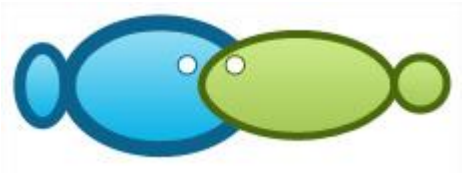

Improving resistance against *Aeromonas hydrophila* and growth performance by oral administration of fucoidan from *Padina boergesenii* Allender & Kraft, 1983 in catfish (*Clarias* sp.)

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Abstract. Fucoidan is a polysaccharide sulfate compound that can only be obtained from marine organisms such as brown seaweed and echinoderms, with the main structure of L-fucose. This research aimed to evaluate the non-specific immune responses, disease resistance, and growth of catfish (*Clarias* sp.) fed with a fucoidan-supplemented diet. Fucoidan was extracted from a tropical brown alga, *Padina boergesenii*, indigenous to the Jepara coast, Central Java, Indonesia. The result proved that dietary fucoidan significantly increased nonspecific immune responses such as phagocytic activity (PA), nitro blue tetrazolium (NBT) activity, superoxide dismutase (SOD) activity, serum antibacterial and agglutination activity ($p<0.05$). It did not change significantly the phagocytic index (PI) ($p>0.05$). The challenge test also clearly indicated that fucoidan improved the fish resistance against *Aeromonas hydrophila* infection by improving the survival rate (SR) ($p<0.05$). The growth of length (L) and weight (W) as well as its specific growth rate (SGR) were also significantly increased by fucoidan supplementation in the feed ($p<0.05$). The diet with fucoidan from *P. boergesenii* at 4000-6000 mg kg^{-1} effectively increased the non-specific immunity, resistance against bacterial infection, and growth of length and weight as well as SGR of the fish. These results suggest that fucoidan is a prospective substance that can not only improve growth performance but also enhance disease resistance in catfish.
Key Words: brown algae, challenge test, disease resistance, non-specific immune response.

Introduction. Fucoidan is a sulfated polysaccharide found in brown seaweeds, being a typical substance produced by marine organisms, brown algae, and echinoderms, but not by terrestrial organisms (Isnansetyo et al 2016). The macromolecule contains α-1,3-linked sulfated L-fucose as the main sugar unit and sulfate ester groups (Song et al 2012). Bioactivity and the structural features of fucoidan vary depending on seaweed species and extraction method (Ale et al 2011). It has anti-inflammatory, antiviral, anti-tumor, and antioxidative activities (Song et al 2012), and immunostimulating activity (Teruya et al 2010; Ramberg et al 2010; Isnansetyo et al 2016). Fucoidan is a heteropolysaccharide composed of several monosaccharides such as fucose, galactose, xylose, arabinose, glucose, etc., and contains a small amount of protein, aldonic acid, Na, K, Ca, and other metal ions (Anastyuk et al 2009).

Fucoidan extracted from brown algae, especially from *Sargassum* sp. has been widely used for immune research (Ale et al 2011). Injection of fucoidan from *S. cristaefolium* increases phagocytic activity (PA), total plasma protein (TPP), and leukocyte count in tilapia (*Oreochromis niloticus*) (Isnansetyo et al 2016), and increases hemocyte count, PA, PO, and superoxide anion (SOA) by oral administration in vannamei shrimp (*Litopenaeus vannamei*) (Setyawan et al 2018). Moreover, dietary fucoidan from *S. wightii* increases hemoglobin, white blood cells (WBCs), and red blood cell (RBCs) counts but

reduces superoxide dismutase (SOD) and catalase activities of *Labeo rohita* (Gora et al 2018). Additionally, fucoidan extracted from other brown algae (*Padina* sp.), showed potential selective cytotoxicity and is promising for the development of an anti-cancer compound (Isnansetyo et al 2017). Macro algae extracts containing fucoidan are reported to enhance performance in olive flounder (*Paralichthys olivaceus*) (Kim et al 2014) and the immune system in juvenile yellow catfish (*Tachysurus fulvidraco*) (Yang et al 2014). In addition to fucoidan, dietary alginate from *S. siliquosum* enhanced the innate immunity as well as the expression of immune-related genes in *L. vannamei* (Yudiati et al 2016).

Catfish (*Clarias* sp.) is one of the widely cultured freshwater fish, including in Indonesia. This species is highly susceptible to the pathogenic bacterium *Aeromonas hydrophila* causing motile *Aeromonas* septicemia (MAS) disease. MAS is a common freshwater bacterial disease that causes high mortality in infected fish. MAS is epizootic and the outbreak of the disease is widely spread in Southeast Asia. This disease is a serious and important disease known to affect juvenile and adult fish in the culture systems, acting as primary pathogens (Sivagnanavelmurugan et al 2014). Treatment with antibiotics has been abandoned for causing bacterial resistance, and the contamination of the environment and hosts. An available alternative to avoid the use of chemicals and prevent economic losses is the administration of immunostimulants, which act by increasing the innate immune system.

Although fucoidan has been studied for enhancing immunity, there has been no report of fucoidan from *Padina* increasing the immunity of catfish. Little research related to the oral administration of fucoidan (dietary) directly evaluating the resistance to infectious diseases was conducted. Our previous study in sub-adult catfish demonstrated that fucoidan from *P. boergesenii* improved the non-specific immune system (Purbomartono et al 2019). The purpose of this study was to evaluate the non-specific immune response, resistance, and growth performance of catfish after the administration of dietary fucoidan from *P. boergesenii*.

Material and Method

Isolation of fucoidan. A brown seaweed, *P. boergesenii* was obtained from Bandengan's coast, Jepara, Central Java, Indonesia. Identification of species *Padina* sp. was conducted based on morphological observation. Fucoidan was extracted by the method described by Kim et al (2007). Fresh brown seaweed was washed with fresh water and then air-dried and cut into approximately 0.5 cm pieces. A sample of 100 g was macerated in 1 L of 0.1 N HCl for 24 h at room temperature and filtered with a rough filter. The retentate was macerated in 1 L 0.2 N HCl for 2 h 70^{o}C, then filtered as in the first process. The collected filtrates were combined, then filtered through Whatman paper number 40, and evaporated using a rotary evaporator to obtain a final volume of 150 mL. Purification with ethanol precipitation was conducted. 3 x volumes of 95% of cold ethanol were added while stirring and allowed to stand for 2 h at room temperature. The mix was centrifuged at 8000g, at 4°C for 15 min. The precipitate obtained dissolved with equates which pH was arranged into 2 with the addition of HCl. HCl was added to the obtained precipitate until the pH became 2. $CaCl_2$ 2 M was added to the final concentration and recentrifuged (8000g*,* 4^{o}C for 15 min).

Experimental design. The experiment was conducted for 40 days to evaluate the nonspecific immune response and growth performance. Fish (6.5±0.5 cm) were administered 3 different doses of fucoidan with three replications. Experimental fish were reared in 12 rounded plastic tanks, each containing 15 fish for non-specific immunity and growth determinations. The challenge test used 12 fiberglass tanks 30x40 cm, each tank containing 15 fish. After 40 days of treatment, the blood of the fish was sampled, and the fish were measured for length and weight. Intraperitoneal injection with 10^4 cells mL^{-1} of *A. hydrophila* was conducted after 10 days of treatment for the challenge test and clinical signs were observed for 7 days.

Preparation of feed diets. The feed diet containing 4000 (T1), 6000 (T2), and 8000 (T3) mg fucoidan kg^{-1} feed was prepared by homogenizing with 0.1% Progol (PT INDOSCO, Surabaya, Indonesia) as a binder in 100 mL distilled water for 1 kg feed. The mixture was sprayed on the feed pellet (P.T. Central Proteina Prima, Sidoardjo, Indonesia). The control was feed pellet sprayed with 0.1% Progol only. The feed was air-dried and stored in a refrigerator until use.

Adaptation and feeding trial. The fish were acclimatized to laboratory conditions and to the treatment feed for 5 days. After being acclimatized, the fish were treated according to their respective dose. Feed was given twice a day at a 3% feeding rate.

Evaluation of non-specific immune response. A peripheral blood sample was collected on day 40 after treatment for non-specific immune parameters evaluations. The blood was collected from the caudal vein using a 1 mL syringe after wetting with 10% EDTA to evaluate non-specific immunity.

Phagocytic activity (PA) and phagocytic index (PI). 50 µL of bacterial suspension (10^8 cells mL^{-1}) was mixed with 50 µL leucocytes in a 96-well microplate and incubated for 30 min at room temperature. After incubation, 5 µL of each sample was smeared on object glass, added with ethanol 96%, allowed to air-dry, and stained with 0.15% safranin for 30 min. The PA and PI were determined by microscopic observation at 1000x magnification and calculated from 100 phagocytes on each slide.

Respiratory burst activity. Heparinized whole blood with a volume of 15 µL was put in a microtube and 15 µL of 0.2% nitro-blue tetrazolium NBT (Sigma) in 0.85% NaCl was added. The mix was incubated for 30 min at room temperature. 600 µL of N-N dimethylformamide (Merck) was added, then centrifuged at 3000 rpm for 5 min. The absorbance of the supernatant was measured by a spectrophotometer (UV mini-1240, Shimadzu) at 540 nm (Anderson & Siwicki 1994).

Superoxide dismutase (SOD) activity test. Superoxide dismutase (SOD) activity was measured according to Sarathi et al (2007) using NBT (Sigma) in 0.85% NaCl by addition of riboflavin. The sample was measured at 560 nm absorbance (UV mini-1240, Shimadzu), and the results were expressed as relative enzyme activity.

Serum antibacterial activity. An overnight *A. hydrophila* culture in a tryptic soy broth (TSB) medium (Merck) was counted and diluted to obtain a density of 10^6 cells mL^{-1}. 5 µL of bacterial suspension was added to 55 µL of serum sample and incubated at 25°C for 30 min. After incubation, 20 µL were collected and added into 180 µL of sterile and serially diluted 0.85% NaCl. The bacteria were counted on GSP (Merck) by the pour plate method and further incubated at 30°C for 24 h. The bacterial colony was counted and compared between treatments and control (Sritunyalucksana et al 1999).

Bacterial agglutination activity. 20 µL of serum sample was aliquoted in a 96-microwell, and serially diluted with the same volume of sterile phosphate buffer saline (PBS). *A. hydrophila* bacterial suspension at a density of 10^8 cells mL^{-1} with a volume of 20 µL was added to each well, and incubated at 25°C for 1 h. Agglutination was observed using a microscope (Sritunyalucksana et al 1999).

Challenge test. A challenge test of catfish was conducted using *A. hydrophila.* The pure isolate of *A. hydrophila* was obtained from the Laboratory of Fish Health Management, Department of Fisheries, Faculty of Agriculture, Universitas Gadjahmada. The bacterial isolate was grown in TSB medium (Merck) and incubated at 30°C, for 20 h. The bacterial density was estimated by a spectrophotometer (UV mini-1240, Shimadzu 165) at 630 nm using McFarland standard solution. The challenge test was conducted in 12 fiberglass tanks, 20x40 cm, each tank stocked with 15 fish. After feeding with the fucoidan-supplemented diet for 10 days, each fish was intraperitoneally injected with 0.1 mL of *A. hydrophila*

bacterial suspension at a density of 10^5 cells mL^{-1}. The observation of clinical signs and fish mortality was performed for 7 days after injection.

Growth performance. To evaluate the effect of fucoidan on the growth rate, the total length and weight of fish were measured on days 10, 20, 30, and 40 during the feeding trial. The parameters measured in this study are the survival rate (SR), mean time to death (MTD), cumulative survival rate (CSR), and relative percent survival (RPS). Length and weight gain (LG and WG), as well as the specific growth rate (SGR), were determined with the formula described by Fuchs et al (2015):

LG = Lt - Lo

Where: LG - length gain (cm); Lt - final length; Lo - initial length.
WG = Wt – Wo

Where: WG - weight gain (g); Wt - final weight; Wo - initial weight.

$$SGR = \frac{\ln Lt - \ln Lo}{t}$$

Where: SGR - specific growth rate (% body length day^{-1}); t - feeding time; Lt - final length (cm); Lo - initial length (cm).

$$SGR = \frac{\ln Wt - \ln Wo}{t}$$

Where: SGR - specific growth rate (% body weight day^{-1}); t - feeding time; Wt - final weight (g); Wo - initial weight (g).

Statistical analysis. The data were analyzed by one-way analysis of variance (ANOVA) with post hoc Duncan test using SPSS 21.0 (IBM).

Results. Challenge test by injecting *A. hydrophila* at a dose of 10^4 cells mL^{-1} showed that dietary fucoidan significantly increased ($p<0.05$) SR, MTD (Table 1), and CSR ($p<0.05$) (Figure 1). However, an increase in the fucoidan dose did not affect the RPS ($p>0.05$) (Table 1). After injection, diseased fish showed typical clinical signs of MAS, such as sluggishness, loss of appetite, upright down body position on the water surface, skin erosion, pale skin color, and erosion of the caudal fin.

Table 1

Survival rate (SR), relative percent survival (RPS), and mean time to death (MTD) of catfish (*Clarias* sp.) supplemented with fucoidan during 10 days and challenged with *Aeromonas hydrophila* 10^4 cell mL^{-1}

Treatment	*SR (%)*	*RPS (%)*	*MTD (day)*
Control	26.67 ± 6.67^{a}		1.35 ± 0.13^{b}
4000	42.22 ± 3.85^{b}	24.67 ± 4.62^{b}	1.00 ± 0.00^{a}
6000	44.44 ± 10.18^{b}	27.33 ± 13.20^{b}	1.22 ± 0.22^{ab}
8000	26.67 ± 0^{a}	4.00 ± 0.00^{a}	1.13 ± 0.06^{ab}

Note: different superscripts in the same column indicate significant differences ($p<0.05$).

Nonspecific immune parameters. Results indicated that dietary fucoidan from *P. boergesenii* significantly increased the PA, NBT, SOD, serum antibacterial, and bacterial agglutination activity of catfish ($p<0.05$) (Figures 2-6). However dietary fucoidan did not affect the PI ($p>0.05$). However, at doses of 6000 and 8000 mg kg^{-1} (2.21-2.28 bacteria $cell^{-1}$), it was higher than the control (1.79).

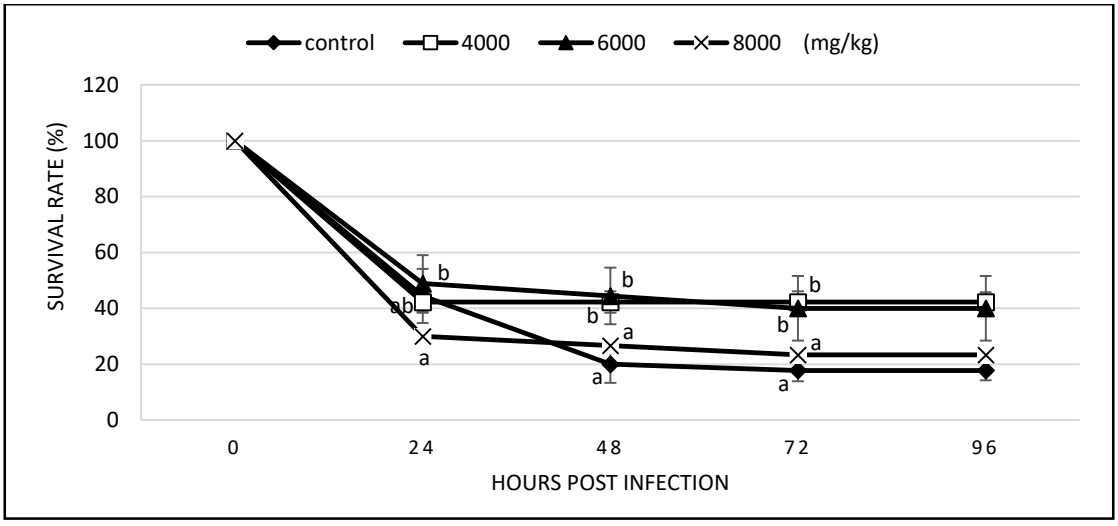

Figure 1. Cumulative survival rate (CSR) of catfish (*Clarias* sp.) fed a diet supplemented with fucoidan for 10 days and challenged with *A. hydrophila* 10^4 cells mL^{-1}.

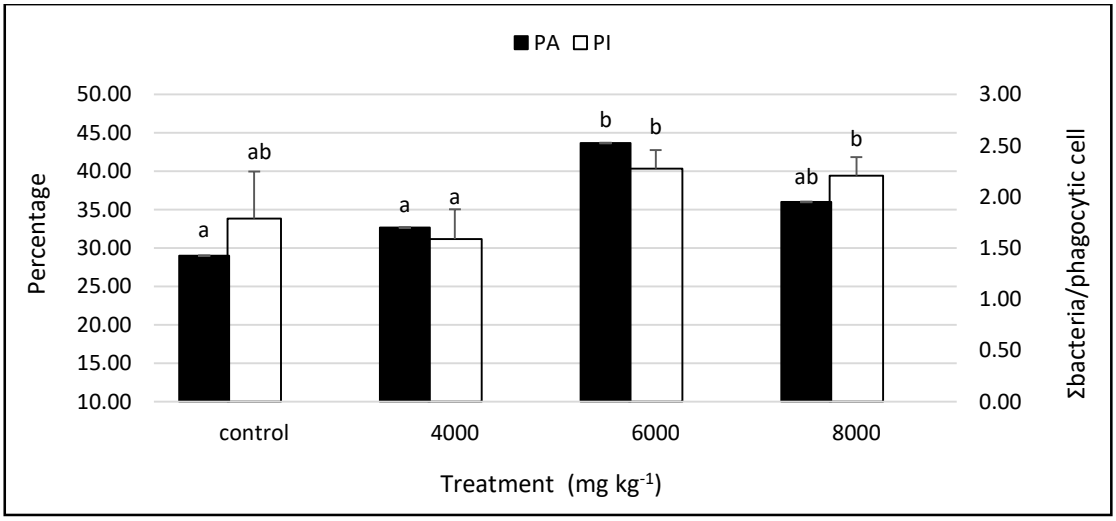

Figure 2. Phagocytic activity (PA) and phagocytic index (PI) of catfish (*Clarias* sp.) fed diets with fucoidan at various doses.

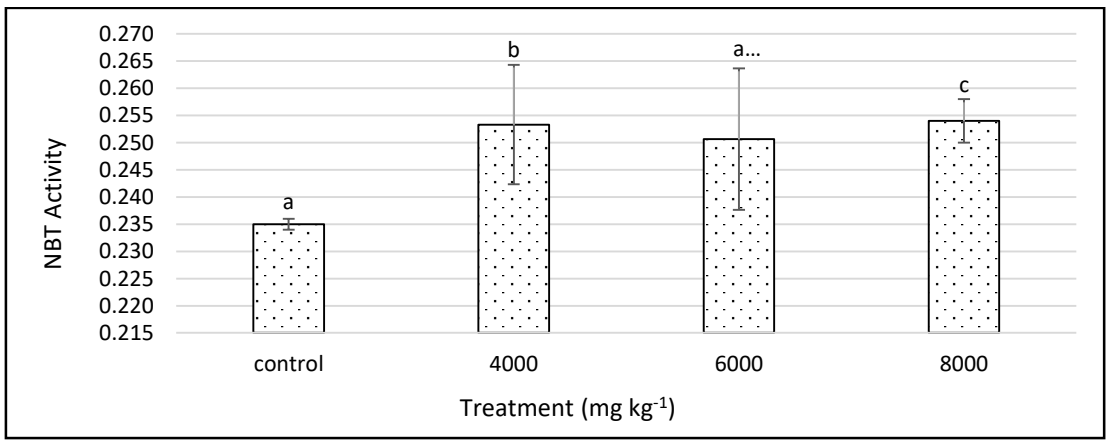

Figure 3. Nitro blue tetrazolium (NBT) activity of catfish (*Clarias* sp.) fed diets with fucoidan at various doses.

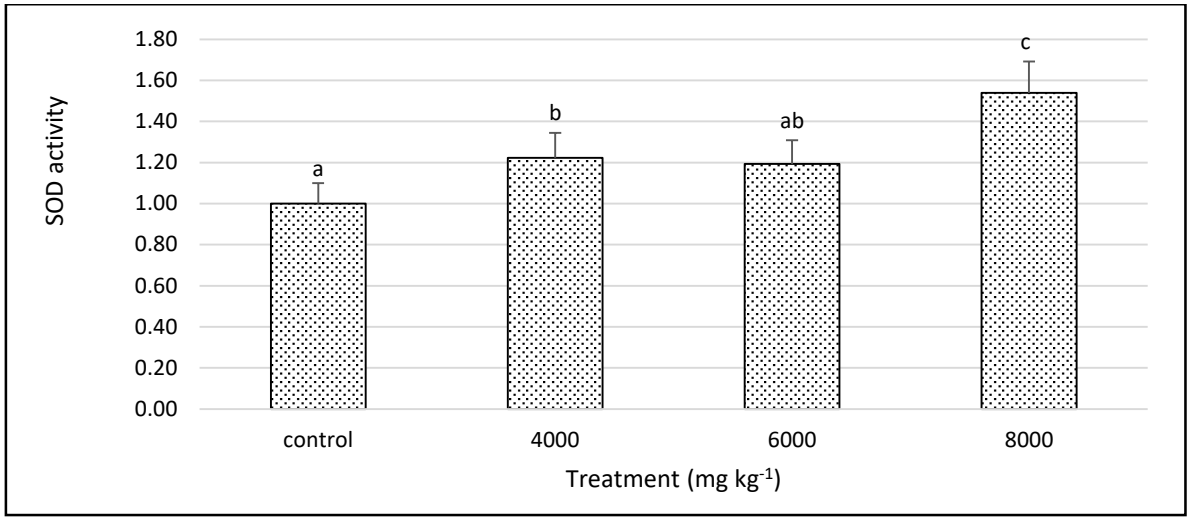

Figure 4. Superoxide dismutase (SOD) activity of catfish (*Clarias* sp.) fed diets with fucoidan at various doses.

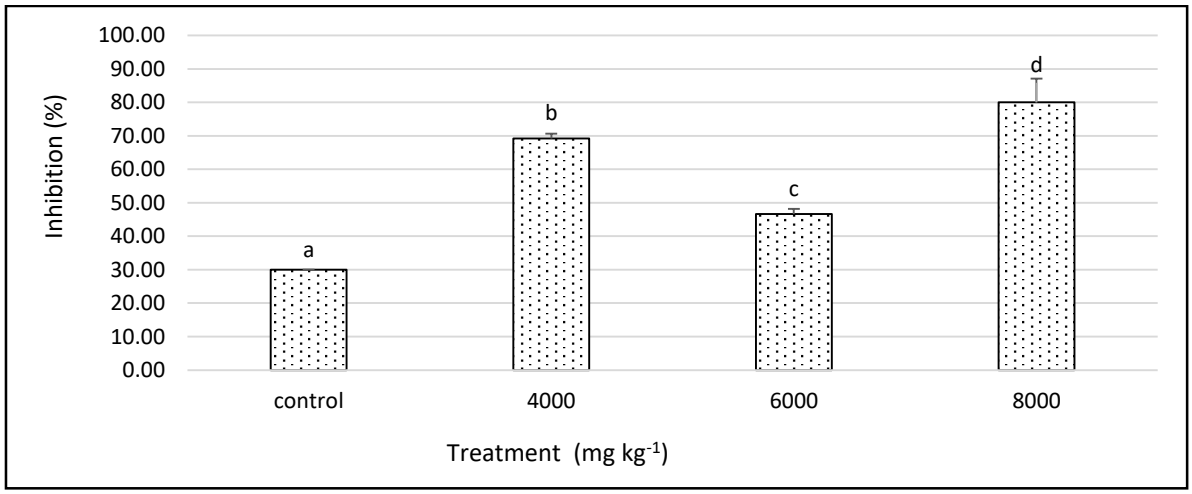

Figure 5. Serum antibacterial activity of catfish (*Clarias* sp.) fed diets with fucoidan at various doses.

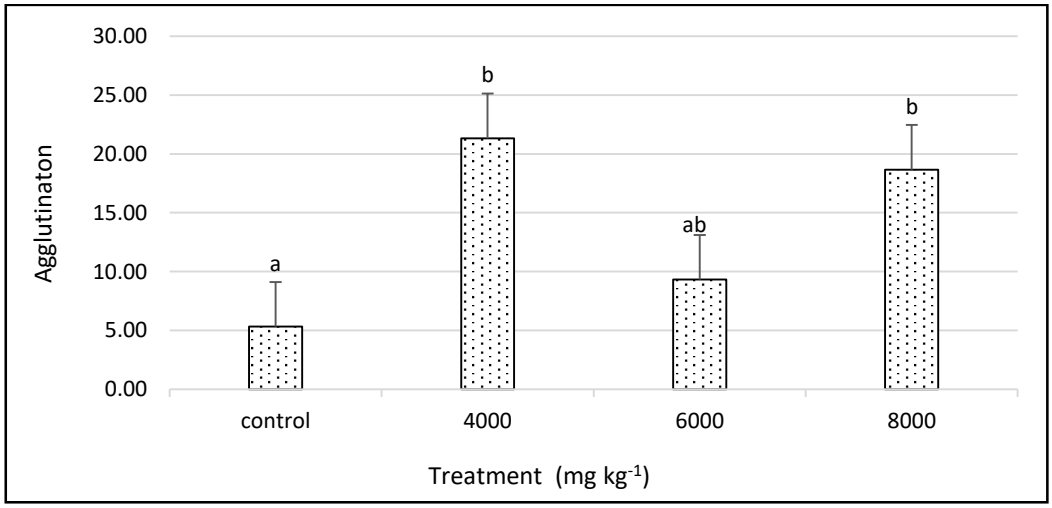

Figure 6. Bacterial agglutination activity of catfish (*Clarias* sp.) fed diets with fucoidan at various doses.

Growth performance. The results showed that dietary fucoidan for 40 days significantly increased the growth of length and weight, including the SGR of catfish ($p<0.05$) (Figures 7 and 8). During 40 days of experiments, the length gain and SGR in the control group were 3.48 cm and 1.07 cm, respectively. The treatment groups exhibited higher length gain and SGR ranging from 4.12 to 4.22 cm and from 1.2 to 1.24 cm, respectively. Similar results were also found for weight gain and SGR, ranging from 5.57 to 6.38 g and from 3.27 to 3.51 g, respectively.

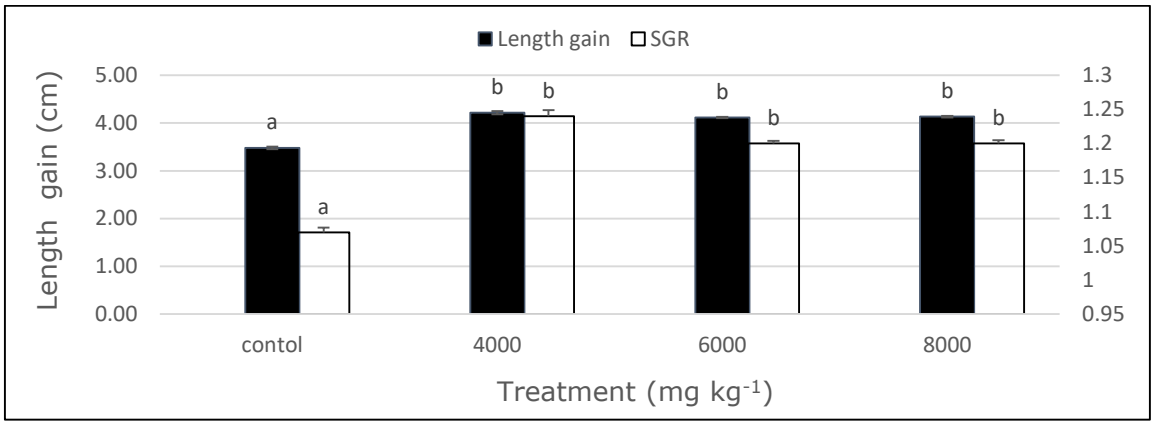

Figure 7. Length gained of catfish (*Clarias* sp.) fed diets with fucoidan at various doses for 40 days; SGR - specific growth rate.

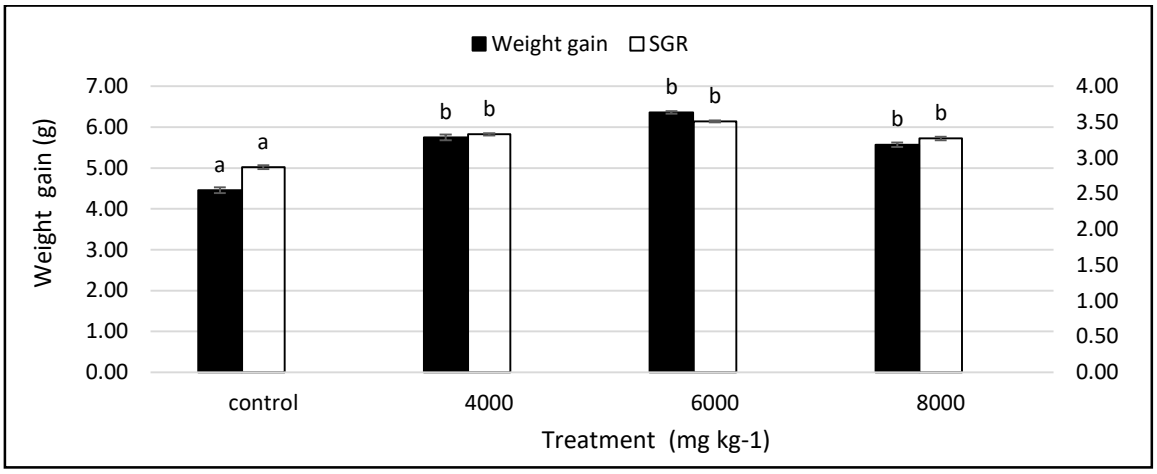

Figure 8. Weight gained of catfish (*Clarias* sp.) fed diets with fucoidan at various doses for 40 days; SGR - specific growth rate.

Discussion. During the 7-day period of the challenge test, the fish mortality was first observed at 24 h after the intraperitoneal injection. The catfish fed with dietary fucoidan showed higher disease resistance compared to fish from the control. After the 3rd day (72 h) post-challenge, there was no fish death until the end of the challenge test (7th day). Supplementation of fucoidan increased significantly SR and MTD (Table 1) at 4000 and 6000 mg kg^{-1}. These results support the results of El-Boshy et al (2014), who have reported that dietary fucoidan significantly decreases the cumulative mortality rate and increases the SR in catfish, *C. gariepinus*, after challenge with pathogenic *A. hydrophila*. Our study showed that from 24 h to 48 h, the percent cumulative survival rate of control decreased. At 48 h and further, until the end of the experiment (96 h), the CSR in all treatments was higher than in the control. A similar experiment on rainbow trout (*Oncorhynchus mykiss*) revealed that supplementation of futerpenol (containing fucoidan) decreases cumulative mortality and increases the SR and RPS post-infection with *Piscirickettsia salmonis* (Hernández et al 2016).

Observations of clinical signs during the 7-day period of the challenge test showed a change in behavior and morphological features. The infected fish were sluggish, lost appetite, and had upside-down body positions on the water surface. Morphologically, the abdomen was swollen, and there was desquamation and hemorrhages In the control group, the clinical symptoms were clearer than in the treatment groups, and the number of dead fish was higher (73.33%) compared to treatments, 57.78% at 4000 and 8000 mg fucoidan kg^{-1}, and 55.56% at 6000 mg fucoidan kg^{-1}. This shows that fucoidan can reduce the number of fish deaths and increase the SR. The results are similar to those of Rajendran et al (2016), with fucoidan from *Padina gymnosperm* significantly decreasing the mortality of *C. carpio* after being challenged with *A. hydrophila* and *Edwardsiella tarda*. Reduction in mortality can be caused by fucoidan, which can increase non-specific immune response and disease resistance (Kim et al 2014). Different reports are supported by Marudhupandi & Kumar (2013), who noted that fucoidan from brown algae showed potential antibacterial activity against various marine ornamental fish pathogens such as *A. hydrophila*. As an immune response modulator, fucoidan is used as a therapeutic agent for infectious diseases (Zhang et al 2015). In general, fucoidan is a sulfated polysaccharide composed of fucose and sulfate ester groups, capable of increasing immunity in yellow catfish, depending on the composition of the polysaccharide (Yang et al 2014).

Fucoidan from *P. boergesenii* significantly increased the PA but did not have significant effects on PI. Fucoidan stimulated the PA significantly at 6000 mg kg^{-1} (Figure 2). In this experiment, the increase in PA was followed by an increase in NBT activity. Fucoidan from brown algae is an activator of macrophages (Yang et al 2008), enhances respiratory burst activity, and can increase phagocytosis (El-Boshy et al 2014). Fucoidan from *Sargassum cristaefolium* intraperitoneally injected in tilapia increased phagocytic activity, Leucocrite, and total plasma protein (Isnansetyo et al 2016).

NBT (Figure 3) and SOD (Figure 4) activity significantly increased after supplementation with fucoidan from *P. boergesenii*. An increase in NBT activity indicates the existence of higher respiratory burst (RB) activity. Some research showed that the supplementation of brown seaweed affects RB activity. Significantly higher RB activity occurred in a fish diet supplemented with fucoidan (Kim et al 2014), including for parrot fish (*Scarrus javanicus*) fed a *Sargassum fusiforme*-containing diet (Song et al 2012). RB activity of the yellow catfish significantly increases if feeding diets with fucoidan (Yang et al 2014). The sulfate group in fucoidan is essential in binding macrophage cell surface receptors and plays a key role in the immune-regulating activity of macrophages (Teruya et al 2010). RB can increase oxygen consumption, so it can lead to the formation of superoxide anions (SOA). In this experiment, an increase in NBT was followed by increasing SOD activity. The relative increase of SOD is a response to the increase in NBT respiratory burst. Serum SOD is a common antioxidant enzyme that can protect the organism against damage by reactive oxygen species (ROS), which may lead to many disorders through attacking macromolecules (Seifried et al 2007). Fucoidan has a positive effect on the antioxidant capacity of the yellow catfish by increasing the antioxidant enzymes and subsequently decreasing ROS. SOD increased, decreasing ROS and reducing MDA contents in yellow catfish (Yang et al 2014).

All treatments with dietary fucoidan significantly increased the serum antibacterial activity of catfish (Figure 5). In this study, dietary fucoidan significantly increased NBT activity (Figure 3) and serum agglutination (Figure 6). The sulfate in fucoidan enhances NBT activity by phagocyte oxidative burst and induces serum bactericidal activity (Uribe et al 2011). Enhanced PA, RB activity, and nitric oxide may be observed in the fucoidan-treated groups (El-Boshy et al 2014) in African catfish (*Clarias gariepinus*). Bacterial agglutination for inactivation is caused by natural fish haemagglutinins/agglutinins that bind to carbohydrate moieties of microbes (Srivastava & Pandey 2015). Agglutination is a process that results in a clump or precipitate. Agglutination is mediated by proteins such as antibodies and complements. Both antibodies and complements have a receptor to bind with a pathogen and cause particulate agglutination of bacteria. Complements in many fishes might be activated by immunostimulants to induce bactericidal activity (Srivastava & Pandey 2015). Mannose-binding lectin (MBL) firmly bound to foreign or altered self-surfaces can participate in host defense response by the activation of the lectin complement

pathway, phagocytosis, apoptotic cell clearance, and inflammatory processes. MBL can function directly as an opsonin by binding to a pathogen, or indirectly by producing opsonin like C3b and promoting phagocytosis (Dinasarapu et al 2013).

Fish growth was significantly influenced by fucoidan for the 40 days rearing period. Length and weight gain, as well as SGR, were significantly higher in catfish fed dietary fucoidan in comparison to the control ($p<0.05$). Fucoidan significantly increases the growth performance of aquatic animals (Sivagnanavelmurugan et al 2014). Prabu et al (2014) reported that dietary fucoidan improves significantly the weight gain and SGR of the fingerlings of the *Pangasianodon hypothalamus*. The FCR was also significantly lower in the treatment diets due to different concentrations of fucoidan. A different report demonstrated that the supplementation of fucoidan at 500-2000 mg kg^{-1} enhances the growth performance and prevents or reduces mortality in *Penaeus monodon* infected with *Vibrio harveyi*.

Conclusions. The oral administration of fucoidan extracted from *P. boergesenii* significantly increased PA, NBT, SOD, serum antibacterial activity, and agglutination activity ($p<0.05$). Dietary fucoidan also increased significantly the resistance of catfish against *A. hydrophila* infection by improving SR and prolonging MTD. Furthermore, dietary fucoidan also increased the growth performance of length and weight, as well as SGR. Briefly, dietary fucoidan of *P. boergesenii* at a dose of 4000-6000 mg kg^{-1} in catfish can be used to increase non-specific immunity and resistance against *A. hydrophila*, as well as growth.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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