# Effects of Bacillus subtilis on Growth Performance, Immune Organ Index, Intestinal Flora and Intestinal Morphology of Cherry Valley Meat Ducks (Postprint)

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## Abstract

This study was conducted to investigate the effects of Bacillus subtilis on growth performance, immune organ indices, intestinal microbiota, and intestinal morphology of Cherry Valley meat ducks. A total of 600 healthy 2-week-old Cherry Valley meat ducks with similar body weight were randomly allocated into 3 groups, with 4 replicates per group and 50 ducks per replicate. The control group was fed a basal diet, while the experimental groups were fed the basal diet supplemented with 2 g/kg Bacillus subtilis (BS group) and 1 g/kg compound Bacillus (CB group), respectively. The experimental period lasted 4 weeks. The results showed that: 1) Compared with the control group, the average daily feed intake of ducks in both BS and CB groups was extremely significantly decreased in week 5 and during weeks 3-6 ($P < 0.01$), and the feed conversion ratio in week 5 was significantly decreased ($P<0.05$); the feed conversion ratio of CB group during weeks 3-6 was significantly decreased ($P<0.05$). 2) The thymus index of ducks in BS and CB groups was significantly higher than that of the control group ($P < 0.05$), while there was no significant difference in spleen index and bursa of Fabricius index among all groups ($P>0.05$). 3) The total bacterial count and Bacillus count in the cecum of ducks in BS and CB groups were significantly higher than those of the control group ($P<0.05$), while the Escherichia coli count was significantly lower ($P<0.05$); the Lactobacillus count in the cecum of ducks in BS group was significantly higher than that of the control group ($P<0.05$). 4) The duodenal villus height, mucosal thickness, villus height/crypt depth ratio, and jejunal mucosal thickness of ducks in BS and CB groups were significantly higher than those of the control group ($P<0.05$), while the jejunal crypt depth was significantly lower than that of the control group ($P<0.05$). In conclusion, dietary supplementation with Bacillus subtilis

can improve intestinal morphology, increase the number of beneficial bacteria in the intestine, stimulate the development of immune organs, and promote the growth of meat ducks.

## Full Text

## Effects of Bacillus subtilis on Growth Performance, Immune Organ Indices, Intestinal Flora, and Intestinal Morphology of Cherry Valley Meat Ducks

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**Abstract:** This study investigated the effects of *Bacillus subtilis* on growth performance, immune organ indices, intestinal flora, and intestinal morphology of Cherry Valley meat ducks. A total of 600 healthy two-week-old Cherry Valley meat ducks with similar body weight were randomly divided into three groups with four replicates per group and 50 ducks per replicate. The control group was fed a basal diet, while the experimental groups received basal diets supplemented with 2 g/kg *Bacillus subtilis* (BS group) or 1 g/kg composite *Bacillus* (CB group). The trial lasted for four weeks. The results showed: (1) Compared with the control group, the average daily feed intake (ADFI) of ducks in both BS and CB groups was significantly reduced at week 5 and during weeks 3-6 ($P<0.01$), and the feed-to-gain ratio was significantly decreased at week 5 ($P<0.05$). The CB group also showed a significantly lower feed-to-gain ratio during weeks 3-6 ($P<0.05$). (2) The thymus index of ducks in BS and CB groups was significantly higher than that of the control group ($P<0.05$), while no significant differences were observed in spleen index or bursa of Fabricius index among groups ($P>0.05$). (3) The total bacterial count and *Bacillus* population in the cecum were significantly higher in BS and CB groups compared to the control ($P<0.05$), while *Escherichia coli* counts were significantly lower ($P<0.05$). The cecal *Lactobacillus* count in the BS group was significantly higher than in the control group ($P<0.05$). (4) Villus height, mucosal thickness, villus height-to-crypt depth ratio in the duodenum, and mucosal thickness in the jejunum were significantly greater in BS and CB groups than in the control ($P<0.05$), while crypt depth in the jejunum was significantly shallower ($P<0.05$). In conclusion, dietary supplementation with *Bacillus subtilis* can improve intestinal morphology, increase beneficial intestinal bacteria, stimulate immune organ

development, and promote growth in meat ducks.

**Keywords:** *Bacillus subtilis*; Cherry Valley meat ducks; immune organ index; intestinal flora; intestinal morphology

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The use of antibiotics in animal production has improved growth performance but introduced numerous problems. Antibiotic use, whether for therapeutic or prophylactic purposes, has led to escalating global concerns regarding bacterial resistance, disruption of normal microbial flora, and drug residues in food products. Probiotics have emerged as successful antibiotic alternatives, with demonstrated effects in preventing disease and promoting growth when added to animal diets. This shift has transformed disease management in modern livestock production from chemical therapy dominated by antibiotics to a new field of microscopic biological immunization. Research indicates that probiotics not only regulate commensal bacteria that maintain intestinal homeostasis but also enhance intestinal epithelial barrier function and promote gut health. Dietary probiotic supplementation can reduce pathogenic bacteria such as *Escherichia coli*, *Salmonella*, and *Clostridium* in the intestinal tract.

- Bacillus* represents a novel probiotic genus whose endospores remain stable under various physical stress factors including low temperature, chemicals, and UV radiation. These organisms produce robust proteases, lipases, and amylases that effectively degrade complex carbohydrates. They exhibit acid, salt, and heat tolerance, along with ease of storage, processing, and transport. Additionally, *Bacillus* species possess pathogen elimination, antioxidant, broad-spectrum antimicrobial, immunomodulatory, and food fermentation capabilities, making them widely studied and utilized probiotics in livestock production. *Bacillus subtilis* and *Bacillus licheniformis* have been approved as feed additives in China. Previous studies demonstrate that *B. subtilis* promotes nutrient digestion and absorption, improves feed utilization, reduces harmful intestinal bacteria, regulates gastrointestinal microecological balance, and enhances disease resistance. However, systematic research on *B. subtilis* in meat ducks remains limited. Therefore, this study evaluated the effects of a previously screened *B. subtilis* strain, added to meat duck diets with a commercial composite *Bacillus* as control, on growth performance, immune organ indices, intestinal morphology, and intestinal flora to provide scientific evidence for probiotic application in meat duck feed.

### 1.1 Test Materials

The *Bacillus subtilis* strain was isolated, screened, and prepared in our laboratory (viable count: 1×10  CFU/g). The composite *Bacillus* product (containing *B. subtilis* and *B. licheniformis*, viable count  $3\times10^{1}$  CFU/g) was provided by a commercial company.

### 1.2 Experimental Design

A total of 600 healthy two-week-old Cherry Valley meat ducks with similar body weight were randomly allocated into three groups with four replicates per group and 50 ducks per replicate. The control group received a basal diet, while the experimental groups received basal diets supplemented with 2 g/kg *Bacillus subtilis* (BS group) or 1 g/kg composite *Bacillus* (CB group). The composition and nutrient levels of the basal diet are presented in Table 1 .

**Table 1 Composition and nutrient levels of the basal diet (air-dry basis)**
**%**

| Ingredients | Content | Nutrient levels | Content |
|---|---|---|---|
| Corn | | Metabolic energy (ME)/(MJ/kg) | |
| Wheat middlings | | Crude protein (CP) | |
| Soybean meal | | Crude fiber (CF) | |
| NaCl | | Crude ash | |
| CaHPO | | Available phosphorus (AP) | |
| Ca(HCO ) | | Methionine (Met) | |
| Premix | | Lysine (Lys) | |
| Total | | | |

*Premix provided the following per kg of diet: VA 8,000 IU, VD 3,000 IU, VE 25 IU, VK 4 mg, VB 2.5 mg, VB 5 mg, VB 5 mg, VB 0.01 mg, nicotinic acid 50 mg, pantothenic acid 20 mg, folic acid 1 mg, biotin 0.03 mg, choline chloride 500 mg, Cu 10 mg, Fe 30 mg, Zn 79.35 mg, Mn 63.6 mg, I 0.5 mg, Se 0.2 mg.*

### 1.3 Management Practices

The trial was conducted at the experimental duck farm of Wuhan Academy of Agricultural Sciences using a fermented litter net-rearing system. Ducks had ad libitum access to feed and water, and other management procedures followed the farm’ s routine protocols. The experimental period lasted four weeks.

### 1.4.1 Growth Performance Measurement

At the end of weeks 1, 2, 3, and 4, ducks were weighed after fasting. Thirty ducks per replicate were randomly selected for individual weighing. Weekly feed consumption and mortality were recorded to calculate average daily feed intake (ADFI), average daily gain (ADG), feed-to-gain ratio, and mortality rate throughout the trial period.

### 1.4.2 Immune Organ Indices and Intestinal Flora Measurement

At 42 days of age, three ducks per replicate (12 per group) with similar body condition were selected for slaughter. The thymus, spleen, and bursa of Fabricius were collected, fat was removed, and organs were weighed to calculate immune organ indices.

**Immune organ index = immune organ weight (g) / live body weight (kg)**

Cecal contents were diluted in a laminar flow hood and plated at 2–3 appropriate dilutions onto nutrient agar, MRS agar, and eosin-methylene blue agar (all media purchased from Qingdao Hope Bio-Technology Co., Ltd.) to determine total bacterial count, *Lactobacillus*, and *E. coli* populations. For *Bacillus* enumeration, 2–3 appropriate dilutions were heat-treated at 80°C for 15 minutes before plating on nutrient agar. Colony counts were performed using the plate count method, with results expressed as log (CFU/g) of intestinal content.

### 1.4.3 Intestinal Morphology Measurement

At 42 days of age, three ducks per replicate (12 per group) with similar body condition were selected for slaughter. Two-centimeter tissue samples were collected from the mid-sections of the duodenum, jejunum, and cecum, rinsed with physiological saline, and fixed in 4% paraformaldehyde solution. Paraffin sections were prepared using conventional methods. Five non-consecutive sections were prepared per intestinal segment, and five typical fields of view (with complete, straight villi) were randomly selected per section. Five longest villi were measured for villus height, crypt depth, and mucosal thickness, and villus height-to-crypt depth ratio (V/C) was calculated.

### 1.5 Data Processing

Experimental data were analyzed using Excel 2007 and SPSS 18.0 software. Results are expressed as "mean ± standard deviation." $P<0.05$ was considered statistically significant, and $P<0.01$ was considered highly significant.

### 2.1 Effects of Bacillus subtilis on Growth Performance of Meat Ducks

The effects of *Bacillus subtilis* on growth performance are shown in Table 2 . During weeks 3-6, ADG, ADFI, and feed-to-gain ratio showed increasing trends across all groups. Specifically, at week 3, the feed-to-gain ratio in the BS group was significantly higher than in the control group ($P<0.05$), with no significant difference between CB and BS groups ($P>0.05$). At week 5, ADFI in the control group was significantly higher than in BS and CB groups ($P<0.01$), and the feed-to-gain ratio was significantly higher than in BS and CB groups ($P<0.05$). At week 6, ADFI in the CB group was significantly lower than in the control and BS groups ($P<0.01$). Overall, during weeks 3-6, ADFI in the control group was significantly higher than in BS and CB groups ($P<0.01$), and the feed-to-gain ratio was significantly higher than in the CB group (P<0.05). No significant differences were observed in ADG or mortality among groups (P>0.05).

These results indicate that dietary supplementation with *Bacillus subtilis* or composite *Bacillus* can promote growth performance in meat ducks during the later growth period.

**Table 2 Effects of Bacillus subtilis on growth performance of meat ducks**

*Note: In the same column, values with different lowercase letter superscripts indicate significant difference (P<0.05), different uppercase letter superscripts indicate highly significant difference (P<0.01), and same or no letter superscripts indicate no significant difference (P>0.05). The same notation applies to subsequent tables.*

## 2.2 Effects of Bacillus subtilis on Immune Organ Indices of Meat Ducks

The effects of *Bacillus subtilis* on immune organ indices are presented in Table 3 . At 42 days of age, the thymus index in BS and CB groups was significantly higher than in the control group (P<0.05). No significant differences were observed in spleen index or bursa of Fabricius index among groups (P>0.05), though BS and CB groups showed increasing trends.

These findings suggest that dietary supplementation with *Bacillus subtilis* or composite *Bacillus* can enhance immune organ indices and stimulate immune organ development in meat ducks.

**Table 3 Effects of Bacillus subtilis on immune organ indices of meat ducks**

| Groups | Thymus index | Spleen index | Bursa of Fabricius index |
|---|---|---|---|
| Control group | 1.73±0.18 | 1.21±0.11 | 0.74±0.08 |
| BS group | 2.01±0.21 | 1.30±0.18 | 0.83±0.09 |
| CB group | 1.94±0.12 | 1.27±0.17 | 0.81±0.07 |

## 2.3 Effects of Bacillus subtilis on Intestinal Flora Population of Meat Ducks

The effects of *Bacillus subtilis* on intestinal flora are shown in Table 4 . At 42 days of age, total bacterial count and *Bacillus* population in the cecum were significantly higher in BS and CB groups compared to the control (P<0.05), while *E. coli* counts were significantly lower (P<0.05). No significant differences were observed between BS and CB groups (P>0.05). The cecal *Lactobacillus* count in the BS group was significantly higher than in the control group (P<0.05), while the CB group showed a slightly higher count than the control without significant difference (P>0.05).

These results demonstrate that dietary supplementation with *Bacillus subtilis* can promote *Lactobacillus* growth, inhibit *E. coli* proliferation, maintain intestinal microbial balance, and promote growth in meat ducks.

**Table 4 Effects of Bacillus subtilis on intestinal flora number of meat ducks (log CFU/g)**

| Groups | Total bacterial count | Lactobacillus | Escherichia coli | Bacillus |
|---|---|---|---|---|
| Control group | 7.78±0.08 | 7.43±0.12 | 6.82±0.09 | 6.06±0.11 |
| BS group | 7.95±0.07 | 7.71±0.13 | 6.54±0.13 | 6.34±0.10 |
| CB group | 8.01±0.06 | 7.65±0.21 | 6.57±0.11 | 6.39±0.12 |

### 2.4 Effects of Bacillus subtilis on Intestinal Morphology of Meat Ducks

The effects of *Bacillus subtilis* on intestinal morphology are presented in Table 5 . At 42 days of age, the villus height-to-crypt depth ratio (V/C) in the duodenum was significantly higher in BS and CB groups compared to the control ($P<0.01$), while villus height and mucosal thickness were significantly greater ($P<0.05$). Crypt depth in the duodenum was significantly lower in the BS group ($P<0.05$) and slightly lower in the CB group without significant difference ($P>0.05$). In the jejunum, crypt depth was significantly lower in BS and CB groups ($P<0.05$), while mucosal thickness was significantly higher ($P<0.05$). The V/C ratio in the jejunum was significantly higher in the CB group ($P<0.05$) and slightly higher in the BS group without significant difference ($P>0.05$). In the ileum, the V/C ratio was significantly higher in the CB group ($P<0.01$) and slightly higher in the BS group without significant difference ($P>0.05$).

As shown in Figure 1 [Figure 1: see original paper], BS and CB groups exhibited more regular and orderly villus structures with longer, denser villi and shallower crypts compared to the control group, which showed shorter, sparser villi and deeper crypts.

These findings indicate that dietary supplementation with *Bacillus subtilis* or composite *Bacillus* can increase duodenal villus height, reduce crypt depth in the duodenum and jejunum, enhance mucosal thickness in the duodenum and jejunum, improve intestinal structure, and promote small intestine development.

**Table 5 Effects of Bacillus subtilis on intestinal morphology of meat ducks**

| Items | Groups | Villus height ( m) | Crypt depth ( m) | V/C ratio | Mucosal thickness ( m) |
|---|---|---|---|---|---|
| **Duodenum** | Control group | 825.18±53.91 | 128.20±8.68 | 6.45±0.36 | 906.26±72.35 |
| | BS group | 871.48±57.91 | 121.65±8.04 | 7.17±0.34 | 961.13±74.14 |
| | CB group | 873.23±56.35 | 121.45±7.27 | 7.19±0.25 | 974.18±90.60 |
| **Jejunum** | Control group | 799.43±62.85 | 123.59±8.07 | 6.50±0.72 | 903.11±84.35 |
| | BS group | 771.78±67.74 | 115.57±10.99 | 6.73±0.83 | 973.18±58.94 |
| | CB group | 811.86±63.87 | 116.39±9.60 | 7.00±0.61 | 970.46±61.46 |
| **Ileum** | Control group | 736.91±62.82 | 107.48±11.56 | 6.89±0.60 | 857.01±57.38 |
| | BS group | 758.96±71.81 | 106.92±6.68 | 7.10±0.58 | 874.85±66.30 |
| | CB group | 770.33±58.67 | 104.25±7.91 | 7.40±0.32 | 862.94±78.72 |

**Figure 1** Morphology of duodenum, jejunum, and ileum of meat ducks (40×). A, B, C: control group; D, E, F: BS group; G, H, I: CB group; A, D, G: duodenum; B, E, H: jejunum; C, F, I: ileum.

### 3.1 Effects of Bacillus subtilis on Growth Performance of Meat Ducks

Numerous studies have demonstrated that dietary supplementation with appropriate levels of *Bacillus subtilis* can improve feed utilization and growth performance in meat ducks. The present study showed that *B. subtilis* supplementation reduced average daily feed intake and feed-to-gain ratio in 42-day-old meat ducks during the later period (week 5) and overall period (weeks 3-6). These findings align with those of Qi et al. The higher feed-to-gain ratio observed in BS and CB groups during the early period (week 3) may be attributed to the time required for *Bacillus* colonization and regulation of the intestinal microecosystem. Probiotics primarily enhance animal growth performance by providing nutrients and digestive enzymes. *Bacillus* can supply certain nutrients, vitamin K, vitamin B , extracellular enzymes, and growth factors that improve intestinal digestive function and enhance nutrient digestibility.

### 3.2 Effects of Bacillus subtilis on Immune Organ Indices of Meat Ducks

The thymus, bursa of Fabricius, and spleen are crucial for avian immune function. The thymus, a central immune organ, plays a vital role in inducing T lymphocyte differentiation and maturation. The bursa of Fabricius is a specialized avian humoral immune organ, while the spleen, the largest peripheral immune organ, participates in both humoral and cellular immunity. Heckert et al. noted that measuring immune organ weight is a common method for evaluating chicken immune status. *Bacillus subtilis* produces various beneficial substances in the intestine, such as vitamins and amino acids, that promote immune organ development. This study demonstrated that dietary *B. subtilis* significantly promoted thymus development in 42-day-old meat ducks and showed a non-significant promoting effect on spleen and bursa of Fabricius development. These results are similar to those of Zhao et al. However, other studies have reported that probiotic supplementation in meat ducks significantly increased bursa of Fabricius index or both thymus and spleen indices. These discrepancies may be related to animal health status, immunologically active components produced by probiotics in the intestine, strain biological characteristics, and supplementation concentration.

### 3.3 Effects of Bacillus subtilis on Intestinal Flora of Meat Ducks

Balanced intestinal microbiota directly affects animal growth and development. Inevitable exposure during rearing management allows pathogenic bacteria such as *E. coli*, *Salmonella*, and enterococci to enter and proliferate in the intestinal tract. These pathogens reduce microbial diversity and species richness by producing toxins and harmful substances, thereby decreasing intestinal homeostasis and pathogen resistance. Studies show that probiotics enhance pathogen resistance not only by stimulating innate and adaptive immunity but also by regulating intestinal epithelial permeability, mucus secretion, and antimicrobial compound production. Li et al. reported that *Bacillus* can inhibit harmful bacteria while promoting beneficial bacterial growth. This study found that dietary *B. subtilis* supplementation significantly increased total bacterial count and populations of *Bacillus* and *Lactobacillus* in the cecum while significantly decreasing *E. coli* counts, indicating that *B. subtilis* can colonize the meat duck intestine and exert probiotic effects by promoting microbial balance.

The probiotic efficacy of *B. subtilis* primarily stems from its ability to be ingested as spores that rapidly proliferate in the intestine, creating an anaerobic environment that promotes the growth of anaerobic beneficial bacteria such as *Lactobacillus* and *Bifidobacterium*. It inhibits pathogen growth by reducing intestinal pH, secreting antimicrobial peptides and bacteriocin-like antimicrobial substances, and reducing pathogen colonization through competitive exclusion, thereby regulating intestinal homeostasis and promoting meat duck growth and development.

### 3.4 Effects of Bacillus subtilis on Intestinal Histomorphology of Meat Ducks

The small intestine plays a crucial role in nutrient absorption. Villus height, mucosal thickness, and crypt depth are primary indicators of small intestinal function. Increased villus height expands the contact area with nutrients, enhancing digestion and absorption capacity. Metabolites produced by probiotics in the intestine (lactic acid, succinic acid, short-chain volatile fatty acids) not only meet host energy requirements but also stimulate submucosal blood vessels to promote villus development. Crypts are sites of intestinal cell proliferation and differentiation, with continuous differentiation from crypt base to villus tip driving villus growth. Crypt depth varies with basal cell generation rate; reduced generation rate leads to shallower crypts, increased intestinal epithelial cell maturation rate, and enhanced nutrient absorption capacity. Shallower crypt depth also facilitates rapid intestinal epithelial cell renewal to counteract pathogen-induced inflammation. Therefore, V/C ratio can evaluate changes in intestinal physiology, with increased V/C indicating improved intestinal mucosal structure and enhanced digestive capacity. Probiotics improve intestinal mucosal structure by continuously stimulating mucosal development and epithelial cell renewal, increasing V/C ratio, and by competitively inhibiting harmful bacteria and producing antimicrobial peptides and bacteriocins that reduce mucosal damage and promote intestinal health. Studies show that dietary probiotic *Bacillus* supplementation in Cherry Valley ducks (28 days old) significantly increased duodenal and jejunal villus height and V/C ratio while reducing duodenal crypt depth. Supplementation with $5.0\times10^{1}$ CFU/kg *B. subtilis* in Linwu ducks significantly affected jejunal and ileal V/C ratios. This study demonstrated that dietary *B. subtilis* supplementation increased duodenal villus height and V/C ratio while reducing crypt depth in the duodenum and jejunum, indicating that *B. subtilis* can improve intestinal structure and promote small intestine development, particularly in the duodenum and jejunum.

## 4 Conclusion

1. Dietary supplementation with *Bacillus subtilis* can improve growth performance in meat ducks.
2. Dietary supplementation with *Bacillus subtilis* can inhibit harmful bacteria, promote beneficial bacterial proliferation, maintain intestinal microbial balance, and stimulate immune organ development in meat ducks.
3. Dietary supplementation with *Bacillus subtilis* can improve intestinal mucosal structure and promote small intestine development in meat ducks.

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