> | 0.04 | .036    |
| *E. coli*       | 6.65 | 6.51 | 6.51 | 6.50 | 0.08 | .390    |
| *Salmonella*    | 2.85<sup>a</sup> | 2.66<sup>ab</sup> | 2.36<sup>b</sup> | 2.43<sup>b</sup> | 0.04 | .043    |

<sup>1</sup>NC, basal diet with no antibiotics or *B. subtilis*; PC, basal diet + 40 ppm avilamycin; T1, basal diet + *B. subtilis* RX7; T2, basal diet + *B. subtilis* C14.

<sup>2</sup>Standard error of the mean.

<sup>3</sup>Means in the same row with different superscripts differ significantly (P < .05).
responses, as indicated by changes in blood haptoglobin. In addition, we showed that the reduction in the number of Salmonella in B. subtilis-fed groups was concomitant with a decrease in serum haptoglobin levels. Live birds are constantly exposed to various stresses, including harmful bacteria and infections, throughout their life. B. subtilis supplementation reduced serum haptoglobin levels, even though the broilers in this study were not subjected to any stress. Further studies are needed to confirm these positive effects in poultry.

In the present study, nutrient retention was significantly improved by B. subtilis supplementation. Some previous studies have suggested that the addition of probiotics to broiler diets might improve nutrient retention. Some probiotics, including B. subtilis, have been shown to increase jejunal villus height and decrease villus crypt depth in the intestinal villi, leading to an increase in the feed utilization of chickens (Samanya and Yamauchi 2002; Chichilowski et al. 2007; Awad et al. 2010). Hossain et al. (2015) observed significant improvements in DM and N retention when broilers were fed a diet supplemented with a probiotic containing three bacterial strains (B. subtilis, C. butyricum, and L. acidophilus) continuously for 35 days. Sen et al. (2011) demonstrated that broilers fed B. subtilis LS 1–2 showed greater retention of DM, crude protein, and energy than the control group. The increased caecal Lactobacillus counts (Table 5) observed after B. subtilis supplementation could be considered beneficial to the host in terms of the metabolic processes of digestion and nutrition utilization, as it could potentially increase the activity of useful enzymes (Fuller et al. 1978). Thus, the finding of the current study is consistent with the previous studies, which suggests that positive effects due to the improvement of nutrient retention could be expected following supplementation with B. subtilis in broilers.

In our study, the Salmonella and Lactobacillus populations in the caecum were significantly decreased and increased, respectively, by B. subtilis supplementation. Many studies have demonstrated that probiotics can competitively exclude pathogens in poultry (La Ragione et al. 2001; Higgins et al. 2007; Mountzouris et al. 2007), and that B. subtilis spores may be successful competitive exclusion agents (La Ragione and Woodward, 2003). This has been well documented for several Lactobacillus strains, and some evidence exists that Bacillus strains may have similar mechanisms of action (Barbosa et al. 2005). B. subtilis modulates the intestinal microflora and selectively favours the growth of lactic acid bacteria (Knarreborg et al. 2008). Populations of beneficial bacteria, such as Lactobacillus and Bifidobacterium, decrease the pH of the gastrointestinal tract due to increased production of lactic acid and volatile fatty acids. Therefore, the environment of the gastrointestinal tract becomes unsuitable for the proliferation of pathogens such as Salmonella. Furthermore, this inhibition is believed to be associated with the secretion of various antimicrobial compounds, such as organic acids, hydrogen peroxide, and bacteriocins (Reid et al. 2003). Thus, these probiotic effects, such as an improved intestinal environment and modulation of enteric inflammatory/immune responses, might influence the proliferation of the Lactobacillus and Salmonella populations as well as the serum haptoglobin concentration.

5. Conclusions

Feeding B. subtilis to broilers reduced serum haptoglobin levels and caecal Salmonella colonization, whereas it increased energy retention and caecal Lactobacillus colonization. Therefore, these B. subtilis strains have beneficial effects and can be used as alternatives to antibiotics for growth promotion in broiler production.

Disclosure statement

No potential conflict of interest was reported by the authors.

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