

Phycocyanin from *Spirulina Platensis* Promotes Growth, Immunity, and Resistance to *Vibrio parahaemolyticus* in Shrimp

Waheeb Sami Aggad^{1*}, Anwar Bushnaq², Abeer S. Albalawi³, Kholoud A Seferji³, Asmaa Sayed Abdelgeliel⁴, Moaheda E.H. Eissa⁵, El-Sayed Hemdan Eissa^{6*}

¹Division of Anatomy, Department of Basic Medical Sciences, College of Medicine, University of Jeddah, Jeddah 23890, Saudi Arabia.

²Department of Biology, College of Science in Yanbu, Taibah University, Yanbu Governorate, Saudi Arabia.

³Department of Biology, College of Science, Taibah University, Madinah, 42353, Saudi Arabia.

⁴Department of Botany & Microbiology, Faculty of Science, Qena University, Qena 83523, Egypt.

⁵Biotechnology Department, Fish Farming and Technology Institute, Suez Canal University, Ismailia 41522, Egypt.

⁶Fish Research Centre, Faculty of Environmental Agricultural Sciences, Arish University, El-Arish, Egypt.

*Corresponding to: Waheeb Sami Aggad (E-mail: wsaggad@uj.edu.sa (W.S.A)); El-Sayed Hemdan Eissa (E-mail: sayedhemd@gmail.com (E.H.E.))

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Abstract

Objective: The present study evaluated whether dietary phycocyanin extracted from *Spirulina platensis* could improve growth performance, feed utilization, intestinal microbial balance, immune response, antioxidant status, and resistance to *Vibrio parahaemolyticus* infection in Pacific white shrimp (*Litopenaeus vannamei*).

Methods: A feeding experiment was conducted for 70 days using 240 shrimp randomly assigned to four dietary treatments with three replicates each. Experimental diets included a control diet without supplementation and diets supplemented with phycocyanin at 0.1, 0.2, and 0.3 g/kg diet. Growth indices, feed efficiency, intestinal bacterial populations, immune parameters, antioxidant enzyme activities, and the expression of selected immune- and antioxidant-related genes were analyzed. At the end of the feeding period, shrimp were challenged with *Vibrio parahaemolyticus* to evaluate disease resistance.

Results: Shrimp receiving phycocyanin-supplemented diets showed significantly better growth performance and feed utilization than the control group. Improvements were also observed in survival rate and intestinal microbial composition, with higher counts of beneficial bacteria and lower counts of harmful microorganisms. Dietary supplementation enhanced immune responses, as indicated by increased lysozyme and prophenoloxidase activities. Antioxidant enzyme activities, particularly catalase and superoxide dismutase, were significantly elevated in supplemented groups. In addition, the expression levels of immune- and antioxidant-related genes were upregulated in shrimp fed phycocyanin-containing diets. Following bacterial challenge, supplemented groups exhibited lower mortality rates than the control group, with the strongest protective effect observed at the highest supplementation level.

Conclusion: The findings indicate that phycocyanin derived from *Spirulina platensis* can serve as a beneficial dietary supplement for *Litopenaeus vannamei*. Supplementation improved growth, supported intestinal microbial stability, enhanced immune and antioxidant responses, and increased resistance to *Vibrio parahaemolyticus* infection. These results suggest that phycocyanin may represent a useful natural feed additive for sustainable shrimp aquaculture.

Keywords: Algae, penaeidae, *Vibrio parahaemolyticus*, health status, colony count, microbial

Introduction

Shrimp aquaculture is an important contributor to global aquatic-food production, and Pacific white shrimp, *Litopenaeus vannamei*, is one of the main penaeid species used in intensive and semi-intensive systems.¹ The high productivity of this species depends not only on feed efficiency but also on the capacity of shrimp to tolerate repeated husbandry stressors. Functional feeds have therefore become a practical strategy for improving resilience, provided that their biological effects are supported by clear dose-response data and by reliable immune and health indicators.

Vibriosis remains a major constraint in shrimp farming. Pathogenic *Vibrio* spp., including *V. parahaemolyticus*, can cause severe disease, and some strains are associated with acute hepatopancreatic necrosis disease through plasmid-borne virulence factors.^{2,3} Although antimicrobial treatments have historically been used to manage bacterial outbreaks, their overuse in aquaculture is linked with antimicrobial-resistance risks and environmental concerns.^{4,5} Consequently, nutrition-based disease prevention is increasingly favored over repeated therapeutic intervention.

Microalgae and their pigments are attractive feed additives because they may provide bioactive proteins, pigments, vitamins, minerals, and antioxidant compounds. *Spirulina platensis*, now widely referred to as *Arthrospira platensis*, contains phycobiliproteins such as C-phycocyanin. This pigment has documented radical-scavenging, anti-inflammatory, and antimicrobial activities, which may be relevant to animals exposed to oxidative and infectious stress.^{6–8} In aquatic animals, dietary phycocyanin has been associated with changes in growth, digestive enzyme activity, immune biomarkers, and antioxidant responses.^{9,10}

Despite these findings, the biological response to microalgal additives is not always consistent across species, life stages, feed matrices, or inclusion levels. Whole *Spirulina* meal and purified or semi-purified phycocyanin may also differ in bioavailability and mode of action. For this reason, studies that combine growth performance, bacterial-count data, innate immune markers, antioxidant indices, gene expression, and pathogen challenge within the same feeding trial can provide a clearer assessment of practical value.

The present study was therefore designed to evaluate the effects of dietary PSP on growth performance, feed utilization,

intestinal bacterial counts, innate immunity, antioxidant status, immune- and antioxidant-related gene expression, and resistance of *L. vannamei* to *V. parahaemolyticus*.

Materials and Methods

Experimental Design

L. vannamei juveniles were obtained from a private farm and acclimated for 15 days before the feeding trial. A total of 240 shrimp (average initial body weight, approximately 4.20 ± 0.00 g) were randomly distributed into 12 cement ponds ($1 \times 1 \times 1$ m), with 20 shrimp per pond. Four diets were prepared using the same basal formulation and supplemented with PSP at 0.0 (control; PSP0.0), 0.1 (PSP0.1), 0.2 (PSP0.2), or 0.3 (PSP0.3) g/kg diet. Each treatment had three replicate ponds. Shrimp were fed to apparent satiation three times daily (08:00, 12:00, and 16:00) for 70 days. The composition of the basal diet is presented in Table 1. Approximately 25% of the water was exchanged daily. Water quality was maintained at $26.33 \pm 0.33^\circ\text{C}$, pH 7.93 ± 0.03 , salinity 32.67 ± 0.33 ppt, dissolved oxygen 5.89 ± 0.03 mg/L, unionized ammonia 0.52 ± 0.00 mg/L, nitrite 0.04 ± 0.00 mg/L, and nitrate 0.02 ± 0.00 mg/L under natural photoperiod conditions.

Growth Parameters

All shrimp in each experimental group were individually weighed using an electronic analytical balance with 0.001 g precision. Growth performance, feed utilization, and survival were calculated as follows:

Weight gain (g) = FW – IW; Weight gain (%) = [(FW – IW) / IW] $\times$ 100.

Specific growth rate (SGR; %/day) = $100 \times [\ln(\text{FW}) - \ln(\text{IW})] / \text{days}$.

Feed conversion ratio (FCR) = FI / WG.

Average daily gain (ADG; g/day) = (FW – IW) / days.

Survival (%) = (final shrimp number / initial shrimp number) $\times$ 100. FW = final weight; IW = initial weight; FI = feed intake; WG = weight gain.

Intestinal Bacterial Counts

Intestinal samples (six shrimp per treatment) were aseptically collected and homogenized in 1 mL sterile saline. For serial

dilution, 500 μL of the homogenate was transferred into 5 mL sterile saline and diluted stepwise to the fifth dilution. One milliliter from each dilution was plated on the appropriate medium. Nutrient agar was used for total aerobic bacteria and incubated aerobically at 30°C for 24–48 h. MacConkey agar was used for enteric bacterial counts and incubated aerobically at 37°C for 48 h. MRS agar was used to estimate lactic-acid/probiotic-associated bacteria according to APHA (2005),¹¹ with incubation at 37°C for 48–72 h. Tryptic sulfite cycloserine agar was used for *Clostridium perfringens* counts and incubated anaerobically at 37°C for 48 h.

Sampling and Immune Parameter Analysis

Hemolymph samples (250 μL) were collected from the ventral sinus of nine randomly selected shrimp per treatment using a 25-gauge needle and a 1 mL syringe containing pre-cooled anticoagulant (4°C), following the approach described by Liu and Chen (2004).¹²

For total hemocyte count (THC), 50 μL of anticoagulated hemolymph was mixed with 150 μL of 4% formaldehyde. A 20 μL aliquot was counted using a Neubauer hemocytometer under light microscopy, and THC was calculated as cells/mL using the equation: count $\times 10^4 \times$ dilution factor. Lysozyme activity was measured by the turbidimetric method described by Engstad et al. (1992).¹³ Phenoloxidase activity was measured spectrophotometrically using a modified method based on El Asely et al. (2010).¹⁴ Nitroblue tetrazolium (NBT) activity was determined according to Siwicki et al. (1985).¹⁵

Antioxidant Activity and Lipid Peroxidation

Commercial colorimetric assay kits (BioVision, Inc., California, USA) were used to measure glutathione peroxidase (GPx; K762-100), catalase (CAT; K274-100), superoxide dismutase (SOD; K335-100), and malondialdehyde (MDA), a lipid-peroxidation marker. All assays were conducted according to the manufacturer's instructions.

Gene Expression Methodology

Total RNA Extraction

Total RNA was extracted from hepatopancreas tissue from each treatment group using an RNA purification kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol. RNA purity was assessed by the A260/A280 ratio using a NanoDrop Lite spectrophotometer (Thermo Scientific, USA). Complementary DNA (cDNA) was synthesized from 1 μg total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen, USA) and oligo-dT primers. The cDNA samples were stored at -20°C until quantitative PCR analysis.

Real-time Quantitative PCR (RT-qPCR)

The relative mRNA expression of immune-related genes (prophenoloxidase and lysozyme) and antioxidant-related genes (SOD and CAT) was quantified using a SensiFast SYBR Lo-ROX kit (Bioline, London, UK). Primer sequences are listed in Table 2. The thermal profile was 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 62°C for 30 s, with a final melting step at 85°C for 5 min. Gene expression was normalized against β -actin and calculated using the $2^{-\Delta\Delta\text{CT}}$ method.¹⁶

Table 1. Ingredients and proximate chemical composition of the basal experimental diet

Ingredients	g/kg diet	Proximate chemical analysis	
Shrimp meal	250.0	Dry matter	90.68
Fish meal	300.0	Crude protein ($N \times 6.25$)	38.34
Soybean meal	150.0	Crude fat	10.73
Rice bran	70.0	Crude fiber	1.71
Wheat flour	120.0	Ash	6.05
Fish oil	60.0		
CMC	10.0		
Vitamin mix1	20.0		
Mineral mix2	20.0		

Challenge Assay

After the 70-day feeding trial, 80 shrimp were randomly selected and distributed into eight transparent plastic tanks (10 shrimp per tank). A *V. parahaemolyticus* stock culture was grown in brain heart infusion broth at 25°C for 24 h and centrifuged at 1000 × g. The supernatant was discarded, and the bacterial pellet was resuspended in sterile saline solution (1.5% NaCl). The stock suspension was adjusted spectrophotometrically at OD600 to 1 × 10⁷ CFU/mL, and then diluted with sterile saline to prepare the working inoculum used for challenge (1 × 10⁴ CFU/mL). Each shrimp was injected with the working inoculum, and cumulative mortality was recorded for 6 days after challenge.

Statistical Analysis

Data were analyzed using SPSS version 26. One-way analysis of variance (ANOVA) was used to test treatment effects, followed by Duncan's multiple range test when significant differences were detected. Differences were considered significant at *P* < 0.05. Results are presented as mean ± SEM.

Results

Growth and Survival

After 70 days, shrimp fed PSP-supplemented diets had significantly higher final body weight, weight gain, specific growth rate, and biomass than shrimp fed the control diet (*P* < 0.05; Table 3). FCR was significantly lower in all PSP-supplemented groups than in the control group (*P* < 0.05). Feed intake did not differ significantly among most groups. The PSP0.3 group showed the highest final body weight, weight gain, SGR, and ADG, whereas survival and biomass were highest in the PSP0.2 and PSP0.3 groups.

Intestinal Microbial Counts

Dietary PSP significantly affected intestinal bacterial counts (Figure 1). Counts of total aerobic bacteria, enteric bacteria, and *C. perfringens* decreased as dietary PSP increased (*P* < 0.05). In contrast, counts on MRS agar increased with PSP supplementation (*P* < 0.05), with the highest values observed in the PSP0.3 group.

Table 2. Forward and reverse primer sequences used for qPCR analysis

Gene	Forward primer	Reverse primer	Product size (bp)	Accession no / ref.
Immunity				
Prophenoloxidase	GAGATCGCAAGGGAGAACTG	CGTCAGTGAAGTCGAGACCA	243	XM_027381752.1 Wang et al. (2010) ¹⁷
Lysozyme	GAAGCGACTACGGCAAGAAC	AACCGTGAGACCAGCACTCT	216	XM_027352840.1 Wang et al. (2010) ¹⁷
Antioxidant				
Catalase (CAT)	TCAGCGTTTGGTGGAGAA	GCCTGGCTCATCTTTATC	147	XM_027383088.1 Liu et al. (2023) ¹⁸
Superoxide dismutase (SOD)	ATCCACCACACAAAGCATCA	AGCTCTCGTCAATGGCTTGT	229	XM_027376216.1 Wang et al. (2010) ¹⁷
β-actin	CTTGTGTGCGACAATGGCTC	TCGATGGGGTACTTGAGGGT	194	XM_027371505.1 (Liu et al., 2023) ¹⁸

Table 3. Growth performance and feed utilization of *L. vannamei* fed diets supplemented with graded levels of PSP for 70 days (means ± SEM)

Parameters	PSP0.0	PSP0.1	PSP0.2	PSP0.3
Initial shrimp weight (g)	4.17 ± 0.03 ^a	4.17 ± 0.03 ^a	4.20 ± 0.00 ^a	4.17 ± 0.03 ^a
Final shrimp weight (g)	11.63 ± 0.23 ^d	13.33 ± 0.09 ^c	14.67 ± 0.27 ^b	15.40 ± 0.06 ^a
Weight gain (g)	7.47 ± 0.20 ^d	9.17 ± 0.07 ^c	10.47 ± 0.27 ^b	11.23 ± 0.03 ^a
Feed intake	14.58 ± 0.12 ^a	14.58 ± 0.12 ^a	14.70 ± 0.00 ^a	14.58 ± 0.12 ^a
Feed conversion ratio (FCR)	1.96 ± 0.04 ^a	1.59 ± 0.01 ^b	1.41 ± 0.04 ^c	1.30 ± 0.01 ^d
Specific growth rate (SGR; %/day)	1.47 ± 0.02 ^d	1.66 ± 0.01 ^c	1.79 ± 0.03 ^b	1.87 ± 0.01 ^a
ADG	0.11 ± 0.00 ^c	0.13 ± 0.00 ^b	0.15 ± 0.01 ^a	0.16 ± 0.00 ^a
Initial shrimp number	20.00 ± 0.00	20.00 ± 0.00	20.00 ± 0.00	20.00 ± 0.00
Final shrimp number	17.00 ± 0.58 ^c	18.00 ± 0.58 ^{cb}	19.67 ± 0.33 ^a	19.33 ± 0.33 ^{ab}
Shrimp biomass (g/m ³)	197.60 ± 5.25 ^c	240.07 ± 8.86 ^b	288.57 ± 9.35 ^a	297.70 ± 4.19 ^a
Survival rate (%)	85.00 ± 2.89 ^c	90.00 ± 2.89 ^{cb}	98.33 ± 1.67 ^a	96.67 ± 1.67 ^{ab}

Immunological Parameters

THC increased significantly in the hemolymph of shrimp fed PSP-supplemented diets, and the response increased with the inclusion level ($P < 0.05$; Figure 2). PO activity was significantly higher in the PSP0.3 group than in all other groups ($P < 0.05$). Lysozyme activity also increased, with the highest activity detected in shrimp fed 0.3 g PSP/kg diet. NBT activity was significantly higher in all PSP-fed groups than in the control group ($P < 0.05$).

Antioxidants Status and Lipid Peroxidation

As shown in Figure 3, SOD, CAT, and GPx activities increased significantly in shrimp fed 0.2 and 0.3 g PSP/kg diet compared

with the control group ($P < 0.05$). CAT activity was highest in the PSP0.3 group and differed significantly from all other treatments. MDA levels decreased significantly in the PSP0.2 and PSP0.3 groups relative to the control group ($P < 0.05$), indicating reduced lipid peroxidation.

Gene Expression Analysis

Gene expression analysis showed significant upregulation of prophenoloxidase and lysozyme in PSP-supplemented groups compared with the control group ($P < 0.05$; Figure 4). Expression of CAT and SOD was also significantly higher in PSP-fed shrimp, with a dose-related increase across treatments.

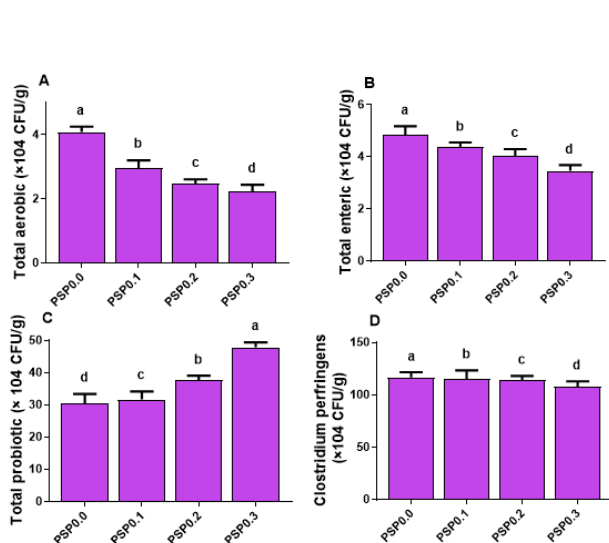


Fig. 1 Intestinal bacterial counts of *L. vannamei* fed diets supplemented with graded levels of PSP for 70 days. Different letters indicate significant differences among treatments ($P < 0.05$).

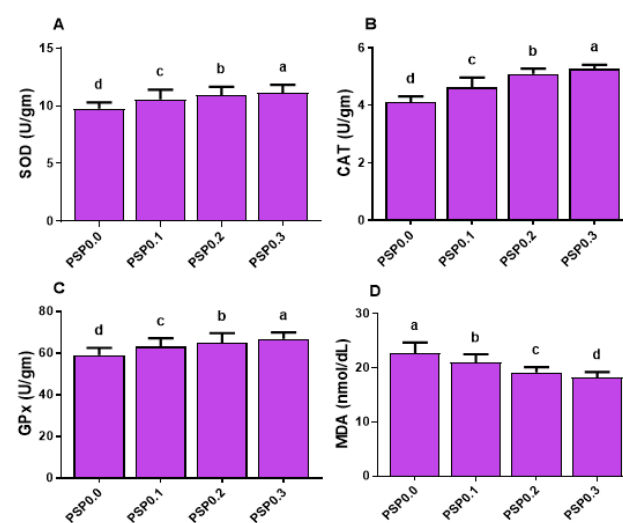


Fig. 3 Antioxidant status and lipid peroxidation of *L. vannamei* fed diets supplemented with graded levels of PSP for 70 days. Different letters indicate significant differences among treatments ($P < 0.05$).

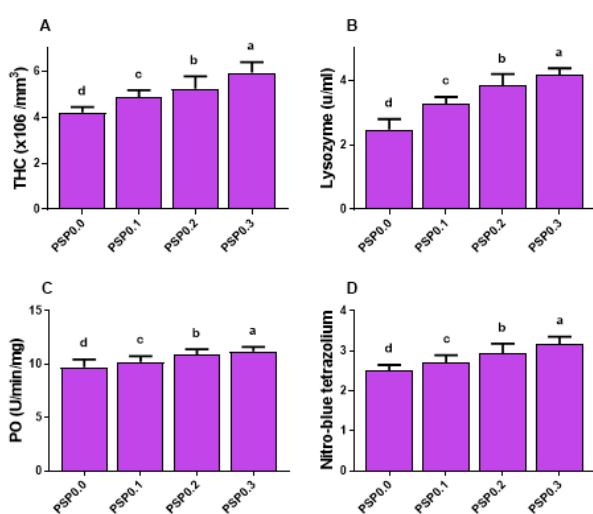


Fig. 2 Immunological parameters of *L. vannamei* fed diets supplemented with graded levels of PSP for 70 days. Different letters indicate significant differences among treatments ($P < 0.05$).

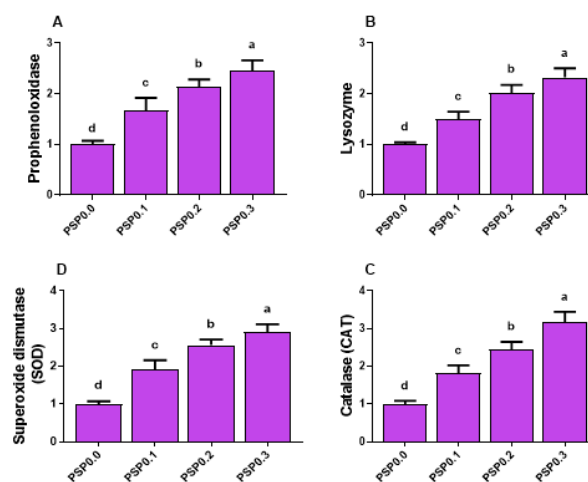


Fig. 4 Relative gene expression of immune- and antioxidant-related genes in *L. vannamei* fed diets supplemented with graded levels of PSP for 70 days. Different letters indicate significant differences among treatments ($P < 0.05$).

Challenge Assay Against *Vibrio parahaemolyticus*

Following challenge with *V. parahaemolyticus*, cumulative mortality was lower in all PSP-fed groups than in the control group (Figure 5). Mortality declined with increasing PSP level: 60% in PSP0.0, 45% in PSP0.1, 30% in PSP0.2, and 25% in PSP0.3.

Discussion

The present study shows that dietary PSP improved growth and feed utilization of *L. vannamei* under the tested conditions. The reduction in FCR together with higher final weight and biomass suggests that PSP did not merely increase feed intake but may have improved nutrient utilization. This interpretation is consistent with studies showing that *Spirulina*-derived products can improve shrimp performance and immune status^{19,20} and with a meta-analysis indicating that *Spirulina* meal can support feed efficiency in fish and shrimp.²¹ However, the response to microalgal additives is not universal. Gora et al. (2019),²² for example, reported limited growth benefits when crude pigment-rich microalgal extracts were fed to Asian seabass larvae. Such differences may reflect species-specific digestive physiology, additive purity, age, basal diet formulation, and the distinction between whole biomass and isolated phycocyanin.

Several mechanisms may explain the growth response observed here. Phycocyanin contains chromophore-bearing protein subunits with antioxidant properties, and *Spirulina*-derived products may supply compounds that influence digestive enzyme activity and intestinal microbial balance.^{6,9} In shrimp, improved gut conditions can increase nutrient availability and reduce the energetic cost of immune activation. Nevertheless, the present study did not directly measure digestive enzymes, intestinal morphology, or nutrient digestibility; therefore, these mechanisms should be considered plausible explanations rather than proven pathways.

The intestinal bacterial-count results support a possible microbiota-related contribution to health status. PSP decreased culturable total aerobic, enteric, and *C. perfringens* counts while increasing colonies on MRS agar. Phycocyanin and *Arthrospira*-derived compounds have documented antimicrobial activity *in vitro*,^{8,23} which may partially explain the lower counts of potentially undesirable bacteria. However, culture-based enumeration provides only a coarse view of the intestinal community. MRS counts should not be interpreted as definitive probiotic identification without

molecular confirmation, and future work should use 16S rRNA sequencing or targeted qPCR to determine whether PSP selectively enriches beneficial taxa.

The immune results indicate activation of key innate defense components rather than a nonspecific stress response alone. Hemocytes are central to crustacean cellular immunity, while the prophenoloxidase cascade contributes to melanization, encapsulation, and pathogen restriction.^{24,25} The parallel increases in THC, PO activity, lysozyme activity, NBT response, and expression of prophenoloxidase and lysozyme suggest that PSP enhanced both cellular and humoral immune capacity. Similar immune stimulation after exposure to *Spirulina* extracts has been reported in *L. vannamei* challenged with *V. alginolyticus*,¹⁹ and dietary C-phycocyanin improved immune indices and survival in *P. vannamei*.¹⁰

The antioxidant data provide a complementary explanation for the challenge results. SOD, CAT, and GPx act sequentially to limit reactive oxygen species, whereas MDA is a marker of lipid peroxidation. PSP increased antioxidant enzyme activities and upregulated SOD and CAT expression while reducing MDA, especially at 0.2 and 0.3 g/kg. This pattern suggests that PSP improved the antioxidant defense network and reduced membrane lipid damage. Phycocyanin can scavenge reactive species and modulate oxidative and inflammatory processes.^{6,26} In shrimp, better antioxidant capacity may preserve hemocyte function during bacterial challenge, thereby supporting immune effectiveness rather than acting independently of immunity.

The *V. parahaemolyticus* challenge supports the biological relevance of the immunological and antioxidant findings. Mortality was 60% in the control group but decreased to 25–30% in shrimp fed 0.2–0.3 g PSP/kg. These results are consistent with previous reports that *Spirulina*-derived extracts or C-phycocyanin can improve resistance to *Vibrio* infection in shrimp.^{10,19} The revised challenge protocol clarifies that the 1×10^7 CFU/mL suspension was the stock culture and that shrimp were injected with a diluted 1×10^4 CFU/mL working inoculum. This clarification is important because inconsistent challenge-dose reporting can lead to misinterpretation of virulence and survival data.

Although PSP0.3 produced the strongest overall response in this trial, the data do not establish an optimum dietary inclusion level beyond the tested range. The study also has limitations: bacterial counts were culture based; histological and digestive-enzyme endpoints were not included; the challenge test was short-term; and performance was evaluated under controlled experimental conditions rather than commercial farm conditions. Therefore, PSP should be viewed as a promising additive that warrants further validation, including broader dose-response testing, microbial-community profiling, and cost-benefit analysis.

Conclusion

Under the conditions of this 70-day trial, dietary PSP at 0.1–0.3 g/kg improved growth performance, feed utilization, selected innate immune markers, antioxidant status, and post-challenge survival of *L. vannamei*, with the most consistent responses observed at 0.2 and 0.3 g/kg. The results support further evaluation of PSP as a functional feed additive for shrimp aquaculture. However, conclusions should be limited to the tested conditions until farm-scale trials, microbiome

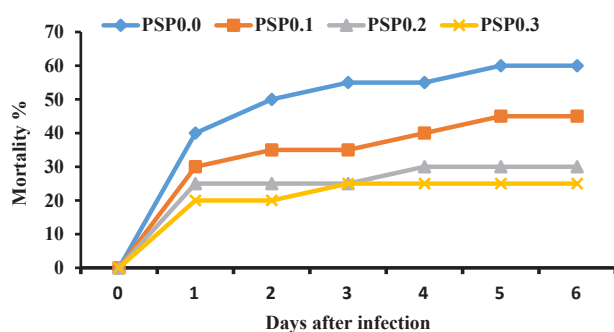


Fig. 5 Cumulative mortality of *L. vannamei* fed the control and PSP-supplemented diets for 70 days and challenged with *V. parahaemolyticus* for 6 days.

sequencing, digestibility measurements, and additional pathogen-challenge studies confirm the practical dose and mechanism of action.

Conflicts of Interest

None. ■

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