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Effect of *Hermetia illucens* Larvae on the Hematology of Tilapia (*Oreochromis niloticus*) Infected with *Edwardsiella tarda*

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Abstract

Non-specific defense is the main defense in fish. One of the natural ingredients as a source immunostimulant is *H. illucens* larvae (HIL) with a protein content of up to 30%. Immunostimulants are biological compounds that can boost the immune system body. The essential amino acid content of HIL such as alanine as an energy substrate for leukocytes affects immune function in addition to the amino acid content in HIL, also serine could stimulate lymphocyte proliferation. The purpose of this study is to find out the effect of HIL in increasing immunity non-specific in tilapia (*O. niloticus*) which are infected with *E. tarda*. In giving HIL feed ad libitum with concentrations of A(30%), B(40%), C(50%), K(0%). In this research method, a completely randomized design (CRD) was used with 5 treatments and 3 replications in which each aquarium contained 10 tilapia. The parameters tested are leukocytes, differential leukocytes, erythrocytes, and hemoglobin. The most optimal results were obtained in treatment C (50%) because there was an increase in total erythrocytes up to $2.83x10^6$ cells/ml^3, while an increase in total leukocytes reached 39.910 cells/ml^3. On the parameters of monocytes and neutrophils, there was an increase of 12% and 17%, respectively. The results showed that intake of 50% HIL on tilapia could increase non-specific immunity, such as total leukocytes, total erythrocytes, differential leukocytes, and hemoglobin.

INTRODUCTION

Indonesia is the second major producer of tilapia worldwide, accounting for above 50.000 tons in 2018 (Mawardi *et al.,* 2023), a fish commodity with the highest production in aquaculture in Indonesia in the first quarter of 2022, namely 258.000 tons is tilapia (Nasyiruddin *et al.,* 2023). According to Phillips *et al*. (2015), Indonesia's largest tilapia production is on the islands of Sumatra and Java. However, the increasing production of tilapia is followed by the emergence of problems such as disease. According to Han *et al*. (2020), *E.*

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tarda can cause tilapia infection with mortality reaching 60%.

The natural resistance of tilapia against *E. tarda* through the innate and adaptive immune system. According to Mustafa and Al-Taee, (2020), the innate immune system as natural killer cells such as leukocytes is the first line of defense. However, if the fish's immune system has been defeated, farmers tend to give antibiotics as a form of treatment that has an impact on resistant bacteria and the accumulation of antibiotic residues in fish and the environment (Farooqi and Qureshi, 2018). Therefore, the use of immunostimulants that increase innate immunity in fish is considered a form of prevention.

HIL which are bioconverted with protein content reaching 38.22% (Widianingrum *et al*., 2021). The essential amino acid content of insects ranges from 46-96%. Alanine is an energy substrate for leukocytes that affects immune function in addition to the amino acid content in HIL, also serine could stimulate lymphocyte proliferation (Li *et al*., 2007). According to Sándor *et al*. (2022), insect meals have an immunostimulatory effect due to their high levels of the essential amino acid lysine (LYS), methionine (MET), and leucine (LEU), which are usually limiting in plants. Therefore, HIL is considered suitable as an immunostimulant with complex amino acids. Thus, this research purpose was to determine the impact of HIL on the hematology of tilapia infected with *E. tarda*.

METHODOLOGY

Ethical Approval

The Research Ethics Commission, University of Brawijaya has carefully studied the research design and provided ethical approval (194-KEP-UB-2023).

Place and Time

This research was conducted in January-April 2022. The proximate test was carried out at the Nutrition Laboratory, Faculty of Animal Husbandry, Brawijaya University. The hematology test for tilapia was carried out at Fish Cultivation Laboratory, Fish Reproduction Division, Brawijaya University.

Research Materials

The materials used in this study were HIL, commercial feed (CF) using CP Prima 781-1, tilapia (±10 cm and ± 7,81 g in weight), and *E. tarda*. The bacteria were cultured on TSA and TSB (Oxoid), hayem's solution, Turk's solution, and Giemsa's solution. While tools used are aquarium size 50×40×40 cm, microscope (Olympus C×41), autoclave (GEA LS-B100), incubator (RED Line), aeration, sectio set, syringe, EDTA tube, glass preparations, hemocytometer, and centrifuge.

Research Design

The research design used an experimental method with a Completely Randomized Design (CRD) which was divided into 3 treatments, 2 controls and each consisted of 3 replications. The placement of treatment containers and replications was carried out randomly (Hanafiah, 2012). The method used in this study is experimental validation or testing, namely testing the effect of one or more variables on other variables. The data collection technique in this study used a descriptive method based on molecular studies. The descriptive method describes phenomena that occur naturally without any intervention from an experiment (Hamdi and Bahruddin, 2015). Dose determination based on research by Meitiyani and Erwin (2017) based on Table 1 and applied to tilapia reared for 30 days with a total of 10 fish/pond. The method of feeding ad libitum for 2 times a day (Amoush *et al.*, 2022) is as follows:

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Table 1. HIL dosage and infection treatment.

Treatment	HIL (%)	CF (%)	Infected *E. tarda*
A	30	70	+
B	40	60	+
C	50	50	+
K+	-	100	+
K-	-	100	-

Work Procedure

H. illucens Larvae

HIL was obtained from Talun, Blitar, Indonesia. Larvae were harvested from residents who did bioconversion and were fed tofu dregs for 14 days. Tofu dregs fermentation will accelerate decay and produce amino acids that can remodel fiber and food essence in feed so that protein is easily absorbed by HIL (Rofi *et al*., 2021). The research treatment was obtained from a combination of HIL flour and CF from CP Prima-781 feed. CF was ground into powder then mixed with 3% tapioca flour as an adhesive added HIL powder and finally dried by placing it in the sun.

Proximate Analysis

Proximate analysis for HIL was carried out in Simplicia according to the Standard Operating Procedure (SOP) of the Nutrition Laboratory of the Faculty of Animal Husbandry, Universitas Brawijaya, Malang.

Amino Acid

Amino acid analysis for HIL was carried out in Simplicia according to the Standard Operating Procedure (SOP) of Saraswanti Indo Genetech, Surabaya.

Lethal Dose 50 (LD_{50})

E. tarda was obtained from the Fish Quarantine Standard Testing Center, Quality Control and Safety of Fishery Products, East Jakarta. LD_{50} was carried out with bacterial density 10^5, 10^6, 10^7, 10^8 cells/ml[3].

Erythrocyte

Blood that has been given anticoagulant is sucked with a hemocytometer pipette to the 0.5 mark, then the hayem solution (for erythrocytes) is sucked up to the 101 mark. To mix the blood evenly, the pipette is shaken to form a figure of eight for 3-5 minutes. After that, two drops of blood were removed to remove air cavities, then the blood was dripped into the hemocytometer box and covered with a cover glass, then the number of erythrocytes was observed using a microscope in 5 small boxes on the hemocytometer, based on the formula Zissalwa *et al*. (2020) as follows:

Total Erythrocytes = $N \times 10^4$

Where:

N : the number of erythrocytes in 5 visual fields

10^4 : dilution factor

Leukocyte

The total leukocyte count was carried out through the blood in the vacutainer that had been mixed with EDTA sucked with a pipette up to the 0.5 mark and the tip of the pipette was cleaned with a tissue. Then the Turk solution was sucked in with the same pipette until it reached the number 11. The pipette was then shaken for about three minutes until homogeneous. Next, two or three drops of the solution are discarded before being put into the counting chamber. After that, leukocytes were counted using a magnification of 10 or 40 times on the objective lens (Lubis *et al*., 2016) then leukocytes were calculated using the formula based on Arlanda *et al.* (2018) as follows:

Total Leukocytes = $N \times 50$

Where:

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N : the number of leukocytes in the 4 large boxes of the counting room
10^4 : dilution factor

Differential Leukocytes

The differential calculation of tilapia leukocytes was carried out by observing blood smear preparations. The preparation of smear preparations was carried out with two glass objects to form a thin layer of blood, then the preparations were fixed with 95% methanol for 5 minutes, then removed and allowed to air dry. The preparations were stained with Giemsa's solution for 15 minutes. Then removed, rinsed, and allowed to air dry. The finished preparations were then placed under a microscope and observed with a magnification of 400 times. The leukocyte differential (lymphocytes, monocytes, and neutrophils) was counted up to 100 leukocyte cells, and then the number of differential leukocytes was counted based on Salim *et al*. (2016), as follows:

Lymphocytes = number of cells × 100%
Monocytes = number of cells × 100%
Neutrophils = number of cells × 100%

Hemoglobin

The calculation of hemoglobin levels was carried out by referring to the Sahli method. Hemoglobin levels were measured using a salinometer tube filled with 0.1 N HCl solution to 0 (the bottom line on the salinometer tube), then the tube was placed between 2 tubes with standard colors, then fish blood was taken from the microtube tube with a Sahli pipette. as much as 0.02 ml and put into the Sahli tube and allowed to stand for 3 minutes. Then add distilled water with a dropper droplet little by little while stirring with a glass stirrer until the color is the same as the standard color. Hemoglobin levels are expressed in g/dL (Zissalwa *et al*., 2020).

Calculation of erythrocyte, leukocyte, and hemoglobin using the ImageJ application. ImageJ is an application that can be used to measure objects by comparing the number of pixels with a scale bar of known length. Measurement of the number of pixels on a scale bar whose length is known is done by drawing a line of a certain length when using the straight line tool (Hardian *et al*., 2020).

Survival Rate

The survival rate of test fish according to Hasanah *et al*. (2019) compares the number of living test fish at the end of the study with the number of test fish at the beginning of the study which can be calculated using the following formula:

$$SR = \frac{Nt}{N0} \times 100\%$$

Where:
SR : Degree of survival of fish (%)
Nt : Number of fish at the end of maintenance
N0 : Number of fish at the beginning of maintenance

Water Quality

The water quality parameters observed were temperature, pH, and dissolved oxygen. Water quality measurements were carried out every day during the maintenance period, namely in the morning and evening.

Data Analysis

The data results were then analyzed using the One-Way ANOVA test. The assumption of the analysis of variance to be carried out is homogeneity of variance. Furthermore, the measurement of the total variability of the existing data can be grouped into three parts, namely between groups, within groups, and totals. Post Hoc follow-up analysis was Duncan because it tested the differences between all the treatment pairs available from each trial and still maintained the set level of significance.

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RESULTS AND DISCUSSION

Proximate Analysis

The dry matter which is high enough up to 91.89% can be caused by roast the larvae beforehand to kill and preserve the larvae also makes it easier for the larvae to be mixed into feed. The protein in the larvae reached 30.52% based on Table 2, it was found that treatment C had the highest protein content (31.32%) and CF had a fairly low protein content ranging from (28.63%).

Table 2. Proximate result of HIL and feed mixture.

Sample	Dry Matter (%)	Ash (%)	Crude Protein (%)	Crude Fiber (%)	Crude Fat (%)
A (30%)	91.16	10.49	29.20	7.04	12.98
B (40%)	88.95	11.91	30.25	7.59	12.99
C (50%)	88.73	11.82	31.32	6.88	13.09
CF	88.38	10.40	28.63	8.78	13.33
HIL	91.89	14.93	30.52	7.79	34.03

According to Iskandar and Elrifadah (2015), the feed needed by fish is feed that has a protein content of 20-60% while the optimum is between 20-25%. So the protein needs of tilapia have been fulfilled using HIL. The protein content possessed by maggots comes from the protein found in the maggot growing media, this is because maggot has a storage organ called trophocytes which functions to store the nutrient content contained in the culture media it eats so that the protein that enters the fish's body makes the fish grow get high (Sepang *et al.*, 2021). The protein in the larvae reaches 30.52% whereas the live media of tofu dregs already contains 12.77% protein (Putri and Sumardiono, 2020).

Amino Acid

The results of HIL and CF amino acid analysis are presented in Table 3.

Table 3. Amino acid on HIL and CF.

Amino Acid	HIL (%)	CF (%)
Essential		
Histidine	1,29±0,01	0,60
Arginine	2,18±0,01	1,81
Threonine	2,13±0,01	1,28
Valine	2,56±0,01	1,25
Isoleusine	1,82±0,01	1,02
Leusine	2,83±0,01	2,11
Lisine	2,87±0,02	1,40
Fenilalanine	1,93±0,01	1,20
Non Essential		
Serine	2,15±0,01	1,75
Glisine	2,84±0,01	2,16
Alanine	2,83±0,01	1,51
Proline	2,25±0,01	2,01
Tirosine	2,23±0,01	0,67
Aspartatic Acid	4,00±0,02	2,15
Glutamate Acid	5,70±0,03	4,35

Fermentation resulting from tofu waste will accelerate decay and produce amino acids that can remodel fiber and food essence in the feed so that protein can be easily absorbed by HIL (Rofi *et al.*, 2021). Insect essential amino acid content scores ranged from 46 to 96%. Alanine is an energy substrate for leukocytes that

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affects immune function in addition to the amino acid content in HIL such as serine which stimulates lymphocyte proliferation (Li *et al*., 2007). The first limiting essential amino acid in most insects is lysine (Verkerk *et al*., 2007). The most dominating amino acids in HIL are presented in Table 3. are glutamic acid and aspartic acid. One of the important roles of glutamic and aspartic acid is to accelerate wound healing (Chasanah *et al*., 2015).

Lethal Dose 50

LD_{50} analysis was carried out with bacterial densities of 10^5, 10^6, 10^7, and 10^8 cells/ml, it was found that the LD_{50} was 10^6 cells/ml for 72 hours with the death of 15 fish from 30 tilapia. So the density of bacteria used in this study was 10^6 cells/ml.

Total Erythrocytes

After 30 days of maintenance, the total erythrocyte yields before and after infection with *E. tarda* bacteria were obtained by feeding HIL (Figure 1).

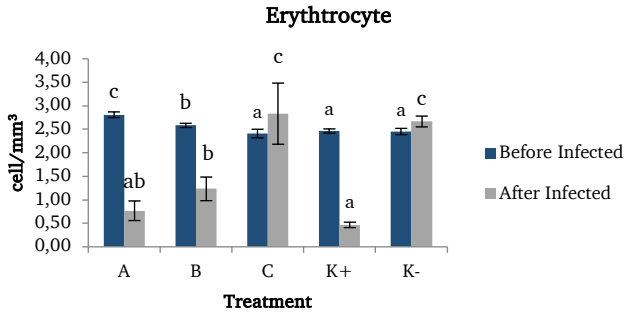

Figure 1. Erythrocyte result of tilapia fed HIL.
Description: Different alphabets (a,b,c) indicate significantly different means for different groups of diets at $p<0.05$. A(30% HIL); B(40% HIL); C (50% HIL); K+(0% HIL); K- (0% HIL, not infected).

Based on Figure 1, it can be concluded that before infection treatment A had the highest yield followed by treatment B. Treatment C, K+, and K- had results that were not significantly different but all treatments were in the range of $2.41\text{-}2.81\times10^6$ cells/mm^3. Treatment K+ and treatment A were the lowest yields on tilapia erythrocytes. According to Hassan *et al*. (2020) the presence of hemolysin toxin in *E. tarda* which causes the lysis of erythrocyte cells which affects the supply of food to cells, tissues, and organs will be reduced, and the metabolic process of fish becomes inhibited (A'yunin *et al*., 2020), followed by low or the absence of the addition of immunostimulants to increase the fish's immune system. According to Maryani and Rosdiana (2020), immunostimulants can improve the fish's immune system. Feed without immunostimulants causes the immune system to lose, and erythrocyte cells lyse/burst.

The process of forming red blood cells is influenced by several factors, and one of them is influenced by the hormone erythropoietin, which is a hormone produced by the kidneys to trigger the process of forming red blood cells (Sianturi *et al*., 2013). Where *E. tarda* tends to attack the digestive organs, including the kidneys, which produce hormones that form red blood cells, causing anemia (Liu *et al*., 2014) as in the K+ and A treatments. The normal level of total erythrocytes in the body of tilapia is $1.05\times10^6 - 3.0\times10^6$ cells/mm^3 (Royan *et al*., 2014). Treatment C is the treatment with the best results. This is presumably because the levels of amino acids in the feed consumed are to the needs of the fish.

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According to Rosita *et al*. (2019), the outer structure of erythrocytes in the form of hemoglobin is composed of amino acids and iron for erythropoiesis so that enough amino acids are needed by fish, where hemoglobin is closely related to erythrocytes so that the production of red blood cells is not hampered. The normal level of total erythrocytes in the body of tilapia is $1.7\pm0.45\times10^{6}$ cells/mm^3 (Sukenda *et al*., 2018).

Total Leukocytes

The results of total leukocytes in tilapia treated with HIL can be seen in Figure 2.

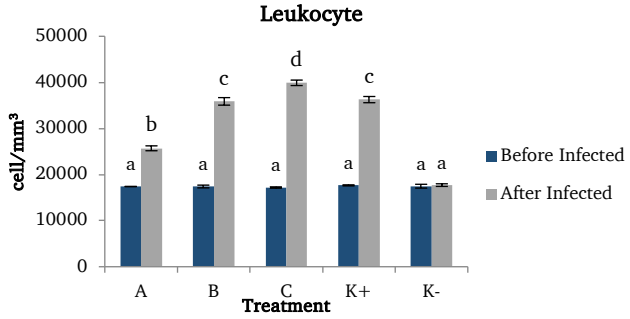

Figure 2. Leukocyte result of tilapia fed HIL.
Description: Different alphabets (a,b,c) indicate significantly different means for different groups of diets at p<0.05. A(30% HIL); B(40% HIL); C (50% HIL); K+(0% HIL); K- (0% HIL, not infected).

The results showed that there were no significant differences in all treatments with a range of 17,190-17,494 cells/mm^3 which was still included in the normal erythrocyte range. The normal range of tilapia leukocytes is 16,240-24,620 cells/mm^3 (Riauwaty and Syawal, 2016). According to Maftuch *et al*. (2012), the increase in the number of white blood cells occurs because the fish are trying to increase their body resistance to bacterial infection so that leukocytes move actively towards the infected site. In treatments A and K+, the total leukocyte count was low, this was presumably due to the virulence of *E. tarda*. According to Xie *et al*. (2015), *E. tarda* can attack and even replicate in phagocytic cells or primary macrophages of fish.

Effector eseJ is an effector that supports the process of replication in macrophages and inhibits ROI (Reactive Oxygen Intermediate) which helps the process of phagocytes. Based on this statement, fish need immune system-enhancing stimuli such as immunostimulants that can increase the number of leukocytes in the fish's body as a first line of defense. According to Prasetya (2015), *E. tarda* bacteria are Gram-negative bacteria with cell wall components in the form of LPS. This cell wall component contains peptidoglycan which can bind to macrophage cells in the tissue so that it will trigger the secretion of proinflammatory cytokines with omega-6 from HIL as a medium.

In treatment C, 50% HIL got the highest yield, which was 39,910±596.5 cells/mm^3. This shows that treatment C is optimal in increasing leukocyte cells in the fish body. Provision of HIL which contains complex amino acids that affect leukocytes in fish such as alanine as an energy substrate for leukocytes that affects immune function in addition to amino acid content in HIL such as serine which stimulates lymphocyte proliferation (Li *et al*., 2007). The normal range of tilapia leukocytes is 16,240-24,620 cells/mm^3 (Riauwaty and Syawal, 2016). Results of total leukocyte tilapia after intake of HIL before and after *E. tarda* infection (Table 4).

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Differential Leukocyte

The results of differential leukocytes in tilapia fed HIL in Figure 3 before infection showed lymphocyte results were not significantly different in all treatments with a range of 81±0.58-83±1.00%. Normal levels of lymphocytes in tilapia range from 60-80% (Riauwaty and Syawal, 2016). However, the results of lymphocytes after infection showed that the graphic results decreased from the pre-infection treatment with the highest results from treatment A and K+ and the lowest being treatment C which was significantly different. It is suspected that *E. tarda* induces systemic immunosuppression through lymphocyte apoptosis, which suppresses the systemic immune response during the early stages of septicemia (Park *et al*., 2012).

The resistance activity of white blood cells causes a decrease in the number of lymphocyte cells because these components function to provide immune substances for the body's defense. An increase in the intensity of infection by certain pathogens will trigger an increase in the need for white blood cells, especially phagocytic cells. This increased need results in a reduction in the number of cells providing immune substances, namely lymphocytes (Taukhid *et al*., 2010).

The number of monocytes in the white blood cell population is small, but the number will increase if there are foreign substances in the tissue. The normal level of tilapia monocytes is 3-5% (Arlanda *et al*., 2018). Monocytes in tilapia after infection have increased with the best results from C treatment, this is presumably because monocytes play a role in phagocytosis of antigens that enter the body and provide information about infectious diseases to leukocytes (A'yunin *et al*., 2020). According to Wulandari *et al*. (2018), monocyte cells can provide themselves quickly in inflammatory areas, consuming wound-causing agents in the event of an attack of a disease, so that monocytes can increase drastically.

The normal proportion of neutrophils in fish blood is 6-8%. The high number of neutrophils in tilapia infected with *E. tarda* indicates an immune system response to fight infection. This follows the statement of Utami *et al*. (2016), that the increase in the number of neutrophils is the result of an immune mechanism that works in response to an infection in the body which indicates a phagocytosis process (A'yunin *et al*., 2020). An increase in the percentage of the number of neutrophils is thought to be due to stimulation by immunostimulants so that the production activity by these cell-forming organs increases (Taukhid *et al*., 2010).

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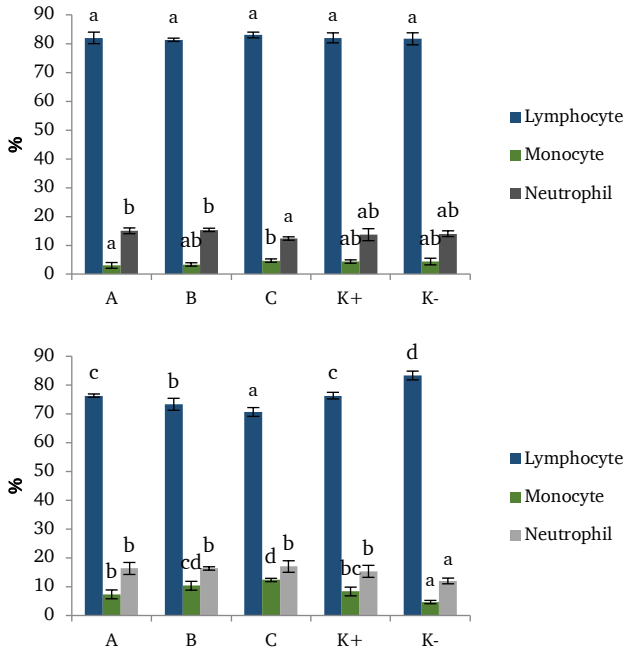

Figure 3. The results of differential leukocyte before infected *E. tarda* (up) and after infected *E. tarda* (down) of tilapia fed HIL.

Description : Different alphabets (a,b,c,d) indicate significantly different means for different groups of diets at $p<0.05$. A(30% HIL); B(40% HIL); C (50% HIL); K+(0% HIL); K- (0% HIL, not infected).

Hemoglobin

The results of hemoglobin in tilapia fed HIL in Figure 4 before infection had results in the range of 8.23±0.23-8.74±0.21G%. The normal range of tilapia hemoglobin is 5.05-8.33 G% (Royan *et al*., 2014). In treatments A and K+, low hemoglobin results were obtained, this is presumably because hemoglobin is a protein in red blood cells that contains a cofactor with heme which plays an important role in transporting and storing oxygen (Zhao *et al*., 2021). Hemoglobin is closely related to erythrocyte levels in the body, where the total erythrocytes in fish bodies also have low results in the A and K+ treatments.

The decrease in the value of hemoglobin in the blood is related to the low value of erythrocytes, which is thought to be because the fish undergoes lysis in the blood. Lysis is caused by the rupture of red blood cells due to the presence of a bacterial toxin, especially *E. tarda* in the blood, called hemolysin. This toxin will lyse hemoglobin and release hemoglobin. Low hemoglobin levels can be an indication in fish of infection, in this case, bacteria (Minaka and Hastuti, 2012). In treatment C, the results were not significantly different from the treatment before infection because the immunostimulant from HIL was successful in phagocytosing *E. tarda* bacterial cells.

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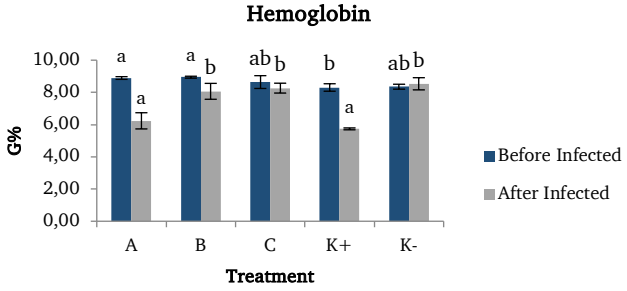

Figure 4. Hemoglobin result of tilapia fed HIL.
Description: Different alphabets (a,b,c,d) indicate significantly different means for different groups of diets at p<0.05. A(30% HIL); B(40% HIL); C (50% HIL); K+(0% HIL); K- (0% HIL, not infected).

Survival Rate

The results of the survival rate in tilapia fed with HIL in Table 4 after infection had results in the range of 40-93%. The results of the lowest tilapia survival rate were found in the K+ treatment and the highest in the C treatment. Factors affecting the survival of the fish were external factors and internal factors. External factors were caused by bacterial infection from *E. tarda* which was able to cause mortality up to 60% in tilapia (Ibrahem *et al*., 2011), which was seen in the results of K+ treatment which had a survival rate of 40%.

In treatment C, the highest results were obtained after being infected with *E. tarda* bacteria which showed that the immunostimulant of HIL succeeded in increasing the fish's immune system against bacteria and had a good ability to utilize food.

Table 4. The results of the survival rate of tilapia after intake of HIL before and after infection with *E. tarda*.

Treatment	Fish Before Infected	Fish After Infected	Survival rate (%)
A	30	14	47
B	30	21	70
C	30	30	100
K+	30	11	40
K-	30	30	100

Water Quality

The results of water quality in tilapia ponds fed with HIL within a 30-day rearing period are presented in Table 5.

Table 5. Results of tilapia pond water quality for 30 days.

Parameters	A	B	C	K+	K-
Temperature (°C)	25.16	25.11	25.05	25.09	25.14
pH	7.03	7	7.03	7	7
DO (mg/L)	6.9	6. 14	6. 15	6. 10	6. 12

According to Yanuar (2017), the optimal water quality parameter for rearing tilapia is a temperature of 25-30 °C. A good pH value for fish breeding and growth is 7-8.5. The minimum DO in tilapia culture is >4 mg/L. In *E. tarda* bacteria, the optimum temperature is 35 °C and pH is 5.5 (Zheng *et al*., 2004). However, in the range of data as attached, *E. tarda* can still grow but is not optimal.

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CONCLUSION

This study concluded that the active compound content of HIL affected erythrocytes, leukocytes, differential leukocytes, and hemoglobin of tilapia infected with *E. tarda* with the best treatment of 50% HIL and 50% CF.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

All authors have contributed to the final manuscript. Contributions of individual authors are ET, M, MA, and MF: Conception and design of the study. ET and M: data acquisition. M, MA, and MF: Data analysis and interpretation. ET, M, and MF: Drafted the manuscript. ET, M, and MF: Critically revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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