

# Enhancing immunity and antioxidant activity in green mud crab (*Scylla paramamosain*) by dietary sea lettuce (*Ulva rigida*)

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**ABSTRACT:** This study investigated the effects of dietary supplementation with sea lettuce (*Ulva rigida*) on growth performance, immunity, and antioxidant parameters of green mud crab (*Scylla paramamosain*). Three replicate groups of green mud crabs were fed for 90 days with fish flesh (FF), commercial shrimp feed (CSF), and four alternative diets supplemented with 0, 5, 10, and 15% powdered sea lettuce (PSL). The results showed that growth was significantly higher and the whole-body proximate composition of mud crabs was significantly different from the other treatment groups when they were fed the FF ( $p < 0.05$ ). However, no significant differences in growth or whole-body proximate composition were observed in mud crabs fed diets containing 0 and 5% PSL in comparison to the CSF ( $p > 0.05$ ). A second-order polynomial regression fit indicated the optimal PSL level as 3.21%. Hemolymph analysis revealed significantly higher total hemocyte count and hemolymph protein levels in mud crab fed diets containing 5 or 10% PSL compared to FF ( $p < 0.05$ ). Moreover, mud crabs fed diets supplemented with 15% PSL exhibited significantly higher phenoloxidase activity than the other treatment groups ( $p < 0.05$ ). In addition, dietary PSL enhanced antioxidant enzyme activity in mud crabs, as evidenced by increased catalase and glutathione peroxidase activities, particularly in the 15% PSL group. The present findings indicate that PSL supplementation enhances immunity and antioxidant activity levels in green mud crab.

**KEYWORDS:** mud crab, *Ulva rigida*, aquafeed, immunity, antioxidant activity

## INTRODUCTION

Mud crabs (*Scylla* spp.) are important economic portunid species with high demand in domestic and international markets [1]. At present, four species of mud crabs, *S. serrata*, *S. tranquebarica*, *S. paramamosain*, and *S. olivacea* are the focus of both commercial fisheries and aquaculture [2]. Among the portunid crab species, green mud crab (*S. paramamosain*) is one of the most valuable marine aquaculture crustaceans due to its large size and meat quality [3]. This carnivorous crab is widely distributed in tropical, subtropical, and temperate waters of Southeast Asia [4].

Although the farming of mud crabs still relies on fresh feeds such as trash fish and shellfish [5], information on the nutritional requirements by mud crabs has been expanding as researchers gain better knowledge of this commercially important crab [6, 7]. Previously, macroalgae have been investigated as a dietary ingredient for a wide range of aquafeeds, due to their nutritional value and functional properties [8]. Among the various green macroalgae, *Ulva* sp. (Ulvaceae) or sea lettuce is widely distributed along the coastal areas of Thailand [9] and is rich in essential nutrients, including proteins, carbohydrates, vitamins, minerals, as well as bioactive compounds [9, 10]. Taboada et al [11] reported that *U. rigida* contains 17.8% protein (dry weight), while Thun-

yawanichnonndh et al [12] found the higher 32.24% (dry weight) protein content. Given these findings, *U. rigida* has been recognized as a promising alternative nutrient source for aquafeeds [13]. Multiple roles of *Ulva* sp. and its extract in aquatic animals, such as enhancing growth, feed utilization, survival, antioxidant activity, immune responses, and disease resistance, have been reported [14–16]. Therefore, the present study aimed to investigate the optimal level of dietary sea lettuce supplementation for green mud crabs to maximize growth performance, immune responses, and antioxidant parameters. The results from this study will be useful information for mud crab aquaculture worldwide.

## MATERIALS AND METHODS

### Ethics statement

This study was performed in agreement with the “Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes”, National Research Council, Thailand, and has been approved by the Institutional Animal Care and Use Committee, Prince of Songkla University, under permission number Ref. AQ070/2022.

### Experimental design

For a 90-day trial period, 6 experimental diets were prepared. Sea lettuce (*U. rigida*) was obtained from

Royal Sea Farming and Aquaculture Demonstration Project Under the Initiatives of Her Majesty Queen Sirikit (Phetchaburi) Thailand. The experiment followed a completely randomized design with 6 treatments distributed in 18 (6 treatments  $\times$  3 replications) glass aquaria. The dietary treatments were as follows: Treatment 1 (T1: FF), in which mud crabs were fed throughout the experiment with flesh of fish from the family Scombridae (proximate composition on a wet weight basis: 63.17% moisture, 24.17% crude protein, 3.34% crude lipid, and 3.34% ash); Treatment 2 (T2: CSF), in which mud crabs were fed throughout the experiment with commercial shrimp feed (protein not less than 36% and lipid not less than 4%); and Treatments 3–6 (T3–T6), in which mud crabs were fed throughout the experiment with dietary supplementation with powdered sea lettuce (PSL) at 0% (T3: PSL0), 5% (T4: PSL5), 10% (T5: PSL10), and 15% (T6: PSL15), respectively.

#### Preparation of dietary powdered sea lettuce

The sea lettuce was extensively washed with distilled water and air-dried at 60 °C. The dried raw material was ground in a blender, sieved, and stored at –20 °C prior to use in the experiment. The experimental diets were formulated to be isonitrogenous (42%) and isolipidic (8%) (Table 1). All dry ingredients were sieved using a 100-mesh sieve and blended thoroughly in a mixer. Fish oil and soybean lecithin were then added into the mixer and mixed for 15 min. Finally, distilled water (about 30% weight of dry ingredient mixture) was added to the mixer and mixed thoroughly. All mixtures were pelleted (2 mm diameter) using a Hobart mixer (Legacy model, USA), oven-dried at 60 °C, and stored in plastic bags at –20 °C. Chemical composition analysis of the experimental diets was conducted according to AOAC [17].

#### Experimental animals

Healthy juvenile green mud crabs (*S. paramamosain*) with an average weight of 1–1.2 g obtained from the Thung Talae Forest Royal Development Study and Crab Conservation Center (Krabi), Thailand, were used in this study. All the mud crabs were acclimated and reared until they reached an individual weight of about 8 g in fiberglass tanks under continuous aeration before starting the experiment. During the acclimatization and rearing period, they were fed to satiation with commercial shrimp feed twice daily.

The experiment was conducted in 6 treatments with 3 replications using 15 mud crabs (an initial average weight of  $8.00 \pm 1.17$  g/crab) per replication of a treatment group. Growth performance and survival rate were evaluated for 90 days. The experimental mud crabs were fed to satiation at 09.00 a.m. and 17.00 p.m. Uneaten feed was removed by siphon and dried at 60 °C until a constant weight was achieved. The mortality was recorded daily during the growth

trial. The water quality was maintained by an 80% water exchange every 3 days, with water temperature range of 24–26 °C, salinity range of 18–20 ppt, alkalinity of more than 100 mg/l, pH range of 7.5–9.0, dissolved oxygen of more than 5 mg/l, nitrite of less than 0.1 mg/l, and total ammonia nitrogen of less than 1.0 mg/l.

#### Growth performance

All mud crabs in each tank were counted and weighed at the beginning of the experiment and at 30, 60, and 90 days thereafter, following 24 h of feed deprivation. The weight measurements and counts were used for the growth performance indicators [18, 19], which were calculated as follows:

Average daily gain (g/day) = (final weight–initial weight)/days;

Specific growth rate (%/day) =  $[(\ln \text{ final weight} - \ln \text{ initial weight}) / \text{days}] \times 100$ ;

Feed conversion ratio = feed intake (as fed basis)/weight gain;

Survival rate (%) = (final number of crabs/initial number of crabs)  $\times$  100.

#### Whole-body proximate composition of mud crabs

At the end of feeding trial, 3 mud crabs from each replication (9 crabs/treatment) were collected randomly and analyzed for moisture, crude protein, crude lipid, and ash, using the methods of AOAC [17].

#### Hemolymph parameters

At the end of feeding trial, 2 mud crabs from each replication (6 crabs/treatment) were randomly sampled and anesthetized on ice. The hemolymph was collected from the 4th pereopod with 1 ml syringes and 25G needles for analysis of hemolymph parameters. Briefly, the hemolymph sample was diluted in a microcentrifuge tube containing trypan blue. Total hemocytes were counted using a hemacytometer under a compound microscope [20]. Glucose level was examined following a method of Hyvärinen and Nikkilä [21].

For the quantification of hemolymph protein, mud crab hemolymph was homogenized and centrifuged at  $13,225 \times g$  for 10 min at 4 °C. The supernatant was transferred into a new microcentrifuge tube, and total hemolymph protein was quantified colorimetrically by the method of Lowry et al [22].

Phenoloxidase (PO) activity was assayed using the method of Wanlem et al [23]. Briefly, mud crab hemolymph was mixed in a tube containing cacodylate-citrate buffer. The sample was homogenized and centrifuged at  $13,225 \times g$  for 10 min at 4 °C. The supernatant was transferred into 96-well

**Table 1** Ingredients (g/100 g diet) and proximate compositions of experimental diets.

Ingredient	Experimental diet			
	PSL0	PSL5	PSL10	PSL15
Fishmeal	45.00	45.00	45.00	45.00
Powdered sea lettuce	0.00	5	10	15
Defatted-soybean meal	16.00	14.50	13.00	11.50
Wheat flour	12.00	12.00	12.00	12.00
Rice flour	10.78	7.28	3.78	0.28
Wheat Gluten	6.00	6.00	6.00	6.00
Soy lecithin	2.00	2.00	2.00	2.00
Fish oil	2.00	2.00	2.00	2.00
Vitamin premix <sup>1</sup>	0.50	0.50	0.50	0.50
Mineral premix <sup>2</sup>	2.00	2.00	2.00	2.00
Choline chloride	0.70	0.70	0.70	0.70
BHT (Butylated hydroxytoluene)	0.02	0.02	0.02	0.02
CMC (Carboxymethyl cellulose)	2.00	2.00	2.00	2.00
Di-calcium phosphate	1.00	1.00	1.00	1.00
Proximate composition (%) <sup>3</sup>				
Moisture	6.31 ± 0.29	6.31 ± 0.16	6.43 ± 0.13	6.41 ± 0.12
Crude protein	42.16 ± 0.07	42.28 ± 0.31	41.92 ± 0.36	41.79 ± 0.26
Crude lipid	8.03 ± 0.15	8.08 ± 0.32	7.99 ± 0.17	7.82 ± 0.11
Ash	9.35 ± 0.18	9.19 ± 0.06	9.25 ± 0.08	9.71 ± 0.02

<sup>1</sup> Vitamin premix provided the following per kg of diet: Retinal (A) 500,000 IU; Cholecalciferol (D3) 100,000 IU; Tocopherol (E) 5,000 mg; Menadione sodium bisulfite (K3) 2,000 mg; Thiamine (B1) 2,500 mg; Riboflavin (B2) 1,000 mg; Pyridoxine (B6) 1,000 mg; Cobalamin (B12) 10 mg; Ascorbic acid (C) 10,000 mg; Inositol (B8) 1,000 mg; Niacin 3,000 mg; Calcium pantothenate 3,000 mg; Biotin 10 mg; and Folic acid 300 mg.

<sup>2</sup> Mineral premix provided the following per kg of diet: Cu 5 mg; Fe 2.4 mg; Zn 3.08 mg; Mn 2.4 mg; Co 0.48 mg; and Mg 4.32 mg.

<sup>3</sup> Proximate compositions are expressed as mean ± SD of triplicates.

microplate and incubated with trypsin. Cacodylate-citrate buffer and L-dihydroxyphenylalanine were then added. The optical density at 490 nm was recorded in kinetic mode every 2 min for a total of 30 min using a Multiskan™ GO microplate spectrophotometer (Thermo Fisher Scientific, USA). The protein content in crab hemolymph was evaluated by Lowry's method using bovine serum albumin as a standard. One unit of PO activity was defined as an increase in absorbance of 0.001/min/mg protein.

#### Antioxidant parameters

At the end of the feeding trial, 2 mud crabs from each replication (6 crabs/treatment) were randomly sampled and anesthetized on ice for the determination of antioxidant enzyme activities and thiobarbituric acid reactive substances (TBARs). The hepatopancreas was collected and preserved in liquid nitrogen. The samples were then analyzed for catalase (CAT) activity by using the method of Trasviña-Arenas et al [24]. The activities of glutathione peroxidase (GPx) were measured by Glutathione Peroxide Assay Kit (Cayman, USA). TBARs, determined as malondialdehyde (MDA) present in the sample, were measured by the method of Nuntapong et al [19].

#### Data analysis

Data are expressed as mean ± standard deviation (SD). Polynomial regression analysis was conducted between

specific growth rate and dietary PSL level. Statistical differences among mean values were analyzed by one-way analysis of variance (ANOVA), and differences between treatments were analyzed using Duncan's multiple range test and were considered significant at  $p < 0.05$ .

## RESULTS

### Growth performance and survival

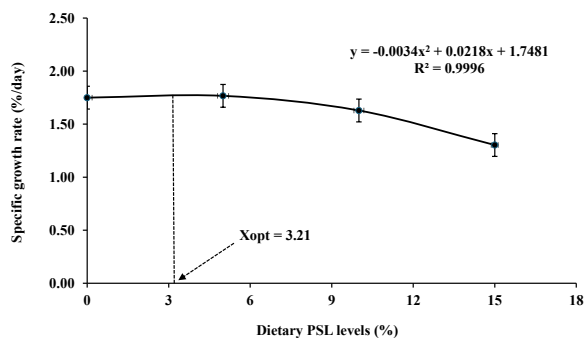
Growth performance and survival rate of the mud crabs fed the experimental diets for 90 days are shown in Table 2. After 30, 60, and 90 days of the feeding trial, mud crabs fed the FF had significantly higher final weight, average daily gain, specific growth rate, and feed conversion ratio than those in the other treatment groups ( $p < 0.05$ ). In addition, no significant differences in final weight, average daily gain, specific growth rate, and feed conversion ratio were observed in mud crabs fed diets supplemented with 0 or 5% PSL compared to the CSF at 30, 60, and 90 days of the feeding trial ( $p > 0.05$ ). In this study, no significant differences were found in survival rates among all treatment groups at 30, 60, and 90 days of the feeding trial ( $p > 0.05$ ). Water quality parameters were maintained throughout the experiment, with temperature at  $24.97 \pm 0.13$  °C, salinity at  $19.63 \pm 0.49$  ppt, total alkalinity at  $119.55 \pm 2.16$  mg/l, pH at  $8.11 \pm 0.08$ , dissolved oxygen at  $6.89 \pm 0.01$  mg/l, nitrite at

**Table 2** Growth performance, feed conversion ratio, and survival rate of green mud crabs fed experimental diets supplemented with PSL at various concentrations compared to FF and CSF for 90 days of dietary treatment (mean  $\pm$  SD).

Treatment	Experimental diet						p value
	T1: FF	T2: CSF	T3: PSL0	T4: PSL5	T5: PSL10	T6: PSL15	
<i>Average body weight (g/crab)</i>							
Initial weight	8.14 $\pm$ 0.96	8.04 $\pm$ 1.17	7.96 $\pm$ 1.42	7.96 $\pm$ 1.33	8.12 $\pm$ 1.09	7.81 $\pm$ 1.00	0.786
Day 30	18.11 $\pm$ 1.20 <sup>b</sup>	15.59 $\pm$ 2.15 <sup>a</sup>	15.39 $\pm$ 2.38 <sup>a</sup>	15.63 $\pm$ 1.95 <sup>a</sup>	15.61 $\pm$ 2.20 <sup>a</sup>	14.92 $\pm$ 2.13 <sup>a</sup>	<0.001
Day 60	34.10 $\pm$ 2.89 <sup>d</sup>	25.17 $\pm$ 2.60 <sup>c</sup>	24.64 $\pm$ 4.21 <sup>bc</sup>	24.80 $\pm$ 2.65 <sup>c</sup>	23.35 $\pm$ 3.14 <sup>b</sup>	19.70 $\pm$ 2.68 <sup>a</sup>	<0.001
Day 90	50.09 $\pm$ 6.91 <sup>d</sup>	36.67 $\pm$ 2.36 <sup>b</sup>	37.79 $\pm$ 3.33 <sup>c</sup>	38.56 $\pm$ 3.17 <sup>c</sup>	35.12 $\pm$ 4.38 <sup>b</sup>	24.84 $\pm$ 2.06 <sup>a</sup>	<0.001
<i>Average daily gain (g/day)</i>							
Day 30	0.33 $\pm$ 0.03 <sup>b</sup>	0.25 $\pm$ 0.06 <sup>a</sup>	0.25 $\pm$ 0.05 <sup>a</sup>	0.26 $\pm$ 0.06 <sup>a</sup>	0.25 $\pm$ 0.06 <sup>a</sup>	0.24 $\pm$ 0.06 <sup>a</sup>	<0.001
Day 60	0.43 $\pm$ 0.05 <sup>d</sup>	0.28 $\pm$ 0.04 <sup>c</sup>	0.28 $\pm$ 0.06 <sup>c</sup>	0.28 $\pm$ 0.04 <sup>c</sup>	0.25 $\pm$ 0.05 <sup>b</sup>	0.20 $\pm$ 0.04 <sup>a</sup>	<0.001
Day 90	0.47 $\pm$ 0.08 <sup>d</sup>	0.32 $\pm$ 0.03 <sup>bc</sup>	0.33 $\pm$ 0.04 <sup>c</sup>	0.34 $\pm$ 0.04 <sup>c</sup>	0.30 $\pm$ 0.05 <sup>b</sup>	0.19 $\pm$ 0.02 <sup>a</sup>	<0.001
<i>Specific growth rate (%/day)</i>							
Day 30	2.68 $\pm$ 0.32 <sup>b</sup>	2.22 $\pm$ 0.50 <sup>a</sup>	2.21 $\pm$ 0.40 <sup>a</sup>	2.27 $\pm$ 0.51 <sup>a</sup>	2.17 $\pm$ 0.45 <sup>a</sup>	2.15 $\pm$ 0.46 <sup>a</sup>	<0.001
Day 60	2.38 $\pm$ 0.20 <sup>d</sup>	1.90 $\pm$ 0.21 <sup>c</sup>	1.88 $\pm$ 0.24 <sup>c</sup>	1.90 $\pm$ 0.30 <sup>c</sup>	1.76 $\pm$ 0.28 <sup>b</sup>	1.54 $\pm$ 0.20 <sup>a</sup>	<0.001
Day 90	2.01 $\pm$ 0.20 <sup>d</sup>	1.71 $\pm$ 0.16 <sup>bc</sup>	1.75 $\pm$ 0.18 <sup>c</sup>	1.77 $\pm$ 0.20 <sup>c</sup>	1.63 $\pm$ 0.18 <sup>b</sup>	1.30 $\pm$ 0.13 <sup>a</sup>	<0.001
<i>Feed conversion ratio</i>							
Day 30	2.32 $\pm$ 0.24 <sup>b</sup>	1.01 $\pm$ 0.02 <sup>a</sup>	1.01 $\pm$ 0.02 <sup>a</sup>	1.00 $\pm$ 0.01 <sup>a</sup>	1.03 $\pm$ 0.02 <sup>a</sup>	1.03 $\pm$ 0.02 <sup>a</sup>	<0.001
Day 60	2.09 $\pm$ 0.10 <sup>b</sup>	1.05 $\pm$ 0.03 <sup>a</sup>	1.04 $\pm$ 0.05 <sup>a</sup>	1.10 $\pm$ 0.03 <sup>a</sup>	1.11 $\pm$ 0.02 <sup>a</sup>	1.11 $\pm$ 0.10 <sup>a</sup>	<0.001
Day 90	2.14 $\pm$ 0.01 <sup>b</sup>	1.03 $\pm$ 0.01 <sup>a</sup>	1.04 $\pm$ 0.01 <sup>a</sup>	1.03 $\pm$ 0.04 <sup>a</sup>	1.11 $\pm$ 0.08 <sup>a</sup>	1.15 $\pm$ 0.15 <sup>a</sup>	<0.001
<i>Survival rate (%)</i>							
Day 0–90	77.78 $\pm$ 3.85	73.33 $\pm$ 6.67	82.22 $\pm$ 3.85	88.89 $\pm$ 7.70	84.44 $\pm$ 7.70	75.55 $\pm$ 3.85	0.061

FF, fish flesh; CSF, commercial shrimp feed; and PSL, powdered sea lettuce.

Values within the same row with different superscripts are significantly different ( $p < 0.05$ ).



**Fig. 1** Relationship between the specific growth rate and dietary PSL level.  $X_{opt}$  is the model-based optimal dietary PSL level maximizing the specific growth rate.

0.06  $\pm$  0.00 mg/l, and total ammonia nitrogen at 0.32  $\pm$  0.07 mg/l.

A polynomial regression analysis showed that the specific growth rate has a strong second order polynomial relationship with dietary PSL level ( $R^2 = 0.9996$ ) and based on this model, it obtained its maximum value when the dietary PSL level was 3.21% (Fig. 1).

#### Whole-body proximate composition of mud crabs

During the 90 days of the feeding trial, body composition (% wet weight basis) was significantly affected by dietary supplementation with PSL (Table 3). The mud crabs fed the FF had significantly higher crude protein and crude lipid contents than those in the other

treatments ( $p < 0.05$ ). In addition, the concentration of crude protein in the whole body of mud crabs significantly decreased with the level of PSL supplementation ( $p < 0.05$ ). However, no significant differences in crude protein and crude lipid were observed in mud crabs fed diets supplemented with 0 or 5% PSL compared to the CSF treatment at 90 days of the feeding trial ( $p > 0.05$ ). There were no statistical differences in moisture or ash content of the whole body of mud crabs examined in this study ( $p > 0.05$ ).

#### Hemolymph parameters

After 90 days of the feeding trial, mud crabs fed diets supplemented with CSF, 5 and 10% PSL had significantly higher total hemocyte counts than those fed the FF or diets supplemented with 0 or 15% PSL ( $p < 0.05$ ). Moreover, mud crabs fed diets supplemented with 5 and 10% PSL exhibited significantly higher hemolymph protein than those fed the FF, CSF, or diets supplemented with 15% PSL ( $p < 0.05$ ). In addition, mud crabs fed diets supplemented with 15% PSL had significantly higher PO activity than those in the other treatments ( $p < 0.05$ ). There were no significant differences in glucose levels of mud crabs by the dietary treatments observed in this study ( $p > 0.05$ ) (Table 4).

#### Antioxidant parameters

After 90 days of the feeding trial, mud crabs fed diets supplemented with 15% PSL had significantly higher CAT activity than the other treatment groups ( $p < 0.05$ ) (Fig. 2a). Moreover, mud crabs fed diets

**Table 3** Whole-body proximate compositions (% wet weight basis) of green mud crabs fed experimental diets supplemented with PSL at various concentrations compared to FF and CSF, at 90 days of dietary treatment (mean ± SD).

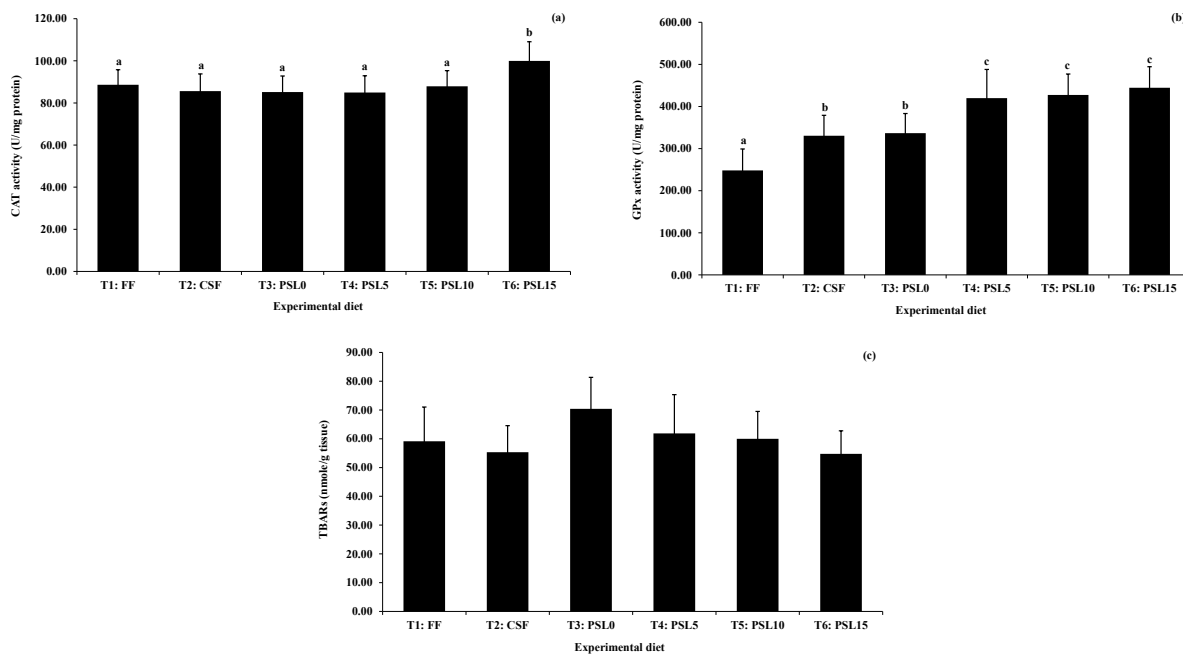
Treatment	Experimental diet						p value
	T1: FF	T2: CSF	T3: PSL0	T4: PSL5	T5: PSL10	T6: PSL15	
Moisture	78.70 ± 2.54	77.85 ± 3.07	78.67 ± 3.26	78.24 ± 2.60	78.79 ± 3.01	78.09 ± 3.32	0.998
Crude protein	9.61 ± 0.13 <sup>d</sup>	8.51 ± 0.41 <sup>c</sup>	8.19 ± 0.43 <sup>c</sup>	7.94 ± 0.31 <sup>c</sup>	7.20 ± 0.14 <sup>b</sup>	6.40 ± 0.23 <sup>a</sup>	0.035
Crude lipid	1.92 ± 0.11 <sup>b</sup>	1.49 ± 0.07 <sup>a</sup>	1.41 ± 0.31 <sup>a</sup>	1.32 ± 0.17 <sup>a</sup>	1.27 ± 0.14 <sup>a</sup>	1.14 ± 0.02 <sup>a</sup>	<0.001
Ash	9.76 ± 1.70	9.36 ± 1.63	9.58 ± 2.73	9.53 ± 2.34	9.14 ± 3.45	8.11 ± 2.21	0.965

FF, fish flesh; CSF, commercial shrimp feed; and PSL, powdered sea lettuce.  
 Values within the same row with different superscripts are significantly different (*p* < 0.05).

**Table 4** Hemolymph parameters of green mud crabs fed experimental diets supplemented with PSL at various concentrations compared to FF and CSF, at 90 days of dietary treatment (mean ± SD).

Treatment	Experimental diet						p value
	T1: FF	T2: CSF	T3: PSL0	T4: PSL5	T5: PSL10	T6: PSL15	
Total hemocyte count (x10 <sup>7</sup> cell/ml)	3.28 ± 0.61 <sup>ab</sup>	5.78 ± 1.22 <sup>c</sup>	3.85 ± 0.76 <sup>b</sup>	5.34 ± 1.30 <sup>c</sup>	5.22 ± 0.28 <sup>c</sup>	2.49 ± 0.77 <sup>a</sup>	<0.001
Glucose (mg%)	12.19 ± 0.54	12.80 ± 0.76	13.04 ± 1.07	12.93 ± 1.14	12.65 ± 0.59	12.40 ± 0.46	0.218
Hemolymph protein (mg/ml)	18.05 ± 4.84 <sup>a</sup>	21.16 ± 4.15 <sup>a</sup>	22.01 ± 5.95 <sup>ab</sup>	25.92 ± 3.75 <sup>b</sup>	26.11 ± 4.33 <sup>b</sup>	18.14 ± 4.14 <sup>a</sup>	<0.001
PO activity (unit/min/mg protein)	19.85 ± 3.43 <sup>a</sup>	21.28 ± 3.16 <sup>a</sup>	20.48 ± 2.07 <sup>a</sup>	21.78 ± 2.34 <sup>a</sup>	22.42 ± 3.50 <sup>a</sup>	30.86 ± 3.73 <sup>b</sup>	<0.001

FF, fish flesh; CSF, commercial shrimp feed; and PSL, powdered sea lettuce.  
 Values within the same row with different superscripts are significantly different (*p* < 0.05).



**Fig. 2** CAT activity (a), GPx activity (b), and TBARS (c) of green mud crabs fed experimental diets supplemented with PSL at various concentrations, compared to FF and CSF, after 90 days of feeding.

supplemented with 5, 10, or 15% PSL had significantly higher GPx than those in FF, CSF and 0% PSL groups (Fig. 2b). No significant differences were found in TBARs among the treatments at 90 days of the feeding trial ( $p > 0.05$ ) (Fig. 2c).

## DISCUSSION

Mud crabs can be successfully raised on a variety of diets including fish and fish wastes, mussels, snails, shrimp, bivalves, animal hides, entrails, kitchen leftovers, and formulated shrimp feeds [2]. The present study investigated the optimal level of dietary PSL on growth performance, immune responses, and antioxidant parameters in green mud crab, when receiving a diet with fish meal and defatted-soybean meal as a protein source, and wheat flour and rice flour as a carbohydrate source. In this study, the dietary PSL treatments were prepared to contain 42% protein and 8% fat, which are apparently similar to those reported by Zhong et al [25]. The results from the present study showed that the mud crabs fed the FF had significantly better growth and whole-body proximate composition than those in the other treatment groups ( $p < 0.05$ ). Previously, Thepphanich et al [5] reported that the juvenile mud crabs (*S. paramamosain*) fed sardine meat attained a higher growth rate ( $p < 0.05$ ) than those fed an artificial diet, in terms of weight gain. The lower growth as well as reduced whole-body protein and lipid contents observed in mud crabs fed dietary PSL, compared with those fed FF, indicate that the formulated feed may not be suitable as a complete replacement for natural feed in mud crab aquaculture. The natural feed may contain certain nutrients that were lacking in the formulated feed, thereby enhancing growth, whereas the formulated feed exhibited poor acceptability to mud crabs [26]. Nevertheless, the use of fresh feed has several limitations such as seasonal availability and difficulties in maintaining water quality, difficulties in storage, and risks related to the spreading of diseases [5, 27]. Taking into account the growth of mud crabs, the results showed no significant differences between mud crabs fed dietary PSL (0 or 5% PSL) and CSF, suggesting that our formulated diets used in this study supported mud crab growth to a level comparable with that of the commercial feed.

Algal meals or algal extracts have long been studied as feed supplement candidates for improving growth performance and feed utilization of aquaculture species. Most nutritional studies with *Ulva* sp. meal or extract indicate that the inclusion of these algae in aquafeeds improves growth and feed utilization. Akbary and Aminikhoei [16] reported that water-soluble polysaccharide extract of *U. rigida* (WPU) was effective as a growth promoter for Pacific white shrimp (*Litopenaeus vannamei*), and that the dietary supplementation with 1.5 g/kg of WPU improved growth, antioxidant activity, and immune responses

in shrimp. The results of the current study showed that 3.21% PSL supplementation in mud crab diet enhanced growth performance over a 90-day feeding trial. This is consistent with Cruz-Suárez et al [8], who concluded that 3.3% *U. clathrata* meal provided better performance in juvenile Pacific white shrimp when compared with those fed brown seaweed meals. In addition, Ergün et al [15] reported that 5% *U. rigida* meal increased growth performance in Nile tilapia (*Oreochromis niloticus*). However, it should be noted that the growth of mud crabs that received diets supplemented with 10 or 15% PSL was significantly reduced. Similarly, Qiu et al [28] reported that dietary supplementation with *U. lactuca* meal at 10 and 15% as a substitute for fish meal significantly reduced the weight gain of Pacific white shrimp. This would be due to nutritional availability, shifts in palatability, and anti-nutrient and mineral excesses [28]. Thus, the utilization of polysaccharide extract of *U. rigida* may provide better growth than PSL, as extraction effectively removes undesirable components.

Generally, survival above 80% is considered good in the study of crustaceans [29, 30]. The present study revealed the highest survival in mud crabs fed a diet supplemented with 5% PSL (88.89%), while no significant differences were observed among the alternative treatments ( $p > 0.05$ ), demonstrating that the experimental diets as well as the rearing conditions used throughout the experiment were appropriate for green mud crab aquaculture. Throughout the experimental period, no disease infection or abnormal clinical signs were observed. Some mortality observed during the experiment was possibly associated with environmental factors, particularly water temperature, which was  $24.97 \pm 0.13$  °C. The optimal temperature range for *S. paramamosain* aquaculture is 28–30 °C, and temperature below or above this range may adversely affect growth performance, survival, or the gill lamella structure of *S. paramamosain* crablets during long-term nursery phase [31].

*Ulva* seaweed contains sulfated polysaccharides that have been reported to have antioxidant, antiviral, immunomodulatory, anticancer, anticoagulant, and anti-inflammatory activities [32]. Although reduced growth was observed in mud crabs fed diets supplemented with 10–15% PSL, this study demonstrated that dietary supplementation with 5–15% PSL enhanced immune responses and antioxidant parameters. The mud crabs fed 5% and 10% PSL diets exhibited higher hemolymph parameters, i.e., total hemocyte count and hemolymph protein, than those fed the FF. Basically, crustacean hemocytes play vital roles in the host immune responses, including in recognition, phagocytosis, melanization, cytotoxicity, and cell-to-cell communication [33]. Suleman et al [34] demonstrated that Pacific white shrimp fed polysaccharide extracts of *U. lactuca* had significantly higher total hemocyte count and PO activity than those received

the control diet. Similarly, shrimp fed *U. fasciata* extract exhibited significantly increased total hemocyte count, differential hemocyte counts, agglutination index, phagocytic rate, bacterial clearance, and serum bactericidal activity than the control group [35]. Glucose in crustaceans is regulated by the crustacean hyperglycemic hormone (cHH). Increased circulating cHH and hyperglycemia are fundamental responses to various types of stress [36]. In this study, no significant differences in hemolymph glucose were observed among the treatments, indicating that dietary supplementation with PSL had no adverse effect on mud crabs.

This study detected the highest PO activity in mud crabs fed diets supplemented with 15% PSL compared to the other treatment groups. The enzyme PO serves as a key component in pathogen defense and recognition [16]. In the study by Akbary and Aminikhoei [16], increased levels of PO activity were observed in Pacific white shrimp fed diets containing WPU at 1 and 1.5 g/kg.

The antioxidant enzymes are important to the non-specific immune responses and have been widely tested to evaluate the health condition of crustaceans [37]. It has been reported that Pacific white shrimp fed 1.5 g/kg of WPU had significantly increased CAT and GPx [16]. CAT and GPx are major antioxidant enzymes that can decompose hydrogen peroxide into water and oxygen, protecting tissue against oxidative damage [38, 39]. In this study, stimulatory effects of PSL on CAT and GPx activities were demonstrated in mud crabs fed diets supplemented with 15 and 5–15% PSL, respectively. These results suggest that the antioxidant properties of these marine algae could enhance immune function. Basically, lipid peroxidation in the hepatopancreas was evaluated via the TBARs level, based on the reaction of 2-thiobarbituric acid and MDA [19]. The present study revealed no significant differences in TBARs among the treatments. MDA is a final product of lipid peroxidation that induces toxic stress in cells and serves as a biomarker for oxidative stress in organisms [40]. Lower MDA levels are attributed to a reduction in free radicals, indicating improved oxidative stress conditions [16].

Although improvements in immunity and antioxidant capacity may not be sufficient to encourage farmers to adopt dietary PSL when growth performance is inferior to that of FF, the present study indicates additional benefits of dietary PSL. Mud crabs fed dietary PSL tended to exhibit higher survival rate than those fed the FF. Moreover, PSL supplementation resulted in maintained growth and enhanced immune responses and antioxidant capacity compared with the CSF. These findings suggest that dietary PSL may provide advantages beyond immunomodulation, including enhanced antioxidant capacity, and highlight its potential as a functional feed ingredient in mud crab aquaculture.

Overall, the inclusion of 3.21% PSL optimized

growth performance in mud crab, while the inclusion of less than 15% resulted in superior immune and antioxidant capabilities. These results highlight dual potential of PSL to enhance growth and boost health, confirming its promise as an alternative nutrient source for sustainable mud crab aquaculture. Further investigation of mixed feeding regimes combining FF and dietary PSL, as well as studies on the effects of polysaccharide extracts from *U. rigida* on growth performance, immune responses, and antioxidant parameters may provide more insights into their potential applications in aquafeed.

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