Effects of Bacillus licheniformis Supplementation in Feed or Culture Water on Growth Performance and Immunity of Litopenaeus vannamei Postprint

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Abstract

This experiment investigated the effects of dietary or aquaculture water supplementation with Bacillus licheniformis on the growth performance and immunity of Pacific white shrimp (Litopenaeus vannamei) through two sub-experiments. The initial average weight of L. vannamei used in both sub-experiments was approximately 1.06 g. In Experiment 1, five groups of shrimp were fed five experimental diets containing 0 (Group D1, as blank control), 0.3 (Group D2), 3.0 (Group D3), 30.0 mg/kg Bacillus licheniformis (Group D4), and 80.0 mg/kg Probiotic A (a commercial compound probiotic preparation; Group D5, as positive control) supplemented to the basal diet; in Experiment 2, four groups of shrimp were all fed the diet of Group D1 from Experiment 1, and at the beginning of the experiment, 0 (Group d1, i.e., Group D1), 0.4 (Group d2), 4.0 mg Bacillus licheniformis (Group d3), and 40.0 mg Probiotic B (a commercial compound probiotic water purifier; Group d4, as positive control) were added to the water of 1 m$\times 1m \times$1 m concrete tanks (effective water depth 0.7 m), followed by supplementary additions of 0, 0.2, 2.0 mg Bacillus licheniformis and 20.0 mg Probiotic B every 7 days thereafter. Each group had six concrete tanks, with 80 shrimp stocked per tank. The culture duration for both Experiment 1 and Experiment 2 was 8 weeks. The results of Experiment 1 showed: The final average weight of shrimp in Groups D1–D4 was significantly higher than that in Group D5 ($P<0.05$), but there was no significant difference among Groups D1–D4 ($P>0.05$); the weight gain rate and specific growth rate of shrimp in Group D2 were significantly higher than those in Group D5 ($P<0.05$) but showed no significant difference from Groups D1, D3, and D4 ($P>0.05$); the feed conversion ratio of shrimp in Groups D2, D3, and D4 was significantly lower than that in Group D1 ($P<0.05$), but showed no significant difference from Group D5 ($P>0.05$); the survival rate of shrimp in Group D4 was significantly higher than that in all other groups ($P>0.05$). Hepatopancreatic superoxide dismutase (SOD) activity of shrimp was higher in Groups D2 and D5, and significantly higher than

that in all other groups (P<0.05); hepatopancreatic total antioxidant capacity (T-AOC) of shrimp was highest in Group D4, and significantly higher than that in all other groups (P<0.05); hepatopancreatic malondialdehyde (MDA) content of shrimp decreased with increasing dietary Bacillus licheniformis supplementation, with the lowest MDA content observed in Group D4, which was significantly lower than that in Groups D1, D2, and D3 (P<0.05), but showed no significant difference from Group D5 (P>0.05). The results of Experiment 2 showed: Supplementation of Bacillus licheniformis in the culture water had no significant effect on the final average weight, weight gain rate, specific growth rate, or feed conversion ratio of shrimp (P>0.05); hepatopancreatic alkaline phosphatase (AKP), SOD activity, and T-AOC of shrimp in Group d3 were highest, and significantly higher than those in all other groups (P<0.05); hepatopancreatic lysozyme (LZM) activity of shrimp in Group d2 was highest, and significantly higher than that in all other groups (P<0.05); hepatopancreatic MDA content of shrimp in Group d1 was highest, and significantly higher than that in all other groups (P<0.05). These results indicate that Bacillus licheniformis, whether added directly to the feed or applied directly to the culture water, can enhance the immunity of L. vannamei; comprehensively considering both growth performance and immunity of L. vannamei, the supplementation effect of Bacillus licheniformis in feed or culture water was superior to that of Probiotic A or Probiotic B; based on the comprehensive results of Experiment 1 and Experiment 2, the dietary supplementation level of Bacillus licheniformis in L. vannamei feed should be controlled at 0.3–3.0 mg/kg.

Full Text

Effects of Dietary or Waterborne Supplementation of *Bacillus licheniformis* on Growth Performance and Immunity of *Litopenaeus vannamei*

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Abstract: Two trials were conducted to investigate the effects of dietary or waterborne supplementation of *Bacillus licheniformis* on the growth performance and immunity of *Litopenaeus vannamei*. In both trials, the shrimp had an average initial body weight of approximately 1.06 g. In Trial 1, five groups of shrimp were fed five experimental diets: a basal diet without probiotics (D1, blank control), basal diets supplemented with 0.3 mg/kg (D2), 3.0 mg/kg (D3), or 30.0 mg/kg *B. licheniformis* (D4), and a basal diet supplemented with 80.0

mg/kg Probiotic A (a commercial compound probiotic preparation; D5, positive control). In Trial 2, four groups of shrimp were all fed the D1 diet, and their culture water in 1 m × 1 m × 1 m concrete tanks (effective water depth 0.7 m) was treated initially with 0 (d1, i.e., D1), 0.4 (d2), 4.0 mg *B. licheniformis* (d3), or 40.0 mg Probiotic B (a commercial compound probiotic water purifier; d4, positive control), followed by supplementary additions of 0, 0.2, 2.0 mg *B. licheniformis*, or 20.0 mg Probiotic B, respectively, every 7 days. Each group consisted of six replicate tanks, with 80 shrimp per tank. Both trials lasted for 8 weeks.

The results of Trial 1 showed that the final body weight of shrimp in groups D1–D4 was significantly higher than that in D5 ($P < 0.05$), with no significant differences among D1–D4 ($P > 0.05$). The weight gain rate and specific growth rate of D2 shrimp were significantly higher than those of D5 ($P < 0.05$) but did not differ significantly from D1, D3, or D4 ($P > 0.05$). The feed conversion ratio of D2, D3, and D4 was significantly lower than that of D1 ($P < 0.05$) but comparable to D5 ($P > 0.05$). The survival rate of D4 was significantly higher than all other groups ($P < 0.05$). Hepatopancreas superoxide dismutase (SOD) activity was elevated in D2 and D5, significantly higher than in the remaining groups ($P < 0.05$). Hepatopancreas total antioxidant capacity (T-AOC) peaked in D4, significantly exceeding other groups ($P < 0.05$). Hepatopancreas malondialdehyde (MDA) content decreased with increasing dietary *B. licheniformis* supplementation, reaching its lowest level in D4, which was significantly lower than D1, D2, and D3 ($P < 0.05$) but not significantly different from D5 ($P > 0.05$).

Trial 2 results indicated that waterborne supplementation of *B. licheniformis* had no significant effects on final body weight, weight gain rate, specific growth rate, or feed conversion ratio ($P > 0.05$). However, hepatopancreas alkaline phosphatase (AKP), SOD activity, and T-AOC were highest in d3, significantly surpassing other groups ($P < 0.05$). Hepatopancreas lysozyme (LZM) activity peaked in d2, significantly higher than in all other groups ($P < 0.05$). The hepatopancreas MDA content was highest in d1, significantly exceeding that of other groups ($P < 0.05$). These findings demonstrate that *B. licheniformis* enhances the immunity of *L. vannamei* whether administered through feed or directly into culture water. Considering both growth performance and immunity, *B. licheniformis* supplementation outperformed Probiotic A or Probiotic B. Based on the combined results of both trials, the optimal dietary inclusion level of *B. licheniformis* for *L. vannamei* should be 0.3–3.0 mg/kg.

Keywords: *Bacillus licheniformis*; *Litopenaeus vannamei*; growth performance; immunity

Introduction

Litopenaeus vannamei, commonly known as the Pacific white shrimp, ranks among the three most commercially farmed shrimp species worldwide. Since 2001, its cultivation area in China has expanded continuously due to favorable economic returns. However, the pursuit of high yields through intensive and high-density farming practices has led to large-scale disease outbreaks. Traditional treatment methods relying heavily on antibiotics have resulted in the emergence of drug-resistant bacterial strains, secondary water pollution, and concerns regarding antibiotic residues in aquaculture products, raising serious food safety issues. Consequently, the search for green, safe, and environmentally friendly antibiotic alternatives has become a research priority.

Probiotics are live microbial feed supplements that, at appropriate concentrations, benefit animal health and growth by modulating host-associated microbial communities, improving nutrient utilization, enhancing disease resistance, or ameliorating water quality. *Bacillus licheniformis*, a probiotic bacterium belonging to the genus *Bacillus*, has been extensively studied in aquaculture due to its excellent tolerance to high temperatures and pressures. Previous research by Cao et al. demonstrated that waterborne supplementation of *B. licheniformis* significantly increased hepatopancreas heat shock protein 70 (HSP70) mRNA expression and enhanced fecal degradation in *L. vannamei*. Li et al. reported that dietary *B. licheniformis* significantly reduced intestinal *Vibrio* counts in Pacific white shrimp, a finding later confirmed by Hu et al. While most studies on *B. licheniformis* have focused on fish species such as yellowfin seabream (*Acanthopagrus latus*), grass carp (*Ctenopharyngodon idellus*), gilt-head seabream (*Sparus sarba*), and Asian seabass (*Lates calcarifer*), research on shrimp remains limited. Therefore, we designed two complementary trials: Trial 1 evaluated graded levels of dietary *B. licheniformis* against a commercial probiotic product for improving growth performance and immunity in *L. vannamei*, while Trial 2 assessed graded levels of waterborne *B. licheniformis* against another commercial probiotic water purifier for the same purpose, providing valuable insights for developing probiotic applications in shrimp aquaculture.

1.1 Experimental Materials

The *B. licheniformis* preparation used in this study was a high-concentration aquaculture-specific probiotic provided by DSM (Netherlands), containing $ $1 $\times 10^{11}$ CFU per gram. Commercial Probiotic A, produced by Chr. Hansen (Denmark), contained *B. licheniformis* and *Bacillus subtilis* as a homogeneous spray-dried compound preparation widely used in terrestrial animal production (pigs) but not previously reported in aquaculture. Commercial Probiotic B, manufactured by Vinovo Bio (Denmark), was a compound probiotic water purifier formulated from seven different *Bacillus* species at specific ratios, which has demonstrated efficacy in improving water quality for Chinese white shrimp (*Fen-*

neropenaeus chinensis), Chinese mitten crab (*Eriocheir japonica*), and kuruma shrimp (*Penaeus japonicus*).

1.2 Experimental Methods

Healthy *L. vannamei* with an average initial body weight of approximately 1.06 g were selected as experimental animals. Prior to the trials, shrimp were acclimated for one week in 1 m × 1 m × 1 m concrete tanks (effective water depth 0.7 m) while being fed a commercial diet. After acclimation, 3,840 uniformly sized shrimp were randomly stocked into 48 concrete tanks at a density of 80 shrimp per tank, with six tanks constituting one experimental group. The study comprised two trials. In Trial 1, five groups were randomly selected and fed five experimental diets: basal diet without probiotics (D1, blank control), basal diets supplemented with 0.3 mg/kg (D2), 3.0 mg/kg (D3), or 30.0 mg/kg *B. licheniformis* (D4), and basal diet supplemented with 80.0 mg/kg Probiotic A (D5, positive control). In Trial 2, four groups were all fed the D1 diet, and their culture water was initially treated with 0 (d1, i.e., D1, blank control), 0.4 (d2), 4.0 mg *B. licheniformis* (d3), or 40.0 mg Probiotic B (d4, positive control), followed by supplementary additions of 0, 0.2, 2.0 mg *B. licheniformis*, or 20.0 mg Probiotic B, respectively, every 7 days. Groups D1 and d1 served as a common blank control for both trials.

1.3 Experimental Diets

Five experimental diets were formulated for Trial 1, with composition and nutrient levels shown in . All feed ingredients were ground and passed through an 80-mesh sieve before being weighed according to the formula proportions and preliminarily mixed. The mixture was blended in a commercial feed mixer (A-200T Mixer Bench Model Unit, Canada) for 15 minutes, after which premixed fish oil, soybean oil, and soybean lecithin were added and blended for an additional 15 minutes. Approximately 40% distilled water (v/w) was then incorporated and mixed for another 15 minutes before extrusion into 1.2 mm pellets using a twin-screw extruder (developed by South China University of Technology). The pellets were polished, dried in an oven at 60 °C for 2 hours with turning every 30 minutes, further air-dried in an air-conditioned room to a moisture content below 10%, packaged in sealed plastic bags, labeled, and stored at –20 °C until use.

1.4 Feeding Management

During the experimental period, shrimp were fed three times daily at 07:00, 15:00, and 22:00. No water exchange occurred during the first three weeks; thereafter, one-third of the water volume was exchanged weekly. The initial feeding rate was set at 6% of body weight and adjusted daily based on actual consumption. Water temperature was maintained at 27 ± 2 °C, salinity at 32 ± 2, with continuous aeration. Water quality parameters including temperature, salinity, pH, dissolved oxygen, and ammonia concentration were monitored

throughout the trials to ensure they remained within normal ranges. Both trials lasted for 8 weeks.

1.5.1 Growth Indices

At the end of each trial, shrimp were fasted for 24 hours before measuring the final total weight and recording survival numbers for each tank to calculate weight gain rate (WGR), specific growth rate (SGR), survival rate (SR), and feed conversion ratio (FCR) using the following formulas:

- WGR (%) = 100 × (final average weight -initial average weight) / initial average weight
- SGR (%/d) = 100 × (ln final average weight -ln initial average weight) / experimental days
- SR (%) = 100 × final shrimp number / initial shrimp number
- FCR = dry feed intake / (final body weight -initial body weight)

1.5.2 Proximate Composition

After the 24-hour fasting period, three shrimp were randomly collected from each tank and stored at -80 °C for whole-body proximate analysis, while another three shrimp were sampled for muscle proximate analysis. Proximate composition of diets, whole shrimp, and muscle was determined according to AOAC (1995) methods.

1.5.3 Hepatopancreas Immune Indices

Following the 24-hour fasting period, six shrimp were randomly sampled from each tank. Hepatopancreases were excised, placed in 2 mL centrifuge tubes, immediately frozen in liquid nitrogen, and subsequently stored at -80 °C until analysis. For analysis, hepatopancreas samples were accurately weighed and homogenized (1:9, w/v) in ice-cold physiological saline under ice-bath conditions to prepare 10% tissue homogenates, which were centrifuged at 2,500 r/min for 10 minutes at 4 °C. The supernatant was collected for determination of total protein, malondialdehyde (MDA) content, total antioxidant capacity (T-AOC), and activities of superoxide dismutase (SOD), alkaline phosphatase (AKP), acid phosphatase (ACP), and lysozyme (LZM) using assay kits purchased from Nanjing Jiancheng Bioengineering Institute.

1.6 Statistical Analysis

All data were subjected to one-way ANOVA using SPSS 21.0 software. When significant differences were detected, Duncan' s multiple range test was applied. Significance was set at $P < 0.05$. Data are presented as means ± standard error.

2.1.1 Effects of Dietary *B. licheniformis* or Probiotic A on Growth Performance of *L. vannamei*

As shown in , final body weight, weight gain rate, and specific growth rate of *L. vannamei* increased initially and then decreased with rising dietary *B. licheniformis* levels. The D2 group exhibited the highest final body weight, weight gain rate, and specific growth rate among all groups, significantly exceeding those of D5 ($P < 0.05$) but not differing significantly from D1, D3, or D4 ($P > 0.05$). Compared with D1, dietary supplementation of *B. licheniformis* in D2, D3, and D4 significantly reduced feed conversion ratio ($P < 0.05$) and improved survival rate to varying degrees, with D4 achieving the highest survival rate (94.25%), significantly greater than D1 ($P < 0.05$).

2.1.2 Effects of Dietary *B. licheniformis* or Probiotic A on Body Composition of *L. vannamei*

reveals that whole-body crude protein content in D1–D4 was significantly higher than in D5 ($P < 0.05$), with no significant differences among D1–D4 ($P > 0.05$). Whole-body crude lipid content in D2 was significantly higher than in D1 and D4 ($P < 0.05$) but comparable to D3 and D5 ($P > 0.05$). Whole-body ash content in D2 and D3 was significantly higher than in D1, D4, and D5 ($P < 0.05$), though D2 and D3 did not differ significantly from each other ($P > 0.05$). No significant differences in whole-body ash content were observed among all groups ($P > 0.05$). Regarding muscle proximate composition, crude protein content in D2, D3, and D5 was significantly higher than in D1 and D4 ($P < 0.05$), while no significant differences were detected among groups for muscle moisture, crude lipid, or ash content ($P > 0.05$).

2.1.3 Effects of Dietary *B. licheniformis* or Probiotic A on Hepatopancreas Immune Indices of *L. vannamei*

demonstrates that hepatopancreas AKP activity in D4 was significantly lower than in D1 and D5 ($P < 0.05$), while ACP activity in D3 was significantly lower than in all other groups ($P < 0.05$). Hepatopancreas LZM activity was significantly higher in D1 and D4 compared to other groups ($P < 0.05$), with D3 and D5 also significantly exceeding D2 ($P < 0.05$). Dietary *B. licheniformis* supplementation in D2, D3, and D4 and Probiotic A supplementation in D5 significantly elevated hepatopancreas SOD activity compared to D1 ($P < 0.05$), with D2 and D5 significantly higher than D3 and D4 ($P < 0.05$). Hepatopancreas MDA content decreased progressively with increasing dietary *B. licheniformis* levels, while T-AOC increased accordingly. Specifically, MDA content in D3 and D4 was significantly lower than in other groups ($P < 0.05$), and T-AOC in D4 was significantly higher than in all other groups ($P < 0.05$).

2.2.1 Effects of Waterborne *B. licheniformis* or Probiotic B on Growth Performance of *L. vannamei*

shows that waterborne supplementation of *B. licheniformis* or Probiotic B had no significant effects on final body weight, weight gain rate, specific growth rate, feed conversion ratio, or total feed intake ($P > 0.05$). The survival rate of shrimp in d4 (Probiotic B) was significantly higher than in d2 (*B. licheniformis* at 0.4 mg) ($P < 0.05$) but did not differ significantly from other groups ($P > 0.05$).

2.2.2 Effects of Waterborne *B. licheniformis* or Probiotic B on Hepatopancreas Immune Indices of *L. vannamei*

As presented in , hepatopancreas AKP activity in d3 was significantly higher than in all other groups ($P < 0.05$), whereas no significant differences were observed in ACP activity among groups ($P > 0.05$). Hepatopancreas LZM activity peaked in d2, significantly exceeding other groups ($P < 0.05$). Hepatopancreas SOD activity in d2 and d3 was significantly higher than in d1 and d4 ($P < 0.05$). With increasing waterborne *B. licheniformis* concentration, hepatopancreas MDA content decreased while T-AOC increased. Specifically, MDA content in d2 and d3 was significantly lower than in other groups ($P < 0.05$), and T-AOC in d3 was significantly higher than in d1 and d4 ($P < 0.05$) but comparable to d2 ($P > 0.05$).

This study comprised two trials investigating *B. licheniformis* supplementation in feed and culture water. Trial 1 results showed that dietary *B. licheniformis* at 0.3–30.0 mg/kg (D2–D4) produced significantly higher final body weight, specific growth rate, and weight gain rate compared to Probiotic A (D5), with improvements over the blank control (D1) that were not statistically significant. However, feed conversion ratio in D2–D4 was significantly lower than in D1. These findings align with previous studies reporting that dietary probiotics enhanced weight gain and specific growth rate in *L. vannamei* without significant differences from controls. Similar results have been documented in other aquatic species such as obscure pufferfish (*Fugu obscurus*) and common carp (*Cyprinus carpio*).

The growth-promoting effects of dietary probiotics in *L. vannamei* likely relate to their ability to secrete digestive enzymes and enhance endogenous digestive enzyme activity, as confirmed by numerous studies. Additionally, *B. licheniformis* improved survival rate, with the highest supplementation level (D4) achieving significantly greater survival than other groups, probably due to competitive exclusion of pathogenic microorganisms by the probiotic bacteria colonizing the shrimp intestine. Notably, Probiotic A (D5) resulted in lower final body weight, weight gain rate, and specific growth rate compared to the blank control, possibly reflecting product purity or quality issues.

In Trial 1, higher *B. licheniformis* levels (D3 and D4) showed reduced final body weight, specific growth rate, and weight gain rate compared to D2, suggesting

potential negative effects at excessive supplementation rates. In contrast, waterborne *B. licheniformis* in Trial 2 did not significantly affect growth performance, differing from some previous reports, possibly due to variations in experimental animals or culture conditions. The lower survival rate in d2 compared to the blank control and d4 may be attributed to heavy rainfall entering some tanks during the trial.

Disease prevention is crucial in *L. vannamei* aquaculture, and lysosomal enzymes constitute a vital component of humoral immunity, including LZM, ACP, AKP, and SOD. Dietary probiotics have been shown to effectively improve immune function in various aquatic animals, including rainbow trout (*Oncorhynchus mykiss*), gilt-head seabream (*Sparus aurata*), and triangle sail mussel (*Hyriopsis cumingii*). In Trial 1, dietary *B. licheniformis* at 0.3 or 3.0 mg/kg significantly elevated hepatopancreas SOD activity and reduced MDA content compared to the blank control, consistent with previous findings and indicating enhanced stress resistance. Waterborne *B. licheniformis* has been reported to effectively degrade shrimp feces, reducing chemical oxygen demand and nitrate-nitrogen levels. In Trial 2, waterborne *B. licheniformis* increased hepatopancreas SOD activity and T-AOC while decreasing MDA content, likely by improving water quality parameters and thereby enhancing shrimp immune capacity.

Under the conditions of this study: (1) *B. licheniformis* enhances *L. vannamei* immunity whether administered through feed or directly into culture water; (2) Considering both growth performance and immunity, *B. licheniformis* supplementation is superior to Probiotic A or Probiotic B; and (3) Based on the combined results, the optimal dietary inclusion level of *B. licheniformis* for *L. vannamei* should be 0.3–3.0 mg/kg.

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