

Dietary *Bacillus subtilis* C-3102 Supplementation Enhances the Exclusion of *Salmonella enterica* from Chickens

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Among the reported probiotic *Bacillus* strains, *B. subtilis* C-3102 has the unique potential to improve feed uptake under stress conditions in the broilers, piglets, and cows. In this study, we sought to evaluate the protective effect of feed additive probiotic *Bacillus subtilis* C-3102 against *Salmonella enterica* infection of specific pathogen-free (SPF) chicks in floor pens in two experiments. In the experiment-1, the chicks in the control group ($n=32$) were fed a basal diet and those in the C-3102 group ($n=32$) were fed a basal diet supplemented with 1×10^6 CFU/g of feed for 28 days. On day 7 post-challenge with *S. enterica*, there was no significant change in the body weight between both the groups throughout the test period, whereas detection rates of *S. enterica* in the C-3102 group were significantly lower in the cecum and liver on days 21 and 14 post-challenge, respectively. In the experiment-2, minimum dosage of C-3102 cells required to protect *Salmonella* infection was evaluated using 3 dosages. Chicks were divided into four groups, fed with different dosages of C-3102 (1×10^6 , 5×10^5 , 3×10^5 , and 0 CFU/g of feed), and challenged with *S. enterica* (2.8×10^8 CFU/chicken). *S. enterica* infection was completed within 7 days post-challenge and was almost excluded from the liver and spleen on day 21 post-challenge in the control group. Average values showed a trend for higher infection rates in the control group $>3 \times 10^5 > 5 \times 10^5 > 1 \times 10^6$ CFU/g on days 14 and 21 post-challenge. These results suggest that *B. subtilis* C-3102 supplementation has the potential to reduce *S. enterica* infection rates and/or to accelerate the exclusion of *S. enterica* from the chicks.

Key words: chicken, detection rate, probiotic *Bacillus subtilis* C-3102, *Salmonella enterica* infection, specific pathogen-free (SPF)

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Introduction

Prevention of pathogenic bacterial infection is the most important challenge for chicken production in the broiler industry. *Salmonella* species are often associated with poultry salmonellosis, which results in an acute inflammation of the intestines, severe morbidity, and mortality in the poultry (Foley *et al.*, 2011; Leeson, 2012). Most *Salmonella* serovars are considered to be transferable from broilers to other livestock animals, resulting in the development of food-borne diseases and diarrhea in humans (Matulova *et al.*, 2013; Videnska *et al.*, 2013). Poultry-derived products can

be contaminated with many serovars of *Salmonella enterica*, including *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella pullorum*, and *Salmonella gallinarum* (Leeson, 2012). The serovar *S. gallinarum* has a restricted host range and is usually associated only with the poultry; however, it can cause significant losses in the profit and low production yields. Under unhygienic conditions, poultry is susceptible to upper respiratory tract infections and gastrointestinal (GI) diseases, such as diarrhea.

Probiotics can enhance the immune system and protect against the pathogenic bacterial infections (Fang *et al.*, 2000; Corthesy *et al.*, 2007). Probiotics are live microorganisms that confer a wide range of benefits to the animals, such as stimulation of immune responses, maintenance of gut barrier function, and prevention of pathogen invasion into gut tissues (Leeson, 2012; Yeoman *et al.*, 2012). Accordingly, they were found to be suitable for the chickens for improving mucosal and general immunity (Cox *et al.*, 2010; Gleeson *et al.*, 2012). Gram-positive probiotic *Lactobacillus* and *Bacillus* strains have been used for immunostimulation and prevention of *Salmonella* infection in the broiler chickens (Park

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and Kim, 2015; Oh *et al.*, 2017; Nakphaichit *et al.*, 2018; Liu *et al.*, 2018; Zhen *et al.*, 2018). Among the reported probiotic *Bacillus* strains, *B. subtilis* C-3102 is used in the commercial product Calsporin[®], which has the unique potential to improve feed uptake under stress conditions in the broilers, piglets, and cows (Silley, 2006). Enhancement of the eggshell quality was achieved by *B. subtilis* C-3102 supplementation (Nishiyama *et al.*, 2020). Possible reasons for the probiotic effects of C-3102 are considered to be the protected gut barrier function by increasing bifidobacteria and lactic acid bacteria and preventive effect against the pathogenic bacterial infections (Maruta *et al.*, 1996; Hooge *et al.*, 2004; Jeong and Kim, 2014). Recent clinical studies with *B. subtilis* C-3102 spores revealed an increase in the bone mineral density in the postmenopausal women by inhibiting bone resorption (Takimoto *et al.*, 2018), and improved stool frequency (Hatanaka *et al.*, 2018) due to control of intestinal microbiota and increased germination in the gut (Hatanaka *et al.*, 2012).

In this study, the anti-pathogenic effect of *B. subtilis* C-3102 on chicks was evaluated, with the expected enhancement of immunostimulation and gut barrier function. For the evaluation of a preventive effect of C-3102 against *S. enterica*, the specific pathogen-free (SPF) chicks were challenged with *Salmonella* to eliminate the possibility of different microbial backgrounds interfering with the experiment.

Materials and Methods

Management of Birds and Diet

Hatching SPF eggs (vaccination-free) were obtained from a vaccine company (Nisseiken Co. Ltd., Japan) and hatched in an incubator P-05 (Showa Furanki Co. Ltd., Japan). All the animal studies were conducted from November 2016 to August 2017. In experiment-1, total 64 0-d-old SPF chicks (Lohmann valo, Germany) were randomly divided into two groups on day 0 (Fig. 1). The chicks in the control group ($n=32$) were fed a basal diet (Table 1) and those in the C-3102 group ($n=32$) were fed a basal diet supplemented with 1×10^6 CFU/g feed of *B. subtilis* C-3102 (commercially available as Calsporin[®]) (Silley, 2006) for 28 d. In experiment-2, total 120 0-d-old SPF chicks (Lohmann valo, Germany) were randomly divided into five groups (24 chicks each) on day 0 (Fig. 1), and the trials lasted for 28 d. The infection control and untreated groups were fed a basal diet (Table 1; Control and Normal, respectively). The third group was supplemented with a low dosage of C-3102 in the basal diet (3×10^5 CFU/g) (Low), the fourth group with a medium dosage (5×10^5 CFU/g) (Mid), and the fifth group with a high dosage (1×10^6 CFU/g) (High). All the chicks were housed in separate isolators of identical size (1.00 m \times 0.75 m) for each treatment group, and allowed *ad libitum* access to water and feed. The temperature at hatching was 32°C, which was reduced to 25°C until the end of the trial. All the

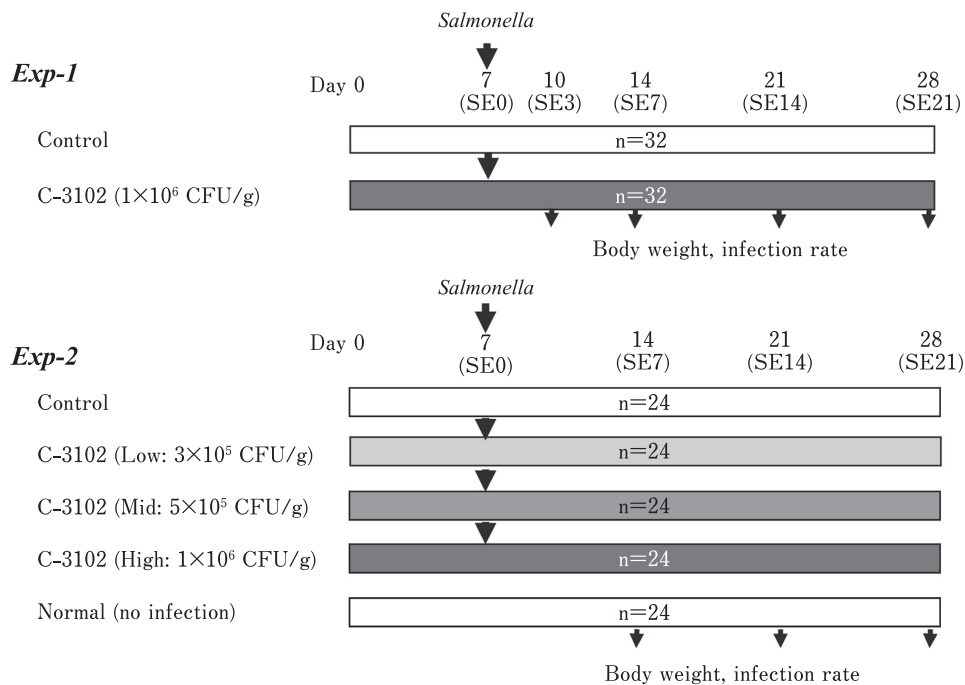


Fig. 1. Treatment schedule for the challenge study with *Salmonella enterica* on the specific pathogen-free chicks in experiment-1 and -2. After feeding the *Bacillus subtilis* C-3102-containing feeds on Day 0, *S. enterica* was administered to the chicks on day 7 (SE0). Body weights, infection rates, and histology of the liver, spleen, and cecum were subsequently analyzed according to the scheme.

Table 1. Nutrient composition of the basal diet used in the present experiment

Ingredient (%)	Composition
Corn	67.00
Soybean meal	22.00
Fish meal	5.00
Other ¹	6.00
Analyses	
Metabolisable energy (kcal/kg)	2,850
Crude protein (%)	19.00
Crude fat (%)	2.50
Crude fiber (%)	5.00
Crude ash (%)	8.00
Calcium (%)	0.70
Phosphorus, available (%)	0.55

¹Other ingredients were composed of Alfalfa meal, Calcium carbonate, Salt, Vitamin premix, Mineral premix and Methionine. Vitamine premix: vitamine A, vitamine D₃, vitamine E, vitamine K₃, vitamine B₁, vitamine B₂, vitamine B₆, vitamine B₁₂, nicotinic acid, d-pantothenic acid, choline chloride and biotin. Mineral premix: manganese sulfate, zinc carbonate, iron sulfate, copper sulphate, cobalt sulfate and calcium iodate.

chicks were exposed to 12 h cycles of light and dark. The experiments were approved by the animal welfare committee of Shoku-kan-ken, Inc.

Salmonella Infection

For the evaluation of a preventive effect of C-3102 against *S. enterica*, the specific pathogen-free (SPF) chicks were administrated with *Salmonella* by a single dosage study with a diet containing 1×10^6 CFU/g of diet (Experiment-1). Then, a study with three different dosages of C-3102 was conducted on SPF chicks for 28 d (Experiment-2). *Salmonella enterica* serovar *enteritidis* LM-7 (nalidixic acid-resistant) were pre-cultured on nalidixic acid agar plates at 37°C for 24 h, followed by sub-culturing in the heart infusion broth at 37°C with shaking for 5 h. To determine the concentration of *S. enterica* in the infection solution, the inoculum was diluted with PBS, plated on a nalidixic acid agar plate, and incubated at 37°C for 24 h. The colonies grown were counted as CFUs. In experiment-1, 64 SPF chicks were divided into two groups on day 0, i.e., the control group ($n=32$) and the C-3102 group ($n=32$) supplemented with 1×10^6 CFU of C-3102/g of feed. All the chickens in both the groups were challenged with *S. enterica* (1.5×10^7 CFU/chicken) on day 7, and body weight and detection rate of *S. enterica* in the cecum, liver, and spleen were measured on days 3, 7, 14, and 21 post-challenge (Fig. 1). In experiment-2, 120 chicks (SPF) were divided into five groups (24 chicks/group) on day 0, and chicks of four groups were fed different dosages of C-3102 (1×10^6 : High, 5×10^5 : Med, 3×10^5 : Low, and 0: Control, CFU/g of feed). On day 7, chickens in the C-3102 groups were challenged with *S. enterica* (2.8×10^8 CFU/chicken) by an oral gavage; however, the fifth group was not treated with *S. enterica* (Normal group) (Fig. 1).

Body Weight Measurement and Sample Collection

On days 3, 7, 14, and 21 of experiment-1 and on days 7, 14, and 21 of experiment-2 post-challenge of *S. enterica* (SE3, SE7, and SE14 in experiment-1, SE7, SE14, and SE21 in experiment-2), eight chicks per group were randomly selected, and samples (liver, spleen, and cecum content) were isolated for *Salmonella* analysis. All the collected samples were stored at 4°C and *Salmonella* count was analyzed within the day of sampling. All the chicks were weighed on day 0 and 7, and the eight selected chicks were weighed on days 3, 7, 14, and 21 in experiment-1, and on days 7, 14, and 21 in experiment-2 post-challenge.

Salmonella Measurement

Intestinal contents were collected from the cecum, serially diluted with PBS, and plated on a nalidixic acid agar plate. Then, the number of viable *Salmonella* cells on the agar plate was counted after incubation at 37°C for 24 h, followed by calculation of CFU/g. Residual cecum contents were diluted 10 times in nalidixic acid-containing broth, incubated at 37°C for 24 h, and subsequently inoculated in Hajna tetrathionate broth, followed by incubation at 42°C for 24 h. For *S. enterica* detection, each culture was plated on a nalidixic acid agar plate, and incubated at 37°C for 24 h. The collected liver and spleen tissues were chopped, diluted 10 times in nalidixic acid-containing broth, and homogenized by a stomacher (Seward, UK). Then, the homogenized samples were incubated at 37°C for 24 h, followed by inoculation and incubation in Hajna tetrathionate broth at 42°C for 24 h. For *S. enterica* detection, each culture was plated on a nalidixic acid agar plate and incubated at 37°C for 24 h. The detection rate of *S. enterica* was calculated as [the number of positive chick/8 chicks].

Statistical Analysis

In experiment-1, Student's *t*-test (equal variance) or Welch's test (unequal variance) was performed for the significant effects of *B. subtilis* C-3102 on body weight. In experiment-2, analysis of variance and Tukey's test were performed for the significant effects of *B. subtilis* C-3102 on body weight. Chi-square test was used for the effects on *S. enterica*-positive ratios in both the experiments. An alpha (α) level of 0.05 was used as threshold for statistical significance, and a P-value of 0.10 was considered to represent a trend. All the analyses were conducted using the statistical software "Statistix 10" (Analytical Software, USA).

Results

General Observations

To eliminate the influence of microbial background on the *S. enterica* infection, SPF chickens in floor pens were used to evaluate the protective effect of probiotic *B. subtilis* C-3102 against *S. enterica* infection. There were no significant differences in feed intake, performance, and fecal appearance between SPF chickens of the C-3102 and control groups throughout the experimental period (data not shown).

Experiment-1

In experiment-1, 64 SPF chicks were randomly divided into two groups on day 0, i.e., the control group ($n=32$) and

Table 2. Change of body weight of broiler chickens after challenging of *Salmonella enteritidis* in the 1st experiment

	Body weight (g)		P-value
	Control	C-3102	
Day 0	39.2±2.1	39.2±2.4	0.98
Day 7	58.2±3.4	60.2±6.0	0.11
Day 3 after challenge	75.2±4.0	77.0±7.2	0.55
Day 7 after challenge	111.2±7.5	113.3±7.2	0.59
Day 14 after challenge	187±17	194±17	0.47
Day 21 after challenge	257±24	266±31	0.51

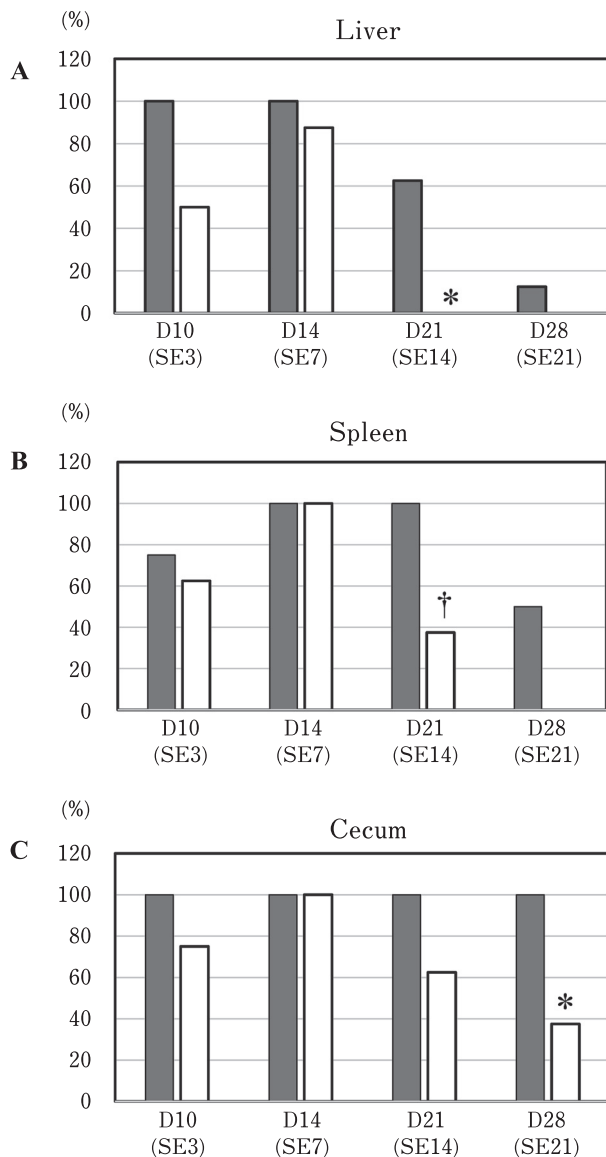


Fig. 2. Changes in the infection rates in the liver (A), spleen (B), and cecum (C) of specific pathogen-free chicks at different time points after challenging ($n=32$ /group) with *Salmonella enterica* on day 7. The experimental diet was either a basal diet (■) or a basal diet containing *Bacillus subtilis* C-3102 (□). Significant differences between the control and C-3102 groups are indicated as † $P<0.10$ and * $P<0.05$.

the C-3102 group ($n=32$) supplemented with 1×10^6 CFU of C-3102/g of the feed (Table 1). All the chickens in both the groups were challenged with *S. enterica* (1.5×10^7 CFU/chicken) on day 7, and body weight and detection rate of *S. enterica* in the cecum, liver, and spleen were measured on days 3, 7, 14, and 21 post-challenge (Fig. 1). There was no significant difference in body weight between the control and C-3102 groups during the test period (Table 2). After challenging with *S. enterica*, high rates of infection were observed in the liver, spleen, and cecum of all the chickens in the control group on days 3, 7, 14, and 21 post-challenge (Fig. 2). These infection rates decreased to 12.5% in the liver and 50% in the spleen on day 21 post-challenge in the control group (Fig. 2). However, no infection in the liver was observed in the C-3102 group on day 14 post-challenge, and the infection rate was significantly lower than that of the control group. In the spleen, the infection rate on day 14 post-challenge showed a lower tendency than that of the control group. In the cecum, the infection rate observed in the C-3102 group was significantly lower than that of the control group on day 21 post-challenge. These results indicated that *S. enterica* was excluded from the liver within 3 weeks of infection, but these beneficial effects were delayed in the spleen and cecum. These results also revealed that the treatment with C-3102 might accelerate the exclusion of *S. enterica* from the liver, spleen, and cecum after an infection.

Experiment-2

In experiment-2, 120 SPF chicks were divided into five groups (24 chicks/group) on day 0, and chicks of four groups were fed different dosages of C-3102 (1×10^6 : High, 5×10^5 : Med, 3×10^5 : Low, and 0: Control, CFU/g of feed). On day 7, chickens in the C-3102 groups were challenged with *S. enterica* (2.8×10^8 CFU/chicken), while the fifth group was kept untreated (Normal group) (Fig. 1). Interestingly, the C-3102 (High) group showed significant increases in the body weight compared to the control group on day 7 post-chal-

Table 3. Change of body weight of broiler chicken after challenging of *Salmonella enteritidis* in the 2nd experiment

	Body weight (g)				
	Control	C-3102 (Low)	C-3102 (Med)	C-3102 (High)	Normal
Day 0	42.36±2.5	42.3±2.7	42.4±2.6	42.4±2.6	42.4±2.5
Day 7	59.33±4.9	61.2±6.1	59.5±4.6	60.9±4.5	60.2±4.5
Day 7 after challenge	108.42±4.6 ^b	113.0±3.5 ^{ab}	113.5±3.7 ^{ab}	114.4±2.7 ^a	110.0±4.9 ^{ab}
Day 14 after challenge	197.04±17	205±15	206.6±5.5	210.3±7.9	208.3±7.9
Day 21 after challenge	284.06±20	292±17	288±20	293±16	294±20

^{a,b} Within the same column, means significant difference with different superscripts ($P < 0.05$).

challenge (Table 3).

In experiment-2, *S. enterica* was detected in all the samples isolated from liver, spleen, and cecum from *S. enterica*-challenged groups on day 7 post-challenge (Fig. 3). On day 14 post-challenge, infection rates of *S. enterica* were significantly decreased in the liver. While the infection rate in the control group was 62.5%, *S. enterica* was completely excluded from the liver in the group treated with the highest dosage of C-3102 (1×10^6 CFU/g feed). The liver infection rate in the C-3102 (Med) group was insignificantly different from the control group on day 14 post-challenge, with the average rate of 50%. In the spleen, no significant differences were detected among all the groups in the infection rates on days 7, 14, and 21 post-challenge. However, averaged values showed a trend for higher infection rates in the control group (without C-3102 treatment) > Low group > Mid group > High group on days 14 and 21 post-challenge. In the cecum samples, a trend was observed for faster exclusion of *S. enterica* from the liver than from the cecum. Considering the above observations, *S. enterica* infection was completed within 7 days post-challenge, and was almost excluded from the liver and spleen on day 21 post-challenge in the control group. In contrast, supplementation of C-3102 was associated with shorter times for the exclusion of *S. enterica* from the chickens, in a dose dependent manner.

Discussion

In this study, the anti-pathogenic effect of the spores formed by *Bacillus subtilis* C-3102 on *Salmonella*-challenged SPF chicks was examined. As a result, pre-treatment with C-3102 for 7 days induced increased shedding of *S. enterica* from the SPF chicks.

S. enterica detected in all the chickens 7 days post-challenge tended to be excluded within 3 weeks, probably due to the basic host immune response. However, shedding of *S. enterica* from the cecum, liver, and spleen was accelerated by pre-treatment with C-3102 (Figs. 2 and 3). Upon evaluating the treatment with probiotic *Bacillus* strains against *Salmonella* infection, supplementation of *B. subtilis* DSM17299 to the broiler chickens showed a reduction in the *Salmonella* counts in the cecum compared to that in the control group (Knarreborg *et al.*, 2008). *Salmonella* infection in the broilers fed with *B. subtilis* DSM17299 diet was reduced to 58% compared to that in the positive control birds

(100% infection), where a decrease in the intestinal pH by an increase of lactic acid bacteria provided by DSM17299 was a suggested reason (Knap *et al.*, 2011). In our study, no change in the gut microflora was detected; however, an increase in the lactic acid bacteria and a decrease in the fecal pH in the broilers supplementation with C-3102 were observed in the previous study (Khaksefidi and Ghoorchi, 2006). Exclusion of *S. enteritidis* and *Clostridium perfringens* by the *B. subtilis* supplementation was considered to be caused by a change in the microflora composition and microbial competition (Videnska *et al.*, 2013). Reduced lateral spread and improved shedding of *S. enterica* from the chicks by pre-treatment with *B. subtilis* PY79hr was also explained by competitive infection of *S. enterica* in the GI tract (La Ragione and Woodward, 2003). The idea that chicks pre-treated with the probiotics are potentially protected against pathogenic bacterial infections is an important concept to utilize the probiotic strains as feed additive ingredient.

For *Salmonella* infection in various tissues, the disruption of tight junctions between the epithelial cells in the GI tract may be a crucial event in the translocation and invasion of pathogenic bacteria into the blood stream. In the birds, the barrier function of the intestinal epithelium is considered to be modulated by the commensal microbiota (Van Deun *et al.*, 2008; McCarville *et al.*, 2016). Orally administered *Bacillus licheniformis* and *Bacillus flexus* spores induced the germinal centers of Peyer's patches (Xin *et al.*, 2012). In another study, birds challenged with *B. subtilis* showed decreased crypt depth and increased villus height relative to control and *Escherichia coli*-challenged broilers (Manafi *et al.*, 2017). Further, C-3102 supplementation showed a reduction in the bacterial translocation and protective gut barrier function in mice (Marubashi *et al.*, 2001). In this study, *S. enterica* was detected in the cecum, liver, and spleen on day 3 post-challenge. This may have resulted from the damaged gut barrier function by *S. enterica* infection, its frequent invasion into the blood stream, and its delivery to the spleen and liver through the circulation. To understand the relationship between barrier function and *Salmonella* infection, histological analysis of the chicken guts will be addressed in the future studies.

Another possible explanation for reduced *S. enterica* counts in the SPF chickens upon C-3102 treatment, as observed in this study, is an immunostimulatory effect. In a study of

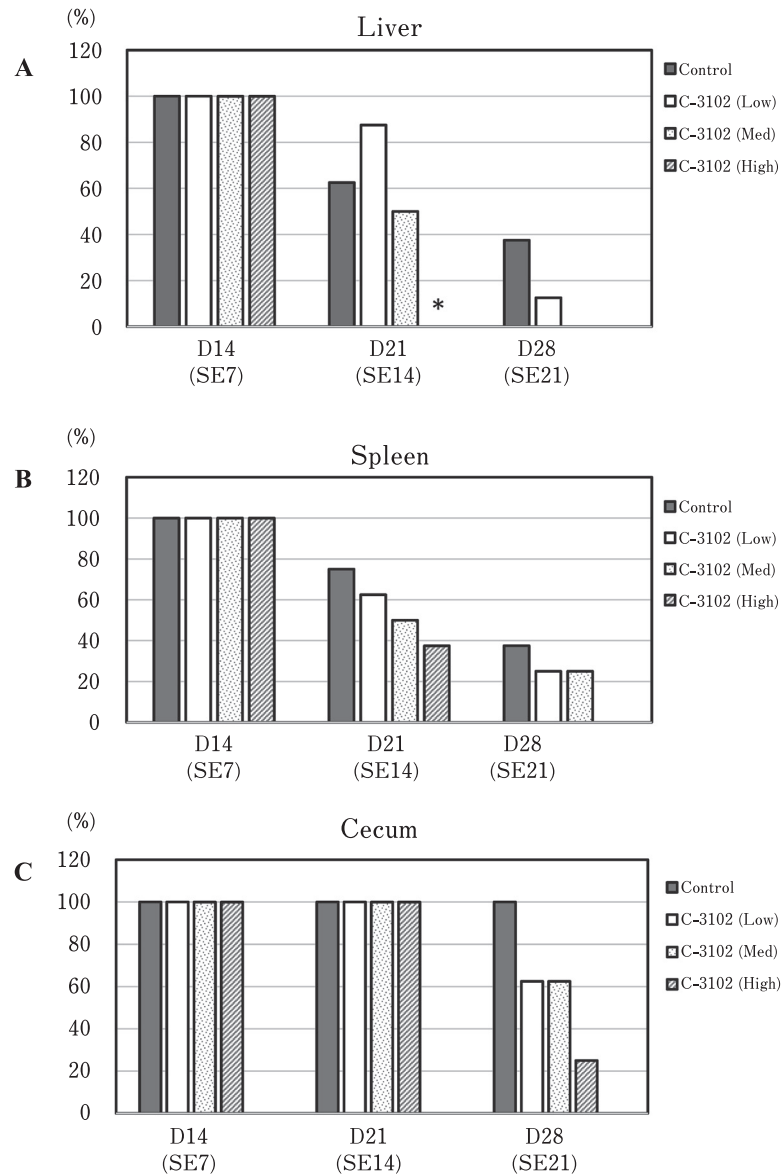


Fig. 3. Changes in the infection rates in the liver (A), spleen (B), and cecum (C) of specific pathogen-free chicks at different time points after challenging ($n=24/\text{group}$) with *Salmonella enterica* on day 7. The experimental diet was either a basal diet or a basal diet containing low-dosage (3×10^5 CFU/g of diet), medium-dosage (5×10^5 CFU/g of diet), or high-dosage (1×10^6 CFU/g of diet) of C-3102. Significant differences between the control and C-3102 groups are indicated as * $P < 0.05$.

laying breeders, the concentration of IgM and the level of influenza virus titer in the serum increased by dietary supplementation of *B. subtilis* C-3102 (Liu *et al.*, 2019). Another study showed a stimulation of antigen presenting cells and T lymphocytes, which were markedly enhanced by a challenge with *Bacillus* species owing to enhanced expression of toll-like receptor (TLR) 2 and 4 genes (Xin *et al.*, 2012). Pro-inflammatory cytokines, such as TNF- α , IFN- γ , IL-1 β , IL-6, and IL-12 were induced by *B. subtilis* B4 treatment (Xi *et al.*,

2012) through the stimulation of lipoteichoic acids (Opitz *et al.*, 2001). However, the impact of C-3102 on the chicken immune system and the subsequent reduction of *S. enterica* infection in this study are unclear.

In conclusion, *B. subtilis* C-3102 supplementation over 3 weeks accelerates the exclusion of *S. enterica* from the cecum, liver, and spleen of the chickens in a dose-dependent manner. Further studies are needed to investigate the relationship between gut barrier function and gut immune re-

sponses against *Salmonella* infection during C-3102-induced protection.

Conflicts of Interest

All authors declare no conflict of interest in this study.

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