

Effects of *Bacillus subtilis* on Growth Performance, Intestinal Morphology, and Microbial Count in Broiler Chickens (Postprint)

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Abstract

This experiment was conducted to investigate the effects of *Bacillus subtilis* on growth performance, slaughter performance, intestinal morphology, and microbial population in broiler chickens. A total of 320 healthy 1-day-old Arbor Acres (AA) male broiler chicks with similar body weight were randomly allocated to 4 groups, with 8 replicates per group and 10 birds per replicate. The control group was fed a basal diet, while the treatment groups were fed the basal diet supplemented with 500 mg/kg *Bacillus subtilis* (BS group), 20 mg/kg colistin sulfate (CS group), and 500 mg/kg *Bacillus subtilis* + 10 mg/kg colistin sulfate (BS+CS group), respectively. The results showed that: 1) Compared with the control group, the BS and CS groups significantly increased the body weight of broilers at 42 days of age ($P < 0.05$), improved average daily gain during the late phase (22-42 days of age), and significantly decreased feed conversion ratio during the late phase and the overall period (1-42 days of age) ($P < 0.05$); the BS+CS group had no significant effect on growth performance at any experimental stage ($P > 0.05$). 2) No significant changes in slaughter performance were observed in any treatment groups ($P > 0.05$). 3) Compared with the control group, the BS group significantly increased duodenal villus height and the villus height to crypt depth ratio in broilers ($P < 0.05$). 4) Compared with the control group, the BS, CS, and BS+CS groups all significantly increased cecal *Lactobacillus* count at 42 days of age ($P < 0.05$), and the BS and BS+CS groups significantly increased the *Lactobacillus* to *Escherichia coli* ratio in the cecum at 42 days of age ($P < 0.05$). These results indicate that *Bacillus subtilis* can improve intestinal morphology, increase *Lactobacillus* population in the intestine, and promote growth in broiler chickens. Overall, *Bacillus subtilis* was superior to colistin sulfate, but the combination showed poor efficacy.

Full Text

Effects of *Bacillus subtilis* on Growth Performance, Intestinal Morphology and Bacterial Enumeration of Broilers

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Abstract: This study investigated the effects of *Bacillus subtilis* on growth performance, slaughter performance, intestinal morphology, and bacterial enumeration in broilers. A total of 320 one-day-old healthy male Arbor Acres broilers with similar body weight were randomly allocated into 4 groups with 8 replicates per group and 10 broilers per replicate. The control group received a basal diet, while experimental groups received basal diets supplemented with 500 mg/kg *Bacillus subtilis* (BS group), 20 mg/kg colistin sulfate (CS group), and 500 mg/kg *Bacillus subtilis* + 10 mg/kg colistin sulfate (BS+CS group), respectively. The results showed: (1) Compared with the control group, BS and CS groups significantly increased body weight at 42 days of age ($P < 0.05$), improved average daily gain during the later period (22-42 days), and significantly decreased feed-to-gain ratio during both the later period and the entire experimental period (1-42 days) ($P < 0.05$). The BS+CS group showed no significant effects on growth performance at any stage ($P > 0.05$). (2) No significant differences in slaughter performance were observed among all groups ($P > 0.05$). (3) Compared with the control group, BS group significantly increased duodenal villus height and the villus height-to-crypt depth ratio ($P < 0.05$). (4) Compared with the control group, BS, CS, and BS+CS groups all significantly increased cecal *Lactobacillus* counts at 42 days of age ($P < 0.05$), while BS and BS+CS groups significantly increased the *Lactobacillus*/*Escherichia coli* ratio ($P < 0.05$). These findings indicate that *Bacillus subtilis* can improve intestinal morphology, increase cecal *Lactobacillus* enumeration, and promote broiler growth. Overall, *Bacillus subtilis* was more effective than colistin sulfate, but the combination showed suboptimal results.

Keywords: *Bacillus subtilis*; colistin sulfate; growth performance; intestinal morphology; bacterial communities

Introduction

Antibiotics have played an extremely important role in the development of modern livestock and poultry production, delivering substantial economic benefits for animal growth while also posing significant risks. Long-term antibiotic use has led to enhanced bacterial resistance, causing serious impacts on animal health and the ecological environment [1]. Currently, many countries have pro-

hibited the use of antibiotic growth promoters in animal feed. Therefore, identifying antibiotic alternatives is crucial for maintaining healthy animal growth and ecological stability.

Bacillus subtilis is a green, non-polluting aerobic probiotic with resistance to pressure, acid, and alkali. After passing through the stomach acid into the intestine, it can rapidly germinate and proliferate in spore form, consuming large amounts of oxygen in the gut, inhibiting harmful bacteria, promoting beneficial bacterial proliferation, improving feed efficiency and intestinal mucosal structure, and playing a positive role in disease prevention and animal growth promotion. Previous studies have shown that adding *Bacillus* to broiler diets can achieve growth-promoting effects and improve feed efficiency comparable to antibiotics [2].

The development of novel feed additives represents a hotspot in animal nutrition research. Studies have demonstrated that dietary supplementation with 0.1% *Bacillus subtilis* can increase average daily gain (ADG) and decrease feed-to-gain ratio (F/G) in broilers [3]; 0.3% *Bacillus subtilis* can reduce intestinal *Escherichia coli* counts and increase *Bifidobacterium* numbers, maintaining intestinal microbial balance and animal health [4]; and 500 mg/kg *Bacillus subtilis* can increase duodenal and jejunal villus height while decreasing crypt depth, promoting small intestinal digestive and absorptive function [5]. Given that antibiotic restriction and prohibition represent major trends in China's animal husbandry development, and the unique benefits of *Bacillus* as a probiotic have attracted widespread attention, research on *Bacillus* substituting for antibiotics and combined usage remains insufficient. Therefore, this study aimed to investigate the effects of *Bacillus subtilis* as an antibiotic substitute and in combination with antibiotics on broiler growth performance, intestinal morphology, and bacterial enumeration, providing theoretical basis and reference for future research and application of *Bacillus* in broilers.

Materials and Methods

Experimental Materials

Bacillus subtilis preparation: *Bacillus subtilis* (DSM 17299) with viable count of 1.6×10^9 CFU/g, provided by Evonik Degussa (China) Co., Ltd.

Colistin sulfate premix: Active ingredient content 10% (Kangdisu), purchased from Jiangxi Tianjia Animal Pharmaceutical Co., Ltd.

Experimental Animals and Design

A total of 320 one-day-old healthy male Arbor Acres broilers with similar body weight were randomly divided into 4 groups with 8 replicates per group and 10 broilers per replicate. The control group received a basal diet, while experimental groups received basal diets supplemented with 500 mg/kg *Bacillus subtilis*

(BS group), 20 mg/kg colistin sulfate (CS group), and 500 mg/kg *Bacillus subtilis* + 10 mg/kg colistin sulfate (BS+CS group). The experimental design is shown in Table 1 . A four-tier cage system was used, and the experimental period lasted 6 weeks.

Experimental Diets

A single-factor randomized design was employed. The basal diets were formulated according to NRC (1994), Chinese Feeding Standards (NY/T 33–2004), and the AA Broiler Management Manual. Composition and nutrient levels of basal diets are shown in Table 2 .

Management Practices

During the experiment, standard management protocols were strictly followed. Routine immunization procedures were implemented, and experimental facilities and chicken houses were maintained clean and hygienic with regular disinfection. The chicken houses were well-ventilated, and feed was kept from moisture, caking, and mold without any additional antibiotics or growth promoters. Drinking water was of good quality and contamination-free. Healthy broilers were used with ad libitum access to feed and water. House temperature and humidity were recorded daily at 08:30 and 14:30, and regular cleaning was performed.

Measurements

Growth Performance At 21 and 42 days of age, broilers were weighed after fasting by replicate to calculate average body weight and average daily gain. Feed consumption was recorded by replicate during the experimental period to calculate average daily feed intake (ADFI) and feed-to-gain ratio for the early, later, and entire periods. Mortality was recorded daily to calculate mortality rate.

Slaughter Performance At 42 days of age, one broiler with body weight close to the replicate average was randomly selected from each replicate, slaughtered by jugular exsanguination, and breast muscle, leg muscle, and abdominal fat were separated and weighed. Dressing percentage, breast muscle rate, leg muscle rate, and abdominal fat rate were calculated according to the “Poultry Performance Calculation Methods” of the National Poultry Breeding Committee.

Intestinal Morphology At 42 days of age, one broiler with body weight close to the replicate average was randomly selected from each replicate and slaughtered. The duodenum and jejunum were isolated, and approximately 3 cm mid-sections were cut, gently rinsed with cold physiological saline, and immersed in pH 7.4 formalin solution at 4°C for paraffin section preparation. Intestinal samples were processed into paraffin sections following the method of Li Hui [6] and stained with toluidine blue method. Under an optical microscope,

5 non-consecutive fields were randomly selected from each section with 3 sets of data per field. Villus length was measured from villus tip to base, and crypt depth was measured from intestinal gland bottom to the opening between two villi bases.

Bacterial Enumeration Sample collection and dilution: At 21 and 42 days of age, 8 broilers were randomly selected from each group, anesthetized with sodium pentobarbital injection, and rapidly dissected. The terminal 2 cm of cecum was collected and stored in liquid nitrogen. Samples were removed from liquid nitrogen and diluted 10^{-4} to 10^{-6} in sterile diluent in a sterile operation room, then thoroughly mixed with a small vortex mixer.

Inoculation: Using a micropipette, 10 mL of diluted sample was dropped onto culture media (Eosin Methylene Blue agar for *Escherichia coli*; Lactobacillus selective medium for *Lactobacillus*) at appropriate gradients. Each sample had 3 replicates, and each replicate used 3 dilutions (10^{-3} , 10^{-4} , 10^{-5} for *E. coli*; 10^{-4} , 10^{-5} , 10^{-6} for *Lactobacillus*). Inoculated plates were carefully spread with sterile curved glass rods.

Incubation: *E. coli* was aerobically incubated at 37°C for 24 h; *Lactobacillus* was anaerobically incubated at 37°C for 48 h.

Colony counting: After incubation, plates with appropriate concentration gradients (10-20 colonies preferred) were selected. Based on the principle of countability (one bacterium produces one colony), plate colony counting was performed for both microorganisms.

Statistical Analysis

Data are expressed as mean \pm standard deviation. One-way ANOVA was performed using SPSS 16.0 software, followed by F-test and Duncan's multiple comparison. $P < 0.05$ was considered statistically significant.

Results

Effects of *Bacillus subtilis* on Growth Performance

Table 3 shows the effects of *Bacillus subtilis* on broiler growth performance. During the early period (1-21 days), no significant differences were observed among BS, CS, and BS+CS groups compared with the control group ($P > 0.05$). At 42 days of age, BS and CS groups increased broiler body weight by 3.55% and 4.30%, respectively, with significant differences from the control group ($P < 0.05$). Average daily gain during the later period (22-42 days) was increased in all treatment groups, with CS group showing a significant 7.25% increase compared with the control group ($P < 0.05$). BS and CS groups significantly decreased feed-to-gain ratio during both the later period and the entire period (1-42 days) ($P < 0.05$), while BS+CS group showed no significant effect ($P > 0.05$). BS group

reduced average daily feed intake during all periods, but differences were not significant ($P>0.05$).

These results indicate that BS and CS groups significantly improved 42-day body weight ($P<0.05$), increased later-period average daily gain, and significantly decreased later-period and overall feed-to-gain ratio ($P<0.05$). The BS+CS group showed no significant effects on growth performance at any stage ($P>0.05$). No significant differences in mortality were observed among groups during any period ($P>0.05$). These findings suggest that *Bacillus subtilis* or colistin sulfate can promote later-stage growth and development in broilers, but no synergistic effect exists between them.

Effects of *Bacillus subtilis* on Slaughter Performance

Table 4 presents the effects of *Bacillus subtilis* on slaughter performance. No significant differences were observed in dressing percentage, breast muscle rate, leg muscle rate, or abdominal fat rate among BS, CS, and BS+CS groups compared with the control group ($P>0.05$).

Effects of *Bacillus subtilis* on Intestinal Morphology

Table 5 shows the effects of *Bacillus subtilis* on intestinal morphology, with duodenal sections illustrated in Figure 1 [Figure 1: see original paper]. In the duodenum, BS group significantly increased villus height by 40.98% ($P<0.05$) and villus height-to-crypt depth ratio by 36.97% ($P<0.05$) compared with the control group. BS+CS group showed a trend toward increased crypt depth ($P=0.094$), while BS and CS groups had no significant effect on crypt depth ($P>0.05$). In the jejunum, BS group showed a trend toward increased villus height ($P=0.051$), while CS and BS+CS groups had no significant effects on villus height, crypt depth, or their ratio ($P>0.05$).

These results suggest that *Bacillus subtilis* can increase duodenal and jejunal villus height at 42 days of age, improve intestinal morphology, and enhance intestinal digestive and absorptive function, but shows no synergistic effect when combined with colistin sulfate.

Effects of *Bacillus subtilis* on Cecal Bacterial Enumeration

Table 6 shows the effects of *Bacillus subtilis* on cecal bacterial enumeration. At 21 days of age, CS group increased cecal *Lactobacillus* counts by 5.76% ($P=0.085$), and BS group increased the *Lactobacillus*/*E. coli* ratio by 10.58% ($P=0.091$) compared with the control group. At 42 days of age, BS, CS, and BS+CS groups all significantly increased cecal *Lactobacillus* counts ($P<0.05$) without significantly affecting *E. coli* counts ($P>0.05$). BS and BS+CS groups significantly increased the *Lactobacillus*/*E. coli* ratio by 9.45% and 8.66%, respectively ($P<0.05$).

These findings indicate that *Bacillus subtilis* significantly increased cecal *Lactobacillus* counts and *Lactobacillus*/*E. coli* ratio at 42 days of age ($P < 0.05$). Colistin sulfate and the combination of *Bacillus subtilis* with colistin sulfate also increased cecal *Lactobacillus* counts at 42 days ($P < 0.05$), while the combination additionally improved the *Lactobacillus*/*E. coli* ratio ($P < 0.05$). These results suggest that either *Bacillus subtilis* or colistin sulfate can increase intestinal *Lactobacillus* counts and promote broiler growth.

Discussion

Effects of *Bacillus subtilis* on Broiler Growth Performance

Numerous studies have reported that *Bacillus subtilis* can improve body weight gain and feed conversion ratio, thereby enhancing growth performance in broilers. Research has shown that 0.1% *Bacillus subtilis* can increase average daily gain and decrease feed-to-gain ratio during 1-6 weeks of age [3]. Dietary *Bacillus subtilis* supplementation can also increase average daily gain and decrease feed-to-gain ratio during 1-3 weeks of age [7]. The present study demonstrated that *Bacillus subtilis* increased 42-day body weight and decreased feed-to-gain ratio during the later and entire periods, consistent with the findings of Molnár et al. [8]. This effect may be related to the time required for digestive tract and intestinal development to reach maturity. The growth-promoting and feed efficiency-improving effects of *Bacillus subtilis* may be attributed to its ability to produce various digestive enzymes after entering the digestive tract, thereby enhancing nutrient absorption [9].

Effects of *Bacillus subtilis* on Broiler Slaughter Performance

Currently, limited research exists on the effects of *Bacillus subtilis* on broiler slaughter performance. Slaughter performance represents an important indicator of carcass quality. Studies have shown that 500 mg/kg *Bacillus subtilis* can significantly increase dressing percentage at 35 days of age without affecting other slaughter performance indicators [10], while 1% *Bacillus subtilis* can increase breast muscle mass in silky fowl [11]. However, the present study found no effect of *Bacillus subtilis* on broiler slaughter performance, consistent with Molnár et al. [8]. These inconsistent results indicate that the effects of *Bacillus subtilis* on broiler slaughter performance remain inconclusive and require further investigation.

Effects of *Bacillus subtilis* on Broiler Intestinal Morphology

Villus height and crypt depth are primary indicators of small intestinal digestive function and cell maturation rate. Increased villus height expands the contact area with intestinal chyme, enhancing digestive and absorptive function. As the digestive tract matures, crypt epithelial cell activity decreases and crypt depth becomes shallower [12-13]. An elevated villus height-to-crypt depth ratio indicates enhanced small intestinal digestive function and improved intestinal

mucosal structure. Research has demonstrated that 500 mg/kg *Bacillus subtilis* can significantly increase duodenal villus height and villus height-to-crypt depth ratio while decreasing crypt depth at 42 days of age [14]. Combined supplementation of *Bacillus subtilis* with oligosaccharides and enzymes can increase duodenal and jejunal villus height and the villus height-to-crypt depth ratio in yellow-feathered broilers [5]. The present study found that *Bacillus subtilis* increased the duodenal villus height-to-crypt depth ratio and villus height, consistent with Wu et al. [5]. These results indicate that *Bacillus subtilis* can enhance small intestinal digestive and absorptive function, thereby promoting animal growth.

Effects of *Bacillus subtilis* on Broiler Cecal Bacterial Enumeration

The intestinal tract maintains microbial balance primarily through beneficial bacteria (*Lactobacillus*, *Bifidobacterium*, etc.). However, management practices inevitably promote proliferation of harmful bacteria (*E. coli*, *Salmonella*, etc.). Excessive proliferation of harmful bacteria disrupts microbial balance, causing intestinal diseases and potentially death. *Bacillus subtilis*, an aerobic bacterium, rapidly consumes oxygen after entering the digestive tract, promoting proliferation of anaerobic beneficial bacteria such as *Lactobacillus* while inhibiting aerobic harmful bacteria like *E. coli* [15]. Additionally, *Bacillus subtilis* produces biologically active antimicrobial substances (lipopeptides, peptides, etc.) that inhibit pathogenic bacteria [16]. Studies have shown that *Bacillus subtilis* can significantly reduce intestinal *E. coli* counts and increase *Lactobacillus* content, with 0.1% *Bacillus subtilis* significantly increasing *Lactobacillus* content and decreasing *E. coli* content in 1–6 week-old broilers [3]. The present study found that *Bacillus subtilis* increased cecal *Lactobacillus* counts and *Lactobacillus*/*E. coli* ratio at 42 days of age, consistent with Sun et al. [17], possibly related to the time-dependent effect of *Bacillus subtilis* function in the intestine.

Combined Effects of *Bacillus subtilis* and Colistin Sulfate

The compatibility of probiotics and antibiotics in animal production has long been a topic of interest. This study demonstrated no synergistic effect between *Bacillus subtilis* and colistin sulfate on broiler growth promotion. Possible reasons include: (1) Mutual influence between colistin sulfate and *Bacillus subtilis*—colistin sulfate may reduce *Bacillus subtilis* survival in the intestine, while *Bacillus subtilis* metabolites may affect the antimicrobial spectrum, intensity, and duration of colistin sulfate. (2) Combined use may affect certain intestinal bacteria—colistin sulfate, composed of multiple amino acids, binds to free phosphates in pathogenic cell membranes, increasing membrane permeability and exerting bactericidal effects that promote growth [18]. *Bacillus subtilis* produces phospholipids and amino acids in the intestine [15], and their combined use may create competitive inhibition against beneficial intestinal bacteria. These metabolic changes and altered intestinal microbiota from combined use may impact growth performance.

Conclusions

1. Both *Bacillus subtilis* and colistin sulfate can promote later-stage growth and development and improve feed efficiency in broilers. Considering effects on intestinal morphology and cecal microbiota, *Bacillus subtilis* is superior to colistin sulfate, but combined use yields suboptimal results.
2. *Bacillus subtilis* can increase duodenal and jejunal villus height, enhancing small intestinal digestive and absorptive capacity.
3. *Bacillus subtilis* can increase cecal *Lactobacillus* counts, maintain microbial balance, and promote broiler growth.

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