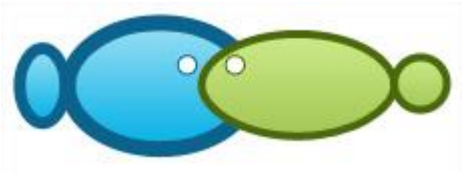

Effects of dietary algae, fungi and herb on the growth and innate immunity of Nile tilapia *Oreochromis niloticus* challenged with *Streptococcus agalactiae*

Fifin B. Kumala, Dinamella Wahjuningrum, Mia Setiawati, Sukenda

Department of Aquaculture, Faculty of Fishery and Marine Science, Bogor Agricultural University, Bogor, Indonesia. Corresponding author: D. Wahjuningrum, dinamella@yahoo.com

Abstract. This study was performed to determine the efficacy of three immunomodulators viz., algae, fungi and herb on hematology, an innate immune response of Nile tilapia, *Oreochromis niloticus*. Tilapias were divided into 4 groups and each group was fed with diets supplemented with or without immunostimulant for 30 days. Red blood cells (RBC), white blood cells (WBC), hematocrit, and hemoglobin, the non-specific cellular (phagocytic capacity and respiratory burst activity) responses were determined and compared with control (no supplement) after 30 days of feeding. The results of 30 days feeding trial showed that fungi supplementation significantly enhanced tilapia growth. Variation in the levels of responses was evident among different supplements. Compared with algae or herb, fungi could maintain the immunity of tilapia at a higher level during the experimental period. However, continuously applying fungi, algae or herb into the diet caused immunity fatigue in tilapia. After feeding for 30 days the experimental groups significantly ($P<0.05$) enhanced hematocrit, white blood cell counts, and red blood cell counts. Respiratory burst activity, phagocytic activity, and hemoglobin were none significantly ($P>0.05$) changed during the whole experiment. After 30 days of feeding, fish were injected intraperitoneally with 100 µL of *Streptococcus agalactiae* (7×10^7 colonies forming unit). The cumulative mortality was 73.33%, 64.44% and 51.11% in fish receiving diets supplemented with 20 mL kg^{-1} fungi, 8 g kg^{-1} algae and 0.3 g kg^{-1} herb, respectively, compared to 35.56% mortality in the control group. Especially supplementation with fungi to the tilapia for 30 days showed considerable improvement in the growth, survival and immune response of the tilapia.
Key Words: bacteria, *Gracilaria verrucosa*, *Nodulisporium* sp. KT29, *Anredera cordifolia.*

Introduction. Outbreaks of Streptococcal diseases are being increasingly recognized as a significant constraint in aquaculture production. Streptococcosis can cause mass mortality in tilapia. Previous studies showed that algae, fungi, and herbs are successfully used in fish culture. The use of immunostimulants has increased due to increased incidence of pathogenicity in aquaculture production. In this study, three immunostimulants, *Gracilaria verrucosa*, *Nodulisporium* sp. KT29, and *Anredera cordifolia* were chosen for the experiments. Various supplementation of *G. verrucosa*, *Nodulisporium* sp. KT29 and *A. cordifolia* have been used as an immunostimulant and there are many reports supporting (Kanjana et al 2011; Wahjuningrum et al 2016; Sari et al 2015). Bansemir et al 2006 reported that Gracilaria species contain antiviral, antifungal compounds and also has antibacterial activities. Whereas *A. cordifolia* contains alkaloids, flavonoids, phenols, steroids and essential oils (Astuti et al 2011; Citarasu 2010). Yuliani et al (2012) reported that the binahong leaf has formulated in wound healing gel. Flavonoids protect cells from oxidative damage and have immunostimulatory properties. The antioxidant effects of many components have been reported to activate the fish immune system, according Saputra et al (2016b) *Nodulisporium* sp. KT29 contained phytocemical compounds such as saponin, polyphenol, phytosterol and β-Glucan for bioactive compound which is composed of a heterogeneous group of glucose polysaccharides that are connected by β glycosidic bonds or D-glucose molecules which

are repetitively linked at a specific position. These complex carbohydrates also have branching glucose side-chains and are found in plants, algae, fungi and some bacteria where they are a major structural component of the cell wall. β glucan forms derived from yeast mainly comprise D glucose units with b-1.3-linkages and side-chains of D-glucose at position six. These homopolysaccharides are denominated as β-1.3/1.6-glucans and have been shown to reduce the susceptibility to infection (Dalmo & Bogwald 2008; Chen & Seviour 2007). β-1.3-glucan is known to have a potent stimulatory effect on the immune system of fish.

The resistance conferred by flavonoid and β-glucans against pathogens has been reported in several fish species. In general, the protective activities induced by flavonoid and β-glucans in fish include an increase in the number of immune parameters such as number of leucocytes, phagocytic activity and also macrophage bactericidal activity. Therefore, this study aimed to determine the efficacy of three immunomodulators viz., algae, fungi and herb on haematology, an innate immune response of Nile tilapia, *Oreochromis niloticus*.

Material and Method

Preparation of G. verucossa powder. The *G. verrucosa* used were obtained from Muara gembong west java, which was dried in an oven at 40°C. Dried leaves were grinded and sieved to produce *G. verrucosa* powder.

The preparation of Nodulisporium sp. KT29 metabolites induced by killed Vibrio harveyi cells. *Nodulisporium* sp. KT29 isolate was rejuvenated on Potato Dextrose Agar (PDA) for seven days. *V. harveyi* was cultured for 18 hours (10^8 CFU mL^{-1}) on SWC broth medium and shaked at 160 rpm for 18 hours. Before *Nodulisporium* sp. KT29 was induced by killed *V. harveyi* cells (*Nodulisporium* sp. KT29 + 5% (12.5 mL) *V. harveyi* was sterilized using autoclave (temperature 121°C). The mass culture was carried out by taking 5% mold inoculum from pre-culture medium then transferred into 250 mL PDB medium and incubated (shaked at 120 rpm) during 14 days. Harvesting was done by filtration using Whatmann filter paper to separate mycelia and culture broth. The evaporated metabolites were then added to the feed.

Preparation of binahong leaf powder. The binahong (*A. cordifolia*) leaves used were obtained from Bogor, West Java, which was dried in an oven at 40°C. Dried leaves were grinded and sieved to produce binahong leaf powder.

Feed preparation. The feed used was commercial feed that was treated by the addition of *G. verrucosa* 8 g kg^{-1}, *Nodulisporium* sp. KT29 metabolites 20 mL kg^{-1}, binahong leaf powder (*A. cordifolia*) 0.3 g kg^{-1}. Commercial feed (26-28% protein content) was re-pelleted and was added with 0.1% vitamin C and 30 g kg^{-1} of feed carboxyl methyl cellulose as a binder for control. The treatment feed was a commercial feed, which was repelleted and supplemented with 0.1% vitamin C, CMC 30 g kg^{-1}, alga, fungi, and herb. The feeds were treated as follows:

Control: Commercial Feed + 0 g kg^{-1} of feed treatment (control)
Alga : Commercial Feed + 8 g kg^{-1} of feed treatment *G. verrucosa*
Fungi : Commercial Feed + 20 mL kg^{-1} of feed treatment *Nodulisporium* sp. KT29 metabolites
Herb : Commercial Feed + 0.3 g kg^{-1} of feed treatment binahong leaf powder *(A. cordifolia*)

Experimental design. The experiment was performed on healthy tilapia fish (*O. niloticus*) cultured for 30 days in ponds (2×1×1.5 m) with the mean weight of 2.95±0.13 g. The fish were produced at Bogor Agricultural University, Faculty of Fisheries, Department of Aquaculture in Indonesia. Before the experiment started, the fish were fed

a diet containing 8 g kg^{-1} algae (*G. verrucosa*), 20 mL kg^{-1} fungi (*Nodulisporium* sp. KT29) and 0.3 g kg^{-1} herb (*A. cordifolia*). During the experiment, water properties were measured daily with the following values obtained: temperature 26-28°C, pH 6.2–7.29, and dissolved oxygen 6.8–7.7 mg L^{-1}. Five experimental diets were formulated for the fish. Three of them contained algae, fungi and herb. The control group was without supplementation with algae, fungi or herb. The feed was produced with a standard pelleting machine. Fish were fed ad satiation × 3 a day at 9:00, 13:00 and 17:00 h for 30 days.

After 30 days, the fish were randomly divided into 15 glass aquaria with 15 fish per aquaria and equipped with aeration system. The *Streptococcus agalactiae* strain was originally isolated from infected *O. niloticus*. The seven day LD_{50} was determined by intraperitoneal injection of 50 fish with graded doses of *S. agalactiae* (10^4, 10^5, 10^6, 10^7 and 10^8 CFU/fish) at 24°C, and the result showed that the LD_{50} on day 14 was 10^7 CFU/fish. Challenge tests were conducted in triplicate with 15 fish per replicate. Each fish was injected intraperitoneally with 0.1 mL phosphate buffered saline (PBS) for negative control, for positive control and other experimental injected intraperitoneally with 0.1 mL containing live *streptococcus*. The fish were then kept in separate 63 L glass aquaria (15 fish each). A total of 225 fish (45×5) were used for the study. Commercial feed was given to the animals during the test. *O. niloticus* was observed for the presence of disease manifested. Mortality of fish in each tank was observed over 14 days, and the average of the triplicate tanks was used to express cumulative mortality.

Data collection. At the termination of the experiment, the fish have fasted for 24 h before harvest. The total number was counted and mean body weight of fish was measured. Based on recording the weight of each fish and counting the number of tilapia, specific growth rate (SGR) (Guo et al 2012), feed conversion ratio (FCR) (Huisman 1987) and survival rate (Goddard 1996) was determined. For hematology assays such as red blood cells (RBC, 10^6 mm^3), white blood cells (WBC, 10^4 mm^3), hematocrit (Hc, %) and hemoglobin (Hb, g dL^{-1}) were determined using standard methods according to Blaxhall & Daisley (1973), Siwicki et al (1994) and Wedemeyer & Yasutake (1977). Immunological assays such as phagocytic activity were estimated using the modified method of Siwicki et al (1994). The respiratory burst activity of the phagocytes was carried out by nitroblue tetrazolium following the method of Siwicki et al (1994). The absorbance at 630 nm was measured with a Model Multiskan spectrum using KOH/DMSO alone as a blank.

Statistical analysis. All data were subjected to one-way ANOVA (analysis of variance) using SPSS 16.0 for Windows. Differences between the means were tested by Duncan multiple range tests. The level of significance was chosen at $P<0.05$ and the results are presented as means±S.E.M. (standard error of the mean).

Results and Discussion

Growth parameters. After the 30 day feeding period, *O. niloticus* fed the diets supplemented with immunostimulants tended to have better growth performance (Table 1). Fish supplemented with fungi showed the highest final weight ($P<0.05$) and SGR in comparison with the algae or herb and control group ($P>0.05$). However, there were no differences among fish fed fungi, algae and herb. Feed conversion ratio (FCR) values were not significantly ($P>0.05$) affected by the diets supplemented with immunostimulants, although fish fed the diets supplemented with fungi had the highest numerical values. The mortality of fish fed diet supplemented with algae, fungi, herb was higher than that of fish fed ordinary control feed. Similarly, no significant difference ($P>0.05$) was observed in survival.

Table 1
Growth response and survival of *Oreochromis niloticus* fed the three immunostimulants at the end of 30 days of feeding trial (means ± S.E.M)

Parameters	*Control*	*Algae*	*Fungi*	*Herb*
Initial weight (g)	3.02±0.16	3.01±0.03	3.02±0.03	3.00±0.08
Final weight (g)	5.74±0.06^{a}	6.27±0.03^{b}	7.22±0.47^{c}	6.63±0.17^{b}
SGR (%day^{-1})	2.18±0.05^{a}	2.47±0.04^{b}	2.94±0.25^{a}	2.68±0.05^{b}
FCR	2.29±0.09^{a}	1.94±0.11^{b}	1.58±0.13^{c}	1.90±0.04^{b}
Survival (%)	89±5.3	95±1	97.67±1.53	94.67±2.52

Means±S.E.M having the different letter in the same row are significantly different at P<0.05.

According to Olivier et al (1986), there are alternative strategies to vaccination and uses of antibiotics which are represent by applications of various immunostimulatory substances as dietary supplements. Even though is known about the mechanism of their action in fish, some of them appear to enhance the non-specific killing of pathogenic microbes. The present study showed that growth was significantly increased by feeding dietary fungi supplementation, whereas algae or herb supplementation did not affect the growth of the *O. niloticus*. Growth-enhancing effects of fungi have been reported earlier in shrimps. These results are in accord with previous studies which demonstrated that the application of fungi improves growth rates of shrimp (Saputra et al 2016a; Wahjuningrum et al 2016). Whereas, feeding dietary algae supplementation on growth have been evaluated with varied results. A similar result for algae was obtained by Peixoto et al (2017), who found that dietary supplementation of *Gracilaria* sp*.* showed no differences between groups of dietary treatment into *Dicentrarchus labrax* diets. This immunostimulant may contain a compound with anti-nutritional effects. However, the effect of algae supplementation on growth was negative.

The results showed final weight and SGR of *O. niloticus* within fungi treatment was better when compared with other treatments. Van Hai & Fotedar (2009) reported that β-glucan on *Penaeus lasticulatus* prawn caused the structure of intestinal surface becoming wider so that the absorption of nutrient feed becomes higher and shrimp growth rate was better. In the present study, might be a possible reason for the improved digestion and absorption, as shown by feed efficiency ratio and protein gain results in the fish fed diet supplemented with *Nodulisporium* sp. KT29. According to Lopez et al (2003) β-glucan was degraded by glucanases in the digestive gland, which function to produce energy, therefore protein is used more optimally for growth.

Haematology assays. After 30 days of feeding total red blood count (RBC) in fish fed diets with fungi and herb were significantly higher than of the control group and that of algae feeding group. The RBC of the fungi supplemented group was significantly higher compared with algae or herb supplementation throughout the experimental time (P<0.05). Total RBC started to decrease after injection with *S. agalactiae.* Figure 1 show, that fungi supplementation produced significantly higher RBC compared with algae or herb supplementation (P<0.05) after 7 days of injection and 10 days of injection. On the other hand, on day 7 and 10 post-challenge hemoglobin level with fungi and algae were significantly higher compared with herb (P<0.05) (Figure 2). The highest hematocrit level was significantly different (p<0.05) between three supplemented diets and control (Figure 3).

The administration of the three treatments influenced the hematological parameters of the fish, especially after the challenge test, in which there were fluctuations of hematological parameters including hemoglobin level, hematocrit level, and red blood count (RBC). In our experiment, feeding dietary fungi supplementation showed the highest hemoglobin level, hematocrit level, red blood count (RBC) before the challenge test. On day 7 post-challenge test of hemoglobin level, hematocrit level, and erythrocytes count decreased then increased again on day 10 post-challenge test. The decreased RBC, hemoglobin level, and hematocrit level after challenge suggested that anemia in fish, can be known from Hc value. According to Clauss et al (2008), if the

value of Hc is below 20% then it can be considered the occurrence of anemia in Teleostei. Streptococcosis in fish caused by *S. agalactiae* causes septicemia and meningoencephalitis (Mian et al 2009). Septicemic organs are eyes, brain, and kidneys (Abdullah et al 2013), so decreasing in the number of erythrocytes and fluctuations in hemoglobin level and hematocrit level values is caused by the damage of kidneys causing disturbed blood production.

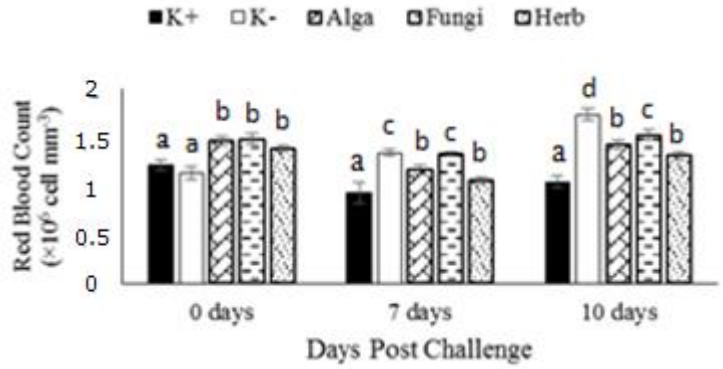

Figure 1. The red blood count of *Oreochromis niloticus* fed the three immunostimulants at the end of 30 days of the feeding trial, 7 days of injection and 10 days of injection. Absence of letters indicates significant difference between treatments (P<0.05).

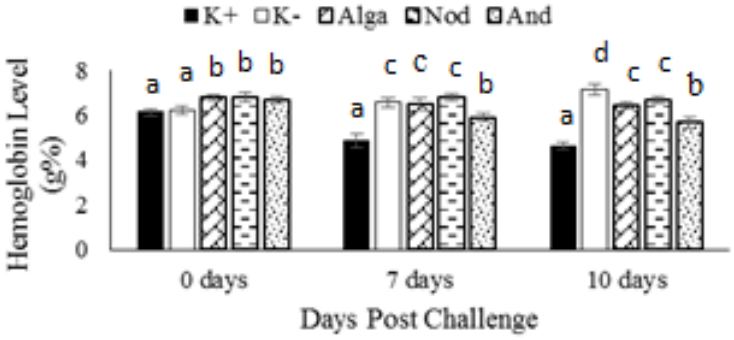

Figure 2. Hemoglobin level of *Oreochromis niloticus* fed the three immunostimulants at the end of 30 days of the feeding trial, 7 days of injection and 10 days of injection. Absence of letters indicates significant difference between treatments P<0.05).

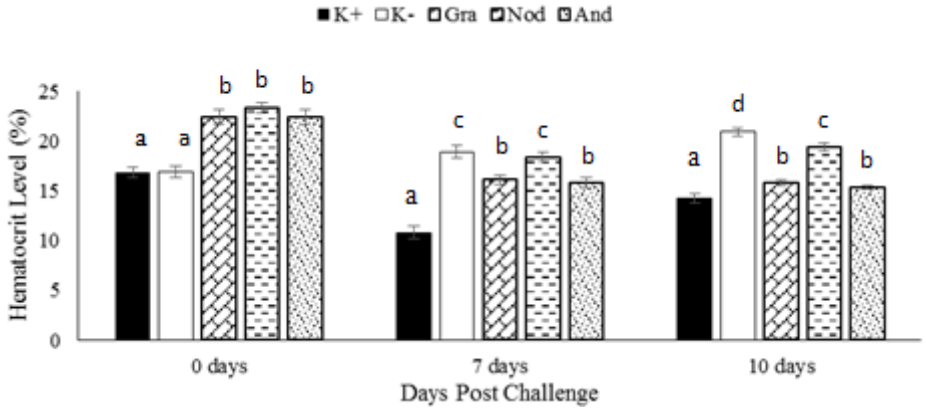

Figure 3. Hematocrit level of *Oreochromis niloticus* fed the three immunostimulants at the end of 30 days of the feeding trial, 7 days of injection and 10 days of injection. Absence of letters indicates significant difference between treatments (P<0.05).

Immunological assays. The total white blood counts (WBC) in fish fed the three immunostimulants supplemented diets were significantly higher than in the control group ($P<0.05$) after 30 days of feeding. However, the WBC increased after 7 days injection with *S. agalactiae*. Figure 4 show that WBC after 7 days injection was significantly increased ($P<0.05$), the benefit for WBC was obtained with immunostimulant supplemented diet during the early stage (after 30 days) of the feeding.

The respiratory burst activity of the phagocytes in algae, fungi groups increased significantly ($P<0.05$) relative to that of control and herb after 7 days of injection, and the respiratory burst activity in fungi groups was higher ($P<0.05$) than that of algae and herb groups (Figure 5). There was no significant difference in respiratory burst activity and phagocytic capacity at the end of 30 days of feeding trial before bacteria injection. However, the maximum activities of phagocytic capacity were observed after 7 days injection. There are differences among the three treatments either algae, fungi or herb. Algae and fungi supplemented diets showed significantly higher phagocytosis activity than the herb group ($P<0.05$) (Figure 6).

Phagocytic cells are cellular components of innate immunity that represents various types such as the evolutionarily ancient macrophages, and natural killer (NK) cells (Vetvicka et al 2013). According to Mac Arthur & Fletcher (1985), the most important cellular components of the innate immune system of fish are phagocytic cells. Its produce toxic oxygen forms during a process called respiratory burst (Neumann et al 2001). Increasing respiratory burst activities in all treated was measured while 7 days post challenge, that indicates bacterial pathogen killing activity by phagocytes and hence a better immunity (Kumar et al 2011).

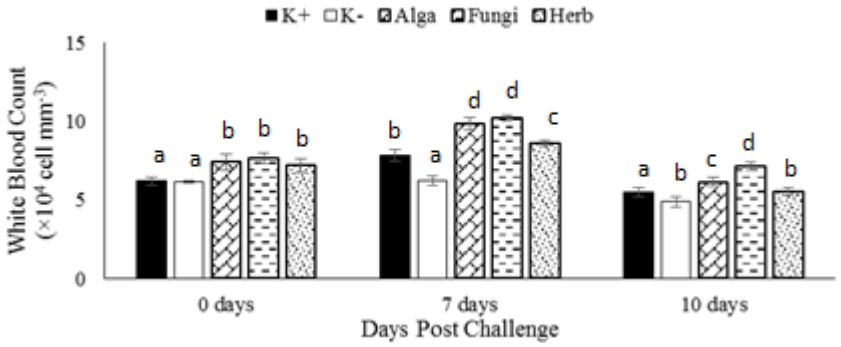

Figure 4. The white blood count of *Oreochromis niloticus* fed the three immunostimulants at the end of 30 days of the feeding trial, 7 days of injection and 10 days of injection. Absence of letters indicates significant difference between treatments ($P<0.05$).

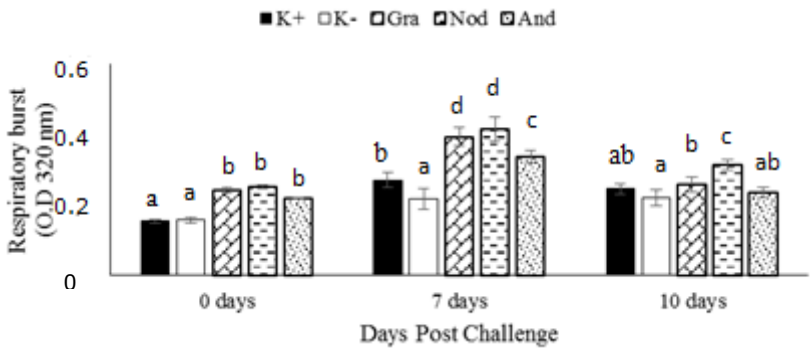

Figure 5. Respiratory burst activity of *Oreochromis niloticus* fed the three immunostimulants at the end of 30 days of the feeding trial, 7 days of injection and 10 days of injection. Different letter indicate significan different ($p<0.05$) and the same letter indicate are not significant different ($p>0.05$).

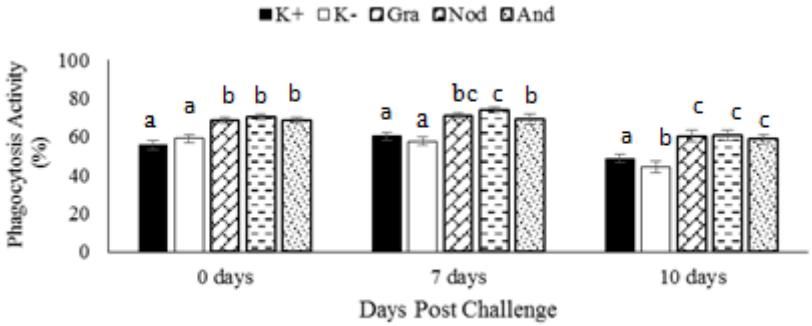

Figure 6. Phagocytosis activity of *Oreochromis niloticus* fed the three immunostimulants at the end of 30 days of the feeding trial, 7 days of injection and 10 days of injection.

Table 2 showed that feeding dietary algae, fungi, and herb supplementation can be improved the resistance to pathogens compared to control. Three immunostimulants such as algae, fungi and herb has been used to enhance innate immunity and improve resistance to pathogens in shrimp and other fish species. The survival rate parameter in the immonostimulant treatments (algae, fungi and herb) showed higher values than that of control. In this study, compared with two other immunostimulants, feeding dietary fungi by using marine fungal *Nodulisporium* sp. KT29 metabolites improved the production performance. However, the comparative efficacy of these immunostimulants widely studied substances on the non-specific immunity of fish has been attempted first time in *O. niloticus*. This study confirmed parameters of innate immunity studied, each substance showed maximum efficacy for each parameter. It was indicated that non-specific humoral and cellular responses show differential specificity to a given immunomodulatory substance.

Table 2

Total survival rate (%) 20 days after challenge with *Streptococcus agalactiae* in *Oreochromis niloticus* fed with three immunostimulants supplemented diets

Treatment	*Survival rate (%)*
Positive control	35.56^{d}
Negative control	93.33^{ab}
Algae	64.44^{bc}
Fungi	73.33^{b}
Herbal	51.11^{cd}

Data are Means ± S.E.M from 36 tilapia. Values with different superscripts are significantly different at P<0.05.

The inclusion of the three immunostimulants into the diets had effect on the immunity of tilapia. Similar results were also reported in Pacific white shrimp (Zahra et al 2017; Saputra et al 2016b), *Penaeus monodon* (Utomo et al 2015), the different types of immunostimulants also have different effect response and different efficacy among species or within species. Among the immunostimulants studied, fungi showed better immune activity compared with other immunostimulants. The result showed that marine fungal was the most effective one in enhancing innate immune response of *O. niloticus*. The improved resistance of *O. niloticus* after challenge may be partly attributable to the increased respiratory burst and phagocytosis activity of *O. niloticus* compared to the control. Therefore, the findings indicated that the increased resistance to *S. agalactiae* of *O. niloticus* was related to the enhanced immune status. More studies are needed in order to find out the mechanism.

Conclusions. In conclusion, the result of our study provided substantiation that dietary algae, fungi and herb supplementation could significantly enhance the non-specific immunity of *O. niloticus* through improvement of various immune parameters such as

WBC, respiratory burst activity, and phagocytic activity of blood phagocytes. The administered supplements also enhanced the survival rate after challenge with *S. agalactiae.* Thus, they can be used as immunostimulants to enhance immune response and disease resistance of fish. Furthermore further researches are needed to be conducted to clarify the mechanisms of all these substances action(s) in the health and immunity improvements of *O. niloticus*.

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Authors:
Fifin Binta Kumala, Bogor Agricultural University, Faculty of Fishery and Marine Science, Department of Aquaculture, Bogor, Indonesia, West Java, Bogor 16680, Dramaga Campus, Jalan Agatis, e-mail: fifinbk1@gmail.com
Dinamella Wahjuningrum, Bogor Agricultural University, Faculty of Fishery and Marine Science, Department of Aquaculture, Bogor, Indonesia, West Java, Bogor 16680, Dramaga Campus, Jalan Agatis, e-mail: dinamella@yahoo.com
Mia Setiawati, Bogor Agricultural University, Faculty of Fishery and Marine Science, Department of Aquaculture, Bogor, Indonesia, West Java, Bogor 16680, Dramaga Campus, Jalan Agatis, e-mail: miasetia@apps.ipb.ac.id
Sukenda, Bogor Agricultural University, Faculty of Fishery and Marine Science, Department of Aquaculture, Bogor, Indonesia, West Java, Bogor 16680, Dramaga Campus, Jalan Agatis, e-mail: sukenda67@gmail.com

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