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Research Article

Sodium Alginate from *Padina australis* Modulates Innate Immune and Immune Gene Expression in Red Tilapia (*Oreochromis* sp.)

Toni Kuswoyo, Alim Isnansetyo*, Murwantoko, Amir Husni, and Indah Istiqomah

Department of Fisheries, Faculty of Agriculture, Gadjah Mada University, Yogyakarta, 55281. Indonesia

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*) Corresponding author:
E-mail: isnansetyo@ugm.ac.id

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Abstract

The study of immunostimulant derived from natural sources has received considerable attention in recent years. Indonesian coasts have various sources of immunostimulant, especially brown algae, which are rich in alginate. This research investigated innate immune response and immune genes in red tilapia (*Oreochromis* sp.) after being fed diets supplemented with sodium alginates (SA) from a brown alga, *Padina australis*, originating from Awur Bay, Jepara, Indonesia. This work provided comprehensive information regarding the interaction of cellular-humoral immunity and immune gene expression. Red tilapia was fed with SA-containing diet at doses of 0, 2, 3 and 4 g/kg for 12 days, then blood samples were collected on days 0, 3, 6, 9, and 12 to evaluate the total leucocyte count (TLC), phagocytic activity/index (PA/PI), respiratory burst, superoxide dismutase (SOD), lysozyme, alternative complement (ACH_{50}) activity, total plasma protein (TPP), and immune genes expression (blood sample on day 6). The immunological parameters improved in response to the administered doses and period. Dietary SA enhanced total leucocyte count, phagocytic activity, respiratory burst, superoxide dismutase, lysozyme, and ACH_{50} activity, whereas the phagocytic index and total plasma protein were not significantly different. Furthermore, the mRNA levels of antioxidant-related gene (GPx), pro-inflammatory cytokines (TNF-α and IFN-γ, except for IL-1β), and antimicrobial peptides (Hepcidin) were upregulated. Meanwhile, anti-inflammatory cytokines (IL-10 and TGF-β1) were downregulated. Supplementation SA diet at 2.0 g/kg as the lowest dose revealed the most effective effects on innate immune and immune genes expression. Dietary SA was a potential immunostimulant in red tilapia aquaculture.

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1. Introduction

One type of tilapia that is popular and has high economic value besides Nile tilapia (*Oreochromis niloticus*) is red tilapia (*Oreochromis* spp.) (Robisalmi *et al*., 2021). Tilapia is widely cultivated worldwide with total production reached 5.58 million metric tons in 2020 (FAO, 2020), and 7.3 million metric tons of total production is predicted by 2030 (Behera *et al*., 2018). Tilapia is the most cultivated freshwater fish in Indonesia, with a total production of 1.34 million tons in 2019 (MMAF-RI, 2020). The intensive tilapia culture has frequently faced a catastrophic disease outbreak. Antibiotics and chemotherapies are widely used to control disease in tilapia farms, but they may have adverse effects such as the increase in antibiotic resistance of bacterial pathogen, environmental hazards, and food safety issues (Arsène *et al*., 2021). Therefore, developing immunostimulants based on natural resources to improve the tilapia immune system is a potential alternative approach (Doan *et al*., 2016; Gobi *et al*., 2018; Yengkhom *et al*., 2019).

Immunostimulants are natural compounds and synthetic substances that modulate the non-specific immune system (Bricknell and Dalmo, 2005). Immunostimulants promote host resistance to diseases by activating innate defense mechanisms rather than adaptive immune responses (Sakai, 1999). The recognition of a pathogen-associated molecular pattern (PAMP) by the pattern recognition receptor (PRR) from the host is required for the innate immunity to eliminate the pathogen (Medzhitov, 2007). Immunostimulants have a similar molecular pattern with pathogens thus they can be recognized by its receptors. PAMP recognition activates the innate immune system extensively, including the secretion of soluble molecules and humoral and cellular elements mediated by circulating hemocytes (Yudiati *et al*., 2016). A brown algae, *Padina australis*, is abundant along Indonesian coast. This seaweed is rich in alginate with yields ranging from 16.93% (Susanto *et al*., 2001) to 30.3% (Mushollaeni, 2011), so it has potential as an immunostimulant. Alginate is a polysaccharide composed of linear homopolymeric and heteropolymeric blocks of α-L-guluronate and **β**-D-mannuronate (Vasudevan *et al*., 2021). This polysaccharide has been reported as a supplement in aquafeed to trigger the immune system. Alginate in *Clarias* sp. diet at 4.0 g/kg increases respiratory burst and phagocytic activity (PA) (Isnansetyo *et al*., 2014). Sodium alginate (SA) has also been reported to elevate the immune status of shrimp (Yudiati *et al*., 2016) and finfish (Yeh *et al*., 2008; Ashouri *et al*., 2018, 2020; Doan *et al*., 2016, 2017; Harikrishnan *et al*., 2011). However, the publication of simultaneous evaluations of the effects of SA on the immune system and immune gene expression in tilapia is scarce. In addition, to the best of our knowledge, SA from *P. australis* has not been reported to improve immune status in tilapia.

This research aimed to analyze the influence of SA originating from *P. australis* on innate immune and immune gene expression in red tilapia. Exploration of SA from underutilized brown seaweed, particularly in the tropics, and their application as an immunostimulant will contribute to better knowledge of its effectiveness, function, and mechanisms in enhancing innate immune defenses.

2. Materials and Methods

2.1 Material

This research was conducted from March to May 2020 at the Fish Disease Laboratory and Aquaculture Laboratory of Department of Fisheries, Universitas Gadjah Mada, Yogyakarta. The materials used were 60 red tilapias with weight of 135 ± 12.5 g/fish, indoor container 0.2 m^3, and water quality measuring tools (thermometer, pH meter, and DO meter).

2.2 Method

2.2.1 SA extraction

P. australis was collected from Awur Bay, Jepara, Indonesia. SA was produced in accordance with the method by Isnansetyo *et al.* (2014). Air dried *P. australis* was cut and ground to obtain coarse powder at approximately 355 microns, depigmented in ethanol (95%) for 24 hours (repeated two times), and filtered. The filtrate was filtered after being submerged in 0.1 N HCl for 24 hours at ambient temperature. Repeated maceration in 0.2 N HCl at 70°C for two hours was followed by filtering. The retentate was suspended in distilled water, precipitated with 0.5 M Na_2CO_3, heated in a water bath at 60°C until a slurry was formed, and then filtered. The slurry was heated to 60°C, evaporated, and precipitated with chilled 95% ethanol before being centrifuged at 3,500 rpm for 15 minutes. SA was dried overnight at 60° C and then stored at 4°C until used.

2.2.2 The FTIR spectroscopy

Obtained SA was mixed with Potassium Bromide (KBr) and measured by using a Nicolet 380 FTIR (Fourier Transform Infrared) spectrometer (Nicolet 380 FTIR, Germany) at 4000–500 cm^{-1}.

2.2.3 Experimental design

The study used a completely randomized design (CBD) with four treatments in triplicate. Briefly, 60 red tilapias were used then stocked in each container according to the treatment of five fish/container. The fish were acclimatized for two weeks and fed with control diet three times a day (3% of total biomass). The water was continuously aerated and kept at 27.10 ± 1.05°C, 7.25 ± 0.25, and 5.0 ± 0.5 mg/L for temperature, pH, and DO (dissolved oxygen), respectively.

A commercial feed (Charoen Pokphand Indonesia) with 32% protein content, 5% fat, and 4% fiber was supplemented with SA at doses of 0 (control group), 2.0, 3.0, and 4.0 g/kg. The SA was diluted in distilled water (100 mL/kg feed), then added with a binder (Progol®, PT. Indosco, Indonesia), then sprayed homogenously over the entire feed, then air-dried at ambient temperature and ready to use. Fish were fed according to the SA level three times a day (3% of total biomass) for 12 days.

Blood samples from each replication consisted of one fish which was sampled randomly and alternately on days 0, 3, 6, 9, and 12 from the treatment and control groups. To minimize stress, fish were anesthetized using clove oil (0.01% v/v). Blood was drawn from caudal veins using a 1 mL syringe flushed with 10% Ethylenediaminetetraacetic acid (EDTA) as described previously (Doan *et al*., 2016). Blood was stored at 15°C in a cooler box without anticoagulant and fish immediately returned to indoor containers for healing. Blood samples were used to evaluate total leucocyte count (TLC), phagocytic activity/index (PA/PI), respiratory burst activity, and gene expression, while the total plasma protein (TPP), superoxide dismutase (SOD), lysozyme, and alternative complement activity were measured from serum.

2.2.4 Parameters of innate immune

2.2.4.1 Total leukocyte count (TLC)

A microtube containing 50 µL of blood was mixed with 500 µL of Turk's solution (1/10 v/v). The mixture was slowly homogenized and then maintained at ambient temperature for 3–5 minutes. The solution was pipetted onto a hemocytometer (Neubauer, Germany) covered with a cover glass. Leukocytes were counted under a light microscope (Zeiss Axioskop, Germany) (Anderson and Siwicki, 1995).

2.2.4.2 Phagocytic activity/index (PA/PI)

PA/PI was assessed as described by Anderson and Siwicki (1995). Blood was filled into a capillary hematocrit tube to the red line, then coated with wax on one side, then centrifuged for five minutes at 1,500 rpm. After cutting the tubes at the plasma-leukocyte boundary, leukocytes were collected using a micropipette. PA/PI was examined by mixing 20 µL of leukocytes and 20 µL of *Bacillus subtilis* (formalin neutralized) at a bacterial density of 10^8 cells/mL in a microplate. At ambient temperature, the mixture was kept for 30 minutes. The mixture was smeared carefully to the slides in five microliter quantities, allowed to air dry, then was fixed with 95% ethanol for 10 minutes before being stained for 20 minutes with 10% Giemsa. Before being examined with a light microscope (Zeiss Axioskop, Germany), the slides were washed with tap water and dried. PA was measured based on the number of phagocytic cells per 100 adhered cells. Then, PI was computed by dividing the ingested bacterial cells by the total phagocytosed cells.

2.2.4.3 Respiratory burst

With minor adjustments, the test was carried out according to the procedure described by Anderson and Siwicki (1995). The same quantity of 0.2% nitro blue tetrazolium (NBT) solution was added to 100 µL of blood (v/v), then incubated for 30 minutes at 25°C. The mixture was then poured into a glass tube with 1 mL of dimethylformamide (DMF), homogenized, and centrifuged (3,000 *g*, 5 minutes). The optical density was determined at 540 nm. For the blank, pure water was used instead of blood.

2.2.4.4 Superoxide dismutase (SOD)

The test was analyzed in accordance with the method by Beauchamp and Fridovich (1971) with minor changes. Prior to centrifugation (6,000 *g*, 4°C, five minutes), 100 µL of blood was poured into 500 µL of phosphate buffer (PB). Then 100 µL of supernatant was used, evaporated (65°C, five minutes), then added with 50 µL of reaction mixtures which contained 20 µM of riboflavin, 13 µM of methionine, 0.75 mM of NBT, 0.1 mM of EDTA, and 50 mM of PB (pH 7.8, 25°C). To measure the optical density, a spectrophotometer (Well Reader-Elisa Reader R-Biopharm, Germany) was employed at 630 nm.

2.2.4.5 Lysozyme

The test was assessed through a method by Ellis (1990). Ten microliters of serum was added to 200 µL of *Micrococcus lysodeikticus* (Sigma, USA) solution (0.2

mg/mL of *M. lysodeikticus* in 0.05 M of PBS, pH 6.2) and stored at 27°C. The optical density was measured at 530 nm using a microplate reader (Well Reader-Elisa Reader R-Biopharm, Germany) after 0.5 and 4.5 minutes. The amount of enzyme required to decrease the absorbance of 0.001/min was defined as one unit of lysozyme activity. Lysozyme standard curve from chicken egg white (Sigma, USA) was used to calculate the concentration of lysozymes.

2.2.4.6 Alternative complement (ACH_{50})

The targets for the experiment were sheep red blood cells (SRBC) (Ortuño *et al*., 2002). SRBC were rinsed in Hank's balanced salt solution (HBSS), which contains Mg^{2+} and ethylene glycol tetra acetic acid, without phenol, and then resuspended to 3% in HBSS (v/v). One hundred microliters of SRBC were added after 100 µL of serum (as a complement source) were diluted with HBSS for final concentrations of 10, 5, 2.5, 1.25, 0.625, 0.312, and 0.156% (v/v). The tubes were stored for 1 hour at 22°C to avoid unlinking of erythrocytes before centrifugation (400 *g*, 5 minutes, 4°C), then 100 µL of supernatant was poured to a 96-well plate. The concentration of relative hemoglobin was determined using a microplate reader (Well Reader-Elisa Reader R-Biopharm, Germany) at 450 nm. Maximum and minimum hemolysis was determined by mixing 100 µL of dH_2O and HBSS to 100 µL of SRBC. Heat-inactivated serum was utilized as a control sample in each experiment. The lysis curve was estimated by creating a graph with Y/(1-Y) plotted versus the quantity of plasma added (mL). This graph was then plotted on a $\log_{10}$-$\log_{10}$ scale, and the level of hemolysis (Y) was measured. The volume of plasma required to yield 50% hemolysis (ACH_{50}) was used to determine the ACH_{50} units/mL in each group.

2.2.4.7 Total plasma protein (TPP)

The assay followed the Bradford's method (Bradford, 1976), with minor changes. Two microliters of serum and 798 µL of distilled water were mixed with 200 µL of Bradford reagent (Biorad, USA), homogenized, then stored for 15 minutes. The optical density was examined at 600 nm. The standard curve of bovine serum albumin (Merck, USA) at 0, 25, 50, 75, and 100 mg/mL were used to calculate the TPP values.

2.2.5 Expression of immune genes

As described in the package instructions, mRNA was isolated from blood using a TRIzol® reagent (Thermo Fisher Scientific, USA). Fifty microliters of blood were used in mRNA extraction, then 500 µl TRIzol® reagent was added following the instructions. The purity and concentration of the extracted mRNA were checked using 260/280 nm readings from a nanodrop spectrophotometer (Thermo Fisher Scientific, USA). Additionally, mRNA was validated using 1% agarose

Table 1. qPCR primer of immune genes of red tilapia *Oreochromis* sp.

Immune genes	Primer sequence forward (5′–3′)	Primer sequence reverse (5′–3′)	Temp (°C)	Ref.
TNF-α	GAGGTCGGCGTGCCAAGA	TGGTTTCCGTCCACAGCGT	58.5	(Yilmaz, 2019)
IL-1β	TGCTGAGCACAGAATTCCAG	GCTGTGGAGAAGAACCAAGC	57	(Yilmaz, 2019)
IFN-γ	TGACCACATCGTTCAGAGCA	GGCGACCTTTAGCCTTTGT	60.5	(Yilmaz, 2019)
TGF-β1	TTGGGACTTGTGCTCTAT	AGTTCTGCTGGGATGTTT	57	(Feng *et al*., 2017b)
IL-10	CCCTGA AGAGAGATGTCA	GTGTCGTTTAGAAGCCAG	57	(Ting *et al*., 2018)
GPx	CCAAGAGAACTGCAAGAACGA	CAGGACACGTCATTCCTACAC	61.5	(Yilmaz, 2019)
Hepcidin	AGCAGGAGCAGGATGAGC	GCCAGGGGATTTGTTTGT	56	(Feng *et al*., 2017b)
β-actin (internal control)	CAGCAAGCAGGAGTACGATGAG	TGTGTGGTGTGTGGTTGTTTTG	61.5	(Salah *et al*., 2017)

gel electrophoresis. cDNA was synthesized from the mRNA by using the SensiFAST cDNA Synthesis Kit (Bioline, USA). A SensiFAST SYBR No-ROX Kit (Bioline, USA) was used to analyze the quantitative real-time PCR (RT-qPCR) utilizing the designated primers (Table 1). Pre-denaturation at 95°C (1 minute); 40 cycles of denaturation at 95°C (15 s), annealing at 60–65°C (15 s), and extension at 72°C (10 s); following melting curve at 95°C for 30 minutes were used in an RT-qPCR thermocycler (Biorad, USA) analysis. Moreover, the amplified value was analyzed using comparative methods ($2^{-\Delta\Delta CT}$) (Livak and Schmittgen, 2001) and standardized using β-actin (internal control) to evaluate the level of gene expression.

2.2.6 Statistical analyses

Statistical tests were carried out using SPSS 18.0 (SPSS Inc., USA), which included ANOVA followed by DMRT, to identify significant differences between treatment groups. The means and standard deviations were used to present the data.

3. Result and Discussion

3.1 Analysis of FTIR spectra

The spectra of SA standard (Sigma) (A) and SA from *P. australis* (B) were evaluated, and a specific alginate wavenumber was positively fingerprinted at 950–750 cm^{-1} (Yudiati and Isnansetyo, 2017) (Figure 1). The results showed the characteristic wavenumber for hydroxyl, ether, and carboxylate groups. The hydroxyl O–H bonds were found at 3435 and 3443 cm^{-1}, while the hydrocarbon C–H bonds were discovered at 2360 and 2284 cm^{-1}. The bands at 1611 and 1648 cm^{-1} were attributed to asymmetric carboxylate O–C–O salt ion, while C–OH bonds with contribution of symmetric carboxylate O–C–O were found at 1418 and 1411 cm^{-1} (Silverstein *et al*., 2014). The weak peak at 1302 and 1096 cm^{-1} has been attributed to C–O/C–C of pyranose formations and C–C–H/O–C–H. The peak at 1032 cm^{-1} might be attributed to uronic acid residues C–O. In the fingerprint area, uronic acid residues C–O and β-mannuronic acid residues C1–H were found at 949 and 890 cm^{-1}. The bands at 820 and 849 cm^{-1} may also be due to β-mannuronic acid residues C1–H (Leal *et al*., 2008; Silverstein *et al*., 2014).

The composition and structural properties of brown seaweed polysaccharides vary according to species, season, latitude, and ecological habitat (Menshova *et al*., 2012). Environmental factors such as water temperature, nutrient salts, salinity, waves, ocean currents, and depth of immersion can affect their structure and biological activity (Hurd *et al*., 2014). This also influences the yields of alginate in *P. australis* from Awur Bay, Jepara, Indonesia yielded 21% SA, with slightly different results from other scientists (Susanto *et al*., 2001; Mushollaeni, 2011). This study found that FTIR spectrum of SA from *P. australis* was positively fingerprinted at 949 - 849 cm^{-1} and conformed to the SA standard (Sigma) fingerprint at 949 - 820 cm^{-1}. The asymmetric carboxylate group at 1648 cm^{-1} was associated with sodium ion of alginate from *P. australis*, while for SA standard (Sigma) at 1611 cm^{-1}. This finding might indicate a difference in the way the typical polymeric chain interacts with sodium ion. In addition, SA from *P. australis* exhibited certain distinct bands, particularly in the fingerprint region, probably because of the absence of β-mannuronic acid residues.

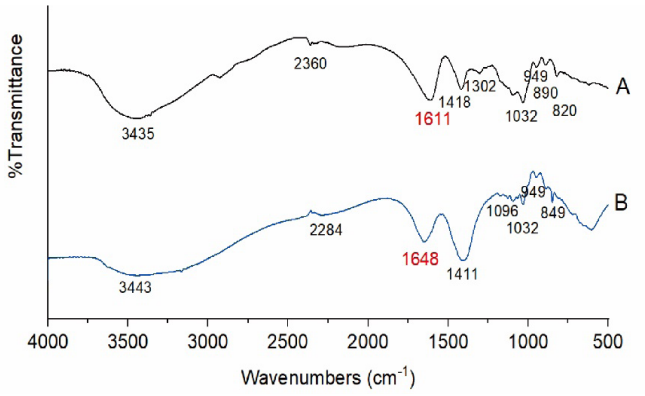

Figure 1. FTIR spectra of (A) standard (Sigma) and (B) SA from *Padina australis*.

3.2 Immune responses of Oreochromis sp. with dietary SA

Growth factors or colony-stimulating factors (CSF) regulates the increase of the number of leukocytes in response to infection. These factors affect the granulocyte-monocyte (GM) line (as well as the erythrocyte and megakaryotic lines) in which hematopoietic stem cells differentiate. GM-CSF, G-CSF, and M-CSF are three types of cytokines. GM-CSF stimulates the proliferation rate and activity of granulocyte-macrophage precursors, G-CSF improves granulocyte maturation and quantity, and M-CSF increases the activity of monocytes and macrophages, respectively (Playfair and Chain, 2009). The present study showed the TLC increased with the increase of SA dosage. On days 6 and 12, supplementary diet at 2.0, 3.0, and 4.0 g/kg enhanced ($P < 0.05$) the TLC, but on days 3 and 9 no significant differences ($P >$

0.05) were found. Relative to other treatments, dietary 2.0 g/kg SA was the most effective dose to improve the TLC (Figure 2a). The results indicated an increase in leucocyte proliferation and differentiation. Leukocyte is a vital cellular component of the innate immune defense against diverse invading pathogens; thus, the results became quite noteworthy. Monocytes and neutrophils differentiate into macrophages and polymorphonuclear (PMN), meanwhile granulocytes differentiate into neutrophils, eosinophils, and basophils, respectively. The three primary phagocytes involved in phagocytosis are monocytes, macrophages, and neutrophils (Abbas *et al*., 2007; Playfair and Chain, 2009). Similarly, dietary SA at ≥5 g/kg feed increased the TLC in *E. fuscoguttatus* (Cheng *et al*., 2008). Another study on shrimp found that oral administration of three types of *S. siliquosum* alginate enhanced the total hemocyte count in *L. vannamei* (Yudiati *et al*., 2016). The significant improvement of the TLC in this experiment demonstrated the effectiveness of alginate supplementation in modulating non-specific immune responses of red tilapia.

Phagocytosis is a fundamental fish defense mechanism to eliminate invading particles and introduce them into specific cells that stimulate the production of immunoglobulins. Phagocytes and certain cells can perform phagocytosis by utilizing membrane receptors to identify foreign particles (Neumann *et al*., 2001). As a polysaccharide, the molecular pattern of SA conforms to the structure of pathogenic organisms (PAMP), such as lipopolysaccharides (LPS) in bacterial cell walls. Therefore, we assumed that alginate could be recognized by its receptors (PRR), thereby enhancing antimicrobial responses. It is known that receptors especially TLR4, CD14, and MD-2 recognize LPS (Medzhitov and Janeway, 2000). Surface receptors and phagosomes also recognize additional molecular structures in bacterial membranes. Macrophages and PMN express pathogen-binding surface receptors such as lectin, mannose, and C3 receptors. The pathogen-receptors linkage activates intracellular killing and digesting, as well as the secretion of chemokines and inflammatory cytokines (Playfair and Chain, 2009). This study reported that the PA increased significantly ($P < 0.05$) on days 3 (except for the treatment group of 4.0 g/kg), 6, and 12. On day 9, there was no noticeable difference in PA ($P > 0.05$) between the treatment groups. After the trial, dietary SA at 2.0 g/kg feed was the lowest dose to effectively increase the PA (Figure 2b). Throughout the experiment, no significant variations ($P > 0.05$) in the PI were found (Figure 2c). Phagocytosis begins with the movement of monocytes, neutrophils, and serum components from the blood vessels into the infected tissue

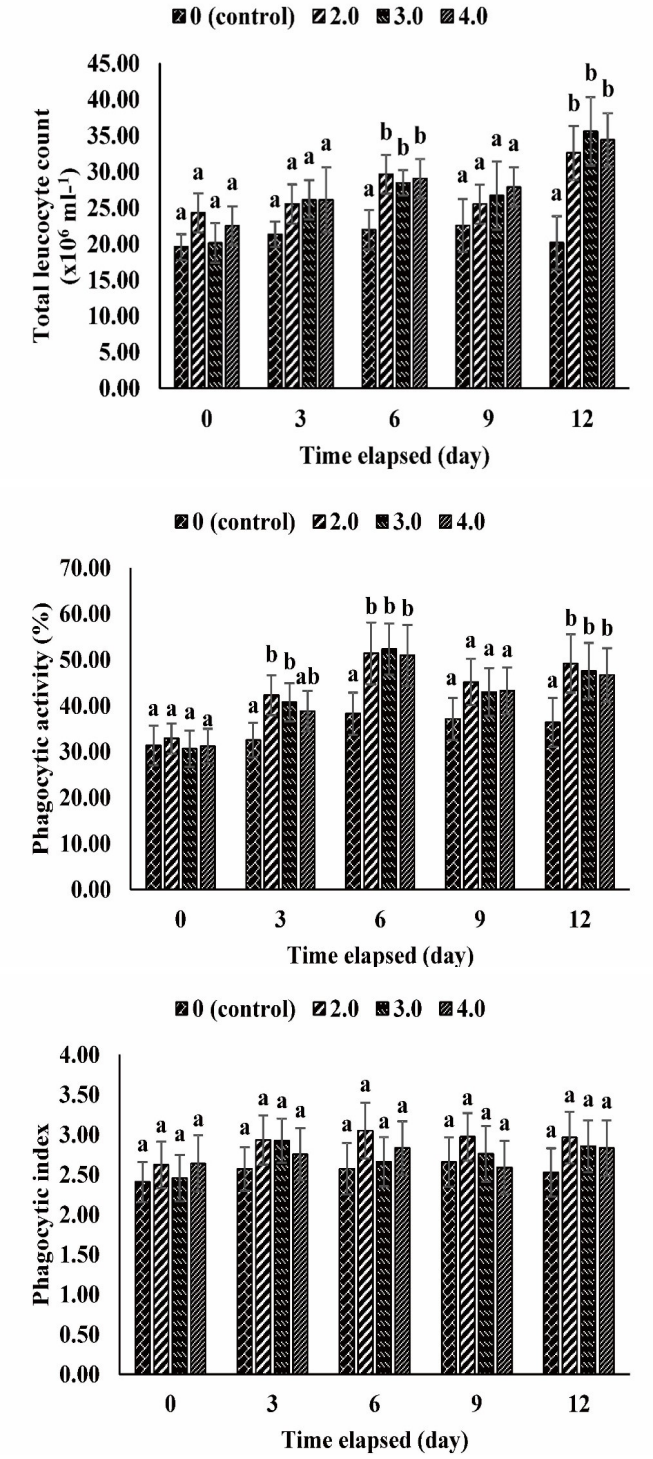

Figure 2. (a) Total leucocyte count (TLC), (b) phagocytic activity (PA), and (c) phagocytic index (PI) of red tilapia *Oreochromis* sp. given diet with SA from *Padina* sp. at doses of 0 (control), 2.0, 3.0, and 4.0 g/kg for 0, 3, 6, 9, and 12 days. Different superscript letters indicate statistically significant values ($P < 0.05$).

in response to inflammation. Chemokines mediate chemotaxis and traffic regulation of phagocytes. Monocytes and neutrophils activated into macrophages and PMN, respectively. Macrophages enhance pathogens adhesion

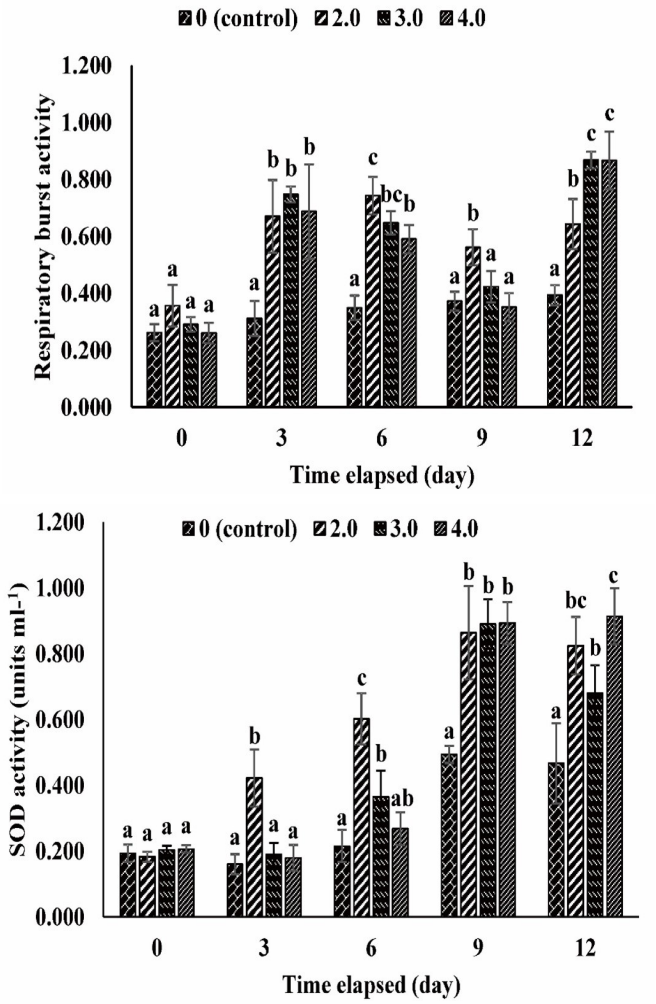

Figure 3. (a) Respiratory burst and (b) SOD activity of red tilapia *Oreochromis* sp. given diet with SA from *Padina* sp. at doses of 0 (control), 2.0, 3.0, and 4.0 g/kg for 0, 3, 6, 9, and 12 days. Different superscript letters indicate statistically significant values ($P < 0.05$).

with the assist of complement through opsonization. Phagosomes ingest the pathogens, then fuse with lysosome to form phagolysosome (Abbas *et al*., 2007; Playfair and Chain, 2009). During phagocytosis, bacterial killing occurs inside the phagolysosome, involving superoxide radicals, lysozyme, and antioxidant enzymes in a process known as respiratory burst. In addition, macrophages release inflammatory cytokines such as TNF-α, IL-1, and IL-6 (Playfair and Chain, 2009), eliminate damaged tissue and initiate tissue healing processes (Abbas *et al*., 2007). In addition, the increment of PA was reported on dietary LMWSA (Low Molecular Weight of Sodium Alginate) in *O. niloticus* (Doan *et al*., 2016); and dietary kefir and LMWSA in *O. niloticus* (Doan *et al*., 2017). The significant effect of dietary SA on PA in this study indicated that red tilapia has an effective mechanism for destroying invasive pathogens and protecting against infectious diseases after the application of SA.

Through NADPH oxidase, phagocytes increase their oxygen consumption and produce reactive oxygen species (ROS) during respiratory burst. ROS play an important role in killing invasive pathogen, while causing serious damage to host cells (lipids, proteins, carbohydrates, and nucleotides). ROS is highly toxic to a broad number of microorganisms, but it is also essential in digestive enzymes release in phagolysosomes, such as elastase and cathepsin (Playfair and Chain, 2009). Arginine catalyzed into a microbicide, nitric oxide (NO), through inducible NO synthase (iNOS). In addition, lysosomal proteases degrade microbial proteins. Lysosomes and phagolysosomes produce these three microbicidal substances to kill ingested microbes without harming phagocytes (Abbas *et al*., 2007). Antioxidant enzymes, such as SOD, GPT, GPx, and CAT, have been known to promote ROS scavenging, control spatial location, and restrict production levels to avoid the oxidative damage (Tassanakajon *et al*., 2013). This study showed that dietary SA at 2.0, 3.0, and 4.0 g/kg enhanced ($P < 0.05$) respiratory burst activities on days 3, 6, and 12. However, only SA diet at 2.0 g/kg feed increased ($P < 0.05$) respiratory burst activity on day 9 (Figure 3a). Furthermore, we found that dietary SA at 2.0, 3.0, and 4.0 g/kg induced ($P < 0.05$) the SOD levels on days 6 (except for supplementary diet at 4.0 g/kg), 9, and 12. Meanwhile, on day 3, only SA diet at 2.0 g/kg feed consistently improved ($P < 0.05$) the SOD level (Figure 3b). The SA diet at 2.0 g/kg feed was generally the most effective dose to enhance respiratory burst activity and SOD level. Increased respiratory burst activity and PA indicated higher ROS production, whereas improved SOD activity prevented oxidative damage by suppressing excessive ROS. We presumed that red tilapia fed with SA diet stimulated the innate immune system, particularly in the process of eliminating invasive pathogens through intracellular killing mechanisms. Another study reported that dietary LMWSA (prebiotic) or combined with kefir improved respiratory burst in *O. niloticus* (Doan *et al*., 2017, 2016); dietary LMWSA and *P. acidilactici* elevated the level of antioxidant enzymes (SOD, CAT, and GST) in the liver in juvenile Asian *L. calcarifer* (Ashouri *et al*., 2020).

Lysozyme (muramidase) is an innate immune defense molecule produced by phagocytes during an oxygen-independent response (Ellis, 1990; Playfair and Chain, 2009; Doan *et al*., 2016). It enhances phagocytosis against Gram-negative bacteria through direct activation of polymorphonuclear leucocytes and macrophages or indirectly by opsonic effects of the complement system. Meanwhile, it leads to the destruction of the peptidoglycan outer membrane of Gram-positive

bacteria by rupturing the β (1–4) links between N-acetylglucosamine and N-acetylmuramic acid (Saurabh and Sahoo, 2008). Certain bacteria can directly trigger lysozyme activation and cause bacterial lysis. Lysozyme, along with other digestive enzymes, contributes to the destruction of ingested microbes in phagolysosomes (Playfair and Chain, 2009). Tilapia *O. niloticus* given singular LMWSA or combined with kefir shows an increase in lysozyme activity (Doan *et al*., 2017, 2016). Asian *L. calcalifer* (Ashouri *et al*., 2018) received dietary SA elevated lysozyme activity. The present study indicated that SA supplementation at 2.0, 3.0, and 4.0 g/kg consistently enhanced ($P < 0.05$) serum lysozyme activity on days 3 and 6. The significant increments ($P < 0.05$) in lysozyme activity were also observed at 2.0 and 3.0 g/kg on day 9, as well as 2.0 g/kg on day 12, respectively (Figure 4a). The results showed that dietary SA in red tilapia, directly and indirectly, modulates innate immunity against a broad range of pathogens.

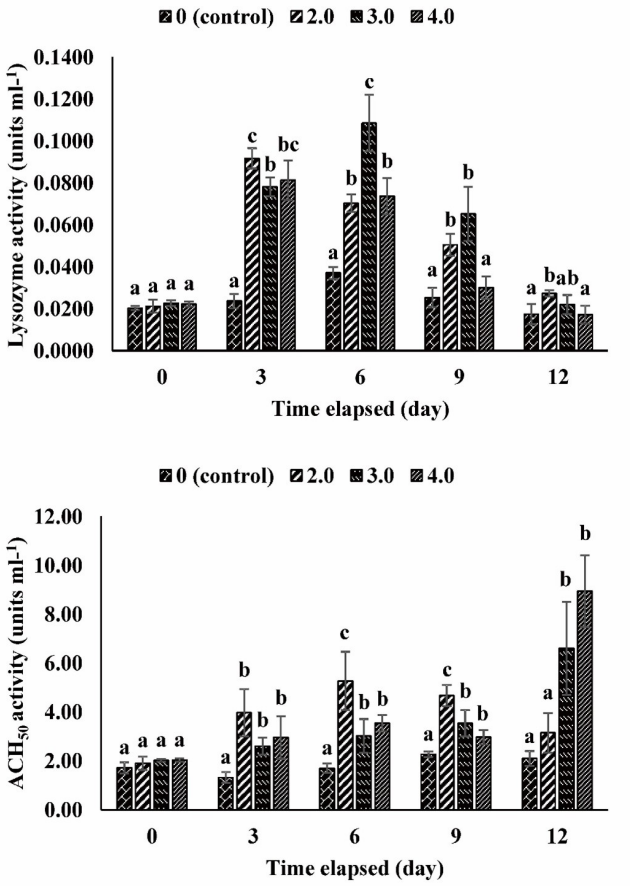

Figure 4. (a) Lysozyme and (b) ACH_{50} activity of red tilapia *Oreochromis* sp. given diet with SA from *Padina* sp. at doses of 0 (control), 2.0, 3.0, and 4.0 g/kg for 0, 3, 6, 9, and 12 days. Different superscript letters indicate statistically significant values ($P < 0.05$).

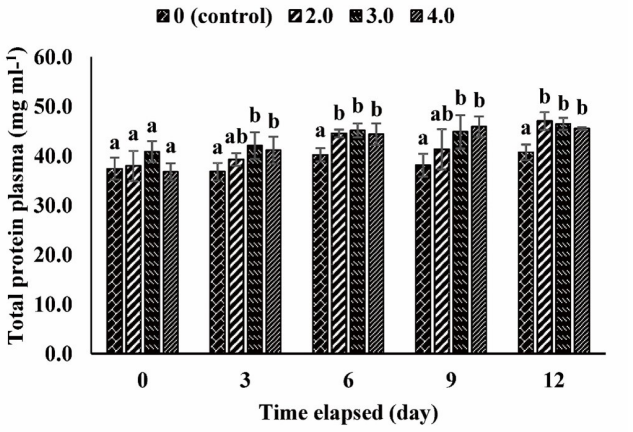

Figure 5. TPP of red tilapia *Oreochromis* sp. given diet with SA from *Padina* sp. at doses of 0 (control), 2.0, 3.0, and 4.0 g/kg for 0, 3, 6, 9, and 12 days. Different superscript letters indicate statistically significant values ($P < 0.05$).

Complement is essential in several aspects of innate immunity, including immunocytic activation, phagocytosis, chemotaxis, inflammation, and opsonization (Ashouri *et al*., 2018). In fish, the alternative complement (ACH_{50}) pathway is the most important complement system mechanism (Doan *et al*., 2016). Complement is directly activated by the surface of bacterial cells (such as LPS) and their products, or by complement receptors (CR), including mannan-binding proteins (MBP) and C reactive proteins (CRP), which promote bacterial lysis, known as alternative pathways. Classical pathway of complement activation mediated by its interaction with antibodies, IgG and IgM. In the absence of antibodies, the linkage between microbial polysaccharides and circulating lectins, such as the mannan-binding lectin (MBL), activates the lectin pathway (Abbas *et al*., 2007; Playfair and Chain, 2009). Compared to the control group, we discovered that dietary SA at 2.0, 3.0, and 4.0 g/kg enhanced ($P < 0.05$) ACH_{50} activity on days 3, 6, and 9. On day 12, dietary SA at 3.0 and 4.0 g/kg SA boosted ($P < 0.05$) ACH_{50} activity, otherwise there was no significant impact ($P > 0.05$) of SA diet at 2.0 g/kg feed (Figure 4b). This finding was consistent with other investigations of dietary SA in *O. niloticus* (Doan *et al*., 2017, 2016) and Asian *L. calcalifer* (Ashouri *et al*., 2018). Complement promotes opsonization and phagocytes activation, inflammation, and cell lysis (Abbas *et al*., 2007). Complement opsonizes the microbes, then presents it to phagocytes and promotes phagocytosis. Certain proteolytic fragments of complement, specifically C5a and C3a, are inflammatory anaphylatoxins. Membrane attack complex (MAC) formation in microbial cells impairs membrane permeability and leads to osmotic lysis or apoptosis. Similar to bacteria, alginate (polysaccharide) has the ability to activate complement and trigger an immunological response. This study reported that dietary SA in red tilapia

boosted complement activity. The results revealed that SA diet triggered an innate immune mechanism to protect fish from a diverse number of invading pathogens, including bacteria, fungus, viruses, and parasites.

TPP is a common diagnosis indicator of fish health, stress, and nutritional status. In particular, globulins correlate with blood proteins that are important for organism's defenses, including lysozyme, immunoglobulins, complement, cytokines, acute-phase proteins, transferrin, and lectins (Magnadóttir, 2006). Increased TPP, albumin, and immunoglobulin levels in fish are associated with a strong innate response (Alexander *et al*., 2011). This study showed that SA diet at 2.0, 3.0, and 4.0 g/kg elevated ($P < 0.05$) TPP at days 6 and 12. Meanwhile, dietary SA at 3.0 and 4.0 g/kg enhanced ($P < 0.05$) TPP at days 3 and 9. The supplementary SA at 2.0 g/kg feed indicated the lowest dose, which could effectively elevate the TPP level (Figure 5). In line with this study, dietary administration of three types of alginates in *L. vannamei* could increase TPP (Yudiati *et al*., 2016). Additionally, information regarding the impact of dietary SA related to TPP in red tilapia is still lacking.

3.3 Expression of immune genes

We used individual replication of blood samples to evaluate the immune genes expression. Immune parameters test revealed that SA from *P. australis* significantly increased the immune response as early as the 6th day of feeding trial. Therefore, the blood taken at 6th day of the feeding trial was evaluated for immune genes expression (Figure 6).

The inflammatory response to various challenges is primarily regulated by cytokines (Alvarez-Pellitero, 2008). Cytokines were reported to be regulated by nuclear factor κB (NF-κB) in rainbow trout *O. mykiss* (Mariano *et al*., 2013) and target of rapamycin (TOR) in human (Weichhart *et al*., 2008), similarly in other fish (Xu *et al*., 2016). It was observed that IKK complex promoted IκBα *de*struction, which led to the activation of NF-*κB* (Dolcet *et al*., 2005) and elevated the level of pro-inflammatory cytokines genes in human (Shakibaei *et al*., 2007). TOR activated S6K1 and inhibited 4E-BP expressions to enhance the level of anti-inflammatory cytokines in mice (Hay and Sonenberg, 2004). Pro-inflammatory cytokines genes initiate and accelerate the inflammation by activating lymphocytes, macrophages, and natural killer cells, leading to increase the respiratory burst activity, nitric oxide production, and phagocytosis. Anti-inflammatory cytokines genes suppress the production of pro-inflammatory cytokines, thereby preventing the over reaction of inflammatory responses in fish (Jiang *et al*., 2017). We found that dietary SA at 2.0 and 3.0 g/kg enhanced ($P < 0.05$) TNF-α gene expression, while IFN-γ gene transcription increased ($P < 0.05$) at 2.0 g/kg. By contrast, dietary SA at ≥3.0 g/kg decreased ($P < 0.05$) IL-1β gene expression. IL-10 and TGF-β1 gene expression decreased ($P < 0.05$) at ≥2.0 and ≥3.0 g/kg. The results demonstrated that dietary

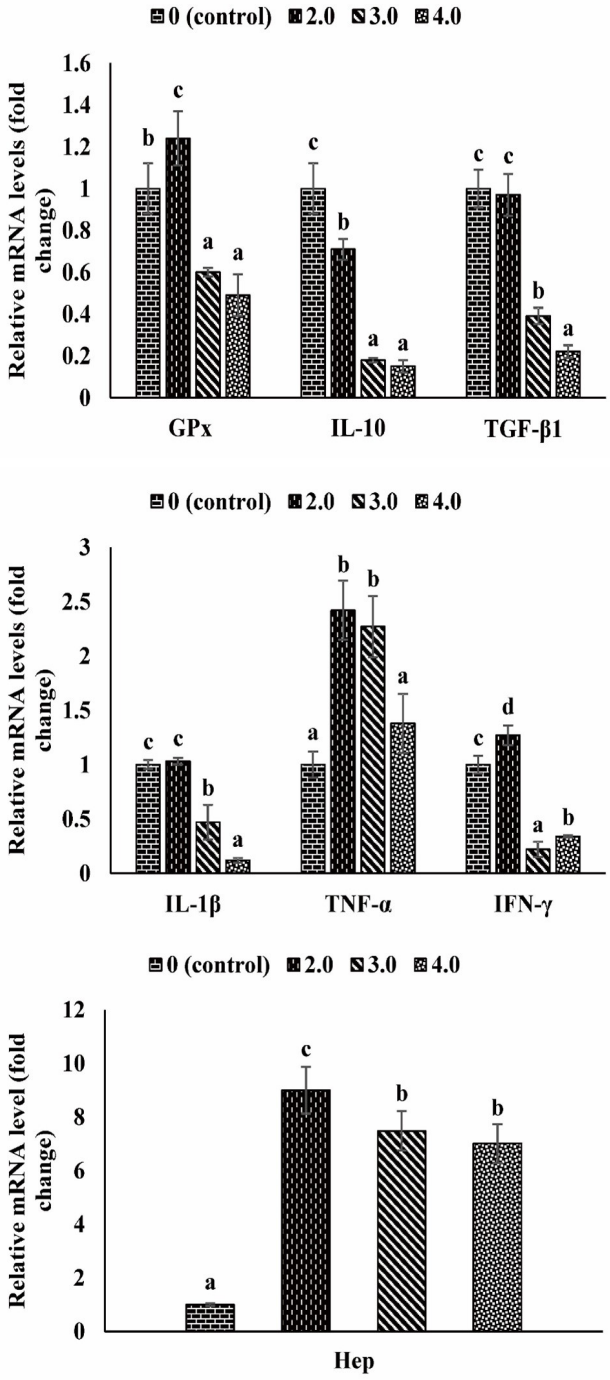

Figure 6. Relative mRNA transcript levels of red tilapia *Oreochromis* sp. given diet with SA from *Padina* sp. at doses of 0 (control), 2.0, 3.0, and 4.0 g/kg on day 6. Different superscript letters indicate statistically significant values ($P < 0.05$)

SA induced inflammation, as indicated by the increased transcription of pro-inflammatory cytokines genes (TNF-α and IFN-γ, except for IL-1β) and the decreased expression of anti-inflammatory cytokines genes (IL-10 and TGF-β1).

Apart from inflammation, the cellular integrity can be harmed by apoptosis and oxidative stress because of excessive ROS (Jiang *et al*., 2017). Thus, fish develop antioxidant defense consisting of antioxidant and antioxidant enzymes (Feng *et al*., 2017a). The expression of antioxidant enzymes was positively correlated with the Nrf2 mRNA levels, and conversely negatively correlated with Keap1a mRNA levels (Song *et al*., 2017; Wu *et al*., 2022). The present study reported that dietary SA at 2.0 g/kg improved ($P < 0.05$) GPx gene expression. This finding was relevant to the improvement of SOD activity, which may be correlated with the increment of respiratory burst and phagocytosis activities. The enhancement of antioxidant-related genes and antioxidant enzymes activities may adjust its immune-stimulatory ability to control excessive ROS production.

Antibacterial substances, such as antimicrobial peptides, lysozyme, and complement, as well as cytokines are essential for immune function (Chen *et al*., 2012). Antimicrobial peptides (AMPs) are important in regulating the immunological response to pathogens. Hepcidin gene (previously known as LEAP-1) is a small and cationic cysteine-rich AMP, containing approximately 21–28 amino acids, and it has been linked to iron regulation and antimicrobial response in the innate immunity (Barnes *et al*., 2011). This study revealed that dietary SA at ≥2.0 g/kg improved ($P < 0.05$) Hep gene expression, leading to its function in iron regulation and antimicrobial activity.

In addition, information regarding the effect of dietary SA on the genes expression in red tilapia may still be insufficient. Another study has reported that dietary *Agaricus bisporus* polysaccharides (ABPs) enhanced IFN-γ2, TNFα, IL-1β, NF-κB p65, IL-6, IL-10, hepcidin, β-defensin, LEAP-2A, and LEAP-2B mRNA expression in grass carp *C. Idella* (Harikrishnan *et al*., 2021b). β-1,3-glucan and ascorbic diet increased Nrf2, Cu–Zn SOD, Mn SOD, GSH-Px, and CAT genes expression, as well as decreased Keap gene expression after transport simulation in live tiger grouper (Wu *et al*., 2022). Ulvan diet induced expression of Nrf2, SOD, GPx, IL-1β, TNFα, hepcidin, Lyz, IgM, and β-2M genes in *L. rohita* against columnaris disease (Harikrishnan *et al*., 2021a). Additionally, we found that the expression of immune genes decreased gradually with the escalation of dietary SA levels. This finding might indicate that dietary SA in red tilapia at 2.0 g/kg was the most effective dose in regulating immune genes expression. We also found that certain parameters were significantly improved at days 3 and 6, not at days 9 and 12. We assumed that innate immune system was instantly triggered in response to various pathogens or immunostimulants.

4. Conclusion

In summary, our study confirmed that SA dietary boosted the innate immune system in red tilapia. Briefly: dietary SA significantly improved TLC, PA, respiratory burst, SOD, lysozyme, and ACH_{50} activity, but PI and TPP were not significantly different; mRNA levels of antioxidant-related gene (GPx), pro-inflammatory cytokines (TNF-α and IFN-γ, except for IL-1β), and antimicrobial peptides (Hepcidin) were significantly upregulated, in contrary, anti-inflammatory cytokines (IL-10 and TGF-β1) were significantly downregulated. The SA supplementation of brown alga, *P. australis*, native to the Indonesian coast has been proven to enhance the innate immunity and immune-related gene expression. These findings provided important information for future investigations and promoted the use of alginate as an immunostimulant in fish disease prevention.

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Authors' Contributions

The contribution of each author is as follows, Toni Kuswoyo and Alim Isnansetyo; participated in conception, experimental design, and manuscript preparation. Toni Kuswoyo and Indah Istiqomah; contributed to conducting experiments, data collection and analysis. Murwantoko and Amir Husni; involved in supervising laboratory work, drafting the manuscript and revision. All authors have read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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