Synergistic effects of *Bacillus subtilis* probiotic and brown macroalgae premix extract on growth performance and digestive enzymes activity in whiteleg shrimp, *Litopenaeus vannamei*

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Abstract

In recent decades, proper and high-quality food rations have been important factors in the development of the shrimp industry, its growth and maintaining its health. Since, macroalgae and probiotics play significant role in aquaculture development.This study investigates the synergistic effect of a premixed extract of brown macroalgae (*Sargassum ilicifolium*،*Nizimuddinia zanardini*،*Padina australis*, and *Cystoseira indica*, MPE) and probiotic *Bacillus subtilis* IS0 (P) on the growth performance, and digestive activity in *Litopenaeus vannamei*. In this study, 2400 post-larvae with an average weight of 1.29±0.28 mg were randomly distributed at a density of 100 individuals in 8 experimental groups with 3 replications for each group. The control group received a diet without MPE and P, while the other groups were fed diets containing 15 g kg-1 of MPE, 1 g 100g-1 of P (P1), 2 g 100g-1 of P (P2), 3 g 100g-1 of P (P3), MPE+P1, MPE+P2, and MPE+P3 respectively for 60 days. The results showed that the groups fed MPE+P2 and MPE+P3 had the highest specific growth rates (SGR), final weights (FW) and weight gains (WG), which were significantly different from the control and other experimental groups ($p<0.05$). The MPE+P2 group also had the highest enzyme activities for lipase (2.02±1.37 U mL-1 protein) and protease (76.37±883.33 U mL-1 protein) ($p<0.05$). In conclusion, the Simultaneous use of extracts from brown macroalgae and probiotic *Bacillus subtilis* at a concentration of 2 g 100-1 g of diet improved growth performance and digestive enzyme activity of shrimp.

Keywords: Extract of macroalgae premix, *Bacillus subtilis*, *Litopenaeus vannamei*, Growth performance, Digestibility

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Introduction

As the world's population continues to grow, the demand for sustainable food sources has become increasingly urgent. Therefore, production has emerged as a fundamental challenge for agriculture, and in the near future, there will be strong competition for arable land and other limited resources such as fossil carbon, water, and certain nutrients (phosphorus) among feed/food, fuel, and fiber. According to statistics provided by FAO, the global population is expected to rise to about 7 to 9 billion by 2050, while the demand for milk and meat during this time is estimated to nearly double, prompting humanity to cultivate and consume aquatic animals, including fish, crustaceans, mollusks, and other aquatic species (Steinfeld *et al*., 2006; Akbary *et al*., 2023). Today, shrimp farming, as a crustacean species, has seen rapid growth in Iran and around the world. The shrimp farming industry in the country, especially in the southern regions, holds significant economic importance as it not only provides healthy animal protein but also contributes greatly to job creation and foreign exchange earnings. In Iran, shrimp farming began in 1984 in Bushehr, and eight years later, in 1992, it continued with the farming of *Penaeus monodon* from imported post-larvae from Malaysia in Ahvaz, a trend that continued for several consecutive years. In the following years, an outbreak of white spot disease in Iranian shrimp farms caused severe damage to farmers, leading to the start of farming shrimp *L. vannamei* in Bushehr in 2004, which completely replaced *P. semisulcatus* and *P. indicus* by 2010. Currently, the breeding and cultivation of shrimp, specifically *L. vannamei*, are currently under way along the southern and northern coasts of Iran, including the shores of Sistan and Baluchestan Province.. Although the breeding and cultivation of aquatic animals, including shrimp, hold significant economic importance, the expansion and development of this industry in coastal areas have faced various problems and challenges, particularly the presence of diseases and strategies to combat them. Recently, production in the aquaculture industry has increased by approximately 7.8% compared to the catch of aquatic animals, which has placed global shrimp farming under pressure due to the rise and emergence of shrimp diseases. Consequently, most aquatic producers have shown a strong inclination to use chemicals such as antibiotics to control diseases and enhance the resistance of aquatic animals. However, in recent years, the use of antibiotics has faced numerous restrictions, as these substances have destructive effects on the environment and on end consumers. Moreover, long-term use of these chemicals can lead to bacterial resistance in shrimp and can also negatively affect growth and other immune factors at higher doses (Romano *et al*., 2015). One of the most effective methods to enhance the health and immune system performance of aquatic animals is the use of dietary additives, which often lack nutritional value but can alter the physical and

chemical properties of the diet, as well as influence immune response performance and growth in aquatic organisms (Castillo *et al*., 2014). The search for alternative methods has facilitated the development and use of probiotic as effective agents in promoting health and hygiene management in aquaculture. By enhancing health status, growth performance, optimal feed consumption, disease resistance, response to stressors, or overall body capacity, probiotic benefit the host. This effect is achieved through improvements in host microbial factors or the microbial balance of the environment (Merrifield *et al*., 2010; Butt *et al*., 2021). The use of probiotics in aquaculture has emerged as a relatively new concept over the past two decades. Due to their disease-reducing effects, the application of probiotics in aquaculture is rapidly expanding (Butt *et al*., 2021). Consuming foods containing live bacteria is the oldest and most widespread method for increasing beneficial bacteria in the gut (Merrifield *et al*., 2010). Dietary probiotics are microorganisms introduced into the digestive system through food, and they can benefit gut microflora as well as host health. Additionally, the most commonly used probiotics in crustaceans belong to the genus Bacillus (Farzanfar, 2006). Generally, the Bacillus species tested in shrimp farming have been selected based on their antimicrobial activities against pathogenic *Vibrio* strains and laboratory antagonism tests (Regpipat *et al*., 1998; Decamp, 2008; Liu *et al*., 2010; Zokaeifar *et al*., 2012; Lim *et al*., 2019). Currently, the largest segment of the functional food market consists of foods aimed at improving the balance and activity of gut microbiota. Shrimp farming is a productive and economic activity where cost reductions can significantly impact profitability. The primary costs associated with shrimp farming are feed, which accounts for 60 to 70 percent, and larvae supply, which constitutes about 10 to 15 percent. Together, these two factors represent approximately 70 to 85 percent of the total operational costs. Probiotics play several roles in aquaculture, including reducing the feed conversion ratio (FCR) and increasing survival rates (Tacon *et al*., 2002; Xue *et al*., 2016; Adel *et al*., 2017). The benefits of probiotics include competing with pathogenic bacteria for space, food, and oxygen, stimulating appetite which leads to optimal food intake through the action of protease and amylase enzymes, breaking down indigestible components of the diet, and producing vitamins such as riboflavin and K, which enhance growth, survival, and immunity in the host. As a result, improvements in immune functions, disease resistance, stress reduction, survival rates, growth indices, nutritional efficiency, and meat quality are observed (Bita *et al*., 2017). Numerous studies have explored the effects of various probiotics on the growth and digestive enzyme activity of Pacific white shrimp, including the probiotic AquaStar® Biomin GmbH (Gruber *et al*., 2023), Bacillus species known as SANOLIFE®PRO-W (which

includes *B. subtilis* and *B. licheniformis*) (Monier *et al.*, 2023), and the probiotic *Bacillus subtilis* (Keysami *et al*., 2012; Zokaeifar *et al*., 2012). Recent studies have shown that seaweeds have the potential to be incorporated into aquaculture feed as dietary supplements due to their availability, low cost, and high nutritional value (Kazemi *et al*., 2016). Seaweeds are rich in vitamins, minerals, and various carotenoids, making them valuable high-value functional materials (Periera, 2012). Among the most significant biological and natural resources in the country, seaweeds hold considerable economic value and numerous fisheries applications, particularly along the southern coasts (Rabiei *et al*., 2007). Benefits of using algal extracts as food additives in diets include increased growth, improved health, enhanced immune system function, and natural improvement of gut and stomach flora (Chojnacka *et al*., 2012). The use of seaweeds in aquaculture is on the rise due to their nutrient content, including antioxidants, essential fatty acids (omega-3 and -6), essential amino acids, vitamins, minerals, carbohydrates, and beta-carotene (Rajapakse and Kim, 2011; Arumugama *et al*., 2017). Incorporating them into the diets of aquatic animals not only reduces feeding costs but also enhances feed efficiency, digestion, and strengthens the immune system of fish (Tabarsa *et al*., 2012). Furthermore, by improving digestion efficiency, they also positively impact water quality (Banerjee *et al*., 2010). The digestibility of algae by shrimp indicates the total amount of this substance that shrimp can effectively digest. Numerous studies have examined the effects of different algae species on various shrimp species, including the macroalgae *Chaetomorpha clavata* (Borges *et al*., 2024), native macroalgae along the tropical coasts of China (Zhang *et al*., 2023), 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 shown improvements in growth parameters, survival, and intestinal digestibility of shrimp. Given the abundant distribution of macroscopic algae along the coasts of the Persian Gulf and the Sea of Oman, and recognizing that sustainable and successful aquaculture depends on maintaining the health of aquatic organisms while optimizing farming conditions to maximize growth and minimize production costs, researchers and farmers are actively seeking innovative solutions to achieve their aquaculture goals. Firstly, the Pacific white shrimp is one of the most popular shrimp species globally, significantly contributing to the aquaculture industry. Understanding how the combined macroalgae and probiotic extract affects their growth and digestibility can lead to improved farming practices and increased profitability for shrimp farmers. Therefore, this research aims to investigate the Synergisticeffects of the

extract from brown macroalgae (*Sargassum ilicifolium*،*Nizimuddinia zanardini*،*Padina australis*, and *Cystoseira indica*, MPE) and probiotic *Bacillus subtilis* on growth performance and digestive enzyme activity in *Litopenaeus vannamei*.

Materials and methods

Collection of macroalgae

Seaweed biomass specimens from three distinct species, namely *Padina australis*, *Sargassum ilicifolium*, and *Stoechospermum marginatum* were systematically collected from the coastal regions of Chabahar in November 2023 during tidal activities; subsequent to their transfer to the laboratory, these specimens underwent thorough washing with deionized water multiple times to ensure the complete removal of epiphytic organisms and sediment. Following this process, the specimens were desiccated in a shaded environment at ambient temperature (25˚C) away from direct sunlight for 7 days. Upon grinding the macroalgae utilizing an electric grinder, a composite powder comprising the aforementioned three macroalgal species (in a 1:1:1 ratio) was formulated and preserved at -4˚C for future utilization (Choi *et al*., 2014). Additionally, before drying, the identification of all three species of algae was conducted using credible references (Gharanjik and Rohani Qadikalayi, 2009).

Preparation and extraction of macroalgae premix extract

To generate an aqueous extract from the macroalgal premix (conducted over three iterations), 5 g of the resultant powder was amalgamated with 100 mL of distilled water and subjected to vigorous agitation for duration of 20 minutes. Subsequently, the containers were sealed and maintained in obscurity for a period of 72 hours, after which the supernatant was carefully extracted and the solution was concentrated for 6 hours utilizing a rotary evaporator (IKA, Germany) at a temperature of 45˚C, following filtration through Whatman paper No. 1. Ultimately, the obtained extract was positioned in a sterile Petri dish beneath a laminar flow hood until the residual solvent had fully evaporated. This extract was then stored at -20˚C until further application (Choudhury *et al*., 2005).

Preparation of feed rations containing macroalgae premix extract (MPE) and probiotic Bacillus subtilis IS0 (P)

To prepare a ration containing 15 g kg^{-1} of MPE, the total amount of feed for the entire period (60 days) for each group was first calculated. Upon the integration of MPE with the nutritional components, oil and 30% distilled water were subsequently incorporated. The resultant mixture was then processed into pellets measuring 1 mm in diameter and dried utilizing a chopper, after which it was stored at a temperature of -20˚C (Choi *et al*., 2014). Additionally, for the preparation of experimental rations containing *Bacillus subtilis* bacteria at a dose of 2.5×10^{12} $CFUg^{-1}$ (Tak-Gen Biotech Company, Iran), and

experimental rations containing both MPE and P, the method of Wache *et al*. (2006) was employed. Specifically, 1, 2, and 3 g of probiotic were mixed with every 100 g of feed along with algae extract and fish oil (32 mL kg^{-1} feed), then dried at room temperature, and stored at -20ºC for use throughout the experimental period. 15 g kg^{-1} MPe and different concentrations of probiotic *B. subtilis* , as an edible additive, were added to the basic control diet (Table 1).

Table 1: Formulation and analysis of the proximate composition of the basic control diet (basic food percent).

Ingredients	(%)
Fish meal	30.0
Soybean meal	8.0
Wheat meal	7.0
Squid meal	35.0
Shrimp meal	10.0
Yeast	2.0
Fish oil	1.0
Lecithin	4.0
Vitamins and minerals [a]	2.0
Proximate composition	(%)
Protein	46.7
Lipid	9.7
Moisture	9.3
Ash	10.5
Fiber	1.1
Nitrogen free extract	22.7

[a] Vitamins and minerals supplied per Kg. Vitamins: Vitamin A, 2500 U; vitamin D, 2500 U; vitamin E,2000 U. Minerals: 501 mg $CuSO_4$, 1500 mg $ZnSO_4$; 0.01 $MnSO_4$ 500 $CoSO_4$; 500 KI; 35 Na_2SeO_3

The supply of post-larvae of witheleg shrimp, L. vannamei, and the groups

A total of 2400 PL15 shrimp *L. vannamei* needed for this research, with an average weight of 1.28±0.09 mg, were obtained from a private shrimp breeding center (Chabahar, Iran). They were transported to the fish breeding and research hall of the Chabahar Marine Research Center in a double-layer plastic bag, with one-third filled with water and the remainder with air. The post larvae were randomly divided into 8 groups, each consisting of three replicates with 100 PL per replicate. The control group received a diet without MPE and P, while the other groups were fed diets containing 15 g kg^{-1} of MPE, 1 g $100g^{-1}$ of P (P1), 2 g $100g^{-1}$ of P (P2), 3 g $100g^{-1}$ of P (P3), MPE+P1, MPE+P2, and MPE+P3 respectively for 60 days (Ghaedenia *et al*., 2020). Every tank had one-third of its water replaced every two days, and waste materials were removed from each tank using a siphon. During the testing period, the physical and chemical conditions of the water, including temperature measured with a mercury thermometer accurate to 0.1ºC, dissolved oxygen measured with a digital oxygen measuring device (TECPEL DO-1609), and pH measured electrically (Ebro, PHT-3140), were measured daily. The average water temperature was maintained at 30ºC, with a variation of ±2ºC; dissolved oxygen was kept at 2.8±0.5 mg mL^{-1}; acidity was at 7.5; and salinity was maintained at 35±0.47 g L^{-1}.

Shrimp Biometric

At the end of the experimental period (60 days), the length and weight of all shrimp were measured with accuracies of 1 millimeter and 0.001 grams, respectively. Using the data obtained from the biometrics, the final weight

(Wf), weight gain percentage (WG), survival rate, feed conversion ratio (FCR), specific growth rate (SGR), and protein efficiency ratio (PER) are presented in equations 1 to 5 (Harikrishnan *et al*., 2011; Akbary *et al*., 2020):

SGR (%.day-1) = [(LnWf–LnWi) / t] × 100
Wi: initial weight (g), Wf: final weight (g), t: duration of rearing (days)
WG (%) = (Wf - Wi/Wi) × 100
Wf: final weight (g), Wi: initial weight (g)
FCR = WG / F
F: amount of feed consumed (g), WG: weight gained
Survival rate (%)= (number of larvae stored at the beginning of the period / number of larvae remaining at the end of the period)×100

Sampling of the intestine of shrimp

To assess the activity of digestive enzymes at the end of the experimental period (day 60), feeding of the shrimps was halted 48 h prior to sampling. Six shrimp from each replicate of each test group were then randomly selected and dissected while kept on ice to prevent any changes in enzymatic activity. Their intestines were carefully separated, the contents were emptied, and they were thoroughly washed with distilled water. The intestinal samples were immediately stored at -20ºC (Chitsaz *et al*., 2018), and half of the intestinal samples from each group were placed in 10 % formalin for histological analysis.

Measurement of digestive enzymes activity

To measure the levels of digestive enzymes, intestinal samples were thawed and weighed. Then, the intestines of each shrimp were homogenized with a 100 mmol Tris-HCl buffer, 0.1 mmol EDTA, and 0.1% Triton 100-X in a 9:1 ratio at pH 7.8, at a weight-to-volume ratio of 10 to 1, using a homogenizer (model UP200S, Hielscher, Germany) while kept on ice (Chitsaz *et al*., 2018). To isolate the extract containing the enzymes, the homogenized mixtures were centrifuged at 25,000 rpm for 20 minutes (Gawlika *et al*., 2000). Finally, the supernatant was collected in 1.5mL microtubes (with three replicates for each group) for enzyme activity measurement. Kits provided by Pars Azmoun Tehran, Iran and a spectrophotometer (model DR600, HACH, USA) were used to measure the activity of digestive enzymes. The amylase enzyme activity was measured based on the method of Natalia *et al*. (2004) at a wavelength of 540 nanometers, protease enzyme activity was measured using the King method (1972) at a wavelength of 280 nm, and lipase enzyme activity was assessed using the method of Furne *et al*. (2005) at a wavelength of 480 nanometers. The enzyme activities were calculated based

on U mg^{-1} protein with three replicates for each group.

Statistical analysis

Analysis of the data (three iterations for each treatment) was performed using the one-way ANOVA test, and the mean values of the treatments were compared based on Duncan's multiple range test (MRT) at the significance level of 5%. Kolmogorov-Smirnov test and Levene's test were used to test the data normality and the equality of variances, respectively. Also, analysis of the entire data was performed using SPSS (V19) software. Moreover, statistical computations were performed in Excel 2010 software.

Results

Growth performance

The growth and survival performance of shrimp *L. vannamei* fed different dietary regimes at the end of the experimental period (day 60) is presented in Table 2. The survival rate did not show a significant difference among the tested groups (p>0.05). The highest SGR, FW, and WG were observed in the groups fed with MPE+P2 and MPE+P3, which exhibited a significant difference compared to the control group and the other tested groups (p<0.05). In contrast, the growth performance among the groups fed with MPE, P1, P2, P3, and MPE+P1 did not show a significant difference, although it was significant when compared to the control group (p<0.05). The lowest FCR was observed in the groups fed with P2, P3, and MPE+P2, as well as MPE+P3. Additionally, the feed efficiency in the groups fed with MPE and P1 demonstrated a significant difference compared to the control group and the other tested groups (p<0.05).

Table 2: The growth performance of *Litopenaeus 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 rate (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 *Stoechospermummarginatum*, 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.

Activity of digestive enzymes

Table 3 shows the activity of digestive enzymes in *L. vannamei* shrimp fed with

experimental diets after 60 days. Accordingly, the Simultaneous use of extracts from brown macroalgae and probiotic significantly increased the activity levels of lipase, amylase, and protease enzymes compared to the control group (p<0.05). The highest activity levels were recorded for lipase (37.01±2.02 U mg^{-1} protein), and protease (883.33±76.37 U mg^{-1} protein) in the group fed with MPE+P2 (p<0.05). The highest amylase enzyme activity was observed in the groups fed with MPE+P2 and MPE+P3, which did not show a significant difference from one another (p<0.05). Among the three groups fed solely with single-cell probiotics, the activities of lipase, amylase, and protease increased with the rising concentration of probiotics in the food, reported as 19±0.01, 413±33.14, and 464±33.07 U mg^{-1} protein in group P3 respectively, showing a significant difference compared to the control group and other tested groups (p>0.05). Furthermore, in the group fed with MPE, the levels of lipase, amylase, and protease were 8±2.03, 273±15.27, and 196±15.27 U mg^{-1} protein respectively, which did not show a significant difference from the control group (p<0.05).

Table 3: The mean (± SD, n=3) of the activity of digestive enzymes (U mg^{-1}protein) of *Litopenaus vannamei* shrimp fed with experimental diets after 60 days.

Experimental groups	Lipase	Amylase	Protease
Control	6.00±0.00^{g}	214±1^{f}	150.33±1.52^{f}
MPE	8.00±1.00^{fg}	279.00±25.94^{e}	196.66±15.27^{ef}
P1	10.33±1.52^{ef}	273.33±15.27^{e}	265.70±22.36^{de}
P2	12.30±1.53^{e}	330.00±20.00^{d}	307 ±11.26^{d}
P3	15.20±0.72 ^{d}	413.33±32.15 ^{c}	436.33±55.07^{c}
MPE+P1	19.00±1.00^{c}	526.62±37.85^{b}	586.63±23.09^{b}
MPE+P2	37.00±2.01^{a}	736.66 ±32.17^{a}	833.36±76.23^{a}
MPE+P3	30.33±2.51^{b}	716.04 ±29.46^{a}	816.63±76.36^{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 *Stoechospermummarginatum*, 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.

Discussion

One of the important aspects of aquaculture is the use of low-value food sources to increase nutritional efficiency as well as reduce costs (Tacon *et al*., 2002; Xue *et al*., 2016; Adel *et al*., 2017).The study of probiotics and macroalgae extracts on the *L. vannamei* shrimp is to increase growth performance, improve health and safety, and promote sustainable aquaculture practices.These factors collectively contribute to the economic viability and environmental sustainability of shrimp farming (Omar *et al*., 2024).Theresults of this study showed that the use of only the combined blueextract of the brown macroalgae(*P.astraulis* ،*S.ilicifolium*and *S.marginatum* (MPE) in the shrimp dietary ration led to increase of WG and

SGR and decrease FCR compare to the control group Akbary *et al*. (2023) examined the different levels of MPE (*Sargassum ilicifolium*, *Nizimuddinia zanardini*, *Padina australis* and *Cystoseira indica*) on growth, biochemistry, and the antioxidant status of shrimp *L. vannamei* showed the highest growth, and improved antioxidant status at the level of 15 g kg^{-1} of food, which was consistent with the results of this study.Putra*et al*. (2019) showedthat the WG of *P.monodon* shrimp fed *Caulerpa lentillifera*was significantly morethan the controlgroup. Also, feeding *L.vannamei* shrimp with *Sargassum ilicifolium* also significant increase in fina lbiomass and FCR compared tothe group (Hafezieh *et al*., 2017).In this experiment, wecon firmed that the macroalgae diet can promote growth performance including WG and SGR shrimp, which maybe because macroalgae are rich in essential fatty acids and amino acids, and minerals and vitamins C and E can be used as dietary supplements for shrimps (Akbary *et al*., 2023).However, it is necessary to further examine the specific effective components of macroalgae in promoting shrimp growth.On the other hand, the use of only 1, 2 and 3 g 100 g^{-1} of *Bacillus subtilis* in the food ration of the tested shrimp also led to a significant increase in growth performance and food efficiency compared to the control group.Gruber *et al*. (2023) reportedthat probiotic additives can help improvethe intestinal health of shrimp, thereby increasing the yield, production efficiency and resistance to diseases.Adilah *et al*. (2022) investigated the improvement of the effectiveness of the probiotic *Bacillus subtilis*E20 in the growth stimulant and health status of *L. Vannamei* shrimp which was consistent with the results of this study.This is probably related to the nutritional effects of probiotic additives and its ability to improve protein use for tissue growth or synthesis (Gurber *et al*., 2023).Although shrimp cannot use glucose monomers well, they are 92% effective at using starch.Therefore, the activity of probiotic α amylasecannot help shrimps.But hydrolysis of glutenmolecules by the *B. subtilis* strain of bacteria may have a positive effect on the feeding efficiency and growth rate of shrimps (Yalcin *et al*., 2021).Oment *et al*. (2019)investigating the effect of three types of seaweed extract (*Ulva lactuca*, *Eisenia* sp.and *Porphyra*sp) On improving the growth and digestive enzymes of the *L. vannamei* shrimp showed that by increasing the level of seaweed extract, they significantly improved FW, WG, SGR and feed intake (FI) compared to the control regime.In general, diets containing *Ulva* had the best growth performance. Novriadi *et al*. (2022) showed improved shrimp feed efficiency by examining dietary supplements containing mixed probiotics such as *Lactobacillus reuteri*, *Pediococcus acidilactici*, *Enterococcus faecium* and *Bacillus subtilis*.The highest rate of SGR, FW, and WGin the groups fed with MPE+P2 and MPE+P3 and the lowest rate of FCR was observed in the groups Fed with P2, P3, andMPE+P2,and MPE+P3.It can be said

that seaweed plays a role as an alternative protein source for aquaculture due to its relatively high protein value, essential amino acid content, vitamins and rare metals (Zhang *et al*., 2023), probiotics, on the other hand, act as an effective defense against harmful microbes (Mirbakhsh *et al*., 2022; Gruber *et al*., 2023).So they can help improve shrimp growth, feed conversion ratios, and overall animal survival, and as functional feed, they can lead to improved digestive enzyme function, control the abundance of pathogenic bacteria, balance the immune system, and increase growth parameters in aquaculture.On the other hand, one of the economic factors of aquaculture is the amount of FCR, which was observed in the group fed with MPE+P2, the lowest FCR, which indicates that it can not only reduce the cost of food and feeding due to the lower amount of food, but can also prevent the creation of secondary water pollution in the breeding environment and subsequently reduce water quality.Therefore, all these functions in the form of high feeding efficiency, better growth and reduced production costs can be considered a prominent advantage in sustainable aquaculture (Lara-Flores, 2011).This suggests that shrimp growers can incorporate this supplement into their diet to achieve better performance and profitability.The results of the study showed that survival rates among groups fed different diets and the control group did not show a significant difference, which was consistent with the study conducted by Mazlum *et al*. (2021).

There is a strong correlation between the type and amount of nutrients in the feed and the number and function of digestive enzymes in aquatic animals (Monier *et al*., 2023). Monier *et al.* (2023) examines the effects of commercial probiotic use of *Bacillus bacteria* species, called SANOLIFE®PRO-W(including *B.subtilis* and *B. licheniformis*), on digestive enzymes, shrimp *L. vannamei* showed that the use of water containing *Bacillus subtilis* increased the activity of digestive enzymes relative to the control group. It is also reported that *B.licheniformis* can improve the digestibility of nutrients in aquatic animals by increasing the production of enzymes such as amylase, Protease and cellulase.The increase in protease, amylase and lipase enzymes appears to be the main factor in improving growth performance. Oment *et al*. (2019) investigating the effect of three types of seaweed extract (*Ulva lactuca*, *Eisenia* sp.and *Porphyra*sp) On improvingthe growth and digestive enzymes of *L. Vannamei* shrimps howed that in the case of the activity of the enzymes chemotrypsin, lipase and amylase, a meaningful interaction was observed between the type of seaweed and the level of inclusion,inmostcases, the inclusion of 5% ofeach type of seaweed increased enzymatic activities. The results of the study also showed that the use of MPE also led to a significant increase in the activity of amylase and protease synthesis and secretion of digestive enzymes in shrimp is regulated depending on the composition of the diet (Omont *et al*., 2019).In crustacea, they

form a complex of different proteases of the proteolytic system, and proteolytic isoforms(such as trypsin phenotypes) differ in catalytic properties or properties that help organisms digest better (Perera *et al*., 2010).In the case of the lipase enzyme, all algae-containing diets (MPE+P1, MPE+P2 and MPE+P3) were more active than the control diet. Algal polysaccharides can be said to have positive effects on fat metabolism in different species and may also alter the fat content in shrimp (Jimenez-Escrig and Sanchez-Muniz 2000; Sathivel *et al*., 2008; Tsuge *et al*.,2004; Oment *et al*., 2019).In *P.monodon* shrimp, which was fed with prephyotone (6% capacity) containing 37 genera of algae and 5 genera of zooplankton, was observed to have higher digestive enzyme activity, including amylase, cellulase, Protease and trypsin (Anand *et al*., 2013). Peixoto *et al*. (2016) noted that the high ratio of amylase to protease means greater ability to digest carbohydrates, which helps store protein for growth. Another possible explanation for improving shrimp growth factors by *B.subtilis* maybe due to induction of digestive enzymes including Protease and amylase, which thereby stimulates the activity of the host's natural digestive enzyme (Liu *etal*., 2009; Zokaeifar *et al*., 2012; Monier *et al*., 2023). Similar results were reported by Ziaei Nejad *et al*. (2006), who observed higher digestive enzyme activity in *Bacillus* spp treated *Fenneropenaeus indicus* shrimp than control. Monier *et al*. (2023) examines the effects of commercial probiotic use of Bacillus bacteria species, called SANOLIFE®PRO-W(including *B.subtilis* and *B. licheniformis*), showed that probiotics, especially in concentrations of 0.02 and 0.03 g $(m^3)^{-1}$, increased the activity of the digestive enzymes lipase, amylase and protease compared to the control group. Probiotics may promote digestive activity through the production of vitamins, cofactors or improvement of the enzyme's own activity (Monier *et al*., 2023).

In general, the results showed that the highest SGR, FW,and WG were observed in groups fed with MPE+P2 and MPE+P3. The MPE+P2 group also had the highest enzyme activities for lipase (2.02±1.37 U mL^{-1} protein) and protease (76.37±883.33 U mL^{-1} protein) (p<0.05). In conclusion, the Simultaneous use of extracts from brown macroalgae and probiotic *Bacillus subtilis* at a concentration of 2 g 100^{-1} g of diet improved growth performance and digestive enzyme activity of shrimp. Today, to reduce the problems of disease and achieve high and sustainable production, high-quality post larval (PL) need to be stored, so the use of local macroalgae (with bioactive and medicinal substances) along with *Bacillus subtilis* as a dietary supplement can provide the dietary needs of post larvae (PL).This is very important in the quality of the PL and ultimately the sustainable production of shrimp. More detailed studies of the mechanism by which probiotic *Bacillus subtilis* and macroalgae compounds increase the activity of digestive enzymes in *L.*

vannamei shrimp can provide a better understanding of it.This could include molecular studies to clarify the physiological function of shrimp against this supplement. On the other hand, conducting comparative studies with other strains or probiotic compounds along with this compound macroalgae extract can help detect the best formulations to improve growth and digestibility in shrimp.

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