Effects of Bacillus subtilis Combined with Alfalfa Polysaccharide on Production Performance, Egg Quality, Blood Parameters, and Fecal and Intestinal Microbiota in Laying Hens (Postprint)

Authors: Guo Junrui, Dong Xiaofang, Tong Jianming

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Abstract

This experiment aimed to investigate the effects of dietary supplementation of Bacillus subtilis and its combination with alfalfa polysaccharide on production performance, egg quality, blood indices, and fecal and intestinal microflora in laying hens, and to observe whether the combination of Bacillus subtilis and alfalfa polysaccharide has superior effects compared to Bacillus subtilis alone. A total of 288 healthy 27-week-old Hy-Line Brown laying hens were randomly divided into 4 groups with 6 replicates per group and 12 hens per replicate. Group 1 was the control group fed a basal diet; groups 2-4 were fed the basal diet supplemented with $1.0\$\times 107 CFU/g Bacillus subtilis, 1.0\times 107 CFU/g Bacillus subtilis + 250 mg/kg alfalfa polysaccharide, and 1.0\times\$107$ CFU/g Bacillus subtilis + 4 000 mg/kg alfalfa polysaccharide, respectively, with an experimental period of 24 weeks. The results showed that: 1) There were no significant differences among groups in feed intake, egg weight, egg production per hen, and mortality rate (P>0.05). However, compared with the control group, the egg production rate of groups 3 and 4 during weeks 9-16 was significantly increased (P<0.05), and the feed-to-egg ratio of groups 2, 3, and 4 during weeks 9-16, 17-24, and 1-24 was significantly decreased (P<0.05). 2) Dietary supplementation of Bacillus subtilis and its combination with alfalfa polysaccharide had no significant improving effect on eggshell color, eggshell thickness, eggshell strength, egg shape index, albumen height, and Haugh unit (P>0.05), but significantly increased yolk color in weeks 1, 2, 4, and 12 (P<0.05). 3) Dietary supplementation of Bacillus subtilis and its combination with alfalfa polysaccharide had significant improving effects on serum glucose in weeks 2, 3, 4, 12, 16, and 20 and serum urea content in weeks 2, 3, 8, 12, and 20 (P<0.05), and serum glucose content in group 3 during weeks 2, 3, 4, and

8 and in group 4 during weeks 3, 4, 8, and 20 was significantly higher than that in group 2 (P<0.05); dietary supplementation of Bacillus subtilis and its combination with alfalfa polysaccharide significantly increased blood white blood cell, lymphocyte, intermediate cell, and neutrophil counts (P<0.05), intermediate cell counts in group 3 during weeks 1, 3, 4, 8, and 12 and in group 4 during week 3 were significantly higher than those in group 2 (P<0.05), and neutrophil counts in group 3 during weeks 1, 3, 4, and 8 and in group 4 during weeks 1, 3, and 8 were significantly higher than those in group 2 (P<0.05); dietary supplementation of Bacillus subtilis and its combination with alfalfa polysaccharide significantly increased platelet counts in weeks 3, 16, and 24 (P<0.05). Dietary supplementation of Bacillus subtilis and its combination with alfalfa polysaccharide significantly increased serum immunoglobulin A in week 16, immunoglobulin G in weeks 1 and 4, and immunoglobulin M in weeks 2, 4, and 8 (P<0.05), and groups 3 and 4 were superior to experimental group 2 (P<0.05). 4) Dietary supplementation of Bacillus subtilis and its combination with alfalfa polysaccharide significantly reduced fecal Escherichia coli count and cecal Escherichia coli/Lactobacillus ratio (P<0.05), and significantly increased Bifidobacterium counts in the jejunum, ileum, and cecum (P<0.05). In conclusion, dietary supplementation of Bacillus subtilis and its combination with alfalfa polysaccharide can improve production performance and egg quality in laying hens, reduce fecal and cecal Escherichia coli counts, increase intestinal Bifidobacterium counts, and the combination has certain advantages over Bacillus subtilis alone in improving serum biochemical indices and regulating immune function.

Full Text

Effects of Bacillus subtilis and Alfalfa Polysaccharide on Performance, Egg Quality, Blood Indices and Fecal and Intestinal Microbial Flora of Laying Hens

Guo Junrui, Dong Xiaofang*, Tong Jianming
(Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, China)

Abstract

This study investigated the effects of dietary Bacillus subtilis and its combination with alfalfa polysaccharide on performance, egg quality, blood indices and fecal and intestinal microbial flora of laying hens, and evaluated whether the combination produced superior effects compared to Bacillus subtilis alone. Two hundred and eighty-eight healthy 27-week-old Hy-Line Brown laying hens were randomly allocated into 4 groups with 6 replicates of 12 birds each for a 24-week feeding trial. Group 1 served as the control and received a basal diet. Groups 2, 3 and 4 received the basal diet supplemented with 1.0$×10^{7}$ CFU/g Bacillus subtilis, 1.0$×10^{7}$ CFU/g Bacillus subtilis + 250 mg/kg alfalfa

polysaccharide, and 1.0$×10^{7}$ CFU/g Bacillus subtilis + 4,000 mg/kg alfalfa polysaccharide, respectively. The results showed: 1) No significant differences were observed among groups in feed intake, egg weight, egg weight per hen or mortality (P>0.05). However, groups 3 and 4 exhibited significantly higher egg production during weeks 9–16 compared to the control (P<0.05), while groups 2, 3 and 4 showed significantly lower feed conversion ratio during weeks 9–16, 17–24 and 1–24 (P<0.05). 2) Dietary supplementation with Bacillus subtilis alone or combined with alfalfa polysaccharide had no significant effects on eggshell color, thickness, strength, egg shape index, albumen height or Haugh unit (P>0.05), but significantly improved yolk color at weeks 1, 2, 4 and 12 (P<0.05). 3) The treatments significantly improved serum glucose content at weeks 2, 3, 4, 12, 16 and 20 and serum urea content at weeks 2, 3, 8, 12 and 20 (P<0.05). Serum glucose in group 3 at weeks 2, 3, 4 and 8 and in group 4 at weeks 3, 4, 8 and 20 was significantly higher than in group 2 (P<0.05). Supplementation significantly increased counts of leukocytes, lymphocytes, intermediate cells and neutrophils (P<0.05). Intermediate cell counts in group 3 at weeks 1, 3, 4, 8 and 12 and in group 4 at week 3 were significantly higher than in group 2 (P<0.05). Neutrophil counts in group 3 at weeks 1, 3, 4 and 8 and in group 4 at weeks 1, 3 and 8 were significantly higher than in group 2 (P<0.05). Platelet counts were significantly elevated at weeks 3, 16 and 24 (P<0.05). Serum immunoglobulin A content at week 16, immunoglobulin G content at weeks 1 and 4, and immunoglobulin M content at weeks 2, 4 and 8 were significantly increased (P<0.05), with groups 3 and 4 showing superior effects compared to group 2 (P<0.05). 4) The treatments significantly reduced fecal Escherichia coli count and the cecal E. coli/Lactobacillus ratio (P<0.05), while significantly increasing Bifidobacterium counts in jejunum, ileum and cecum (P<0.05). In conclusion, dietary Bacillus subtilis alone or combined with alfalfa polysaccharide can improve performance and egg quality, reduce fecal and cecal E. coli counts, and increase intestinal Bifidobacterium counts in laying hens. Moreover, the combination showed superior effects on improving serum biochemical indices and enhancing immune function compared to Bacillus subtilis alone.

Keywords: laying hens; Bacillus subtilis; alfalfa polysaccharide; performance; egg quality; blood indices; microbial flora

Introduction

With the development of animal husbandry, livestock and poultry production has become increasingly intensive. Many countries have now banned the use of antibiotics in animal feed, leaving animals vulnerable to environmental stressors and disease pressures. Consequently, the search for safe and effective feed additives has attracted widespread attention. Probiotics are among the most commonly used additives, as they can improve intestinal microflora, enhance immunity and increase animal performance. Prebiotics have also demonstrated

important roles in improving host health. While oligosaccharides that selectively stimulate the growth of Lactobacillus and Bifidobacterium are well known, numerous studies have confirmed that polysaccharides play important roles in improving growth performance, antioxidant capacity, promoting beneficial bacteria growth and immunomodulation. Although many reports have documented the application of probiotics combined with oligosaccharides in animal production, research on the synergistic effects of probiotics and polysaccharides in laying hens remains limited. This study aimed to investigate the effects of Bacillus subtilis alone and in combination with alfalfa polysaccharide on performance, egg quality, blood indices and fecal and intestinal microbial flora of laying hens, and to determine whether the combination produces synergistic effects superior to Bacillus subtilis alone, thereby providing experimental evidence for their application in laying hen production.

1.1 Experimental Animals and Design

Two hundred and eighty-eight healthy Hy-Line Brown laying hens aged 27 weeks with similar laying rates (92.2%, 91.2%, 91.7% and 92.3%) and body weights (1.658–1.679 kg) were randomly divided into 4 groups with 6 replicates of 12 birds each. Group 1 served as the control and received a basal diet formulated according to NRC (1994) requirements. The composition and nutrient levels of the basal diet are presented in . Groups 2, 3 and 4 received the basal diet supplemented with 1.0$×10^{7}$ CFU/g Bacillus subtilis, 1.0$×10^{7}$ CFU/g Bacillus subtilis + 250 mg/kg alfalfa polysaccharide, and 1.0$×10^{7}$ CFU/g Bacillus subtilis + 4,000 mg/kg alfalfa polysaccharide, respectively. The experimental period lasted 24 weeks.

1.2 Experimental Materials

The bacterial strain used was Bacillus subtilis CGMCC 1.921, purchased from the China General Microbiological Culture Collection Center and prepared by Cangzhou Huayu Pharmaceutical Co., Ltd., with viable count $ 1.0×10^{10}$ CFU/g. The alfalfa polysaccharide composition was: 24.28% polysaccharides, 2.90% flavonoids, 3.47% saponins, 15.55% crude protein and 24.35% crude ash.

1.3 Management

Birds were housed in two-tier cages with each replicate consisting of two adjacent cages (80 cm × 60 cm × 50 cm) housing 6 birds per cage. Feed was provided three times daily ad libitum, and water was freely available via nipple drinkers. The lighting schedule was 16 h light (06:00–22:00) with automatic temperature control, heating and ventilation.

1.4 Measurements

1.4.1 Performance During the experimental period, daily egg number, egg weight and mortality were recorded per replicate. Feed remaining was weighed

weekly to calculate feed intake. Egg production, feed intake, egg weight, feed conversion ratio, egg weight per hen and mortality rate were calculated.

1.4.2 Egg Quality At the start of the experiment (week 0) and at weeks 1, 2, 3, 4, 8, 12, 16, 20 and 24, all eggs laid on the sampling day were collected for analysis. Eggshell color was measured using a QCR colorimeter (TSS, UK). Eggshell strength was determined with a Model- tester (Robotmation, Japan). Eggshell thickness was measured using a Model P-1 gauge (Ozaki MFG, Japan). Egg shape index was assessed with an NFN384 device (FHK, Japan). Albumen height, Haugh unit and yolk color were measured using an EMT-2500 egg quality analyzer (Robotmation, Japan).

1.4.3 Blood Indices At the start of the experiment and at weeks 1, 2, 3, 4, 8, 12, 16, 20 and 24, 4 birds were randomly selected from each replicate after 12 h fasting (water ad libitum). Blood was collected from the wing vein into regular vacuum tubes, allowed to clot, centrifuged at 2,000 r/min for 10 min, and serum was stored in EP tubes for analysis. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) activities and total protein (TP), albumin (ALB), total bilirubin (TBIL), glucose (GLU), urea (UREA), uric acid (UA), creatinine (CREA), triglyceride (TG) and cholesterol (CHOL) contents were determined using a Toshiba TBA-120FR automatic biochemical analyzer (Japan).

Additional blood samples were collected into 5 mL EDTA-anticoagulant tubes and 2 mL sodium citrate tubes. White blood cell (WBC), lymphocyte (LYM), intermediate cell (MID), neutrophil (GRA) and red blood cell (RBC) counts, hematocrit (HCT), hemoglobin (HGB) content and platelet (PLT) count were measured by electrical impedance using a TEK- mini automatic hematology analyzer (Jiangxi Tekang, China). Erythrocyte sedimentation rate (ESR) was determined by the Westergren method using a XC-40B automatic ESR analyzer (Beijing, China). Serum immunoglobulin A (IgA), IgG and IgM contents were measured by double-antibody sandwich ELISA using kits from Shanghai Langdun Biotechnology.

1.4.4 Microbial Flora Analysis At the start of the experiment and at weeks 1, 2, 3, 4, 8, 12, 16, 20 and 24, fresh feces were collected from 6 birds per replicate and E. coli counts were determined by plate counting on eosin methylene blue agar. At the end of week 24, one bird per replicate (6 per group) was euthanized by cervical dislocation. The abdomen was opened, and jejunum, ileum and cecum were ligated, excised and placed in 50 mL sterile tubes. Contents were immediately collected in sterile EP tubes under aseptic conditions. Lactobacillus, Bifidobacterium, E. coli, Enterococcus, Clostridium perfringens and Campylobacter were enumerated by plate counting, and the Lactobacillus/E. coli ratio was calculated. Lactobacillus was cultured on MRS agar, Bifidobacterium on TPY agar, E. coli on eosin methylene blue agar, Enterococcus on Pfizer selective

enterococcus agar, C. perfringens on TSC agar and Campylobacter on modified Camp-BAP agar.

1.5 Statistical Analysis

Data were analyzed using SPSS 19.0. One-way ANOVA and least significant difference (LSD) tests were used for multiple comparisons. Significance was set at $P<0.05$. Data are expressed as means ± standard deviation.

Results

2.1 Effects on Performance

As shown in [Figure 1: see original paper], dietary Bacillus subtilis alone or combined with alfalfa polysaccharide had no significant effects on egg weight, feed intake, egg weight per hen or mortality ($P>0.05$). For egg production, groups 3 and 4 were significantly higher than the control during weeks 9–16 ($P<0.05$), but did not differ significantly from group 2 ($P>0.05$). For feed conversion ratio, groups 2, 3 and 4 were significantly lower than the control during weeks 9–16, 17–24 and 1–24 ($P<0.05$), with no significant differences among the three treatment groups ($P>0.05$). These results indicate that the combination of Bacillus subtilis and alfalfa polysaccharide did not produce superior effects on performance compared to Bacillus subtilis alone.

2.2 Effects on Egg Quality

As shown in [Figure 2: see original paper], dietary supplementation had no significant effect on eggshell color ($P>0.05$). For eggshell thickness, groups 2, 3 and 4 were significantly higher than the control at week 1 ($P<0.05$). Group 2 was significantly higher than the control and group 4 at week 3 ($P<0.05$), but did not differ from group 3 ($P>0.05$). For eggshell strength, group 2 was significantly higher than the control and group 4 at week 8 ($P<0.05$), while group 3 did not differ significantly from either the control or group 2 ($P>0.05$). For egg shape index, group 2 was significantly lower than group 3 at week 20 ($P<0.05$), with no significant differences between groups 2 and 3 versus group 4 and the control ($P>0.05$). Haugh unit and albumen height in groups 2 and 3 were significantly lower than the control at week 4 ($P<0.05$), with group 2 significantly higher than group 3 ($P<0.05$) and both significantly lower than group 4 ($P<0.05$). Groups 2 and 3 had significantly lower Haugh unit than the control at week 8 ($P<0.05$), with no significant difference from group 4 ($P>0.05$). Group 3 had significantly higher Haugh unit than groups 2 and 4 at week 24 ($P<0.05$), but did not differ from the control ($P>0.05$).

For yolk color, groups 2, 3 and 4 were significantly lower than the control before the experiment ($P<0.05$), with group 4 significantly higher than group 3 ($P<0.05$) but significantly lower than group 2 ($P<0.05$). At week 1, groups 2, 3 and 4 were significantly higher than the control ($P<0.05$), with group 3 signif-

icantly higher than group 4 (P<0.05) but not differing from group 2 (P>0.05). At week 2, groups 2 and 4 were significantly higher than the control (P<0.05), with no significant difference from group 3 (P>0.05). At week 4, groups 2, 3 and 4 were significantly higher than the control (P<0.05) with no significant differences among the three treatment groups (P>0.05). At week 12, groups 2, 3 and 4 were significantly higher than the control (P<0.05), with group 2 significantly higher than groups 3 and 4 (P<0.05). Group 3 was significantly lower than all other groups at week 20 (P<0.05). These findings indicate that Bacillus subtilis alone or combined with alfalfa polysaccharide had some effect on improving yolk color, but the combination showed no clear advantage over Bacillus subtilis alone.

2.3 Effects on Hematological Indices

As shown in [Figure 3: see original paper], RBC counts in group 2 (weeks 2, 12), group 3 (weeks 1, 3) and group 4 (weeks 1, 2, 3, 12) were significantly lower than the control (P<0.05), with group 4 significantly lower than groups 2 and 3 at week 3 (P<0.05). No significant differences were observed among treatment groups at other time points (P>0.05), though group 2 was significantly higher than other groups at weeks 16 and 20 (P<0.05). For HCT, groups 2 (weeks 1, 2, 3, 12), 3 (week 1) and 4 (weeks 1, 2, 3, 12) were significantly lower than the control (P<0.05), with group 4 significantly lower than groups 2 and 3 at week 3 (P<0.05). Groups 2 and 3 were significantly higher than the control at week 20 (P<0.05), while group 4 was significantly lower than group 2 (P<0.05).

For HGB content, groups 2 (weeks 1, 3, 12), 3 (weeks 8, 20) and 4 (weeks 1, 2, 3, 8, 12, 16, 20) were significantly lower than the control (P<0.05), while groups 2 (week 16) and 3 (week 4) were significantly higher (P<0.05). Groups 3 (week 16) and 4 (weeks 2, 8, 16, 20) were significantly lower than group 2 (P<0.05), while group 3 was significantly higher than group 2 at week 4 (P<0.05). For PLT count, groups 2 (weeks 3, 16, 24), 3 (weeks 3, 24) and 4 (weeks 3, 24) were significantly higher than the control (P<0.05).

For WBC count, groups 2 (weeks 1, 3, 4, 8, 16, 20, 24), 3 (weeks 8, 12, 20, 24) and 4 (weeks 0, 2, 16, 20, 24) were significantly higher than the control (P<0.05). Groups 3 (weeks 1, 3, 4, 16, 20) and 4 (weeks 1, 3, 4, 20) were significantly lower than group 2 (P<0.05), while groups 3 (week 12) and 4 (weeks 0, 2, 12) were significantly higher than group 2 (P<0.05). For LYM count, groups 2 (weeks 3, 4, 8, 20, 24), 3 (weeks 20, 24) and 4 (weeks 20, 24) were significantly higher than the control (P<0.05), while groups 3 (weeks 3, 4, 20) and 4 (weeks 3, 4, 8, 20) were significantly lower than group 2 (P<0.05).

For MID count, groups 2 (weeks 0, 12, 16, 20, 24), 3 (weeks 1, 3, 4, 8, 12, 16, 24) and 4 (weeks 0, 1, 3, 12, 16) were significantly higher than the control (P<0.05). Groups 3 (weeks 1, 3, 4, 8, 12) and 4 (weeks 0, 3) were significantly higher than group 2 (P<0.05), while groups 3 and 4 were significantly lower than group 2 at week 16 (P<0.05). For neutrophil count, groups 2 (weeks 0, 12, 16, 24), 3

(weeks 1, 4, 8, 12, 16, 20, 24) and 4 (weeks 8, 12, 16, 24) were significantly higher than the control ($P<0.05$). Groups 3 (weeks 1, 3, 4, 8) and 4 (weeks 1, 3, 8) were significantly higher than group 2 ($P<0.05$). For ESR, groups 2 (weeks 3, 20), 3 (weeks 8, 20) and 4 (weeks 3, 8, 20, 24) were significantly higher than the control ($P<0.05$), with group 2 significantly higher than all other groups at week 3 ($P<0.05$). These results demonstrate that compared to Bacillus subtilis alone, the combination with alfalfa polysaccharide significantly increased blood MID and neutrophil counts without significantly affecting other hematological indices.

2.4 Effects on Serum Biochemical Indices

As shown in [Figure 4: see original paper], dietary supplementation had no significant effects on serum ALT activity or TG content ($P>0.05$). For AST activity, groups 2 and 4 were significantly higher than the control at week 8 ($P<0.05$), with no significant difference from group 3 ($P>0.05$). For TP and GLB contents, groups 3 and 4 were significantly higher than other groups at week 3 ($P<0.05$). For ALB content, group 3 was significantly lower than the control at week 4 ($P<0.05$), and group 4 was significantly lower than group 3 ($P<0.05$). For ALB/GLB ratio, groups 2, 3 and 4 did not differ from the control at week 2 ($P>0.05$), while groups 3 and 4 were significantly lower than other groups at week 3 ($P<0.05$), and group 4 was significantly lower than the control at week 4 ($P<0.05$).

For TBIL content, group 3 was significantly higher than other groups at week 1 ($P<0.05$). Groups 2, 3 and 4 were significantly lower than the control at week 3 ($P<0.05$). Groups 3 and 4 were significantly lower than the control at week 4 ($P<0.05$), with group 4 significantly lower than groups 2 and 3 ($P<0.05$). For GLU content, groups 2 (week 8), 3 (weeks 1, 8, 24) and 4 (week 24) were significantly lower than the control ($P<0.05$). Groups 2 (weeks 2, 3, 12, 16), 3 (weeks 2, 3, 4, 12) and 4 (weeks 2, 3, 4, 8, 12, 16, 20) were significantly higher than the control ($P<0.05$), with groups 3 (weeks 2, 3, 4, 12) and 4 (weeks 3, 4, 8, 12, 20) significantly higher than group 2 ($P<0.05$).

For UREA content, groups 3 (week 4) and 4 (weeks 4, 16) were significantly higher than the control ($P<0.05$), while groups 2 (weeks 3, 12), 3 (weeks 2, 3, 12) and 4 (weeks 3, 8, 12, 20) were significantly lower ($P<0.05$). Groups 3 (week 2) and 4 (weeks 8, 20) were significantly lower than group 2 ($P<0.05$). For UA content, groups 2 and 4 were significantly lower than the control at week 3 ($P<0.05$). For CHOL content, group 3 was significantly higher than the control and group 4 at week 1 ($P<0.05$). For CREA content, group 2 was significantly lower than the control at weeks 1 and 2 ($P<0.05$), while groups 3 (weeks 2, 3, 4) and 4 (week 4) were significantly higher than the control ($P<0.05$). In summary, dietary Bacillus subtilis alone or combined with alfalfa polysaccharide had no significant effects on serum ALT and AST activities or TP, ALB, GLB, TBIL, UA, CREA, CHOL and TG contents or ALB/GLB ratio, but showed some improvement in serum GLU and UREA contents, with the combination

demonstrating advantages in improving serum GLU content.

2.5 Effects on Serum Immunoglobulin Content

As shown in [Figure 5: see original paper], for IgA content, group 4 was significantly lower than group 3 at week 1 ($P<0.05$) but did not differ from the other two groups ($P>0.05$). Groups 2, 3 and 4 were significantly higher than the control at week 16 ($P<0.05$). For IgG content, groups 3 (weeks 1, 4) and 4 (week 4) were significantly higher than the control ($P<0.05$), with groups 3 (week 1) and 4 (week 4) significantly higher than group 2 ($P<0.05$). For IgM content, groups 3 and 4 were significantly higher than the control and group 2 at weeks 2, 4 and 8 ($P<0.05$). These results indicate that compared to Bacillus subtilis alone, the combination with alfalfa polysaccharide significantly increased serum immunoglobulin content.

2.6 Effects on Fecal and Intestinal Microbial Flora

As shown in [Figure 6: see original paper], compared with the control, fecal E. coli counts were significantly reduced in group 2 (weeks 1, 12, 20), group 3 (week 12) and group 4 (weeks 3, 20) ($P<0.05$). Groups 3 and 4 were significantly higher than group 2 at week 1 ($P<0.05$). As shown in [Figure 7: see original paper], dietary supplementation had no significant effects on cecal Lactobacillus, Campylobacter, Clostridium perfringens and Enterococcus counts, or on jejunal and ileal Lactobacillus and E. coli counts ($P>0.05$). Cecal Bifidobacterium counts were significantly higher in groups 2 and 4 compared to the control ($P<0.05$). Cecal E. coli counts in groups 2 and 3 were significantly lower than in group 4 ($P<0.05$), and the cecal E. coli/Lactobacillus ratio was significantly lower than the control ($P<0.05$), while group 4 did not differ significantly from the control ($P>0.05$). Jejunal and ileal Bifidobacterium counts were significantly higher in groups 2, 3 and 4 compared to the control ($P<0.05$), with no significant differences among the three treatment groups ($P>0.05$). These findings indicate that in terms of fecal and intestinal microbial flora, the combination of Bacillus subtilis and alfalfa polysaccharide did not produce superior effects compared to Bacillus subtilis alone.

Discussion

3.1 Effects on Performance

Dietary probiotics can enhance digestive enzyme activity and improve nutrient digestion and absorption, thereby increasing animal performance. Previous studies from our laboratory demonstrated that alfalfa polysaccharide can improve egg production rate and egg weight per hen. Geng et al. reported that dietary chitosan combined with Bacillus subtilis significantly increased specific growth rate in cobia, while Fan et al. observed similar effects with sea cucumber fed a combination of Astragalus polysaccharide, Poria polysaccharide and Bacillus

subtilis. Synbiotics have shown clear benefits for animal growth, possibly because prebiotics promote probiotic growth, creating conditions for improved intestinal structure and microflora. This study found that Bacillus subtilis alone or combined with alfalfa polysaccharide significantly reduced feed conversion ratio, but no significant differences existed between the two treatments, indicating no superior effect of the combination over Bacillus subtilis alone. Li reported that probiotics alone or combined with Astragalus polysaccharide (36.12% content) significantly improved weekly weight gain and reduced feed conversion ratio in broiler chicks, but found no significant difference between synbiotic and probiotic groups, consistent with our results. The effects of polysaccharide-probiotic combinations on animal performance may relate to rearing environment, physiological status and polysaccharide properties. Limited reports exist on this topic, and the underlying mechanisms remain unclear.

3.2 Effects on Egg Quality

Reports on probiotic effects on egg quality are inconsistent. Lei found that dietary Bacillus licheniformis improved eggshell thickness, strength, Haugh unit, albumen height and yolk color in laying hens, while Tang reported that a probiotic preparation (Lactobacillus acidophilus, L. casei, Bifidobacterium bifidum, Streptococcus faecalis, Aspergillus oryzae) had no significant effects on eggshell thickness, Haugh unit or yolk color. Wang and Xin reported that Astragalus polysaccharide and alfalfa polysaccharide improved yolk color, albumen height, Haugh unit and eggshell thickness. However, no reports exist on the combined effects of polysaccharides and probiotics on egg quality. This study found that Bacillus subtilis alone or combined with alfalfa polysaccharide had no significant effects on eggshell color, thickness, strength, shape index, albumen height or Haugh unit, but improved yolk color to some extent. Yolk color depends on dietary carotenoid content, and any factor hindering carotenoid absorption and deposition will result in lighter yolk color. Therefore, the improved yolk color in this study may be attributed to enhanced carotenoid absorption and deposition. Additionally, both Bacillus subtilis and alfalfa polysaccharide have antioxidant properties, which may have protected carotenoids from oxidation and increased pigment deposition, though the specific mechanisms require further investigation.

3.3 Effects on Serum Biochemical Indices

ALT and AST are primarily located in hepatocytes, with ALT in the cytoplasm and AST in mitochondria. When hepatocytes are damaged, ALT enters the bloodstream first; if damage is severe enough to affect mitochondria, AST is also released, increasing serum ALT and AST activities. This study showed that Bacillus subtilis alone or combined with alfalfa polysaccharide had minimal effects on ALT and AST. Serum TP, ALB, GLB contents and ALB/GLB ratio are key indicators of normal liver function. No major differences were observed among groups for these parameters. Groups 2 (week 3) and 3 and 4

(weeks 3, 4) showed significantly reduced serum TBIL content, suggesting that supplementation did not cause adverse liver effects. Studies have reported that dietary probiotics (Lactobacillus and Bacillus) and synbiotics (probiotics + Astragalus polysaccharide) had no significant effects on serum AST activity or TP content, consistent with our findings.

Dietary Bacillus subtilis alone or combined with alfalfa polysaccharide increased serum GLU content, with groups 3 (weeks 2, 3, 4, 8) and 4 (weeks 3, 4, 8, 20) significantly higher than group 2. This suggests that the combination enhanced glycogenolysis or gluconeogenesis, possibly by increasing intestinal amylase activity. However, Li reported that dietary probiotics (Bacillus and Lactobacillus) and synbiotics (probiotics + Astragalus polysaccharide) reduced serum GLU content in broilers. This study found no significant effects on TG and CHOL contents, consistent with Li' s findings. Additionally, supplementation had minimal effects on UA and CREA contents but significantly reduced UREA content, with group 3 (week 2) and group 4 (weeks 8, 20) significantly lower than group 2. These results suggest that supplementation improved dietary nitrogen utilization and may have provided some kidney protection, with synergistic effects between Bacillus subtilis and alfalfa polysaccharide. Limited reports exist on the effects of polysaccharide-probiotic combinations on serum UREA and UA contents, though Fallah reported that dietary Enterococcus faecium + fructooligosaccharide + trehalose increased serum GLU and reduced UA in ostrich chicks, suggesting alfalfa polysaccharide may act as a potential prebiotic.

3.4 Effects on Immune Function

Leukocytes are important components of the defense system, playing roles in pathogen phagocytosis and disease prevention. Lian reported that probiotics (Bacillus, Lactobacillus, Bifidobacterium) combined with yeast polysaccharide significantly increased WBC counts in 10-day-old Hy-Line chicks, while probiotics combined with Astragalus polysaccharide significantly increased WBC counts at 40 and 50 days, though the probiotic alone group did not differ from the control. This study demonstrated that Bacillus subtilis alone or combined with alfalfa polysaccharide increased WBC, lymphocyte, MID and neutrophil counts, with the combination groups showing significantly higher MID (weeks 1, 3, 4, 8, 12) and neutrophil (weeks 1, 3, 4, 8) counts than the Bacillus subtilis alone group. Hassaan reported that Bacillus licheniformis combined with yeast extract significantly increased WBC counts compared to Bacillus licheniformis alone, with some effects on RBC and HGB counts and variable effects on HCT. Lian found that probiotics combined with yeast or Astragalus polysaccharides had no significant effects on RBC counts. This study showed that supplementation had no significant effects on RBC, HGB and HCT, but increased PLT counts. Elzey reported that platelets also play important roles in the immune system. Furthermore, supplementation increased serum IgA (weeks 1, 16), IgG (weeks 1, 4) and IgM (weeks 2, 4, 8) contents. Immunoglobulins are produced by B cells after antigen stimulation and participate in complement activation

and toxin neutralization; their levels reflect immune status. Wang reported that ginseng polysaccharide combined with Lactobacillus plantarum C88 significantly increased serum IgG content in immunosuppressed mice. Shao found that Bacillus subtilis combined with Acanthopanax polysaccharide significantly increased serum IgA, IgM and IgG contents in chicks. This study demonstrated that the combination of Bacillus subtilis and alfalfa polysaccharide had clear advantages over Bacillus subtilis alone, indicating that the combination can enhance humoral immunity.

3.5 Effects on Fecal and Intestinal Microbial Flora

This study showed that Bacillus subtilis alone or combined with alfalfa polysaccharide significantly reduced fecal and cecal E. coli counts and increased intestinal Lactobacillus and Bifidobacterium counts. Hu reported that combined Lentinan polysaccharide and Lactobacillus plantarum C88 significantly increased Lactobacillus and Bifidobacterium counts while reducing Enterococcus and Enterobacter counts in mice, with superior effects compared to Lactobacillus alone. Li found that probiotics (Lactobacillus and Bacillus cereus) and synbiotics (probiotics + Astragalus polysaccharide) significantly increased Lactobacillus and Bifidobacterium counts while reducing E. coli counts in ileum and cecum of chicks at 21 and 42 days, with synbiotic groups showing superior effects in cecum. Calik reported that in ovo injection and post-hatch feeding of Enterococcus faecium + inulin increased cecal Lactobacillus counts at 21 days and reduced Enterobacter counts at 42 days, with combined in ovo and post-hatch administration showing superior effects to post-hatch feeding alone, suggesting that animal physiological status may influence synbiotic efficacy in modulating microbial flora.

Conclusions

Dietary supplementation with Bacillus subtilis alone or combined with alfalfa polysaccharide reduced feed conversion ratio and improved egg production rate and yolk color, though the combination showed no superior effects compared to Bacillus subtilis alone. The treatments improved serum biochemical and hematological indices, and enhanced immune function to some extent, with the combination showing clear advantages over Bacillus subtilis alone. Supplementation significantly reduced fecal and cecal E. coli counts, increased intestinal Bifidobacterium counts, decreased the cecal E. coli/Lactobacillus ratio, and improved intestinal microenvironment, though no significant differences were observed between combined and individual supplementation.

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