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Combination of Moringa Leaf Meal and Probiotics in Feed for Tilapia (*Oreochromis niloticus*) Seeds Survival and Immune System

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Abstract

Prevention of disease in tilapia fish seeds can be done by increasing non-specific defense systems by improving and supplementing the nutritional content of the feed. This study aims to determine the effect of adding Moringa leaf meal combined with probiotics to feed on the survival rate of tilapia seeds and the number of seed blood cells that have been infected with *Aeromonas hydrophila*. The method used was an experimental method using a Completely Randomized Design (CRD) with 5 treatments and 3 replications. Treatment A = addition of 4% moringa leaf meal + 6 ml/kg of probiotic feed + *A. hydrophila* injection; Treatment B = addition of 6% moringa leaf meal + 6 ml/kg of probiotic feed + *A. hydrophila* injection; Treatment C = addition of 8% moringa leaf meal + 6 ml/kg of probiotic feed + *A. hydrophila* injection; Treatment D = feed + probiotic 6 ml/kg of feed (positive control) + injection of *A. hydrophila*; Treatment E = feed + probiotic 6 ml/kg of feed (negative control) + injection of physiological solution. The results showed the addition of Moringa leaf meal combined with probiotics got the best results in treatment C with a survival value of 86.67%, hemoglobin levels of 2.3 g%, erythrocytes of 2.20×10^6 cells, leukocytes of 11.5×10^4 cells, and the total number of intestinal bacteria was 10.34×10^6 cfu/ml.

INTRODUCTION

A common problem in tilapia cultivation is a disease that often causes mass mortality. According to Ashari *et al.* (2014), the emergence of disease in fish is the result of host factors, the presence of pathogenic organisms, and unsuitable environmental quality. *Aeromonas hydrophila* was a type of pathogenic bacteria that attack many fish seeds with clinical symptoms caused by red patches on the body of fish (Firnanda *et al.*, 2013). Disease prevention can be pursued by improving the non-specific defense system which is part of the fish immunity, which

can be done by improving and supplementing the nutritional content in the feed. Maskur (2004) states that feed is an important aspect of cultivation activities because it provides energy to support fish growth. The addition of Moringa leaf meal to fish feed can complement commercial feed nutrition with protein, iron, calcium, and vitamin (Oluduro, 2012). In addition, the content of flavonoids, saponins, and vitamins A, B, C, and E can help improve the immune system of fish (Rosidah *et al.*, 2019).

However, feed with good nutrition cannot support fish growth if it is not fully absorbed by fish (Arief *et al.*, 2012). So that feed needs to be combined with probiotics which can improve feed efficiency by releasing enzymes that can help the digestion process. According to Azhar (2014), the microbes contained in probiotics can produce digestive enzymes such as amylase, protease, and lipase to facilitate the digestion process in the fish intestines. Probiotics also help increase fish immunity because they can prevent disease by balancing the gut microflora by suppressing the growth and development of pathogenic micro-organisms in the fish gut. This study aims to determine the effect of adding Moringa leaf meal combined with probiotics to feed on the survival rate of tilapia seeds and the number of seed blood cells that have been infected with *Aeromonas hydrophila*.

METHODOLOGY

Place and Time

This research was conducted in August-October 2019 at the Aquaculture Laboratory, Faculty of Agriculture, Mataram University.

Research Material

The tools used in this research were analytical scales with an accuracy of 0.01 g, micropipette, Eppendorf tube, syringe, autoclave, scoop, plastic sill, thoma pipette, microscope, hemocytometer, ose needle, test tube, trident, hand tally counter, centrifuge, Bunsen, petri dishes, measuring cups, and cool box. The materials used are distilled water, 90% alcohol, Turk solution, hayem's solution, physiological solution, pure culture of *Aeromonas hydrophila*, TSB media, and EDTA.

Research Design

The research was conducted by experimental methods using a Completely Randomized Design (CRD). The aspect studied is the effect of Moringa leaves flour and probiotic (6 ml/kg) addition in

the feed to the survival rate of fish that have been infected by *Aeromonas hydrophila*. The CRD consist of five treatments and three replications as follows:

Treatment A = addition of 4% moringa leaf meal + 6 ml/kg of probiotic feed + *A. hydrophila* injection. Treatment B = addition of 6% moringa leaf meal + 6 ml/kg of probiotic feed + *A. hydrophila* injection. Treatment C = addition of 8% moringa leaf meal + 6 ml/kg of probiotic feed + *A. hydrophila* injection. Treatment D = feed + probiotic 6 ml/kg of feed (positive control) + injection of *A. hydrophila*. Treatment E = feed + probiotic 6 ml/kg of feed (negative control) + injection of physiological solution.

Work Procedures

Survival Rate

Survival Rate (SR) is the percentage of tilapia fry that are alive during rearing. The survival rate is calculated based on Azhar (2014) as follows:

$$SR = \frac{N_i}{N_o} \times 100\%$$

Note:

SR = survival rate (%)

N_i = the final quantity of fish fry

N_o = the initial quantity of fish fry

Bacterial Injection

The bacteria used in this study was *Aeromonas hydrophila*, the injection was done after 35 days of maintenance with bacterial population 10⁶ cfu/ml and injected 0.1 ml/test fish. The fish that have been injected were stored for 5 days for a challenge test, and the dead fishes are counted during the challenge test process.

Blood Sampling

Blood sampling was done after the challenge test. The EDTA solution was put into the syringe as much as 0.1 g and blood was drawn through the base of the tail as much as 0.1 ml with a ratio of 1:1. After that, the blood was transferred into an Eppendorf tube and stored in a cool

box. Furthermore, the blood sample was observed in the laboratory using Thoma pipette with Turk and Hayem's solutions.

Hemoglobin Levels

Hemoglobin levels were analyzed using Sahli's Hemoglobinometer. Blood samples were absorbed using Sahli's pipette as much as 0.2 ml. After that, the blood was put into a Hb-meter tube that has been filled with HCl 0,1 N up to a scale of 10 (red). Furthermore, the blood was stirred with a stirring rod for 3-5 minutes. Then distilled water is added to the tube until the blood color is like the color of the standard solution in the Hb-meter.

Erythrocytes Cell Count

The samples were taken from an Eppendorf tube with an erythrocyte pipette as much as 0.5 ml. Then, the Hayem's solution was sucked up to 101 ml. The solutions were homogenized by shaking in '8' figure form. After that, a drop was inserted into hemocytometer and observed under a microscope using small block method with the equation as follows:

$$\Sigma \text{erythrocytes} = n \times 10^4 \text{ cells/mm}^3$$

Note:

n = number of erythrocytes in 5 counting chambers

10^4 = dilution factor

Leukocytes Cell Count

Blood samples were taken from the Eppendorf tube with an erythrocyte pipette about 0.5 ml, then the Turk solution was sucked up to 11 ml. After that homogenized the solution by shaking in a figure-eight form. The first drop was discarded and then put a drop into a hemocytometer. Sample was observed under a microscope with a magnification of 10 x 40. Leukocytes were calculated by

the big block method with the equation below:

$$\Sigma \text{leukocytes} = n \times 500 \text{ cells/mm}^3$$

Note:

n = number of leukocytes in 4 counting chambers

500 = dilution factor

Total Bacteria in Intestine

The calculation of bacteria in the intestine was carried out using the spread method with NA (Nutrient Agar) media. Total bacteria in the intestine was counted at the end of the study using 0.1 ml of the contents of the digestive tract of tilapia seeds in each treatment. Furthermore, an Eppendorf tube containing 0.9 ml of physiological solution and pieces of fish intestine were prepared, then crushed until smooth. After that, serial dilutions were carried out 9 times. The results of the dilution used were the 7th, 8th, and 9th dilutions. Then it was distributed to nutrient media to use the trident, after 24 hours the number of colonies on the media was counted.

$$\Sigma \text{Bacteria} = \Sigma \text{colony} \times \frac{1}{\text{dilution}}$$

Data Analysis

The data obtained were tested using Analysis of Variance (ANOVA) at the 95% confidence level through the SPSS program to determine the effect of each treatment. If the results of the statistical analysis show a significantly different effect, the Tukey HSD test is performed to determine which one is the best treatment.

RESULTS AND DISCUSSION

Survival Rate

The survival rate of tilapia seeds from the five treatments are shown in Figure 1.

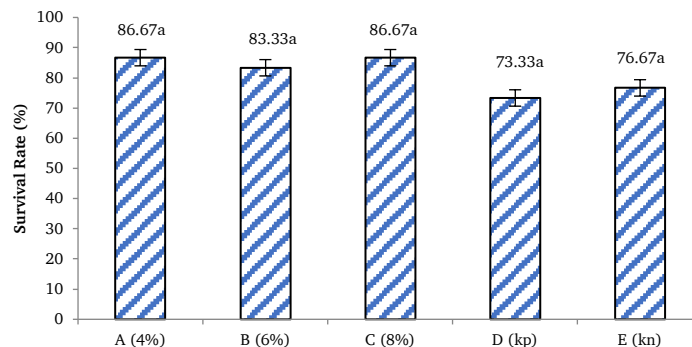


Figure 1. The survival rate of tilapia seeds from the five treatments.

The highest survival rate of tilapia seeds after *Aeromonas hydrophila* injection are shown in treatment A (4%) and treatment C (8%) with a value of 86.67%, while the lowest SR is found in treatment D (positive control) namely 73.33%. Based on the results of the ANOVA test and the Tukey follow up test, there was a significant difference ($P>0.05$) between treatment D and the rest of the treatments; A (4%), B (6%), C (8%) and E (negative control).

The addition of Moringa leaf meal increases the survival rate which indicates that the consumption of Moringa leaf meal sufficient nutritional needs so that feed consumption can increase (Astuti *et al.*, 2005). The effect of Moringa leaves on feed is quite good because it contains essential protein and amino acids needed by fish seeds for cell development. The content of Moringa leaf compounds varies, including minerals, proteins, vitamins (A, C, E, B-carotene), amino acids, flavonoids, B-sitosterol, and polyphenols (Setiyowati *et al.*, 2018). Astuti *et al.* (2005) stated that Moringa leaves contain anti-pathogenic bacteria and antioxidants, and the content of essential amino acids is quite balanced. So that the quality of feed using Moringa leaf substitution is better in increasing the survival rate.

The good bacteria in probiotics have a role in feed digestibility so that the absorption of nutrients in the feed is more optimal. As stated by Irianto *et al.* (2006),

the microbes contained in probiotics are helpful in regulating the microbial environment in the gut, preventing intestinal pathogens, and improve feed efficiency by releasing enzymes that will all aid the process of digestion. The application of probiotics in feed can reduce the mortality rate of fish caused by *A. hydrophila* bacteria by 50%.

In general, fish mortality in each treatment can be caused by several factors. This is strengthened by the statement of Fahrizal and Nasir (2017), where factors that affect the survival of the fish during maintenance are stocking density, feeding, disease, and water quality which includes temperature, ammonia levels, dissolved oxygen, and water pH. Mortality in each treatment due to the different adaptability of fish. Death can also be caused by physiological disturbances or stress. Fish that experience stress can be characterized by the unresponsiveness to the feed given, the fish banging themselves against the wall of the rearing tank, or jumping to the water surface. According to Noviana *et al.* (2014), stress causes a drastically decreased appetite and it will be difficult for fish to do activities such as swimming and breathing.

Hemoglobin

Figure 2 shows the amount of hemoglobin (Hb) from all treatments.

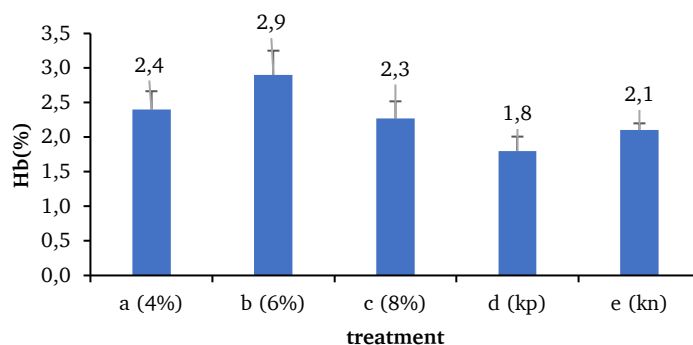


Figure 2. Total amount of hemoglobin in tilapia seeds.

Figure 2 shows the highest amount of Hb is in treatment B (2.9 g%), followed by treatment A (2.4 g%), then treatment C (2.3 g%), treatment E (2.1 g%), and the lowest Hb amount was treatment D (1.8%). Based on ANOVA and Tukey test results, there was a significant difference ($P < 0.05$) between treatment D from other treatments. This indicates that the addition of Moringa leaf meal in the feed effects on the hemoglobin level of tilapia. According to Wahjuningrum *et al.* (2008), the activity of flavonoids in the content of plant active compounds can increase the work of blood-producing organs so that blood production increases. However, these data show that tilapia Hb levels are still in the low range. Salasia *et al.* (2001) stated that normal Hb levels in tilapia ranged from 5.05-8.33 g%. Stressful conditions can affect the physiological activity and hemoglobin levels in fish. The physiological state of fish blood varies greatly, depending on environmental conditions such as humidity, temperature, and pH (Safitri *et al.*, 2013). Low Hb levels will have an impact on the amount of oxygen that exists in the blood. This is reinforced by the statement of Royan *et al.* (2014), which states that the hemoglobin

concentration in blood has a positive correlation with the number of erythrocytes. That is, the lower the concentration of hemoglobin in the blood then the lower the erythrocytes level will be. Erythrocytes contain Hb to supply oxygen to all body tissues, Hb levels determine fish endurance (Fauzan *et al.*, 2017). This is because hemoglobin is related to the oxygen-holding capacity of the blood. So, the low level of Hb can cause decreased metabolic rate and energy production. In addition, environmental conditions such as water temperature also affect the metabolic rate of fish. During an increase in temperature, metabolism in the body takes place rapidly so that it requires a lot of oxygen (O_2), while carbon dioxide (CO_2) in the blood decreases (Roberts *et al.*, 2002). Stress due to increased water temperature in fish has an impact on the performance and health of the fish in the form of impaired function of blood cells.

Erythrocytes

Figure 3 shows the numbers of erythrocytes from all treatments.

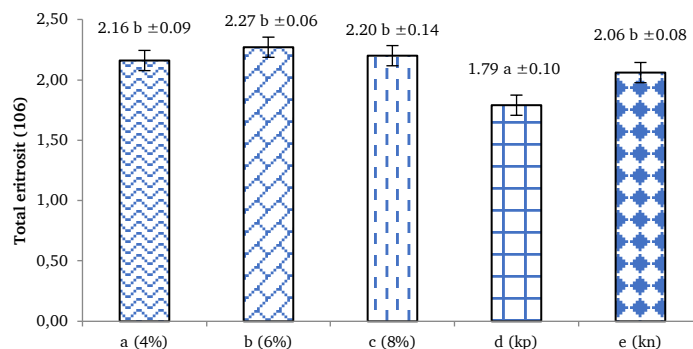


Figure 3. Total numbers of erythrocytes.

Erythrocytes are a type of blood cell that supplies body tissues with oxygen. According to Hartika *et al.* (2014), the optimal numbers of erythrocytes in tilapia ranged from 20,000 to 3,000,000 cells/mm³. The numbers of erythrocytes from all treatments still categorized as normal. Based on the data, the highest number of erythrocytes found in treatment B (2.27x10⁶), followed by treatment C (2.20x10⁶), treatment A (2.16x10⁶), treatment E (2.06x10⁶), and the lowest was treatment D (1.79x10⁶).

Based on the results of ANOVA and Tukey test, there was a significant difference ($P < 0.05$) between treatment D and treatment A, B, C, E. The differences caused by flavonoids and saponins content in Moringa leaves extract that increases fish immunity. Bamishaiye *et al.* (2011) state that the active compound flavonoids have a role as an antioxidant and ward off free radicals, while saponin is one of immunostimulant agent.

In treatment D, the number of erythrocytes decreased due to bleeding caused by infected fish organs. Fujaya

(2002) states that the low numbers of erythrocytes cause the fish unable to take large amounts of oxygen even though the levels of dissolved oxygen are sufficient, this condition leads to anorexia (oxygen deficiency).

Several factors affect the numbers of erythrocytes in fish namely; age, gender, environment, nutrition, and oxygen deficiency condition (Yanto *et al.*, 2015). Temperature also has an impact on erythrocyte levels, high temperature will decrease erythrocytes numbers. In addition, disease and fish appetite also influenced erythrocyte levels. When the fish catches a disease and loses appetite, the hematocrit value becomes abnormal followed by decreased erythrocyte numbers. The low number of erythrocytes is an indicator of anemia, while the high number of erythrocytes indicates that the fish are under stress.

Leukocytes

Figure 4 shows the numbers of erythrocytes from all treatments.

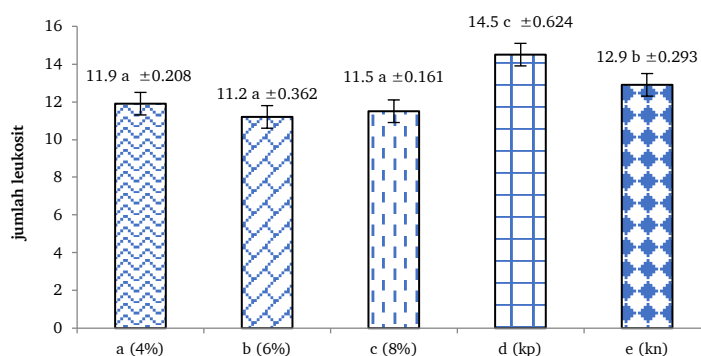


Figure 4. Total numbers of leukocytes.

Mathematically, the highest number of leukocytes (Figure 4) was in treatment D (14.5×10^4), followed by treatment E (12.9×10^4), treatment A (11.9×10^4), treatment C (11.5×10^4), and the lowest number of leukocytes was in treatment B (11.2×10^4). The results of ANOVA and Tukey test showed significant differences ($P < 0.05$) between treatment D (positive control) from treatment E, A, B, and C. This happened because in treatment D *Aeromonas hydrophila* was injected so that the number of leukocytes in fish increased as a form of self-defense and response to the pathogens. The function of leukocytes in the immune system is to produce a special type of protein known as antibodies that identify and fight foreign substances that attack the body. These cells are classified as granulocytes and agranulocytes.

The addition of Moringa leaf meal to the test feed can stabilize the number of leukocytes in the blood. Moringa leaves contain flavonoids, alkaloids, terpenoids, and saponins as immunostimulant agents (Subryana *et al.*, 2020). Immunostimulants are biological compounds that can boost the immune system. The number of leukocyte cells (Figure 4) on the treatment provided additional moringa leaf meal tends to be stable with

values ranging from $11.2-11.9 \times 10^4$. From the data it is also known that the number of leukocytes in the blood is still in normal circumstances, this follows Kurniawan *et al.* (2019), which states that the normal amount of tilapia leukocytes ranges from 20,000 to 150 000 cells/mm³. In treatment D where the fish injected with *Aeromonas hydrophila* there was no dramatic increase in leukocyte counts was seen due to the addition of probiotics to the feed. According to Adams (2009), probiotic can produce numbers of essential nutrients in the host immune system and metabolism, such as vitamin B (pantothenic acid), pyridoxine, niacin, folic acid, cobalamin, biotin, and important antioxidant such as vitamin K. The number of leukocytes in fish influenced by several factors such as species and age. According to Sugito *et al.* (2014), leukocytes will decrease when the fish are under stress and will increase when the fish are infected by pathogens as a form of the immune response.

Total Number of Bacterial Colonies

Figure 5 shows the total amount of bacteria colonies obtained from the intestine.

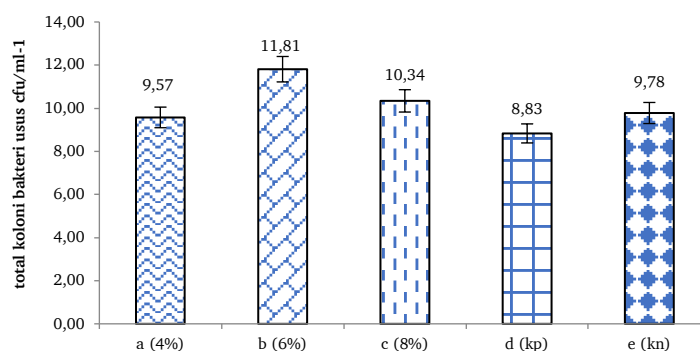


Figure 5. Total number of intestinal bacteria colonies.

The total bacteria count from the fish gut was carried out 5 days after the challenge test. The highest total yield of intestinal bacteria colonies (Figure 5) was in treatment B (11.81×10^6 cfu/ml), followed by treatment C (10.34×10^6

cfu/ml), treatment E (9.78×10^6 cfu/ml), treatment A (9.57×10^6 cfu/ml), and the lowest total bacteria was in treatment D (8.83×10^6 cfu/ml). The lowest total bacteria present in treatment D indicated that the normal flora in the intestines of

the tested fish was lower than the other treatments. Total levels of bacteria in the gut can also be affected by the invasion of pathogenic bacteria from the environment which can cause low growth rates and high susceptibility to pathogens. Microbial density can also be caused by food eaten, the more complex nutrients content the growth of intestinal microflora will increase. The addition of probiotics to fish feed also supports the high bacteria present in the fish intestines. According to Setyawan *et al.* (2014), the density of lactic acid bacteria will increase if the substrate contains sufficient nutrients. Lactic acid bacteria in the intestines of fish can balance the digestive tract microbes which can increase the digestibility of food in the intestines of fish by converting carbohydrates through a series of enzymatic into lactic acid. Factors that affect bacterial growth in the digestive tract of fish are the intestine surface area, feed nutrient availability, and normal microflora content of the intestine so that bacteria can enter and thrive in the digestive tract (Kesarcodi-Watson *et al.*, 2008). Besides environmental factors can also affect the growth of bacteria in the intestines of fish. Changes in water temperature affect the digestive activity, growth, and reproduction of fish (Cruz *et al.*, 2002). The growth rate and total bacterial count are strongly influenced by temperature, pH, and oxygen gas. Each species of bacteria grows over a range of temperatures and can tolerate temperature changes well.

CONCLUSION

It is concluded that the addition of Moringa leaf meal combined with probiotics to tilapia seeds that have been infected by *Aeromonas hydrophila* obtained the best results in treatment C with a survival rate of 86.67%, hemoglobin levels of 2.3 g%, erythrocytes of 2.20×10^6 , leukocytes of 11.5×10^4 , and the total number of intestinal bacteria was 10.34×10^6 cfu/ml.

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