HAYATI
Journal of Biosciences

Vol. 31 No. 6, November 2024 1116-1129
DOI:10.4308/hjb.31.6.1116-1129
ISSN: 1978-3019
EISSN: 2086-4094

The Effect of Enhancing Ulvan's Antioxidant Properties in Supplemented Diets on Accelerating The Phenoloxidase Immune Response in White Shrimp

Nuril Azhar[1,2], Ervia Yudiati[1,2*], Ambariyanto[1], Agus Trianto[1]

[1]*Department of Marine Science, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang 50275, Indonesia*
[2]*Tropical Marine Biotechnology, Faculty of Fisheries and Marine Science, Diponegoro University, Semarang 50275, Indonesia*

ARTICLE INFO

Article history:
Received June 5, 2024
Received in revised form June 15, 2024
Accepted July 5, 2024

KEYWORDS:
Antioxidant,
Phenoloxidase,
Shrimp,
Growth,
Ulvan

ABSTRACT

This study investigated the total phenol content (TPC) and DPPH inhibition of ulvan from *Ulva lactuca*, along with its supplementation effects on the specific growth rate (SGR) and phenoloxidase (PO) activity of *Litopenaeus vannamei*. *Ulva lactuca* samples were processed to obtain four ulvan extracts using different methods such as P-HWE (Polysaccharide-Hot Water Extract), O-HWE (Oligosaccharide-Hot Water Extract), P-A-HWE (Polysaccharide-Acid-Hot Water Extract), and O-A-HWE (Oligosaccharide-Acid-Hot Water Extract). The *U. lactuca* powder was treated with 80% ethanol overnight, then centrifuged and dried. Depigmented *U. lactuca* was extracted with water (65°C, 2 hours), and polysaccharides were precipitated with 99% ethanol, yielding P-HWE. P-HWE was heated at 145°C for 4.5 hours to yield O-HWE. Another extraction after depigmentation, using water containing H_2O_2 and ascorbic acid (65°C, 2 hours), precipitated P-A-HWE, which was dried and heated to yield O-A-HWE. FTIR analysis provided insights into the chemical composition and structural characteristics of ulvan extracts. TPC and DPPH inhibition were measured spectrophotometrically, and PO activity using a colorimetric assay with L-DOPA. Ulvan supplementation in shrimp feed was evaluated for growth and immune response. Results showed significant differences in TPC, DPPH inhibition, and PO activity among extracts, with O-A-HWE having the highest phenol content and DPPH inhibition. Ulvan supplementation significantly influenced the SGR of *L. vannamei*, with the highest SGR in the O-A-HWE treatment. This study suggests that ulvan extracts, especially O-A-HWE, could be effective natural immunostimulants for enhancing the health and growth of *L. vannamei*, warranting further research to optimize extraction methods and understand the underlying mechanisms.

1. Introduction

Phenoloxidase (PO) is a key enzyme in the immune system of *Litopenaeus vannamei*, primarily found in hemocytes, with activity also detectable in serum and trypsin, and can be stimulated by lipopolysaccharide (LPS) (Perazzolo and Barracco 1997). When pathogens attack, β-Glucan-Binding Protein (βGBP) or Lipo-polysaccharide-Binding Protein (LGBP) triggers a serine proteinase cascade, activating the proPO system (Cerenius *et al.* 2008). Various pattern recognition proteins (PRPs), including LGBP, identify PAMPs like LPS, initiating this cascade and converting inactive proPO to active PO, leading to mechanisms that produce toxic melanin compounds to fight pathogens (Amparyup *et al.* 2013). Hemocytes contribute to the immune response through phagocytosis, melanization, production of cytotoxic species, particle encapsulation, and nodule formation (Cerenius and Söderhäll 2004; Kanost *et al.* 2004; Nappi and Christensen 2005; Cerenius *et al.* 2010).

Immunostimulants are substances that enhance the immune system (Barman *et al.* 2013; Yudiati *et al.* 2024), offering a promising alternative to antibiotics for bolstering shrimp immunity in an eco-friendly manner. Research by Kumar *et al.* (2023) highlights the potential of immunostimulants in safeguarding

* Corresponding Author
E-mail Address: erviayudiati@lecturer.undip.ac.id

global shrimp aquaculture sustainably. It has been shown that shrimp supplements that enhance the immune system can increase survival rates and immunity to various diseases, including bacteria and viruses. Given significant concerns about disease in aquaculture, especially in shrimp production that is vulnerable to infection, innovative approaches have become crucial. Immunostimulants emerge as a promising alternative to antibiotics, strengthening shrimp immune systems in an environmentally friendly manner.

Ulvan, a water-soluble sulfated polysaccharide derived from the Ulvales order, remains relatively underexplored compared to other polysaccharides despite its broad range of applications in biotechnology and the food industry (Cindana Mo'o *et al.* 2020), particularly as an immunostimulant. The effectiveness of ulvan extract in boosting shrimp immunity faces several challenges. The primary concern is the time-consuming nature of enhancing phenoloxidase (PO) activity, which may not provide a rapid enough response to acute disease outbreaks. For instance, infections such as Choi *et al.* (2017) reported that diseases such as *Vibrio parahaemolyticus* AHPND infection could lead to the death of *L. vannamei* juveniles by up to 100% within 24 hours. Another issue is the variability in ulvan's immunostimulant properties due to differences in extraction methods and the intrinsic characteristics of ulvan. Research has shown that while ulvan can enhance PO activity, this process can take between 14 to 21 days (Declarador *et al.* 2014; Lauzon and Serrano Jr 2015), which may not be practical for rapid disease response. Oligosaccharides demonstrated superior immune enhancement against *Vibrio* spp. compared to polysaccharides in the artemia model (Yudiati *et al.* 2020, 2021). Our study confirmed elevated trypsin activity in the intestines of *L. vannamei* following ulvan supplementation (Azhar *et al.* 2024). Nathan *et al.* (2023) reported that immunostimulants affect immune-related metabolites. Abbas *et al.* (2023) found that dietary polysaccharide supplementation enhances the expression of growth-related genes.

Furthermore, the total phenolic content (TPC) and antioxidant properties of ulvan are critical factors that influence its efficacy as an immunostimulant. Enhancing these properties could potentially improve its performance, but this requires additional processing and optimization. Antioxidant supplementation in the diet has been shown to enhance immune responses and growth (Eldessouki *et al.* 2022; Mansour *et al.* 2022), growth performance, and digestive enzymes in *L. vannamei* (Akbary *et al.* 2020). The performance of ulvan extract treatment is influenced by the nature and characteristics of immunostimulants, the time-consuming nature of PO activity enhancement, the need for rapid disease response, the enhancement of TPC and antioxidant properties, and the effectiveness of oligosaccharides. Improving these factors can enhance the efficacy of ulvan as an immunostimulant in shrimp aquaculture. This study aimed to investigate the total phenol content (TPC) and DPPH inhibition of ulvan extracted from Ulva lactuca, along with its supplementation effects on the specific growth rate (SGR) and phenoloxidase (PO) activity of *L. vannamei*.

2. Materials and Methods

2.1. Collection of *Ulva lactuca*

U. lactuca samples (Figure 1), the main source of ulvan sulfated polysaccharides, were collected from intertidal zones in Ngrehan Coastal, Gunungkidul Regency, Yogyakarta Special Region, during low tide. Ulva lactuca attached to rocks, shells, or other hard surfaces using a holdfast. The thallus of *U. lactuca* is thin and delicate, translucent due to its two-cell thick layer. The seaweed has a smooth texture and vibrant green color, which can range from light to dark green. The edges of the thallus are usually wavy and irregularly lobed, with a broad, leaf-like shape that often forms a ruffled margin. Upon arrival, the samples underwent a thorough cleaning process to remove impurities and epiphytes, followed by a rinse with fresh water. Subsequently, the samples were air-dried and finely powdered. The components of *U. lactuca* consisted of carbohydrates, proteins, ash, fat, and crude fiber using protocols by Horwitz and Latimer (1990).

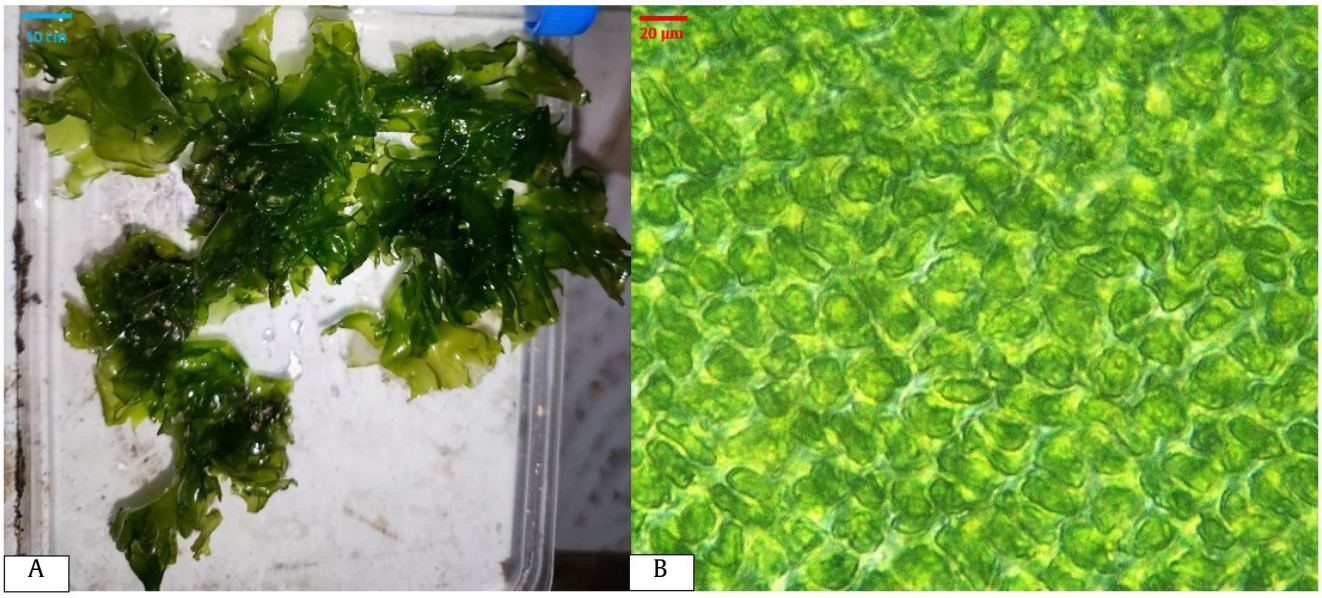

Figure 1. (A) *Ulva lactuca* characterized by thin, flat, green thalli with strap-shaped blades, and (B) microscopic features at 400x magnification

2.2. Preparation of Ulvan from *U. lactuca*

2.2.1. P-HWE Extraction Method and O-HWE Production

The modified P-HWE method involved processing 20 g of dried *U. lactuca* powder to remove lipids, pigments, and low molecular weight compounds using 80% ethanol (200 ml, Merck, Germany) with constant stirring overnight Tabarsa *et al.* (2018). After centrifugation at 8,000 rpm for 10 minutes, the depigmented *U. lactuca* powder was air-dried. Distilled water (400 ml) was then added to the depigmented powder (20 g), and extraction was conducted at 65°C with stirring for 2 hours. The supernatant obtained after centrifugation at 10,000 rpm for 10 minutes underwent polysaccharide precipitation using 99% ethanol to reach a final ethanol concentration of 70%. The mixture was refrigerated at 4°C overnight, and the precipitate was obtained by centrifugation at 10,000 rpm for 10 minutes. The polysaccharide precipitate was subsequently dried at room temperature. The polysaccharide yield was calculated from the depigmented powder obtained after ethanol precipitation. Later, the dried polysaccharide (P-HWE) underwent heating in an oven at 145°C for 4.5 hrs to produce the oligosaccharide extract (O-HWE) following the method of Yudiati *et al.* (2018). The flow chart of ulvan extraction is depicted in Figure 2.

2.2.2. P-A-HWE Extraction Method and O-A-HWE Production

Dried *U. lactuca* powder (20 g) was treated with ethanol (80% EtOH, 200 ml, Merck, Germany) overnight to remove lipids, pigments, and low molecular weight compounds. After centrifugation at 8,000 rpm for 10 mins, the depigmented *U. lactuca* powder was air-dried. Then, distilled water (400 ml) containing hydrogen peroxide (H_2O_2) and ascorbic acid (1:1 ratio, concentration 17.26 mM) was added to the depigmented powder (20 g) for extraction at 65°C with stirring for 2 hours (Chen *et al.* 2016) Polysaccharide precipitation was done using 99% ethanol to achieve a final concentration of 70% EtOH after collecting the supernatant through centrifugation at 10,000 rpm for 10 mins. The mixture was refrigerated overnight, and the precipitate was obtained after another centrifugation. The resulting polysaccharide precipitate was dried at room temperature, and its yield was calculated from the depigmented powder after ethanol precipitation. Next, the dried acidic polysaccharide (P-A-HWE) was heated in an oven at 145°C for 4.5 hours to obtain the hot acidic oligosaccharide extract (O-A-HWE) following the method by (Yudiati *et al.* 2018). The flow chart of ulvan extraction is presented in Figure 2.

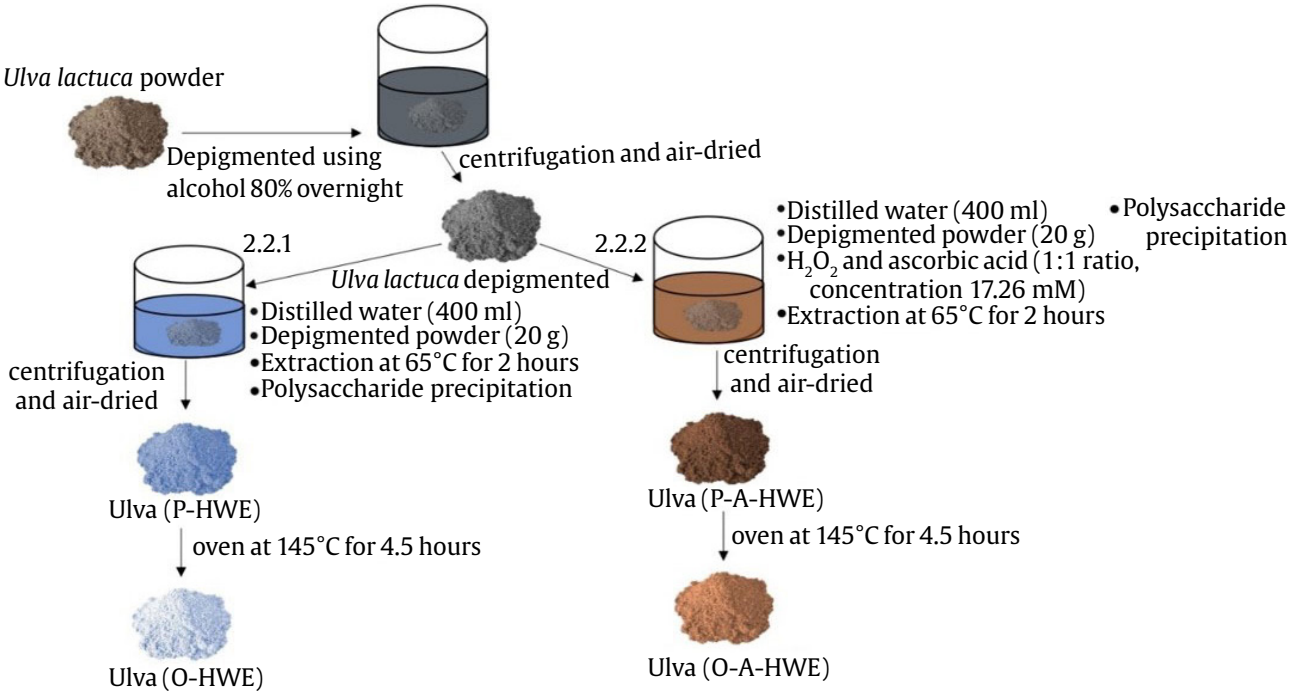

Figure 2. Flow chart of ulvan extraction

2.3. Characterization of Ulvan

2.3.1. Total Phenolic Content

The Folin-Ciocalteu method (Gutfinger 1981; Brighente *et al.* 2007) was employed. A reaction mixture comprising 0.5 ml extract, 5.0 ml distilled water, and 0.5 ml Folin-Ciocalteu reagent (Merck, Germany) was prepared. After allowing it to stand for 3 minutes, 1.0 ml of a saturated sodium carbonate solution was added. This mixture was shaken and left to stand for 1 hour. The absorbance was measured at 725 nm using a UV-Vis spectrophotometer Evolution 600 (Thermo Fisher Scientific, USA). Results were expressed as mg GAE (gallic acid equivalent) per gram of dry extract based on a gallic acid calibration curve.

2.3.2. Free Radical Scavenging Activity (DPPH)

The calculation of 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical inhibition followed the method described by Mohd Fauziee *et al.* (2021). Samples and standards (125 µL, 2 mg ml^{-1}) were mixed with 50 µL of 0.15 mM DPPH reagent in methanol (Merck, Germany) and allowed to react in the dark for 30 minutes. Absorbance was measured at 517 nm using a UV-Vis spectrophotometer Evolution 600 (Thermo Fisher Scientific, USA). The DPPH assay was performed in triplicate, and results were compared to ascorbic acid standards. DPPH scavenging activity was calculated using the equation:

$$\text{Free radical scavenging activity of DPPH (\%)} = \left[\frac{A1 - A2}{A1}\right] \times 100$$

Where:
A1 : blank absorbance
A2 : sample absorbance

2.3.3. Fourier Transform Infrared (FTIR) Analysis

Polysaccharide spectra were obtained using Fourier transform infrared (FT-IR) analysis. Sample scanning occurred from 500 to 4,000 cm^{-1}, and the analysis was performed using a Tensor 27 spectrometer (Bruker Instruments, Billerica, USA) with KBr pellet preparation (Tabarsa *et al.* 2018).

2.4. Ulvan Supplementation

SGH® commercial pellets, equivalent to 5% of the average body weight per day, were fed four times daily (at 04:00, 10:00, 16:00, and 22:00). The proximate analysis of SGH® feed revealed water content of 11%, protein 32-36%, fat 6.5-7.0%, crude fiber 3%, ash 12%, and energy 16.5-17.0 MJ kg^{-1}. Ulvan was dried and added to shrimp feed at a concentration of 0.15% (1,500 mg kg^{-1} of feed). Each of the four types of extracts (P-HWE, O-HWE, P-A-HWE, and O-A-HWE) was prepared with the same supplementation level of 1,500 mg per kg of feed. This step involved dissolving 1,500 mg of the extract in 50 ml of sterile

water and evenly spraying it onto the feed, which was then dried at room temperature. A control group without supplementation (0 mg kg^{-1}) was included in the study. The feed with the extract was weighed, prepared as a stock, and administered according to the daily feeding schedule for each treatment.

2.5. Experimental Design

The experimental setup utilized a Completely Randomized Design. Three hundred and seventy-five healthy *L. vannamei* (5.76-6.66 g) were obtained from the Brackish Water Aquaculture Development Center (BBPBAP), Ministry of Maritime Affairs and Fisheries, Jepara. Shrimp were housed in rectangular plastic tanks (145 liters) with 100 liters of seawater, rearing a density of 30 shrimp per tank. The remaining 15 shrimp were used for hemolymph sampling on day 0 for the PO test. Tanks were aerated, and approximately 10% of water renewal occurred daily (Yudiati *et al.* 2019; Azhar and Yudiati 2023). Water parameters were monitored for pH (7.72-8.11), dissolved oxygen (6.03-6.71 mg L^{-1}), salinity (30 ppt), temperature (28.23-30.02°C), and light intensity (42920-46143 lux), with nitrate, nitrite, and ammonia levels undetected using Merck colorimetric kits. Water parameters were collected at 08:00 and 16:00. A 5-day acclimatization period was observed until the shrimp displayed normal behavior and consumed the provided feed. The survival rate and specific growth rate were calculated as follows (Klongklaew *et al.* 2021):

$$\text{Survival rate (\%)} = \frac{\text{Number of live shrimps}}{\text{The initial number of total shrimp}} \times 100$$

$$\text{Specific growth rate (SGR) (\%)} = \frac{\text{ln of final weight} - \text{ln of initial weight}}{\text{Duration of the experiment}} \times 100$$

Where:
ln: natural log

Each treatment was replicated three times, and three individual shrimp samples from each replicate were collected for the PO test.

2.6. Sampling Hemolymph

Hemolymph (200 µL) was aspirated from individual shrimp from the pleopod (swimming leg located on the abdomen of the shrimp) using a sterile 1 ml syringe (Onemed) equipped with a 25G needle. The shrimp from which blood had been taken were not reused but then placed in a container with sterilized seawater to observe their survival rate and continued to be provided with supplementary feed. Before aspiration, the syringe was coated with an anticoagulant solution (10% sodium citrate, Merck, Germany) (Yudiati *et al.* 2016). Sampling was conducted before rearing and continued daily until the 10th day of the shrimp rearing period.

2.7. Phenoloxidase Activity Assay

Phenoloxidase (PO) activity was measured spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) (Sigma-Aldrich, USA) (Liu *et al.* 2004; Yudiati *et al.* 2016). The assay involved mixing 100 µL of hemolymph with 100 µL Phosphate Buffer Saline (PBS) (Sigma-Aldrich, USA) in a 1:1 ratio and centrifuging at 700 g for 20 mins at 4°C. The supernatant was discarded, and the pellet was centrifuged again in 100 µL of cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, 0.26 M magnesium chloride, pH 7). After reacting with 100 µL of cacodylate buffer, 100 µL of trypsin (Sigma-Aldrich, USA) was added, mixed, and resuspended before incubating for 10 minutes. Finally, 50 µL of L-DOPA was added, and the ELISA spectrophotometer measured the optical density at 490 nm (R-Biopharm Well Reader; Germany). PO enzyme activity was evaluated before rearing and continued daily until the 10th day of the shrimp rearing period.

2.8. Data Analysis

Data underwent analysis of variance (ANOVA) followed by LSD (Least Significant Difference) test for further comparisons. Variables examined included phenolic content, DPPH scavenging, survival rate, specific growth rate, and PO.

3. Results

3.1. Proximate Composition, Yield, and Sulfate Content in *U. lactuca*

Proximate composition, yield, and sulfate content in *U. lactuca*, as shown in Figure 3. In Figure 3A, *U. lactuca* comprises carbohydrates (10.99±2.62%), proteins (28.76±0.09%), ash (37.67±0.15%), fat

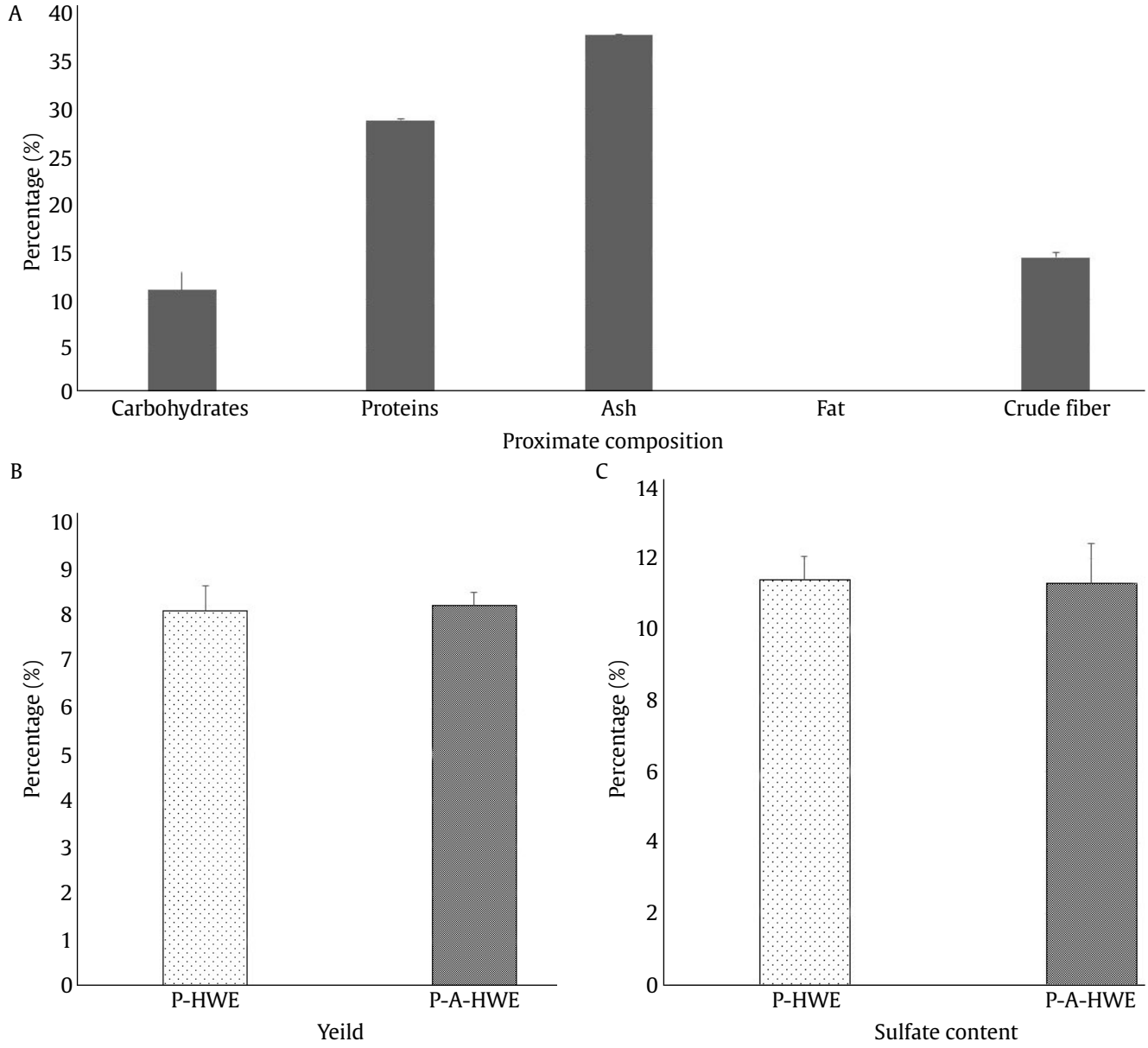

Figure 3. (A) Proximate composition, (B) yield, and (C) sulfate. Content in *Ulva lactuca*

(0.00±0.0%), and crude fiber (14.37±0.49%). According to Figure 3B, the yields of ulvan from P-HWE and P-A-HWE resulting from these extraction methods were 8.11±0.53% and 8.21±0.30%, respectively. In Figure 3C, the sulfate content of ulvan from P-HWE and P-A-HWE resulting from these extraction methods was 11.4±0.67% and 11.3±1.14%, respectively. O-HWE and O-A-HWE were not assessed for yield and sulfate content as they originate from their respective polysaccharides, P-HWE and P-A-HWE.

3.2. Total Phenol Content (TPC) of Ulvan and its DPPH Inhibition

Figure 4 showed significant differences in the TPC ($p = 0.0467$) and DPPH inhibition ($p = 0.0309$) among the presented ulvan samples. O-A-HWE demonstrated the highest phenol and DPPH inhibition compared to the other groups. However, no significant difference was observed between O-HWE and P-A-HWE for both phenol and DPPH content. P-HWE exhibited the lowest phenol and DPPH content.

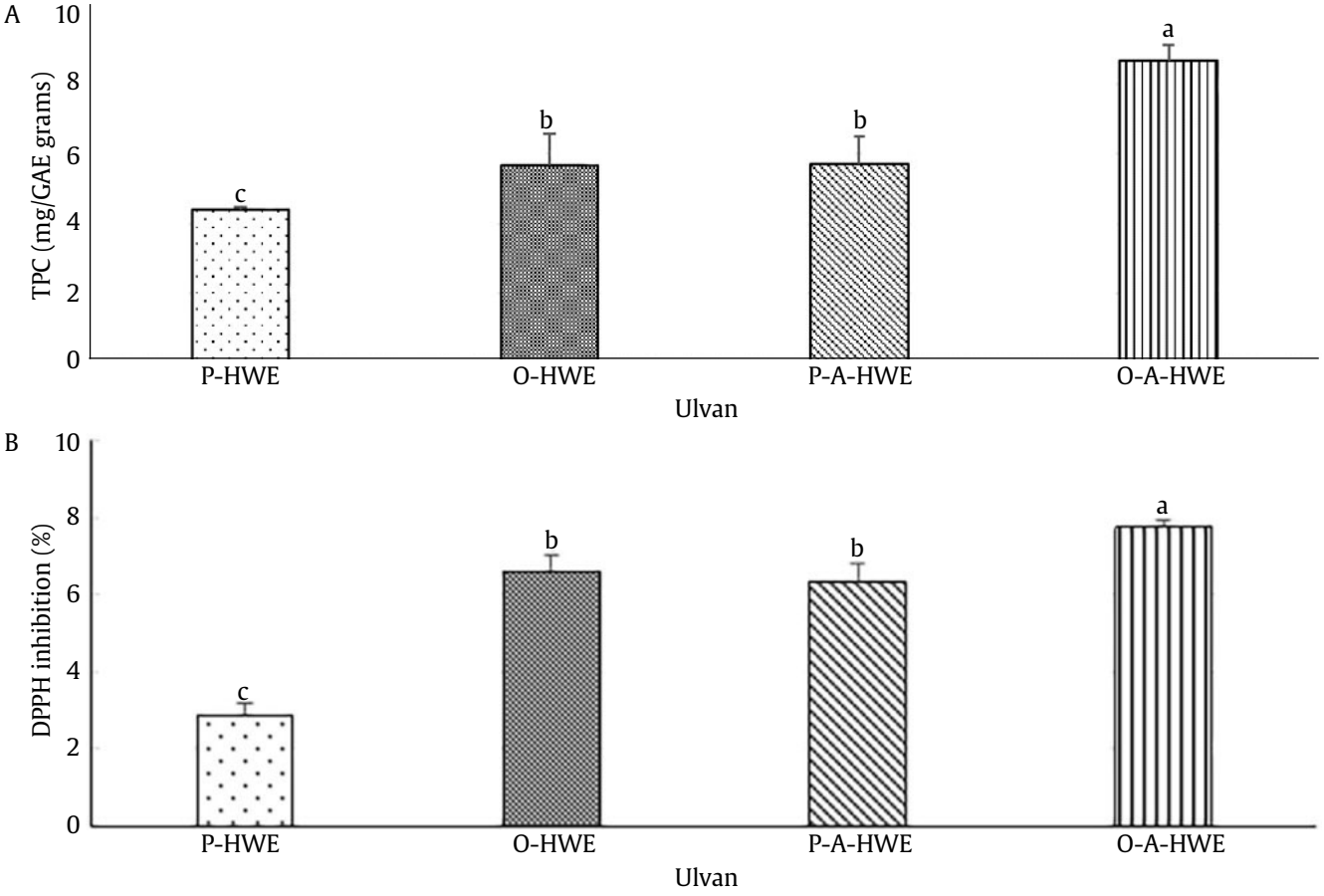

Figure 4. (A) Total phenol of ulvan and (B) DPPH radical scavenging activity by different ulvan polysaccharide and oligosaccharide extracts. Letters behind the results indicate significant differences ($p<0.05$, LSD test)

3.3. FTIR Analysis

The assignment of the IR bands was conducted based on previously published ulvan data, and the results are summarized in Figure 5. The FTIR spectrum of P-HWE (Figure 5A) exhibits prominent peaks at 2962 cm^{-1}, 2932 cm^{-1}, 1648 cm^{-1}, 1451 cm^{-1}, 1232 cm^{-1}, 1035 cm^{-1}, 874 cm^{-1}, and 855 cm^{-1}. Moving to the FTIR spectrum of O-HWE (Figure 5B), notable peaks are observed at 3390 cm^{-1}, 2928 cm^{-1}, 1641 cm^{-1}, 1428 cm^{-1}, 1082 cm^{-1}, and 855 cm^{-1}. Significant peaks in the FTIR spectrum of P-A-HWE (Figure 5C) are found at 3437 cm^{-1}, 2962 cm^{-1}, 1648 cm^{-1}, 1452 cm^{-1}, 1036 cm^{-1}, and 856 cm^{-1}. Meanwhile, the FTIR spectrum of O-A-HWE (Figure 5D) displays peaks at 3413 cm^{-1}, 2930 cm^{-1}, 1640 cm^{-1}, 1450 cm^{-1}, 1033 cm^{-1}, and 855 cm^{-1}. These distinctive peaks provide insights into the unique chemical composition and structural characteristics of ulvan extracts obtained through different methods.

3.4. Phenoloxidase (PO) Activity assay in *L. vannamei*

In Figure 6 and 7, Phenoloxidase (PO) activity showed significant differences among the different ulvan extract treatments. Oligosaccharide O-A-HWE showed the fastest and most substantial increase in activity on the 4^{th} ($p = 0.0112$), 7^{th} ($p = 0.0092$), and 8^{th} ($p = 0.0081$) days compared to the control group, suggesting a potentially positive impact on *L. vannamei's* immune response during these intervals. Additionally, O-HWE exhibited significant differences in PO activity on the 6^{th} ($p = 0.0211$), 9^{th} ($p = 0.034$), and 10^{th} ($p = 0.0123$) days compared to the control, indicating a distinctive influence on the shrimp's immune system during these days. P-A-HWE displayed significant differences in PO activity on the 8^{th} ($p = 0.0099$) day, suggesting a potential immunomodulatory effect at this time point. Finally, P-HWE showed significant differences from the

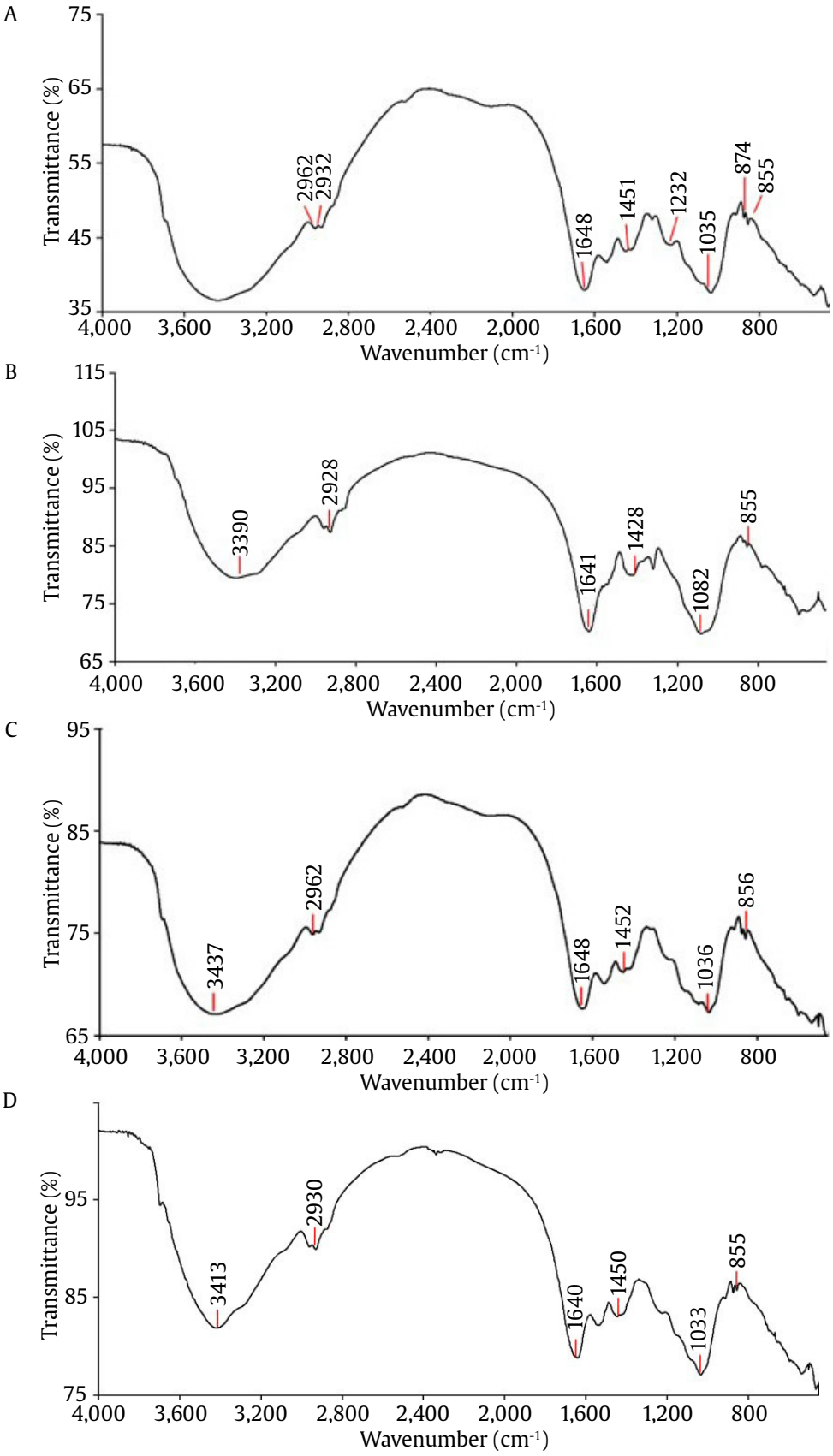

Figure 5. (A) FT-IR spectra of P-HWE, (B) O-HWE, (C) P-A-HWE, and (D) O-A-HWE. The spectra reveal peaks at 3390 cm^{-1} (O-HWE), 3437 cm^{-1} (P-A-HWE), and 3413 cm^{-1} (O-A-HWE), indicating the presence of hydroxyl groups that contribute to the formation of the antioxidant compound

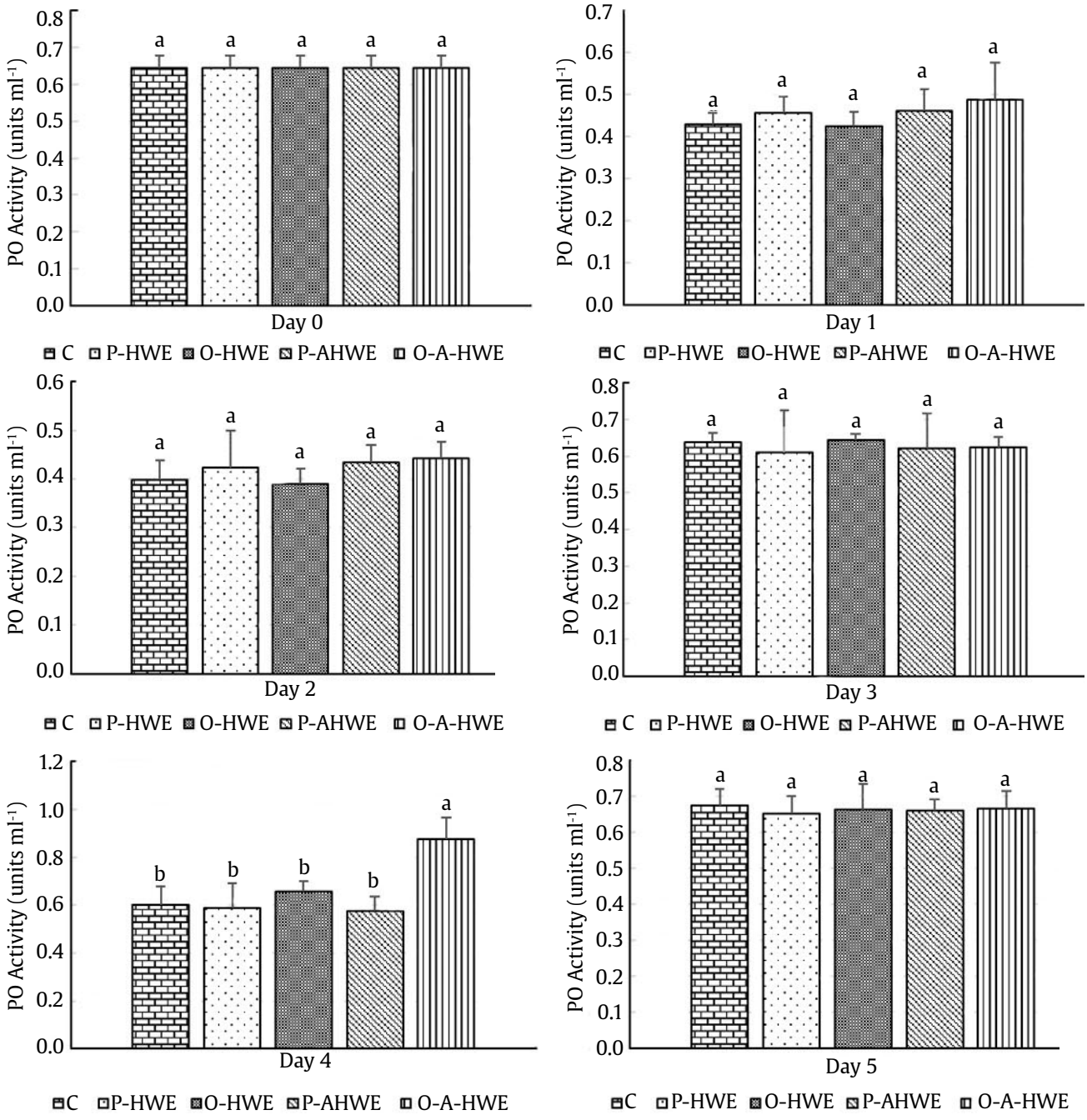

Figure 6. The phenoloxidase enzyme immune-related activity in *L. vannamei* 0-5th day of rearing with oral supplementation of P-HWE, O-HWE, P-A-HWE, and O-A-HWE in feed. Letters behind the results indicate significant differences ($p<0.05$, LSD test)

control on the 10th ($p = 0.0245$) day, indicating a notable impact on the shrimp's immune response towards the end of the experimental period. These findings highlight the dynamic and treatment-specific modulation of PO activity in *L. vannamei*, emphasizing the potential immunostimulatory effects of ulvan extracts at different stages of the experiment.

3.5. Survival Rate (SR) and Specific Growth Rate (SGR) of *L. vannamei*

The results showed no significant differences ($p>0.05$, LSD test) in survival rate (100%) among *L. vannamei* exposed to different ulvan extract treatments (Table 1). However, there were significant differences in Specific Growth Rate (SGR) ($p = 0.0001$). The highest SGR was observed in the O-A-HWE

Table 1. Survival Rate and Spesific Growth Rate of *L. vannamei*

Sample	Survival rate (%)	Spesific growth rate (%)
Control	100±0[a]	6.53±0.13[b]
P-HWE	100±0[a]	6.63±0.23[b]
O-HWE	100±0[a]	6.02±0.14[c]
P-A-HWE	100±0[a]	5.93±0.18[c]
O-A-HWE	100±0[a]	6.93±0.17[a]

All values given are means of three determinations (x ± SD); SD: standard deviation
Means in column with different capital letters are significantly different (p<0.05, LSD test) between the Ulvan

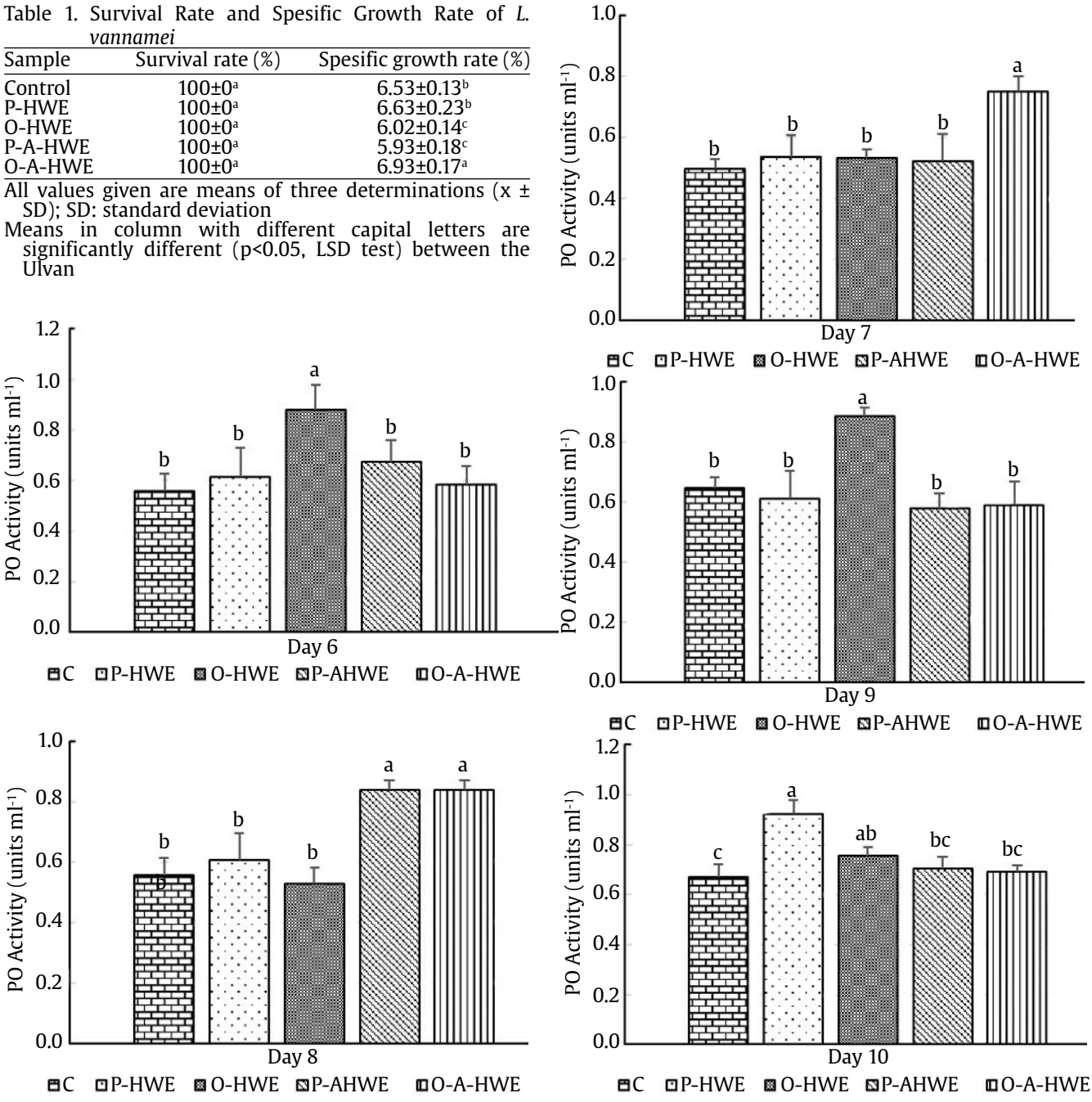

Figure 7. The phenoloxidase enzyme immune-related activity in *L. vannamei* 6-10th day of rearing with oral supplementation of P-HWE, O-HWE, P-A-HWE, and O-A-HWE in feed. Letters behind the results indicate significant differences (p<0.05, LSD test)

treatment, which was significantly different from all other treatments. The P-HWE treatment showed no significant difference compared to the control but was significantly different from P-A-HWE and O-HWE. The control group also showed significant differences compared to P-A-HWE and O-HWE.

4. Discussion

Ulvan, a natural sulfated polysaccharide found in green algae cell walls of the Ulvale group, is associated with algae proliferation in coastal and eutrophic lagoon waters (Cindana Mo'o *et al.*

2020). The extraction method significantly affects ulvan's antioxidant potential, with Oligosaccharide O-A-HWE showing superior activity compared to other extracts. This underscores the importance of extraction conditions in preserving bioactive compounds and adds to the evidence supporting Ulvan's antioxidant properties. The highest phenol content and DPPH inhibition were observed in the O-A-HWE group, demonstrating the superior antioxidant potential of this particular extract. This finding aligns with previous studies suggesting that the method of extraction significantly influences the bioactive compound profile and antioxidant capacity of ulvan (Qi *et al.* 2005). The total phenol content is a critical indicator of the antioxidant potential of natural extracts. Phenolic compounds are known for their ability to scavenge free radicals, thereby mitigating oxidative stress, which is crucial for maintaining cellular integrity and function in aquatic organisms (Cindana Mo'o *et al.* 2020). Research on *U. clathrata* indicates its enhanced antioxidant potential, with a low IC_{50} value (0.881 mg mL^{-1}) and the highest phenolic content (5.080 mg GAE g^{-1}) compared to other species (Farasat *et al.* 2014). Ascorbic acid (0.01 mg ml^{-1}) demonstrated a DPPH scavenging ability of 73.86%. The scavenging activity of ulvan Oligosaccharides is dose-dependent, ranging from 1.25 mg ml^{-1} to 10 mg ml^{-1}. Notably, at 10 mg ml^{-1}, ulvan extract (52.46%) exhibited superior DPPH scavenging ability compared to ulvan Oligosaccharides (Hung *et al.* 2021). The uronic acid content of the polysaccharide conjugate fractions influences their antioxidant properties, with high uronic acid fractions showing greater scavenging activities (Cheng *et al.* 2014).

Hydroxyl groups (O-H) are identified in the spectra at 3390 cm^{-1} (O-HWE), 3437 cm^{-1} (P-A-HWE), and 3413 cm^{-1} (O-A-HWE), indicating the presence of hydroxyl groups that form the antioxidant compound. Carbon-hydrogen bonds (C-H) manifest at 2962 cm^{-1}, 2932 cm^{-1} (P-HWE), 2928 cm^{-1} (O-HWE), 2962 cm^{-1} (P-A-HWE), and 2930 cm^{-1} (O-A-HWE), confirming the existence of organic compounds within the ulvan samples. Carbonyl and carboxylate groups (C=O and COO-) emerge at 1648 cm^{-1}, 1451 cm^{-1} (P-HWE), 1641 cm^{-1}, 1428 cm^{-1} (O-HWE), 1648 cm^{-1}, 1452 cm^{-1} (P-A-HWE), and 1640 cm^{-1}, 1450 cm^{-1} (O-A-HWE), signifying the presence of uronic acid and sulfated polysaccharides. Sulfate groups (S=O) are noticeable at 1232 cm^{-1} (P-HWE), suggesting the occurrence of sulfate groups within the polysaccharide structures. Carbon-oxygen bonds (C-O) are discerned at 1035 cm^{-1} (P-HWE), 1082 cm^{-1} (O-HWE), 1036 cm^{-1} (P-A-HWE), and 1033 cm^{-1} (O-A-HWE), indicating the presence of ether linkages in the molecular composition. The presence of C-O-S bonds is confirmed by the characteristic peaks observed at 874 cm^{-1}. In addition, specific peaks are detected in the samples: 855 cm^{-1} for both P-HWE and O-HWE, 856 cm^{-1} for P-A-HWE, and 855 cm^{-1} for O-A-HWE (Andrade Figueira *et al.* 2020; Zhao *et al.* 2020; Barakat *et al.* 2022; Ibrahim *et al.* 2022). The ulvan Signature Region, spanning from 1150 to 750 cm^{-1}, consistently corresponds to typical ulvan signatures (Andrade Figueira *et al.* 2020; Ibrahim *et al.* 2022). These peaks indicate the presence of specific functional groups, such as C-H stretching, C=O stretching, and S=O stretching, corresponding to the ulvan components reported by Lahaye and Robic (2007).

This rapid increase in PO activity indicates that the O-A-HWE extract may enhance the shrimp's ability to respond to pathogenic challenges more effectively, providing a quicker and more robust immune response (Cerenius *et al.* 2008). A study on ulvan from *Enteromorpha intestinalis* at dietary levels of 1,000 to 1,500 mg kg-1 showed its ability to induce hemocytic degranulation and convert proPO into PO in juvenile Penaeus monodon (Declarador *et al.* 2014). Optimal dietary ulvan levels were estimated at 0.21% for *L. vannamei* and 0.15% for *P. monodon* based on a quadratic model, suggesting ulvan as an effective immunostimulant for both species (Lauzon and Serrano Jr 2015). Other studies have shown a correlation between antioxidants and the immune system in *L. vannamei*. Additionally, according to Mansour *et al.* (2022), dietary supplementation with the natural antioxidant astaxanthin improved growth performance, immune response, and antioxidant levels in *L. vannamei*. Eldessouki *et al.* (2022) found dietary astaxanthin effective at concentrations of 100–200 mg kg^{-1} of feed in enhancing growth, antioxidant status, immune response, and resistance against *Vibrio harveyi* infection in *L. vannamei*. These findings highlight the dynamic and treatment-specific modulation of PO activity in *L. vannamei*, emphasizing the potential immunostimulatory effects of ulvan extracts at different stages of the experiment. The ability of these extracts to enhance PO activity suggests their potential as valuable tools

in improving shrimp health and resilience against diseases (Barman *et al.* 2013).

During an immune response, reactive oxygen species (ROS) are generated as part of the defense mechanism. However, high levels of ROS can be detrimental to host tissues, initiating autoimmune responses and damaging cells (Tavassolifar *et al.* 2020). Ulvan's immunomodulatory effect has been quantified by measuring levels of signaling molecules released from LPS-stimulated macrophages (Kidgell *et al.* 2020). Inflammatory processes induce oxidative stress and reduce cellular antioxidant capacity (Khansari *et al.* 2009). In this research, ulvan, with its antioxidant properties, scavenges ROS, protecting host cells from oxidative damage and maintaining cellular integrity and function. This ensures the proper functioning of immune components such as the phenoloxidase (PO) system. ROS can inactivate key proteins involved in the PO cascade. By neutralizing ROS, ulvan preserves the activity of prophenoloxidase and the serine proteases that convert proPO to its active form. Similarly, antioxidant N-acetylcysteine has been shown to remove and inhibit intracellular ROS, significantly up-regulating the expression of proPO (Zhou *et al.* 2023).

The addition of ulvan extract enhances immune response, improves hemolymph nutrient distribution, and boosts digestive enzyme activity. Specifically, O-A-HWE treatment shows the highest Specific Growth Rate (SGR), suggesting it enhances *L. vannamei* growth via increased nutrient intake or enhanced metabolic efficiency. Our study confirms increased trypsin activity in *L. vannamei* intestines post-ulvan supplementation (Azhar *et al.* 2024). Nathan *et al.* (2023) found oleic acid as an immunostimulant in fish feed affects more immune-related metabolites in vibriosis-infected grouper, with spleen glycine (20.9%), l-threonine (1.0%), l-serine (0.8%) and liver l-glutamine (1.8%), aspartic acid (0.6%) showing high prevalence.

These findings demonstrated the potential of ulvan as a dietary supplement to enhance shrimp immunity and health, presenting opportunities to enhance overall aquaculture performance. Further investigation could delve into the mechanisms underlying ulvan-induced immune responses and their efficacy against various shrimp diseases. Moreover, this study lays the groundwork for devising more efficient disease management strategies in aquaculture. It is also crucial to assess the impact of ulvan usage on aquatic ecosystems and the sustainability of shrimp farming and water conservation efforts. By highlighting ulvan's potential as a safe and effective feed additive, this research significantly contributes to the advancement of sustainable and efficient shrimp farming practices.

Acknowledgements

We would like to state our special thanks and gratefulness to Diponegoro University and the Indonesian Government's Ministry of Research and Higher Education. This research was supported by the Indonesian Government's Ministry of Research and Higher Education through the PDD scheme, with grant number 449A-04/UN7/D2/PP6/2023 and 601-38/UN7.D2/PP/VI/2024.

References

Abbas, E.M., Al-Souti, A.S., Sharawy, Z.Z., El-Haroun, E., Ashour, M., 2023. Impact of dietary administration of seaweed polysaccharide on growth, microbial abundance, and growth and immune-related genes expression of the pacific whiteleg shrimp (*Litopenaeus vannamei*). *Life* 13, 344. https://doi.org/10.3390/life13020344

Akbary, P., Adeshina, I., Jahanbakhshi, A., 2020. Growth performance, digestive enzymes, antioxidant activity and immune responses of Litopenaeus vannamei fed with Jania adhaerens J.V. Supplemented diet against *Photobacterium damselae* infection. *Anim Feed Sci Technol.* 270, 114696. https://doi.org/10.1016/j.anifeedsci.2020.114696

Amparyup, P., Charoensapsri, W., Tassanakajon, A., 2013. Prophenoloxidase system and its role in shrimp immune responses against major pathogens. *Fish Shellfish Immunol.* 34, 990-1001. https://doi.org/10.1016/j.fsi.2012.08.019

Andrade Figueira, T., Da Silva, A., Enrich-Prast, A., Yoneshigue-Valentin, Y., Oliveira, V., 2020. Structural characterization of ulvan polysaccharide from cultivated and collected *Ulva fasciata* (Chlorophyta). *Adv Biosci Biotechnol.* 11, 206-216. https://doi.org/10.4236/abb.2020.115016

Azhar, N., Yudiati, E., 2023. Outbreak simulation of *Litopenaeus vannamei* recovery rate with oral alginate and spirulina diet supplementation against *Vibrio parahaemolyticus* AHPND. *Aquacult Int.* 31, 1659-1676. https://doi.org/10.1007/s10499-023-01050-6

Azhar, N., Yudiati, E., Ambariyanto, A., Trianto, A., 2024. Immunostimulatory effects of ulvan on trypsin-mediated protein digestion in the gut of pacific whiteleg shrimp (*Litopenaeus vannamei*). *Jurnal Riset Akuakultur*. 19, 45-56. https://doi.org/10.15578/jra.19.1.2024.45-56

Barakat, K.M., Ismail, M.M., Abou El Hassayeb, H.E., El Sersy, N.A., Elshobary, M.E., 2022. Chemical characterization and biological activities of ulvan extracted from *Ulva fasciata* (Chlorophyta). *Rend Lincei Sci Fis Nat.* 33, 829-841. https://doi.org/10.1007/s12210-022-01103-7

Barman, D., Nen, P., Mandal, S., Kumar, V., 2013. Immunostimulants for aquaculture health management. *J Mar Sci Res Dev.* 3, 134. https://doi.org/10.4172/2155-9910.1000134

Brighente, I.M.C., Dias, M., Verdi, L.G., Pizzolatti, M.G., 2007. Antioxidant activity and total phenolic content of some Brazilian species. *Pharm Biol.* 45, 156-161. https://doi.org/10.1080/13880200601113131

Cerenius, L., Söderhäll, K., 2004. The prophenoloxidase-activating system in invertebrates. *Immunological Reviews.* 198, 116-126. https://doi.org/10.1111/j.0105-2896.2004.00116.x

Cerenius, L., Lee, B.L., Söderhäll, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology*. 29, 263-271. https://doi.org/10.1016/j.it.2008.02.009

Cerenius, L., Babu, R., Söderhäll, K., Jiravanichpaisal, P., 2010. *In vitro* effects on bacterial growth of phenoloxidase reaction products. *J Invertebr Pathol.* 103, 21-23. https://doi.org/10.1016/j.jip.2009.09.006

Chen, B.J., Shi, M.J., Cui, S., Hao, S.X., Hider, R.C., Zhou, T., 2016. Improved antioxidant and anti-tyrosinase activity of polysaccharide from *Sargassum fusiforme* by degradation. *International Journal of Biological Macromolecules.* 92, 715-722. https://doi.org/10.1016/j.ijbiomac.2016.07.082

Cheng, J., Zhang, W., Zhang, X., Han, F., Li, X., He, X., Li, Q., Chen, J., 2014. Effect of angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers on all-cause mortality, cardiovascular deaths, and cardiovascular events in patients with diabetes mellitus: a meta-analysis. *JAMA Intern Med*. 174, 773-785. https://doi.org/10.1001/jamainternmed.2014.348

Choi, M., Stevens, A.M., Smith, S.A., Taylor, D.P., Kuhn, D.D., 2017. Strain and dose infectivity of *Vibrio parahaemolyticus*: the causative agent of early mortality syndrome in shrimp. *Aquac Res*. 48, 3719-3727. https://doi.org/10.1111/are.13197

Cindana Mo'o, F.R., Wilar, G., Devkota, H.P., Wathoni, N., 2020. Ulvan, a polysaccharide from macroalga *Ulva* sp.: a review of chemistry, biological activities and potential for food and biomedical applications. *Appl Sci.* 10, 5488. https://doi.org/10.3390/app10165488

Declarador, R., Serrano Jr, A., Corre, V., 2014. Ulvan extract acts as immunostimulant against white spot syndrome virus (WSSV) in juvenile black tiger shrimp *Penaeus monodon. AACL Bioflux.* 7, 153-161.

Eldessouki, E.A.A., Diab, A.M., Selema, T.A.M.A., Sabry, N.M., Abotaleb, M.M., Khalil, R.H., El-Sabbagh, N., Younis, N.A., Abdel-Tawwab, M., 2022. Dietary astaxanthin modulated the performance, gastrointestinal histology, and antioxidant and immune responses and enhanced the resistance of *Litopenaeus vannamei* against *Vibrio harveyi* infection. *Aquac Int.* 30, 1869-1887. https://doi.org/10.1007/s10499-022-00876-w

Farasat, M., Khavari-Nejad, R.A., Nabavi, S.M., Namjooyan, F., 2014. Antioxidant activity, total phenolics and flavonoid contents of some edible green seaweeds from northern coasts of the persian gulf. *Iran J Pharm Res.* 13, 163-170. https://doi.org/10.5812/jjnpp.7736

Gutfinger, T., 1981. Polyphenols in olive oils. *J Am Oil Chem Soc.* 58, 966-968. https://doi.org/10.1007/BF02659771

Horwitz, W., Latimer, G.W., 1990. *Official Methods of Analysis of AOAC International*, fifteenth ed. AOAC International Gaithersburg, Arlington, Virginia, USA.

Hung, Y.H.R., Chen, G.W., Pan, C.L., Lin, H.T.V., 2021. Production of ulvan oligosaccharides with antioxidant and angiotensin-converting enzyme-inhibitory activities by microbial enzymatic hydrolysis. *Fermentation*. 7, 160. https://doi.org/10.3390/fermentation7030160

Ibrahim, M.I.A., Amer, M.S., Ibrahim, H.A.H., Zaghloul, E.H., 2022. Considerable production of ulvan from *Ulva lactuca* with special emphasis on its antimicrobial and anti-fouling properties. *Appl Biochem Biotechnol.* 194, 3097-3118. https://doi.org/10.1007/s12010-022-03867-y

Kanost, M.R., Jiang, H., Yu, X.Q., 2004. Innate immune responses of a lepidopteran insect, *Manduca sexta. Immunological Reviews.* 198, 97-105. https://doi.org/10.1111/j.0105-2896.2004.0121.x

Khansari, N., Shakiba, Y., Mahmoudi, M., 2009. Chronic inflammation and oxidative stress as a major cause of age-related diseases and cancer. *Recent Patents on Inflammation & Allergy Drug Discovery*. 3, 73-80. https://doi.org/10.2174/187221309787158371

Kidgell, J.T., Glasson, C.R.K., Magnusson, M., Vamvounis, G., Sims, I.M., Carnachan, S.M., Hinkley, S.F.R., Lopata, A.L., de Nys, R., Taki, A.C., 2020. The molecular weight of ulvan affects the *in vitro* inflammatory response of a murine macrophage. *International Journal of Biological Macromolecules*. 150, 839-848. https://doi.org/10.1016/j.ijbiomac.2020.02.071

Klongklaew, N., Praiboon, J., Tamtin, M., Srisapoome, P., 2021. Chemical composition of a hot water crude extract (HWCE) from *Ulva intestinalis* and its potential effects on growth performance, immune responses, and resistance to white spot syndrome virus and yellowhead virus in Pacific white shrimp (*Litopenaeus vannamei*). *Fish Shellfish Immunol.* 112, 8-22. https://doi.org/10.1016/j.fsi.2021.02.004

Kumar, S., Verma, A.K., Singh, S.P., Awasthi, A., 2023. Immunostimulants for shrimp aquaculture: paving pathway towards shrimp sustainability. *Environ Sci Pollut Res.* 30, 25325-25343. https://doi.org/10.1007/s11356-021-18433-y

Lahaye, M., Robic, A., 2007. Structure and functional properties of ulvan, a polysaccharide from green seaweeds. *Biomacromolecules.* 8, 1765-1774. https://doi.org/10.1021/bm061185q

Lauzon, Q., Serrano Jr, A., 2015. Ulvan extract from *Enteromorpha intestinalis* enhances immune responses in *Litopenaeus vannamei* and *Penaeus monodon* juveniles. *ABAH Bioflux.* 7, 1-10.

Liu, C.H., Yeh, S.T., Cheng, S.Y., Chen, J.C., 2004. The immune response of the white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio* infection in relation with the moult cycle. *Fish Shellfish Immunol.* 16, 151-161. https://doi.org/10.1016/S1050-4648(03)00058-5

Mansour, A.T., Ashour, M., Abbas, E.M., Alsaqufi, A.S., Kelany, M.S., El-Sawy, M.A., Sharawy, Z.Z., 2022. Growth performance, immune-related and antioxidant genes expression, and gut bacterial abundance of pacific white leg shrimp, *Litopenaeus vannamei*, dietary supplemented with natural astaxanthin. *Frontiers in Physiology*. 13, 874172. https://doi.org/10.3389/fphys.2022.874172

Mohd Fauziee, N.A., Chang, L.S., Wan Mustapha, W.A., Md Nor, A.R., Lim, S.J., 2021. Functional polysaccharides of fucoidan, laminaran and alginate from Malaysian brown seaweeds (*Sargassum polycystum, Turbinaria ornata* and *Padina boryana*). *International Journal of Biological Macromolecules.* 167, 1135-1145. https://doi.org/10.1016/j.ijbiomac.2020.11.067

Nappi, A.J., Christensen, B.M., 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. *Insect Biochemistry and Molecular Biology*. 35, 443-459. https://doi.org/10.1016/j.ibmb.2005.01.014

Natnan, M.E., Low, C.F., Chong, C.M., Bunawan, H., Baharum, S.N., 2023. Oleic acid as potential immunostimulant in metabolism pathways of hybrid grouper fingerlings (*Epinephelus fuscoguttatus* × *Epinephelus lanceolatus*) infected with *Vibrio vulnificus*. *Scientific Reports*. 13, 12830. https://doi.org/10.1038/s41598-023-40096-7

Perazzolo, L.M., Barracco, M.A., 1997. The prophenoloxidase activating system of the shrimp Penaeus paulensis and associated factors. *Dev Comp Immunol.* 21, 385-395. https://doi.org/10.1016/S0145-305X(97)00022-0

Qi, H., Zhang, Q., Zhao, T., Chen, R., Zhang, H., Niu, X., Li, Z., 2005. Antioxidant activity of different sulfate content derivatives of polysaccharide extracted from *Ulva pertusa* (Chlorophyta) *in vitro*. *International Journal of Biological Macromolecules*. 37, 195-199. https://doi.org/10.1016/j.ijbiomac.2005.10.008

Tabarsa, M., You, S., Dabaghian, E.H., Surayot, U., 2018. Water-soluble polysaccharides from *Ulva intestinalis*: molecular properties, structural elucidation and immunomodulatory activities. *J Food Drug Anal.* 26, 599-608. https://doi.org/10.1016/j.jfda.2017.07.016

Tavassolifar, M.J., Vodjgani, M., Salehi, Z., Izad, M., 2020. The influence of reactive oxygen species in the immune system and pathogenesis of multiple sclerosis. *Autoimmune Diseases.* 2020, 5793817. https://doi.org/10.1155/2020/5793817

Yudiati, E., Isnansetyo, A., Murwantoko, Ayuningtyas, Triyanto, Handayani, C.R., 2016. Innate immune-stimulating and immune genes up-regulating activities of three types of alginate from *Sargassum siliquosum* in Pacific white shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 54, 46. https://doi.org/10.1016/j.fsi.2016.03.022

Yudiati, E., Pringgenies, D., Djunaedi, A., Arifin, Z., Sudaryono, A., 2018. Free radicals scavenging activities of low molecular weight sodium alginate (LMWSA) from *Sargassum polycystum*, produced by thermal treatment. *Aquac Indones.* 19, 21-27. https://doi.org/10.21534/ai.v19i1.121

Yudiati, E., Isnansetyo, A., Murwantoko, Triyanto, Handayani, C.R., 2019. Alginate from *Sargassum siliquosum* simultaneously stimulates innate immunity, up-regulates immune genes, and enhances resistance of Pacific white shrimp (*Litopenaeus vannamei*) against white spot syndrome virus (WSSV). *Mar Biotechnol.* 21, 503. https://doi.org/10.1007/s10126-019-09898-7

Yudiati, E., Subagiyo, S., Djarod, M.S.R., 2020. Preliminary study of polysaccharide and oligosaccharide alginate (AOS) as prebiotic of probiotic bacteria. *Jurnal Kelautan Tropis.* 23, 234-238. https://doi.org/10.14710/jkt.v23i2.7674

Yudiati, E., Wijayanti, D.P., Azhar, N., Chairunnisa, A.I., Sedjati, S., Arifin, Z., 2021. Alginate oligosaccharide/polysaccharide and lactic acid bacteria (*Lactobacillus bulgaricus* FNCC-0041 & *Streptococcus thermophilus* FNCC-0040) as immunostimulants against pathogenic *Vibrio* spp. using Artemia bio model. *IOP Conference Series: Earth and Environmental Science*. 919, 012060. https://doi.org/10.1088/1755-1315/919/1/012060

Yudiati, E., Kurniawan, P.N.R., Putri, A.N., Al Falah, I.H., Azhar, N., Sedjati, S., Arifin, Z., Santosa, G.W., 2024. Benefits of alginate and spirulina supplementation to the growth and resistance against salinity exposure in *Litopenaues vannamei* post-larvae. *Ribarstvo.* 82, 65-72. https://doi.org/10.2478/cjf-2024-0008

Zhao, C., Lin, G., Wu, D., Liu, D., You, L., Högger, P., Simal-Gandara, J., Wang, M., da Costa, J.G.M., Marunaka, Y., Daglia, M., Khan, H., Filosa, R., Wang, S., Xiao, J., 2020. The algal polysaccharide ulvan suppresses growth of hepatoma cells. *Food Frontiers.* 1, 83-101. https://doi.org/10.1002/fft2.13

Zhou, X., Chen, Q., Chen, L., Liao, X., Wang, Z., Zhu, F., 2023. The effect of reactive oxygen species (ROS) in immunity and WSSV infection of *Scylla paramamosain*. *Fish Shellfish Immunol.* 141, 109075. https://doi.org/10.1016/j.fsi.2023.109075