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Effect of vitamin C supplementation on the survival rate and histopathological changes of gills and kidneys of tilapia *(Oreochromis niloticus)* infected by *Aeromonas hydrophila*

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Abstract

This study was conducted to determine the effect of vitamin C supplementation on the survival rate and histopathological changes of gills and kidneys of tilapia infected by *Aeromonas hydrophila*. Three doses of vitamin C were tested (150; 300; and 450 mg/kg) with two control groups. Tilapia with uniform size (average weight of 14 grams) as the criteria for inclusion were randomly distributed in five ponds with 15 tilapia fish per pond. Tilapia was fed with hands until full for two weeks. Tilapia was then infected with *A. hydrophila* to find out the survival rate and histopathological changes of gills and kidneys at the end of the experimental period or 7 days after infection. The supplementation of 150 mg/kg vitamin C in feed increased Tilapia's survival rate (%) by 86.67% or higher than other treatment and control groups. The damage to gills in terms of lamella separation was found in all treatment groups and kidneys. The results found that the supplementation of 150 mg/kg vitamin C in feed increased survival rate but did not give effective protection on gills and kidneys.

INTRODUCTION

Globally, in addition to cereal and milk products, animal products such as fish and seafood are the third largest protein food source consumed by humans up to 6.4% of the total protein supply (19.8% of the total supply from animal protein) (Ning *et al*., 2023). Capture Fisheries have not developed rapidly over the past few years in addition to several factors considered from this sector because of the potential for over-fishing(Naylor *et al*., 2021). The development of the aquaculture sector is an alternative to reduce the capture of aquatic biota with economic value to support economic productivity (Henriksson *et al*., 2021).

One of the widely cultivated aquaculture commodities is Tilapia *(Oreochromis niloticus)* an African native fish that has grown significantly since it was introduced to China Until Indonesia (Wiradana *et al*., 2022b; Yuan *et al*.,

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2017). As one of the main species of tilapia, the Genetic Engineering of Nile Tilapia is carried out through a crossing of eight different tilapia strains (Prabu *et al*., 2019). Tilapia is also very commonly cultivated because it can adapt and has a relatively fast reproduction (Miao *et al*., 2020). However, the rapid expansion of the aquaculture industry with high-density results in an increase in the rate of aquatic animal disease infections (Kamaruddin *et al*., 2021; Wiradana *et al*., 2022a). The application of antibiotics is generally used in a mixture of feeds because it can increase resistance to disease infections and trigger growth (Manyi-Loh *et al*., 2018). However, the use of antibiotics in the long run not only endangers human ecosystems and health but can also result in the development of resistance to pathogenic and non-pathogenic bacteria (Karakaya *et al*., 2019).

A. hydrophila is the most common infectious bacteria in the cultivated tilapia species and can cause mass death in extreme situations (Lu *et al*., 2021). *A. hydrophila* is a gram-negative bacterium, rod-shaped, and facultative anaerobes that can be found in all bodies of water worldwide (Igbinosa *et al*., 2012). The outbreak of infectious diseases due to *A. hydrophila* has been detected in many countries, especially the exporters of aquaculture products (Ferri *et al*., 2022) including tropical countries like Indonesia. The pathogen causes septicemia with open skin ulcers, gastrointestinal bleeding, ascites, and cloaca bleeding in several types of fish (Elsheshtawy *et al*., 2019; Pridgeon and Klesius, 2011; Zhang *et al*., 2013). However, differences in isolates or bacterial strains cause different symptoms and pathological disorders in infected fish. Researchers revealed that the presence of various virulence factors such as aerolysin (AER), Serine Protease (SER), Elastase (AHYB), Cholesterol Acyltransferase (GCAT), Type III Secretion System (ASCV), DNases (Exu), Polar Flagella (FLA), Cytotonics, Cytotonics Enterotoxins (ACT, ALT, AST), and Lipase (Lip) are the causes of differences in clinical symptoms caused by each isolate or strain from *A. hydrophila* (Bakiyev *et al*., 2022; Pattanayak *et al*., 2020).

Various approaches have been taken to study the pathogenicity caused by *A. hydrophila* in cultivated animals. Polymerase Chain Reaction (PCR) is used for early detection of infection through the identification of strains and virulence gene generation (Li *et al*., 2022) in addition to studying clinical symptoms and tissue histopathology in aquatic animals as the host of *A. hydrophila*. Sturgeon fish infected by *A. hydrophila* is sluggish and tends to swim near the surface of the water, experiences multiple ulcers in various surface areas until the muscles, has bleeding in the abdominal area, has pale gills, has kidney disorders, has hemorrhagic spots in the liver to accumulated bloody exudate in the abdominal cavity (Bakiyev *et al*., 2022). The study also found mononuclear leukocyte stasis in sinusoids and local hepatocytes, edema, infiltration of inflammatory cells in the parenchyma, and glomerular necrotic (Bakiyev *et al*., 2022). Histological change of fish organs is also a typical characteristic of *Aeromonas* spp., for example, local hepatocyte necrosis in the liver (Abdelhamed *et al*., 2017; Chen *et al*., 2018).

Preventive measures can be used as an alternative effort in aquaculture management activities (Carballeira Braña *et al*., 2021). The utilization of immunostimulants to prevent infectious diseases from more popular. Several immunostimulants including lipopolysaccharides, glucan, peptidoglycan, vitamin C, and other natural ingredients have been reported extensively used in several species of aquaculture (Popoola *et al*., 2023; Rahardjo *et al*., 2022). Tilapia requires certain nutrients to meet its needs, such as protein, fat, carbohydrates, vitamins, and minerals. Nutrition is needed to produce energy and replace damaged cells for growth such as vitamin C in the right amount. Vitamin C

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deficiency can cause spinal bending, growth delays, and imbalances in the body. A study found the recommended levels of vitamin C and vitamin E for juvenile *Piaractus mesopotamicus* infected by *A. hydrophila* were 500 and 250 mg/kg of feed (Garcia *et al*., 2007).

However, until now, there has been no study revealing histopathological changes and survival rate (SR) in Tilapia infected by *A. hydrophila* with the supplementation of vitamin C in feed. This study will be very useful as initial information for related authorities regarding the management of aquaculture, especially tilapia through the addition of vitamin C in feed to prevent *A. hydrophila* infection based on histopathological changes of gills and kidneys, and survival rate.

METHODOLOGY

Ethical Approval

Test animals were not harmed or treated inappropriately during the research. The test animals in this study were given proper treatment by adapting to optimal environmental conditions which included air quality and feeding according to the needs of the test animals. This has been approved by the Institute for Research and Community Service (LPPM), Udayana University, and the Faculty of Marine Affairs and Fisheries, Udayana University through due diligence sessions and seminars.

Place and Time

This research was conducted in September – October 2022 at the Aquatic Animal Experiment Laboratory, Faculty of Marine Affairs and Fisheries, Udayana University. The tilapia fish (*O. niloticus*) used as test animals had an average size of 25 cm with a weight ranging from 200 – 250 gr obtained from the Mina Bakti Fish Cultivation Group, Serampingan Village, Tabanan Regency, Bali Province.

Research Materials

The materials and equipment used in this research included tilapia *(O. niloticus*), *A. hydrophila* isolate (ATCC, USA), Nutrient Broth (NB) (Merck, USA), sterile Aquadest (Merck, USA), 70% alcohol (Merck, USA), Nutrient Agar (NA) (Merck, USA), commercial fish feed (PT. Charoen Phokpand), Vitamin C in granule form (CSPC Pharma, China), Hematoxylin-Eosin (HE) dye (Merck, USA), histology instrument equipment, maintenance container, aerator (Yamano LP 60, Japan), ADB 200-4 analytical balance (Kern, Germany), thermometer, pH meter, Petri dish (Iwaki, Japan), Ose, GEA LS-50 HD autoclave, and Erlenmeyer (Iwaki, Japan).

Research Design

This study used a completely randomized design (CRD) consisting of 5 treatment groups and 3 replications with the following details:

Treatement Group A (Positive control): commercial feeds + *A. hydrophila* infection (10^6 CFU/mL); Treatement Group B (Negative control): commercial feeds without *A. hydrophila* infection (10^6 CFU/mL); Treatment Group C: commercial feeds + vitamin C supplementation (150 mg/kg) + *A. hydrophila* infection (10^6 CFU/mL); Treatment Group D: commercial feeds + vitamin C supplementation (300 mg/kg) + *A. hydrophila* infection (10^6 CFU/mL); Treatment Group E: commercial feeds + vitamin C supplementation (450 mg/kg) + *A. hydrophila* infection (10^6 CFU/mL).

Work Procedure

Fish Rearing Conditions

15 juvenile tilapia meeting the inclusion criteria (average weight of 14 grams with healthy and physical conditions) were used in this study. Tilapia was placed in a chlorine-cleaned glass aquarium measuring 80 × 40 × 30 cm filled around 25 L and equipped with aerators and tap water that has been dechlorinated where about 20% of the

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water is replaced every day. Tilapia were acclimatized for 2 weeks before the experiment was conducted and given commercial feeds during this period. The parameters of the water quality were measured and adjusted to the recommendations (Walter, 1961). The parameters namely dissolved oxygen levels, temperatures, ammonia, and nitrite content were monitored twice a day during the experimental period to adjust to the recommended value (Ibrahim *et al*., 2020).

Diet Preparation

Feed and vitamin C were mixed mechanically to be then converted to a pellet using a meat chopper. Pellet feed was aired with regular rotation to ensure uniform drying. Dry pellets were stored at 4 °C during the experimental period. Pellet Feed Was was given to Tilapia using hands until tilapia was full three times a day (09.00 am; 12.00 pm and 4.00 pm) for twenty days.

Culture Preparation and *A. hydrophila* Infection

The pure *A. hydrophila* isolates used in this study were culture collections from the Faculty of Marine Affairs and Fisheries, Udayana University. *A. hydrophila* isolates were prepared and propagated in a medium with 70% Tryptic Soy Broth (TSB) (Sigma-Aldrich, USA) then incubated at 37 °C for 24 hours. The concentration of *A. hydrophila* was set to 10^6 CFU/ml and calculated using a McFarland standard tube (Khalil *et al*., 2017). Bacteria as much as 0.1 ml was then injected intraperitoneally in tilapia to determine the survival rate and histopathological changes in the gills and kidneys during the seven days of rearing.

Survival Rate

The survival rate of tilapia can be calculated using the following formula (Rahardjo *et al*., 2022):

$$SR = \frac{Nt}{No} \times 100$$

Where:

SR = survival rate (%)

Nt = number of fish in each group after a feeding period of seven days post-infection

No = initial number of fish

Histological Examination

Histopathological preparations for gills and kidneys were prepared according to the procedures reported in the study of AlYahya *et al*. (2018) and Abdelhamed *et al*. (2017). The gills and kidneys of each treatment group were taken using a sterile dissecting set. Each organ was fixed in a 10% formalin solution to then dehydrated to eliminate formalin from the tissue with the alcohol solution (35; 70; 80; 80; and 90%) each for 1 hour. Purification was then carried out by placing a sample in xylol alcohol solution (1: 1) for 1 hour and Xylol I and Xylol II, each for 1 hour. Furthermore, infiltration of paraffin was carried out by soaking each sample in a mixture of xylol-paraffin (1: 1) for 1 hour, then pure paraffin 1 and pure paraffin 2 for 1 hour. All infiltration procedures were carried out at the Tissue Embedding Center at 56 ° C.

Sample embedding was carried out by instilling a sample in a paraffin block at 62 °C, then covered with a tissue cassette and left until it cools/hardens in the freezer for 2 to 24 hours. Sample sectioning and cutting were carried out using a microtome. The sample was placed in the microtome holder, then tidied and cut with a thickness of 5 microns. The paraffin band containing a sample was placed in a water bath at 45 ° C. After the sample expanded, the sample was removed and attached to the albumin glycerol-lubricated glass object. The sample was then incubated in the oven at 45 ° C for 24 hours. The sample was then colored with HE solution (hematoxylin-eosin). After that, mounting was carried out by closing the sample using cover glass. The sample was dropped with an entellan new, then covered with a

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cover, to then incubated in an oven at 45 ° C. The sample then was observed with a binocular microscope with a 400 × enlargement and the displayed image was taken using a digital camera.

RESULTS AND DISCUSSIONS

The results found a different survival rate of tilapia in each treatment group (Figure 1). The highest percentage of survival rate was found in group C at 86.67%, then group B at 76.67%, group D at 66.67%, group E at 53.33%, and group A at 36.67% after *A. hydrophila* infection. The optimum supplementation of vitamin C for protection against *A. hydrophila* infection was 150 mg/kg. A relevant study found that the supplementation of 400 mg/kg of vitamin C increased the survival rate of tilapia infected by *A. sobria*. The supplementation of vitamin C or combined with *Echinacea purpurea* (EP) significantly increased the survival rate and immunological response from tilapia (Rahman *et al*., 2018). Likewise, The Supplementation of Vitamin C in the feed can increase the resistance of young cobia *(Rachycentron canadum)* through an increase in the survival rate for bacterial infections (Zhou *et al*., 2012). Vitamin A supplementation of 3910 IU/kg of feed protects juvenile tilapia infected with *Streptococcus iniae* with a survival rate of 99% and the production of antibodies against this bacterium (Guimarães *et al*., 2014). Trials of feeding containing Vitamin C at a dose of 150 mg/kg for 70 days also significantly affected the growth and survival rate of GIFT tilapia (Baroi *et al.*, 2019).

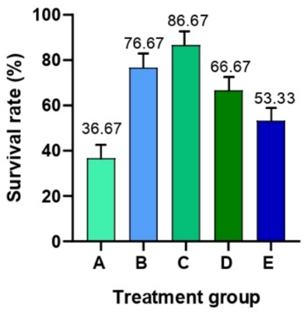

Figure 1. The survival rate of tilapia *(Oreochromis niloticus)*. A (commercial feeds + infected by 10^6 CFU/ml bacteria); B (commercial feeds); C (commercial feeds + 150 mg/kg vitamin C + infected by 10^6 CFU/ml bacteria); D (commercial feeds + 300 mg/kg vitamin C + infected by 10^6 CFU/ml bacteria); and E (commercial feeds + 450 mg/kg vitamin C + infected by 10^6 CFU/ml bacteria).

Vitamin C or ascorbic acid (AA) is an essential vitamin that can dissolve in water and is proven to improve the health and performance of aquatic animals (Dawood and Koshio, 2018). Vitamin C also acts as a natural antioxidant that has a strong category so it is useful in scavenging the level of reactive oxygen species (ROS) in the body of aquatic animals. These results confirmed that the supplementation of vitamin C influenced the resistance of tilapia to *A. hydrophila* infection. However, further study is still needed especially in measuring the ability of vitamin C to be combined with other natural ingredients for bacterial co-infection in tilapia.

Vitamin C can be involved in several biological aspects such as enzyme activity, hormone production, collagen production, and anti-stress oxidative activity. Most aquatic animals, especially those that are cultivated need an ideal supply of vitamin C because they cannot synthesize it due to a lack of L-Gulonolactone oxidase (Fracalossi *et al*., 2001). The recommended value for vitamin C in feeds varies greatly from 10 - 10,000 mg/kg. It

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should be noted that this is very dependent on the type of animal, age, size, and maintenance conditions (Chen and Chang, 1994).

Based on the observations on histopathology of gills and kidneys, in group A, tilapia has damaged gill lamella and kidneys, when compared to group B (without *A. hydrophila* infection) (Figures 2 and 3). Interestingly, the damaged gills and kidneys in other treatment groups were also still visible due to *A. hydrophila* infection (Figure 4). This is alleged because vitamin C has not been absorbed optimally in the body of the fish so the period of supplementation needs to be extended. On the other hand, the water quality and physiology of each fish can affect the severity of damage to the two organs. Another comprehensive study is still needed to test the oxidative stress marker associated with infection from this bacterium so that the action mechanism that results in organ damage can be explained in detail.

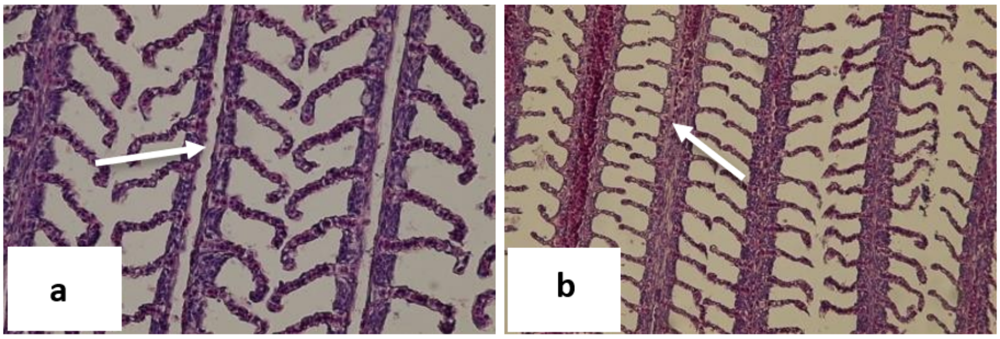

Figure 2. Histological change of gills in treatment group A (commercial feeds + *A. hydrophila* infection (10^6 CFU/mL)) and treatment group B (commercial feeds without *A. hydrophila* change). The arrows indicate the part that has changed in the form of widening of the lamellae on the gills (A) and normal gills (B).

In general, pathomechanism caused by bacteria raises clinical symptoms such as decreased appetite, bleeding in gills, enlarged abdominal fluid, exfoliation of scales, damage to tail fins, and swelling in internal organs (liver, kidney, and spleen). Gills are one of the vital organs in fish that interact directly with the external environment. If attacked by infectious diseases, gills will show changes to pale red. If it occurs in chronic conditions, the color of the gills will become more concentrated to be browned. Gills with physiological disorders will experience Telangiectasia or capillary blood dilation so that the fish have difficulty breathing.

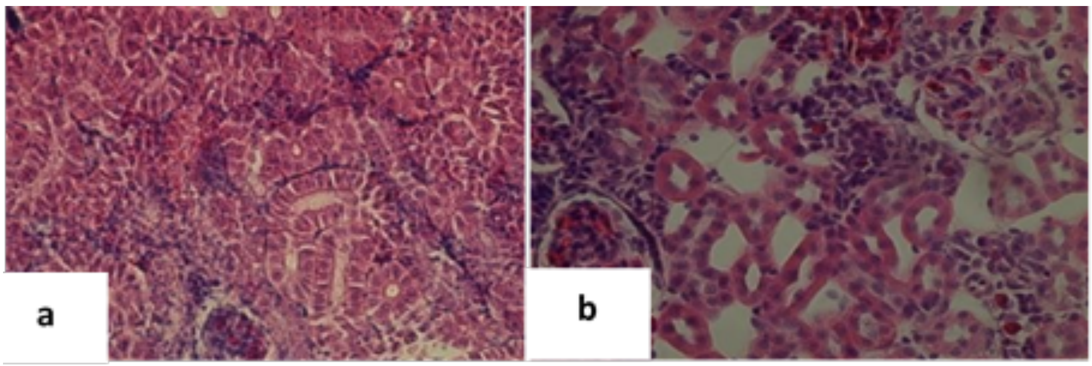

Figure 3. Histological change of kidneys in treatment group A (commercial feeds + *A. hydrophila* infection (10^6 CFU/mL)) and treatment group B (commercial feeds

without *A. hydrophila* change). Arrows indicate necrosis and swelling in tilapia kidneys (A) and normal tilapia kidneys (B).

Necrosis can be defined as uncontrolled cell death and is closely related to cell swelling and inflammation (Nikinmaa, 2014). Research has revealed the ability of *A. hydrophila* as an opportunistic pathogen in aquatic environments and capable of causing necrotizing fasciitis and gas gangrene in fish (Mohanty *et al*., 2022). *A. hydrophila* infection and spleen infection and kidney necrosis virus in Siniperca chuatsi can act antagonistically and synergistically. The study also confirmed that infection with the two pathogens resulted in serious clinical symptoms and clear histopathological changes (Liu *et al*., 2020). Other similar studies also revealed that *S. iniae* infection in tilapia causes a funnel-shaped renal corpuscle and a convoluted corpuscle which is a coiled canal (Nopilita *et al*., 2016). Necrosis that causes swelling of the glomeruli causes inflammation at the edges of blood vessels, and degeneration of epithelial cells in the kidneys caused by pathogenic infections (Agarwal *et al*., 2013).

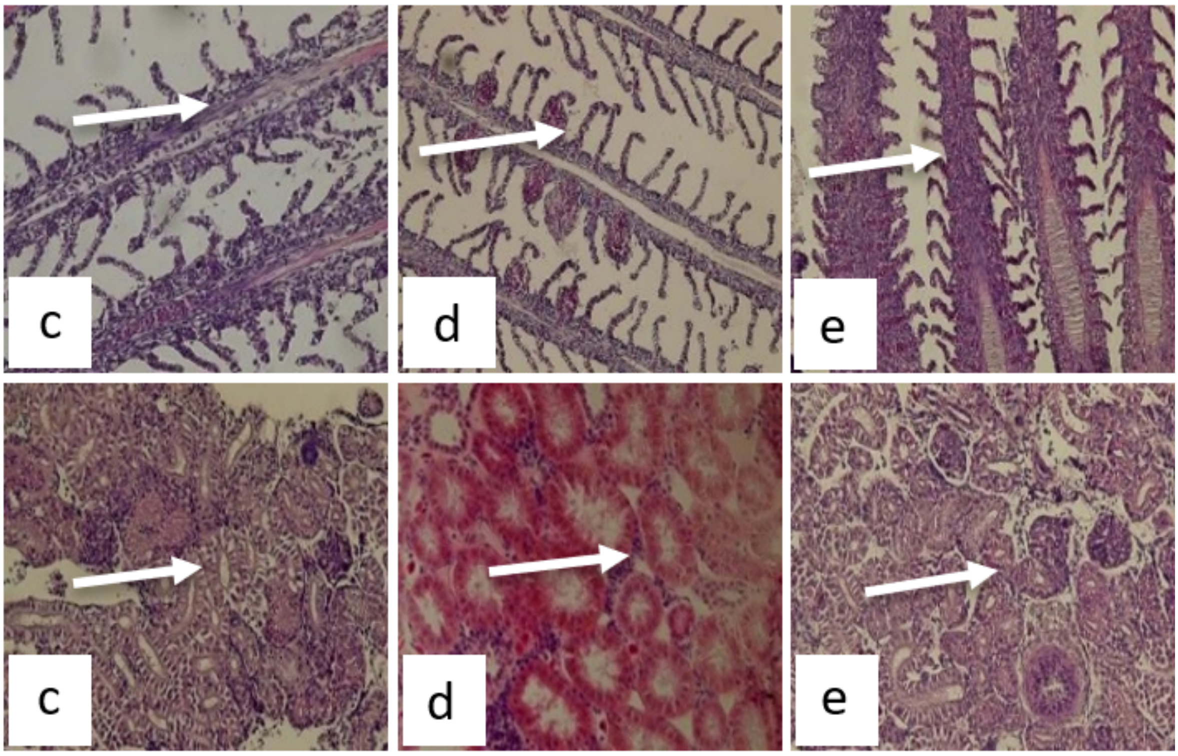

Figure 4. Histological change of gills and kidneys in Treatment Group C (commercial feeds + 150 mg/kg vitamin C + 10^6 CFU/ml *A. hydrophila* infection); D (commercial feeds+ 300 mg/kg vitamin C + 10^6 CFU/ml *A. hydrophila* infection); and E (commercial feeds + 450 mg/kg vitamin C + 10^6 CFU/ml *A. hydrophila* infection). The arrows indicate the gills and kidneys of tilapia which are not damaged by *A. hydrophila* infection and are given vitamin C in the feed.

CONCLUSION

Overall, the supplementation of 150 mg/kg of vitamin C on the feed was able to increase the survival rate of tilapia infected by *A. hydrophila* up to 86.67%. The damage to kidneys and gills causes them to widen, swell, and separate. However, the supplementation of vitamin C in tilapia can be used as a feed additive as a precautionary act against bacterial diseases such as *A. hydrophila*. Further

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study is still needed to assess the effectiveness of vitamin C in immunity, digestive enzyme activity, to the maintenance of oxidative stress due to *A. hydrophila* infection.

CONFLICT OF INTEREST

There is no conflict of interest in this manuscript between all authors upon writing and publishing the manuscript.

AUTHOR CONTRIBUTION

The contribution of each author is as follows: Dewa Ayu Angga Pebriani and I Ketut Wija Negara conceptualization, experimental design, and project acquisition. Ni Putu Putri Wijayanti and Putu Eka Sudaryatma collecting and formally analyzing data. Putu Angga Wiradana drafting, manuscript preparation, and revision.

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