Synergistic effects of a brown macroalgae premix extract and Bacillus subtilis on growth performance, haemolymph characteristics, antioxidant responses, and disease resistance in whiteleg shrimp (*Litopenaeus vannamei*)

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Abstract

The use of functional feed additives is a key strategy for improving growth performance, health status, and disease resistance in shrimp aquaculture. This study evaluated the effects of a brown macroalgae premix extract (MPE), composed of *Sargassum ilicifolium*, *Nizimuddinia zanardini*, *Padina australis*, and *Cystoseira indica*, in combination with the probiotic *Bacillus subtilis* (P), on growth performance, haemolymph chemistry, antioxidant capacity, and resistance to *Vibrio parahaemolyticus* in whiteleg shrimp (*Litopenaeus vannamei*). A total of 2,400 post-larvae (initial weight: 1.29 ± 0.28 mg) were randomly assigned to eight dietary treatments, including a control, MPE alone, probiotic alone at three inclusion levels, and combinations of MPE with each probiotic level, for 60 days. Shrimp fed the MPE+P2 and MPE+P3 diets showed significantly higher final weight, weight gain, and specific growth rate than the control and other treatments ($p < 0.05$). These groups also exhibited significantly elevated total haemocyte counts, while the MPE+P2 treatment resulted in the highest haemolymph protein level and the lowest glucose concentration ($p < 0.05$). Combined supplementation significantly enhanced superoxide dismutase and glutathione peroxidase activities and improved post-challenge survival against V. parahaemolyticus, with the highest survival rate observed in the MPE+P2 group. Overall, dietary supplementation with brown macroalgae extracts in combination with B. subtilis exerted synergistic effects on growth performance, immune-related haemolymph parameters, antioxidant status, and disease resistance in *L. vannamei*.

Keywords: Brown macroalgae extract; *Bacillus subtilis*; *Litopenaeus vannamei*; Growth performance; Haemolymph parameters; Antioxidant enzymes; Disease resistance

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Introduction

Currently, shrimps are regarded as a vital shellfish product both globally and domestically. The shrimp sector plays a significant role in the nation's economy, particularly in the southern regions, as it not only provides essential animal protein but also contributes substantially to addressing malnutrition, unemployment and creating value (Kumar *et al*., 2020). However, the sector faces numerous challenges, including the issue of bacterial infections. The emerging disease known as Early Mortality Syndrome (EMS), or Acute Hepatopancreatic Necrosis Disease (AHPND), poses a serious threat to shrimp populations. This disease is characterized by the sudden disappearance of recently stocked shrimp in ponds and is the leading non-viral illness in shrimp farming, causing considerable losses within the first 35 days (Lim *et al*., 2020). EMS first appeared in certain East Asian countries in 2009, spreading rapidly and inflicting significant damage on the shrimp industry in those regions (Kumar *et al*., 2020).

Historically, antibiotics were commonly employed as a strategy to prevent and control disease outbreaks; however, some countries continue to use them indiscriminately despite established preventive measures (Santos *et al*., 2019; Lim *et al*., 2020). A major concern surrounding this practice is the increase in drug resistance among bacteria, which has led to the proliferation of resistant strains and a decrease in the efficacy of antibiotic treatments. Additionally, the presence of antibiotic residues in breeding ponds is considered a significant environmental hazard. Consequently, there has been a demand for reduction in the excessive use of antibiotics for disease control in shrimp farming, with a shift towards exploring alternative strategies (Lazado *et al*., 2015).

The search for alternative methods has led to the adoption of probiotics as a key element for health and hygiene in aquaculture. Probiotics enhance the microbial balance of the host or the environmental microbial factors, ultimately benefiting the host's health, growth, feed intake, disease resistance, stress response, and overall vitality (Merrifield *et al*., 2010; Butt *et al*., 2021). The use of probiotics in aquaculture has gained momentum over the last twenty years, as they have been shown to mitigate the impact of bacterial diseases (Butt *et al*., 2021). Oral probiotics consist of living organisms added to the digestive system via feed, promoting the well-being of intestinal microflora and the host's health. Among these, *Bacillus* species are the most commonly utilized probiotics in crustaceans (Farzanfar, 2006). Typically, *Bacillus* species used in shrimp aquaculture have been selected for their ability to combat pathogenic *Vibrio* strains through their antimicrobial properties, as demonstrated in laboratory antagonism tests (Decamp *et al*., 2008; Liu *et al*., 2010; Zokaeifar *et al*., 2012; Lim *et al*., 2020).

The primary expenditures in shrimp farming generally account for 60-70% on feed and 10-15% on larvae, collectively comprising approximately 70-85% of total farming costs (Zokaeifar *et al*., 2012). One of the roles of probiotics in aquaculture is to lower the food conversion ratio (FCR) and improve survival rates (Tacon *et al*., 2002; Xue *et al*., 2015; Adel *et al*., 2017), consequently reduces feed, cost of feed, and culturing periods.

Recent research indicates that seaweeds can be incorporated into aquaculture feed as a dietary supplement due to their availability, cost-effectiveness, and nutrient density (Kazemi *et al*., 2018; Akbary *et al*., 2020). Seaweeds are rich in vitamins, minerals, and carotenoids, making them valuable functional ingredients (Periera, 2011). Regarded as valuable resources along Iran's southern coasts, seaweeds are crucial biological assets with high economic value and diverse applications in fisheries. The benefits of incorporating algal extracts into diets include improved health and growth, enhanced immune responses, and natural enrichment of gut and stomach flora (Yeh *et al*., 2010; Wongprasert *et al*., 2014; Akbary *et al*., 2023; Zhang *et al*., 2023).

Numerous researchers have investigated the effects of probiotics on the growth and immune indices of crustaceans. For example, juvenile freshwater crayfish (*Astacus leptodactylus*) that fed *Pediococcus acidilactici* based diets demonstrated notable benefits (Vaezi *et al*., 2018), as did shrimp (*L. vannamei*) fed *Bacillus subtilis* and *Bacillus licheniformis* probiotic bacteria (Monier *et al*., 2023). Zhang *et al*. (2023) highlighted the influence of nine native macroalgae species from China's tropical coast on the growth and health of *Litopenaeus vannamei*, finding that shrimp fed macroalgae diets exhibited enhanced immune defense capabilities than the control group. Additionally, various studies have examined the effects of different algae species on several shrimp species, including the macroalga *Chaetomorpha clavata* (Borges *et al*., 2024), native macroalgae along the tropical coasts of China (Zhang *et al.,* 2023), *Jania adhaerens* extract (Akbary *et al*., 2020), a combined extract of brown macroalgae (*Sargassum ilicifolium*, *Nizimuddinia zanardini*, *Padina australis*, and *Cystoseira indica*) (Akbary *et al*., 2023), *Gracilaria pygmaea* (Ojifar *et al*., 2017), and polysaccharides extracted from seaweed (*Enteromorpha*) (Liu *et al*., 2020). These studies have reported improvements in growth parameters, survival rates, and intestinal digestibility in shrimp. In spite of these advantages, there are paucity of information on the use of indigenous macroalgae combined with probiotics, because the mixtures could perform better than their individuals due to complementarity and synergistic potentials of the bioactive components in them, hence, the need for this study.

In contemporary aquaculture, it is essential to maintain high-quality post-larvae to reduce disease prevalence and ensure efficient, sustainable production.

Therefore, the incorporation of indigenous macroalgae—rich in bioactive and medicinal compounds—alongside the probiotic *Bacillus subtilis* as a supplement can meet the nutritional needs of post-larvae while minimizing bacterial infections and contamination. Furthermore, this approach helps mitigate the risks associated with drug residues and the environmental and health issues linked to the use of chemical disinfectants and antibiotics. This consideration is vital for the quality of post-larvae and ultimately for the sustainable production of shrimp. This study aims to investigate how the simultaneous use of a premixed extract of brown macroalgae (*Sargassum ilicifolium*, *Nizimuddinia zanardini*, *Padina australis*, and *Cystoseira indica*) and the probiotic *Bacillus subtilis* IS0 (P) enhances growth performance, haemolymph chemistry, and resistance to *Vibrio parahaemolyticus* in shrimp, *Litopenaeus vannamei*.

Material methods

Collection of seaweed biomass samples

Samples of seaweed biomass from three different species—*Sargassum ilicifolium*, *Nizimuddinia zanardini*, *Padina australis*, and *Cystoseira indica*—were collected in November 2023 from the coastal areas of Chabahar during tidal movements and identified in the Faculty of Marine Science, Chabahar Maritime University, Iranaccording to Gharanjik and Rouhani Qadiklai (2009). Upon arrival in the laboratory, the specimens were thoroughly rinsed multiple times with deionized water to eliminate all epiphytic organisms and sediment. Following this procedure, the samples were dried in a cool, shaded area at room temperature (25˚C) without exposure to sunlight for one week. After drying, the macroalgae were ground using an electric grinder to produce a powdered mixture containing equal proportions (1:1:1) of the three macroalgal species, which was then stored at -4˚C for later use (Choi *et al*., 2014a).

Preparation and extraction of nacroalgae premix extract

To create a aqueous extract from the mixed macroalgae, cold extracting method was used.Briefly, 5 g of the powdered mixture was dissolved in 100 mL of distilled water and vigorously stirred for 20 minutes. The containers were then sealed and kept in darkness for 72 hours. Subsequently, the filtrate was separated using No. 1 Whatman paper while the water was eliminated in a rotary evaporator (IKA, Germany) at 45˚C 6 hours. The collected sample was placed in a clean Petri dish within a laminar flow hood until all remaining solvent had completely evaporated. The sample was then stored in a freezer at -20˚C for future use (Choudhury *et al*., 2005).

Formulation of feed rations with macroalgae premix extract (MPE) and probiotic Bacillus subtilis IS0 (P)

To prepare a ration containing 15 g kg^{-1} of macroalgae premix extract (MPE), the total feed amount for each group over the entire 60-day period was calculated.

Following the combination of MPE with nutritional elements, oil and 30% distilled water were added sequentially (Akbary *et al*., 2023). The final mixture was formed into pellets with a diameter of 1 mm, dried using a chopper, and stored at -20˚C (Choi *et al*., 2014). Furthermore, the method described by Wache *et al*. (2006) was employed to prepare experimental rations with *Bacillus subtilis* at a dosage of 2.5×10^{12} CFU g^{-1} from Tak-Gen Biotech Company, Iran, as well as experimental rations containing both MPE and the probiotic. Specifically, probiotic amounts of 1, 2, and 3 g were combined with each 100 g of feed, alongside algae extract and fish oil (32 mL kg^{-1} feed), then dehydrated at room temperature and preserved at -20°C for the duration of the study. The standard diet included 15 g kg^{-1} of MPE and varying levels of probiotic *Bacillus subtilis* as an edible supplement (Table 1).

Table 1: Formulation and assessment of the proximate composition of the standard control diet.

Ingredients	(%)
Fish meal	29.0
Soybean meal	10.0
Wheat meal	6.0
Squid meal	34.0
Shrimp meal	9.0
Yeast	3.0
Fish oil	2.0
Lecithin	5.0
Vitamins and minerals [a]	2.0
Proximate composition	
Protein	36.7
Lipid	14.7
Moisture	14.3
Ash	8.5
Fiber	3.1
Nitrogen free extract	22.7

[a] Vitamins and minerals are provided per kilogram. Vitamins include 2500 U of Vitamin A, 2500 U of Vitamin D, and 2000 U of Vitamin E. Minerals consist of 501 mg of CuSO4, 1500 mg of ZnSO4, 0.01 mg of MnSO4, 500 mg of CoSO4, 500 mg of KI, and 35 mg of Na2SeO3.

Shrimp culture

The study required 2400 post-larval (PL) shrimp (*L. vannamei*) with an average weight of 1.28 ± 0.09 mg, obtained from a shrimp breeding center in Chabahar, Iran. They were transported to the fish breeding and research section of the Chabahar Marine Research Center in a double-layer plastic bag containing one-third water and the remainder air. The post-larvae were randomly divided into eight groups, with each group containing three replicates of 100 PL each. The control group did not receive MPE or the probiotic in their diet, while the other groups were fed diets containing 15 g kg^{-1} of MPE, 1 g 100 g^{-1} of the probiotic (P1), 2 g 100 g^{-1} of the probiotic (P2), 3 g 100 g^{-1} of the probiotic (P3), MPE+P1, MPE+P2, and MPE+P3, respectively, for 60 days (Ghaednia *et al*., 2020). Each

tank had its water partially replaced every other day, and waste was siphoned using a handmade hose. Throughout the testing period, the physical and chemical attributes of the water were assessed daily, including temperature (measured with a mercury thermometer accurate to 0.1°C), dissolved oxygen (using a digital oxygen measuring device, TECPEL DO-1609), and pH (measured electrically with an Ebro PHT-3140). The average water temperature was maintained at 30.0±2.0 °C; dissolved oxygen level was sustained at 2.8±0.5 mg mL^{-1}; pH was recorded at 7.5; and salinity was kept at 35±0.47 g L^{-1}.

Growth performance parameters

At the conclusion of the 60-day trial period, the length and weight of the shrimp were recorded with a precision of 1 mm and 0.001 g, respectively. Equations 1 to 5 present the calculations for final weight (FW), weight gain percentage (WG), survival rate, feed conversion ratio (FCR), specific growth rate (SGR), and protein efficiency ratio (PER) using data obtained from biometrics (Harikrishnan *et al*., 2011; Akbary *et al*., 2021):

(1) SGR (%.day-1) = [(LnWf–LnWi) / t] × 100

Wi: initial weight (g), Wf: final weight (g), t: duration of rearing (days)

(2) WG (%) = (Wf - Wi/Wi) × 100

Wf: final weight (g), Wi: initial weight (g)

(3) FCR = WG / F

F: amount of feed consumed (g), WG: weight gained

(4) Survival rate (%) = (number of larvae stored at the beginning of the period / number of larvae remaining at the end of the period) × 100

Haemolymph sampling

At the conclusion of the experimental period, haemolymph was collected from all groups. Approximately 10 minutes before collection, 10 shrimp from each tank were placed in a container with dry ice at 4°C to minimize stress and movement. To prevent haemolymph coagulation, a cooling anticoagulant solution consisting of 10 mM HCl - Tris, 250 mM sucrose, and 100 mM sodium citrate at a pH of 6.7 was used in equal parts. Considering the rapid clotting of shrimp blood, a 1 mL insulin syringe was filled with 0.4 mL of the anticoagulant solution at 4°C. The syringe needle was then carefully inserted beneath the cuticle at a 45degree angle near the first and second legs, adjacent to the ventral nerve cord, to obtain 0.4 mL of haemolymph. Following collection, the syringe's contents were promptly transferred into a sterile microtube for immediate analysis (Yang et al. 2014). The samples were divided into two portions. One portion was used immediately to assess haemolymph cell parameters, while the remaining portion was centrifuged at 1000 g for 5 minutes at 4 °C using a Centrifuge 5810/5810R (Eppendorf®). The plasma was then stored in a freezer

at -80°Cuntil required for biochemical parameter analysis and enzyme activity assessments.

Haemolymph cellular indices

Total Hemocyte Count (THC)

To calculate the total hemocyte count (THC), samples were thawed at room temperature and mixed using a vortex for 20 to 30 seconds. A 100 µL aliquot of the haemolymph sample with anticoagulant was mixed with an equal volume of 10% formalin buffer and incubated for 30 minutes. The hemocyte count was then performed using a Neubauer chamber (Fortuna, Germany) and a light microscope equipped with a 40 × objective lens. The total number of hemocytes was determined by applying the Neubauer chamber formula, taking into account the dilution factor after the counting process (Song *et al*., 2003).

$$\text{THC } (mL{-}1) = \left(\frac{\text{number of counted hemocytes}}{5}\right) = 10^5$$

Differential hemocyte count (DHC)

To calculate the DHC, haemolymph smears were stained using the Giemsa - May-Grunwald method and analyzed under a light microscope at 100 × magnification (Ghaednia *et al*., 2020).

Haemolymph biochemical indices

The activity of lysozyme in haemolymph samples was measured following the method proposed by Ellis in 1990. Initially, 175 mL of a *Micrococcus lysodeikticus* suspension (from Sigma), containing 0.375 g mL^{-1} in sodium phosphate buffer with a molarity of 0.05 and a pH of 6.2, was combined with 250 µL of each sample and incubated at 22 °C. The optical density was analyzed using a spectrophotometer at a wavelength of 600 nm at both 15 and 180 seconds. The variation in optical density from the initial to the subsequent readings was recorded, and the findings were quantified in millilitre units through the development of a standard curve using dilutions made with egg white lysozyme (Sigma product) in sodium phosphate buffer (with the starting dilution at 1.6 g mL^{-1}). Sodium phosphate buffer was also employed as a negative control. Protein quantification was conducted using a colourimetric technique with a biochemical kit from Pars Azmoon Co. and a 200-BS biochemical autoanalyser manufactured by Mindray, China, as reported by Yu *et al*. (2008). Glucose was measured using an enzymatic test involving glucose oxidase and peroxidase, according to Trinder (1969). The spectrophotometer was used to measure the activity of phenoloxidase (PO) with L-4-3-dihydroxyphenylalanine (Sigma) as the substrate and trypsin as the product (Rengpipat *et al*., 1998). Two hundred µL of L-4-3-dihydroxyphenylalanine (0.3%) was added to 200 µL of the sample supernatant and incubated for 30 minutes at room temperature. Subsequently, it was mixed with 600 µL of buffer (pH 7), and its absorbance was measured at a wavelength of 490 nm. A single enzyme activity unit is specified as U mg^{-1} protein.

Haemolymph antioxidant status indices

The mixture for testing superoxide dismutase (SOD) included HEPES-KOH at pH 7.8 with 0.1 mM EDTA, sodium carbonate buffer at 50 mmol and pH 7.2, metallothionein at 12.7 mmol, nitro blue tetrazolium at 75 µmol, riboflavin at 1 µmol, and 200 µL of the extracts. The specimens were exposed to light for 15 minutes, after which the absorbance at 560 nm was determined using a spectrophotometer; a control tube containing all reaction components except the enzyme served as the blank sample (Winterbourn *et al*., 1975). The assessment of catalase (CAT) activity involved measuring the decrease in light absorbance at 240 nm over 30 seconds, using hydrogen peroxide as the substrate, with results normalised to mg of protein in the enzyme extract. A 20 mmol sodium phosphate buffer at pH 7 and 20 µL of 30% hydrogen peroxide were used as electron acceptors in the procedure (Dazy et al., 2008). The functionality of glutathione peroxidase (GPX) was evaluated following the protocol outlined by Lawrence and Burk (1976). Briefly, 0.9 mL of the reaction mixture was prepared with 50 mmol phosphate buffer at pH 7.0, 1 mmol EDTA, 1 mmol sodium azide (a catalase inhibitor), 0.2 mmol β-NADPH, 20 µmol glutathione reductase, and 1 mmol GSH. The mixture was then incubated at 25°C for 15 minutes. Afterwards, 0.1 mL of 0.25 mmol hydrogen peroxide and 50 µL of enzyme extract were added, and light absorbance was measured at 440 nm using a spectrophotometer. The test for malondialdehyde (MDA) was conducted in accordance with the procedure established by Baluchnejadmojarad *et al*. (2010). Initially, 375 mg of thiobarbituric acid (TBA) was dissolved in 2 mL of HCl, then mixed with 15% (100 mL) trichloroacetic acid (TCA), and the mixture was kept in a hot water bath at 50°C to fully dissolve any sediment particles. Following this, 1 mL of the extract was added to 2 mL of the solution and boiled in water for 45 minutes until it turned orange. The samples were then centrifuged at 1000 rpm for 10 minutes, and the absorbance was recorded at 535 nm.

Cultivation of the pathogenic agent Vibrio parahaemolyticus

A lyophilised bacterial isolate was used to cultivate *Vibrio parahaemolyticus* (ID 17802). The isolate was obtained from the Faculty of Veterinary Medicine at the University of Tehran, Iran. The freeze-dried bacteria were reconstituted in Tryptic Soy Broth (TSB) with 3% NaCl to create a consistent suspension. Linear cultures were prepared on nutrient agar and selective culture medium TCBS from the obtained suspension and then incubated at $37^{\circ C}$ for 24 hours. Following the identification of green colonies, a bacterial suspension was prepared using the McFarland method at concentrations of 10^8, 10^7, and 10^6 CFU mL^{-1}, as described by Farmer and Hickman-Brenner (2006).

Determination of LC50

Following a 48-hour fasting period, this test was conducted to determine the

LC50 (Akbary and Aminikhoei, 2018). In this experiment, each concentration of the bacterium *Vibrio parahaemolyticus* (10^6, 10^7, 10^8 CFU mL^{-1}) was tested in three replicates (10 shrimp each). The shrimp were then transferred to the main holding tanks after being challenged by immersion with the pathogenic agent, and their mortality was assessed over a period of 96 hours (Akbary and Aminikhoei, 2018).

Shrimp challenge with Vibrio parahaemolyticus bacteria

Forty-eight hours prior to exposure to the pathogen, feeding of the shrimp was halted (Burbank *et al*., 2011; Choi *et al*., 2014b). Ten shrimp from each group in the different treatments were exposed to the pathogen (LC50) using the immersion technique in 60L tanks. The daily mortality rate was tracked for each group over a period of 10 days, and the survival rate was computed using the following formula:

Survival Rate (SVR) = [Number of shrimp tested - Number of deaths / Number of shrimp tested] × 100

Statistical analysis

The data were analyzed using one-way ANOVA with three replicates for each group, and mean comparisons between groups were based on Duncan's multiple range test at a 5% significance level. In R software, three non-parametric tests—Breslow (Generalised Wilcoxon), Log Rank (Mantel-Cox), and Tarone-Ware—were employed to assess survival rates of groups given varying diets following exposure to *Vibrio parahaemolyticus*. The normality of the data was evaluated using the Kolmogorov-Smirnov test, while Levene's test was used to check for equal variances. SPSS 19 was used for all data analysis, while Excel 2010 was employed for statistical calculations.

Results

Growth performance

Table 2 provides information on the growth parameters and feed utilization of *L. vannamei* shrimp after 60 days on different diets. There was no significant difference in survival rates among the groups studied (p>0.05). The highest specific growth rate (SGR), final weight (FW), and weight gain (WG) were observed in the groups that received MPE+P2 and MPE+P3, showing a significant difference compared to the control group and other experimental groups.

Table 2: The growth performance of *Litopenaus vannamei* shrimp fed with experimental diets after 60 days (mean± SD (n=3)).

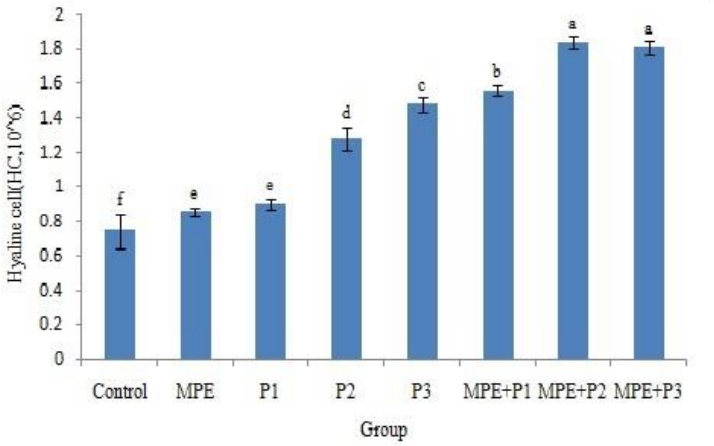
Experimental groups	Initial weight (mg)	Final weight (FW, mg)	Weight gain (WG)	Specific growth factor (SGR, %)	Feed conversion ratio (FCR)	Survival (%)
Control	29±1	1026.66±212.01^{c}	40.60±7.57^{c}	6.21±0.25^{c}	1.93±0.33^{a}	97.66±0.51
MPE	29.13±0.91	1526.66±190.73^{b}	51.41±6.44^{b}	6.58±0.20^{b}	1.75±0.11^{b}	100±0
P1	28.86±1.06	1513.13±287.51^{b}	51.40±9.85^{b}	6.57±0.31^{b}	1.54±0.24^{c}	98.33±0.71
P2	28.93±0.96	1566.60±205.86^{b}	53.03±7.94^{b}	6.63±0.24^{b}	1.29±0.13^{d}	100±0
P3	28.80±1.80	1540±244.36 ^{b}	52.44±8.16^{b}	6.61±0.26^{b}	1.28±0.15^{d}	98.00±0.19
MPE+P1	29±0.92	1633.30±266.26^{b}	55.36±9.34^{b}	6.69 ± 0.29^{b}	1.25±0.00^{d}	98.66±1
MPE+P2	28.80±1.08	1926.56 ± 138.70^{a}	66.36±4.93^{a}	7.00 ± 0.13^{a}	1.17±0.13^{d}	100±0
MPE+P3	28.66±1.29	1806.64 ± 122.27^{a}	61.70±5.15^{a}	6.89±0.12^{a}	1.15±0.00^{d}	100±0

Different letters in the same columns indicate significant differences between groups ($p<0.05$). MPE: premix extract of brown *Padina australis*, *Sargassum ilicifolium*, and *Stoechospermum marginatum*, P1, P2 and P3: diets containing the concentrations of 1, 2 and 3 g 100 g^{-1}*Bacillus subtilis* probiotic respectively, MPE+P1, MPE+P2 and MPE+P3 diets containing the simultaneous use of MPE and the concentrations of 1, 2 and 3 g 100 g^{-1}P.

Haemolymph chemistry

Haemolymph cellular indices

Figure 1 displays the haemolymph cell indices of *L. vannamei* shrimp in the tested groups after a 60-day period. Changes in diet resulted in a significant increase in total haemocyte count (THC), granular cells (GC), semi-granular cells (SGC), and hyaline cells (HC) in comparison to the control group ($p<0.05$). The highest THC levels were observed in the groups receiving MPE+P2 and MPE+P3, with values of 15.36 ± 15.36 and 0.36±15.10 (10^{6} mL^{-1}) respectively, demonstrating significant differences from the other groups ($p<0.05$). The control group exhibited the lowest THC, measuring 0.30±0.09 (10^{6} mL^{-1}). The increase in probiotic *B. subtilis* concentration was associated with a rise in THC, indicating a significant distinction among groups ($p<0.05$). The presence of varying levels of probiotics mixed with macroalgae extracts resulted in a notable increase in the quantities of GC, SGC, and HC compared to the control group and other test groups. The MPE+P2 and MPE+P3 groups exhibited the highest counts, showing significant differences from the remaining groups ($p<0.05$). However, there was no significant difference between MPE+P2 and MPE+P3 ($p>0.05$).


Hyaline cell(HC,10^6)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
f
e
e
d
c
b
a
a
Control
MPE
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P2
P3
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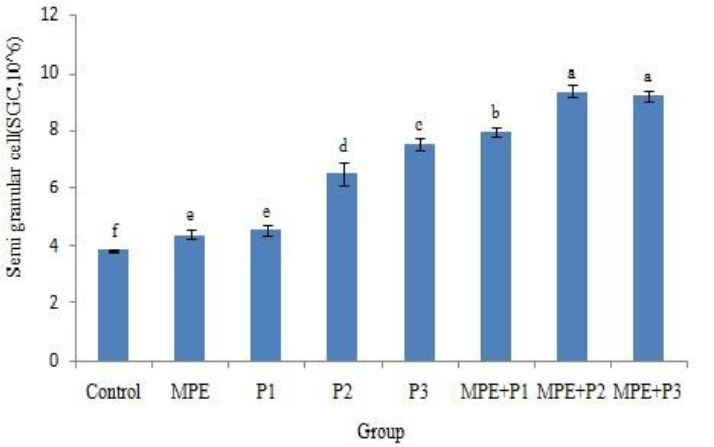
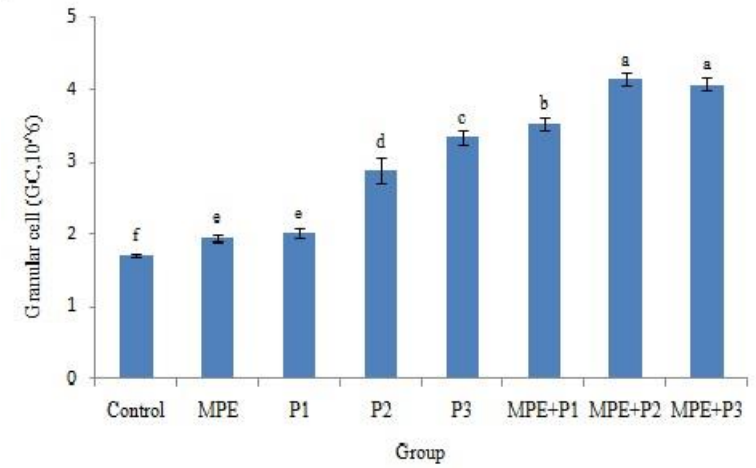
MPE+P3
Group


Semi granular cell(SGC,10^6)
12
10
8
6
4
2
0
f
e
e
d
c
b
a
a
Control
MPE
P1
P2
P3
MPE+P1
MPE+P2
MPE+P3
Group


Granular cell (GC,10^6)
5
4
3
2
1
0
f
e
e
d
c
b
a
a
Control
MPE
P1
P2
P3
MPE+P1
MPE+P2
MPE+P3
Group

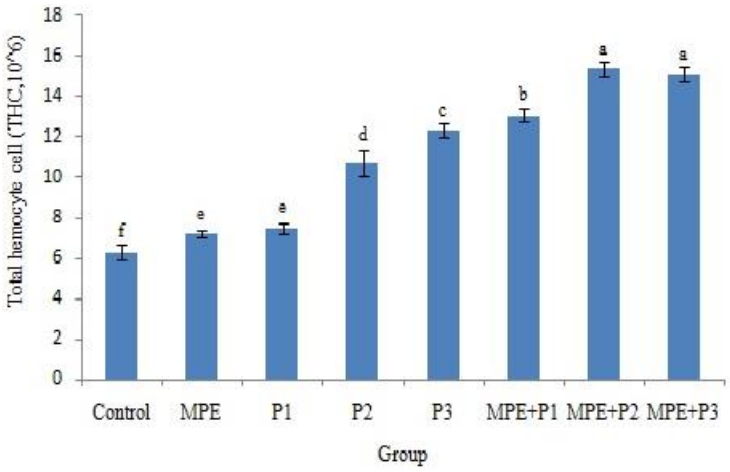
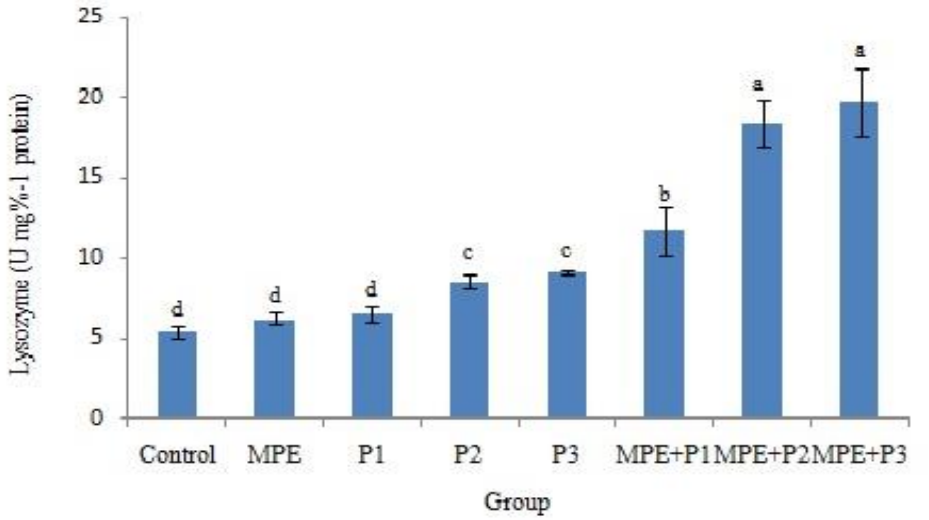


Figure 1: Variations of the mean (± SD) hemolymph indices (total hemocyte count (THC), granular cell (GC), semi granular cell (SGC), and hyaline cell (HC), 10^6 mL^{-1}) *of L. vannamei* shrimp fed with experimental diets after 60 days. MPE: premix extract of brown *Padina australis*, *Sargassum ilicifolium*, and *Stoechospermum marginatum*, P1, P2 and P3: diets containing the concentrations of 1, 2 and 3 g 100 g^{-1}*Bacillus subtilis* probiotic respectively, MPE+P1, MPE+P2 and MPE+P3 diets containing the simultaneous use of MPE and the concentrations of 1, 2 and 3 g 100 g^{-1}P. The significant difference between the groups is indicated by non-similar letters (p>0.05).

Haemolymph biochemical indices

Figure 2 illustrates the levels of total protein, glucose, phenoloxidase (PO), and lysozyme in *L. vannamei* shrimp after being fed different diets throughout the experiment. The addition of macroalgae extract alongside varying amounts of probiotic *B. subtilis* significantly increased lysozyme, PO, and protein levels, while notably reducing glucose levels compared to the control group (p<0.05). The group receiving MPE+P2 showed the highest protein levels (111.47±9.0 mg mL^{-1}) and PO (88.53±1.79 U mg^{-1} protein), along with the lowest glucose level (9.0±0.91 mg dL^{-1}) compared to other groups in the study. This difference was significant when compared to the control group and other experimental groups (p<0.05)



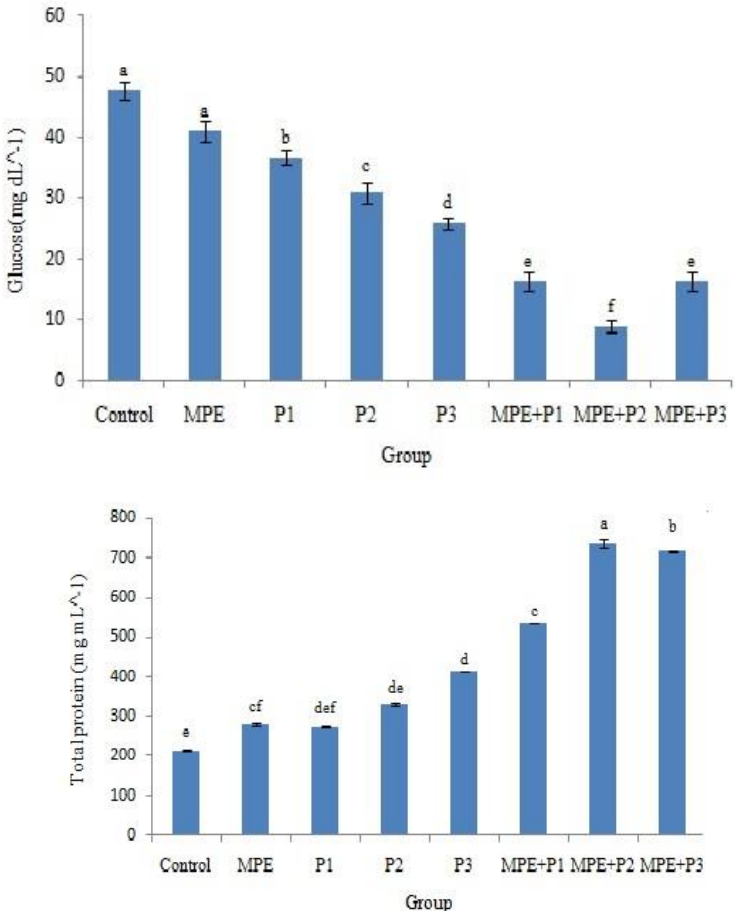


Figure 2: Variations of the mean (± SD) non immunity parameters (total protein, glucose, lysozyme, and phenol oxidase *of L. vannamei* shrimp fed with experimental diets after 60 days. MPE: premix extract of brown *Padina australis*, *Sargassum ilicifolium*, and *Stoechospermum marginatum*, P1, P2 and P3: diets containing the concentrations of 1, 2 and 3 g 100 g^{-1} *Bacillus subtilis* probiotic respectively, MPE+P1, MPE+P2 and MPE+P3 diets containing the simultaneous use of MPE and the concentrations of 1, 2 and 3 g 100 g^{-1}P. The significant difference between the groups is indicated by non-similar letters (p>0.05).

The groups fed MPE+P2 and MPE+P3 displayed the highest levels of lysozyme, with no significant difference observed between them (p>0.05). There was no significant difference in the levels of lysozyme, PO, and total proteins between the MPE and P1 groups (p>0.05). Furthermore, there were no notable variances in the levels of lysozyme and glucose between the MPE group and the control group (p>0.05).

Haemolymph antioxidant status indices

Table 3 shows the variations in antioxidant levels in shrimp haemolymph after being fed different diets throughout the experiment. The combined extract of macroalgae and varying concentrations of probiotic *B. subtilis* led to higher levels of superoxide dismutase (SOD) and glutathione peroxidase (GPX) enzymes compared to other groups, demonstrating a significant variance from the control group (p<0.05). Both the MPE+P2 and MPE+P3 groups exhibited levels of

catalase (CAT) and SOD at 12.66±3.21, 12.80±0.72, and 23.33±1.52 and 22±1 U mg^{-1} protein, respectively, with no significant variations between them (p>0.05). The groups fed MPE+P2 and MPE+P3 had the lowest levels of Malondialdehyde (MDA) at 0.99±0.01 and 1.04±0.16 U mg^{-1} protein respectively, compared to the highest level in the control group at 6.50±0.43 U mg^{-1} protein, showing a significant difference between the diets and the control group (p<0.05). No notable variations were observed in the quantities of SOD and CAT between the groups that consumed different levels of probiotic (p<0.05).

Resistance to disease in *L. vannamei* when exposed to infection caused by *Vibrio parahaemolyticus bacteria.*

Table 3: Comparison of the mean (mean ± SD, n=3) antioxidant status (glutathione peroxidase (GPX), catalase (CAT), malondialdehyde (MDA), and superoxide dismutase (SOD)) of*Litopenaus vannamei* shrimp fed with experimental diets after 60 days.

Experimental groups	GPX	CAT	MDA	SOD
Control	73.19±0.23^{g}	0.82±0.05^{d}	6.50±0.43^{a}	6.80±0.53^{c}
MPE	76.00±1.00^{f}	2.74±0.67^{d}	4.63±0.05^{b}	11.49±0.23^{d}
P1	76.00±1.00^{f}	3.45±0.47^{cd}	4.33±0.17^{bc}	12.60±0.53^{cd}
P2	79.66±0.57^{e}	3.80±0.52^{c}	4.29±0.07^{c}	13.00±1.00^{cd}
P3	82.66±1.03 ^{d}	5.10±0.85 ^{bc}	3.74±0.08^{d}	13.33±1.15^{c}
MPE+P1	88.66±1.52^{c}	7.66±2.51^{b}	2.13±0.03^{e}	18.59±0.52^{b}
MPE+P2	99.33±0.57^{a}	12.66 ±3.21^{a}	0.99±0.01^{f}	23.33±1.52^{a}
MPE+P3	94.95±1.69^{b}	12.80 ±0.72^{a}	1.04±0.16^{f}	22.00±1.01^{a}

Different letters in the same columns indicate significant differences between groups (p<0.05). MPE: premix extract of brown *Padina australis*, *Sargassum ilicifolium*, and *Stoechospermum marginatum*, P1, P2 and P3: diets containing the concentrations of 1, 2 and 3 g 100 g^{-1}*Bacillus subtilis* probiotic respectively, MPE+P1, MPE+P2 and MPE+P3 diets containing the simultaneous use of MPE and the concentrations of 1, 2 and 3 g 100 g^{-1}P.

At the end of the 60-day feeding trial, *L. vannamei* shrimp fed the control and different diets were infected with *V. parahaemolyticus* bacteria for 10 days. Table 4 and Figure 3 display the percentage of cumulative survival over this period.

Table 4: The mean (± SD, n=3) values of cumulative survival (%) for *L. vannamei* shrimp fed different diets after being challenged with *Vibrio parahaemolyticus* (LC50= 8×106 CFU mL^{-1})

Experimental groups	Survival
Control	30.01±7.65^{h}
MPE	39.80±12.52^{gh}
P1	43.50±21.37^{fgh}
P2	50±15.82^{cef}
P3	61.50±24.52 ^{cbe}
MPE+P1	58.50±14.22^{bd}
MPE+P2	78.00±23.12^{a}
MPE+P3	65.45±24.03^{b}

Different letters in the same columns indicate significant differences between groups (p<0.05). MPE: premix extract of brown *Padina australis*, *Sargassum ilicifolium*, and *Stoechospermum marginatum*, P1, P2 and P3: diets containing the concentrations of 1, 2 and 3 g 100 g^{-1}*Bacillus subtilis* probiotic respectively, MPE+P1, MPE+P2 and MPE+P3 diets containing the simultaneous use of MPE and the concentrations of 1, 2 and 3 g 100 g^{-1}P.

There was no significant difference in survival rates between the P2 group and the control group (p>0.05). No notable disparity was observed in the survival rates between the P3, MPE+P1, and MPE+P3 groups (p>0.05). The group receiving MPE+P2 showed the highest survival rate at 78.82±25.52 percent, significantly different from both the control group and the other groups (p<0.05). There was no significant difference between the control group and the groups fed with MPE+P1 and P2 (p>0.05).

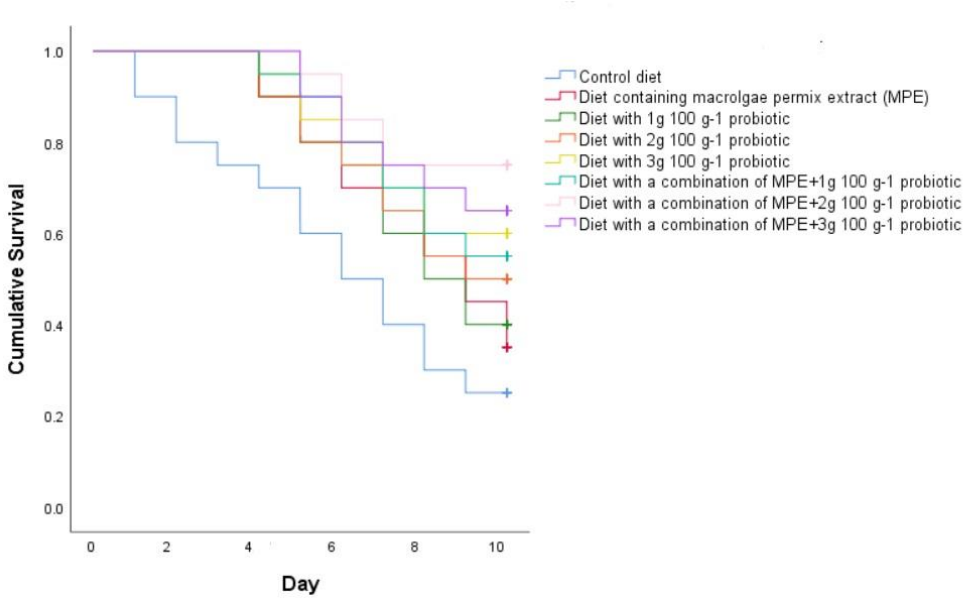


Figure 3: Kaplan-Meier survival curves illustrating the survival rates of *L. vannamei* shrimp subjected to various experimental diets subsequent to exposure to *Vibrio parahaemolyticus* bacteria (LC50= 8×10^6 CFU mL^{-1}) for 10 days.

Discussion

The utilisation of inexpensive food resources is a critical aspect of aquaculture aimed at enhancing nutritional efficiency and reducing costs (Tacon *et al*., 2002; Xue *et al*., 2015; Adel *et al.*, 2017). The findings of this research indicate that feeding Pacific white shrimp exclusively with a water-based extract derived from a mixture of brown macroalgae (*Sargassum ilicifolium, Nizimuddinia zanardini, Padina australis, and Cystoseira indica*) resulted in increased weight and growth rates, alongside a lower feed conversion ratio, when compared to the control group. Akbary *et al*. (2023) discovered that evaluating various concentrations of a blend of brown macroalgae on shrimp's growth, nutritional content, biochemical health, and antioxidant capacity revealed optimal growth and antioxidant enhancement at a concentration of 15 g kg^{-1} of feed, which aligns with the results of this investigation. It can be inferred that the utilisation of low-value food sources can enhance nutritional efficiency and decrease costs in aquaculture. Previous studies corroborate these findings,

demonstrating improved growth and biomass in shrimp fed with macroalgae compared to control groups (Hafezieh *et al*., 2017; Putra *et al*., 2019; Akbary *et al*., 2023). This study substantiates that feeding shrimp with macroalgae can enhance their growth parameters, such as weight gain and specific growth rate, likely due to the high levels of fatty acids and essential amino acids, as well as the provision of minerals and vitamins C and E as supplements for shrimp (Akbary *et al*, 2023). Nevertheless, further research is warranted to explore the specific active components of macroalgae that promote shrimp development.

Conversely, the application of levels 1, 2, and 3, corresponding to 1 g 100 g^{-1} diet of probiotic *Bacillus subtilis* (P1, P2, and P3), resulted in improved growth and feed efficiency in the tested shrimp compared to the control group, although no significant difference was observed with the MPE group. In alignment with our results, Gruber *et al*. (2023) found that incorporating 3 g $kg^{-1}$of a probiotic (AquaStar® Biomin GmbH) in combination with varying levels of marine flour (15% and 32%) led to higher survival and growth rates in shrimp, alongside reduced feed intake and enhanced feed conversion ratio, irrespective of diet type, compared to the control group. Novriadi *et al*. (2022) demonstrated that shrimp feed efficiency was improved through dietary supplementation with a combination of probiotics, including *Lactobacillus reuteri*, *Pediococcus acidilactici*, *Enterococcus faecium*, and *Bacillus subtilis*. Furthermore, Adilah *et al*. (2022) found that encapsulating the probiotic *Bacillus subtilis* E20 in alginate and coating it with chitosan enhanced its efficacy as a growth stimulant and health promoter for *Litopenaeus vannamei*. This could be linked to the dietary advantages of the probiotic supplement and its ability to improve protein absorption for growth or tissue development (Gruber *et al*., 2023).

Although shrimp exhibit limited efficacy in utilizing glucose monosaccharides, they can utilize starch with an efficiency rate of 92%. Therefore, the presence of alpha-amylase from probiotics may not be beneficial for shrimp. Nonetheless, the degradation of gluten molecules by *B. subtilis* bacteria could potentially enhance the nutritional efficacy and growth rate of shrimp (Yalcin *et al*., 2020). The groups fed with MPE+P2 and MPE+P3 exhibited the highest specific growth rate (SGR), final weight (FW), and weight gain (WG), along with the lowest feed conversion ratio (FCR). Seaweed is regarded as an alternative protein source for aquaculture due to its high protein content, essential amino acids, vitamins, and trace elements (Zhang *et al*., 2023). Concurrently, probiotics serve as a robust barrier against harmful microorganisms (Mirbakhsh *et al*., 2022; Gruber *et al*., 2023). Thus, enhancing shrimp growth, FCR, and overall survival can yield a synergistic positive effect by functioning as functional feed that boosts digestive enzyme activity, regulates pathogenic

bacteria levels, maintains immune system balance, and enhances growth parameters in aquaculture. This suggests that it is capable of not only reducing feed costs through decreased consumption but also mitigating secondary water pollution in farming areas, thereby preserving water quality. Consequently, these functions provide substantial benefits to sustainable aquaculture by improving feeding efficiency, promoting enhanced growth, and lowering production costs (Lara-Flores, 2011). The findings of the study indicated that the survival rate was comparable among groups receiving varied diets and the control group, consistent with the study conducted by Mazlum *et al*. (2020).

This research focuses on the role of SGC and GC in shrimp's immune response to pathogens, underscoring the beneficial effects of probiotic *B. subtilis* and macroalgal extract on enhancing shrimp immunity. In this study, the administration of P1, P2, and P3 diets resulted in a significant increase in total haemocyte count (THC), as well as GC, SGC, and HC. The highest number of hemocytes was observed in SGC. Furthermore, Abdollahi Arpanahi *et al*. (2019) and Ghaednia *et al*. (2020) reported results similar to ours when investigating the impact of the probiotic *Bacillus subtilis* on hemocytes in *L. vannamei*. The studies indicate that hemocytes play a crucial role in the immune defence mechanisms of shrimp (Ghaednia *et al*., 2011). Numerous studies also support the prevalence of SGC in the haemolymph of shrimp (Jiravanichpaisal *et al*., 2006; Ghaednia *et al*., 2011). The substantial increase in hemocyte counts in the group administered the probiotic suggests a highly beneficial impact on the haemolymph system of shrimp. Hemocytes in shrimp perform phagocytosis, the process of ingesting foreign particles. SGCs are primarily involved in enclosing migratory microorganisms, while large granular cells serve as the main storage for the prophenoloxidase (PO) system's function (Jiravanichpaisal *et al*., 2006). Research indicates that GCs and SGCs respond to pathogens, such as peptidoglycans and lipopolysaccharides in bacterial cell walls, by releasing components from the PO system, including amino compounds for melanin production, cell adhesion, encapsulation, and phagocytosis. These responses are integral to the humoral immunity of invertebrates, including crustaceans (Yeh *et al*., 2010; Sudaryono *et al*., 2018). Hence, the notable increase in hemocyte count in shrimp administered P1, P2, P3, MPE+P2, and MPE+P3, compared to the control group, indicates an enhanced immune system due to the synergistic effect of the permixed extract of brown macroalgae. Consistent with current research, Ghaednia *et al*. (2011) observed minimal effects of *Sargassum glaucescens* extract on hemocyte counts in *Fenneropenaeus indicus*. The increase in hemocyte numbers across several studies is linked to the growth-promoting effects of antioxidants and lipopolysaccharides (Cheng, 2019; Su *et al.*, 2020). This study demonstrated a

significant enhancement in immune indicators in cohorts administered MPE+P2 and MPE+P3 compared to the control group and other experimental groups, indicating that a combination of probiotics and macroalgal extract could potentially bolster immunity in the haemolymph of western white shrimp (Monier *et al*., 2023). Several research studies have provided evidence of the stimulating effects of algae (Tayag *et al*., 2010; Yeh *et al*., 2010; Sudaryono *et al*., 2018). Additionally, the composition of the extract can influence hemocyte counts, which were not examined in this research. Moreover, the hemocyte counts in this study exhibited less variability compared to other studies, possibly due to differences in physical and chemical factors such as temperature and salinity (Ghaednia *et al*., 2011). Therefore, further research is necessary to accurately comprehend how the blended water extract of macroalgae affects haemolymph cell counts.

Stress from handling and transport, environmental conditions, seasonal changes, nutritional status, and sexual maturity significantly influence glucose and protein levels, which serve as plasma metabolic indicators (Yu *et al*., 2008; Modarresi, 2015). This study revealed that the addition of varying concentrations of probiotics to MPE resulted in a significant decrease in glucose levels compared to the control group, with the group fed MPE+P2 exhibiting the lowest glucose level (9.01 ± 1.00mg dL^{-1}). Salehpour *et al*. (2022) demonstrated that incorporating fucoidan from the brown macroalga *Cystoseira trinodis* into the diet of Pacific white shrimp resulted in a significant reduction in glucose levels when compared to the control group. Glucose serves as a critical stress indicator, and lower levels in shrimp fed macroalgae and probiotics suggest heightened resistance to common stressors in laboratory settings and optimal physiological health (Barton, 2002; Yu *et al*., 2008; Omar *et al*., 2024). This is particularly crucial in aquaculture, as environmental stressors can adversely affect the well-being and development of shrimp. The combined utilization of macroalgae and single-cell probiotics can benefit shrimp populations by alleviating stress, ultimately leading to improved survival and productivity rates. The findings from this study indicated that the group receiving MPE+P2 had a notably elevated protein level. Shrimp, akin to other crustaceans, rely on their innate immune system, which is predominantly composed of proteins and their enzymatic components (Pascual *et al*., 2006; Arumugama *et al*., 2017). Chen *et al*. (2014) and Salehpour *et al*. (2022) provided evidence that the incorporation of fucoidan and carrageenan derived from brown macroalgae into the diet resulted in a significant increase in protein levels in shrimp. Another study found that following eight weeks of feeding shrimp with two strains of *Bacillus subtilis*, there was a notable rise in total protein levels in shrimp administered $10^5$and 10^8CFU mL^{-1} of these bacterial strains, in contrast to the

control group, consistent with the findings of this study (Zokaeifar *et al*., 2012). The increase in overall protein in shrimp receiving a combination of macroalgae and probiotics likely contributes to enhanced growth due to protein accumulation, as concluded by Chen *et al.* (2014). Furthermore, the elevation in overall protein levels resulting from dietary modifications signifies a beneficial effect on the immune system of shrimp, which is reliant on various proteins (Yu *et al*., 2008). Key immune enzymes found in shrimp include lysozyme and phenoloxidase (PO), which play roles in the innate immune system (Monier *et al*., 2023). These enzymes serve as biomarkers for assessing environmental and stress conditions (Liu *et al*., 2004) and can also be used as indicators to evaluate immunity and physiology in animals, such as crustaceans (Xue and Renault, 2000). Additionally, lysozyme degrades the cell walls of Gram-positive bacteria and is crucial for protecting aquatic animals from microbial infections and pathogens (Qiu *et al*., 2023). The findings of this study indicated that supplementation with a blend of seaweed extract and varying doses of individual probiotics significantly increased lysozyme levels compared to the control group. The group fed with 2P+MPE exhibited the highest lysozyme levels at 18.33±1.52units per milligram of protein, followed by the group fed with 3P+MPE at 19.66±2.08 units per milligram of protein. In accordance with these results, the introduction of the probiotic *Bacillus subtilis* into the environment of Pacific white shrimp led to increased lysozyme activity compared to the group without it (Monier *et al*., 2023). Furthermore, another study revealed that the application of *B. subtilis* AQAHBS001 resulted in elevated lysozyme levels in Pacific white shrimp (Kewcharoen and Srisapoome, 2019). Additionally, Akbary and Aminikhoei (2018) demonstrated that the use of the macroalga *Ulva rigida* extract enhanced the immunity of Pacific white shrimp, leading to increased lysozyme levels in shrimp administered 1 and 1.5 g kg^{-1} of extract. The presence of polysaccharides is believed to be linked to the stimulating effect of the water-soluble extract. Numerous studies indicate that the composition of polysaccharides in macroalgae varies among species and can be influenced by environmental conditions, potentially affecting their physiological functions (Vatsos and Rebours, 2015; Akbary and Aminikhoei, 2018). Melanisation occurs at the onset of the cellular defence response. The enzyme PO, essential for melanin production, initially appears in the haemolymph in an inactive form known as proPO. Vatsos and Rebours (2015) noted that serine protease converts proPO into PO, which is a vital enzyme for the immune system. As previously mentioned, SGCs also contribute to cellular toxicity and the activation of the proPO system. GCs, the largest type of hemocyte, contain granules in their cytoplasm, yet they do not engage in phagocytosis and play a minor role in

capsule and nodule formation. Instead, they participate in cellular toxicity and play a significant role in activating the PO activation system. Granular hemocytes are recognized as reservoirs for the PO activating system due to this characteristic (Alday-Sanz, 2010). This study observed a substantial increase in PO activity levels in groups administered a mixture of macroalgae and varying amounts of probiotics, compared to the control group, the MPE group, and the groups fed P1, P2, and P3. Given the significant rise in various hemocytes, the increase in the activity of this enzyme was not unexpected.

Macroalgae may possess the capability to trigger antioxidant defence mechanisms as an adaptation to oxidative stress. Consequently, these organisms have the potential to serve as a natural antioxidant resource (Akbary and Aminikhoei, 2018). This study found that the utilisation of a combination of brown macroalgae extract (MPE) increased antioxidant levels in shrimp compared to the control group, which is supported by previous research indicating enhanced antioxidant levels in shrimp fed macroalgae compared to control groups (Akbary and Aminikhoei, 2018; Akbary *et al*., 2023; Zhang *et al.,* 2023). Akbary *et al*. (2023) demonstrated that shrimp fed with an aqueous extract containing *S. ilicifolium, Nizimuddinia zanardini, Cystoseira indica, and Padina australis* exhibited the highest levels of GPX and CAT at 15 g kg⁻¹ of feed. Phenolic compounds in MPE may assist in alleviating oxidative stress by counteracting reactive oxygen species (Shi *et al*., 2005). The levels of malondialdehyde (MDA) reflect detrimental activities stemming from these species and can serve as a reliable measure of lipid peroxidation (Peixoto *et al*., 2016). In line with the findings of this research, Akbary *et al*. (2023) indicated that shrimp fed MPE10 and MPE15 exhibited lower MDA levels compared to the control group. Enzymatic antioxidants play a pivotal role in the immune system by combating lipid oxidation and free radicals in organisms. Certain substances, such as polysaccharides extracted from algae, can activate these physiological antioxidants (Zhang *et al*., 2023). The bioactive properties of polysaccharides are likely responsible for the stimulating and positive effects as antioxidant inducers in organisms (Dore *et al*., 2013; Akbary *et al*., 2023). In this study, feeding groups with varying levels of probiotics (P1, P2, and P3) resulted in decreased MDA levels and increased GPX levels in shrimp diets compared to the control group and MPE, consistent with previous research on juvenile freshwater shrimp *Astacusleptodactylus* (Vaezi *et al*., 2018) and shrimp *L. vannamei* (Monier *et al*., 2023). The capacity of single-cell probiotics to function as antioxidants in the gut microbiome can aid in combating oxidative stresses from both internal and external sources (Monier *et al*., 2023). The superoxide dismutase (SOD) enzyme plays a crucial role as one of the primary antioxidants defence enzymes in protecting the body from damage

caused by free radicals. This enzyme accelerates the conversion of two superoxide anion radicals into hydrogen peroxide and molecular oxygen, thereby mitigating the accumulation of these harmful radicals. Aquatic organisms are safeguarded from oxidative stress through this mechanism (Akbary *et al*., 2023; Monier *et al*., 2023). The elevated levels of SOD in groups receiving a combination of macroalgae and probiotics suggest that this enzyme is likely present in the haemolymph and contributes to protecting host tissues from oxidative damage during the defence response (Castex *et al*., 2009). Feeding shrimp with MPE+P2 and MPE+P3 can enhance antioxidant enzymes and potentially reduce disease-related mortality rates. This can assist farmers in maintaining healthier stocks and decreasing reliance on environmentally detrimental antibiotics.

The findings of this study indicated that the group receiving MPE+P2 achieved the highest survival rate at 12.23±00.78 percent. This suggests that the blend of macroalgal extract may contain immune-boosting compounds such as sulfates and polysaccharides, which can fortify the shrimp's immune response against harmful infections (Wongsasak *et al*., 2015). Moreover, probiotics are essential for enhancing the natural defence system of shrimp, aiding them in combating harmful microorganisms and environmental challenges (Monier *et al*., 2023; Omar *et al.,* 2024), as indicated by various studies (Liu *et al*. 2010; Lim *et al*., 2020; Gruber *et al*., 2023; Omar et al. 2024). This implies that the compound extracts from macroalgae may harbour immune-boosting substances such as sulfates and polysaccharides that can enhance shrimp immunity and fend off pathogen attacks (Wongsasak *et al*., 2015). Additionally, Probiotics are crucial for the development of the immune systems in aquatic species, aiding them in combating harmful microorganisms and environmental pressures. A study conducted by Monier *et al.* (2023) investigated the impact of Bacillus species probiotics, such as SANOLIFE® PRO-W (which includes *B. subtilis* and *B. licheniformis*), on various aspects of shrimp health. The results indicated a significant reduction in shrimp losses to only 35% in the group treated with 0.3 g (m^3) of probiotics. In contrast, the control group ultimately reached a loss rate of 100%.

Conclusion

It is recommended to incorporate a level of 2 g per 100 g of *Bacillus subtilis* probiotic, combined with a premix extract of *Sargassum ilicifolium*, *Nizimuddinia zanardini*, *Padina australis*, and *Cystoseira indica* macroalgae into the shrimp's diet. Overall, the findings suggest that utilizing this combination can enhance growth, cellular health, biochemistry, and antioxidant levels of haemolymph, as well as improve the shrimp's resilience against *Vibrio parahaemolyticus* infections. To address disease issues and achieve high, sustainable production, it is essential to store high-quality larval feed. By

utilizing local macroalgae with bioactive and medicinal properties alongside *Bacillus subtilis* as a dietary supplement, we can not only meet the dietary requirements of shrimp but also reduce infection rates and bacterial contamination. This approach contributes to preventing the accumulation of pharmaceutical residues and mitigating environmental and health issues associated with the use of chemicals such as disinfectants and antibiotics. Such measures play a critical role in enhancing shrimp quality and, consequently, the sustainability of shrimp production. Further research into how probiotics and macroalgae compound extracts enhance digestive enzyme activity, antioxidant levels, and immune responses in shrimp could lead to a deeper understanding of these dynamics. This may involve molecular studies to elucidate the physiological responses of shrimp to these supplements. Additionally, conducting comparative studies with different strains or probiotic compounds, in conjunction with macroalgae extracts, can help identify the most effective formulations for improving shrimp health and combating pathogens.

Conflict of interest and ethical statement

The authors declare no conflict of interest. This article does not contain any studies involving animals conducted by any of the authors.

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