Oral Administration of Hot-water Extract of Tropical Brown Seaweed, Sargassum cristaefolium, to Enhance Immune Response, Stress Tolerance, and Resistance of White Shrimp, Litopenaeus vannamei, to Vibr

by Diana Chilmawati

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Oral Administration of Hot-water Extract of Tropical Brown Seaweed, *Sargassum cristaefolium*, to Enhance Immune Response, Stress Tolerance, and Resistance of White Shrimp, *Litopenaeus vannamei*, to *Vibrio parahaemolyticus*

Agung Sudaryono[1] Diana Chilmawati, and Titik Susilowati

Aquaculture Department, Faculty of Fisheries and Marine Science, Diponegoro University, Jl. Prof Sudharto, SH, Tembalang Campus, Semarang, 50275, Indonesia

Abstract

The efficacy of hot-water extract of tropical brown seaweed, *Sargassum cristaefolium* (SCE), supplemented in diets on immune response, stress tolerance, and disease resistance of *Litopenaeus vannamei* to *Vibrio parahaemolyticus* was evaluated. Shrimp were fed diets containing graded levels of SCE (0, 250, 500, 750, and 1000 mg/kg). The results showed that shrimp fed all diets containing SCE had significantly higher ($P < 0.05$) immune response in total hemocyte count (THC), differential hemocyte count (granular and hyaline cells), and phagocytic activity than those of shrimp fed the control diet. Similarly, in low dissolved oxygen stress tolerance test and the challenge test with *V. parahaemolyticus*, survival rates of shrimp fed all diets containing SCE were significantly higher ($P < 0.05$) (83–93% in stress test and 27–47% in challenge test) than those of shrimp fed the control diet (77 and 3.3%, respectively). These results suggest that oral administration of SCE at 500 and 750 mg/kg can be effectively used to enhance immune response, stress tolerance, and resistance of white shrimp, *L. vannamei*, against *V. parahaemolyticus* infection. These findings also confirm that using dietary SCE as immunostimulant is effective at increasing the nonspecific immune system in penaeid shrimp, *L. vannamei*.

KEYWORDS

hot-water extract, immune response, *Litopenaeus vannamei*, *Sargassum cristaefolium*, *Vibrio parahaemolyticus*

Disease outbreaks caused by various bacteria, fungus, parasites, and viruses are the main problem in the sustainability and profitability of crustacean farming (Flegel et al. 2008; Bai et al. 2014). Recently, the global shrimp farming industry has suffered severe economic losses due to the acute hepatopancreatic necrosis disease (AHPND) or early mortality syndrome (EMS) attributed to certain strains of *Vibrio parahaemolyticus* being identified and suspected as the causative agents that resulted in total mortalities in penaeid shrimp (Tran et al. 2013). Therefore, the use of immunostimulating compounds that could prevent and control diseases is urgently needed. The potential of using medicinal plants such as seaweeds as natural immunostimulants in aquaculture as an alternative to antibiotics and immunoprophylactics has been well documented (Rabia et al. 2013; Al-Saif et al. 2014; Reverter et al. 2014; Thanigaivel et al. 2016). Immunostimulants have been proven to be more effective and safer than chemotherapeutics and their efficacy has become more powerful than vaccination (Sakai 1999). However, it is well known that vaccination and chemotherapeutic treatments are able to improve health status and resistance of farmed fish and shellfish.

The interest in application of seaweeds in the form of meal and/or extract compounds as immunostimulants in aquaculture has recently increased worldwide because they are easy to prepare, inexpensive, and have few side effects

[1] Correspondence to: agungsoed@yahoo.co.id

on aquatic animals and the environment (Thanigaivel et al. 2016). Ulvaceae and Caulerpaceae (green algae); Sargassaceae (brown algae); and Gracilariaceae, Gelidiaceae, and Hypneaceae (red algae) are the most important seaweed families that have been used as immunostimulants in aquaculture (Hafezieh et al. 2014; Thanigaivel et al. 2016). The potency of these bioactive polysaccharide immunostimulants extracted from macroalgae such as carrageenan, laminaran, alginate, and fucoidan has been widely studied and have effectively been proved to enhance growth performance and nonspecific immune system in penaeid shrimp and fish (Cheng et al. 2004, 2005, 2013; Chotigeat et al. 2004; Yeh and Chen 2008; Traifalgar et al. 2009; Immanuel et al. 2010; Ghaednia et al. 2011; Huynh et al. 2011; Kitikiew et al. 2013; Sudaryono et al. 2015; Isnansetyo et al. 2016).

Many studies have been conducted to evaluate the potency of active ingredients of immunostimulatory polysaccharides from several brown algae species, *Sargassum* spp., in the form of meal or extracts in diets for penaeid shrimp. Immune enhancement in penaeid shrimp to *Vibrio alginolyticus* and *Vibrio harveyi* infections has been reported when the shrimp were fed diets containing various species of *Sargassum* either in the form of meal (Felix et al. 2004; Cheng et al. 2005; Hafezieh et al. 2014) or methanolic and hot-water extracts (Chotigeat et al. 2004; Huang et al. 2006; Yeh et al. 2006; Huxley and Lipton 2009; Ghaednia et al. 2011; Huynh et al. 2011; Chang et al. 2013; Yudiati et al. 2016). In addition, dietary administration of hot-water extract of *Sargassum cristaefolium* (SCE) has been proven to improve feed and protein utilization in juvenile *Litopenaeus vannamei* (Sudaryono et al. 2015). To the best of our knowledge, no information to date exists regarding the evaluation of oral administration of SCE obtained from Indonesia on immune response, stress tolerance, and resistance against *V. parahaemolyticus* infection in penaeid white shrimp, *L. vannamei*. This *V. parahaemolyticus* and another three vibrio bacteria such as *V. harveyi*, *Vibrio penaecida*, and *Vibrio vulnificus* are known as dangerous pathogen bacteria that can result in mass mortality up to 80% in cultured penaeid shrimp (Ishimaru et al. 1995; Lavilla-Pitogo 1995; Lightner 1996; Chatterjee and Haldar 2012; Kumaran and Citarasu 2016).

The present study was conducted to evaluate the effects of dietary supplementation of SCE on immune response, stress tolerance (under low dissolved oxygen [DO] conditions), and resistance of white shrimp, *L. vannamei*, against *V. parahaemolyticus* infection. It is hoped that these findings will positively assist in increasing the nonspecific immune system of penaeid shrimp, *L. vannamei,* so that shrimp can survive the high mortality risk caused by bacterial disease outbreaks of *V. parahaemolyticus*.

Materials and Methods

An indoor laboratory feeding trial facility at the Coastal Eco-Development Laboratory of Fisheries and Marine Science Faculty, Diponegoro University, Jepara, Central Java, Indonesia, equipped with a continuous aeration system and a natural photoperiod cycle was used in this study. Seawater (salinity 32 ppt) was pumped to a 10,000-L concrete tank and settled for 7 d, and then the water was moved into another 10,000-L concrete tank as a reserve tank. In the reserve tank, the water was sterilized by adding 15 mg/L chlorine solution and kept for 4 h. Then, 4.5 mg/L thiosulfate solution was added and kept for 24 h to neutralize chlorine contamination in the water. Continuous aeration was provided by electrical blower during the sterilization process.

Preparation of S. cristaefolium *Extract and the Experimental Diets*

Tropical brown seaweed, *S. cristaefolium*, was obtained from the coastal water of Bandengan District, Jepara Regency, Central Java Province, Indonesia. SCE was prepared following the method described previously by Sudaryono et al. (2015). The seaweed was properly washed with tap water and rinsed to let dry naturally overnight on a plastic net at room temperature. The samples were cut into small pieces (ca. 0.5 cm in length) and then air dried in an oven at 40 C for 24 h. The dried samples were ground into powder using an electric grinder and passed through a 250-µm mesh sieve. The milled dried *S. cristaefolium* (100 g) were extracted twice (double boiling) in 3000 and 2000 mL hot distilled water for 3 and 2 h, respectively. The first and second filtrated extracts were mixed and then gradually evaporated using a pan heated on a stove with a small fire until it produced a pasta-like concentrate. The percentage of the residue obtained from the extraction process of *S. cristaefolium* meal in hot water was 20.7 ± 0.4%. Proximate analysis of the extract was determined by following AOAC (2000). It contained 1.4% crude protein, 0.6% crude lipid, 62.9% crude ash, 12.2% moisture, and 22.9% total carbohydrate. The extract was stored in a freezer until being used. Commercial feeds (Shrimp Starter Diet PV1; PT. STP Comfeed) for *L. vannamei* with a minimum 40% crude protein content obtained from suppliers in Jepara, Central Java Province, were used as a basal diet in the present study. SCE was added in the experimental diets as immunostimulant in this study. Four formulated diets containing different SCE levels (250, 500, 750, and 1000 mg/kg diet) and one control diet (0 mg/kg diet) were prepared as experimental diets (Table 1).

All experimental diets were formulated to be isonitrogenous (ca. 40.0% crude protein content on dry matter basis) with contents of crude lipid (minimum 6.5%), crude fiber (maximum 2.2%), and ash (maximum 13.0%). Preparation for making test diets followed that of Sudaryono et al. (2015), with a slight modification. The commercial feeds were ground using an electric grinder and then sieved through a 250-μm sieve, weighed according to each formulation in Table 1, and thoroughly mixed in a bowl mixer for 5 min; then oil was gradually added and mixed well again. A 40% distilled water was slowly added to make a stiff dough consistency. The dough was pelleted through a 2-mm die to produce a noodle-like shape and then air dried in an oven overnight at 50 C. The dry pellets were cut in 5-mm length, packed in sealed plastic bags, and then kept in a freezer until use for immunity and challenge tests.

Experimental Animals and the Experimental Design

Two separate groups of healthy white shrimp, *L. vannamei*, with different sizes obtained from a commercial shrimp farm in Jepara, Central Java, Indonesia, where no disease outbreaks have been recorded were used in the study (through negative polymerase chain reaction test). The first group, 300 small shrimp with a weight range of 0.5–1.0 g were used for the stress tolerance test. The second group consisted of 400 juvenile shrimp in total with a weight range of 10–12 g (mean weight of 10.82 ± 0.7 g) that were used for the immune response test (200 shrimp) and the disease resistance test (200 shrimp). There were no potential effects on the use of two different sizes of shrimp in the present study. The smaller ones were used to study stress tolerance. Any sizes of shrimp can be used for stress tolerance testing without any effects. On the other hand, the use of larger shrimp juveniles in the study could allow for withdrawing 100 μL of hemolymph from the ventral sinus of each shrimp into a 1-mL sterile syringe.

The small shrimp and the juvenile shrimp were stocked in two 200-L round fiber tanks and in a 1500-L concrete tank, respectively. All tanks were equipped with aeration and black plastic blanket to control the temperature (28–30 C). Before feeding the experimental diets, the shrimp were acclimated to commercial shrimp feed (Grower Diet D2, STP Aqua Feed Company, Jakarta, Indonesia) as a control diet and laboratory conditions for 7 d. Only shrimp in the intermolt stage were used in the study following the method of Liu and Chen (2004). During the periods of acclimation and experiment, 10% of the water was exchanged daily to maintain adequate water quality. The water quality was maintained at a range of 28–30 C for temperature, 7.8–8.2 for pH using a pH meter (WTW330I model 202), 32–33 ppt for salinity using a refractometer, 3.7–6.0 mg/L for DO using a DO meter (WTW320I), and total ammonia nitrogen less than 0.01 mg/L using an Ammonia Kit (Tetra Test GmbH, Tetra Warke, Germany). A natural photoperiod of 12 h light and 12 h dark was maintained throughout in all the experiments.

A total of 150 acclimated healthy shrimp juveniles (10.82 ± 0.7 g initial mean weight) from the stock tank were transferred and randomly distributed in 15 black round polyvinyl chloride

TABLE 1. *Composition of the basal diet (g/kg) for* Litopenaeus vannamei.

Ingredients	Hot-water extract of *Sargassum cristaefolium* in diet (mg/kg) Diet A (Control)	Diet B (250)	Diet C (500)	Diet D (750)	Diet E (1000)
SCE[1]	0.00	0.25	0.50	0.75	1.00
CMC (binder) (0.5%)	5.00	5.00	5.00	5.00	5.00
Basal diet[2] (98.5%)	995.00	994.75	994.50	994.25	994.00
Total (100%)	1000.00	1000.00	1000.00	1000.00	1000.00
Proximate analysis (% dry basis)[3]					
Crude protein	406.20	394.50	400.90	392.80	412.00
Crude lipid	90.60	85.60	90.10	89.90	87.50
Crude fiber	20.20	21.60	21.50	22.00	20.90
Crude ash	115.70	112.70	122.60	114.00	111.80
Nitrogen-free extract (calculated by difference)	367.30	385.60	364.90	381.30	367.80

[1] SCE = hot-water extract of Indonesian tropical brown seaweed, *S. cristaefolium*.

[2] Commercial shrimp starter feed PV1 of STP, Comfeed Company, Indonesia (specifications: crude protein minimum, 40,0%; crude lipid minimum, 6.5%; crude fiber maximum, 2.2%; crude ash maximum, 13,0%; moisture maximum, 12,0%).

[3] Values are mean of triplicate samples.

(PVC) tanks (25 L) filled with 20 L of filtered seawater for trial. Shrimp were stocked at a density of 10 shrimp per experimental tank and fed regularly thrice a day (0700, 1200, and 1700 h) with 20% water exchanged daily. There were five treatment groups in triplicate to examine effects of shrimp fed five different experimental diets (Diets A [control], B, C, D, and E; see Table 1) on immune response, that is, total hemocyte count (THC); differential hemocyte count (DHC): hyaline cells (HCs), semi-granular cells (SGCs), and granular cells (GCs); and phagocytic activity (PA).

Immune Parameters: THC and DHC

Only shrimp in the intermolt stage were used for the hemolymph sampling (Liu and Chen 2004). The procedures for hemolymph sampling, preparation of hemolymph, and counting of hemocytes were conducted following the method described previously by Wei et al. (2012) and Chang et al. (2013), with slight modification. Shrimp were fed the test diets thrice a day for 12 d and after 3, 6, 9, and 12 d of feeding, hemolymph (100 µL) was collected from the ventral sinus (a walking leg) of each shrimp by using a 1-mL sterile syringe (25-gauge) containing 0.9 mL precooled (4 C) anticoagulant solution and injected into the Eppendorf microfuge.

The anticoagulant solution was prepared according to Vargas-Albores et al. (1993) (10 mM KCl, 450 mM NaCl, 10 mM ethylenediaminetetraacetic acid, 10 mM HEPES, and pH 7.3). A drop of the hemolymph and anticoagulant mixture (100 µL) was put on a hemocytometer to calculate the number of blood cells as THC/mm^3 using a microscope (Nikon Photolab, Tokyo Japan) with magnification of 400×. Another part of the mixture was used to determine DHC by using morphological criteria such as size and shape of cells and the difference of hemocyte refractivity. This was used to identify and numerate HCs, SGCs, and GCs. Before all samples were observed under the microscope, cells must be stained first by the May–Grünwald–Giemsa reagents. The remaining mixture was used for subsequent tests.

Phagocytic Activity

PA was determined following the method developed by Chang et al. (2013) and Isnansetyo et al. (2014). PA was determined by mixing 20 µL of hemolymph and 20 µL of phosphate-buffered saline in a microwell plate. The mixture was then added with 20 µL of 10^8 cells/mL formalin-killed *Bacillus subtilis* suspension and incubated for 30 min at 30 C. After incubation, 7 µL of this mixture was then smeared gently and followed by fixing with 95% ethanol and staining with 10% Giemsa for 20 min. The slides were then rinsed with tap water and then dried. The slides were observed under a light microscope (Axioskop, Zeiss, Germany) and some photographs were taken. PA was determined by observation under the microscope at a 1000× magnification and calculated from 100 phagocytes per slide. PA, defined as phagocytic rate (PR) was expressed as:

$$PR = \left(\left[\text{sum of active phagocytes (phagocytosis)}\right] / \left[100 \text{ phagocytes}\right]\right) \times 100\%$$

Challenge Test

The method of challenge trials followed a previous study conducted by Chang et al. (2013) with a bit of modification. The challenge test was carried out to assess the effects of dietary alginate of SCE on the resistance of *L. vannamei* to *V. parahaemolyticus* infection. A group of 150 healthy juvenile shrimp ranging from 10–12 g was stocked in fifteen 25-L black circular PVC tanks. Experimental shrimp (10 shrimp in each tank) were fed the test diets for 28 d. During the feeding period, 20% of the water in each tank was exchanged daily. There were five treatments in triplicate and each treatment used 30 shrimp.

Pathogenic bacterial suspension stock of *V. parahaemolyticus* used in the study was the common strain, not the pathogen-causing EMS. The *V. parahaemolyticus* was obtained from the bacterial collection of the Brackishwater Aquaculture Development Research Centre, the Ministry of Marine and Fisheries, Jepara, Indonesia. The virulent bacteria were prepared in sterile 1.5% NaCl and diluted to a certain concentration. After the 28-d feeding period, each experimental shrimp fed the test diets was injected by 20 µL of bacterial suspension (1×10^8 colony-forming units [CFU]/mL), resulting in 2×10^6 CFU/shrimp. However, shrimp fed the control diet were then injected with 20 µL of sterile 1.5% NaCl solution, which served as the unchallenged control. All injected shrimp were maintained in clean PVC tanks with aerated sterilized seawater at 33 ppt and 28 C for 5 d. Mortalities of the infected shrimp were monitored and recorded daily. The survival (%) of white shrimp, *L. vannamei*, challenged with *V. parahaemolyticus* were used for the challenge test data.

Stress Tolerance Test

After acclimatization to laboratory conditions, 20 small shrimp with an individual weight range of 0.5–1 g from each treatment were transferred to duplicate stress test chambers (10-L PVC tanks). A low-DO stress test was carried out following the method described by Supamattaya et al. (2005), with a slight modification. Conditions of low DO in the chamber were maintained by turning off the aeration system and using a plastic sheet overlying on the water surface in each stress test chamber. DO content in the test chamber was monitored and measured using a DO meter (YSI model 57 YSI Incorporated, OH, USA). The stress condition was applied only for 10 h/d from 7000 to 1700 h and then returned to the normal condition at 1700 h. DO levels in the chamber linearly dropped at 0.8–1 mg/L within 10 h. The stress test was conducted for 10 d. During the stress test, shrimp were fed regularly with a 20% daily water exchange rate. The mortality was recorded every day for a period of 10 d.

All data were subjected to one-way ANOVA and Duncan's multiple comparison test to examine differences among treatments using a SPSS version 19.0 computer software package for Windows® (Armonk, NY, US). All probability values were set at a $P < 0.05$ level of significance.

Results

Immune Parameters (THC and DHC) of L. vannamei *to* V. parahaemolyticus

No significant differences in THC were observed among shrimp fed treatment diets containing SCE and the control diet for 3 and 6 d. The shrimp had different THC values after feeding with all experimental diets containing SCE for 9–12 d and they had significantly better THC performances than those fed the control diet. Mean THC ± SE for the

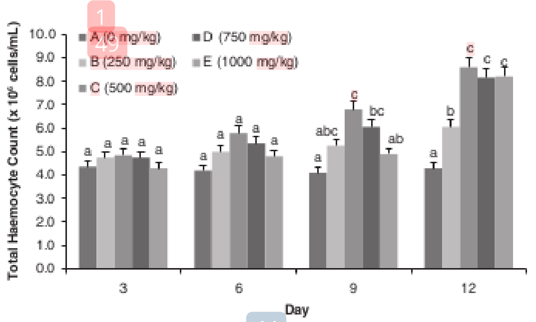

FIGURE 1. *Mean (±SE) total hemocyte count (THC; ×10⁶/mL) of* Litopenaeus vannamei *fed control diet and diets containing hot-water extract of* Sargassum cristaefolium *at 250, 500, 750, and 1000 mg/kg for 12 d. Each bars (mean ± SE;* n = 3*) with the same letters are not significantly different (*P > 0.05*).*

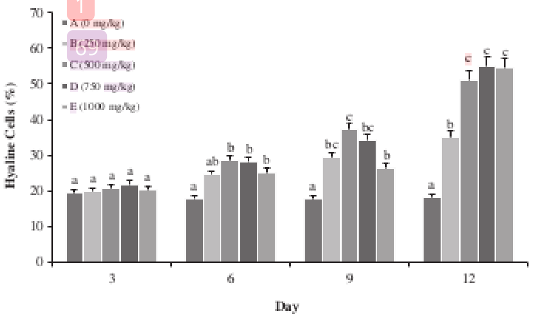

FIGURE 2. *Mean (±SE) hyaline cells (×10⁵/mL) of* Litopenaeus vannamei *fed control diet and diets containing hot-water extract of* Sargassum cristaefolium *at 250, 500, 750, and 1000 mg/kg for 12 d. Each bars (mean ± SE;* n = 3*) with the same letters are not significantly different (*P > 0.05*).*

shrimp fed the control, 250, 500, 750, and 1000 mg/kg SCE-containing diets for 12 d were 4.3 ± 0.4, 6.1 ± 0.4, 8.6 ± 0.7, 8.2 ± 0.9, and $8.2 \pm 0.6 \times 10^6$ cells/mL, respectively (Fig. 1). The shrimp fed SCE-containing diets for 12 d showed 41–100% higher THC performances than those fed the control diet. However, there was an increase in THC when shrimp were fed with increased dietary SCE levels from 0 to 500 mg/kg and then the THC decreased at levels of 750–1000 mg/kg (Table 2).

For the immune parameter of DHC in terms of HCs, there was a significant difference ($P < 0.05$) in HC among the shrimp fed SCE-containing diets and the control diet (0 mg SCE/kg) for 6, 9, and 12 d. An increased HC was found in hemocytes of the shrimp when they consumed the SCE-containing diets for 12 d. After a 12-d feeding period, the shrimp fed with increasing dietary SCE levels at 0, 250, 500, 750, and 1000 mg/kg resulted in increasing the number of HC (18.3 ± 2.4, 35.1 ± 3.4, 51.2 ± 4.3, 55.0 ± 5.2, and $54.5 \pm 4.1 \times 10^5$ cells/mL, respectively). The HC constituted 42.0 to 67.5% of the THC and varied from $17.6 \pm 3.0 \times 10^5$ (mean ± SE) to $55.0 \pm 5.2 \times 10^5$ cells/mL (Fig. 2). It was found that there were increased HCs of 42–61% in the shrimp when they consumed the SCE diets for 6 d compared to the control diet. The number of HCs increased by 67–111% and 92–201% for shrimp fed the SCE diets for 9 and 12 d, respectively, compared with those fed the control diet. However, there were no significant differences in the number of SGCs of the shrimp fed the SCE-containing diets and the control diet for 3, 6, 9, and 12 d ranging from $10.4–16.0 \times 10^5$ cells/mL. The SGC constituted 14.9–28.5% of the THC and varied from $10.4 \pm 2.3 \times 10^5$ to $16.0 \pm 6.2 \times 10^5$ cells/mL (Table 2).

In terms of GCs, the shrimp fed with dietary SCE of different levels up to 9 d did not result in effects on the number of GCs in their hemocytes. In addition, supplementation of

TABLE 2. *Mean (±SE) semi-granular cells (SGC) (×10⁵ cells/mL) of* Litopenaeus vannamei *(*n = 3*) fed different dietary hot-water extract of* Sargassum cristaefolium *(SCE) levels (0, 250, 500, 750, and 1000 mg/kg) for 12 d.*

	SGC (10^5/mL) of shrimp after feeding the SCE containing diets				
Day	A (0 mg/kg)	B (250 mg/kg)	C (500 mg/kg)	D (750 mg/kg)	E (1000 mg/kg)
3	11.8 ± 1.5^a	13.1 ± 2.2^a	12.8 ± 4.1^a	11.7 ± 2.3^a	10.4 ± 2.3^a
6	12.0 ± 2.0^a	12.2 ± 0.6^a	13.6 ± 1.4^a	11.6 ± 2.9^a	10.5 ± 2.3^a
9	11.5 ± 2.0^a	11.0 ± 3.1^a	14.2 ± 4.1^a	12.1 ± 3.6^a	10.4 ± 3.5^a
12	12.2 ± 0.9^a	12.1 ± 2.3^a	16.0 ± 6.2^a	12.1 ± 1.9^a	12.6 ± 3.9^a

TABLE 3. *Mean (±SE) granular cells (×10^5 cells/mL) of* Litopenaeus vannamei *(n = 3) fed different dietary hot-water extract of* Sargassum cristaefolium *(SCE) levels (0, 250, 500, 750, and 1000 mg/kg) for 12 d.*

	Granular cells of shrimp fed the SCE-containing diets[1]				
Day	A (0 mg/kg)	B (250 mg/kg)	C (500 mg/kg)	D (750 mg/kg)	E (1000 mg/kg)
3	12.2 ± 1.5^a	14.4 ± 2.4^a	15.1 ± 4.8^a	13.9 ± 2.7^a	12.4 ± 2.7^a
6	12.4 ± 2.1^a	13.3 ± 0.7^a	16.0 ± 1.6^a	13.8 ± 3.4^a	12.5 ± 2.7^a
9	19.9 ± 2.0^a	12.1 ± 3.3^a	16.7 ± 4.8^a	14.3 ± 4.3^a	12.4 ± 4.2^a
12	12.6 ± 0.9^a	13.3 ± 2.6^{ab}	18.8 ± 7.3^c	14.4 ± 2.3^{ac}	14.9 ± 4.6^{abc}

[1]Data in the same row with different superscripts are significantly different ($P < 0.05$).

dietary SCE significantly increased GC of the shrimp fed the diets for 12 d with the values of 12.6 ± 0.9, 13.3 ± 2.6, 18.8 ± 7.3, 14.4 ± 2.3, and 14.9 ± 4.6 × 10^5 cells/mL for the diets containing 0, 250, 500, 750, and 1000 mg SCE/kg, respectively. The GCs constituted 17.6–29.5% of the THC and varied from 11.9 ± 2.0 × 10^5 to 18.8 ± 7.3 × 10^5 cells/mL (Table 3). Adding SCE in the diets was found to effectively enhance the number of GCs 5.6–49.2% in *L. vannamei* fed the SCE-containing diets for 12 d.

Phagocytic Activity

PA of shrimp fed the SCE-containing diets at different levels of 250, 500, 750, and 1000 mg/kg was significantly higher ($P < 0.05$) than that of shrimp fed the control diet for the 6 to 12 d trial. PA at the end of the trial (12 d) was 71.5 ± 6.4, 88.0 ± 4.2, 87.5 ± 3.5, 90.5 ± 3.5, and 87.0 ± 2.8% for the shrimp fed the control diet (0), 250, 500, 750, and 1000 mg/kg SCE-containing diets, respectively (Fig. 3). Shrimp fed the diets containing SCE resulted in higher PA values than those fed the control diet with no SCE after feeding periods for 6, 9, and 12 d with PA increment of 11.4–16.4, 13.9–20.1, and 21.7–26.6%, respectively.

Disease Resistance Trial

Different dietary SCE levels (0, 250, 500, 750, and 1000 mg/kg) had a significant effect ($P < 0.05$) on survival of the shrimp after the challenge test with *V. parahaemolyticus* for 5 d (Table 4). All treatment groups fed diets containing SCE had higher survival than the control group. The shrimp fed diets containing 500 and 750 mg SCE/kg produced the highest survival

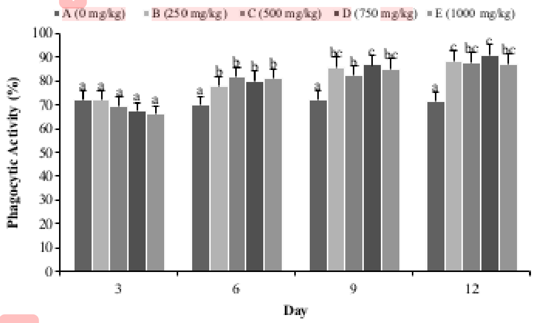

FIGURE 3. *Mean (±SE) phagocytic activity (%) of* Litopenaeus vannamei *fed control diet and diets containing hot-water extract of* Sargassum cristaefolium *at 250, 500, 750, and 1000 mg/kg for 12 d. Each bars (mean ± SE; n = 3) with the same letters are not significantly different (P > 0.05).*

(40 and 46.7%, respectively) at the end of the 5-d challenge trial followed by Diets E (33.3%), B (26.7%), and A (3.3%), respectively. Survival in the unchallenged control group was 100% following the 5-d challenge trial period. The highest dietary SCE level of 1000 mg/kg had no additional benefits in survival and was comparable to those of the 250 and 500 mg/kg diet.

Stress Tolerance

During the low DO stress tolerance trial period, different dietary SCE levels (0, 250, 500, 750, and 1000 mg/kg) had a significant effect ($P < 0.05$) on survival of the shrimp (Fig. 4). Shrimp fed the diet containing SCE of 500 mg/kg had the highest survival rate (93.3%), and similar survival rates (83.3–86.7%) were exhibited by shrimp fed diets containing SCE of 250, 750, and 1000 mg/kg. Moreover, the survival of all groups fed SCE-based

TABLE 4. *Mean (±SE) survival (%) of* Litopenaeus vannamei *after a challenge test with* Vibrio parahaemolyticus.[1]

Day	Unchallenged (control)	Hot-water extract of *S. cristaefolium* levels in diets[2] 0 mg/kg (control)	250 mg/kg	500 mg/kg	750 mg/kg	1000 mg/kg
1	100	33.3 ± 15.3^{a}	63.3 ± 5.8^{b}	66.7 ± 5.8^{b}	80.0 ± 10.0^{c}	63.3 ± 11.5^{b}
2	100	3.3 ± 5.8^{a}	26.7 ± 5.8^{b}	40.0 ± 10.0^{cd}	46.7 ± 15.3^{d}	33.3 ± 5.8^{bc}
3	100	3.3 ± 5.8^{a}	26.7 ± 5.8^{b}	40.0 ± 10.0^{cd}	46.7 ± 15.3^{d}	33.3 ± 5.8^{bc}
4	100	3.3 ± 5.8^{a}	26.7 ± 5.8^{b}	40.0 ± 10.0^{cd}	46.7 ± 15.3^{d}	33.3 ± 5.8^{bc}
5	100	3.3 ± 5.8^{a}	26.7 ± 5.8^{b}	40.0 ± 10.0^{cd}	46.7 ± 15.3^{d}	33.3 ± 5.8^{bc}

[1] Five triplicate groups of 10 shrimp were fed diets containing different dietary levels of hot-water extract of *Sargassum cristaefolium* (SCE) at 0, 250, 500, 750, and 1000 mg/kg for 28 d and challenged with *V. parahaemolyticus* by injection.
[2] Treatment Diets A (0 mg/kg), B (250 mg/kg), C (500 mg/kg), D (750 mg/kg), and E (1000 mg/kg). Data in the same row with different superscripts are significantly different ($P < 0.05$).

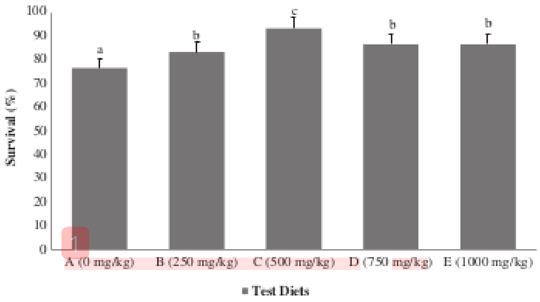

FIGURE 4. *Survival (%) of* Litopenaeus vannamei *observed in each treatment under low dissolved oxygen stress test for 10-d period. Each bar represents the mean ± SE. Each bar (mean ± SE;* n = 3*) with the same letters are not significantly different (*P > *0.05).*

diets (83.3–93.3%) was significantly higher ($P < 0.05$) than the control group (76.7%).

Discussion

Bioactive ingredients of polysaccharide extracted from brown seaweed either in the form of purified compounds or in the form of less purified compounds referred to as "crude extracts" such as alginate and fucoidan have been reported to have similar potential immunostimulatory and antimicrobial properties (Immanuel et al. 2004; Hou and Chen 2005; Fu et al. 2007). In recent studies, the use of algal extracts derived from different brown seaweed species, *Sargassum* spp., has been reported to be effective at enhancing immune response and the resistance of white shrimp, *L. vannamei*, against *Vibrio* bacterial infection (Yeh et al. 2006; Huynh et al. 2011; Chang et al. 2013; Kitikiew et al. 2013; Yudiati et al. 2016). In this study, it was found that the immune response of *L. vannamei* fed with dietary levels of SCE at 250–1000 mg/kg was effectively enhanced compared to those fed the control diet without the seaweed extract. Increasing THC 28–66% and 41–100% in the shrimp hemolymph after they consumed the SCE-containing diets for 9 and 12 d, respectively, showed that there was a substance with immunomodulator activity available in SCE. This is in agreement with previous studies. Similarly, an increased THC, phenoloxidase (PO) activity and respiratory burst was also reported by Yeh et al. (2006) when *L. vannamei* was injected with hot-water extract of *Sargassum duplicatum* (20 μg/g). In addition, increased levels of THC followed by increasing PO activity and respiratory burst was also reported by Fu et al. (2007) when *L. vannamei* were immersed, injected, and fed with hot-water extract of *Gelidium amansii*. Enhancement of the THC (13–73%) compared to the control was also reported by Ghaednia et al. (2011) when *Fenneropenaeus indicus* were immersed in seawater containing hot-water extracts of *Sargassum glaucescens* in 100, 300, and 500 mg/L. Fucoidan from the same species of this brown algae, *S. cristaefolium*, from Indonesia has been proved to effectively increase the nonspecific immune parameters of tilapia, *Oreochromis niloticus* (Isnansetyo et al. 2016). However, a similar study conducted by Chang et al. (2013) reported that there were no effects of dietary SCE on THC in *L. vannamei*. The difference between the results of this study and that of Chang et al. (2013) may have been attributed to the different strains of *S. cristaefolium* obtained from different water areas (tropical and subtropical).

Results of THC are also relevant to pathogen and environmental stress resistance. A relationship between THC and its resistance to parasitic fungus, *Aphanomyces astaci*, in the freshwater crayfish, *Pacifastacus leniusculus*, has been reported by Persson et al. (1987). *Penaeus styliriostris* with low THC were reported to become more susceptible to infections of *V. alginolyticus* (Le Moullac et al. 1998). Declining THC in *P. styliriostris* was found when the shrimp were exposed to ammonia at 3 mg/L (Le Moullac and Haffner 2000). This study confirmed that increasing THC resulted in increasing survival rates when *L. vannamei* consumed the SCE-containing diets under low-oxygen stress. It was found that the highest THC (8.6×10^6 cells/mL) and survival (93%) were displayed by the shrimp fed the SCE-containing diet at 500 mg/kg in this study (see Figs. 1, 4).

Hemocytes play a central role in the immune response in crustaceans (Johansson and Söderhäll 1989). There are three morphologically different cell types in hemocytes, that is, HCs, SGCs, and GCs. HCs of crustaceans play an important role in phagocytosis (Walton and Smith 1999; Giulianini et al. 2007). In the present study, the number of HCs significantly increased after *L. vannamei* were fed the diet containing SCE for 6–12 d ($[24.5–55.0] \times 10^5$ cells/mL). On the other hand, shrimp fed the control diet resulted in a relatively constant number of HCs (ca. 19×10^5 cells/mL). Moreover, an increase in SCE supplementation in the diets (250–1000 mg/kg) could boost HCs of the shrimp up to 92–201% compared to the control diet (see Fig. 2). In fact, shrimp fed the diet containing SCE resulted in an increased HC and was followed by an increased PA at Day 6 after feeding trial started. A higher PA (78–90%) in this study was found in hemocytes treated by SCE compared to untreated control cells with SCE (70–72%). High PA values in both the control shrimp and treated shrimp in the present study may have been attributed to the following: (1) the shrimp were in good health condition, (2) the shrimp were already infected by bacteria but had not yet been sick, and (3) the experimental diets may have contained immunostimulants or probiotics. However, the high PA values in this study were in agreement with Powell et al. (2011), reporting that *L. vannamei* hemocytes injected with *Vibrio* spp. after feeding the control diet (50–70%) compared to those on the treatment diets (80–90%). Higher PA was found in *Peneus monodon* hemocytes treated with garlic extract (78.7%) compared to control cells without preincubation with garlic extract (64.1%) (Wagner 1990).

An increased PA in shrimp fed the SCE diets was attributed to an increased amount of activity in HCs and THC. In the present study, *L. vannamei*, which had been fed a diet containing SCE at 500–750 mg/kg, showed enhanced PA so that the shrimp were more resistant to *V. parahaemolyticus* infection. An improved survival of the shrimp (from 3.3% [control diet] to 40–46.6%) after consuming the diets containing SCE at 500–700 mg/kg and a challenge test with *V. parahaemolyticus* (not AHPND *V. parahaemolyticus* strain) showed an effective increased immune response and disease resistance of the shrimp.

In this study, assessments of immune parameters were consistent with results of the challenge and stress tolerance tests. The immune parameters (THC, HCs, GCs, PA) of *L. vannamei* fed experimental diets containing SCE at 250, 500, 750, and 1000 mg/kg were significantly different from the shrimp fed the control diet (no SCE supplementation). Supplementation of dietary SCE levels significantly improved immune response, stress tolerance, and resistance of *L. vannamei* against *V. parahaemolyticus* infection. Nonspecific immunity ability and activity of the shrimp hemocytes significantly increased due to the intake of SCE supplemented in the diet when the shrimp consumed SCE-containing diets. Supplementation of SCE was effective as immunostimulant to enhance immune response, stress resistance, and resistance of *L. vannamei* to pathogenic *V. parahaemolyticus*. In this study, after feeding for 6–12 d, dietary SCE supplementation could effectively increase THC 41–100%, GCs 5.6–49.2%, HCs 42–201%, and PA 11.4–26.6% higher than those of the control diet with no SCE content.

In general, the results of this study are in agreement with the previous findings of other authors. However, inconsistency of the results of our findings with some other works can be attributed to species differences, method of administration, and doses. In addition, differences in biochemical properties, biologically active compounds of brown seaweed species, and methods used for extracting the alginate may explain these disparities.

In conclusion, several important benefits have been demonstrated in the present study. Supplementation of SCE at any dietary treatment levels (250–1000 mg/kg) enhanced nonspecific immune system in penaeid shrimp, *L. vannamei*. These findings suggest that SCE at doses of 500 and 750 mg/kg can be supplemented in diets as an effective immunostimulant for increasing immune ability, stress tolerance, and resistance of *L. vannamei* against *V. parahaemolyticus* infection. Considering these findings, the use of this functional feed additive may help to minimize the high mortality risk of *L. vannamei* caused by bacterial disease outbreaks of *V. parahaemolyticus*. These findings may contribute to reduction of disease risk of *V. parahaemolyticus* suspected as the causative agent of AHPND/EMS.

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