≪Research Note≫

Effect of Dietary *Bacillus Subtilis* C14 and RX7 Strains on Growth Performance, Blood Parameter, and Intestinal Microbiota in Broiler Chickens Challenged with *Salmonella Gallinarum*

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Sixty broilers (initially 1.6 kg and 35 d-old) were used to determine the effect of *Bacillus subtilis* C14 and RX7 strains on growth performance, blood parameter, and intestinal microbiota in response to experimental challenge with *Salmonell gallinarum*. Broilers were distributed to 4 treatment groups include: C1 (control group; no challenge, no *B. subtilis*), C2 (*Salmonella*-challenged group; *S. gallinarum* 10^8 cfu/bird), T1 (C2+supplemented with of *B. subtilis* C14 (1.0×10^9 cfu/g) at 0.1% in diet) and T2 (C2+supplemented with of *B. subtilis* RX7 (1.0×10^9 cfu/g) at 0.1% in diet). Results indicated that inclusion of *B. subtilis* (T1, T2) in the diet increased ($P < 0.05$) the weight gain and feed intake, and improved feed conversion of challenged broilers compared with no *B. subtilis* supplementation diet (C2). Improvements ($P < 0.05$) in the immunoglobulin A concentration were observed by the addition of *B. subtilis* compared with C2 treatment, whereas tumor necrosis factor-α was decreased ($P < 0.05$). *Latobacillus* number in small and large intestines was higher ($P < 0.05$) by *B. subtilis* additon than C2 treatment but *Salmonella* numbers were lower ($P < 0.05$). The results suggested that dietary supplementation of *B. subtilis* C14 and RX7 improved the growth performance, and affected the blood profiles and intestinal microbiota of broilers against *S. gallinarum* infection. Therefore, *B. subtilis* C14 and RX7 may have beneficial effects, in relieving the stress of broilers infected with *S. gallinarum*.

Key words: *Bacillus subtilis,* blood profile, broiler, challenge, productivity, *Salmonella gallinarum*

Introduction

Salmonella gallinarum (*S. gallinarum*) is the causative agent of fowl typhoid in avian species. Although *S. gallinarum* is not a serious public health concern, fowl typhoid is a severe systemic disease of poultry (Shivaprasad, 2000), causing serious financial losses due to high mortality, septicemia, enteritis, hemolytic anemia, and reduced egg production (Christensen *et al*., 1992). Although this disease has been eradicated from U. S., Canada, Australia, and most European countries, it is still a significant problem in some countries of Asia, Africa, Central America, and South America (Lee *et al*., 2007). In South Korea, increased incidence of fowl typhoid has been reported since 1992. It is an endemic disease in the poultry industry (Kwon *et al*., 2010).

Efforts such as vaccination and therapeutic antibiotics intervention have been made to control *S. gallinarum* colonization. However, antibiotics have been under strict control in animal production worldwide. Since a ban on in-feed antibiotic use was introduced in the European Union and the United States, *Salmonella* colonization in broiler farm has been gradually increasing in these countries (EFSA, 2007; USDA, 2007).

Several antibacterial agents as alternatives of antibiotics have been recommended to protect chickens from *Salmonella* infection, including probiotics, prebiotics, bacteriophages, organic acids, enzymes, essential oils, and phytogenic additives (Patterson and Burkholder, 2003; Van Immerseel *et al*., 2006; Atterbury *et al*., 2007; Johny *et al*., 2010; Amerah *et al*., 2012). These agents have substantiated in the last 3−4 decades. They were found to be potentially useful for preventing *Salmonella* and improving the performance of poultry. To reduce *Salmonella* contamination in broilers, *Bacillus subtilis* strains have been considered as reliable agent with competitive exclusion ability by reducing *Salmonella* load to the gut wall (La Ragione and Woodward, 2003). In this study, *B. subtilis* C14 and *B. subtilis* RX7 strains showing superior inhibitory activity against *Salmonella* were tested for their potential use as probiotics for

poultry. Their ability of preventing *S. gallinarum* infection in broiler chickens was determined in this study.

Materials and Methods

All animal trials were conducted following the animal care protocol ACUCDU 1302406 approved by the University Institutional Animal Care and Use Committee.

Isolation of Bacterial Strain for Probiotics

In this study, a total of 55 isolates were obtained from soil samples taken from Cheonan, Korea to select bacterial strains. Briefly, soil samples were serially diluted, plated onto Nutrient agar (Difco Laboratories, Detroit, MI, USA), and incubated at 37℃ for 24 h. A total of 8~9 colonies were picked from each plate. Among these isolates, two strains showing antagonistic activity against *S. gallinarum* ATCC 9184 were selected. They were identified as *B. subtilis* based on bacteria morphology and 16S rRNA gene sequencing followed by BLAST search against the NCBI (National Center for Biotechnology Information) database. The two strains were named *B. subtilis* C14 and *B. subtilis* RX7, respectively.

Experimental Design

Sixty 35-d-old male *Salmonella*-free Ross 308 broilers were housed in 12 battery cages (124 cm-width×64 cm-length×40 cm-height) for 7 days. These broilers were individually weighed and allocated to 4 treatments (15 birds/treatment) with 3 replicates (5 birds/cage) per treatment based on similar body weights (1608 g). Experimental diet was corn-soybean mash meal containing 3,200 kcal/kg of metabolizable energy (ME) and 20% of crude protein (CP). The different treatment groups included unchallenged control (C1), *Salmonella*-challenged group (C2; *S. gallinarum* 10^8 cfu/bird), *Salmonella*-challenged group supplemented with of *B. subtilis* C14 ($1.0×10^9$ cfu/g) at 0.1% in diet (T1) and *Salmonella*-challenged group supplemented with of *B. subtilis* RX7 ($1.0×10^9$ cfu/g) at 0.1% in diet (T2). On day 1 of the experiment, broilers in C2, T1, and T2 groups were orally challenged through crop using a syringe with plastic injection tube. Each broiler was injected with 2 m*l* suspension containing approximately 10^8 cfu of *S. gallinarum* (ATCC 9184). *S. gallinarum* ATCC 9184 is a non-flagellated, gram-negative non-spore forming rod-shaped bacteria. Clinical signs of *S. gallinarum* in chickens include anorexia, diarrhea, dehydration, reduced immunity, and decreased body weight (Christensen *et al.*, 1992; Lee *et al.*, 2013).

Growth Performance and Blood Collection

Body weight and feed intake per cage were recorded at the end of the experiment. Feed conversion was calculated based on feed intake divided by body weight gain. Blood samples of 15 broilers from each treatment were collected from jugular vein at 12 h after being challenged with *S. gallinarum*. For white blood cell (WBC), red blood cell (RBC), and lymphocyte profiling, approximately 3 mL of blood was collected into tubes containing K_3EDTA (BD Vacutainer®, Plymouth, Devon, UK). Blood was analyzed immediately after collection. To determine the levels of haptoglobin, immunoglobulin G (IgG), immunoglobulin A (IgA), immuno-

globulin M (IgM), cortisol, tumor necrosis factor-α (TNF-α), Interleukin-1beta (IL-1β), Interleukin-6 (IL-6), prostaglandin E2 (PGE$_2$), and glutathione, blood samples were placed in serum separator tubes (BD Vacutainer®) and centrifuged at 3,000 rpm for 15 min. Serum samples were stored at -80℃ until being assayed.

Haematological and Serum Parameters

Haematological parameters such as WBC, RBC, and lymphocytes were estimated using haematology analyzer (HemaVet 850; CDC Technologies, Inc., Oxford, CT, USA). Serum haptoglobin was determined using an enzyme-linked immunosorbent assay (ELISA) kit (TP801; Tri-Delta Diagnostics, Morris Plains, NJ, USA). The levels of serum immunoglobulins (IgG, IgA and IgM) were quantified using commercial ELISA assay Kits (Bethyl Laboratories, Inc., TX, USA). Serum cortisol, TNF-α, IL-6, IL-1β, and PGE$_2$ were determined using ELISA kit (Lifespan Biosciences, Seattle, WA, USA). Serum glutathione levels were measured using a commercial kit from Enzo Life Sciences (Ann Arbor, MI, USA) according to the manufacturer's instructions.

Intestinal Microbial Analysis

Fresh intestinal samples from each treatment were taken at the end of the experiment. A total of 15 broilers per treatment were slaughtered and sampled after a 12-h feed with-

Table 1. **Formulation and chemical composition of basal diet (as-fed basis)**

Ingredients	%
Corn	63.49
Soybean meal (CP 48%)	24.63
Corn gluten meal (CP 60%)	5.20
Soybean oil	3.73
Limestone	1.60
Dicalcium phosphate	0.45
L-lysine-HCl	0.10
DL-Mehionine	0.15
Salt	0.25
Vitamin premix[1]	0.20
Trace mineral premix[2]	0.20
Total	100.00
Calculated compositions, %	
ME, Kcal/kg	3,200
Crude protein	20.09
Crude fat	5.03
Lysine	1.05
Methionine	0.51
Calcium	0.93
Avail. Phosphorus	0.40

[1] Provided per kg of diet: 15,000 IU of vitamin A, 3,750 IU of vitamin D3, 37.5 mg of vitamin E, 2.55 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 pantothenic acid.

[2] Provided per kg of diet: 37.5 mg of Zn, 37.5 mg of Mn, 37.5 mg of Fe, 3.75 mg of Cu, 0.83 mg of I, 62.5 mg of S and 0.23 mg of Se.

drawal. Their large and small intestines were used to enumerate *Lactobacillus*, *E. coli* and *Salmonella*. Small intestinal digesta were collected from a 4–5 cm segment between the front and rear parts of the Meckel's diverticulum. Large intestinal digesta were collected from a 2–3 cm front part of the cloaca. To enumerate intestinal *Salmonella*, approximately 200 mg of sample was diluted 10 fold by blending them with anaerobically sterilized phosphorus buffered saline (PBS, 0.1 M, pH 7.0) and homogenized. Afterwards, a 0.1 m*l* sample was serially diluted 10^3~10^7 and spread onto sterilized flat Rogosa agar (Difco Laboratories, Detroit, MI, USA), *Salmonella-Shigella* (SS) agar (Difco Laboratories, Detroit, MI, USA), and MacConkey's agar (Difco Laboratories, Detroit, MI, USA) for *Lactobaicllus, Salmonella*, and *Escherichia coli* culture. *Lactobacillus* medium agar plates were then incubated anaerobically at 37℃ for 48 h. *Salmonella* and *E. coli* medium agar plates were incubated at 37℃ for 24 h under aerobic conditions. Colonies on each flat medium were counted using a colony counter. Results were transformed as colony-forming units (CFU) at $\log_{10}$ per gram.

Statistical Analysis

The replicate was the experimental unit for growth performance. Each broiler was the experimental unit for blood profiles and intestinal microbiota counts. Tukey's test was performed to detect the significance of differences among groups using the general linear model of SAS. Orthogonal contrasts were used to test the overall effect of 2 *B. subtilis* supplementation (C1 vs. T1+T2 and C2 vs. T1+T2) and the individual effect of *Salmonella* challenge (C1 vs. C2). Variability in data was expressed as pooled SEM. Statistical significance was considered when *p* value was less than 0.05.

Results

Growth Performance

During the 7-day trial, body weight, weight gain, and feed intake were decreased by *S. gallinarum* challenge (C1 vs. C2; $P<0.001$) while feed conversion was increased in the control (Table 2). However, *B. subtilis* C14 and RX7 treatments significantly increased body weight, weight gain, feed intake, and feed conversion of broilers after the *S. gallinarum* challenge (C2 vs. T1+T2; $P<0.001$).

Blood Parameters

No significant difference was observed on WBC, RBC, lymphocyte, haptoglobin, IgG, IgM, cortisol, IL-1β, IL-6, PGE2, or glutathione among all treatments (Table 3). Serum IgA concentration was significantly ($P=0.001$) higher in broilers fed with *B. subtilis* compared to broilers fed with C1 or C2 diet. Challenge with *S. gallinarum* significantly ($P=0.001$) increased the concentration of TNF-α compared to the control. However, the concentration of TNF-α was significantly ($P=0.040$) decreased in challenged groups treated with *B. subtilis* compared to the C2 group.

Intestinal Microflora

Broilers challenged with *S. gallinarum* had significantly lower number of *Lactobacillus* but higher numbers of *E. coli* and *Salmonella* in the small and large intestines compared to the control group (C1 vs. C2; $P<0.001$, $P<0.001$ and $P<0.001$, respectively; Table 4). However, *B. subtilis* supplementation increased the number of *Lactobacillus* in the small and large intestines compared to C2 treatment ($P=0.009$ and $P=0.003$, respectively). Significantly decreased the number of *Salmonella* in the small and large intestines was observed in *B. subtilis* supplementation treatments as compared to C2 treatment ($P<0.001$). However, no significant differences in the number of *E. coli* in the small and large intestines were determined as compared to C2 treatment ($P>0.05$).

Discussion

The primary purpose of this study was to determine the effect of *B. subtilis* (C14 and RX7) as potential inhibitors for *Salmonella* infection. We found that they were effective in inhibiting the growth of *S. gallinarum* through agar well diffusion inhibition assay (data not shown). We then performed *in vivo* broiler experiment followed by experimental challenge/infection.

Salmonellosis can lead to depression, diarrhea, severe body weight loss, and mortality of poultry (Vandeplas *et al*.,

Table 2. **The effect of *B. subtilis* C14 and RX7 on growth performance in broilers challenged with *S. gallinarum*[1]**

| Item | C1 | C2 | T1 | T2 | SEM[2] | P-values | | |
						C1 vs. C2	C1 vs. T1+T2	C2 vs. T1+T2
Body weight								
before inoculation	1610	1605	1609	1607	3.73	0.701	0.913	0.834
after inoculation	2124[a]	1737[c]	1823[b]	1834[b]	11.16	<.001	<.001	<.001
Weight gain, g	514[a]	132[c]	214[b]	227[b]	10.46	<.001	<.001	<.001
Feed intake, g	958[a]	372[c]	564[b]	578[b]	13.25	<.001	<.001	<.001
Feed conversion	1.864[c]	2.819[a]	2.636[b]	2.546[b]	0.06	<.001	<.001	<.001

[1] C1, (No *S. gallinarum*, No *B. subtilis*); C2, C1+*S. gallinarum*; T1, C2+0.1% *B. subtilis* C14; T2, C2+0.1% *B. subtilis* RX7.
[2] Standard error of means.
Values represent least squares means of 3 replicate cages containing 5 birds per cage.
[a, b] Means in the same row with different superscripts differ significantly ($P<0.05$).

Table 3. The effect of *B. subtilis* C14 and RX7 on blood profiles in broilers challenged with *S. gallinarum*[1,2]

Item	C1	C2	T1	T2	SEM[3]	C1 vs. C2	C1 vs. T1+T2	C2 vs. T1+T2
						P-values		
WBC, $10^3/\mu l$	476.4	530.6	495.1	467.3	18.09	0.325	0.919	0.301
RBC, $10^6/\mu l$	2.46	2.64	2.71	2.69	0.04	0.074	0.080	0.479
Lymphocyte, %	59.0	66.2	63.5	64.6	3.76	0.504	0.164	0.135
Haptoglobin, g/dL	5.75	6.17	5.75	4.75	0.34	0.656	0.537	0.262
IgG, mg/dL	33.4	36.2	35.6	34.8	0.60	0.186	0.545	0.440
IgA, mg/dL	32.4[b]	36.1[b]	45.3[a]	46.0[a]	1.29	0.211	0.001	0.001
IgM, mg/dL	6.8	6.4	6.1	6.2	0.24	0.542	0.236	0.622
Cortisol, ug/dL	0.20	0.18	0.19	0.21	0.01	0.313	0.949	0.221
TNF-α, pg/mL	90.6[c]	185.7[a]	151.7[b]	138.2[b]	10.44	0.001	0.023	0.040
IL-1β, pg/mL	44.8	67.5	60.6	78.7	7.97	0.331	0.223	0.917
IL-6, pg/mL	186.2	207.6	211.5	212.2	5.71	0.207	0.083	0.766
PGE2, pg/mL	153.9	131.0	140.8	151.8	6.55	0.226	0.638	0.349
Glutathione, uM	8.07	7.69	9.52	8.68	0.29	0.641	0.147	0.051

[1] C1, (No *S. gallinarum*, No *B. subtilis*); C2, C1+*S. gallinarum*; T1, C2+0.1% *B. subtilis* C14; T2, C2+0.1% *B. subtilis* RX7.
[2] WBC, White blood cells; RBC, Red blood cells; IgG, Immunoglobulin G; IgA, Immunoglobulin A; IgM, Immunoglobulin M; TNF-α, tumor necrosis factor-α; IL-1β, Interleukin-1beta; IL-6, Interleukin-6; PGE$_2$, Prostaglandin E2.
[3] Standard error of means.
Values represent means of 15 birds per treatment.
[a,b] Means in the same row with different superscripts differ significantly ($P<0.05$).

Table 4. The effect of *B. subtilis* C14 and RX7 on intestinal microflora populations in broilers challenged with *S. gallinarum*[1]

Item, $\log_{10}$ cfu/g	C1	C2	T1	T2	SEM[2]	C1 vs. C2	C1 vs. T1+T2	C2 vs. T1+T2
						P-values		
Small intestine								
Lactobacillus	7.57[a]	7.02[c]	7.41[b]	7.38[b]	0.02	<.001	<.001	0.009
E. coli	5.86[b]	6.18[a]	6.03[ab]	6.01[ab]	0.02	<.001	0.172	0.252
Salmonella	2.43[c]	3.79[a]	3.41[b]	3.39[b]	0.08	<.001	<.001	<.001
Large intestine								
Lactobacillus	7.46[a]	6.89[c]	7.31[b]	7.32[b]	0.02	<.001	<.001	0.003
E. coli	5.75[b]	6.24[a]	6.08[ab]	6.05[ab]	0.02	<.001	0.098	0.146
Salmonella	2.59[c]	3.79[a]	3.45[b]	3.51[b]	0.07	<.001	<.001	<.001

[1] C1, (No *S. gallinarum*, No *B. subtilis*); C2, C1+*S. gallinarum*; T1, C2+0.1% *B. subtilis* C14; T2, C2+0.1% *B. subtilis* RX7.
[2] Standard error of means.
Values represent means of 15 birds per treatment.
[a,b,c] Means in the same row with different superscripts differ significantly ($P<0.05$).

2009; Marcq *et al*., 2011). Infection with *S. gallinarum* decreased the weight gain compared to unchallenged broilers in this study. The decrease in weight gain might be mainly due to decreased feed intake. Our results was in consistent with previous reports on reduced weight gain and feed intake in poultry challenged with *S. gallinarum* (Gupta *et al*., 2005; Vandeplas *et al*., 2009; Marcq *et al*., 2011). Our results also demonstrated that the addition of *B. subtilis* into diets could enhance the growth performance of broilers challenged with *S. gallinarum*. Gil de los Santos *et al*. (2005) and Vila *et al*. (2009) also observed increased weight gain and feed conversion of broiler challenged with *Salmonella* following

supplemented with *B. subtilis* in the diet. In the present study, decreased body weight, feed intake, and feed conversion occurred after oral challenge with *S. gallinarum*. However, dietary *B. subtilis* mitigated these performance parameters resulting from *S. gallinarum* infection. We hypothesized that *B. subtilis* in the diet might improve the balance of intestinal microflora and increase the absorption of diet, thereby improving the growth performance of broilers.

Immune responses generated by endotoxins from invasive pathogenic strains are determined by a number of complex interactions, including factors such as immune cell interac-

tions with bacteria and their products. Serum immunoglobulin levels have been determined routinely in clinical practice because they provide key information on humoral immune status of hosts. Infected chickens can produce three principal classes of antibodies: IgG, IgA, and IgM (Ayaz *et al*., 2008). Cytokines are also important in host defense, inflammatory response, and immune-mediated diseases. Of them, TNF-α is one multifunctional cytokine that plays a major role in regulating immune responses and acute phase reactions (Akira *et al*., 1990). In several studies on chicken challenged with *Salmonella*, improvement of humoral immune response to *Salmonella* infections in serum (IgG, IgM, and IgA) has been described (Pasetti *et al*., 2011). It has been suggested that *S. gallinarum* exposure could elevate IgA levels and reflect the activation of immune system in protecting chicks against pathogen colonization. However, no significant change in serum IgA levels during the experimental period was observed in challenged groups compared to unchallenged groups in this study. The levels of IgA were higher in *B. subtilis*-fed groups compared to C1 or C2. Currently there is a lack of useful information regarding the role of *B. subtilis* in *S. gallinarum* infection. However, the response of broilers in T1 and T2 groups was similar to that described by Revolledo *et al*. (2009), who demonstrated that a mixture of *Lactobacillus*, *Enterococcus*, and *Bifidobacteria* could activate the levels of total IgA in the intestinal fluid after infection with *S. typhimurium*. Lee *et al*. (2010) have also reported that dietary *B. subtilis*-based probiotics could reduce the clinical symptoms of coccidiosis and enhance immunity of broilers against challenges with *Eimeria maxima*. In addition, De Simone *et al*. (1993) and Miettinen *et al*. (1996) has reported that the addition of probiotics can increase immune cell proliferation and diminish the production of proinflammatory cytokines such as TNF-α and IL-6. In our study, the levels of IL-1β and IL-6 in broilers fed with *B. subtilis* were not changed. However, increased TNF-α level was observed in challenged broilers, and *B. subtilis* treatment might have led to decreased TNF-α in broilers challenged with *S. gallinarum*. Therefore, dietary *B. subtilis* might be able to enhance immune response and disease resistance of broilers against challenges with *S. gallinarum* by modulating the production of IgA and TNF-α.

It has been reported that *Salmonella* challenge can cause higher *Salmonella* colonization in cecal or fecal contents (Bohez *et al*., 2008; Borsoi *et al*., 2011) but decrease *Lactobacillus* colonies (Audisio *et al*., 2000). In agreement with these results, our study also revealed that *Salmonella* colonies were increased in the small and large intestines of *Salmonella*-challenged broilers whereas the *Lactobacillus* population was significantly decreased. The addition of *B. subtilis* to the diet decreased the *Salmonella* population, suggesting that *B. subtilis* could regulate the intestinal microflora of broilers after *Salmonella* infection. Our results were in agreement with the findings of other studies that resistance to pathogens and bacteria is found in broilers supplemented with *B. subtilis* or other probiotics (La Ragione *et al*., 2003; Higgins *et al*., 2007; Mountzouris *et al*., 2007; Park and

Kim, 2015). In other words, beneficial effects of probiotics seem to be exerted by suppressing the growth of enteric pathogenic bacteria while favoring the growth of beneficial bacterial species, resulting in improvement in intestinal tract health. Therefore, effect of probiotics such as improved intestinal environment and modulation of enteric immune responses might influence the proliferation of *Salmonella*. In addition, *Bacillus subtilis* C14 and RX7 strains could act as antibacterial agent against *S. gallinarum*, thus inhibiting *Salmonella* growth and improving the intestinal health.

In summary, our results suggested that dietary *B. subtilis* C14 and *B. subtilis* RX7 strains could improve the growth performance and immune function of broilers under *S. gallinarum* challenge situation. In addition, these two *B. subtilis* strains were effective in reducing intestinal *Salmonella* population while increasing *Lactobacillus* population after challenge with *S. gallinarum*. Therefore, *B. subtilis* C14 and *B. subtilis* RX7 strains might be able to alleviate the stress of broilers infected by *S. gallinarum*. They might be considered as viable alternatives of antibiotics for broiler diets.

Acknowledgment

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