Immunoprotection of *Sargassum* fucoidan from Lampung coastal toward white spot disease infection in Pacific white shrimp *Litopenaeus vannamei*

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Abstract. The purpose of this study was to know the immunomodulatory effects to protect Pacific white shrmp toward white spot disease. Six hematological parameters such as total hemocytes count (THC); phagocytic activity (PA); phagocytic index (PI); relative superoxide dismutase, and total plasma protein (TPP) in Pacific white shrimp were evaluated after 14 days feeding using fucoidan from *Sargassum* sp that collected from Lampung coastal, Indonesia. The results showed that dietary crude fucoidan from *Sargassum* was significantly able to increase THC, PA, and SOD in Pacific white shrimp. This study revealed that the crude fucoidan from tropical *Sargassum* was very promising to be developed as an immunostimulant in shrimp feed.

Key Words: fucoidan, immune response, immunostimulant, *Litopenaeus vannamei,* tropical brown algae

Introduction. Over the last decade, Pacific white shrimp (*Litopenaeus vannamei*) culture grows fastly worldwide. However, this development prosperities is in conformity with shrimp disease. There have been numerous efforts taken to control the shrimp disease such as by developing new diagnostic methods, probiotics, immunostimulant, quorum sensing control of bacterial virulence, phage therapy, shrimp vaccines, RNA interference, antiviral and antbacterial substances, molecular epidemiology, and shrimp breeding and selection (Flegel et al 2008). We still develop the immunostimulant to control shrimp disease due to some reasons i.e. abundance substance resources, widely target range, propilactic approach, reliability for large scale application, and less side effect or environment pollution. Thus, the exploration of immunostimulant materials for shrimp has been continuing up to present.

The crustaceans immune system have completed by pattern recognition proteins (PRPs) or pattern recognition receptors (PRRs) that have an important role to recognize the foreign molecules derived from microorganisme pathogens like lipopolysacharide (LPS), peptidoglycan (PG), and β-1,3-glucan (BG). Lectin, β-1,3-glucan binding protein (LGBP), and Toll implied in PRR that known incrustaceans. Lectin play important role in innate immunity to recognize and eliminate pathogens efficiently (Wang et al 2009; Wei et al 2012). Lectin play to recognize LPS, PG, bacteria lipoteichoic acid, fungi β-1,3-glucan and viral RNA (Lee & Söderhäll 2002). LGBP have an important role to play as receptor to binding of foreign molecules like lipopolysacharide (LPS) derived from bacteria cell walls and β-1,3-glucan (BG) derived from fungi and then activates the serine proteinase cascade that activates the proPO system (Cerenius et al 2008; Chen et al 2016b). Whereas Tolls and Toll-like receptors (TLRs) play an essential role in recognition of microbes during host defense (Akira et al., 2001; Takeda et al 2003). ProPO, one of the enzyme in proPO cascade that highly important in shrimp immunity. ProPO activation can be triggered by PAMPs after their recognition by specific PRPs, leading to activation of a serine proteinase cascade that results in the activation of proPO-activating enzymes (PPAEs). Then, the activated PPAE(s) convert the zymogen proPO to the functionally active phenoloxidase (PO) by specific proteolytic cleavage. Subsequently, PO catalyzes the formation of quinone-reactive intermediates for melanin synthesis at the injury site or around invading microorganisms (Amparyup et al 2012). These proteins may be triggered by administered immunostimulant or by invading pathogen. The profile of antimicrobial protein in shrimp immune was not enough observed by its activity, but also must enumerated by its genes expression at certain organs. By measuring the amount of cellular RNA, particular gene is being expressed able determined. For many genes, the expression levels change dramatically from gene to gene, cell to cell or during various experimental conditions (Schmittgen & Livak, 2008).

Fucoidan, one of immunostimulant that mainly extracted from brown algae, was known to have multi-bioactivity as antioxidant, growth enhancer, both in finfish and crustacea (Chotigeat et al 2004; Traifalgar & Serrano 2009; Immanuel et al 2012; Traifalgar et al 2012; Kitikiew et al 2013; Sivagnanavelmurugan et al 2014; Yang et al 2014; Isnansetyo et al 2016; Sinurat et al 2016). However, the study about fucoidan from Indonesian brown algae as immunostimulant in shrimp and finfish was still limited i.e. fucoidan from *Sargassum cristaefolium* to tilapia *Oreochromis niloticus* (Isnansetyo et al 2016) and fucoidan from *S. binderi* to white shrimp *L. vannamei* (Sinurat et al., 2016). Beside them, the hematological pramaters that observed were limited. Whereas, the bioactivity of fucoidan varied depending on the species and extraction methods (Ale et al 2011) and very complex chemical compositions and structures of fucoidans from brown algae with variations of structures among species (Li et al 2008).

Recent study was to compare the shrimp immune by dietary fucoidan from three genera of brown algae i.e. *Sargassum, Padina,* and *Turbinaria* that collected from southern of Gunung Kidul Regency, Special Region of Yogyakarta, Indonesia. In addition, the shrimp immune response was observed by both hematology test including total hemocyte count; phagocytic activity and index; PO activity; SOD activity; and total plasma protein; and immune-related genes expression including LGBP, Toll, lectin, and proPO.

Material and Method

Algae collection. The three brown algae samples of the genera *Sargassum*, *Padina*, and *Turbinaria* were collected from Gunungkidul coastal, Special Region of Yogyakarta, Indonesia. Algae were washed by freshwater and dried at room temperature. Dry algae were cut in small size by using blender, packaged into plastic bag and stored in container before being used. Identification of brown algae sample was conducted in Laboratory of Plant Systematica, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta according to algabase.org and marine plant identification (Dawson 1954).

Fucoidan extraction. Crude fucoidan was extracted from brown algae referred to previous study (Doh-Ura et al 2007; Kim et al 2007; Synytsya et al 2010; Isnansetyo et al 2016). Before extraction, the pigments in algae samples were reduced by depigmentation in 96% ethanol (1:10 m/v) for 48 h. After that, fifty grams of each algal sample were macerated in 0.1 N HCl (1:10 m/v) for 24 h. The algal sample was then macerated again in 0.2 N HCl (1:10 m/v) at 70^{0}C for 2 h. The two extracts were combined and filtered through Whatman paper number 40. The extract was evaporated by using the rotary evaporator at 60^{0}C to obtain 100 mL of final volume. Extracts were added with 96 % cold ethanol (1:3 v/v) and stirred with a magnetic stirrer. After being stored in refrigerator for 2 h, the sample was centrifuged at 8000*g*, 4^0C for 15 min. The precipitate was dissolved in distilled water at pH 2, added with $CaCl_2$ at final concentration of 2M and centrifuged at 8000*g*, 4^0C for 15 min. To obtain fucoidan, supernatant was precipitated by ethanol. The crude fucoidan was dialyzed (MW cut off 12,300 Da) in distilled water for 48 h at 4^0C and confirmed using a fourier-transformed infrared (FTIR) spectrophotometer (Thermo Nicolet 380 FTIR, Germany) and thin layer chromatography (TLC). Finally, the obtained fucoidan was freeze-dried and stored in 4^0C until used.

Experimental Design. Healthy white shrimp was collected from intensive pond, brackishwater aquaculture development center (BADC) Jepara. Before set into the experiment tanks, shrimp were acclimated for 2 weeks in 500 L fiberglass tanks. During acclimatization period, shrimp were fed with 3% of total body weight/day using commercial feed four times a day. After acclimatization, shrimp (18 ± 2 g) were transferred into 7 plastic containers (80 L) according to the treatment groups (8 shrimp each container). The seawater on each container was exchanged daily with approximately 30% of filtered seawater. Shrimp were fed with two different doses (250 and 500 mg kg^{-1} feed) of crude fucoidan-coated feed with 0.25% Progol® (PT. INDOSCO, Surabaya, Indonesia), whereas the control shrimp group was fed only with Progol-coated feed. Crude fucoidan were prepared by extraction from *Saegassum* (SF), *Padina* (PF), and *Turbinaria* (TF) that was described below. The hemolymph were randomly collected from three intermoult shrimps of each group at 0, 4th, 8th, and 12th days of treatment. Shrimp were randomly re-blooded to know the trends of its hematology profile at series time during this research.

Hemolymph collection. Hemolymph was collected according to the previously described procedures (Wei et al 2012). Hemolymph was withdrawn individually from the ventral sinus cavity of each group of shrimp into a 1 ml sterile syringe (26-gauge needle) that washed before with anticoagulant solution (10% sodium citrate). Hemolymph was transferred into six microtubes for the hematology assay: 1) total hemocyte count (THC) (20 µL), 2) phagocytic activity (PA) and phagocytic index (PI) (20 µL), 3) phenoloxidase (PO) activity (100 µL), 4) superoxide dismutase (SOD) activity (40 µL), 5) total plasma protein (15 µL), and 6) gene expression (25 µL). Fresh hemolymph was used for THC, phagocytic activity and phagocytic index assays, whereas the others was stored in freezer (-20^0C) until used.

Hematology analysis. *Total hemocyte count (THC)* was conducted by fresh hemolymph (10 µL) was diluted in PBS (20 µL) followed by pipetting and transferred into bright-line hemacytometer (Hausser Scientific, USA) then observed under microscope with 400X magnification. Hemocytes on the entire corner of 1 mm squares were counted by following the Jones's formula (Jones 1962).

Phagocytic activity (PA) test was carried out based on the previously study (Chotigeat et al 2004). Twenty microliters of hemolymph were diluted with PBS (2:1 v/v) then transferred to a 96-well U-bottom microplate and added with the same volume of formalin-killed *Bacillus* sp. After incubated at 30^0C for 20 min, each sample (5 µL) was smeared on glass slide, air dried, and then soaked with 2.5% glutaraldehide for 20 minutes. Finally, the slides were washed with 0.85% NaCl then dried again followed by stained with 10% Wright stain for 20 minutes. The PA was determined by microscopic observation at 400X magnification. Phagocytic activity (PA) and PI were calculated from 100 phagocytes per slide using the previously published equation (Watanuki et al 2009).

Superoxide dismutase (SOD) activity was measured spectrophotometrically by recording the formation of riboflavin by NBT (Beauchamp & Fridovich 1971). Briefly, 40 µL of hemolymph was diluted 10 times with buffer phosphate, and then centrifuged at 6000*g*, 4^0C for 7 min. The supernatant was then heated up to 65^0C for 5 min to get SOD crude extract. Finally, 150 µL of SOD crude extract was added with 50 µL of NBT reagent (0.1

Mm EDTA, 13 µM methionine, 0.75 mM NBT and 20 µM riboflavin in 50 mM phosphate buffer, pH 7.8) and incubated for 2 min.The optical density then was measured at 630 nm using a microplate reader (*R-Biopharm Well Reader, Germany*). Relative SOD activity was achieved by divided OD of each sample with OD of control.

Phenoloxidase (PO) activity was measured spectro-photometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA) (Amparyup et al 2009). The procedure of PO activity was carried out based on the previous study (Liu et al 2004; Yudiati et al 2016). Briefly, 100 µL hemolymph was diluted with PBS (1:1), centrifuged at 700*g*, 4^0C for 20 minutes. After discharging the supernatant, pellet was added with 100 µL cacodylate cytrate buffer (0.1M sodium cacodylate trihydrate; 0.45M NaCl, dan 0.01M sodium cytrate), and centrifuged at 700*g*, 4^0C for 20 minutes. The supernatant was then discharged and pellet was added with 100 µL cacodylate buffer (0.01M sodium cacodylate trihydrate; 0.45M NaCl; 0.01M $CaCl_2.2H_2O$; 0.26M $MgCl.6H_2O$), to be mixed well. Then, 100 µL of mixture was transferred into 96 well microplate and added with 100 µL of Trypsin (Sigma Aldrich), resuspended and incubated at room temperature for 10 minutes. Fifty microliters of L-DOPA were added to the well, and the absorbance was measured using the microplate reader (R-Biopharm Well Reader, Germany) at 490 nm.

Total plasma protein (TPP) was measured by adopted from the Bradford's method (Hammond & Kruger 1988). Briefly, 15 µL of hemolymph was centrifuged at 700 *g* for 10 min, followed by transferring 5 µL of supernatant to 96 well microplate and added with 250 µL of Bradford reagent (Bio-Rad), incubated for 10 minutes. The absorbance was measured at 630 nm using a microplate reader (R-Biopharm Well Reader, Germany). Prior to this, the standard curve of protein level was determined by BSA (Bovine Serum Albumine, Merck) at different concentration (0; 500; 750; 1,000; 1,500; and 2,000 mg mL^{-1}).

Statistical analysis. All data were subjected to one-way analysis of variance (ANOVA) at level of significance of 0.05. A Duncan's multiple range test (DMRT) was used to examine significant differences among treatments using EXCEL macro add-ins DSAASTAT.XLS (Onofri & Pannacci, 2014) and IBM SPSS statistic 23 tools.

Results. Morphology-based identification confirmed that the three brown algae used in this study were *Sargassum crassifolium* J. Agardh, *Padina australis* Hauck, and *Turbinaria ornata* (Turner) J. Agardh. The fourier transformed infrared (FTIR) spectra of *Sargassum* Fucoidan (SF), *Padina* Fucoidan (PF) and *Turbinaria* Fucoidan (TF) were recorded and compared to those of the crude and pure fucoidan standards from *Fucus vesiculosus* (Sigma). The fingerprint areas of the three brown algae fucoidans in this study have similar spectra to the fucoidan standards, both crude and pure fucoidan (Figure 1).

Hematology parameters. Dietary crude fucoidan from three tropical brown algae i.e. *Sargassum*, *Padina*, and *Turbinaria* was able to increase the innate immunity in Pacific white shrimp. This proved by increasing some immune parameters after treatment such as phagocytic activity (PA), total hemocyte count (THC), and relative SOD activity. The total hemocyte count significantly increased at 4th day of treatment (DOT) then dramatically decreased at 8th and 12th DOT. In this study, there was only dietary 500 mg kg^{-1} SF treatment that significantly increased the total hemocyte count in Pacific white shrimp (Figure 2.a). All of fucoidan treatment in this study succeed to increase the PA. The dietary 500 mg kg^{-1} SF treatment was able to increase the PA significantly at 12th DOT (Fig. 2.b). The other immune parameter that increased in this study was SOD activity which significantly increased at 8th DOT (Figure 2.e). However, there was not significant different between fucoidan treatment and control group in total plasma protein (Figure 2.f). Instead, both the phagocytic index (PI) and PO activity significantly decreased, particularly at 12th DOT (Figure 2.c & 2.d).

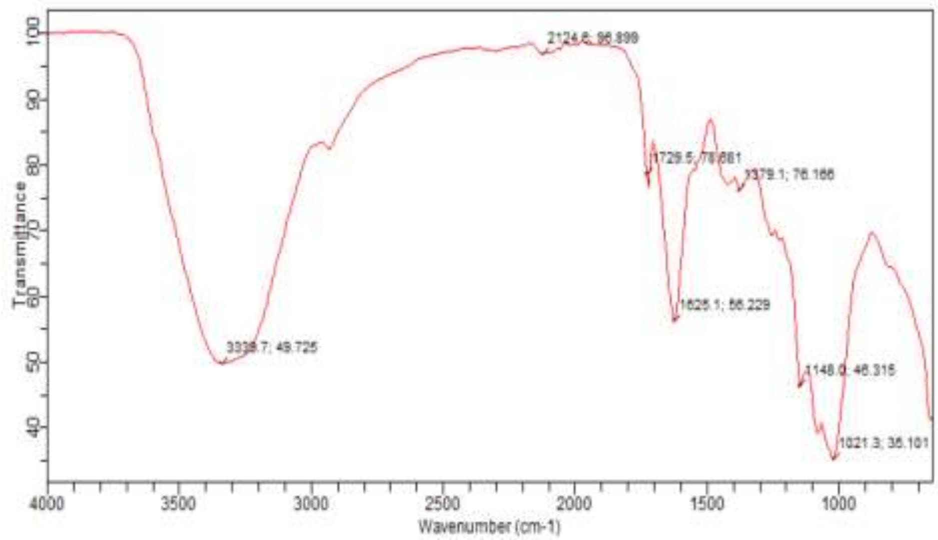


Figure 1. The spectrum of *Sargassum* fucoidan from Lampung Waters by FT-IR spectroscopy

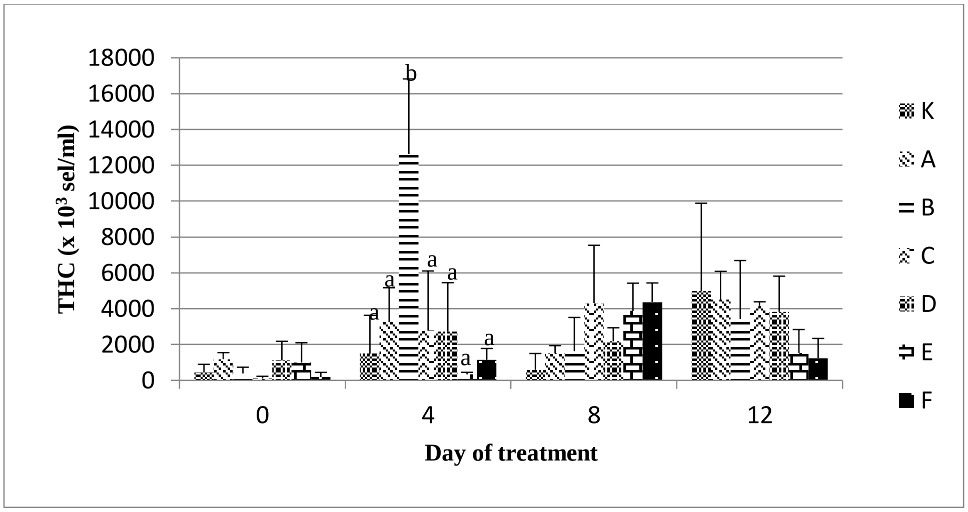
Hematology parameters. Dietary crude fucoidan from three tropical brown algae i.e. *Sargassum*, *Padina*, and *Turbinaria* was able to increase the innate immunity in Pacific white shrimp. This proved by increasing some immune parameters after treatment such as phagocytic activity (PA), total hemocyte count (THC), and relative SOD activity. The shrimp total hemocyte significantly increased ($p < 0.05$) at 4th day of treatment (DOT) by 500 mg kg^{-1} of SF treatment, although it then dramatically decreased at 8th and 12th DOT (Figure 2.a). All of fucoidan treatments in this study significant increase the shrimp phagocytic aactivity (PA) at 4th DOT ($p < 0.05$), but there was only the dietary 500 mg kg^{-1} SF treatment that consistently increased PA until 12th DOT (Figure 2.b). The SOD activity was also significantly increased at 8th DOT ($p < 0.05$) (Figure 2.e). However, there was not significant different between fucoidan treatment and control group in total plasma protein ($P > 0.05$) (Figure 2.f). Instead, both the phagocytic index (PI) and PO activity significantly decreased, particularly at 12th DOT ($P > 0.05$) (Figure 2.c & 2.d).

Immune-related genes expression. Based on the hematology results above, *Sargassum* fucoidan (SF) exhibited higher immunomodulator activity in white Shrimp than that of *Padina* fucoidan (PF) and *Turbinaria* fucoidan (TF). It was indicated by a significant increase in hematological parameters such as THC, PA, and SOD activity. Therefore, in this study, *Sargassum* fucoidan was selected for further evaluation in immune gene expression testing in white shrimp. The hemolymp samples used in the test were selected on day 12 after treatment based on high value in hematologic parameters. It was assumed that at the time was the culmination of RNA synthesis which would then be transcribed into immune proteins.

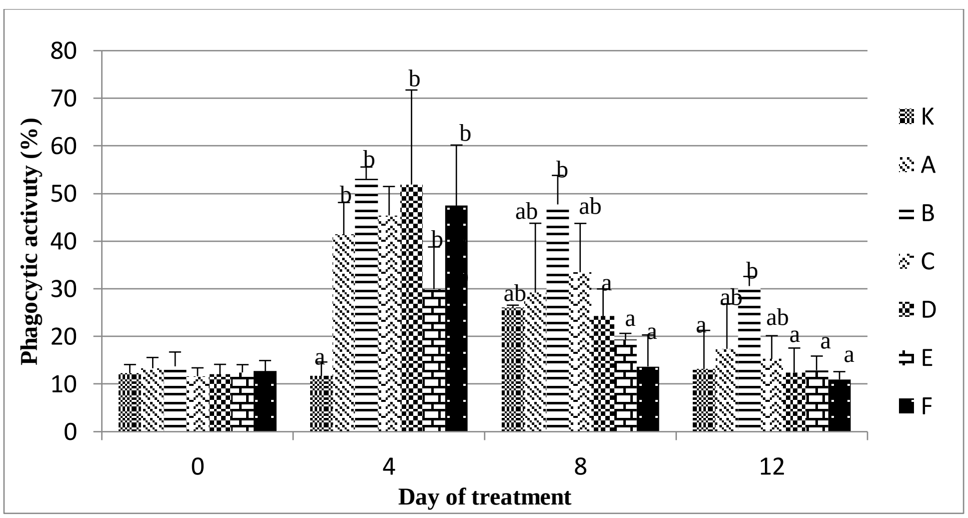
The threshold cycle (C_T) analysis showed that oral administration of fucoidan from tropical *Sargassum* succeed to up-regulate immune genes in Pacific white shrimp such as LGBP, Lectin, Toll, proPO that were transcribed 1.58 - 20.33 folds. However, there was a quite decrease in proPO gene expression in shrimp treated by 500 mg kg^{-1} SF (Figure 3). The Toll gene showed as the gene that highest transcribed in this study, particularly in shrimp fed with 500 mg kg^{-1} SF that achieved until 20 folds. In overall, shrimp that fed with

500 mg kg^{-1} *Sargassum* fucoidan showed higher gene expression than that of in shrimp fed with 250 mg kg^{-1} *Sargassum* fucoidan. Nevertheless, both of treatments consistently increased the immune-genes expression in Pacific white shrimp.

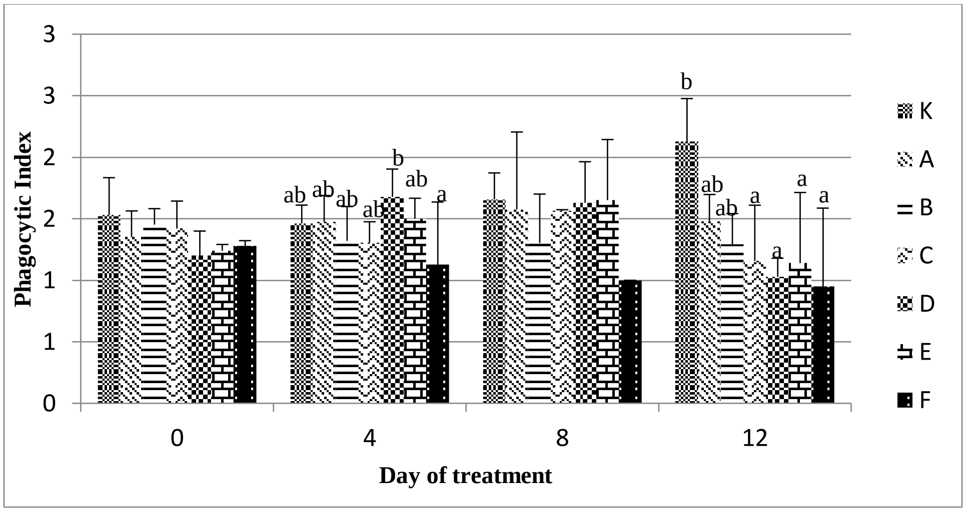
a.



b.

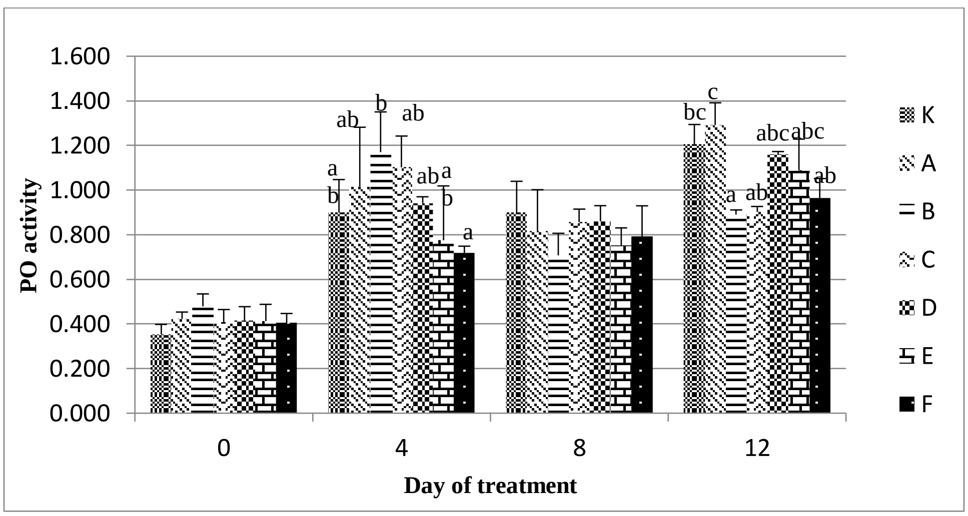


c.



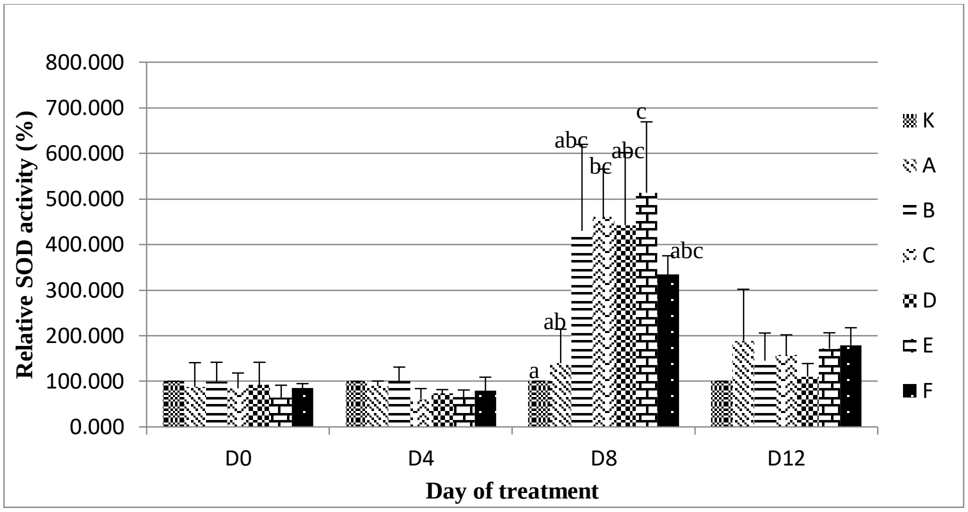
3
3
2
2
1
1
0
Phagocytic Index
0
4
8
12
Day of treatment
K
A
B
C
D
E
F

d.



1.600
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
PO activity
0
4
8
12
Day of treatment
K
A
B
C
D
E
F

e.



f.

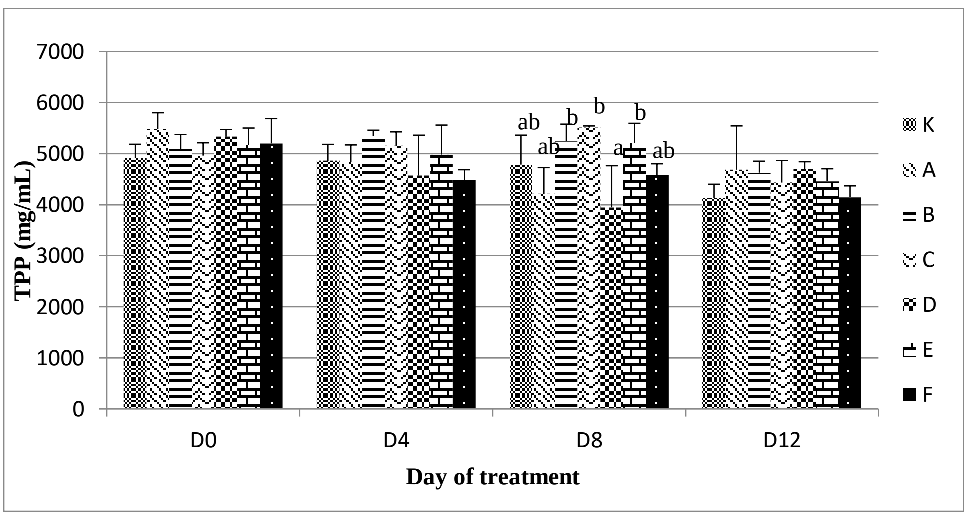


Figure 2. Hematological parameters of shrimp *L. vannamei* fed with different crude fucoidan supplemented diets. Each value is the mean ± SD of three replicates; bars with different letters are statistically significant different (P < 0.05). (K: control group; A: 250 mg kg^{-1} SF; B: 500 mg kg^{-1}SF; C: 250 mg kg^{-1}PF; D: 500 mg kg^{-1} PF; E: 250 mg kg^{-1}TF; and F: 500 mg kg^{-1}TF): a. total hemocyte count (THC); b. phagocytic activity (PA); c. phagocytic index (PI); d. PO activity; e. SOD activity; and f. Total plasma protein (TPP).

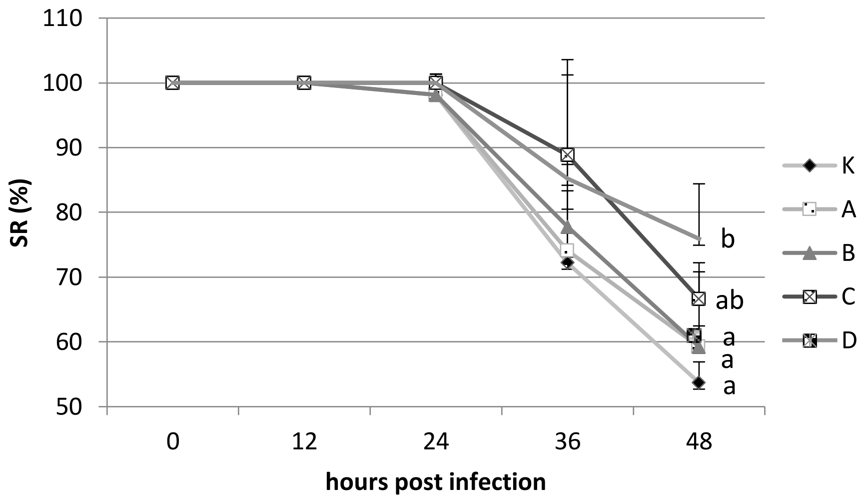


Figure 3. Survival rate (SR) of shrimp *L. vannamei* fed with *Sargassum* fucoidan supplemented diets. Each value is the mean ± SD of three replicates; bars with different letters are statistically significant different ($P < 0.05$). (K: control group; A: 0 mg kg^{-1} SF; B: 500 mg kg^{-1}SF; C: 1000 mg kg^{-1}PF; D: 1500 mg kg^{-1} PF.

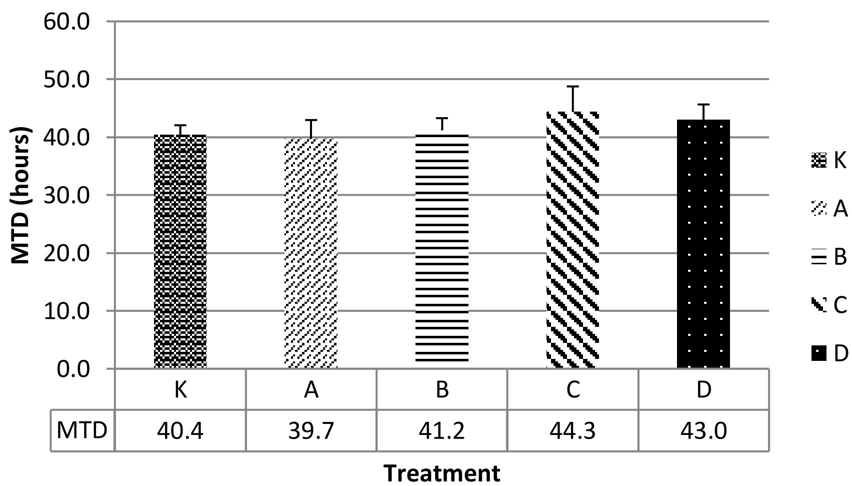


Figure 4. mean time to death (SR) of shrimp *L. vannamei* fed with *Sargassum* fucoidan supplemented diets. Each value is the mean ± SD of three replicates; bars with different letters are statistically significant different ($P < 0.05$). (K: control group; A: 0 mg kg^{-1} SF; B: 500 mg kg^{-1}SF; C: 1000 mg kg^{-1}PF; D: 1500 mg kg^{-1} PF.

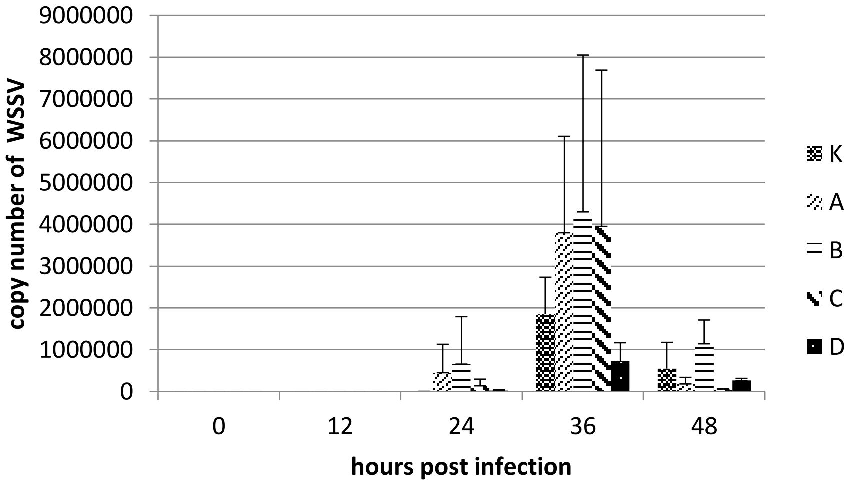


Figure 5. Copy number virus on shrimp *L. vannamei* fed with *Sargassum* fucoidan supplemented diets. Each value is the mean ± SD of three replicates; bars with different letters are statistically significant different ($P < 0.05$). (K: control group; A: 0 mg kg^{-1} SF; B: 500 mg kg^{-1}SF; C: 1000 mg kg^{-1}PF; D: 1500 mg kg^{-1} PF.

Discussion. There were some methods that have succeed to extract fucoidan from brown algae (Ale et al 2011). As the previous study by Isnansetyo et al (2016), the fucoidan of this study was successfully extracted by using 0.1 N HCl followed by addition $CaCl_2$ in acid condition and precipitation with cold ethanol. By this method, the rendements of dry crude fucoidan from three species brown algae extraction were 2.7; 4.8; and 2.6% for *Sargassum* fucoidan (CFS), *Padina* fucoidan (CFP), and *Turbinaria* fucoidan (CFT), respectively. As the comparation, there were the fucoidan rendement that have extracted from brown algae in previous studies such as that extracted from *T. ornata* (4.2±0.33%) (Marudhupandi & Centre 2013), *Costaria costata* (1.87%) (Wang et al 2014b), *Undaria pinnatifida* 3.9% (Kim et al 2007), and from *S. wightii* (4.24%) (Marudhupandi et al 2013).

The FT-IR spectra of three tropical brown algae used in this study showed the typical bands of polysaccharide and have the similar spectra with crude and pure fucoidan standard from *F. vesiculosus* (Sigma). There are 8 bands of FT-IR spectra of *S. cristaefolium* fucoidan (Isnansetyo et al 2016) i.e. 3435 cm^{-1} (O–H); 2939 cm^{-1} (C–H); 1614 cm^{-1} (O-C-O); 1424 cm^{-1} (C–OH); 1258 cm^{-1} (O=S=O) ; 1040 cm^{-1} (C–O–C/C–OH); 820 cm^{-1} (C–O–S); and 580 cm^{-1} (O=S=O) (Fig. 1). However, there were three bands (2360; 2338; and 2153 cm^{-1}) in all of brown algae fucoidan samples that not found in *S. cristaefolium* fucoidan spectra, these three bands was confrimes as H–C=O stretch. The FT-IR spectra proved that the extraction method used in this study was effective to obtain fucoidan from brown algae. The previous study reported that the position and content of sulphate group was the most valuable in fucoidan activity (Li et al 2008). The sulphate groups in all of fucoidan samples in this study appeared at 1258 cm^{-1} (O=S=O) and 820 cm^{-1} (C–O–S), although their intensity were different.

Based on the results, the oral administered of fucoidan-supplemented feed was able to enhance shrimp immunity namely THC, PA, and relative SOD activity (Figure 2). The similar increase occurred in Pacific white shrimp that fed with 0.5 -2 g kg^{-1} *S. wightii* fucoidan diet (Kitikiew et al 2013). Kitikiew et al (2013) also confirmed that only small size of hemocyte increase (11.5% to 36.5%) at 3 hours after *S. wightii* fucoidan feeding to Pacific white shrimp, whereas large size hemocyte precisely decrease (88.5% to 63.5%). There are three classes of hemocyte in crustacea i.e. hyalinocyte, granulocyte, and semi-granulocyte. Only granulocyte and semi-granulocyte that have β-1,3-glucan receptors allow encapsulation, recognizing proPO cascade, phagocytosis, and clothing (Zhang et al 2006). Whereas non-granula cells, hyalinocyte, have no phagocytic activity (Aguirre-Guzmán et al 2009). The high molecular weight of fucoidan from brown seaweed is more easily absorbed by the shrimp compared with crude fucoidan that initiates the hemocyte proliferation (Sinurat et al 2016).

The 500 mg kg^{-1} *Sargassum* fucodian diet treatment also increased in phagocytic activity (PA) in Pacific white shrimp reached 17 - 40%. This result was higher compared to the result that previously studied such as dietary *S. wightii* fucoidan that able to increase the PA 6-7% (Sivagnanavelmurugan et al 2014) and *S. polycystum* fucoidan that able to increase the PA up to 9.1% (Chotigeat et al 2004) in *P. monodon*. The increase in PA also occured in shrimp that were treated with fucoidan supplemented diets after being challenged with either WSSV (Immanuel et al 2012) or *V. alginolyticus* (Kitikiew et al 2013). Phagocytosis is a highly conserved process representing an important component of the innate immune system in multicellular organisms (Stuart & Ezekowitz, 2008). The cascades of phagocytosis start with particle recognition and binding of particles to cell surface receptors, which activate diverse signaling pathways. These signals coordinate an orderly progression of cellular changes, including reorganization of the plasma membrane and cortical cytoskeleton, which results in phagosome formation. The phagosome undergoes fission and limited fusion events with endosomes and lysosomes, resulting in a mature phagolysosome that can destroy pathogens by low pH, hydrolysis, and radical formation (Stuart & Ezekowitz, 2005). Wang et al (2014a) identified molecules that play essential functions in the host antiviral responses, i.e. *Rab* proteins, *Ran* protein, ADP ribosylation factor (*Arf*), and recognition proteins. Such molecules imply in the regulation of phagocytic process. The increase in PA in this study, however, was not followed by increase in phagocytic index (PI). The PI of shrimp fed with fucoidan supplemented diet showed no significantly increase at 4th day of treatment, even it decreased significantly at 12th day of treatment (Figure 2.c). This result was in contrast with previously study such as in dietary alginat (Yudiati et al 2016) and immersion with carragenan (Chen et al 2014) that could still increase in PI in Pacific white shrimp.

Significant increase actually occurred on the shrimp SOD activity, especially on the 8th day of feeding treatment with fucoidan. The relative SOD activity in this study increased significantly ($P < 0.05$) by 360 to 410% in shrimp fed with 250 mg kg^{-1} PF and TF supplementation compared to the control group. However, the SOD activity decreased on the 12th day of treatment (Figure 2.e). Sivagnanavelmurugan et al (2014) reported that feeding containing 0.1-0.3% *S. wightii* fucoidan also increases SOD activity in *P. monodon* from 36.75 Unit ml^{-1} in the control group to 54.96-59.88 Unit mL^{-1}. Immanuel et al (2012) also reported that SOD activity of shrimp fed with 0.1-0.3% *S. wightii* fucoidan increase after 45 days of treatment (56.13 - 62.14 Unit mL^{-1}) compared with control group (36.85 Unit mL^{-1}). Similar results also occur in white shrimp fed with acids, calcium and sodium alginate *S. siliquosum* (Yudiati et al 2016). Superoxide dismutase (SOD) is an antioxidant enzyme that converts superoxide anions ($O2^-$) to hydrogen peroxide (H_2O_2) both of which belong to the reactive oxygen species (ROS) that have played an important role in shrimp immunity by eliminating attacking microbes (Tassanakajon et al 2013). Superoxide dismutase (SOD) (EC 1.15.1.1) is also the primary antioxidant defense produced in response to oxidative stress (Liu et al 2006). In penaeid shrimp, there are two types of MnSOD, cytosolic MnSOD (cytMnSOD) and mitochondrial MnSOD (mtMnSOD), and one CuZnSOD, extracellular CuZnSOD (ecCuZnSOD) (Gómez-Anduro et al 2006; Lin et al 2010). SOD activity is influenced by temperature, salinity, pH, ammonia, and oxygen concentration (Liu & Chen 2004; Cheng et al 2005b; García-Triana et al 2010). In this study, however, water quality such as temperature, salinity, pH, ammonia, and dissolved oxygen were measured continuously and arranged homogeneously in the normal range for Pacific white shrimp culture to minimize any effect on shrimp immunity, specifically SOD activity.

In this study, unfortunately, dietary fucoidan was not able to increase PO activity (P > 0.05) (Figure 2.d). This result was not in accordance with the result reported previously. PO activity in *L. vannamei* improves significantly after treatment of 0.1-0.3% *S. wightii* fucoidan, both after 45 days (Immanuel et al 2012) and 60 days (Sivagnanavelmurugan et al 2014). However, the activity gradually decreased after being challenged with WSSV and *Vibrio parahaemolyticus*. Increase in PO activity also occurs in *L. vannamei* shrimp fed with acid and sodium alginate (Cheng et al 2004; Yudiati et al 2016). Huang et al (2006) reported that administration of 0.5% of *S. fusiforme* (SFPSE) polysaccharide extract for 14 days is also able to increase PO activity in *Fenneropenaeus chinensis*, but at higher SFPSE doses (1 and 2%) significantly decreased PO activity (P <0.05). The proPO system produces one protein called proPO, it plays an important role in defense immune reactions in crustaceans (Söderhäll & Cerenius 1998). In this mechanism, the proPO will be converted to PO by the serine protease enzyme (ppAE) (Wang et al 2014).

Fucoidan from *S. crassifolium* exhibited more effective activity in stimulating the immune parameters in Pacific white shrimp compared to fucoidan from *P. australis* and *T. ornata*. The other studies also reported the increasing of immune activity in both shrimp and fish, that induced by *Sargassum* fucoidan such as *S. hemiphyllum* var. Chinense (Huynh et al 2011) in *L. vannamei*, *S. wightii* fucoidan in *P. monodon* (Immanuel et al 2012; Sivagnanavelmurugan et al 2014; 2015), *S. fusiforme* polysaccharide extract (SFPSE) in *Fenneropenaeus chinensis* (Huang et al 2006), and *S. cristaefolium* fucoidan in Tilapia (*Oreochromis niloticus*) (Isnansetyo et al 2016). Indeed, *S. duplicatum* powder and hot water extract was also reported succesfully to improve the desease resistance in shrimp and fish (Yeh et al 2010).

In previous studies, increased immune-related gene expression in shrimp may also be triggered by other immunostimulants such as laminarin, LPS and poly I:C (Dechamma et al 2015); sodium alginate (Yudiati et al 2016); and also by fucoidan (Chen et al 2016a, Sinurat et al 2016), although the observed genes were different. In this study, the highest gene expression was found in the Toll gene that reached up to 20.3 fold, especially in shrimp fed with a diet containing 500 mg kg^{-1} SF (Figure 3). The Toll gene expression was higher than the same gene expression in Pacific White shrimp fed with 2000 mg kg^{-1} alginic acid added to the diet that reached 5.58 times above the control (Yudiati et al 2016). At the same treatment, strangely, the proPO gene was actually found as the lowest expressed gene implying fucoidan effectively triggers activation of Toll, LGBP, and lectin genes instead of proPO gene. This was also reinforced from the results of PO activity test which showed no significant difference in shrimp treatment (Fig. 2.d). Chen et al (2014) also reported there were sixteen immune-related genes LGBP, lectin, peroxinectin (PX), ppA, proPO I, proPO II, a2-macroglobulin (a2-M), cytMnSOD, mtMnSOD, glutathione peroxidase (GPx), catalase, lysozyme, penaeidin 3a, trans-glutaminase (TGS) I, TGS II, and heat shock protein (HSP)70 genes that are up-regulated in shrimp hemolymph after fed with 500 mg kg^{-1} carrageenan diet. The injection of commercial fucoidan from *Fucus vesiculosus* (Sigma) at 2, 6, and 10 μg g^{-1} is able to increase the copies number of five immune-related genes expression (LGBP, peroxinectin, proPO I, proPO II, astakine, and HHAP) (Chen et al 2016a). The three genes that up-regulated in this study have the important role in the shrimp immune system. Toll is responsible to recognize foreign molecules derived from Gram (+) bacteria and virus, LGBP is responsible to recognize foreign molecules derived from Gram (-) bacteria and fungi (Cheng et al 2005a). Whereas lectins are recognize molecules like LPS, PG, bacteria lipoteichoic acid, *β*-1,3-glucan fungi and RNA virus (Lee & Söderhäll 2002). These PRR (lectin, Toll, and LGBP) will recognized spesific PRPs in virus or bacteria leading to activation of a serine proteinase cascade that results in the activation of proPO-activating enzymes (PPAEs) (Cerenius et al 2008). Then, the activated PPAE(s) convert the zymogen proPO to the functionally active phenoloxidase (PO) by specific proteolytic cleavage. Subsequently, PO catalyzes the formation of quinone-reactive intermediates for melanin synthesis at the injury site or around invading microorganisms (Amparyup et al 2012). Up-regulation of lectin and Toll genes in shrimp by oral administration of fucoidan have not been reported before.

Conclusions. The feeding of crude fucoidan from tropical brown algae could trigger innate immunity of Pacific white shrimp, in particular by increasing the total hemocyte count, phagocytic and SOD activities. Moreover, oral administration of SF at 500 mg kg^{-1} effectively up-regulates immune-related genes such as LGBP, Toll, and lectin. Based on these results, this study was expected to open opportunities for the application of a reasonable fucoidan in shrimp farming activities. This paper is the first report to compare three tropical brown algae as well as a fucoidan source namely *Sargassum, Padina*, and *Turbinaria*, as an immunostimulant by oral administration of Pacific white shrimp. In addition, the hematology and gene expression parameters observed in this study are quite comprehensive and are observed in time series. This allows to know the correlation between parameters observed and the trend of each parameter.

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