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The addition of *Spirulina platensis* extract in feed on gill histopathology and survival rate of *Osphronemus gouramy* after infected with *Aeromonas hydrophila*

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Abstract. Prevention of disease in *Osphronemus gouramy* due to infection with *A. hydrophila* can be done by using immunostimulants, one of which is using *Spirulina platensis* extract. The purpose of this study was to determine the effect of *S. platensis* extract on gill histopathology and survival rate of *O. gourami* after being infected with *A. hydrophila*. The fish were divided into three groups: fish without addition of *S. platensis* extract and infected with PBS (K-); without giving the *S. platensis* extract and infected with *A. hydrophila* (K+); and given extracts of *S. platensis* at 75, 150 and 300 mg/kgof feed and infected with *A. hydrophila*. The results showed the lowest value of gill damage was obtained in fish with the addition of 75 mg/kg of feed of *S. platensis* extract (1.22) and the highest damage was in K+ (2.67). The highest survival rate was found in the addition of 75 mg/kg of feed of *S. platensis* extract (91.67%) and the lowest was in K+ (70.83%). It can be concluded that the addition of *S. platensis* extract75 mg/kg of feed proved to be the most effective in reducing gill damage and increasing the survival rate of fish.

1. Introduction

Giant Gourami (*Osphronemus gouramy*) is a consumption fish that is much favoured by the people of Indonesia [1]. A serious problem in the fish farming of giant gourami is the emergence of diseases caused by *Aeromonas hydrophila*, namely Motile *Aeromonas Septicemia* (MAS). *A. hydrophila* can infect through injured body surfaces or gills [2]. The gills are the main organs that work in the diffusion mechanism between blood and water so that the gills have a great chance of being exposed to pollutants in the waters [3]. Damage to the gills can cause gill function to be not optimal and can interfere with respiration, causing death.

Efforts to control the disease, fish farmer still use antibiotics or other chemicals, but the use of these antibiotics is not environmentally friendly because it causes *A. hydrophila* to be resistant to some chemicals. Disease prevention can be done by using immunostimulants [4]. One of the algae that can be used as an immunostimulant is *Spirulina sp.*[5].

Suggested by [6] that hot water extraction *Spirulina platensis* can release chemical compounds in the form of polysaccharides that can stimulate the immune system in fish and shrimp. Polysaccharides are known to be able to make contact with intestinal epithelial cells or cellular components in the intestine that are associated with lymphoid tissue resulting in immune cell activity [7]. Said by [8] that

lipopolysaccharide (LPS) can stimulate the activity of cellular defence responses, in this case activating phagocytosis, melanisation, encapsulation, nodulation and coagulation activities.

Control of disease expansion must be carried out as early as possible so that disease outbreaks do not occur, causing economic losses [8]. Based on this, it is necessary to conduct research to determine the effect of hot water extract of *Spirulina platensis* on gill histopathology and survival rate of giant gourami after infected with *A. hydrophila*).

2. Methods

2.1. Procedure

The test animals used were giant gourami with a size range of 9-10 cm and a weight of 10-12 grams. Prior to the research, giant gourami was given acclimatization treatment for approximately one week, after which they were put into an aquarium equipped with an aerator. Making hot water extraction of *Spirulina platensis* based on the method of [9] with a ratio of 1:10, namely 20 g of *Spirulina platensis* flour was added to 200 ml of water, then boiled and stirred for 1 hour at 90°C. Then it was centrifuged at 3000 rpm for 30 minutes to separate the precipitate and supernatant. The supernatant was dried using the freeze-drying process and obtained a dry weight of 3.382 g. The resulting extract was mixed with commercial feed according to the treatment dose and was given binder in the form of 2 grams of egg white and 15 ml of PBS (1% of the total feed). The feed mixture was stirred homogeneously and dried in an oven at 50-60°C for 6 hours.

Before being challenged, *A. hydrophyla* was passed (malignant) first. Passage is done by infecting healthy giant gourami with *A. hydrophyla*. *A. hydrophyla* was then isolated on the part of the organ or skin that was injured due to infection with *A. hydrophyla*. The bacteria were ready to be cultured and then inoculated on Tryptic Soy Agar (TSA) media for 24 hours at a temperature of 30°C, after which they were identified through observation of colony shape and biochemical tests. The giant gourami that had been given *Spirulina platensis* extract for 14 days was then challenged with *A. hydrophyla*.

The research treatment given was A (positive control) fish injected with bacteria but not fed extract, B (negative control) where the fish were not injected with bacteria and not fed extract. Treatments were C (75 mg/kg feed), D (150 mg/kg feed), E (300 mg/kg feed) and were infected with 0.1 ml of *A. hydrophyla* with a total of 10^8 CFU/ml [10]. The main parameters observed in this study were gill histopathology and survival rates of giant gourami. Supporting parameters observed were water quality measurements consisting of temperature, pH, dissolved oxygen (DO) and ammonia.

2.2. Data Analysis

The data obtained from the scoring results of the gill histopathology were analysed by the Kruskall-Wallis test and if there was a significant difference between the treatment groups ($p < 0.05$), then continued with the Mann-Whitney test [11]. While the survival rate data were analysed using the Analysis of Variance (ANOVA) statistical test with a 95% confidence interval and continued with Duncan's multiple distance test to determine the difference between treatments [12].

3. Results and Discussion

3.1. Clinical Sign

Giant Gourami (*Osphronemus gouramy*) which has been infected with *A. hydrophila* was observed for clinical signs that appear through observation of wounds and behaviour of giant gourami. The results showed that in treatments A, C, D and E there were wounds and dilation at the injection site, scales fell off and bleeding around the injection site. On the 3rd day post-infection, fish treatment A showed that the injection site was getting wider and the fish flesh was exposed. Observation of behaviour in treatments A, C, D and E, fish experienced decreased swimming movements, tilted swimming and fish tended to cluster at the aeration source and swim on the surface. In treatment B (control -) the fish looked normal and there was no dilation at the injection site, the fish movement was active and there was no change.

A. hydrophila can be isolated from the internal organs of fish and from skin wounds caused by *A. hydrophila* attacks. The isolated bacteria were then cultured on TSA media for 24 hours at 30°C. Colonies of *A. hydrophila* that had been inoculated and grown on TSA media were then identified to

determine whether the species that attacked carp was caused by *A. hydrophila* or not. Bacterial identification was carried out at Balai Karantina Ikan Kelas II Perak Surabaya. The results of the biochemical test showed a positive result that the bacteria that attacked the giant gourami was a type of *A. hydrophila*.

3.2. Gill Histopathology

Gills are the main respiratory organs in fish that work in the diffusion mechanism of respiratory gases between blood and water. The gills are formed from hardened cartilage arches with several filaments consisting of primary lamellae that have many branches and are called secondary lamellae [13]. The results of microscopic examination showed gill damage in the form of secondary lamellae hyperplasia, lamellae congestion and fusion with varying degrees of damage, ranging from slight to severe. Gill damage of giant gourami (*Osphronemous gouramy*) in each treatment can be seen in Figure 1.

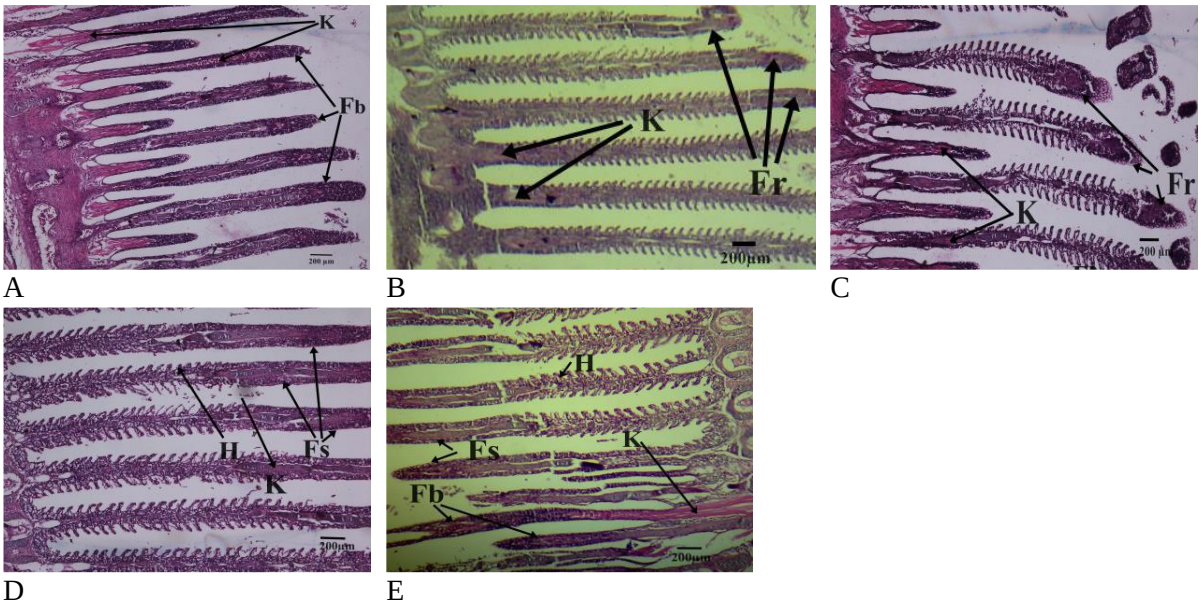

Figure 1. Histopathological Result of Giant Gourami Gill in Treatments A, B, C, D and E. Staining HE, Magnificationon200 times.
Description: A: control +, B: control -, C: dose 75 mg/kg feed, and E: dose 300 mg/kg feed.
⟶ Indicates damage to the gills, F indicates fusion, r: slight, s: moderate and b: severe, K indicates congestion and H indicates hyperplasia.

The results of the examination based on the score and average gill damage are presented in Table 1.

Table 1. Average Histopathological Data on Giant Gurame Gill Damage

Treatments	Average of Gill Scoring ±SD
A (control +)	2.667^{a} ± 0.471
B (control -)	1.000^{c} ± 0.000
C (75 mg/kg feed)	1.222^{b} ± 0.157
D (150 mg/kg feed)	2.222^{ab} ± 0.314
E (300 mg/kg feed)	2.333^{ab} ± 0.942

Note: Different superscript letter notations in the same column indicate that there is a significant difference between treatments. (F count >F table 0.05).

Determination of the level of gill damage at various degrees of infection was carried out using the scoring method. Based on the Kruskal Wallis test, there were significant differences between treatments in histopathological gill damage ($p<0.05$). Treatment B obtained the lowest average damage value of 1,000, this was because in treatment B it was not infected with bacteria so that the fish's functional system and immune system were normal. Gill damage in this treatment is thought to be due to the influence of cultivation media or less sterile equipment, so that contaminants, disease or bacteria can easily attack the gills.

The lowest average level of damage in the treatment was obtained in treatment C (75 mg/kg feed) which was 1.222. Treatment D (150 mg/kg feed) had an average score of 2.222 while treatment E (300 mg/kg feed) was 2.333. Treatment C got the lowest score (slight fusion level), it is suspected that this treatment is the right concentration of *Spirulina platensis* extract added in giant gourami feed. *Spirulina platensis* contains biologically active compounds including polysaccharides and lipopolysaccharides (LPS) [14]. LPS will be recognized by a receptor, TLR-4 which is found on the surface of leukocytes. TLR-4 functions as a dimer, and relies on the small protein MD-2 to recognize LPS [15]. The released LPS binds to lipopolysaccharide binding protein (LBP) with the help of the TLR-4 receptor and forms an LBP-LPS complex [16]. The LBP-LPS complex with the help of the TLR-4 signal will stimulate macrophages, neutrophils and monocytes to produce and release proinflammatory cytokines [17]. These cytokines function to activate phagocytic activity against bacterial cells so that bacteria cannot reproduce.

Treatment D and E had a higher level of damage, namely moderate fusion and some severe fusion, this is presumably because the concentration of *Spirulina platensis* extract given was too much so that it was toxic. This is related to the role of LPS in activating macrophages to produce cytokines. Cytokines in large quantities or uncontrolled products can cause oxidative stress, harm the body and can cause tissue damage in the host [18]. This is thought to cause higher fusion in treatments D and E.

Treatment A (control +) got the highest average score of 2.667, which means the level of secondary lamellae damage is getting higher. This is presumably due to the decreased immunity of fish due to the attack of *A. hydrophila*, so that bacteria can easily attack the target organ, namely the gills. Gill damage due to infection with *A. hydrophila* will cause disturbances in the respiration process so that the transport of respiratory gases (O_2 and CO_2) also does not run normally, which ultimately causes fish death [19].

3.3. Survival Rate

Data on the survival rates of giant gourami (*Osphronemus gouramy*) after infection with *A. hydrophila* are presented in Table 2.

Table 2. Survival Rates of Giant Gourami (*Osphronemus gouramy*) after Infection with *A. hydrophila*

Treatments	Survival Rates (%) ±SD
A (control +)	70.833^{a} ± 0.098
B (control -)	91.667^{b} ± 0.291
C (75 mg/kg feed)	91.667^{b} ± 0.291
D (150 mg/kg feed)	83.333^{ab} ± 0.125
E (300 mg/kg feed)	87.500^{ab} ± 0.000

Note: Different superscript letter notations in the same column indicate that there is a significant difference between treatments. (F count >F table 0.05).

Based on the ANOVA test on the effect of treatment on survival showed a significant value ($p <0.05$). The highest survival rate of giant gourami was obtained in treatment B (negative control) which was 91.667% and C, which was 91.667%. Treatment B (negative control) got the highest survival value because the fish were not infected with *A. hydrophila* so that the fish's body functions continued to run normally. According to [20], in relatively low concentrations LPS can function as an immunomodulator, which can increase the nonspecific immune system to attack bacteria. In this study, the lowest extract dose, namely treatment C (75 mg *Spirulina platensis* extract/kg feed) obtained the highest survival rate compared to other treatments. It is suspected that the treatment is the right concentration which causes the fish's functional system is not disturbed.

Treatment D (150 mg *Spirulina platensis* extract/kg feed) and E (300 mg *Spirulina platensis* extract/kg feed) had lower survival values. This is presumably due to the addition of too much *Spirulina platensis* extract so that the lipopolysaccharide content is toxic to fish. Excessive addition of lipopolysaccharide can induce the production and release of inflammatory cells such as Reactive Oxygen Species (ROS) which can cause chain reactions, and also trigger several types of infection (inflammatory) in macrophage cells and other cells [17];[21].

The lowest survival was found in treatment A (positive control) which was 70.833% where in this treatment the giant gourami were infected with bacteria but were not given feed containing *Spirulina platensis* extract. The low survival rate was influenced by the high level of damage to the gill lamellae in treatment A due to the attack of *A. hydrophila*. This gill damage causes disturbances in the respiration process so that O_2 transport also does not run normally, which ultimately causes fish death.

3.4. Water Quality

Water quality according to [22] is the nature of water and the content of living things, energy substances, or other components in the water. The range of measurement results of water quality parameters during the maintenance period of giant gourami (*Osphronemus gouramy*) is presented in Table 3.

Table 3. Water Quality Data during Research

Water quality parameters		Treatments				
		A (K+)	B (K-)	C	D	E
Temperature (ºC)	Morning	27-29	27-29	27-29	27-29	27-29
	Afternoon	28-30	28-30	28-30	28-30	28-30
DO (mg/l)	Morning	5.4-6.2	5.75-6.15	5.6-6.27	4.96-6.17	5.18-6.05
	Afternoon	5.75-6.25	5.45-6.05	5.5-6.16	5.2-6.12	5.7-6.08
pH	Morning	7	7	7	7	7
	Afternoon	7	7	7	7	7
Ammonia (mg/l)	Morning	0.5-1	0.5	0.5-1	0.5-1	0.5-1
	Afternoon	0.5-1	0.5	0.5	0.5-1	0.5

The results of water quality measurements obtained during the study generally indicate that the water quality is still in the optimal range to support the maintenance of giant gourami. Based on observations of water temperature in the morning, the range is between 27-29°C and in the afternoon between 28-30°C. This condition is in accordance with the statement by Handajani [23] that the optimal temperature for giant gourami is 25°C–30°C. Temperatures below 21°C will reduce the growth rate of fish so that it will reduce the ability of hormones to change sex [24].

DO (dissolved oxygen) is the level of oxygen dissolved in water. Dissolved oxygen (DO) content ranged from 4.96-6.27 mg/L. According to [25] the optimal value of dissolved oxygen in the waters is 4.4-7.6 mg/L. The degree of acidity (pH) during the study was still in the normal range of 7, this is in accordance with the statement by [23] that the optimal pH for giant gourami is 7-8. Giant gourami will experience growth disturbances if the pH is <4 or >11, which is an unfavourable condition for fish/lethal. Ammonia content during the study ranged from 0.5-1 mg/L. This water condition is quite normal for fish growth because according to [25] the ideal ammonia level for life is less than 1 ppm. Toxicity will increase as the pH decreases and the dissolved oxygen decreases.

4. Conclusion

The conclusion of this study is that the addition of hot water extract of *S. platensis* has an effect on the histopathology of giant gourami gills, namely reducing the average damage to gill lamellae and can increase the survival of giant gourami after infected with *A. hydrophila*. The best dose of hot water extract *Spirulina platensis* which affects the histopathology of gills and the survival of giant gourami after infected with *A. hydrophila* is a dose of 75 mg/kg of feed (Treatment C). Based on this research, it is recommended that the application of adding *Spirulina platensis* hot water extract to giant gourami feed should use an effective dose of 75 mg extract/kg feed. Furthermore, it is hoped that there will be

further research on the benefits of *Spirulina platensis* extract in treating diseases and normalizing damage to important fish organs so that fish survival will also increase.

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