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 study aimed to investigate the effects of spore surface display of Salmonella pullorum OmpC by 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 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 CFU/g) Bacillus subtilis 168 preparation and 0.1% (1.0×10 CFU/g) recombinant Bacillus subtilis SE1 preparation, respectively, for a 35-day experimental period. 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 microbiota 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). These results indicate that recombinant Bacillus subtilis SE1 has the same effect as Bacillus subtilis 168, effectively promoting broiler growth, enhancing intestinal lipase and protease activities, regulating broiler gut microbiota, and improving the stability and diversity of gut microbiota.

Full Text

Effects of Recombinant Bacillus subtilis SE1 on Growth Performance, Intestinal Digestive Enzyme Activities and Microflora of Broilers

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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 allocated into three groups with four replicates per group and ten broilers per replicate. Group A (control) received a basal diet, while 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 trial lasted for 35 days. The results showed: 1) Compared with group A, the final body weight and average weight gain of broilers in group C increased by 6.14% and 6.76% (P>0.05), respectively, while 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 counts of *Escherichia coli* in the ileum and cecum of groups B and C were significantly lower than those in group A (P<0.05), while cecal *Lactobacillus* counts were significantly higher (P<0.05). 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 in group A (P<0.05). The similarity of cecal microflora in group C was 26.8% and 15.6% higher than in group A at 28 and 42 days of age, respectively, with no significant difference between groups C and B (P>0.05). These results indicate that recombinant *Bacillus subtilis* SE1 exhibits comparable effects to *Bacillus subtilis* 168, effectively promoting broiler growth, enhancing intestinal lipase and protease activities, regulating intestinal microflora, and improving microflora stability and diversity.

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

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Bacillus subtilis is internationally recognized as a microbial strain that can be directly used in feed. It maintains and adjusts intestinal microflora balance, enhances immunity, secretes various digestive enzymes, improves feed utilization efficiency, and promotes animal growth [**?**, **?**]. *Bacillus subtilis* does not secrete toxins, exhibits good safety profiles, is amenable to genetic manipulation, shows 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 for its use as a secretory expression system. The spores of *Bacillus subtilis* offer advantages including resistance to acid, salt, compression, and high temperatures, with easy purification and low production costs, making spore surface display of exogenous functional proteins a research hotspot [**?**, **?**]. Lian et al. [**?**] surface-displayed human growth hormone (hGH) on *Bacillus subtilis* spore coats and fed it to silkworms, demonstrating that the hGH protein displayed on spore coats could be absorbed into the hemolymph. Zhou et al. [**?**] displayed *Helicobacter pylori* urease B protein on *Bacillus subtilis* spore surfaces and fed it to mice, which resulted in fecal urease B-specific secretory IgA (SIgA) and serum IgG production. Liu et al. [**?**, **?**] constructed recombinant *Bacillus subtilis* SE1 displaying *Salmonella pullorum* outer membrane protein OmpC on spore surfaces and orally administered it to mice, finding that it could induce specific antibodies against *S. pullorum* and provide cross-protection against *Salmonella typhimurium* infection. However, few reports have examined 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* OmpC on broiler 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.

1. Materials and Methods

1.1 Experimental Materials

Both *Bacillus subtilis* 168 preparation (viable spore count of 1.0×10 CFU/g) and recombinant *Bacillus subtilis* SE1 preparation displaying *S. pullorum* OmpC (viable spore count of 1.0×10 CFU/g) were 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) received a basal diet, group B received the basal diet supplemented with 0.1% (1.0×10 CFU/g) *Bacillus subtilis* 168, and group C received the basal diet supplemented with 0.1% (1.0×10 CFU/g) recombinant *Bacillus subtilis* SE1. The supplementation level was determined based on preliminary experiments. The basal diet (antibiotic-free) was provided by a feed mill in Pengzhou, Chengdu, and its composition and nutrient levels are shown in Table 1 .

1.3 Management

Broilers were raised in single-tier cages with separate housing for each group, maintained under infrared heating at approximately 26 °C. They had ad libitum access to feed and water, with regular cage cleaning. Daily observations were made regarding feed consumption, water intake, and health status, with records of feed consumption and morbidity/mortality maintained throughout the 35-day experimental period. Broilers were immunized with Newcastle disease attenuated vaccine via eye drop and intranasal administration at 7 and 21 days of age.

1.4 Measurements

1.4.1 Growth Performance At 42 days of age, broilers were fasted for 8 hours and deprived of water for 2 hours before being weighed to calculate average weight gain and feed-to-gain ratio.

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

1.4.3 Intestinal Microflora Counts Under aseptic conditions, 1 g of ileal and cecal contents was weighed, and plate count methods were used to enumerate *Escherichia coli* and *Lactobacillus* according to reference [**?**].

1.4.4 PCR-DGGE Analysis of Cecal Microflora Diversity Under aseptic conditions, 200 mg of cecal contents was weighed, and PCR-DGGE was used to analyze cecal microflora diversity according to reference [**?**].

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, and significant differences were further analyzed using Duncan's multiple comparison test. The significance level was set at $P<0.05$. All data are expressed as "mean ± standard deviation."

2. Results

2.1 Effects of Recombinant Bacillus subtilis SE1 on Growth Performance

No mortality occurred during the experiment. As shown in Table 2 , compared with group A, the final body weight and average weight gain of broilers in group C increased by 6.14% and 6.76%, respectively ($P>0.05$). 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 not significant ($P>0.05$).

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

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 ($P<0.05$). No significant differences in intestinal digestive enzyme activities were observed between groups B and C ($P>0.05$).

2.3 Effects of Recombinant Bacillus subtilis SE1 on Intestinal Microflora Counts

As shown in Table 4 , at 28 days of age, the counts of *E. coli* in the ileum and cecum of groups B and C were significantly lower than those in group A ($P<0.05$), while cecal *Lactobacillus* counts were significantly higher ($P<0.05$). At 42 days of age, the counts of *E. coli* in the ileum and cecum of groups B and C remained significantly lower than those in group A ($P<0.05$), and *Lactobacillus* counts were significantly higher ($P<0.05$).

2.4 Effects of Recombinant Bacillus subtilis SE1 on Cecal Microflora Diversity

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. Table 5 shows that the richness, evenness, and Shannon-Wiener index of cecal microflora in groups B and C were significantly higher than those in group A ($P<0.05$). According to the clustering results 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 inter-group similarity between groups A and C was 64.1%, while that between groups B and C was 90.9%. At 42 days of age, intra-group similarities were 79.1%, 94.7%, and 94.7% for groups A, B, and C, respectively. The inter-group 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 excised from the PCR-DGGE fingerprint of broiler cecal microflora 16S rDNA V3 region (indicated by arrows in Figure 1). After removing 6 duplicate bands, 12 common bands and 9 specific bands remained. Sequencing and BLAST analysis 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-specific bands corresponded to *Coprococcus eutactus*, while groups B and C showed specific bands for *Clostridium methylpentosum*, *Lactobacillus acidophilus*, *Serratia liquefaciens*, *Ruminococcus albus*, *Clostridium saccharobutylicum*, *Desulfosporosinus acidiphilus*, *Thiobacillus plumbophilus*, and *Lactobacillus rogosae*.

3. Discussion

3.1 Effects of Recombinant Bacillus subtilis SE1 on Broiler Growth Performance

Zhang et al. [**?**] reported that *Bacillus subtilis* can increase daily weight gain and feed intake while reducing feed-to-gain ratio in broilers. Our study demonstrated that dietary supplementation with recombinant *Bacillus subtilis* SE1 increased final body weight and average weight gain while decreasing feed-to-gain ratio compared with the control group. Previous research has also shown that *Bacillus subtilis* can improve animal digestive function, enhance feed utilization, and promote growth [**?**, **?**]. Bai et al. [**?**] found that feeding *Bacillus subtilis* increased broiler daily weight gain, possibly because *Bacillus subtilis* secretes proteases, amylases, lipases, and various vitamins, growth factors, and amino acids that improve intestinal digestive function and promote nutrient absorption [**?**]. Liu [**?**] reported that oral administration of avian influenza recombinant *Bacillus subtilis* significantly enhanced systemic immunity and markedly increased small intestinal villus height in chickens, with superior effects on body weight compared to normal *Bacillus subtilis*. Our results showed that compared with *Bacillus subtilis* 168, recombinant *Bacillus subtilis* SE1 increased final body weight and average weight gain while decreasing 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 [**?**]. The lack of significant differences may be attributed to optimal rearing conditions and broiler breed, resulting in comparable effects between recombinant *Bacillus subtilis* SE1 and *Bacillus subtilis* 168. Guo [**?**] reported that before challenge, feeding *S. pullorum* OmpC-DC recombinant *Lactobacillus* showed no significant differences in 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

Animal intestinal digestive enzymes primarily include amylase, lipase, and protease, which are closely related to growth and metabolism. Zokaeifar et al. [**?**] fed *Bacillus subtilis* to *Litopenaeus vannamei* for 8 weeks and found significantly higher intestinal protease and amylase activities in the treatment group. Bian et al. [**?**] 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. [**?**] found that feeding *Bacillus subtilis* significantly increased protease and amylase activities in the digestive tract of eels. Our study demonstrated that feeding recombinant *Bacillus subtilis* SE1 significantly increased jejunal lipase and ileal protease activities in broilers, consistent with these reports and closely related to the characteristic of *Bacillus subtilis* to produce digestive enzymes such as proteases, lipases, and amylases [**?**, **?**]. However, intestinal amylase activity was not significantly increased, differing 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 enhance intestinal digestive enzyme activities and that displaying *S. pullorum* OmpC on the spore coat surface does not affect this function.

3.3 Effects of Recombinant Bacillus subtilis SE1 on Intestinal Microflora

Studies have confirmed that *Bacillus subtilis* can maintain intestinal microecological balance and increase microflora diversity [**?**, **?**, **?**]. Pei et al. [**?**] found that dietary *Bacillus subtilis* supplementation significantly decreased cecal *E. coli* counts while increasing *Lactobacillus* and *Bifidobacterium* counts in laying hens. Park et al. [**?**] reported that feeding *Bacillus subtilis* significantly increased cecal *Lactobacillus* counts and decreased *Salmonella* counts in broilers. Gao et al. [**?**] demonstrated that dietary *Bacillus subtilis* significantly decreased cecal *E. coli* and *Salmonella* counts while increasing *Lactobacillus* counts in broilers. These effects occur because: 1) *Bacillus subtilis* spores rapidly germinate into vegetative cells in the intestinal tract, creating an anaerobic environment through oxygen consumption that favors anaerobic bacteria [**?**]; 2) it produces organic acids such as volatile fatty acids and lactic acid, lowering intestinal pH and inhibiting pathogen growth [**?**]; and 3) it secretes various antagonistic peptides [**?**]. Our results showed that dietary recombinant *Bacillus subtilis* SE1 significantly reduced ileal and cecal *E. coli* counts while increasing cecal

Lactobacillus counts, consistent with these findings.

Microflora richness indicates species diversity, while evenness and Shannon-Wiener index reflect uniform distribution. Greater species diversity and more uniform distribution contribute to environmental stability, and these parameters can indicate intestinal microecosystem stability [**?**, **?**]. Our results demonstrated that dietary recombinant *Bacillus subtilis* SE1 significantly increased cecal microflora richness, evenness, and Shannon-Wiener index compared with the control group. Intra-group similarity of cecal microflora in the recombinant *Bacillus subtilis* SE1 group was significantly higher than in the control group (26.8% higher at 28 days and 15.6% higher at 42 days), while similarity with the *Bacillus subtilis* 168 group exceeded 90%. PCR-DGGE fingerprint analysis revealed that the recombinant *Bacillus subtilis* SE1 group had the same number of specific bands as the *Bacillus subtilis* 168 group, both exceeding 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 stability, consistent with reports by Zhao et al. [**?**], Wang et al. [**?**], and Zhu [**?**]. However, both recombinant *Bacillus subtilis* SE1 and *Bacillus subtilis* 168 groups harbored the opportunistic pathogen *Serratia liquefaciens*; whether this organism is pathogenic to broilers and the reasons for its presence require further investigation.

In this study, dietary supplementation with recombinant *Bacillus subtilis* SE1 and *Bacillus subtilis* 168 showed essentially identical effects on cecal microflora counts and diversity without significant differences. However, Liu et al. [**?**] demonstrated that oral immunization with recombinant *Bacillus subtilis* spores induced specific serum IgG and intestinal mucosal SIgA antibodies, providing cross-protection against *S. typhimurium* infection in mice. Liu [**?**] found that oral avian influenza recombinant *Bacillus subtilis* significantly increased local mucosal SIgA and serum IgG levels in chickens. Guo [**?**] reported that dietary *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 compromising 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 body weight and average weight gain while decreasing feed-to-gain ratio, thereby promoting broiler growth performance with effects similar to *Bacillus subtilis* 168.

2. Dietary supplementation with recombinant *Bacillus subtilis* SE1 can significantly increase jejunal lipase and ileal protease activities without sig-

nificantly affecting amylase activity, with effects comparable to *Bacillus subtilis* 168.

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

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