Alginate and Spirulina Vast-Promoting Immune Activity and Resistance to Diverse Vibrio Species in *Litopenaeus vannamei* Culture

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Abstract

Litopenaeus vannamei is a key species in global aquaculture; however, its susceptibility to vibriosis presents a persistent challenge. The aim of this study was to investigate the effects of dietary supplementation with alginate and Spirulina hot water extract (SWE) on the immune response and gene expression associated with pathogen resistance. Shrimp fed diets containing 3 g·kg $^{-1}$ of alginate and 5 mg·kg $^{-1}$ of SWE exhibited marked improvements in immune parameters, including total hemocyte count, phagocytic activity, phagocytic index, phenoloxidase activity, and superoxide dismutase levels, as measured on days 3 and 7 (P< 0.05). Following challenge with Vibrio parahaemolyticus, V. vulnificus, and V. harveyi, treated shrimp demonstrated a 90% survival rate compared to 0% in the control group. Gene expression analysis revealed a 2-fold and 1.5-fold upregulation of prophenoloxidase (proPO) and lipopolysaccharide β-glucan binding protein (LGBP), respectively, indicating the activation of the proPO cascade and pathogen recognition pathways. These results provide direct evidence that alginate and SWE act as immunostimulants by modulating hemocyte function and immune-related gene expression. The findings of the present study suggest that oral supplementation with these natural bioactive compounds can serve as a functional dietary strategy to enhance disease resilience in shrimp and promote more sustainable aquaculture systems, in seven days period.

Keywords: *Alginate, Immune, Gene Expression, Shrimp, Spirulina, Vibrio*

Introduction

The cultivation of *Litopenaeus vannamei* has experienced rapid growth, reaching 223 billion tons and constituting 59% of global crustacean production (FAO, 2024), with Asia contributing approximately 80% (Diwan *et al.*, 2022). However, intensive aquaculture practices have led to frequent disease outbreaks, predominantly caused by *Vibrio* species (Rusmana *et al.*, 2021; Yudiati *et al.*, 2021c; Zhang *et al.*, 2021; Azhar and Yudiati, 2023). These outbreaks compromise shrimp immunity, diminish disease resistance, and impair metabolic functions, including protein synthesis and gene expression (Suryono *et al.*, 2024), leading to substantial economic losses (Asche *et al.*, 2021). While antibiotics remain a widely used solution due to their affordability (Shao *et al.*, 2021), their overuse has led to the emergence of antibiotic-resistant pathogens, weakened host immunity (Azhar and Yudiati, 2023; Utami *et al.*, 2025), and resulted in harmful residues in shrimp products (Amalia *et al.*, 2024), in addition to disrupting gut microbiota (Sapkota *et al.*, 2008). Notably, *V. parahaemolyticus*, *V. vulnificus*, and *V. harveyi* from Indonesian ponds have shown resistance to beta-lactams, azithromycin, and ciprofloxacin (Yudiati *et al.*, 2021c). Other *Vibrio* species, including *V. alginolyticus* and *V. owensii*, exhibit multidrug resistance (Isnansetyo *et al.*, 2022; Amalia *et al.*, 2024). Although agents such as pine oil and chloroxylenol provide some inhibition (Subagiyo *et al.*, 2020), antimicrobial use in aquaculture is projected to rise to 13,600 tons by 2030, with Indonesia ranking third in Asia (Schar *et al.*, 2020).

To address this issue, immunostimulants, particularly from seaweeds, offer a promising and eco-friendly approach. A study examining the extract

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of the marine red alga *Galaxaura oblongata* for its angiotensin-converting enzyme (ACE) inhibitory properties (Duat and Gelani, 2023) and the role of sulfated polysaccharides against SARS-CoV-2 (Herida *et al.*, 2024) has been reported. Furthermore, alginate from *Sargassum* spp. enhances shrimp innate immunity and modulates gene expression in 14 days (Yudiati *et al.*, 2019; Suryono *et al.*, 2024), showing efficacy against virulent *V. parahaemolyticus* (Azhar and Yudiati, 2023), improving stress tolerance (Yudiati *et al.*, 2020a; Yudiati *et al.*, 2022a), and acting as a prebiotic (Yudiati *et al.*, 2020b; Yudiati *et al.*, 2021b). *Spirulina platensis* is a sustainable microalga with a high protein content (460–630 g·kg^{-1}), surpassing that of soybeans and wheat (Guo *et al.*, 2022). Similar to other rich pigmented (chlorophyll a, b, and carotenoids) marine microalgae, such as *Nannochloropsis* sp. (Yudiati *et al.*, 2022b), *Spirulina platensis* is rich in essential amino acids and pigments, including chlorophylls, carotenoids, and phycobiliproteins such as C-phycocyanin, phycoerythrin, and allophycocyanin (Hidayati *et al.*, 2020; Lafarga *et al.*, 2020; Rodríguez-Olague *et al.*, 2021). The phenolic compounds and phycocyanins extracted from *Spirulina* exhibit antioxidant properties, contributing to the reduction of reactive oxygen species through superoxide dismutase (SOD) activity (Xu *et al.*, 2025). Similar to alginate, *Spirulina* hot water extract (SWE) enhances immunity and disease resistance in *L. vannamei* in 14 days (Yudiati *et al.*, 2024a; Yudiati *et al.*, 2024b). Lin *et al.* (2010) also reported improved immune recovery and gene expression in shrimp treated with SWE following pH stress. Additionally, *S. platensis* has been shown to enhance immunity and resistance in Nile Tilapia (*Oreochromis niloticus*) (Sherif *et al.*, 2024), Stinging Catfish (*Heteropneustes fossilis*) (Zahan *et al.*, 2024), and Goldfish (*Carassius auratus*) (Yousefi *et al.*, 2022).

This study aimed to investigate the seven-day administration of combined dietary effects of alginate from tropical *Sargassum* spp. and *Spirulina* water extract on the immune response, gene expression (proPO, LGBP). To firm the disease resistance of *L. vannamei* after diet supplemental stimulation, the challenge test against *V. parahaemolyticus, V. vulnificus, and V. harveyi* was also investigated. Immune parameters, gene activity, and survival rates were assessed to evaluate their potential as sustainable health enhancers in shrimp aquaculture.

Materials and Methods

Extraction of sodium alginate from Sargassum sp. and Spirulina via the hot water method

Alginate was synthesized according to the methodology outlined by Yudiati and Isnansetyo (2017). Brown algae (*Sargassum* spp.) collected from Teluk Awur Bay, Jepara, Central Java, Indonesia were were air-dried and processed by crushing them with a commercial blender. *Sargassum* sp. powder (40 g) was incorporated into 1000 mL of distilled water, along with 50 g sodium carbonate and 18.617 g EDTA. The pH was adjusted to 8.5, and the mixture was stirred for 24 h before being filtered. KCl was subsequently added to the filtrate in conjunction with absolute ethanol to facilitate precipitation. The solution underwent centrifugation for 6 min at 4000 rpm to facilitate the separation of the liquid from the pellet. The pellets were allowed to remain in a drying cabinet for 24 h. The Fourier transform-infrared spectra indicated that the obtained sodium alginate (Alg) matched the standard alginate reference material (Sigma, St. Louis, MO, USA), which has a molecular weight of 217.5 KDa and an acetylation level of 89.95% (Yudiati *et al.*, 2018).

SWE was generated as outlined in Yudiati *et al.* 2024b. Dried Spirulina (10 g) was combined with 250 mL of distilled water and subsequently heated at 70°C for 1 h. The extract underwent centrifugation for 15 min at 3500 rpm. Alginate and *Spirulina* extract were solubilized in 100 µL of distilled water. The solution was combined with one g of shrimp feed (Feng Li®) and subsequently dried in an oven at 40°C for 2 h (Azhar and Yudiati, 2023; Azhar *et al.*, 2024).

Preparation of Vibrio species

Vibrio vulnificus, *V. parahaemolyticus*, and *V. harveyi* were sourced from a collection at CV. Hadid Mukti Karya in Semarang, Indonesia. The *Vibrio* species were cultured in alkaline peptone water (Merck, Darmstadt, Germany). One isolate of *Vibrio* spp. was introduced to the liquid alkaline peptone water and allowed to incubate for 24 h. The following day, the culture was subjected to centrifugation at 4200 rpm for 15 min. Sterile saline was utilized for the dilution of the pellet. The density of *Vibrio* spp. in the shrimp challenge test was quantified using a spectrophotometer at 600 nm, with an absorbance adjusted to 2.0, corresponding to a density of 10^9 CFU.mL^{-1} (Kongchum *et al.*, 2022).

Experimental design

Laboratory experiments were performed in a completely randomized experimental design. Based on previous works (Yudiati *et al.*, 2024b), the following treatments were used: Alg (3.0 g·kg^{-1}), SWE (5.0 mg·kg^{-1}), a combination of Alg + SWE (3.0 g·kg^{-1} Alg + 5.0 mg·kg^{-1} SWE), and a control without supplementation. The independent variable was the concentration of the supplemental extract, whereas the dependent variable was the levels of cellular compounds. THC, PA, and PI; humoral compounds

such as PO and SOD, and immune gene expression of prophenoloxidase (proPO) and lipopolysaccharide beta glucan-binding protein (LGBP) were evaluated.

Feeding trial and challenge test

The shrimps utilized in this study were reared and cultivated at the Center for Brackish Water Aquaculture Development located in Jepara, Indonesia. The experiment was performed in a semi-outdoor setting. A total of 300 shrimp (15.3 ± 1.2 g) were transferred to media in 12 tanks (100 L) at a density of 25 individuals per tank, and were fed with Feng Li Gold GR-3L shrimp pellets. Shrimp rearing occurred over a period of 7 days, with a feeding frequency of four times daily (04:00, 10:00, 16:00, and 22:00) at a rate of 4% biomass per day (Yudiati *et al.*, 2019; Azhar and Yudiati, 2023). Siphoning was conducted bi-daily, specifically in the morning and afternoon, prior to feeding. The rearing medium was substituted with 30% seawater. The survival rates of shrimp in both the control and all treatment groups at the conclusion of the feeding trial were 100%. During maintenance, the water temperature was maintained between 29 and 31°C, with a pH of 7.7 to 7.9, salinity levels of 29 to 31 ppt, and dissolved oxygen concentrations ranging from 5.22 to 5.33 mgL^{-1}.

At the conclusion of the 7-day rearing period, ten shrimps from each group underwent a *Vibrio* challenge test, with three replicates conducted for each treatment group. The water volume was calibrated to 50 L with gentle aeration. The shrimps received no feed during the challenge tests. The survival rate of the shrimp was observed and recorded from day 1 until it reached 0%. Shrimp were exposed to 3×10^6 CFU.mL^{-1} of three *Vibrio* species, including *V. parahaemolyticus*, *V. vulnificus*, and *V. harveyi* via the immersion method (Yudiati *et al.*, 2021a; Yudiati *et al.*, 2021c; Azhar and Yudiati, 2023). Survival rates were systematically monitored and documented on a daily basis.

Evaluation of immune parameters in Litopenaeus vannamei: total hemocyte count (THC), phagocytic activity (PA), and phagocytic index (PI)

Total hemocyte count (THC), phagocytic activity (PA), and phagocytic index (PI) assays were conducted as outlined in previous research (Azhar and Yudiati, 2023). Three individual shrimps were randomly sampled from each container. Hemolymph (10 µL) was diluted with 40 µL of phosphate-buffered saline (PBS) (Yudiati *et al.*, 2016). The hemolymph was subsequently introduced into a hemocytometer and enumerated with the aid of a microscope.

Hemolymph (10 µL) and 30 µL PBS were combined for both PA and PI analyses. Subsequently, 20 µL of 10^8 cells.mL^{-1} formalin-killed *Bacillus* sp. was incorporated into the mixture. Following the gentle application of the mixture, 5 µL mixture was fixed using absolute ethanol, stained with 10% Giemsa, and allowed to stand for 20 min. The slides were subsequently rinsed with distilled water and dried. The slides were imaged and analyzed using a light microscope. THC, PA, and PI were evaluated at baseline (day 0) and subsequently on days 4 and 7 of rearing.

PO enzyme activity assay

Three individual shrimps were randomly sampled from three separate containers, representing each replicate. PO activity was quantified spectrophotometrically by monitoring the formation of dopachrome from L-DOPA (Liu *et al.*, 2004; Yudiati *et al.*, 2016; Azhar *et al.*, 2024). Hemolymph (100 µL) was combined with 100 µL PBS in a 1:1 ratio and centrifuged at 700 ×*g* for 20 min at 4°C, after which the supernatant was removed. The pellet underwent a second centrifugation in 100 µL cacodylate buffer. Trypsin (100 µL) was added to the supernatant, mixed, and resuspended prior to a 10-min-incubation, after which 50 µL L-DOPA was introduced. Absorbance was recorded at an optical density of 490 nm using an R-Biopharm Well Reader (Germany). PO enzyme activity was evaluated on days 0, 4, and 7 of the rearing period.

SOD enzyme activity assay

The activity of SOD was assessed by quantifying riboflavin production through the use of nitro blue tetrazolium (Beauchamp and Fridovich, 1971). Three shrimp samples were collected randomly from three distinct tanks. Hemolymph (30 µL) was combined with 270 µL phosphate buffer in a 1:9 ratio, then subjected to centrifugation at 6000 ×*g* for 7 min at 4°C. The supernatant was subsequently heated for 5 min at 65°C. Subsequently, 50 µL nitro blue tetrazolium was introduced. Optical density was assessed at 630 nm using a 96-well reader (R-Biopharm, Darmstadt, Germany). The activity of the SOD enzyme was evaluated on days 0, 4, and 7 of the rearing period.

Immune-Related Gene Expression

On day 7 of rearing, shrimp hemolymph from three treatments and one control was utilized to evaluate the expression of the immune-related genes (proPO and LGBP). Three shrimps were randomly sampled from three distinct tanks. RNA was extracted from shrimp hemolymph using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) in accordance with the manufacturer's guidelines. Total RNA underwent reverse transcription to cDNA utilizing

an AMV Reverse Transcriptase Kit (Promega, Madison, WI, USA) at 42°C for 30 min, followed by inactivation of the transcriptase enzyme at 94°C for 5 min with SYBR Green Master Mix (Kapa SYBER FAST qPCR Master Mix). The expression levels of proPO and LGBP were analyzed in relation to the internal control β-actin (Kapa Biosystems, Woburn, MA, USA). Quantitative analysis was conducted utilizing a 7500 qRT-PCR (AB Instruments, Applied Biosystems, Foster City, CA, USA) along with specific primers (see Table 1). The conditions for quantitative polymerase chain reaction (qPCR) were previously detailed by Yudiati *et al.* (2016), including pre-denaturation at 94°C for 7 min, denaturation at 95°C for 30 sec, 45 cycles of annealing at 60°C for 30 sec, extension at 68°C for 50 sec, and melting curve analysis at 95°C for 30 min. The amplified data were analyzed via the comparative Ct method to ascertain normalized gene expression levels, employing β-actin as the normalization control (Livak and Schmittgen, 2001). Gene expression related to the immune system was evaluated on day 7, concluding the rearing period and prior to the challenge test.

Statistical analysis

Statistical analysis of the data was conducted using R-Studio to assess differences between treatments. One-way analysis of variance (ANOVA) was employed to assess the significance of effects ($p < 0.05$) across the treatments. The least significant difference test was utilized to assess the variations in THC, PA/PI, PO, SOD, survival rate, and gene expression.

Results and Discussion

Total hemocyte count (THC), phagocytic activity (PA), and phagocytic index (PI)

Shrimp rely solely on innate, non-specific immunity, as their adaptive immune system is absent (Amparyup *et al.*, 2013). Hemocytes are central to this response, particularly in phagocytosis, a critical defence mechanism where immune cells engulf and destroy pathogens (Azhar and Yudiati, 2023). Variations in total hemocyte count (THC) are closely linked to phagocytic activity (PA) and phagocytic index (PI), reflecting the functional status of the immune system (Yudiati *et al.*, 2019).

The THC (PA and PI) of shrimp fed different diets are presented in (Figure 1a, b, c). During the 7-day experiment, variations were observed in these cellular immune parameters. Overall, the shrimp treatments were better than the control, and the combination of alginate and SWE reached the highest level of THC (62.10^8 cells·mL^{-1}), PA (64 %), and PI (1.31).

In this study, supplementation with alginate (Alg), Spirulina hot water extract (SWE), and their combination significantly elevated THC, PA, and PI. Alginate extracted from *Sargassum* spp. contains guluronic and mannuronic acids (Yudiati and Isnansetyo, 2017), while *Spirulina platensis* offers high protein content (59–63% of dry weight) and bioactive compounds such as phenols, chlorophylls, β-carotene, and phycobiliproteins with known antioxidant and immunomodulatory activities (Li *et al.*, 2022).

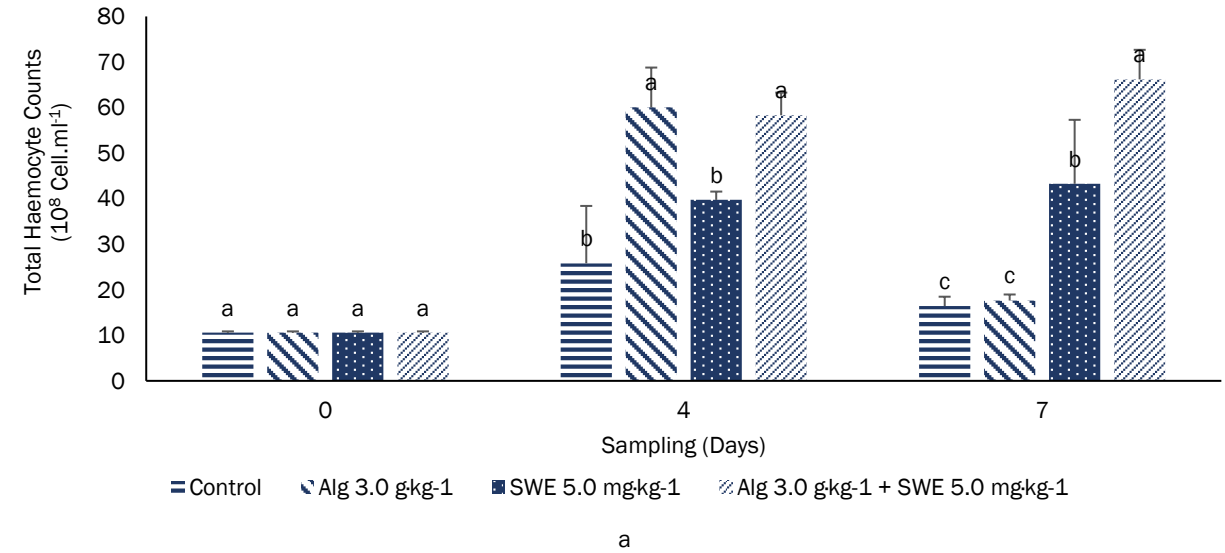
The combined effects of Alg and SWE appeared to promote hemocyte proliferation and enhance pathogen clearance. This is consistent with previous reports of improved phagocytic function following immunostimulant supplementation (Yudiati *et al.*, 2019; Eissa *et al.*, 2024; Yudiati *et al.*, 2024a; Yudiati *et al.*, 2024b). Hematopoietic tissue in shrimp rapidly produces and releases hemocytes into circulation (Abidin *et al.*, 2021), bolstering defence against *Vibrio* spp. (Azhar and Yudiati, 2023).

Phenoloxidase (PO) activity

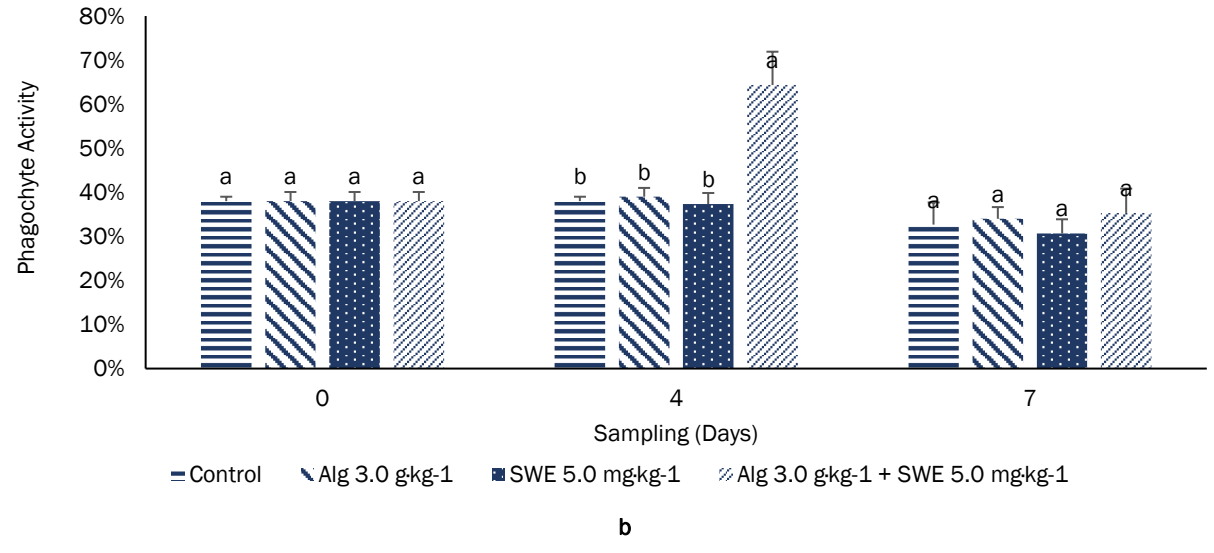
Increased phenoloxidase (PO) activity in treated shrimp suggests the activation of the prophenoloxidase (proPO) cascade. This system is key in invertebrate immunity, mediating melanization and pathogen encapsulation (Amparyup *et al.*, 2013). The PO enzyme activities of shrimp fed different diets are shown in Figure 2. During the 7-day experiment, PO activity of all treatments was found to vary.

Table 1. Sequences of RT-PCR primers specific for proPO and LGBP as *L. vannamei* immune-related genes

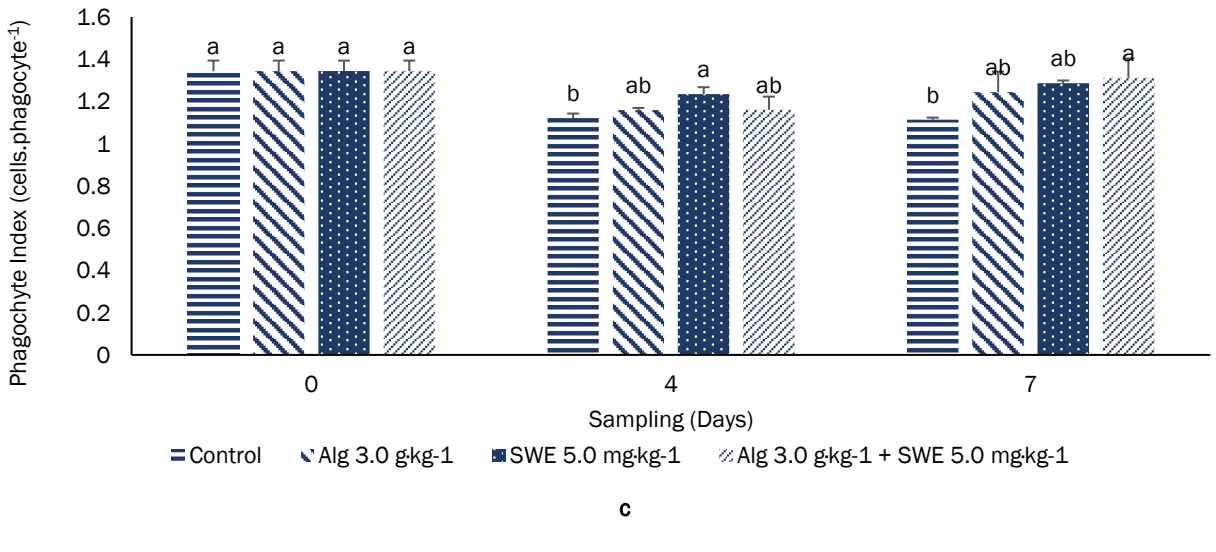
Gene	Primers	Sequences (5′-3′)	Accession no	References
proPO	proPO-F	TTCAACGGTAGACCCGTGATTCTTC	AY723296.1	(Wang *et al.*, 2007)
	proPO-R	TCTTGCCGGGTTTAAGGTGAACAGT		
LGBP	Liva LGBP qPCR F	CGG CAA CCA GTA CGG AGG ACC		(Cheng *et al.*, 2005)
	Liva LGBP qPCR R	GTG GAA ATC ATC GGC GAA GGA G		
β-actin	Lvbac-F	CCTCCACCATGAAGATCAAGATCAT	AF300705.2	(Sun *et al.*, 2007)
	Lvbac-R	CACTTCCTGTGAACAA TTGATGGTC		



a



b



c

Figure 1. Cellular compounds in shrimp fed different diets: (a) Total hemocyte count, (b) Phagocytic activity, and (c) Phagocytic index on days 4 and 7 periods of rearing. Different lowercase letters at each bar indicate significant differences among groups ($P \leq 0.05$).

Overall, the shrimp subjected to treatments showed better results than the control, particularly the Alg treatment alone and combination treatment (Alg and SWE). Alginate may mimic bacterial cell wall components to stimulate pattern recognition receptors, thereby triggering the proPO cascade (Yudiati *et al.*, 2019; Azhar and Yudiati, 2023). Similar effects were reported with other polysaccharides, such as carrageenan from *Kappaphycus alvarezii* (Dhewang *et al.*, 2023).

Superoxide dismutase (SOD) activity

SWE also functioned as a potent immunostimulant through its phenolic and phycocyanin content (Hidayati *et al.*, 2020), which enhanced antioxidant defences via elevated superoxide dismutase (SOD) activity. SOD plays a critical role in neutralizing reactive oxygen species during immune activation (Tassanakajon *et al.*, 2013; Lin *et al.*, 2023). These antioxidants contribute to both cellular and humoral defence through signal transduction pathways, which ultimately stimulate antimicrobial peptide production (Kulkarni *et al.*, 2021).

The SOD activity of shrimp fed different diets is shown in Figure 3. On days 0 and 4 of rearing, all treatment groups showed similar results, with no significant differences ($P>0.05$). On day 7, SOD activity in the Alg and/or SWE combination diet supplementation treatment was higher (0.04–0.05 U·mL^{-1}) than that in the control (0.03 U·mL^{-1}) ($p<0.05$). SOD activity was significantly higher in treated shrimp than in control shrimp, with Alg treatment alone and combination treatment with Alg and SWE showing the strongest SOD-promoting effects. Similar immunostimulatory effects of marine microalgae such as *Phaeodactylum tricornutum* and *Tetraselmis* sp. (Zhang *et al.*, 2025), and sulfated polysaccharides from *Sargassum ilicifolium* (Pourazad *et al.*, 2024) have been documented, further supporting these findings.

Pro phenoloxidase (PO) and lipopolysaccharide beta glucan-binding protein (LGBP) immune-related gene expression

The innate immune response in *L. vannamei* also involves LGBP, a pattern recognition protein that binds to lipopolysaccharides and β-glucans on pathogen surfaces, triggering the proPO system (Amparyup *et al.*, 2013). Our results confirmed that both proPO and LGBP gene expression were significantly upregulated following treatment, consistent with prior studies (Boonchuen *et al.*, 2018; Azhar and Yudiati, 2023; Gustilatov *et al.*, 2024).

Figure 4 shows the RNA transcripts of shrimp fed different supplemented diets before *Vibrio* challenge at the end of rearing. Shrimp fed Alg and the combination of Alg and SWE showed the highest proPO and LGBP gene expression. LGBP and proPO act as early responders to microbial invasion, and their regulation is modulated by immune challenges and environmental factors, including biofloc systems that enhance gene expression while suppressing quorum sensing in pathogens (Gustilatov *et al.*, 2024). In line with previous findings, various immunostimulants, such as seaweed extracts (Ashour *et al.*, 2024), mannan oligosaccharides (Novriadi *et al.*, 2021), and SWE (Lin *et al.*, 2010) have been shown to upregulate immune-related genes like PO and SOD in *L. vannamei*.

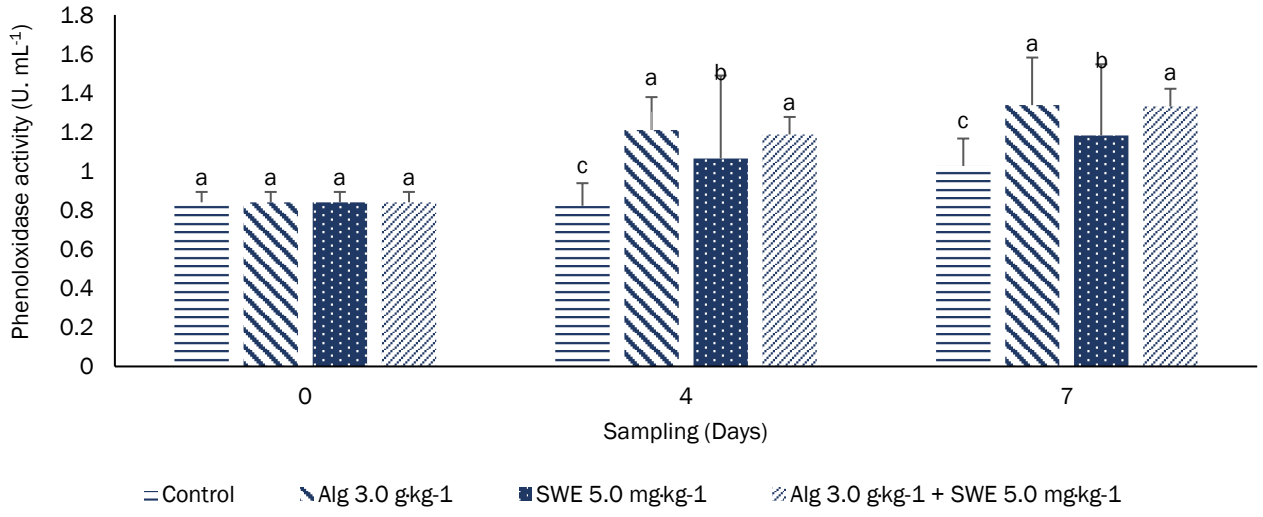


Figure 2. Phenoloxidase enzymatic activity of shrimp fed different supplemented diets on days 4 and 7 of rearing. Different lowercase letters at each bar indicate significant differences among groups ($P\leq0.05$).

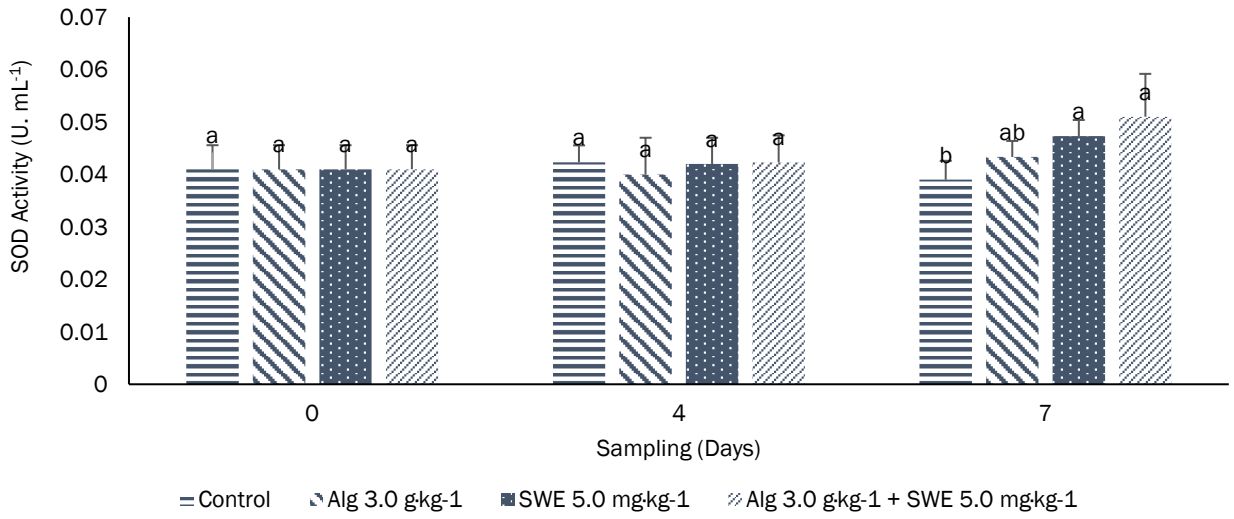


Figure 3. Superoxide dismutase (SOD) activity in shrimp fed different supplemented diets on days 0, 4, and 7 days of rearing. Different lowercase letters at each bar indicate significant differences among groups ($P \leq 0.05$).

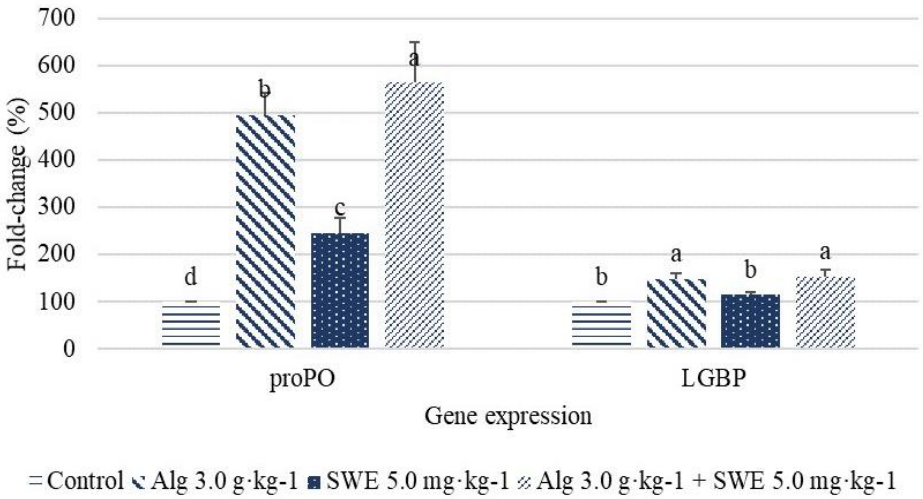


Figure 4. Relative proPO and LGBP gene expression in shrimp fed different supplemented diets at the end of the rearing before *Vibrio* challenge. Gene expression levels were normalized to those of β-actin. Different lowercase letters at each bar indicate significant differences among groups ($P \leq 0.05$).

Survival rate after challenge

Vibriosis remains a major threat in shrimp aquaculture, particularly from *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, and *V. harveyi* (Sarjito *et al.*, 2018; Istiqomah *et al.*, 2020; Haifa-Haryani *et al.*, 2022). The survival rates of shrimp fed the control and treatment diets after *Vibrio* challenge were similar until day 4. On days 5–7, treated groups exhibited a significantly ($P \leq 0.05$) higher survival rate than the control (Figure 5). The control group showed 100% mortality on day 7. Our challenge test demonstrated that shrimp fed Alg + SWE diets had significantly higher survival rates against multiple *Vibrio* spp., supporting the protective efficacy of these immunostimulants. SWE outperformed previous Artemia biomodel challenges in similar trials (Yudiati *et al.*, 2021a).

The use of natural immunostimulants such as alginate (a polysaccharide derived from brown seaweed) and *Spirulina* (a cyanobacterium rich in phycocyanin and bioactive peptides) has gained significant attention in commercial aquaculture. Dietary supplementation of alginate enhances cellular and humoral immune responses by stimulating phagocytic activity, increasing humoral enzyme levels, and modulating the expression of

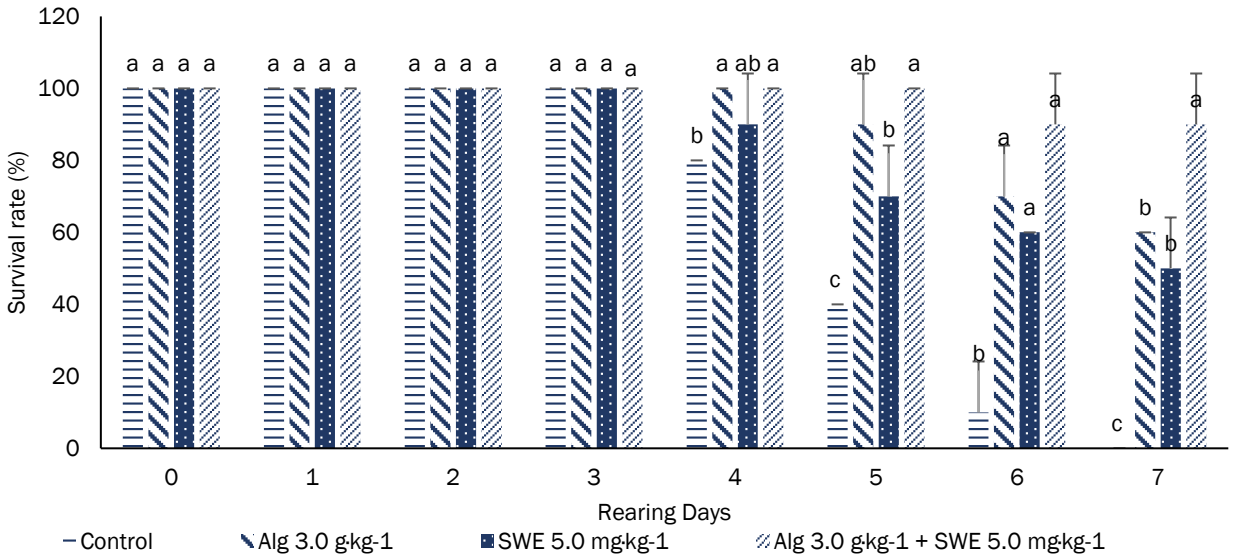


Figure 5. Survival rate of shrimp fed different supplemented diets after *Vibrio parahaemolyticus*, *Vibrio vulnificus*, and *Vibrio harveyi* immersion challenge test. Different lowercase letters at each bar indicate significant differences among groups ($P \leq 0.05$).

immune-related genes in shrimp. *Spirulina* contributes to immune modulation through its high content of essential amino acids, antioxidants, and pigments that improve antioxidant capacity. When incorporated into shrimp feeds, these compounds not only promote better disease resistance against *Vibrio* spp., but also support overall health and growth performance.

However, the bioactivity of alginate and *Spirulina* may vary depending on extraction methods, formulation stability, and dosage accuracy. Over-supplementation can potentially lead to oxidative imbalance or feed palatability issues. In addition, variability in water quality, species-specific responses, and the lack of standardized protocols for field application may hinder consistent results in commercial settings. Future research should focus on optimizing dosage levels, delivery methods (e.g., encapsulation, nanoparticle systems), and synergistic combinations with other bioactive compounds such as probiotics, essential oils, or peptides. Long-term studies under variable environmental conditions are also needed to validate efficacy, safety, and cost-effectiveness at a commercial scale. Furthermore, molecular studies exploring immunomodulatory mechanisms and gut microbiota dynamics are essential to support evidence-based formulation of next-generation functional feeds. In the context of global warming and deteriorating water quality, integrating such immunostimulants into recirculating and biofloc systems presents a sustainable strategy to strengthen shrimp health and aquaculture resilience.

Conclusion

Alginate and *Spirulina* hot water extract (SWE) significantly enhanced cellular and humoral immune parameters, upregulated proPO and LGBP gene expression, and improved survival of *L. vannamei* against *Vibrio* spp. These results indicate their potential as natural dietary immunostimulants to strengthen shrimp health and support sustainable aquaculture practices.

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