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# The effectiveness of honey supplementation in feed for improving goldfish fingerling *Carassius auratus* immune system against *Aeromonas hydrophila* bacteria attack

# Efektivitas penambahan madu ke dalam pakan untuk meningkatkan daya tahan tubuh benih ikan mas koki *Carassius auratus* terhadap serangan bakteri *Aeromonas hydrophila*

**Rosidah[1*], Sriati[1], Ujang Subhan[1], Yuniar Mulyani[1], Rifai Dermawan[1,2]**

[1]Lecturer at Faculty of Fisheries and Marine Sciences, University of Padjadjaran, Jatinangor, West Java
[2]Faculty of Fisheries and Marine Sciences, University of Padjadjaran, Jatinangor, West Java
*Corresponding author: ros_ahdi@yahoo.com

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## ABSTRACT

The attack of *Aeromonas hydrophila* bacteria can cause mortality in goldfish approximately 100%. The controlling of this bacterial attack can be done through increased fish immunity. Honey is one of the natural ingredients that increases body immune system. This study aimed to determine the effective dose of honey supplemented in feed to increase goldfish fingerling resistance for disease prevention. Fish used in this study were goldfish fingerlings with 3.5 g average weight. This study was done using experimental complete randomized design method with five treatments and three replications. Treatments given were honey supplementation in feed with 0 ml/kg (A) as control treatment, 150 ml/kg (B), 200 ml/kg (C), 250 ml/kg (D), and 300 ml/kg (E). The result showed that honey supplementation in feed was effective to improve goldfish fingerlings resistance against *Aeromonas hydrophila* bacterial attack. The supplementation of honey in feed with 200 ml/kg was the best treatment for inducing goldfish fingerlings against *A. hydrophila*. This was proven by the increased white blood cells (leucocytes) (27.84 ± 5.07%) followed with no apparent clinical symptoms after attacked by *A. hydrophila*, such as hemorrhage, necrosis, exophthalmia or dropsy, besides showing the highest survival rate with 73.33 ± 11.5%.

Keywords : *Aeromonas hydophila*, goldfish, honey, leucocyte, resistance

## ABSTRAK

Serangan bakteri *Aeromonas hydrophila* dapat menyebabkan kematian ikan mas koki hingga mencapai 100%. Penanggulangan serangan bakteri tersebut dapat dilakukan dengan meningkatkan ketahanan tubuh (imun) ikan. Madu merupakan salah satu bahan alami yang dapat meningkatkan ketahanan tubuh. Penelitian ini bertujuan untuk menentukan dosis efektif penambahan madu pada pakan untuk meningkatkan daya tahan tubuh benih ikan mas koki dalam upaya pencegahan penyakit aeromonasis. Benih yang digunakan adalah benih ikan mas koki berukuran 3.5 gram. Metode penelitian yang digunakan adalah rancangan acak lengkap (RAL) dengan lima perlakuan dan tiga ulangan. Perlakuan yang digunakan adalah penambahan madu pada pakan dengan dosis 0 mL/kg (A) sebagai kontrol, 150 mL/kg (B), 200 mL/kg (C), 250 mL/kg (D), 300 mL/kg (E). Hasil penelitian menunjukkan bahwa penambahan madu ke dalam pakan efektif dalam meningkatkan ketahanan tubuh ikan mas koki terhadap serangan *Aeromonas hydrophila*. Dosis 200 ml/kg pakan memberikan hasil terbaik dalam meningkatkan ketahanan tubuh ikan mas koki terhadap serangan *A. hydrophila* terlihat dari peningkatan jumlah sel darah putih terbesar (27,84 ± 5,07%), tidak nampak adanya gejala klinis ikan terserang *A. hydrophila* seperti hemoragi, *necrosis*, *exophthalmia* maupun *dropsy* dan menghasilkan kelangsungan hidup benih ikan mas koki tertinggi yaitu sebesar 73,33 ± 11,5%.

Kata kunci: *Aeromonas hydrophila*, ketahanan tubuh, ikan mas koki, madu, sel darah putih.

## INTRODUCTION

*Carassius auratus* is a type of ornamental fish with the common name gold fish that has a diversed body shapes and varied colors such as red, yellow, green, black, and silver. Ornamental fish is served to be enjoyd for its beauty, therefore it is importat to manage its health condition. Disease prevention efforts need to be made early. One of the diseases that can attack both freshwater ornamental and consumed fish, killing up to 100% of the fish is caused by *Aeromonas hydrophila* bacteria infection. According to Lukistyowati and Kurniasih (2011), *A. hydrophila* bacteria greatly affect freshwater fish cultivation and often cause disease outbreaks with high mortality rate (80–100%) in a short time period of time (1–2 week). According to Laith and Najiah (2013), symptoms appeared after infection are increased respiration followed with pale gills, lethargic, skin lesions, discoloration, hemorrhage, and bruises or ulcers on the muscles. Some fish showed fins and genital orifice base bleeding. This infection also caused kidney dropsy, enlarged liver and bile, as well as yellowish fluid accumulation inside the body cavity.

*A. hydrophila* bacteria attack can be prevented by conducting some actions. One of preventive action is mixing feed with supplement as an immunostimulant for resisting the disease attack. Honey is known to be used as an immunostimulator, because it can stimulate lymphocyte cell activities as part of white blood cells. The existence of the lymphocyte activity shows an immune response to infection. This theory is based on the study result conducted by Senas & Linawati (2012), who reported that honey may increase immune system as seen on the proliferation of lymphocytes in test animal. According to Alzahrani *et al*. (2012), honey is known to have the ability as a strong antioxidant, inhibiting the free radical formations, thus protecting the cell components from harmful materials. Antioxidant compounds have closed relation with immune system as it helps to protect immune cells against free radical damages (Puertollano *et al*., 2011, Brambilla *et al*., 2008).

Vitamins contained in honey are thiamin (B1), riboflavin (B2), ascorbic acid (C), Pyridoxine (B6), B3, K, A, E, while minerals in honey are sodium (Na), calsium (Ca), potassium (K), magnesium (Mg), chloride (Cl), iron (Fe), zinc (Zn), niacin, Pantothenic acid, biotin, and folic acid (Bogdanov *et al*., 2008). Fish body needs vitamin C to boost its metabolism and endure environmental changes and diseases (Hemila, 2017, Carr & Maggini, 2017). According to Siswanto *et al*. (2013), Zn in honey enhances the body resilience by increased nucleic acid synthesize. According to Haase and Rink (2009), all immune cell activities are modulated by Zn, therefore Zn plays an important role as the immune system regulator. Immune enhancement supportive substance is related to Zn (Hegazi *et al*., 2009).

Honey also contains Fe which can improve the body immune system. In addition, the presence of organic compounds in the form of polyphenol, flavanoid, inhibin, and glycoside are antibacterial compound, damaging the bacterial cell wall, thus inhibiting or killing the bacteria (Rahman *et al*., 2010). According to Bairwa *et al*. (2012), plants with a lot concentration of phenol and flavanoid compounds are potentially utilized as immunostimulants.

The study conducted by Sunarto *et al*. (2008) reported that vitamin C in the honey increased the body resistance to stress on anabas fish. Vitamin C intake is reported to capable of making the body more resistance to an infection by enabling the production of interferon and leucocyte, besides maintaining inflammatory process (Tewary & Patra, 2008, Van Gorkom *et al*., 2018). Further study was conducted to determine the effective concentration of honey supplemented in feed to increase goldfish fingerlings defense body system, thus providing the best protection for disease prevention on gold fish fingerlings and improve the survival rate of the fish.

## MATERIALS AND METHOD

### Materials and equipments

Materials and equipments used were 15 pieces of aquarium, aerator, sprayer, thermometer, pH meter, DO meter, haemocytometer, 150 goldfish fingerlings aged two months with 3.5 g weight, honey, and *A. hydrophila* isolates. Goldfish fingerlings were obtained from Kalapa Ciung Cimalaka Fish Farmer Group, Sumedang. The honey used was retrieved from forestry department housing complex. *A. hydrophila* bacteria isolates as bacterial challenge test were taken from Center of Freshwater Aqauculture, Sukabumi.

### Experiment design

This study was conducted using complete randomized experimental design, which consisted five treatments and three replications. The

Table 1. Normal and infected *A. hydrophila* fish

| Normal fish | Infected fish | Observed body part |
|---|---|---|
| Complete fins with no body wounds | Wounded fins and body part (inflammation, hemorrhage) | All fin parts<br>Body surface |
| Normal eyes | Shattered eyes, whitened, exopthalmic | Exogenous eyes |
| Bright body color and skin | Darkened body color, hemorrhage and ulcer on skin | Body skin |
| Complete gills | Damaged gills | Gills |
| Responsive swimming | Slow swimming | - |
| Unswelling stomach | Swelling stomach | Fish ventral body part |
| Good feeding response | Anorexia | - |

Reference: Tantu *et al*. (2013)

treatments given were honey supplementation doze in feed, namely A treatment with 0 ml/kg feed (control), B with 150 ml/kg feed, C with 200 ml/kg feed, D with 250 ml/kg feed, and E treatment with 300 ml/kg feed.

## Procedures

*Honey supplementation in feed*

Honey given to the fingerlings was measured using measuring glass based on the treatment dozes given. Honey was then diluted using warm water with 2:1 ratio. Feed was measured according to the fish weight needs per week. Measured feed was mixed with diluted honey by sprayed method using a sprayer and air dried together.

*Test fish treatment application*

Ten test fish (goldfish fingerlings) which had gone through acclimatization process were kept in aquarium with 1 fish/L stocking (Ginting *et al*., 2014). The fish test were given feed with honey supplementation as much as 3% of body weight per day on twice a day feeding frequency, namely at 09.00 and 16.00 (GMT +7). Feed was given for 21 days.

*Test challenge*

The fish were challenged with *A. hydrophila* bacteria with $10^8$ CFU/ml density after given treatments for 21 days. The bacterial density was prepared by following these stages i.e. culture isolate was harvested using Ose needle and put into Falcon tube containing sterile NaCl 0.9%, then homogenized with vortex. Homogenized bacterial density was measured with spectrophotometer at 540 nm wave length under 0.235 absorbance (OD). NaCl 0.9% or isolates were gradually given until reaching 0.235 absorbance value (OD) as assumed that 0.235 OD was equivalent with $10^8$ CFU/ml or Mc Farland No.1 solution.

Challenge test was performed by infecting the fish test using immersion method until clinical symptoms emerged. Clinical symptoms observation and survival rate of test fish were done seven days after challenge test conducted (Wahyuningrum *et al*., 2013).

## Parameters

*The number of white blood cells*

The number of white blood cells in test fish were observed before and after challenged with *A. hydrophila* bacteria. Tesh fish were slashed on the posterior gills until bleeding. Blood was absorbed into Sahli pippette until 0.5 scale continued with Turk's solution until 11 scales, then whipped until homogeneous. Homogenous solution was dropped onto the haemocytometer and covered with a cover glass. White blood cells on the haemocytometer were observed under a microscope and calculated using the following formula (Nabib & Pasaribu, 1989):

*Clinical symptoms*

Clinical symptoms were observed after challenge test using *A. hydrophila*, containing fish physical condition, behavior alteration, and response against feed (Table 1).

*Survival rate*

$$\text{White blood cell} = \frac{\text{Counted white blood cell}}{5} \times 25 \times \frac{1}{\text{square volume}} \times \text{dilution factor}$$

Survival rate percentage of the test fish was observed after treatments given and challenge test for seven days. Survival rate was calculated based on Effendi (2004), as follows :

$$SR\ (\%) = \frac{Nt}{No} \times 100$$

Note :
SR : Fish test survival rate (%)
Nt : Number of fish test on final study
No : Number of fish test on initial study

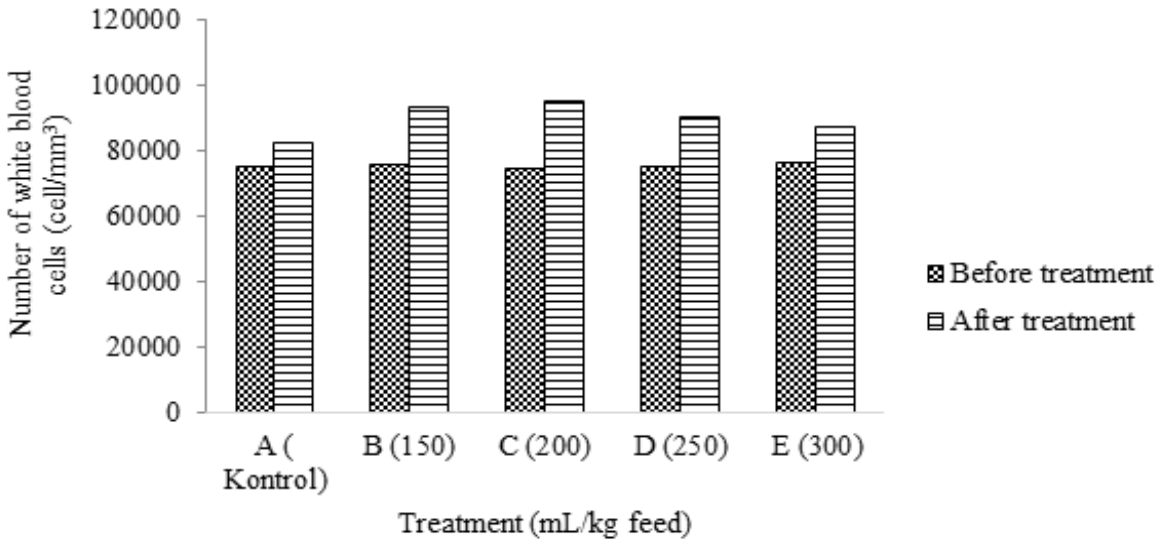


Figure 1. Gold fish fingerlings white blood cells on every treatment

Table 2. Increased number of white blood cells percentage

| Treatment (ml/kg) | Before (cells/mm³) | After (cell/mm³) | Difference (cell/mm³) | Percentage (%) |
|---|---|---|---|---|
| A (Control) | 75200 | 82733 | 7533 | 10.02 ± 4.08[a] |
| B (150) | 76133 | 93533 | 17400 | 22.85 ± 4.76[b] |
| C (200) | 74733 | 95533 | 20600 | 27.6 ± 5.07[b] |
| D (250) | 75533 | 90467 | 14934 | 19.8 ± 8.50[b] |
| E (300) | 76333 | 87333 | 11000 | 14.4 ± 5.05[a] |

Note : Different superscript letter on each percentage shows significant difference (P<0.05).

*Water quality*

Water quality was observed to control the rearing media water condition to be fixed and stabile for fish. Water quality parameters measured were acidity range (pH), temperature, and dissolved oxygen concentration (DO). Measurements were done three times during study, namely first, second, and third week.

**Data analysis**

Survival rate data was analyzed using F-test on 5% confidence level. Analysis was continued using Duncan's multiple range test whether the data showed any significant difference. White blood cells, clinical symptoms, and water quality were analyzed using descriptive method.

## RESULT AND DISCUSSION

**Number of white blood cells**

Number of white blood cells is observed to determine the amount of white blood cell alteration in gold fish fingerlings, which is closely related to the fish immune system. This is in accordance with Ouraji *et al*. (2015), who stated that the function of white blood cells are to attack pathogen by swallowing (phagocytosis) and form antibodies for protecting the body from disease. According to Biller-Takahashi *et al*. (2013), white blood cells (leucocytes) are natural defense system of fish body (innate immunity) indicator. Observations of white blood cells before and after given treatment can be seen in Figure 1.

Figure 1 shows the increased number of white blood cells before and after treatments given. Treatment B (150 mL/kg of feed), C (200 mL/kg of feed), D (250 mL/kg of feed) and E (300 mL/kg) showed great improvement than A treatment (control). This was allegedly due to the existence of honey supplementation in feed that influenced the number of white blood cells in gold fish fingerlings. Difference between increased numbers of white blood cells occured on all treatments can be seen in table 2 along with the percentage on each treatment.

Increased number of white blood cells on treatment A (control) was not due to the honey supplementation, but allegedly due to elevated age and weight of the fingerlings that were kept for 21 days. Elevated age in fish affected the blood system by the occurrence of increased number of blood grains along with increased white blood cell number. This statement was supported by Addass *et al*. (2012), who stated that factors

Table 3. Behavioral alteration and feed response

| Day | (A) | | | (B) | | | (C) | | | (D) | | | (E) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Replication | | | | | | | | | | | | | | |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 2 | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 3 | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| 4 | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - |
| 6 | + | + | + | - | - | - | - | - | - | + | + | - | + | + | + |
| 7 | + | + | + | + | + | + | - | - | - | + | + | + | + | + | + |

Note : (+) = Abnormal swimming and declined feed response; (-) = Normal swimming and feed response

affecting blood system on fish including age, weight, species, and environment.

The highest increased number of white blood cells was observed at treatment C (200 ml/kg) with 27.6%. This shows that the active ingredients contained in honey, such as Fe, β-carotene, Zn, and vitamin C was able to be absorbed by the fish body then elevating the lymphocyte level. The amount of active ingredients in 200 ml honey were 1.26 mg Fe, 7.2 mg β-carotene, 0.8 mg Zn, and 7.04 mg vitamin C (Bogdanof *et al*. 2008). According to Amar et al. (2008), β-carotene in immune system improves the proliferation response of T and B lymphocyte cells, stimulates T cells, and enhances macrophages and T cells production. Based on Pacheco *et al*. (2011), 100 mg/kg addition of β-carotene increased non-specific immune system on shrimp. According to Hernaman *et al*. (2013), 12.64 mg Zn supplementation into animal feed rations improved the antibody response in animal. Vitamin C improves the immune response, as the same with Tewary and Patra (2008), who stated that the intake of vitamin C makes the body more resistant to an infection by enabling the production of interferon, leucocyte, and inflammatory regulating process. This is supported by the result study by Garcia *et al*. (2011), that the supplementation of 500 mg vitamin C during 60 days increased the monocyte production constituting leucocytes and platelets specialized on fish.

High sugar level in honey can also inhibit the bacterial growth of bacteria (Carina *et al*., 2014). The various content of honey support each other for improving the fish immune system of the fish, thus served as as an immunostimulant. Biller-Takashashi *et al*. (2013) stated that increased number of leucocyte happened after fish were treated with immunostimulant. This was in line with the study research conducted by Pasaribu *et al*. (2015), who reported that the total white blood cells was increased after 7 days of immunostimulant supplementation in the form of henna leaves extract.

Treatment E had the lowest increased number of leucocyte (14.6%), compared to other treatments (B, C, and D), which was allegedly due to the high sugar levels in the treatment as the main component of honey. According to Nadhilla (2014), sugar levels contained in honey reached 95-99%. According to Hegazi *et al.* (2013), honey added into the fish body will increase the metabolic energy, however excessive addition will reduce feed intake. The result of this study showed reduced fish response to the feed along with induced honey concentration in the feed, which was significantly observed at treatment E. This consequently resulted in the lack of feed intake from honey, thus impacting and improving the number of leukocytes.

**Clinical symptoms**

Observation on clinical symptoms of gold fish fingerlings as test fish infected with *A. hydrophila* bacteria was done by noticing the fish behavior (swimming motion and feed response) and physical damages. The clinical symptoms of the test fish began to appear within 24 hours after *A. hydrophila* infection with 10 ml doze on each treatment containing $10^8$ CFU/ml. Clinical symptoms did not occur evenly on all fish due to the fish body resistance mechanisms.

Changes in fish behavior occured on the second to seventh day of post challenge test at treatment A (control) included unstablized swimming followed with declined feed response, marked as fish were in the stress state. This was in accordance with Lukistyowati and Kurniasih (2011), who reported that fish infected with motile aeromonas septicaemia would lose appetite in feed. The state of stress on fish reduced the fish body resilience and caused induced clinical symptoms onset massively. This was in accordance with Yardimci and Aydin (2011), who stated that *A. hydrophila*

Table 4. Gold fish fingerlings body damages on post challenge test with *A. hydrophila* bacteria

| Day | Clinical symptoms | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | (A) | | | (B) | | | (C) | | | (D) | | | (E) | | |
| | Replication | | | | | | | | | | | | | | |
| | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 |
| 1 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 2 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 3 | a | a | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 4 | ab | ab | a | - | - | - | - | - | - | - | - | - | - | - | - |
| 5 | abc | abc | abcd | - | - | - | - | - | - | - | - | - | - | - | - |
| 6 | abcde | abcd | abcde | - | - | - | - | - | - | - | - | - | a | a | ab |
| 7 | abcde | abcde | abcde | - | - | - | - | - | - | a | a | ab | abc | abc | abc |

Keterangan : a = hyperemia, b = hemorrhage, c = necrosis, d = exophtalmia, e = dropsy

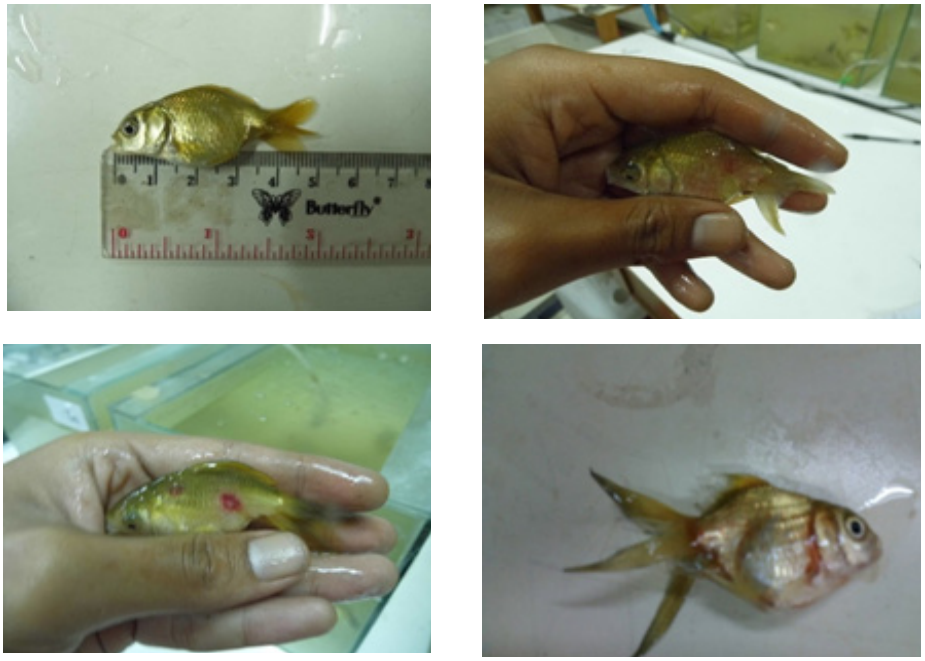

Figure 2. Clinical symptoms on gold fish fingerlings. (A) Normal gold fish; (B) hyperemic fish; (C) hemorrhagic fish; (D) abdominal dropsy, exopthalmic eyes, and fin erosion fish

bacterial attack would look more severe as the fish body resistance decreased due to stress. Behavioral alteration and feed response on all treatments are presented on Table 3.

Table 3 shows C treatment (200 ml/kg) is the best treatment compared all treatments, resulting normal swimming and feed response. This shows that fish at treatment C had good body defense, which was in line with the highest number of white blood cells, capable of inhibiting *A. hydrophila* bacterial attack. The supplementation of honey into the feed was useful as an immunostimulant that suppressed the activity of *A. hydrophila*. Based on the opinion of Selders *et al*. (2017), immunostimulant would increase the fish body defense by stimulating macrophages to produce interleukin, invigorating the lymphocyte cells to divide into T and B lymphocyte cells. T cells would produce interferon that was able to resurrect macrophages, phagocytizing bacteria, viruses, and other foreign particles entered into the body.

Clinical symptoms occured on the test fish at post challenge test were specifically different. Clinical symptoms started to appear on third day post challenge test in the form of red spots (Table 4).

Table 4 shows treatment A (control) had the most severe clinical symptoms compared to other treatments. Test fish was still healthy, yet showing a little clinical symptoms of *A. hydrophila* bacterial infection on first and second day (Figure 2a), then getting worse on the next day observation. Based on the behavior alteration, test fish on treatment

A also experienced early alteration (second day), then continued until the end of observation (Table 3). This happened because the test fish were not given honey that served as a supplement to increase the fish body defense, thus failing to fight *A. hydrophila* attack as only relying on the body natural defenses with insufficient level. Treatment A showed early clinical symptoms occured (third day) with reddish color at the tail base and and body surface (Figure 2b), then inflammation on the fifth day which was developed into hemorrhage (Figure 2c). Tissue damage (necrosis) occurred on the sixth and seventh day followed with protruding eyes (exopthalmia) and abdominal dropsy (Figure 2d). This condition was in accordance with Lukistyowati and Kurniasih (2011), who reported that the clinical symptoms on goldfish occurred due to *A. hydrophila* bacterial infection were hyperremia, inflammation, ulcers, and necrosis on the former bacterial injection area.

Hyperemia occured due to the mobilization of leucocyte as a resistance form after the attack of pathogenic bacteria. Matofani *et al*. (2013) stated that the increased number of leucocytes was allegedly due to enhanced cellular defense to prevent bacterial infection. Inflammation reaction occured as there was a decreased amount of leucocytes undergoing migration to the infected tissue experiencing lysis (Muller, 2011, Carr and Maggini, 2017). The weak body caused reduced disease response and low white blood cell phagocytic ability in response to the bacterial infection, resulting in hyperemia. Phagocytosis is the process of swallowing small foreign particles into some phagocytic cells, such as neutrophils and monocytes. According to Selders *et al*. (2017), neutrophil is the first defensive leucocytes in the body immune system. Neutrophil is the first to reach the inflammation area and host defense against initiated pathogens. Activation of neutrophils also played important role to fight infection effectively with monocytes and macrophages via phagocytosis and microorganisms or through expenditure component of inflammatory activity, such as radical oxygen, protease, or peroxidases (Craig *et al*., 2009). Hence, the addition of immunostimulant was aimed to stimulate the immune system and kill the organism causes infection, besides inactivation of bacterial attack by inducing the leucocyte activity. This was emphasized by the onset of no clinical symptoms occured at treatment B and C.

Sixth until seventh day at treatment D and E began showing clinical symptoms. This was suspected as the compiled feed remains that precipitates during observation with high content of honey, making the feed contain hugh sugar level that coulb be fermented by *A. hydrophila*. This was in accordance with Jayavignesh *et al*. (2011), who stated that *A. hydrophila* was able to ferment some sugars such as glucose, fructose, maltose, and trehalose. The fermented compound could be either acidic compound or acidic compound with gas. This would cause the fish suffered from stress, interrupting the fish body endurance against bacterial attack by occuring clinical symptoms in the form of hyperemia followed with hemorhage and necrosis. Based on the number of white blood cells in test fish at treatment D and E after given the treatment had lower white blood cells than treatment B and C (Figure 1; Table 2).

Treatment that did not show any clinical symptoms occured at treatment C (200 ml/kg) due to optimized fish body immune system to suppress the bacterial activity as seen in the high number of white blood cells observed at treatment C (Table 2). White blood cells are influenced by supplementing honey into the feed that is useful as an immunostimulant. Mechanism action for immunostimulant after entering the body will stimulate macrophages to produce interleukin which will invigorate the lymphocyte cells, dividing into T and B lymphocyte cells. T cells will produce interferon that is able to resurrect macrophages, phagocytizing bacteria, viruses, and other foreign particles entering into the body (Takahashi & Urbinati, 2014). In addition, lymphocyte produces antibodies as specific immune response against disease attack (Arhanari and Dhanapalan, 2016).

The content of vitamin C in honey improved body defense system by stimulating interferon. This same opinion was expressed by Van Gorkom *et al*. (2018), who mentiomed that vitamin C enhanced interferon and activity of immune cells, lymphocytes and macrophages. According to Maggini *et al*. (2016), vitamin C has the ability to modulate body immune system synergically synergistic supporting immune components either innate or adaptive immune system. According to Hemilä (2017), vitamin C reduced the variety of infections. Vitamin C provides beneficial effects on cellular functions of the innate and adaptive immune system, stimulating the neutrophil migration to the site of infection for enhanced phagocytosis (Carr & Maggini, 2017).

Yuniastuti *et al*. (2010) reported that Zn was effective as an immunostimulant. Prasad (2009)

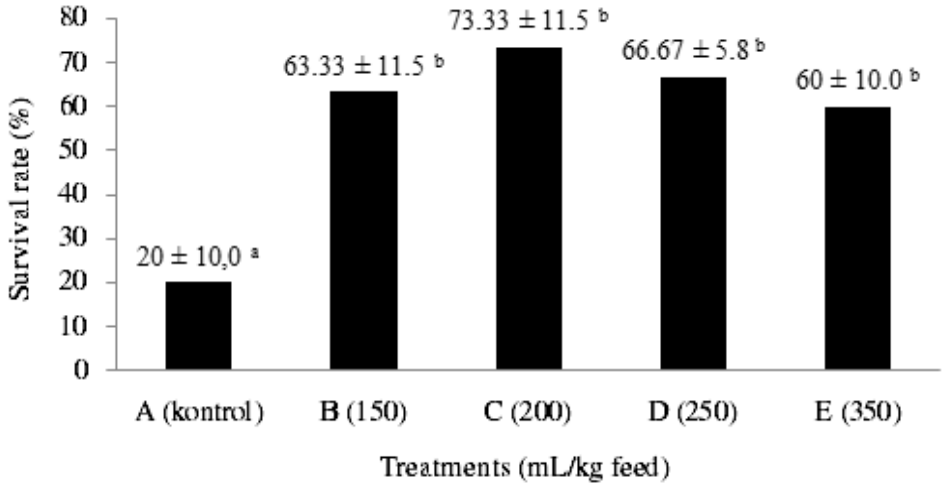


Figure 3. Survival rate of gold fish fingerlings. Note : Different superscript letter on each percentage shows significant

stated that Zn was important to the various development of the cellular immune system components, especially T ymphocyte cells and phagocytic activities. Zn levels in plasma decreased as there are any infections. Fe also plays important role in immunity as the material for lymphocyte cells formation. In addition, two kinds of Fe-binding proteins, lactoferrin and transferin, may prevent the infection occurrence by separating Fe from microorganisms, as it is required by microorganisms to grow. Fe deficiency will have an impact on the reaction of immune system in the form of decreased neutrophil activity, consequently declining the ability to kill intracellular bacteria significantly.

Honey is closely related as antibacterial, i.e. flavanoid organic compounds, whcih undermine the existence of bacterial cell wall, thus inhibiting the bacterial growth (Rahman *et al*., 2010). In addition, honey is also closely related as antioxidants as it contains β-carotene. β-carotene in immune system improves humoral immune system mechanisms regulating both cell that can prevent various disease (Amar *et al.* 2008, Pechinskii and Kuregyan 2014). Amino acid contained in protein honey is arginine to enhance the body immune system. This was in accordance with Briassouli and Briassoulis (2012), who mentioned that arginine increased the proliferation and metabolism of macrophages in the bone marrow for activating innate immunity response. Daslina *et al.* (2015) reported that arginine may boost the immune system by increasing the proliferation and function of macrophages. Nutrients in honey could be used as immunostimulant, making the fish unsusceptible to the disease as proven in this study.

**Survival rate**

Gold fish fingerlings was observed the survival rate after challenged with *A. hydrophila* for seven days showed different survival rate percentages among treatments given (Figure 3)

Treatment B (150 ml/kg), C (200 ml/kg), D (250 ml/kg) and E (300 ml/kg) gave the average survival rate higher than treatment A (control) after challenged with *A. hydrophila* bacteria. This proved that the active organic compounds contained in honey (polyphenol, flavanoid, and β-carotene), Zn, Fe, and vitamin C are capable of increasing the body defense against *A. hydrophila* attack with increased number of white blood cells (Table 2). High white blood cell level indicated the body resistance capability against *A. hydrophila* bacterial attack. According to Rahman *et al*. (2010) the active ingredients may damage the cell wall integrity, thus inhibiting or killing the bacteria. Therefore, *A. hydrophila* infection rate can finally be pressed. Mukti *et al.* (2009) reported that honey supplementation in the feed with various doses (0, 50, 100, 150, 200, 250, and 300 ml/kg ffed) may increase the survival degree of red claw crayfish.

Based on the behavioral alteration and clinical symptoms on post challenge test, fish on each treatment did not show significant damage. Dead fish found on the treatments (B, C, D, and E) were allegedly because the fish were not in a complete healthy state. Based on the survival rate data

Table 5. Temperature, pH, and DO range during rearing

| Treatment (ml/kg) | Parameter | | |
|---|---|---|---|
| | Temperature (°C) | pH | DO (mg/l) |
| A (Control) | 24–26 | 7.9–8.3 | 4.3–5.1 |
| B (150) | 24–26 | 7.9–8.4 | 4.1–4.8 |
| C (200) | 24–26 | 7.9–8.3 | 4.3–4.9 |
| D (250) | 24–26 | 8– 8.2 | 4.2–4.5 |
| E (300) | 24–26 | 7.9 – 8.3 | 4.2–4.9 |

analysis of variance after given treatments and challenge test, continued with Duncan's multiple range test with degree of confience 95% showed that treatment A (controls) differed significantly with other treatments (B, C, D and E), whereas other treatments had no significant difference on each other (Figure 3).

Figure 3 shows the test fish test on treatment of A, where fish gave no honey supplementation, had the lowest survival rate with 20%. The death of the test fish test on treatment of A showed clinical disorder due to ulcer, however some test fish also suffered death because of inflammation, necrosis, and hemorrhage (Table 4). Low survival rate on treatment A was observed as gold fish fingerlings sustained the bacterial attack by solely relying on only natural defenses system, which were unable to fight off the bacteria as seen on the low number of white blood cells (Table 2). According to Triyaningsis *et al*. (2014) and Igbinosa *et al*. (2012), *A. hydrophila* pathogenic bacteria was able to degrade the tissue organs, as well as removing the toxic spread throughout the body via the blood stream, resulting in redness color on the fish body, becoming abdominal ulcer. Exotoxin enzymes from *A. hydrophila,* such as protease and elastase, were suspected of causing damage to the infected body surface of the body, because of high protein content in the muscle tissue and blood vessel. Damages in blood vessels due to exotoxin production would result in hemorrhage on the fish body as the blood came out from the vessel. This happens as the fish body immune system was less responsive to *A. hydrophila* bacteria infection.

The addition of honey in treatment B (150 ml/kg), C (200 ml/kg), D (250 ml/kg) and E (300 ml/kg) did not give any significant difference towards the survival rate of gold fish fingerlings, however treatment C seemed to provide better results than other treatments as seen from the highest survival rate possessed (73.33%) with no clinical symptoms (Table 4) and increased number of white blood cells (Table 2).

**Water quality**

Water quality parameter observation was used as the supported data during this study. Water quality condition as gold fish fingerlings rearing media was in controlled state. Observations were conducted on the first, second, and third weeks of study. The range value of water quality parameters during this study on each treatment showed no different range value (Table 5).

Water quality measurement results obtained during study showed normal range quality for rearing gold fish fingerlings rearing. The observations indicated temperature range between 24 – 26 ° C, pH 7.9-8.4, and DO 4 – 5 mg/l. According to SNI (2011), the range of normal temperature for freshwater ornamental fish rearing should be 24 – 28 ° C, pH 6.5 – 9, and dissolved oxygen (DO) >3 mg/L.

## CONCLUSION

Honey supplemented in feed was effective to increase the immune system of goldfish fingerlings against *A. hydrophila* bacteria. Honey supplementation in feed with 200 mL/kg doze was the best treatment dosage to improve goldfish fingerlings body resilience, showing from increased white blood cells (27.84 ± 5.07%), no presence of clinical symptoms after *A. hydrophila* infection, such as hemorrhage, necrosis, exophthalmic eyes or dropsy, and produced the highest survival rate with 73.33 ± 11.50%.

## REFERENCES

Addass PA, David D, Edward A, Zira KE, Midau A. 2012. Effect of age, sex and management system on some haematological parameters of intensively and semi-intensively kept chicken in Mubi. Adamawa State, Nigeria. Iranian Journal of Applied Animal Science 2: 277–282.

Alzahrani HA, Boukraa L, Bellik Y, Abdellah F, Bakhotmah BA, Kolayli S, Sahin H. 2012. Evaluation of the antioxidant activity of three varieties of honey from different botanical and geographical origins. Global Journal of Health Science 4: 191–196.

Amar EC, Kiron V, Satoh S, Watanabe T. 2008. Effects of dietary β-carotene on the immune response of rainbow trout *Oncorhynchus mykiss*. Fisheries Science 66: 1068–1075.

Arhanari M, Dhanapalan S. 2016. Assesment of the haematological and serumbiochemical parameters of three commercially important freshwater fishesin river Couvery Velur, Namakkal district, Tamil Nadu India. International Journal of Fisheries and Aquatic Studies 4: 155–159.

Bairwa MK, Jakhar JK, Satyanarayana Y, Reddy AD. 2012. Animal and plant originated immunostimulants used in aquaculture. Journal of Natural Product and Plant Resources 2: 397–400.

Biller-Takahashi JD, Takahashi LS, Saita MV, Gimbo RY, Urbinati EC. 2013. Leucocytes respiratory burst activity as indicator of innate immunity of pacu *Piaractus mesopotamicus*. Brazzilian Journal of Biology 73: 425–429.

Bogdanov S, Jurendic T, Sieber R, Gallmann P. 2008. Honey for nutrition and health: a review. Journal of The American College of Nutrition 27: 677–689.

Brambilla D, Mancuso C, Scuderi MR, Bosco P, Cantarella G, Lempereur L, Di Benedetto G, Pezzino S, and Bernardini R**.** 2008. The role of antioxidant supplement in immune system, neoplastic, and neurodegenerative disorders: a point of view for an assessment of the risk/benefit profile. Nutrition Journal 7: 29.

Briassouli E, Briassoulis G. 2012. Glutamine randomized studies in early life: the unsolved riddle of experimental and clinical studies. Clinical and Developmental Immunology 2012: 1-17.

Carina L, Soledad V, Marina B. 2014. Antibacterial activity of honey: A review of honey around the world. Journal of Microbiology and Antimicrobials 6: 51–56.

Carr AC, Maggini S. 2017. Vitamin C and immune function. Nutrients 9: 1211.

Craig A, Mai J, Cai S, Jeyaseelan S. 2009. Neutrophil recruitment to the lungs during bacterial pneumonia. Infection Immunity. American Society for Microbiology 77: 568–575.

Daslina, Darwin E, Djamal AA. 2015. Pengaruh pemberian glutamin pada kemampuan fagositosis makrofag terhadap *Pseudomonas Aeruginosa*. Jurnal Kesehatan Andalas 4: 689–695.

Effendi MI. 2004. Metode Biologi Perikanan. Bogor: Penerbit Dwi Sri.

Garcia F, Pilarski F, Onaka EM, Moraes FR. 2011. Performance and hematology of pacu fed diets supplemented with vitamins C and/or E. Scientia Agriocola 68 : 314–319.

Ginting A, Usman S, Dalimunthe M. 2014. Effect of stocking density on survival and growth rate of goldfish *Carassius auratus* raised by recirculation system. Jurnal Aquacostmarine 5: 118–126.

Haase H, Rink L. 2009**.** Functional significance of zinc related signaling pathways in immune cells. Annual Review of Nutrtion 29: 133–152.

Hegazi AG, El Hady FKA, 2009. Influence of honey on the suppression of human low density lipoprotein (ldl) peroxidation (in-vitro). Journal of Evidence Based Complementary and Alternative Medicine 6: 113–121.

Hegazi A, Abdou AM, Abdallah F. 2013. Influence of honey on immune response against newcastle disease vaccine. International Journal of Basic and Applied Virology 2: 01–05.

Hemilä H. Review Vitamin C and infections. 2017. Nutrients 9: 339.

Hernaman I, Toharmat T, Tarigan S. 2013. Plasma minerals and antibody response in lambs exposed to transportation stress and fed diets supplemented with zinc and fish oil. Jurnal Bionatura 5: 216–226.

Igbinosa IH, Igumbor EU, Aghdasi F, Tom M, Okoh AI. 2012. Emerging *Aeromonas* species infections and their significance in public health. Scientific World Journal. 2012

Laith AR, Najiah M. 2013. *Aeromonas hydrophila*: antimicrobial susceptibility and histopathology of isolates from diseased catfish, *Clarias gariepinus* (Burchell). Journal of Aquaculture Research & Development 5: 1–7.

Jayavignesh V, Sendesh KK, Bhat AD. 2011. Biochemical characterization and cytotoxicity of the *Aeromonas hydrophila* isolated from Catfish. Archives of Applied Science Research 3: 85–93.

Lukistyowati I, Kurniasih. 2011. Kelangsungan hidup ikan mas *Cyprinus carpio* yang diberi pakan ekstrak bawang putih *Allium sativum* dan diinfeksi *Aeromonas hydrophilla*. Jurnal Perikanan dan Kelautan 16 : 144–160

Maggini S, Maldonado P , Cardim P, Newball CF and Latino ERS. 2016. Vitamins C, D and Zinc: synergistic roles in immune function and infections. Vitam Miner an open access journal 6 : 1–10.

Matofani AS, Hastuti S. Basuki F. 2013. Blood profile of tilapia kunti *Oreochromis niloticus* injected by *Streptococcus agalactiae* with different density. Journal of Aquaculture Management and Technology II: 64–72.

Mukti AT, Aprillia FT, Rahmahani J, Arief M. 2009. The addition supplement of honey in feed to increasing growth and survival rate of freshwater crayfish seed red claw *Cherax quadricarinatus*. Jurnal Ilmiah Perikanan dan Kelautan 2: 151–152.

Muller WA. 2011. Mechanisms of leukocyte transendothelial migration. Annual Review of Pathology: Mechanisms of Disease 6: 323–344.

Nabib R, Pasaribu FH. 1989. Patologi dan Penyakit Ikan. Departemen Pendidikan dan Kebudayaan, Direktorat Jendral Pendidikan Tinggi. IPB.Bogor. .

Nadhilla NF. 2014. The activity of antibacterial agent of honey against *Staphylococcus aureus.* Journal Majority 3: 94–101.

Ouraji H, Ahmadivand S, Eagderi S, Shamohammadi S. 2015. Effects of vitamin C and E administration on leukocyte counts in rainbow trout *Oncorhynchus mykiss*. Journal of Nutrition and Health 1: 1–5.

Pacheco R, Scencio F, Zarain M, Gomez G, Campa A. 2011. Enhancement of superoxide dismutase and catalase activity in juvenile brown shrimp, *Farfantepenaeus californiensis* (Holmes, 1900), fed β-1.3 glucan vitamin E, and β-carotene and infected with white spot syndrome virus. Latin American Journal of Aquatic Research 39: 534–543.

Pasaribu W, Longdong, SNJ, Mudeng JD. 2015. The effectiveness of the extract of balsam-weed leaves (*Impatiens balsamina* L.) in enhancing nonspecific immune response of Nile tilapia *Oreochromis niloticus*. Jurnal Budidaya Perairan 3: 83–92.

Pechinskii SV, Kuregyan AG. 2014. The impact of carotenoids on immunity (review). Pharmaceutical Chemistry Journal 47: 3–8.

Prasad AS. 2009. Zinc: role in immunity, oxidative stress, and chronic inflammation. Current Opinion in Clinical Nutrition and Metabolic Care 12 : 646–652.

Puertollano MA, Puertallano E, Cienfuegos GA, Pablo MA. 2011. Dietary antioxidants: immunity and host defense. Current Topics in Medical Chemistry 11: 1752–1766.

Rahman MA, Richardson M, Sofian. 2010. Antibacterial activity of propolis and honey against *Staphylococcus aureus* and *Escheria coli*. Journal of Microbiology Research 4: 1872–1878.

Selders GS, Fetz AE, Radic MZ, Bowlin GL. 2017. An overview of the role of neutrophils in innate immunity, inflammation and host-biomaterial integration. Regenerative Biomaterials 4: 55–68.

Senas KS, Linawati Y. 2012. Pengaruh pemberian madu hutan terhadap proliferasi limfosit pada hewan uji tikus jantan galur wistar. Jurnal Farmasi Sains dan Komunitas 9: 85–90.

Siswanto, Budisetyawati, Ernawati F. 2013. The roles of micronutrients on imunity system. Gizi Indonesia 36: 57–64.

Sunarto, Suriansyah, Sabariah. 2008. Effect of dietary vitamin C ascorbic acid on the growth performance and immune response of betok *Anabas testudineus* Bloch. Jurnal Akuakultur Indonesia 7: 151–157.

SNI. 2011. Syarat mutu dan penanganan ikan hias air tawar. Badan standar nasional/BSN SNI 7734.

Takahashi JDB, Urbinati EC. 2014. Fish Immunology. The modification and manipulation of the innate immune system: Brazilian studies. Annals of the Brazilian Academy of Sciences 86: 1483–1495

Tantu W, Tumbol R, Longdong S. 2013. Detection of the presence *Aeromonas* sp. on Nile tilapia cultured in floating net cage in lake Tondano. Budidaya Perairan 1: 74–80.

Tewary A, Patra BC. 2008. Use of vitamin C as an immunostimulant effecton growth, nutritionalquality and immune respone of Labeo rohita (Ham.). Fish Physiology and Biochemistry 34: 251–259.

Triyaningsih, Sarjito, Prayinto SB. 2014. Patogenisitas *Aeromonas hydrophila* yang diisolasi dari lele dumbo *Clarias gariepinus* yang berasal dari Boyolali. Journal of aquaculture Management and Technology 3:11–17.

Van Gorkom GNY, Wolterink RGJK, Van

Eissen CHMJ, Wietien L, Germeraad WTD, Bos GMJ. 2018. Influences of Vitamin C on Lymphocytes. Nutrients An Overviews 7: 34–44.

Wahyuningrum D, Astrini R, Setiawati M. 2013. Prevention of *Aeromonas hydrophila* on Catfish Juvenile Using Garlic and Shatterstone Herb. Jurnal Akuakultur Indonesia 12: 86 -94.

Yardimci B, Aydin Y. 2011. Pathological findings of experimental *Aeromonas hydrophila* infection in Nile tilapia *Oreochromis niloticus*. Veteriner Fakültesi Dergisi 58: 47–54.

Yuniastuti A, Nugrahaningsih WH, Zunikhah. 2010. Efektivitas seng (Zn) sebagai imunostimulan dalam produksi reactive oxygen intermediate pada mencit balb/c yang diinfeksi *Salmonella typhimurium*. Biosaintifika 2: 53–60.