Utilization of Mycelium sterilium KT31 metabolites with diet for controlling Aeromonas hydrophila infection on catfish Clarias gariepinus

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Abstract. Catfish (Clarias gariepinus) is a freshwater fish that is widely cultivated throughout Indonesia due to its importance commodity and high market prospect. Intensive aquaculture system to increase production has risks, including fish easily infected with the bacteria Aeromonas hydrophila or motile aeromonad septicaemia (MAS) disease in catfish. Alternative efforts to control MAS disease is by using natural source from marine fungal Mycelium sterilium KT31 metabolites. The purpose of this study was to determine the effective dose of fungal Mycelium sterilium KT31 metabolites in feed for preventing and treating infections caused by A. hydrophila in catfish. The feed was prepared by repelleting method and given restricted three times a day. The bacterial concentration used for the challenge test was density of 10⁶ CFU/mL A. hydrophila as much as 0.1 mL per individual. This research was divided into two stage that the preventive experiment and treatment experiment with the different time challenge. Metabolites M. sterilium KT31 dose of 40 mL kg⁻¹ of feed was able to preventing and treating A. hydrophila infection with a better blood value, resulting in the best survival rate and growth rate of catfish.

Keywords: Clarias gariepinus; Aeromonas hydrophila; motile aeromonad septicaemia; Mycelium sterilium KT31 metabolites.

1. Introduction
The African catfish (Clarias gariepinus) is a freshwater aquaculture commodity that is widely cultivated in Indonesia. Catfish becomes one of the major consumption fish with the market demand that continues to increase every year. The data of catfish production from 2012 to 2016 was increased from 441 217 ton to 873 716 ton [1]. Implementation of intensive fish culture system is characterized by increasing stocking density for the reasons to increase mass production of catfish. However, the risk of implementing an intensive system has an impact on declining water quality and the emergence
of diseases. One of the main problems in aquaculture especially for catfish is the emergence of disease motile aeromonad septicemia (MAS), which is caused by bacteria Aeromonas hydrophila [2].

The control solutions through administrating the antibiotics have done often yet. The use of antibiotics for a prolonged period of time will generate negative effects both fish and environment. Along with the ban on the use of antibiotics in catfish farming, the prevention of disease is mostly done by the use of medicines derived from natural ingredients which is environmentally friendly and safe by taking advantage of phytopharmaceutical products to prevent or treat diseases [3]. In the research of study phytopharmaceutical products that have been tested on catfish are meniran leaves extract (Phyllanthus niruri) (4%) and garlic extract (Allium sativum) (1%) for the control of infection A. hydrophila bacteria with survival rate in treating experiment of 45.83% [4]. The administration of Aloe vera powder at a dose of 30 g/kg of diet in fish carp gave a very significant effect on the survival rate of 68.07% after infection with A. hydrophila bacteria [5].

The alternative solution that expected for the reduction of bacterial infections A. hydrophila is marine fungi contain antibacterial compounds and β-glucan [6]. The advantages of marine fungi as treatment is the levels of the active compounds contained in the same range. This is realized that it can be cultured in controlled in the laboratory. Nodulisporium sp. KT29 is a natural endophytic fungus isolated from red alga Euchema edule supplied from Takalar, South Sulawesi. The fungus is reported to contain immune-stimulant compounds, antioxidants such as β-glucan, saponin, polyphenol, and phytosterol [7]. The fungus is reported as source of antibacterial compounds, antioxidants such as β-glucan, saponin, polyphenol, phytosterol and eco-friendly [8].

Beside the Nodulisporium sp. KT29, the marine fungus isolated from red alga Euchema cottoni is Mycelium sterilium KT31. M. sterilium KT31 was proposed to have same potential as Nodulisporium sp. It has antibacterial compounds to inhibitory pathogenic bacteria aquatic organisms, such as Vibrio anguillarum, A. salmonicida, and Yersinia ruckeri [9]. Utilization of marine fungus M. sterilium KT31 never been required for controlling disease aquatic organism. This study was to understand about the best dose effective M. sterilium KT31 was able to controlling A. hydrophila infection.

2. Material and Method

2.1. Place and Time of research
This study was conducted in May until September 2018 at IPB University (Bogor Agriculture University). Research was conducted at the Fish Health Laboratory, Department of Aquaculture and then the water quality analysis at the Environmental Laboratory, Department of Aquaculture. Fungal cultivation of M. sterilium KT31 was conducted at the Aquatic Product Microbiology Laboratory, Department of Aquatic Product Technology, Faculty of Fisheries and Marine Sciences, IPB University.

2.2. Preparation of M. sterilium KT31 metabolite
Fungal isolate M. sterilium KT31 was obtained from red alga (K. alvarezii), Takalar, South Sulawesi, Indonesia. M. sterilium KT31 was cultivated according to a modified method of Tarman. Rejuvenation of M. sterilium KT31 isolate was carried out on potato dextrose agar (PDA) for 7 days, then cut in the shape of a cube for pre-culture in 100 mL of potato dextrose broth (PDB) for 7 days at 28-30°C in static condition. The suspension obtained from the pre-culture process was further transferred into 250 mL of PDB. M. sterilium KT31 suspensions (10 mL) were incubated with thermoshaker at 28-30°C and 120 rpm. After incubation, all suspensions were filtrated using Whatmann paper (mesh size 0.45 μm), then the filtrate were evaporated (40°C, rotary evaporator) to remove the water as much as 80%. The evaporated results (metabolites) were used for added to experimental feed [10].

2.3. Bacteria and Challenge Test
A. hydrophila bacteria was brought from the Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine, IPB University. The bacterium was intramuscularly injected in the fish to evaluate its virulence. The mentioned bacterium was isolated and cultured on 8 mL TSB (Trypticase Soy Broth) medium and incubated in waterbath shaker at 37 °C for 24 hours. One
milliliter broth culture was centrifuged at 5000 × g for 10 min and precipitate was rinsed and supernatant fluid was removed. The bacterial pellet was resuspended in a 1 mL phosphate-buffered saline (PBS) solution for the stock bacterial suspension and then was serial dilution until bacterial density of 1×10⁶ colony-forming units (CFU) per ml as the bacterial suspension for challenge test.

2.4. Preparation of Experimental Feed
This study used the commercial feed containing 31-33% protein by repelleting. The commercial feed was milled and was added 0.1% vitamin C (1g/kg feed) and binder 3% CMC (carboxyl methyl cellulose) as same as the control feed, while the feed for treatments was also supplemented with M. sterilium KT31 metabolites. Each experimental feed was repelleted, dried in an oven and mashed into crumbs then ready to use.

2.5. Research Design
The experiment was conducted in a completely randomized design. Catfishes were arbitrarily divided into four groups K+(feed without M. sterilium KT31 + A. hydrophila challenge test), K- (feed without M. sterilium KT31 + PBS injection), 20 (M. sterilium KT31 20 mL kg⁻¹ of feed + A. hydrophila challenge test), 40 (M. sterilium KT31 40 mL kg⁻¹ of feed + A. hydrophila challenge test) each with 3 replicates of 10 shrimp in aquarium (60 × 30 × 30 cm). The body weight of experimental catfish was 6.69±4.01 g, respectively. Catfish in the experimental feed and positive control (K+) were challenged by injected 0.1 mL fish⁻¹ of a A. hydrophila suspension of 1×10⁶ colony-forming units (CFU) ml⁻¹ into intramuscularly, except negative control injected 0.1 mL PBS. This research was divided into two stage that the preventive experiment and treatment experiment with the different time challenge. The preventive experiment was fed with feed metabolites for ten days, then catfish have challenged with A. hydrophila at 11th day. The treatment experiment was fed commercial feed for ten days and prepared to challenge test at 11th day. Catfish in the treatment experiment was fed with feed metabolites after the challenge test for ten days at the 12th day. The feed was prepared by repelleting method and given restricted three times daily at rate of 3% body weight at 08:00, 12:00 and 16:00 AM at satiation level. During the experimental period, water temperature ranged 25.1 - 27.2°C, pH 6.51 - 6.75, dissolved oxygen (DO) concentration 3.7 – 5.5 mg L⁻¹ and total ammonia nitrogen 0.02-0.24 mg L⁻¹, respectively.

3. Parameter

3.1. Survival Rate
Fish survival rate was observed from the beginning until the end of the rearing period and was determined using the following formula of Effendi [11].

3.2. Feeding Rate
Feeding rate was determined using the following formula of Effendi [11].

3.3. Feed Conversion Ratio
Feed conversion ratio was determined using the following formula of Goddard [12].

3.4. Absolute Growth Rate
The absolute growth rate was determined using the following formula of Goddard [12].

3.5. Hematology Parameters
Fish blood sampling 0.3-0.5 mL was withdrawn of each fish with a 1-ml sterile syringe (25 gauge) previously filled anticoagulant solution homogenized by a hand shake and disposed. Hematological parameters that were observed in the present study consisted of total erythrocytes, hemoglobin, total leukocytes, hematocrit and phagocytic activities. Hematology parameters in the preventive experiment were observed on initial day (H₀), 10 day after experiment fed metabolites (H₁₀), 1st day after challenge test (H₁₂), and 10th day after challenge test (H₂₀). Hematology parameters in the treatment
experiment were observed on initial day (H₀), 10 day after fed commercial feed (H₁₀), 1st day after challenge test (H₁₂), and 10th day after challenge test (H₂₁).

3.5.1. Total Erythrocytes
Fish blood samples were sucked using a pipette that was previously filled with red stirrer up to 0.5. Afterward, Hayem’s solution was added up to a scale of 101, and the mixture was then stirred by shaking the pipette (to form an 8 shape) for 3–5 minutes until the blood was evenly mixed. The first 2 drops of the obtained solution were disposed and the solution was then dropped on a hemocytometer, on which a covering glass was placed. Total erythrocytes were determined based on the following formula of Blaxhall dan Daisley [13].

3.5.2. Total Leukocytes
The blood sample of fish was sucked using a pipette that was previously filled with white stirrer up to 0.5. Afterward, Turk’s solution was added up to a scale of 11 and shook in a way to form an 8 shape for 3–5 minutes until the blood was evenly mixed. The first 2 drops of the obtained solution were disposed and the solution was then dropped on a hemocytometer, on which a covering glass was placed. The solution will capillary meet the counting chamber and leukocyte was determined based on the following formula of Blaxhall dan Daisley [13].

3.5.3. Hemoglobin Level
Hemoglobin level was measured based on Sahli method i.e. by filling haemometer with HCl 0.1 N solution up to the red line that represents the scale of 10 and then placing it between two standard colored tubes. Fish blood in the microtube was collected using a Sahli pipette, about 0.02 mL, and placed in a Sahli tube for a period of 3 minutes. Afterward, distilled water was gradually added using the pipette and mixed until a change in color occurred (same as the standard color). Hemoglobin level was determined in g/dL on a yellow scale.

3.5.4. Hematocrit Level
Blood samples were placed into a hematocrit tube (up to 3/4 of the tube volume), clogged with crystoceal, centrifuged at 3,000 rpm for 5 min, and the precipitation length measured. The formula used to determine hematocrit level was as follows method of Anderson dan Siwicki [14].

3.5.5. Phagocytic Activity Observation
Phagocytic activity was determined by collecting 50 μL of blood and placing it into a microtube and added with 50 μL Staphylococcus aureus suspension in PBS (10⁷ cell/mL). The solution was then homogenized and incubated at room temperature for 20 minutes. A pillowcase was made and dried (air). Afterward, the pillowcase was immersed in methanol solution for 5 minutes, dried, stained (by immersion in Giemsa stain for 15 minutes), cleaned with running water and finally dried. Afterward, cells undergoing phagocytic activity were observed and counted out of 100 observed phagocytic cells was determined based on the following formula of Anderson and Siwicki [14].

4. Data Analysis
Data on parameters such as production performance and hematology test were analyzed using Microsoft Excel 2010. An ANOVA test followed by a Duncan-test if significant differences were observed with an interval of confidence of 95%.
5. Result

5.1. Preventive Experiment

5.1.1. Survival Rate

![Figure 1](image1.png)

The survival rate in the preventive experiment.

The survival rate was observed for 10 days after challenge test in Figure 1. The survival rate of catfish fed with both metabolite *M. sterilium* KT31 dose 20 mL kg$^{-1}$ (66.67±5.77%) and dose 40 mL kg$^{-1}$ (76.67±5.77%) was not significant different effect after challenge test (P>0.05).

5.1.2. Feeding Rate

![Figure 2](image2.png)

Feeding rate in the preventive experiment before and after challenge test.

Feeding rate before challenge test was not significant different effect in Figure 2 (P>0.05). After challenge test, feeding rate of catfish negative control experiment (K-) was significantly higher than that of the other experiments (73.53±2.13 g) (P<0.05).

5.1.3. Feed Conversion Ratio

![Figure 3](image3.png)

Feed conversion ratio in the preventive experiment before and after challenge test.

Feed conversion ratio of catfish fed by metabolite *M. sterilium* KT31 dose 40 mL kg$^{-1}$ was significantly lower (0.81±0.01) than that of the other experiments before challenge test (P<0.05). After challenge test, feed conversion ratio of catfish positive control experiment (K+) was (2.32±0.44) significantly higher than that of the other experiments (P<0.05). Feed conversion ratio of catfish negative control experiment was significantly lower (0.98±0.08) than that of the other experiments (P<0.05) on Figure 3.
5.1.4. Absolute Growth Rate

The absolute growth rate of catfish fed by metabolite *M. sterilium* KT31 dose 40 mL kg$^{-1}$ was significantly higher (0.55±0.01) than that of the other experiments (P<0.05) before challenge test. After challenge test, absolute growth rate of catfish negative control (K-) experiment was significantly higher (0.75±0.04) than that of the other experiments (P<0.05). The absolute growth rate of catfish positive control (K+) was lower, but without significant different with catfish experiment fed metabolite *M. sterilium* KT31 dose 20 mL kg$^{-1}$ (P>0.05) on Figure 4.

![Figure 4. Absolute growth rate in the preventive experiment before and after challenge test.](image-url)

5.1.5 Hematology Parameters

Hematology parameters in the preventive experiment were observed on initial day (H$_0$), 10 day after experiment fed metabolites (H$_{10}$), 1$^\text{st}$ day after challenge test (H$_{12}$), and 10$^\text{th}$ day after challenge test (H$_{21}$) on Table 1.

| Parameters                  | Experiments                      |
|-----------------------------|----------------------------------|
|                            | K- (20 mL kg$^{-1}$) | K+ (40 mL kg$^{-1}$) |                             |
| Total Erythrocytes (×10$^6$ cell mm$^{-3}$) | H$_0$ | 2.79±0.32$^a$ | 2.79±0.32$^a$ | 2.79±0.32$^a$ | 2.79±0.32$^a$ |
|                            | H$_{10}$ | 2.26±0.10$^a$ | 2.19±0.04$^a$ | 2.22±0.05$^a$ | 2.33±0.10$^a$ |
|                            | H$_{12}$ | 2.22±0.08$^a$ | 1.41±0.14$^a$ | 1.48±0.09$^{ab}$ | 1.80±0.18$^b$ |
|                            | H$_{21}$ | 2.27±0.03$^{bc}$ | 1.61±0.18$^a$ | 2.01±0.19$^{abc}$ | 2.28±0.22$^b$ |
| Total Leukocytes (×10$^6$ cell mm$^{-3}$) | H$_0$ | 8.17±0.42$^a$ | 8.17±0.42$^a$ | 8.17±0.42$^a$ | 8.17±0.42$^a$ |
|                            | H$_{10}$ | 8.30±0.26$^a$ | 8.30±0.20$^a$ | 8.30±0.40$^a$ | 8.27±0.42$^a$ |
|                            | H$_{12}$ | 8.20±0.26$^a$ | 12.17±0.42$^{ab}$ | 11.03±0.32$^c$ | 9.87±0.31$^c$ |
|                            | H$_{21}$ | 8.13±0.25$^a$ | 10.67±0.32$^a$ | 9.87±0.31$^c$ | 8.37±0.25$^a$ |
| Hemoglobin level (g%)      | H$_0$ | 8.33±0.58$^a$ | 8.33±0.58$^a$ | 8.33±0.58$^a$ | 8.33±0.58$^a$ |
|                            | H$_{10}$ | 8.27±0.23$^a$ | 8.30±0.10$^a$ | 8.53±0.06$^a$ | 8.47±0.12$^a$ |
|                            | H$_{12}$ | 8.23±0.21$^b$ | 5.53±0.61$^a$ | 5.67±0.46$^a$ | 6.60±0.69$^a$ |
|                            | H$_{21}$ | 8.33±0.29$^{bc}$ | 6.93±0.42$^a$ | 7.60±0.35$^{bc}$ | 7.93±0.23$^b$ |
| Hematocrit Level (%)       | H$_0$ | 27.16±1.12$^a$ | 27.16±1.12$^a$ | 27.16±1.12$^a$ | 27.16±1.12$^a$ |
|                            | H$_{10}$ | 26.51±1.64$^a$ | 27.17±1.16$^a$ | 25.06±3.24$^a$ | 28.70±3.47$^a$ |
|                            | H$_{12}$ | 26.20±0.41$^b$ | 18.73±1.43$^a$ | 21.36±1.09$^{abc}$ | 23.51±1.09$^{bc}$ |
|                            | H$_{21}$ | 26.71±0.82$^b$ | 19.01±1.33$^a$ | 26.18±2.12$^b$ | 28.95±0.34$^b$ |

Total erythrocytes in the preventive experiment at the H$_0$ and H$_{10}$ was not significantly different (P>0.05). Total erythrocytes at the H$_{12}$ were observed to decrease, except negative control. Total erythrocytes of catfish fed by metabolite *M. sterilium* KT31 dose 20 mL kg$^{-1}$ was significantly higher than that of the other experiments at the H$_2$ (P<0.05). Total leukocytes in the preventive experiment at the H$_0$ and H$_{10}$ was not significant different effect (P>0.05). Total leukocytes of catfish fed by metabolite *M. sterilium* KT31 dose 40 mL kg$^{-1}$ was significantly higher than that of the other experiments at the H$_{12}$ (P<0.05). Total leukocytes of catfish fed by metabolite *M. sterilium* KT31 dose 20 mL kg$^{-1}$ was significantly higher than that of the other experiments at the H$_{21}$ (P<0.05).
Hemoglobin level in the preventive experiment at the H₀ and H₁₀ was not significantly different (P>0.05). Hemoglobin level of catfish negative control (K-) experiment at the H₂₁ was higher, but without significant different with catfish experiment fed by metabolite *M. sterilium* KT31 dose 40 mL kg⁻¹ (P>0.05). Hematocrit level in the preventive experiment at the H₀ and H₁₀ was not significantly different (P>0.05). Hematocrit level of catfish fed by metabolite *M. sterilium* KT31 dose 40 mL kg⁻¹ was significantly higher than that of the other experiments at the H₂₁ (P<0.05). Hematocrit level at the H₂₁ of catfish fed with both metabolite *M. sterilium* KT31 dose 40 mL kg⁻¹ and dose 20 mL kg⁻¹ was not significantly different (P>0.05).

5.1.6. Phagocytic activity

![Figure 5. Phagocytic activity in the preventive experim](image)

Phagocytic activity in the preventive experiment at the H₀ and H₁₀ was not significant different effect of the other experiments (P>0.05). Phagocytic activity of catfish fed by metabolite *M. sterilium* KT31 dose 40 mL kg⁻¹ was significantly higher than that of the other experiments at the H₁₂ (P<0.05). Phagocytic activity of catfish fed by metabolite *M. sterilium* KT31 dose 40 mL kg⁻¹ was lower, but without significant different effect with negative control experiment (P>0.05) at the H₂₁ on Figure 5.

5.2. Treatment Experiment

5.2.1. Survival Rate

![Figure 6. The survival rate in treatment experim](image)

The survival rate in the treatment experiment was observed for 10 days after challenge test on Figure 6. The survival rate of catfish fed with both metabolite *M. sterilium* KT31 dose 20 mL kg⁻¹ (73.3±5.8%) and dose 40 mL kg⁻¹ (76.7±5.8%) was not significantly different (P>0.05) after challenge test.
5.2.2. Feeding Rate

![Graph](image1.png)

**Figure 7.** Feeding rate in the treatment experiment before and after challenge test.

Feeding rate in the treatment experiment was not significantly different (P>0.05) before challenge test. After challenge test, feeding rate of negative control (K-) experiment was significantly higher than that of the experiments (P<0.05). Feeding rate of catfish fed with both metabolites of *M. sterilium* KT31 dose 20 mL kg\(^{-1}\) and dose 40 mL kg\(^{-1}\) was not significantly different after challenge test on Figure 7.

5.2.3. Feed Conversion Ratio

![Graph](image2.png)

**Figure 8.** Feed conversion ratio in the treatment experiment.

Feed conversion ratio in the treatment experiment was not significantly different (P>0.05) before challenge test. After challenge test, feed conversion ratio of catfish positive control (K+) experiment was significantly higher than that of the other experiments (P<0.05). Feed conversion ratio of catfish fed with both metabolite *M. sterilium* KT31 dose 20 mL kg\(^{-1}\) and dose 40 mL kg\(^{-1}\) was not significantly different after challenge test on Figure 8.

5.2.4. Absolute Growth Rate

![Graph](image3.png)

**Figure 9.** Absolute growth rate in the treatment experiment.

The absolute growth rate of catfish did not show a significant different effect (P>0.05) all of experiment before challenge test. The absolute growth rate of negative control (K-) experiment was significantly higher than that of the other experiments (P<0.05). The absolute growth rate of catfish
fed with both metabolite *M. sterilium* KT31 dose 40 mL kg$^{-1}$ and dose 20 mL kg$^{-1}$ was not significantly different (P>0.05) on Figure 9.

5.2.5. Hematology Parameters
Hematology parameters in the treatment experiment were observed on initial day (H$_0$), 1$^{st}$ day after challenge test (H$_{12}$), and 10$^{th}$ day after experiment fed metabolites and challenge test (H$_{21}$) on Table 2.

**Table 2.** Hematology catfish in the treatment experiment.

| Parameters                      | Experiments          |
|---------------------------------|----------------------|
|                                 | K-                   | K+                   | 20 mL kg$^{-1}$ | 40 mL kg$^{-1}$ |
| **Total erythrocytes** (x 10$^6$ cell mm$^{-3}$) | H$_0$ 3.07±0.64$^a$ | 3.07±0.64$^a$ | 3.07±0.64$^a$ | 3.07±0.64$^a$ |
|                                 | H$_{12}$ 2.84±0.22$^b$ | 1.60±0.12$^a$ | 1.66±0.72$^a$ | 1.78±0.10$^b$ |
|                                 | H$_{21}$ 2.93±0.41$^b$ | 1.70±0.19$^b$ | 2.23±0.14$^a$ | 2.87±0.05$^a$ |
| **Total Leukocytes** (x10$^3$ cell mm$^{-3}$) | H$_0$ 7.87±0.21$^a$ | 7.87±0.21$^a$ | 7.87±0.21$^a$ | 7.87±0.21$^a$ |
|                                 | H$_{12}$ 7.97±0.51$^a$ | 10.60±0.72$^b$ | 11.20±0.46$^b$ | 11.40±0.46$^b$ |
|                                 | H$_{21}$ 7.93±0.55$^a$ | 10.10±0.26$^c$ | 8.93±0.21$^b$ | 8.03±0.12$^a$ |
| **Hemoglobin Level** (g%)      | H$_0$ 8.33±0.61$^a$ | 8.33±0.61$^a$ | 8.33±0.61$^a$ | 8.33±0.61$^a$ |
|                                 | H$_{12}$ 8.27±0.42$^b$ | 6.40±0.53$^a$ | 6.47±0.42$^a$ | 6.47±0.12$^a$ |
|                                 | H$_{21}$ 8.13±0.31$^{bc}$ | 6.47±0.23$^a$ | 7.73±0.50$^b$ | 8.80±0.20$^c$ |
| **Hematocrit Level** (%)       | H$_0$ 29.15±0.32$^a$ | 29.15±0.32$^a$ | 29.15±0.32$^a$ | 29.15±0.32$^a$ |
|                                 | H$_{12}$ 28.16±0.68$^c$ | 19.64±0.66$^{ab}$ | 18.50±0.96$^a$ | 20.44±0.53$^{b}$ |
|                                 | H$_{21}$ 27.32±0.58$^b$ | 21.72±0.76$^a$ | 23.35±0.74$^a$ | 30.58±0.91$^{c}$ |

Total erythrocytes in the treatment experiment at the H$_0$ was not significantly different (P>0.05). Total erythrocytes at the H$_{12}$ were observed to decrease after challenge test. Total erythrocytes metabolite *M. sterilium* KT31 dose 40 mL kg$^{-1}$ was higher, but without significant different effect with negative control (K-) experiment at the H$_{21}$ (P>0.05).

Total leukocytes in the preventive experiment at the H$_0$ was not significantly different (P>0.05). Total leukocytes at the H$_{12}$ increased after challenge test. Total leukocytes of catfish fed with metabolite *M. sterilium* KT31 dose 40 mL kg$^{-1}$ at the H$_{12}$ was significantly higher than that of the other experiments (P<0.05). Total leukocytes of catfish fed with both metabolites *M. sterilium* KT31 dose 20 mL kg$^{-1}$ and 40 mL kg$^{-1}$ decreased at the H$_{21}$.

Hemoglobin level in the treatment experiment at the H$_0$ was not significantly different (P>0.05). Hemoglobin level of catfish negative control (K-) was significantly higher than that of the other experiments at the H$_{12}$ (P<0.05). Hemoglobin level of catfish fed with metabolites *M. sterilium* KT31 dose 40 mL kg$^{-1}$ was significantly higher than that of the other experiments at the H$_{21}$ (P<0.05). Hematocrit level in the treatment experiment at the H$_0$ was not significantly different (P>0.05). Hematocrit level at the H$_{12}$ was observed to decrease after challenge test. Hematocrit level of catfish negative control was significantly higher than that of the other experiments at the H$_{21}$ (P<0.05). Hematocrit level of catfish fed with metabolite *M. sterilium* KT31 dose 40 mL kg$^{-1}$ was significantly higher than that of the other experiments at the H$_{21}$ (P<0.05).

5.2.6. Phagocytic activity
Phagocytic activity in the preventive experiment at the H$_0$ was not significantly different (P>0.05). Phagocytic activity at the H$_{12}$ of catfish positive control was significantly higher than that of the other experiments (P<0.05). Phagocytic activity of catfish fed with metabolite *M. sterilium* KT31 dose 40 mL kg$^{-1}$ was significantly higher than that of the other experiments (P<0.05) at the H$_{21}$ on Figure 9.
Figure 10. Phagocytic activity in the treatment experiment.

6. Discussions

6.1. Preventive Experiment

*M. sterilium* KT31 is an endophytic fungus isolated from red alga *Kappaphycus alvarezi* [7]. The fungus was reported producing secondary metabolites which can be for any purposes [15]. The fungus contained immunostimulant compound antioxidants such as β-glucan, saponin, polyphenol, and phytosterol [6]. The compounds increased immune system, so that the resistance was increased [16]. The previous research, the compounds contained in the metabolites of *Nodulisporium* sp. KT29 include β-glucan, saponin, polyphenol, and fitosterol [10]. The results of thin layer chromatography tests suggest that the *M. sterilium* KT31 has active compounds such as phenol and steroid, which are characterized by the three color components.

*Aeromonas hydrophila* is a pathogenic bacteria that produced exotoxin and endotoxin that can affect the pathogenicity of these bacteria [16]. Exotoxin products included hemolysin, protease, lipase, cytotoxins and elastase enzymes [2]. The toxin caused 90% mortality in fish [17]. Metabolites of *M. sterilium* KT31 in feed increased the survival rate of catfish during maintenance. The catfish fed with *M. sterilium* KT31 dose of 40 mL kg⁻¹ feed that challenged by *A. hydrophila* had the highest survival rate was 76.67 ± 5.77% which significantly different (P <0.05) with positive control (K+). This result that the administration of *M. sterilium* KT31 contains immunostimulant can inhibit *A. hydrophila* infection and increased the immune system for the survival rate of catfish.

The metabolite of *M. sterilium* KT31 dose 40 mL kg⁻¹ that showed the best feed conversion ratio and absolute growth rate before challenge test. The previous research stated the administration of *Nodulisporium* sp. KT29 added into feed has better growth in Tilapia [18]. The decrease of feeding rate is due *A. hydrophila* infection which can reduce catfish feeding habit (Sukenda et al. 2008). However, the feeding rate at the end maintenance of metabolites *M. sterilium* KT31 dose 40 mL kg⁻¹ was better than positive control. It caused by active compounds in fungus that can improve the catfish feeding habit which impact on increased growth of fish. In addition, the feed conversion ratio of dose of 40 mL kg⁻¹ was not significantly different with negative control (P > 0.05). Therefore, the absolute growth rate showed that the dose was highest compared to other experiments which were also infected with *A. hydrophila*. The use of fungal metabolite can change the surface structure of the white shrimp intestine to increase density of microvilli, nutrient absorption and feed efficiency [19].

Hematology parameters of catfish during maintenance is carried out at in the preventive experiment were observed on initial day (H₀), 10 day after experiment fed metabolites (H₁₀), 1st day after challenge test (H₁₂), and 10th day after challenge test (H₂₁). Total erythrocytes of catfish after the challenge test decreased all of experiment, except negative control. Because that negative control are not given *A. hydrophila* infection. *A. hydrophila* produced toxin, especially exotoxins that can hemolysis on erythrocytes [2]. At the end of maintenance, the erythrocytes increased again caused by the administration of the metabolite *M. sterilium* KT3. Total erythrocytes normal conditions ranges from 1.35-3.00 x10⁶ mm⁻³ cells [20].

Total leukocytes after challenge test increased in each experiment, except negative controls. *A. hydrophila* infection stimulated the formation of certain leukocyte cells such as macrophages,
monocytes, granulocytes, lymphocytes, and platelets. Macrophages, monocytes, and granulocytes function were reduced bacteria [2]. Leukocytes cell function as non-specific immune that destroyed pathogens through the processing of phagocytosis [21]. Therefore, leukocytes cell correlated with phagocytic activity. Leukocytes increased as higher as the phagocytic activity. When leukocytes increased that showed immune system worked against infection bacteria. The range of leukocytes cells in catfish that are not attacked by *A. hydrophila* is 7-8 x10^9 mm^-3 cells [22]. The leukocytes of the end maintenance decreased on dose 40 mL kg^-1. That is caused by the immunostimulant compounds, namely β-glucan and flavonoids contained in the metabolite of *M. sterilium* KT31, so that can improved the immune system of catfish. Flavonoids can enhance fish immune responses [23], as well as β-glucans which act as immunostimulants by stimulating the immune response in shrimp [6].

The hemoglobin level at the 11th day has decreased in each experiment, except negative controls. The hemoglobin level correlated with the erythrocytes cells. Hemoglobin has function of binding oxygen to be used in the catabolism process to produce energy [24]. Decreasing hemoglobin affects the ability to bind oxygen, so that the metabolic process in fish is disrupted. At the end of maintenance, the hemoglobin level is increased. It is known that the metabolites of *M. sterilium* KT31 increased the immunity of catfish after *A. hydrophila* infected. Sukenda *et al.* [25] stated that the hemoglobin level in catfish by 5.6-8.8 g% is still in the normal range.

Hematocrit levels 1 day after challenge test at H12 decreased. This is related to the erythrocytes cells due to *A. hydrophila* infection. Hematocrit levels have decreased by the erythrocytes cells lysis. Therefore, the erythrocytes after challenge test cells decreased will be accompanied by decreased hematocrit levels [25]. Hematocrit levels of normal catfish without *A. hydrophila* infection are 27-30% [21]. Hematocrit levels one day after the challenge test are below the normal range. However, ten days after the challenge test, hematocrit levels increased on the catfish fed with *M. sterilium* KT31 metabolite feed in the normal range and not significantly different (P> 0.05) with negative controls.

Phagocytic activity one day after the challenge test (H12) increased in all experiments injected *A. hydrophila*. The highest phagocytic activity was treated 40 mL kg^-1 which not significantly different from the treatment of 20 mL kg^-1. Based on these results, that immunostimulant compounds contained in the metabolite of *M. sterilium* KT31 increased the fish's immune system against *A. hydrophila* infection. The immune system increased through in the leukocytes cells and phagocytic activity [26]. The hematology parameters of catfish due to metabolites of *M. sterilium* KT31 was better than positive control. In addition, metabolite dose 40 mL kg^-1 showed that were not significantly different from negative controls (catfish was recovery). Therefore, the increasing immune system of fish is able to utilizing the energy obtained from feed for growth.

6.2. Treatment Experiment

Based on the results of this study, the survival rate at the end of maintenance showed the best results in metabolite *M. Sterilium* KT31 doses of 20 mL kg^-1 and doses of 40 mL kg^-1. The survival rate in doses of 20 and 40 mL kg^-1 is higher than control positive. We assumed that metabolite *M. sterilium* KT31 has the ability as an antibacterial, so it can depress the mortality of catfish attacked by disease compared to positive control [27].

The feeding rate of catfish has decreased after challenge test. This is assumed because the fish has decreased response to eat feed. Kurniawan *et al.* (2014) stated that one of the symptoms of *Aeromonas* sp. is low feeding habit for fish. Feeding habit has decreased also thought to be disturbed due to *A. hydrophila* infection. Liver to exposure to toxins from pathogens due will disrupted affect the body's metabolic processes, as a result fish will lose feeding habit [28].

Feed conversion ratio during maintenance was not significantly different for each experiment. This showed that the administration of metabolites *M. sterilium* KT31 mixed with feed does not have a negative impact on fish and the quality of feed given. Absolute growth rate of catfish doses of 20 and 40 mL kg^-1 was significantly different from negative controls. Low weight is thought due the infection from *A. hydrophila* bacteria. This is supported by Octaviana *et al.* [29] research which stated that fish that are attacked by *A. hydrophila* bacteria will be hampered by growth.

The erythrocytes cells, hemoglobin levels, and hematocrit levels in normal catfish were 3.06 x 106 cells/mm3, 10.3-13.5 g%, and 30.8-45.5% [28]. Based on the results of this study, the
erythrocytes cells, hemoglobin levels, and hematocrit levels after the challenge test on control positive, doses of 20 and 40 mL kg$^{-1}$ have decreased when compared with the beginning of maintenance, except negative control which stable. Decreasing the erythrocyte was assumed due to *A. hydrophila* infection. *A. hydrophila* thought to produce toxins that can cause blood cells to decrease. Exotoxins are hemolysin. Hemolysin, it has ability to lyse erythrocytes cells and lost hemoglobin, so that on the surface of the infected body many passes through the wound [30]. This assumed the erythrocytes cells in blood vessels tend to decrease. Decreasing post-infection hemoglobin levels correlates with a decrease in the number of red blood cells, so that the blood's oxygen binding capacity is disrupted and ultimately results in hemoglobin levels dropping. Decreased hematocrit levels are thought to be due to decreased volume of erythrocytes cells due to bleeding around the wound.

After ten days of treatment, doses of 20 and 40 mL kg$^{-1}$ showed an increased in the erythrocytes cells, hemoglobin levels and hematocrit levels. The dose treatment of 40 mL kg$^{-1}$ showed a significant increased and was significantly different (P <0.05) with a treatment dose of 20 mL kg$^{-1}$. Increasing the erythrocytes has indicated that the metabolite given are antibacterial and phenolic compounds contained in metabolite can inhibit the metabolism of *A. hydrophila* bacteria, so that the growth of bacteria in the fish's body can be suppressed and the blood-producing cells targeted by pathogenic bacteria return under normal circumstances.

Based on the results of this study, the leukocytes cells of catfish after the challenge test in the K + treatment, treatment dosages of 20 and 40 mL kg$^{-1}$ tended to increase around 10.60-11.40 x 10$^6$ cells mm$^{-3}$. The increasing leukocytes cells are thought to be the response of fish to maintain their immunity from the attack of *A. hydrophila* bacteria. This is supported by the opinion of Zou et al. [31], which stated that increasing leukocytes cells is caused by increased cell division activities because leukocytes cells play a role in eliminating foreign objects entering the body.

After ten days of treatment (H$_2$), a dose of 40 mL kg$^{-1}$ showed a significant decrease in leukocytes cells compared to the positive control. The leukocytes cells in the positive control at the end of maintenance showed high results, because the fish was still sick and resisting infection with *A. hydrophila* bacteria. Decreasing leukocytes cells in the treatment experiment dose 40 mL kg$^{-1}$ at the end of maintenance, because the fish have recovered the immune system. The parameters for observed leukocytes cells are related to the parameters of phagocytic activity. During the treatment period the phagocytic activity of treatment doses of 20 and 40 mL kg$^{-1}$ have increased and were significantly different from control. Increasing of phagocytic activity is thought to be due to mold metabolites given during treatment containing β-glucan, which acts as an immunostimulant. The mechanism of action immunostimulant is by increasing the activity of phagocytic cells to prey on foreign particles that enter the body [32]. β-glucan compounds have the ability to increase the non-specific immune system in the fish body [33]. β-glucan compounds that function as immunostimulant will bind to receptors that are on the surface of phagocytic cells, so that phagocytic cells become active for phagocytic activity. Phagocytes at the same time release cytokines which in turn stimulate new white blood cell production [33].

7. Conclusions
Metabolites *M. sterilium* KT31 dose of 40 mL kg$^{-1}$ of feed was able to preventing and treating *A. hydrophila* infection with a better blood value, resulting in the best survival rate and growth rate of catfish

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