# Effects of Recombinant Bacillus subtilis SE1 on Growth Performance, Intestinal Digestive Enzyme Activity, and Microbiota in Broiler Chickens: Postprint

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## Abstract

This experiment aimed to investigate the effects of spore surface display of Salmonella pullorum OmpC recombinant Bacillus subtilis SE1 on growth performance, intestinal digestive enzyme activity, and gut microbiota in broiler chickens. A total of 120 7-day-old broiler chickens were selected and randomly divided into 3 groups, with 4 replicates per group and 10 chickens per replicate. Group A (control group) was fed a basal diet, while Groups B and C were fed the basal diet supplemented with 0.1% (1.0×10^6 CFU/g) Bacillus subtilis 168 preparation and 0.1% (1.0×10^6 CFU/g) recombinant Bacillus subtilis SE1 preparation, respectively, for an experimental period of 35 days. The results showed: 1) Compared with Group A, the final body weight and average weight gain of broiler chickens in Group C increased by 6.14% and 6.76% ($P>0.05$), respectively, and the feed conversion ratio decreased by 8.21% ($P>0.05$). 2) At 28 and 42 days of age, the jejunal lipase and ileal protease activities of broiler chickens in Groups B and C were significantly higher than those in Group A ($P<0.05$); there was no significant difference in intestinal digestive enzyme activities between Groups B and C ($P>0.05$). 3) At 28 and 42 days of age, the numbers of Escherichia coli in the ileum and cecum of broiler chickens in Groups B and C were significantly lower than those in Group A ($P<0.05$), while the cecal Lactobacillus numbers were significantly higher than those in Group A ($P<0.05$). The 16S rRNA V3 region polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) results showed that the cecal microbial richness, evenness, and Shannon-Wiener index of broiler chickens in Groups B and C were significantly higher than those in Group A ($P<0.05$); the similarity of cecal microbiota in Group C was 26.8% and 15.6% higher than that in Group A at 28 and 42 days of age, respectively, with no significant difference between

Groups C and B (P>0.05). The results indicated that recombinant Bacillus subtilis SE1 had the same effect as Bacillus subtilis 168, effectively promoting broiler growth, improving intestinal lipase and protease activities, regulating broiler gut microbiota, and enhancing the stability and diversity of intestinal microbiota.

## Full Text

## Abstract

This experiment was conducted to investigate the effects of recombinant *Bacillus subtilis* SE1 with spore surface display of *Salmonella pullorum* OmpC on growth performance, intestinal digestive enzyme activities, and microflora of broilers. One hundred and twenty 7-day-old broilers were randomly divided into three groups with four replicates per group and ten broilers per replicate. Broilers in group A (control group) were fed a basal diet, while those in groups B and C received the basal diet supplemented with 0.1% (1.0×10  CFU/g) *Bacillus subtilis* 168 and 0.1% (1.0×10  CFU/g) recombinant *Bacillus subtilis* SE1, respectively. The experiment lasted for 35 days. The results showed: 1) Compared with group A, the final weight and average gain of broilers in group C increased by 6.14% and 6.76% (P>0.05), respectively, and the feed-to-gain ratio decreased by 8.21% (P>0.05). 2) At 28 and 42 days of age, the activities of jejunal lipase and ileal protease in groups B and C were significantly higher than those in group A (P<0.05), with no significant differences between groups B and C (P>0.05). 3) At 28 and 42 days of age, the number of *Escherichia coli* in the ileum and cecum of broilers in groups B and C was significantly lower than that in group A (P<0.05), while the number of *Lactobacillus* in the cecum was significantly higher (P<0.05). Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis of the 16S rRNA V3 region revealed that the richness, evenness, and Shannon-Wiener index of cecal microflora in groups B and C were significantly higher than those in group A (P<0.05). The similarity of cecal microflora in group C was 26.8% and 15.6% higher than that in group A at 28 and 42 days of age, respectively, with no significant difference between groups B and C (P>0.05). These results indicate that recombinant *Bacillus subtilis* SE1 has the same effect as *Bacillus subtilis* 168, effectively promoting broiler growth, improving intestinal lipase and protease activities, regulating intestinal microflora, and enhancing the stability and diversity of intestinal microflora.

**Keywords:** recombinant *Bacillus subtilis*; broilers; growth performance; digestive enzyme activities; intestinal microflora

## Introduction

*Bacillus subtilis* is an internationally recognized microbial strain that can be directly used in feed. It maintains and adjusts intestinal microflora balance, enhances immunity, secretes various digestive enzymes, improves feed digestion

and utilization, and promotes animal growth [1-2]. *Bacillus subtilis* does not secrete toxins, exhibits good safety, is amenable to genetic regulation, has no codon bias, grows rapidly, and is easy to cultivate. Exogenous proteins expressed through genetic recombination can be directly secreted into the environment, making it an excellent expression system for foreign proteins. However, it also secretes proteases, which presents certain limitations as a secretory expression system. The spores of *Bacillus subtilis* offer advantages such as resistance to acid, salt, extrusion, and high temperatures, with easy purification and low production costs. Consequently, using the spore surface to display exogenous functional proteins has become a research hotspot [3-4].

Lian et al. [5] surface-displayed human growth hormone (hGH) on the spore coat of *Bacillus subtilis* and fed it to silkworms, demonstrating that the hGH protein displayed on the spore coat surface could be absorbed into the hemolymph. Zhou et al. [6] displayed *Helicobacter pylori* urease B protein on the spore coat surface of *Bacillus subtilis* and fed it to mice, observing fecal urease B-specific secretory immunoglobulin A (SIgA) and serum immunoglobulin G (IgG) production. Liu et al. [7-8] constructed recombinant *Bacillus subtilis* SE1 displaying *Salmonella pullorum* outer membrane protein OmpC on the surface and administered it orally to mice, finding that it could induce specific antibodies against *S. pullorum* and provide cross-protection against *Salmonella typhimurium* infection. However, few reports have investigated the effects of such recombinant *Bacillus subtilis* constructed through surface display technology on animal growth performance, intestinal digestive enzyme activities, and microflora. This study used white-feathered broilers as experimental subjects to investigate the effects of feeding recombinant *Bacillus subtilis* SE1 displaying *S. pullorum* outer membrane protein OmpC on growth performance, intestinal digestive enzyme activities, and microflora, and to compare its efficacy with that of *Bacillus subtilis* 168, providing an experimental basis for the application of this recombinant strain.

## Materials and Methods

### 1.1 Experimental Materials

*Bacillus subtilis* 168 preparation (viable spore count of 1.0×10  CFU/g) and recombinant *Bacillus subtilis* SE1 preparation displaying *S. pullorum* outer membrane protein OmpC (viable spore count of 1.0×10  CFU/g) were both provided by our research center.

### 1.2 Experimental Design and Diets

One hundred and twenty 7-day-old Cobb-500 broilers were randomly divided into three groups with four replicates per group and ten broilers per replicate. Group A (control group) was fed a basal diet, group B received the basal diet supplemented with 0.1% (1.0×10  CFU/g) *Bacillus subtilis* 168 preparation, and group C received the basal diet supplemented with 0.1% (1.0×10  CFU/g) re-

combinant *Bacillus subtilis* SE1 preparation. The supplementation level was determined based on preliminary experiments. The basal diet (without antibiotics) was provided by a feed factory in Pengzhou, Chengdu, and its composition and nutrient levels are shown in Table 1 .

### 1.3 Management Practices

Broilers were raised in single-layer cages with separate housing for each group, maintained under infrared lamps for warmth at an average room temperature of approximately 26°C. They had free access to feed and water, with cages cleaned regularly. Daily observations were made regarding feed and water consumption, health status, with feed consumption and morbidity/mortality recorded. The experimental period lasted 35 days. Broilers were immunized with Newcastle disease attenuated vaccine via eye drop and nasal instillation at 7 and 21 days of age.

### 1.4 Sample Collection and Measurements

**1.4.1 Growth Performance** At 42 days of age, broilers were weighed after fasting for 8 hours (feed withdrawal) and 2 hours (water withdrawal). Average weight gain and feed-to-gain ratio were calculated.

**1.4.2 Intestinal Digestive Enzyme Activities** At 28 and 42 days of age, three broilers from each replicate were sacrificed. Jejunum and ileum segments were collected, and contents were rinsed with physiological saline until no obvious contents remained (no specific volume requirement). Intestinal mucosa was carefully scraped onto ice packs, snap-frozen in liquid nitrogen, and stored at -70°C for digestive enzyme activity determination. Amylase, lipase, and protease activities were measured using assay kits from Nanjing Jiancheng Bioengineering Institute according to the manufacturer' s instructions.

**1.4.3 Intestinal Microflora Counts** One gram of ileal and cecal contents was aseptically weighed, and plate count methods were used to enumerate *Escherichia coli* and *Lactobacillus* according to reference [9].

**1.4.4 PCR-DGGE Analysis of Cecal Microflora Diversity** Two hundred milligrams of cecal contents was aseptically weighed, and PCR-DGGE was used to analyze cecal microflora diversity according to reference [9].

### 1.5 Data Processing and Analysis

Experimental data were processed using SPSS 19.0. After calculating means and standard deviations, one-way ANOVA was performed for variance analysis. Significant differences were further analyzed using Duncan's multiple comparison test, with significance level set at $P<0.05$. Data are expressed as "mean ± standard deviation."

## Results

### 2.1 Effects of Recombinant *Bacillus subtilis* SE1 on Growth Performance of Broilers

No mortality occurred during the experiment. As shown in Table 2 , compared with group A, the final weight and average gain of broilers in group C increased by 6.14% and 6.76%, respectively, but the differences were not significant (P>0.05). The feed-to-gain ratio in group C decreased by 8.21% compared with group A and by 6.28% compared with group B, but these differences were also not significant (P>0.05).

### 2.2 Effects of Recombinant *Bacillus subtilis* SE1 on Intestinal Digestive Enzyme Activities of Broilers

As shown in Table 3 , at 28 days of age, the activities of jejunal lipase and ileal protease in groups B and C were significantly higher than those in group A (P<0.05). At 42 days of age, the activities of jejunal lipase, jejunal protease, and ileal protease in groups B and C were significantly higher than those in group A (P<0.05). There were no significant differences in intestinal digestive enzyme activities between groups B and C (P>0.05).

### 2.3 Effects of Recombinant *Bacillus subtilis* SE1 on Intestinal Microflora of Broilers

As shown in Table 4 , at 28 days of age, the number of *E. coli* in the ileum and cecum of broilers in groups B and C was significantly lower than that in group A (P<0.05), while the number of *Lactobacillus* in the cecum was significantly higher (P<0.05). At 42 days of age, the number of *E. coli* in the ileum and cecum of broilers in groups B and C was significantly lower than that in group A (P<0.05), and the number of *Lactobacillus* was significantly higher (P<0.05).

### 2.4 Effects of Recombinant *Bacillus subtilis* SE1 on Cecal Microflora Diversity of Broilers

As shown in Figure 1 [Figure 1: see original paper], groups B and C exhibited more bands and higher band intensity than group A, with several specific bands appearing in groups B and C. As shown in Table 5 , the richness, evenness, and Shannon-Wiener index of cecal microflora in groups B and C were significantly higher than those in group A (P<0.05). As shown in Figure 2 [Figure 2: see original paper], at 28 days of age, the intra-group similarities of cecal microflora were 64.1%, 96.2%, and 90.9% for groups A, B, and C, respectively. The similarity between groups A and C was 64.1%, while that between groups B and C was 90.9%. At 42 days of age, the intra-group similarities were 79.1%, 94.7%, and 94.7% for groups A, B, and C, respectively. The similarity between groups A and C was 79.1%, while that between groups B and C reached 94.7%.

A total of 27 bands were selected for excision and recovery from the 16S rDNA

V3 region PCR-DGGE fingerprint of cecal microflora (indicated by arrows in Figure 1). After removing 6 duplicate bands, 12 common bands and 9 specific bands remained. Sequencing and BLAST comparison in the GenBank database revealed that these bands primarily belonged to *Lactobacillus*, *Clostridium*, *Coprococcus*, *Eubacterium*, *Ruminococcus*, *Acetanaerobacterium*, *Cellulosilyticum*, *Thiobacillus*, *Pseudomonas*, and *Serratia* (Table 6 ). Group A had a specific band for *Coprococcus eutactus*, while groups B and C had specific bands for *Clostridium methylpentosum*, *Lactobacillus acidophilus*, *Serratia liquefaciens*, *Ruminococcus albus*, *Clostridium saccharobutylicum*, *Desulfosporosinus acidiphilus*, *Thiobacillus plumbophilus*, and *Lactobacillus rogosae*.

## Discussion

### 3.1 Effects of Recombinant *Bacillus subtilis* SE1 on Growth Performance of Broilers

Zhang et al. [10] reported that *Bacillus subtilis* can increase daily weight gain and feed intake while decreasing the feed-to-gain ratio in broilers. This study demonstrated that dietary supplementation with recombinant *Bacillus subtilis* SE1 increased final weight and average gain while decreasing the feed-to-gain ratio compared with the control group. Research has also shown that *Bacillus subtilis* can improve animal digestive function, enhance feed utilization, and promote growth [11-12]. Bai et al. [13] found that feeding *Bacillus subtilis* increased daily weight gain in broilers, possibly because *Bacillus subtilis* improves intestinal digestive function by secreting proteases, amylases, lipases, and various vitamins, growth factors, and amino acids, thereby promoting nutrient absorption [14]. Liu [15] reported that oral administration of avian influenza recombinant *Bacillus subtilis* significantly enhanced systemic immune levels and markedly increased small intestinal villus height in chickens, with a superior effect on body weight compared with normal *Bacillus subtilis*. This study showed that compared with *Bacillus subtilis* 168, recombinant *Bacillus subtilis* SE1 increased final weight and average gain while decreasing the feed-to-gain ratio, though these differences were not significant. This may be because recombinant *Bacillus subtilis* SE1 enhanced immune function and improved small intestinal mucosal morphology, particularly affecting villus growth and development, thereby indirectly influencing growth performance [16]. The lack of significant differences may be attributed to the favorable rearing conditions and broiler breed used in this experiment, resulting in no significant difference between the effects of recombinant *Bacillus subtilis* SE1 and *Bacillus subtilis* 168 on growth performance. Guo [17] reported that before challenge, feeding *Salmonella pullorum* OmpC-DC recombinant *Lactobacillus* had no significant effect on chicken body weight compared with the *Lactobacillus* and control groups, consistent with our findings.

### 3.2 Effects of Recombinant *Bacillus subtilis* SE1 on Intestinal Digestive Enzyme Activities of Broilers

Animal intestinal digestive enzymes mainly include amylase, lipase, and protease, which are closely related to growth and metabolism. Zokaeifar et al. [18] fed *Bacillus subtilis* to *Litopenaeus vannamei* for 8 weeks and found that intestinal protease and amylase activities in the treatment group were significantly higher than in the control group. Bian et al. [19] reported that dietary supplementation with 10  CFU/g *Bacillus subtilis* significantly increased jejunal trypsin and amylase activities but significantly decreased lipase activity in Lingnan yellow-feathered broilers. Liu et al. [20] found that feeding *Bacillus subtilis* significantly increased protease and amylase activities in the digestive tract of eels. This study showed that feeding recombinant *Bacillus subtilis* SE1 significantly increased jejunal lipase and ileal protease activities in broilers, consistent with the above results and closely related to the characteristic of *Bacillus subtilis* to produce digestive enzymes such as proteases, lipases, and amylases [21-22]. However, intestinal amylase activity was not significantly improved, which differs slightly from previous studies. This may be because enzymes secreted by *Bacillus subtilis* inhibited endogenous amylase activity, or due to differences in experimental conditions and animal species. No significant differences in intestinal digestive enzyme activities were observed between recombinant *Bacillus subtilis* SE1 and *Bacillus subtilis* 168 groups, indicating that both can improve intestinal digestive enzyme activities and that displaying *Salmonella pullorum* outer membrane protein OmpC on the spore coat surface does not affect this function.

### 3.3 Effects of Recombinant *Bacillus subtilis* SE1 on Intestinal Microflora of Broilers

Studies have confirmed that *Bacillus subtilis* can maintain animal intestinal microecological balance and increase microflora diversity [23-25]. Pei et al. [26] found that dietary *Bacillus subtilis* supplementation significantly decreased cecal *E. coli* counts while increasing *Lactobacillus* and *Bifidobacterium* counts in laying hens. Park et al. [27] reported that feeding *Bacillus subtilis* significantly increased cecal *Lactobacillus* counts and decreased *Salmonella* counts in broilers. Gao et al. [28] demonstrated that dietary *Bacillus subtilis* supplementation significantly decreased cecal *E. coli* and *Salmonella* counts while increasing *Lactobacillus* counts in broilers. This is primarily because: 1) *Bacillus subtilis* spores rapidly germinate into vegetative cells in the animal intestine, creating an anaerobic environment through oxygen consumption that favors anaerobic bacteria growth [29]; 2) it produces organic acids such as volatile fatty acids and lactic acid, lowering intestinal pH and thereby inhibiting pathogenic bacteria [30]; and 3) *Bacillus subtilis* can secrete various antagonistic peptides [31]. This study showed that dietary supplementation with recombinant *Bacillus subtilis* SE1 significantly reduced *E. coli* counts in the ileum and cecum while significantly increasing *Lactobacillus* counts in the cecum, consistent with the above

results.

Microflora richness indicates diversity of intestinal bacterial species, while evenness and Shannon-Wiener index reflect uniform distribution of intestinal microflora. Greater diversity and more uniform distribution indicate a more stable environment, and these parameters can reflect the stability of the intestinal microecological environment to some extent [32-33]. This study demonstrated that dietary supplementation with recombinant *Bacillus subtilis* SE1 significantly increased the richness, evenness, and Shannon-Wiener index of cecal microflora in broilers compared with the control group. The intra-group similarity of cecal microflora in the recombinant *Bacillus subtilis* SE1 group was significantly higher than in the control group, being 26.8% higher at 28 days and 15.6% higher at 42 days of age, while similarity with the *Bacillus subtilis* 168 group exceeded 90%. PCR-DGGE fingerprint analysis showed that the number of specific bands in the recombinant *Bacillus subtilis* SE1 group was identical to that in the *Bacillus subtilis* 168 group and greater than in the control group. Sequenced bands were dominated by beneficial bacteria such as *Clostridium* and *Lactobacillus*, indicating that recombinant *Bacillus subtilis* SE1 can improve cecal microflora diversity and maintain microflora stability, consistent with reports by Zhao et al. [34], Wang et al. [35], and Zhu [36]. However, both recombinant *Bacillus subtilis* SE1 and *Bacillus subtilis* 168 groups contained the opportunistic pathogen *Serratia liquefaciens*, whose pathogenicity to broilers and underlying mechanisms warrant further investigation.

In this study, dietary supplementation with recombinant *Bacillus subtilis* SE1 and *Bacillus subtilis* 168 had essentially identical effects on cecal microflora counts and diversity, with no significant differences. However, Liu et al. [8] reported that oral immunization with recombinant *Bacillus subtilis* spores induced specific serum IgG and intestinal mucosal SIgA antibodies and provided cross-protection against *S. typhimurium* infection in mice. Liu [15] found that oral administration of avian influenza recombinant *Bacillus subtilis* significantly increased local mucosal specific SIgA and serum specific IgG levels in chickens. Guo [17] reported that dietary supplementation with *S. pullorum* OmpC-DC recombinant *Lactobacillus* significantly increased serum IgG and intestinal SIgA levels in chickens. Combined with our results, these findings suggest that recombinant *Bacillus subtilis* SE1 can induce specific immune protection and provide cross-protection against heterologous *Salmonella* infection without altering the probiotic effects of *Bacillus subtilis* 168, offering new insights for further development of *Bacillus subtilis* and novel *Salmonella* vaccines.

## Conclusions

1. Dietary supplementation with recombinant *Bacillus subtilis* SE1 can increase final weight and average gain while decreasing the feed-to-gain ratio in broilers, thereby promoting growth performance with effects similar to *Bacillus subtilis* 168 preparation.

2. Dietary supplementation with recombinant *Bacillus subtilis* SE1 can significantly increase jejunal lipase and ileal protease activities in broilers but has no significant effect on amylase activity, with effects similar to *Bacillus subtilis* 168 preparation.

3. Dietary supplementation with recombinant *Bacillus subtilis* SE1 can significantly reduce *E. coli* counts in the ileum and cecum, increase *Lactobacillus* counts, and significantly improve cecal microflora diversity and stability, with effects similar to *Bacillus subtilis* 168 preparation.

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*Note: Figure translations are in progress. See original paper for figures.*