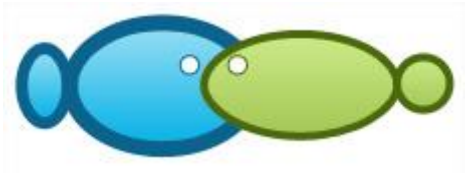

Dietary administration of *Gracilaria verrucosa* extract on *Litopenaeus vannamei* immune response, growth, and resistance to *Vibrio harveyi*

[1,2]Yudiana Jasmanindar, [3]Sukenda Sukenda, [3]Muhammad Jr. Zairin, [3]Alimuddin Alimuddin, [3]Nur B. P. Utomo

[1] Graduated School, Bogor Agricultural University, Jl. Lingkar Kampus Darmaga, Bogor. West Java, Indonesia; [2] Study Program of Aquaculture, Faculty of Marine and Fisheries, Nusa Cendana University, Jl. Adisucipto Penfui, Kupang, East Nusa Tenggara, Indonesia; [3] Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Jl. Lingkar Kampus Darmaga, Bogor, West Java, Indonesia. Corresponding author: S. Sukenda, sukenda@ipb.ac.id

Abstract. *Gracilaria verrucosa* extract was added in diets at concentration of 1, 2, and 3 g kg^{-1}. The supplemented diets were fed to *Litopenaeus vannamei* shrimps for 42 days and the growth performance was assessed. During feeding trial, total hemocyte count, differential hemocyte count, phagocytic activity, phenoloxidase activity, and bactericidal activity were analyzed at 6, 24, 72, 336 and 1,008 hours. After 42 days feeding trial, shrimps were challenged with pathogenic bacteria *Vibrio harveyi*. Total hemocyte count, hyaline cell count, granular cell count, semi-granular cell count, phenoloxidase activity, phagocytic activity and bactericidal activity against *Vibrio harveyi* showed significant differences compared to control group (shrimp fed without *G. verrucosa* extract). The treatment group which benefited of 2 g/kg of *G. verrucosa* extract tended to have a higher immune response than in the case of other doses. The survival rate of shrimp fed *G. verrucosa* extract was significantly higher than control group. No significant differences in growth performance and feed conversion ratios were found among all experimental groups. It was concluded that the addition of *G. verrucosa* extract in the diet was able to increase the shrimp immune response as well as resistance against *V. harveyi* infection.
Key Words: shrimp, immunostimulant, survival, seaweed, sulfated polysaccharide.

Introduction. Shrimps are essential aquaculture commodities in Indonesia. Production of cultured whiteleg shrimp (*Litopenaeus vannamei*) in 2016 reached 500,000 metric tonnes (GAA 2017). However, the production faced problem regarding infectious diseases. Many production sites have been particularly affected by an epidemic of vibriosis and viruses (Chiu et al 2007). The disease which causes mass mortality due to *Vibrio* genus (Felix et al 2011) has been the primary problem faced by Indonesian shrimp culture. Vibriosis disease is impacting the economic loss of shrimp industry, affected by some pathogenic bacteria such as *Vibrio harveyi*, cause mortalities up to 100% (Karunasagar et al 1994; Kanaripan et al 2009).

Appropriate control of disease in shrimp culture is required; either treatment or prophylactic measures to reduce the impact of the disease. It should also be noted that the disease control measures must be safe for consumers, environment and shrimps. Therefore, alternative natural and biologically active substances could be used.

Disease control in crustaceans such as shrimp is usually performed through enhancing immune system so they can be resistant to the pathogen. Therefore, the health of shrimp and improving their innate immunity are of the primary concern of many aquaculturists. Survival against pathogen infection needs to be done quickly and precisely. By doing this, the shrimp innate immune system should give a fast and efficient response (Lee & Söderhäll 2002). Seaweeds potentially have immunostimulant properties, as some studies showed that componds from seaweed increase shrimp innate immunity (Montero-Rocha et al 2006; Fu et al 2007; Yeh & Chen 2009; Kitikiew et al

2013; Wongprasert et al 2014). Moreover, by using natural substances from seaweeds make the shrimp farming environment-friendly.

Seaweed *G*racilaria *verrucosa* is a typical red alga which inhabits Indonesia waters and has been cultivated widely as a source of agar. Besides that, this seaweed can be used as immunostimulant. Immunostimulant molecules contained in seaweed extract of the genus *Gracilaria* may respond to hemocyte in shrimp (Wongprasert et al 2014). Macroalgae *G. verrucosa* has been studied and can improve the immune system of animals, including *L. vannamei* (Yoshizawa et al 1996; Jasmanindar et al 2008). *Gracilaria* sp., containing sulfated galactans (SG) which can increase shrimp immune system (Wongprasert et al 2014).

The use of immunostimulants can be added through formulations, re-pelleted or coated. Some immunostimulatory picograms have been able to stimulate the immune system (Johansson et al 2000). Immunostimulator excess may have an impact on the immune system itself, survival and growth of *L. vannamei*. This study aims to evaluate the innate immune response and growth of *L. vannamei* given diet extract of *G. verrucosa* at different doses. The benefits of this study contribute to the science and information on the utilization of *G. verrucosa* extract as immunostimulant in *L. vannamei*.

Material and Method

Experimental animals and experimental design. Shrimps were obtained from field laboratory in Serang, West Java (at coordinate 6° 1'36.56"S-106° 9'41.51"E) and were shipped to the Laboratory Fish Health, Ministry of Marine Affairs and Fisheries, Depok, Indonesia. Shrimps were acclimatized for ten days before the experiment and were fed with commercial shrimp feed. Four treatments were set up, containing different level of *G. verrucosa* extract (1, 2, and 3 g kg^{-1}), prepared based on Cheng et al (2005). The crude protein content of feed treatment ranged from 45.62 to 47.87% of the dry matter with no significant differences among diets. The composition of the amount of feed raw materials and *G. verrucosa* extract used can be seen in Table 1.

Table 1

Composition basal diet g kg^{-1} for *Litopenaeus vannamei* (Cheng et al 2005)

Ingredients	*Extract in the diet (g kg^{-1})*			
	Control	*1.0*	*2.0*	*3.0*
Fish meal	430	430	430	430
Soybean meal	50	50	50	50
Yeast meal	25	25	25	25
Shrimp shell meal	70	70	70	70
Wheat flour	352	352	352	352
CMC	3	2	1	0
G. verrucosa extract	0	1	2	3
Gluten	25	25	25	25
Fish oil	20	20	20	20
Mineral mixture	21	21	21	21
Vitamin mixture	4	4	4	4

CMC - Carboxymethyl cellulose.

Experimental shrimps groups were feed with different doses of *G. verrucosa* extract, and there were in total 60 shrimps in each experimental group. Shrimp fed diet without addition of the extract served as control. The average weight of the shrimps used in the present study was 6.68±0.9 g with no significant size difference among the groups.

Shrimps were maintained in 60 L of 30‰ seawater in plastic container, maintained at temperature of 28-39°C, pH 7.28-8.22, DO 5.00-6.30 mg L^{-1}. Shrimps were fed their respective diets at the rate of 4% of body weight at 07:00 and 18:00 h. Container received continuous aeration and used a recirculation system.

Pathogenic *V. harveyi* was used in this study as challenge, which was identified using API test kit 20 NE. The challenge test was conducted via injection. Firstly, bacteria

was cultured in TCBSA for 24 h, then cultured in SWC broth for another 24 h, both culture was performed at room temperature. The challenge test was done after administration of diets for 42 days, there two groups were received phosphate-buffered saline (PBS). Shrimp challenge tests were injected with bacteria in 10^6 CFU mL^{-1}. Unchallenged control shrimps were received PBS at 0.1. After injection, the experiment lasted for two weeks. During the challenge, experiment shrimp were fed according to feeding trial.

Evaluation of immune parameters of shrimps. Measurement of immune parameters (total hemocyte count, differential hemocyte count, phenoloxidase activity, phagocytosis activity, bactericidal activity) at 0, 6, 24, 72, 336 h, and 1,008 h. Hemolymph (100 µL) was withdrawn from the ventral sinus at pleopod base first abdomen segment of each shrimp into a 1 mL sterile syringe (25 gauge) containing 0.9 mL anticoagulant (trisodium citrate 30 Mm, sodium chloride 0.34 M, EDTA 10 Mm, Ph 7.55).

Measurement of total hemocyte count (THC) was performed based on method of Immanuel et al (2012) and Sritunyalucksana et al (2005). 50 µL anticoagulant-hemolymph solution was fixed with 4% formalin (50 µL), after 10 minutes 20 µL of 0.5% trypan blue in 2.6% NaCl was added, and then incubated at room temperature for 20 minutes. One drop of this solution was used at hemocytometer to measure THC using an inverted phase contrast microscope (total hemocyte/mL hemolymph). Hemocytes were stained with Giemsa solution for measurement of differential hemocyte count (DHC), using a modified method of Sung et al (1999).

Phenoloxidase (PO) activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) as described previously by Hernández-Lóopez et al (1996). The optical density at 490 nm was measured using a spectrophotometer (Thermo Scientific).

Phagocytic activity was determined by mixing hemolymph and 10^7cells mL^{-1} of *Staphylococcus aureus*. Ten microliters of this mixture were then smeared gently and fixed with 95% ethanol and staining with Giemsa for 25 minutes. The slides were rinsed with tap water and then dried. Slides were observed under a light microscope. Phagocytic activity was defined as the phagocytic rate (PR in %) (Su & Chen 2008).

Bactericidal activity was evaluated based on the method described by Sivagnanavelmurugan et al (2014).

Evaluation of survival rate and growth performance. After feeding trial growth and food conversation rate were calculated. Survival rate was observed after challenge test with pathogenic *V. harveyi* and calculated based on the relative percentage survival (RPS) (Amend et al 1981). Growth was calculated based on the difference between the final body weight and initial body weight. The weight gain of shrimp was determined by deducting the initial weight from final weight. While specific growth rate (SGR) was calculated as suggested by Immanuel et al (2004). Food conversion rate (FCR) was calculated based on Goytortua-Bores et al (2006) method.

Statistical analysis. A multiple-comparison test (Duncan's) test was used to examine significant differences among treatments using SPSS computer software. Percent data (survival rate) were normalized using an arcsin transformation before the analysis. Statistical significance of the difference required that $p<0.05$.

Results

The immune parameter of shrimps. The THC of shrimps received diet containing *G. verrucosa* extract showed significantly higher values than control after 24, 72, 336, and 1,008 h. Shrimps which received *G. verrucosa* extract of 2 g kg^{-1} had THC significantly higher than control shrimp, and other treatment groups after 24 and 72 h. The THC of shrimp fed diets with *G. verrucosa* extract at 1 g kg^{-1} was significantly higher than that of control diet as well as those which contained 2 and 3 g kg^{-1} *G. verrucosa* extract at 336, and 1,008 h. The THC of shrimps received diet with *G. verrucosa* extract at 2 g kg^{-1} were significantly higher than that of the control and the experimental treatments containing *G. verrucosa* extract at 1 and 3 g kg^{-1} at 24 and 336 h (Table 2).

Table 2

Total hemocyte, hyaline cell, semi-granular cell and granular cell of shrimps

Extract concentration (g kg^{-1})	*Hours*					
	0	*6*	*24*	*72*	*336*	*1,008*
	THC (x10^5 mL^{-1})					
0	20.9±7.15	10.5±0.50^{a}	27.6±8.44^{a}	53.2±17.05^{a}	103.4±6.68^{a}	55.8±4.46^{a}
1	20.9±7.15	16.5±0.61^{b}	237.7±19.35^{c}	390.6±18.43^{c}	595.9±14.38^{d}	146.2±3.61^{c}
2	20.9±7.15	15.0±1.19^{b}	510.8±13.59^{d}	584.2±9.1^{d}	301.8±16.94^{c}	131.3±5.87^{b}
3	20.9±7.15	11.8±1.42^{a}	60.7±14.25^{b}	89.9±5.1^{b}	171.6±10.3^{b}	123.5±2.21^{b}
	Hyaline cell (x10^5 mL^{-1})					
0	4.04±1.26	2.29±0.11^{a}	6.08±0.19^{a}	17.13±0.45^{a}	24.68±0.21^{a}	21.42±0.02^{a}
1	4.04±1.26	6.64±0.02^{d}	52.55±0.02^{c}	91.08±0.04^{c}	155.83±0.04^{d}	61.42±0.03^{c}
2	4.04±1.26	3.88±0.21^{c}	142.24±0.07^{d}	188.88±0.07^{d}	107.66±0.07^{c}	64.96±0.02^{c}
3	4.04±1.26	2.90±0.02^{b}	19.73±0.05^{b}	22.46±0.06^{b}	42.61±0.03^{b}	53.53±0.01^{b}
	Semi granular cell (x10^5 mL^{-1})					
0	10.05±0.03	3.94±0.21^{ab}	12.85±0.04^{a}	22.41±0.07^{a}	43.77±0.03^{b}	16.93±0.01^{a}
1	10.05±0.03	4.56±0.26^{ab}	88.51±0.11^{b}	166.72±0.09^{b}	256.14±0.02^{d}	54.57±0.11^{d}
2	10.05±0.03	4.74±0.05^{ab}	181.38±0.07^{c}	200.65±0.09^{c}	73.99±0.54^{c}	26.27±0.18^{b}
3	10.05±0.03	3.53±0.09^{a}	18.32±0.04^{a}	24.89±0.01^{a}	34.64±0.33^{a}	32.10±0.07^{c}
	Granular cell (x10^5 mL^{-1})					
0	6.81±0.68	4.27±0.23^{a}	8.64±2.50^{a}	17.40±0.05^{a}	34.96±0.02^{a}	14.15±0.59^{a}
1	6.81±0.68	5.27±0.12^{ab}	96.61±0.07^{c}	132.83±0.07^{c}	183.89±0.14^{d}	30.21±0.20^{b}
2	6.81±0.68	6.41±0.47^{c}	187.20±0.02^{d}	194.70±0.07^{d}	120.00±0.57^{c}	40.04±0.20^{c}
3	6.81±0.68	4.94±0.61^{ab}	22.62±0.05^{b}	42.59±0.03^{b}	94.39±0.54^{b}	37.89±0.15^{c}

Values are expressed as Mean±SD of three replicate analyses. Within each column, means with different superscript letters are statistically significant from each other (one way ANOVA test; P<0.05 and subsequent post hoc multiple comparisons with the Duncan test).

The differential hemocyte count consists of the hyaline cell (HC), semi-granular cell (SGC) and granular cell (GC) (Table 2). In the recent study, was shown that hyaline cell (HC) of shrimp fed diet containing *G. verrucosa* extract at 1 g kg^{-1} was significantly higher than that of control and of experimental groups containing 2 and 3 g kg^{-1} *G. verrucosa* extract after 6 h (Figure 2). After 24, 72, 336, and 1,008 h the HC of shrimps which received *G. verrucosa* extract was significantly higher than of the control's.

The semi-granular cell of shrimps which were fed diet containing *G. verrucosa* extract shown significantly different values than the control group. The diet containing *G. verrucosa* extract at 2 g kg^{-1} shown substantially higher SGC than other diets after 24 and 72 h. Granular cell of shrimps which received diet containing *G. verrucosa* extract was considerably higher than that of control group after 24, 72, 336, and 1,008 h (Table 2). The GC of shrimp that were fed diets containing *G. verrucosa* extract at 2 g kg^{-1} was significantly higher than that of control after 6, 24, 72, 336, and 1008 h.

Phagocytic activity of shrimp increased directly with the amount of *G. verrucosa* extract in diet only at 24 h. The phagocytic activity of shrimp fed *G. verrucosa* extract diet at 1, 2, and 3 g kg^{-1} was significantly higher than of the control after 24, 72, 336, and 1,008 h (Figure 1).

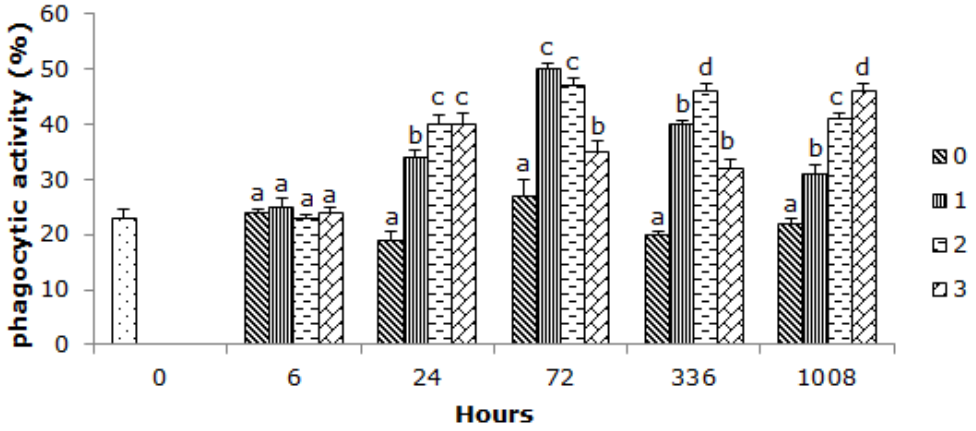

Figure 1. Phagocytosis activity of shrimps fed different concentration of *Gracilaria verrucosa* extract formulated diet. Data at the same sampling time with different letters indicate the highly significant difference ($p<0.05$).

The PO activity of *L. vannamei* fed diets containing extract at 1, 2 and 3 g kg^{-1} was significantly higher than that of shrimp fed control diets at 72 h (Figure 2). The phenoloxidase activity of shrimp that received *G. verrucosa* extract at 2 g kg^{-1} was significantly higher than of shrimp received control diet in 72 and 1,008 h.

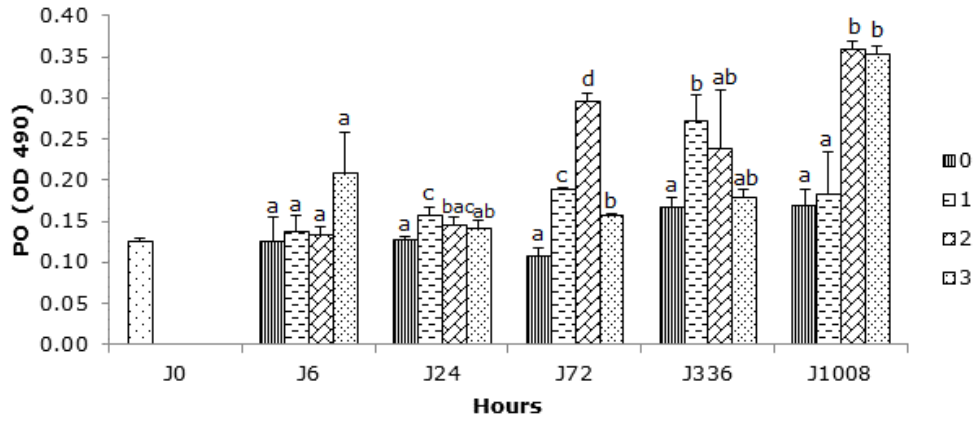

Figure 2. Phenoloxidase activity of shrimps fed different concentration of *Gracilaria verrucosa* extract formulated diet. Data at the same sampling time with different letters indicate the highly significant difference ($p<0.05$).

The result of the bactericidal activity of all experimental groups tested against *V. harveyi* is given in Figure 3. Hemolymph bactericidal activity of shrimps increased directly with the amount of *G. verrucosa* extract in diets at 1,008 h. The bactericidal activity of shrimp fed a diet containing *G. verrucosa* extract at 1, 2, and 3 g kg^{-1} was significantly higher than shrimp fed control diet at 24, 72, 1,008 h. The bactericidal activity of shrimp that had been fed *G. verrucosa* extract diets at 2 g kg^{-1} was significantly higher than that of shrimp received control diet at 24, 72, and 336 h.

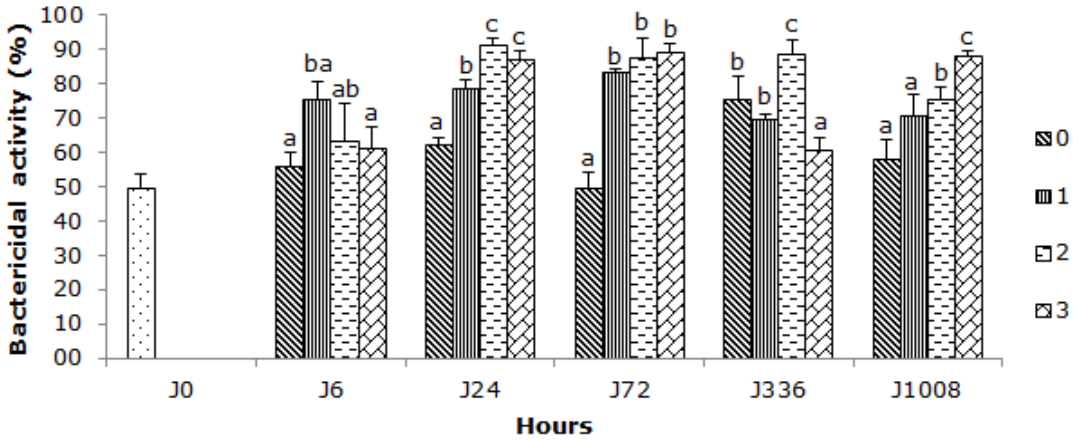

Figure 3. Bactericidal activity of shrimps fed different concentration of *Gracilaria verrucosa* extract formulated diet. Data at the same sampling time with different letters indicate the highly significant difference ($p<0.05$).

Survival rate and growth performance. Survival rate, average body weight, weight gains, SGR, and feed conversion ratios were assessed after the completion of the feeding trial. To determine the effects of the dietary extract of *G. verrucosa* on pathogen resistance, juvenile shrimp from each diet group received injection with *V. harveyi*. Survival rate (SR) was 46.7%, 66.7%, 70.0% and 76.7% of the shrimp that had been fed a diet containing extract at 0 (control), 1, 2 and 3 g kg^{-1}, respectively. The SR of shrimps which received *G. verrucosa* extract diets was significantly higher than of the shrimps which received control diet (Figure 4). When comparing shrimp growth, weight gain, and SGR, there were no significant difference between groups concerning average body weight after the feeding trial (Table 3). However, the growth performance of shrimps fed *G. verrucosa* extract diets at concentration of 2 g kg^{-1} showed higher values than any other experimental group.

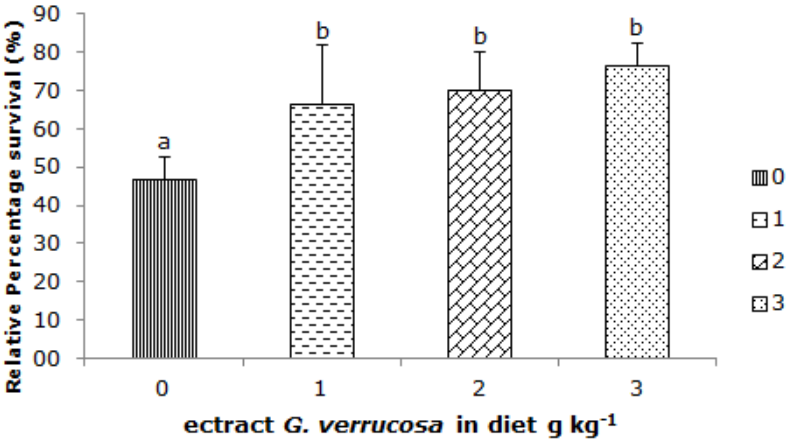

Figure 4. Survival rate. The survival data shown with different letters are significantly different ($p<0.05$).

Table 3
Weight, weight gain, specific growth rate, and food conversion ratio of shrimps until the 42nd day of cultivation

Extract conc. (g kg^{-1})	*W0 (g)*	*Wt (g)*	*W (g)*	*WG (%)*	*SGR (%g/t)*	*FCR*
0	6.59±0.15	8.26±0.02	1.68±0.15^{a}	25.50±2.91^{a}	0.54±0.05^{a}	1.34±0.03^{a}
1	6.66±0.14	8.60±0.06	1.94±0.18^{ab}	29.12±3.33^{a}	0.61±0.06^{a}	1.31±0.04^{a}
2	6.60±0.22	8.69±0.06	2.09±0.22^{ba}	31.70±4.49^{a}	0.65±0.08^{a}	1.28±0.04^{a}
3	6.87±0.18	8.60±0.02	1.73±0.18^{ab}	25.25±3.36^{a}	0.54±0.06^{a}	1.34±0.04^{a}

Each value is a Mean ± SD of three replicate analysis; within each column, means with different superscript letters are statistically significant from each other (one way ANOVA test; P<0.05 and subsequent post hoc multiple comparisons with the Duncan test).

Discussion. The immune system of penaeid shrimps depend on innate immune response including coagulation, phagocytosis, nodule formation, encapsulation, antimicrobial peptide synthesis, and the prophenoloxidase activity (Roweley & Powell 2007). The response of immunity is triggered by immunostimulant molecule contained in seaweed extract such as sulfated polysaccharides (Chotigeat et al 2004; Wongprasert et al 2014).

Dietary immunostimulants effects on increase of shrimp immunity resistance against pathogens have been studied before (Chen et al 2012; Yeh et al 2010; Sirirustananun et al 2011; Kitikiew et al 2013). In the present study, diets containing *G. verrucosa* extract had positive response to *L. vannamei* immune parameters. Extract *G. verrucosa* is a stimulant for innate immunity in shrimp. Previous research showed that extract *G. verrucosa* increased the level of phagocytic activity of mice in vivo (Yoshizawa et al 1994). Furthermore, water-soluble polysaccharide fraction from *G. verrucosa* can increase innate immunity, so it had an immunopotentiator activity for biota (Yoshizawa et al 1996).

Diets containing *G. verrucosa* extract fed to shrimps may interact and activate the hemocytes, this mechanism is important in shrimp immune defense. Hemocytes which are contained in the shrimp hemolymph are involved in immune reactions (Johansson et al 2000). Shrimp has three types of hemocyte cells based on cytoplasmic granules, namely hyaline cell (HC), semi-granular cell (SGC) and granular cell (GC). These three cells of circulating hemocytes have active function and play an important role in immune defense reaction, including phagocytosis, encapsulation, and release of the prophenoloxidase system (Jiravanichpaisal et al 2006).

The result of the total hemocyte observation in the present study showed a remarkable change after 24 h of shrimp fed *G. verrucosa* extract diet. The increase of this parameter occurred until two weeks of culture. However, it decreased on the last day of culture (42 days). The initial observation of total hemocytes (THC) at the sixth hour after feeding, was reduced (Table 2), but THC of shrimps which received diets with *G. verrucosa* extract were significantly higher when compared with control group. According to Ji et al (2009), THC is associated with the status of the immune response of shrimps, where the THC value increased after shrimps received laminarin injection. The mechanism of hemocytes contained on shrimp hemolymph is related to upregulation of Runt gene in crustaceans; hemocytes are synthesized and proliferate prior to be released at the hepatopancreas (van de Braak et al 2002; Söderhäll et al 2003; Sequeira et al 1996). The present study revealed that *G. verrucosa* extract probably can stimulate hemocytes development in the hepatopancreas. Fucoidan as an immune activator can improve proliferation of shrimps hemocytes (Kitikiew et al 2013). Ji et al (2009) reported THC values increase after shrimp received laminarin injection.

In the present study, the number of hyaline cells of shrimps which received *G. verrucosa* extract diet increased significantly against control. The increase number of hyaline cells at 24 h, is an indication that the immune system receptors recognize the stimulant entering the shrimp body, and confirm with quick response via increasing the hyaline cells in shrimp's hemolymph. Fucoidan in the diet at 0.5, 1.0 and 2.0 g kg^{-1} can

increase the hyaline cells amount after 14 and 21 days (Kitikiew et al 2013). In the innate immune system of the shrimps, hyaline cells (HC) are involved in crucial process of phagocytosis for eliminating microorganisms (Bayne 1990; Lin et al 2013). According to Söderhäll & Cerenius (1998), circulating hemocytes also remove foreign particles in the hemocoel by phagocytosis. In decapod crustaceans, phagocytosis is an important process for eliminating microorganisms or foreign particles (Johansson & Söderhäll 1989). Phagocytic activity of shrimps can be stimulated when diet contains seaweed *Gelidium amansii* extract (Fu et al 2007).

In decapod crustaceans, hemocytes play a crucial role in the host innate immune activity. Among the three types of hemocytes, both semi-granular and granular cells are induced to degranulation by microbial of foreign polysaccharides like lipopolysaccharides (LPS), β-glucan and peptidoglycan (Soderhall & Cerenius 1998). Some immunostimulants given to shrimp showed the ability to activate proPO system beginning with lysis of both granular and semi-granular cells (Smith et al 1984; Kitikiew et al 2013). Immunostimulants can enhance immune system. This mechanism is seen in the present study, phenoloxidase activity of shrimps which had been fed diets of extract increased, and the number of granular cells decreased at the end experiment. Therefore *G. verrucosa* extract is a potential immunostimulant to increase innate immunity of shrimps.

In crustaceans, phagocytosis is an important cellular defense mechanism performed by hemocytes (Van de Braak et al 2002). In the present study, phagocytic activity of shrimps which received *G. verrucosa* extract diet showed significantly increase compard to control group. The phagocytic activity of shrimps which were fed diets containing *G. verrucosa* extract at 2 g kg^{-1} was significantly higher than of shrimps which received control diet as well as of shrimp fed different concentration of *G. verrucosa* extract, after 336 h. Fucoidan given through feed at a dose of 1.0 g/kg diet has shown phagocytic activity as well as clearance efficiency against *V. alginolyticus* in *L. vannamei* (Kitikiew et al 2013). Immunostimulants from seaweed can enhance the innate immunity of *L. vannamei*. According to other researchers seaweed immunostimulants are safe and effective in increase shrimps immunity (Sirirustananun et al 2011; Yeh et al 2010; Huynh et al 2011). In fish and shellfish, phagocytic activity is one of the main mediators of innate immune to the pathogens (Nonaka & Smith 2000). Phagocytic activity can release bactericidal product, which is part of innate immune systems and this way can increase the resistance of shrimp against pathogens (Chen et al 2012). Increased phagocytosis of shrimp which received diets with *G. verrucosa* extract at 1, 2 and 3 g kg^{-1} led to significantly higher survival rate than shrimps from control group.

Melanisation is a crucial immune mechanism in arthropods and possibly among many other invertebrate taxa although the latter has been less investigated. This innate immune reaction provides toxic quinone substances and other short-lived reaction intermediates (Cerenius et al 2008). The immunostimulants concentration is not related with the value of PO activity. According to Devaraja et al (1998), highest concentration β-glucan did not increase PO activity. Feeding *Sargassum fusiforme* extract at concentration of 5 g kg^{-1}, increasing PO activity significantly higher than that of 10 g kg^{-1} (Huang et al 2006). This phenomenon was also observed in the present study, where shrimps which were fed diet with *G. verrucosa* extract at 3 g kg^{-1} were not more efficient on the immune response than shrimps fed diet containing *G. verrucosa* extract at 2 g kg-^{1} or 1 g kg^{-1}. In the present study, PO activity of shrimps fed diet with *G. verrucosa* extract at 2 g kg^{-1} or less showed an increased response than that of shrimps fed diet containing 3 g kg^{-1} *G. verrucosa* extract.

Bactericidal activity of shrimps fed *G. verrucosa* extract diets was significantly different compared with control shrimp. The reduction percentage in *Vibrio* load in shrimps fed diet with *G. verrucosa* extract was higher than in control shrimp group. Similarly, Sivagnanavelmurugan et al (2014) reported the effect of fucoidan isolated from *Sargassum wightii* on bactericidal activity of shrimps after feeding trial.

The extract of *G. verrucosa* contains complex sulphated polysaccharides, which consist of sulphate and galactose. At the end of the feeding trial, the growth performance of all groups showed no significant differences. Whilst, the survival rate of all treatment groups fed *G. verrucosa* extract diets was higher than of the control group, and it was

increased with increasing dietary concentration of extract in the diet. According to Sivagnanavelmurugan et al (2014), dietary administration of fucoidan, increase the survival of shrimps against *V. parahaemolyticus*. Similarly, Tayag et al (2010) reported that the administration of immunostimulant *Spirulina platensis* increase innate immunity and resistance against *V. alginolyticus* infection.

The extract of *G. verrucosa* added in diet of shrimps showed an effect on immune response. Immunostimulants from other seaweed (fucoidan, hot-water extract *Gelidium amansii*, *Sargassum wightii* fucoidan) has shown its potential as stimulator on the level of THC, DHC, PO activity, phagocytic activity and bactericidal activity (Immanuel et al 2012; Kitikiew et al 2013; Fu et al 2007; Sivagnanavelmurugan et al 2014). Therefore, fucoidan, *G. amansii,* and extract of *G. verrucosa* all exhibited a positive effect on innate immunity. THC, hyaline cell, semi-granular cell, granular cell, activity PO, activity phagocytic, and bactericidal activity increased after 24 h. According to Anderson (1992), phagocytosis and the production of oxidative radicals are quickly activated by the immunostimulants thus shrimp can survive against a broad spectrum of infectious pathogens attack. In the present study, the peak of immune response effect of the diets containing *G. verrucosa* extract declined at the end of feeding trial (42 days). The immunostimulatory effect of ß-1.3 glucan decreased to the pre-feeding level at the end of the 40 days feeding trial (Chang et al 2000). Immunostimulant effects on the innate immunity have only a short duration (Barman et al 2013). Diets containing *G. verrucosa* extract can improve resistance against *V. harveyi*. However, in the present study the growth of shrimp which have been feed diets containing *G. verrucosa* extract not exhibited significant differences from the control group. Immunostimulants can effect to both innate immunity and resistance against pathogen bacteria but does not have positive correlation with growth improving (Burgents et al 2004).

Conclusions. Diets containing *G. verrucosa* extract have a positive effect on the immune response with concomitant increase of THC, DHC, PO activity, phagocytic activity and bactericidal activity. The increased immune response has an impact on *L. vannamei* resistance against *V. harveyi* infection at the end of the experiment. Extract of *G. verrucosa* at 2 g kg^{-1} gives the optimal immune response. Dietary *G. verrucosa* extract does not improve the growth parameters of *L. vannamei*.

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Authors:
Yudiana Jasmanindar, Bogor Agricultural University, Graduated School, Indonesia, West Java, Bogor, Jl. Lingkar Kampus Darmaga, 16680; Nusa Cendana University, Faculty of Marine and Fisheries, Study Program of Aquaculture, Indonesia, East Nusa Tenggara, Kupang, Jl. Adisucipto Penfui, 85001, e-mail: jasmanindaryudiana@gmail.com
Sukenda Sukenda, Bogor Agricultural University, Faculty of Fisheries and Marine Science, Department of Aquaculture, Indonesia, West Java, Bogor, Jl. Lingkar Kampus Darmaga, 16680, e-mail: sukenda@ipb.ac.id
Muhammad Jr. Zairin, Bogor Agricultural University, Faculty of Fisheries and Marine Science, Department of Aquaculture, Indonesia, West Java, Bogor, Jl. Lingkar Kampus Darmaga, 16680, e-mail: zairinmz@live.com
Alimuddin Alimuddin, Bogor Agricultural University, Faculty of Fisheries and Marine Science, Department of Aquaculture, Indonesia, West Java, Bogor, Jl. Lingkar Kampus Darmaga, 16680, e-mail: alimuddin@yahoo.com
Nur Bambang Priyo Utomo, Bogor Agricultural University, Faculty of Fisheries and Marine Science, Department of Aquaculture, Indonesia, West Java, Bogor, Jl. Lingkar Kampus Darmaga, 16680, e-mail: nurbambang_priyoutomo@yahoo.com

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