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

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Abstract

This study aimed to investigate the effects of Bacillus subtilis on growth performance, immune organ indices, intestinal microflora, and intestinal morphology in Cherry Valley meat ducks. A total of 600 healthy Cherry Valley meat ducks with similar body weight at 2 weeks of age were selected and randomly divided 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 for 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 in 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 were no significant differences 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 than that of the control group ($P<0.05$); 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 supple-

mentation with Bacillus subtilis could 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 experiment was conducted to investigate 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 allocated into three groups, each consisting of four replicates of 50 ducks. The control group was fed a basal diet, while the experimental groups received the basal diet 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 that: (1) Compared with the control group, both BS and CB groups exhibited extremely significant reductions in average daily feed intake during week 5 and weeks 3-6 ($P < 0.01$), and a significant decrease in feed-to-gain ratio during week 5 ($P < 0.05$). The CB group also showed a significant reduction in feed-to-gain ratio during weeks 3-6 ($P < 0.05$). (2) The thymus index in both BS and CB groups was significantly higher than that in 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 group ($P < 0.05$), whereas the *Escherichia coli* count was significantly lower ($P < 0.05$). Additionally, 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 group ($P < 0.05$), while crypt depth in the jejunum was significantly lower ($P < 0.05$). In conclusion, dietary supplementation with *Bacillus subtilis* can improve intestinal morphology, increase beneficial bacterial populations, stimulate immune organ development, and promote growth in meat ducks.

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

Introduction

The use of antibiotics in animal production has improved growth performance while simultaneously introducing numerous problems. Antibiotic usage, 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. Currently, probiotics have been successfully applied as alternatives to antibiotics, with demonstrated effects on disease prevention and growth promotion in animal diets. The emergence of probiotics has also transformed disease management strategies in modern livestock production, shifting from antibiotic-dominated chemical approaches to a new field of microscopic biological immunization.

Research has shown 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 *E. coli*, *Salmonella*, and *Clostridium* in the intestinal tract. *Bacillus* species, as a novel type of probiotic, have gained attention due to their stable endospore properties, which confer resistance to low temperatures, chemicals, UV radiation, and other physical stress factors. These organisms produce robust proteases, lipases, and amylases that effectively degrade complex carbohydrates, and exhibit acid, salt, and heat tolerance, facilitating easy storage, processing, and transportation. Additionally, they 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 have demonstrated that *Bacillus subtilis* can promote nutrient digestion and absorption, improve feed utilization, reduce harmful intestinal bacterial populations, regulate gastrointestinal microecological balance, and enhance disease resistance in livestock and poultry. However, systematic research on *Bacillus subtilis* in meat ducks remains relatively limited. Therefore, this experiment utilized a previously screened *Bacillus subtilis* strain as a probiotic supplement in meat duck diets, with commercial composite *Bacillus* as a control, to investigate its effects on growth performance, immune organ indices, intestinal morphology, and intestinal flora. The aim was to provide a scientific basis for the application and research of this probiotic in meat duck compound feed.

1.1 Experimental Materials

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

1.2 Experimental Design

Six hundred healthy two-week-old Cherry Valley meat ducks with similar body weight were randomly divided into three groups, each comprising four replicates of 50 ducks. The control group received a basal diet, while the experimental groups received the basal diet 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 (MJ/kg)	
Wheat middlings		Crude protein (%)	
Soybean meal		Crude fiber (%)	
NaCl		Crude ash (%)	
CaHPO		Available phosphorus (%)	
Ca(HCO)		Methionine (%)	
Premix		Lysine (%)	
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 experiment was conducted at the experimental duck farm of Wuhan Academy of Agricultural Sciences using a fermented-bed net-rearing system. Ducks had free access to feed and water, and other management practices and immunization programs followed the farm' s routine protocols. The experimental period lasted four weeks.

1.4 Measurements

1.4.1 Growth Performance At the end of weeks 1, 2, 3, and 4, ducks were weighed after fasting. Thirty ducks from each 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 during the experimental period.

1.4.2 Immune Organ Indices and Intestinal Flora At 42 days of age, three ducks from each replicate (12 per group) with similar body condition were selected for slaughter. The thymus, spleen, and bursa of Fabricius were collected, stripped of fat, and weighed to calculate immune organ indices using the formula: 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. Bacterial counts were expressed as log (CFU/g) of intestinal content.

1.4.3 Intestinal Morphology At 42 days of age, three ducks from each replicate (12 per group) with similar body condition were slaughtered. Two-centimeter segments from the mid-sections of duodenum, jejunum, and ileum were collected, rinsed with physiological saline, and fixed in 4% paraformaldehyde. Paraffin sections were prepared using standard methods. Five non-consecutive sections were taken from each intestinal segment, and five typical fields (with intact, straight villi) were randomly selected per section. Villus height, crypt depth, and mucosal thickness were measured at five longest villi, and villus height-to-crypt depth ratio (V/C) was calculated.

1.5 Statistical Analysis

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 extremely significant.

Results

2.1 Effects of Bacillus subtilis on Growth Performance

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, in 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 ($P > 0.05$). In week 5, ADFI in the control group was extremely significantly higher than in BS and CB groups ($P < 0.01$), and the feed-to-gain ratio was significantly higher ($P < 0.05$). In week 6, ADFI in the CB group was extremely significantly lower than in control and BS groups ($P < 0.01$). Overall, during weeks 3-6, ADFI in the control group was extremely significantly higher than in BS and CB groups ($P < 0.01$), while 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 later-stage growth and development in meat ducks.

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

Items	Week 3	Week 4	Week 5	Week 6	Weeks 3-6
Groups	**ADG (g)**	**ADFI (g)**	**F/G**	**Mortality (%)**	**ADG (g)**
Control	72.72±4.30	121.69±6.46	1.67±0.05	0.00±0.00	88.55±3.41
BS group	72.48±2.96	127.97±6.08	1.77±0.04	0.00±0.00	88.69±2.73
CB group	72.26±3.57	123.72±3.44	1.71±0.06	0.00±0.00	89.61±6.82

In the same column, values with different lowercase superscripts indicate significant difference (P < 0.05), different uppercase superscripts indicate extremely significant difference (P < 0.01), and same or no superscripts indicate no significant difference (P > 0.05). The same applies below.

2.2 Effects of Bacillus subtilis on Immune Organ Indices

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

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	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

The effects of *Bacillus subtilis* on intestinal flora are shown in Table 4 . At 42 days of age, both BS and CB groups exhibited significantly higher total bacterial counts and *Bacillus* populations in the cecum compared to the control group ($P < 0.05$), while *E. coli* counts were significantly lower ($P < 0.05$). No significant differences were observed between BS and CB groups ($P > 0.05$). The *Lactobacillus* count in the BS group was significantly higher than in the control group ($P < 0.05$), whereas the CB group showed a slight but non-significant increase ($P > 0.05$).

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

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	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

The effects of *Bacillus subtilis* on intestinal morphology are presented in Table 5 . At 42 days of age, BS and CB groups showed extremely significantly higher villus height-to-crypt depth ratio (V/C) in the duodenum ($P < 0.01$), along with significantly greater villus height and mucosal thickness ($P < 0.05$). Crypt depth in the duodenum was significantly lower in the BS group ($P < 0.05$) and slightly but non-significantly reduced in the CB group ($P > 0.05$). In the jejunum, crypt depth was significantly lower in both BS and CB groups ($P < 0.05$), while mucosal thickness was significantly higher ($P < 0.05$). The V/C in the jejunum was significantly higher in the CB group ($P < 0.05$) and slightly elevated in the BS group ($P > 0.05$). In the ileum, the CB group showed extremely significantly higher V/C ($P < 0.01$), while the BS group exhibited a slight but non-significant increase ($P > 0.05$).

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

These findings indicate that dietary *Bacillus subtilis* and composite *Bacillus* supplementation can increase duodenal villus height, reduce crypt depth in duodenum and jejunum, enhance mucosal thickness, 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	Mucosal thickness (m)
Duodenum	Control	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±0.31	964.13±74.14
	CB group	873.23±56.35	121.45±7.27	7.19±0.25	974.18±90.60
Jejunum	Control	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	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

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.

Figure 1 Morphology of duodenum, jejunum, and ileum of meat ducks (40×)

Discussion

3.1 Effects of Bacillus subtilis on Growth Performance

Numerous studies have shown that appropriate dietary supplementation of *Bacillus subtilis* in meat duck diets can improve feed utilization and growth performance. The present study demonstrated that *Bacillus subtilis* supplementation reduced average daily feed intake and feed-to-gain ratio during the later stage (week 5) and overall period (weeks 3-6) in 42-day-old meat ducks. These findings align with those of Qi et al. The higher feed-to-gain ratio observed in BS and CB groups during the early stage (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* species 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

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, while the bursa of Fabricius is a specialized avian humoral immune organ. 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 showed that dietary *Bacillus subtilis* significantly promoted thymus development in 42-day-old meat ducks and exhibited a non-significant promoting effect on spleen and bursa of Fabricius development. These results are consistent with Zhao et al. However, other studies have reported that probiotic supplementation in meat duck diets 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, biological characteristics of the bacterial strains, and supplementation concentrations.

3.3 Effects of Bacillus subtilis on Intestinal Flora

Balanced intestinal microbiota directly affects animal growth and development. Inevitable exposure to pathogenic bacteria such as *E. coli*, *Salmonella*, and enterococci during rearing can reduce microbial diversity and intestinal homeostasis, compromising pathogen resistance. Research indicates 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 *Bacillus subtilis* significantly increased total bacterial count, *Bacillus*, and *Lactobacillus* populations while decreasing *E. coli* counts in the cecum, demonstrating that *Bacillus subtilis* can colonize the meat duck intestine and exert probiotic effects by promoting microbial balance.

The probiotic efficacy of *Bacillus 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 obligate anaerobes such as *Lactobacillus* and *Bifidobacterium*. This occurs through multiple mechanisms: lowering intestinal pH to inhibit pathogen growth, secreting antimicrobial peptides and bacteriocins to suppress pathogen proliferation, and competitively excluding pathogens from intestinal colonization, thereby regulating intestinal homeostasis and promoting meat duck growth.

3.4 Effects of Bacillus subtilis on Intestinal Morphology

The small intestine plays a critical role in nutrient absorption, with villus height, mucosal thickness, and crypt depth serving as primary indicators of intestinal function. Increased villus height expands the contact area with nutrients, enhancing digestion and absorption capacity. Probiotic metabolites (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 rates result in shallower crypts, increased enterocyte maturation, and enhanced nutrient absorption. Shallower crypts also facilitate rapid epithelial renewal to counteract pathogen-induced inflammation. Therefore, V/C ratio serves as an indicator of intestinal physiological changes, with elevated V/C reflecting improved mucosal structure and enhanced absorptive capacity. Probiotics improve intestinal mucosal structure by continuously stimulating mucosal development and epithelial cell renewal, thereby increasing V/C ratio, while competitive inhibition and production of antimicrobial peptides and bacteriocins reduce pathogen colonization and mucosal damage, promoting intestinal health.

Studies have shown 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. Similarly, supplementation with 5.0×10^1 CFU/kg *Bacillus subtilis* in Linwu ducks significantly affected jejunal and ileal V/C ratios. The present study demonstrated that *Bacillus subtilis* increased duodenal villus height and V/C ratio while reducing crypt depth in duodenum and jejunum, indicating that *Bacillus subtilis* can improve intestinal structure and promote small intestine development, particularly in the duodenum and jejunum.

Conclusion

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