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Immunostimulation of Nile Tilapia Through the Provision of Synbiotic Feed (*Eleutherine bulbosa* and Probiotic) to Prevent Motile Aeromonas Septicemia (MAS) Disease

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ABSTRACT

Motile Aeromonas Septicemia (MAS) is one of the bacterial diseases that attacks and causes mass death of nile tilapia. Disease control in fish farming using synthetic antibiotics has caused problems in aquaculture, such as bacterial resistance to antibiotics, drug residues in fish, and water pollution. Therefore, alternative measures to control MAS that are safe and environmentally friendly, such as the application of immonostimulant that use organic ingredients, are needed. This research aims to determine potency of immunostimulation of nile tilapia trough feeding of synbiotic feed namely prebiotic *Eleutherine bulbosa* powder (Ebp) and probiotic containing *Lactobacillus casei* and *Saccharomyces cerevisiae* as for prevention MAS disease. The fish were reared for 21 days, in reared for the first 14 days, the fish were given synbiotic feed then for the next 7 days they were given feed without synbiotics. The fish were challenged with *Aeromonas hydrophila* on day 15th. Experimental design used was completely randomized design with four treatments: 0 g Ebp and 0 ml probiotics in 1 kg of feed (synbiotic free feed), 7.5 g, 10 g, and 12.5 g Ebp each with 15 ml probiotics in 1 kg of feed. The results showed that feeding the fish with synbiotic feed, especially the treatment Ebp12.5, give significantly different results on parameters prevalence, fish recovery, survival rate of the fish test. This research concluded that treatment of Ebp 12.5 was the best dosage of immunostimulant to prevent MAS disease in nile tilapia.

INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is one of the freshwater commodities that has high economic value. In line with the development of fish farming, there are several inhibiting problems, including pests and diseases. Disease problems are the main obstacle because they can harm aquaculture businesses. One of the bacterial diseases that attacks and causes mass death of nile tilapia is Motile Aeromonas Septicemia (MAS) (Rodrigues *et al*., 2019). The agent of this disease is the bacteria *Aeromonas hydrophila* (Christy *et al*., 2019). Fish infected with this disease show symptoms scales coming off, fins being torn, wounds or ulcers on the surface of the body, hemorrhage in the area of infection, and exophthalmia (Hardi *et al*., 2014).

Disease control in fish farming using synthetic antibiotics has been widely carried out by farmers. However, this effort can cause problems such as bacterial resistance to antibiotics (Stratev & Odeyemi, 2015) and can leave residues in the fish's body that are potentially dangerous if consumed by humans (Liu *et al*., 2017). Therefore, alternative steps are needed to overcome Motile Aeromonas Septicemia (MAS) disease that are more environmentally friendly, safe and easy, for example the use of synbiotic feed. Synbiotics are a technology that combines the application of probiotic and prebiotic bacteria. The provision of synbiotic feed was reported by Munaeni *et al*. (2014) to have succeeded in increasing survival, specific growth rate, immune response of aquaculture organisms and suppressing the growth of pathogenic bacteria.

Probiotic bacteria basically produce enzymes that are able to break down complex compounds into simple ones so that the nutrients contained in the feed can be optimally absorbed by the fish (Alemayehu *et al*., 2018). In increasing the absorption of feed nutrients, probiotic bacteria produce several enzymes for feed digestion such as amylase and protease (Afrilasari *et al*., 2016). The use of probiotics is also one of the safe and environmentally friendly methods of controlling fish diseases (Bharati *et el*., 2019). The administration of probiotics in this research is expected to have an impact on increasing the digestibility of plant-based feed, so that the active compounds in *E. bulbosa* onions are more easily absorbed in the fish's digestive system.

Eleutherine bulbosa is a plant that has been cultivated because it can be utilized and has medicinal properties. This plant contains oligosaccharides including inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), and raffinoses so that *E. bulbosa* has great potential to be used as a prebiotic (Munaeni *et al*., 2020a). The use of *E. bulbosa* extract or powder in feed was also reported by Munaeni *et al*. (2020b) to successfully increase the number of bacteria in the digestive tract and increase the activity of protease, amylase and lipase enzymes in the digestive tract of white shrimp (*Litopenaeus vannamei*). *E. bulbosa*, in addition to its potential as a prebiotic, is also supported by the active compounds contained in the onion bulbs. The active compounds are flavonoids, tannins, saponins, steroids, triterpenoid groups (Munaeni *et al*., 2020b). These active compounds are known to have immunostimulant, antimicrobial and anti-inflammatory properties.

Immunostimulants are a group of natural active compounds that can modulate pathogens by increasing the function of phagocytic cells, as well as stimulating natural killer cells (NK), complement, lysozyme, and antibody responses in fish (Mehana *et al*., 2015). Therefore, the use of immunostimulants in aquaculture is a good solution to control fish diseases. Based on this description, researchers tested the potential of immunostimulation of synbiotic feed (prebiotic *E. bulbosa* powder and probiotics) in controlling MAS disease in nile tilapia.

METHODS

Location and Time of Research

This research was conducted from November 2021 to January 2022, at the Laboratory of Cultivation, Seeding and Fisheries Production, Faculty of Fisheries and Marine Sciences, Halu Oleo University, Kendari.

Experimental Design

Eleutherine bulbosa onion was taken from Buton Island, Southeast Sulawesi Province. *Eleutherine bulbosa* powder was made by drying *E. bulbosa* onion bulbs in the sun. The dried

onion bulbs were then ground into powder. *E. bulbosa* powder (Ebp) was mixed into the feed by repelleting. Probiotics were given by spraying evenly on the feed according to Karel *et al*. (2019). The probiotics used are Effective Microorganisms-4 (EM4) fisheries containing *Lactobacillus casei* and *Saccharomyces cerevisiae*.

This research was designed based on a completely randomized design, 4 treatment levels and 3 replications. The treatments given were Ebp0 (synbiotic free feed), Ebp7.5 treatment (7.5 g Ebp and 15 ml probiotics in 1 kg of feed), Ebp10 treatment (10 g Ebp and 15 ml probiotics in 1 kg of feed), and Ebp12.5 treatment (12.5 g Ebp and 15 ml probiotics in 1 kg of feed). Determination of doses of *E. bulbosa* powder and probiotics, respectively, based on Munaeni *et al*. (2020c) and Karel *et al*. (2019).

Fish

The nile tilapia was obtained from community hatcheries in Konda District, South Konawe Regency, Southeast Sulawesi. The nile tilapia weighed is around 23.9-39.1 g. The fish were kept in an aquarium with a water volume of 100 liters for 21 days. Each aquarium contained 5 fish. Fish are fed with synbiotic feed for 14 days, on days 15-21 they are not given synbiotic feed (Figure 1). Feed was given in the morning and evening with a feed weight of 5% of the weight of the fish's body biomass. All fish were injected intramuscular with *Aeromonas hydrophila* with a bacterial density of 10^7 CFU/ml as much as 0.1 ml per test fish (Armin *et al.*, 2023). The level of bacterial density was determined by the indirect method using McFarland standard solution, referring to Rosmania & Yanti (2020).

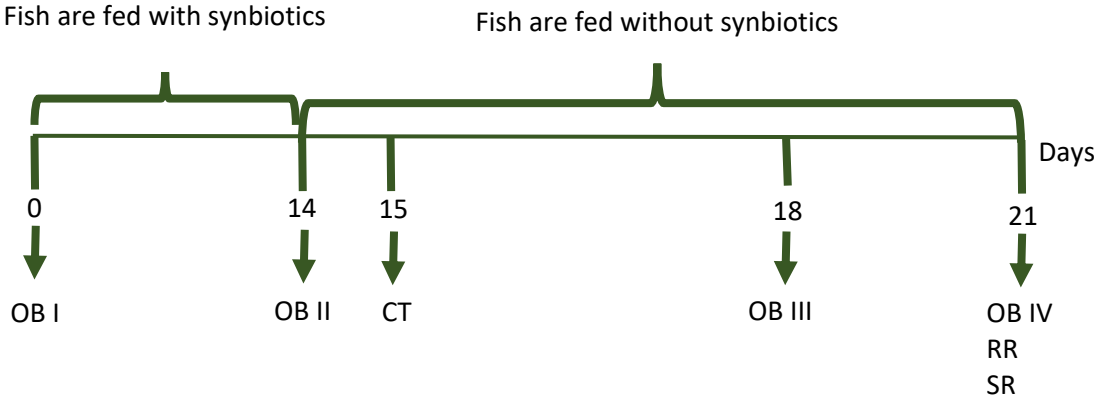

Figure 1. Design of Treatment Time and Observation of Research Parameters

Where:
CT : challenge test with *Aeromonas hydrophila*
OB I : first observation of blood profile
OB II : second observation of blood profile
OB III : third observation of blood profile
OB IV : fourth observation of blood profile
SR : survival rate
RR : recovery rate

Parameters

The research parameters measured were prevalence, recovery rate, survival rate, and blood profile. The blood profile observed consisted of hematocrit, leukocyte and hemoglobin levels. The blood profile observations were carried out on days 0, 14, 18, and 21 (Figure 1).

a. Prevalence

$$\text{Prevalence } (\%) = \frac{\text{number of sick fish}}{\text{number of fish}} \times 100\%$$

Where: the number of sick fish is determined by observing clinical symptoms of sick fish (Harlina *et al*., 2019).

b. Recovery Rate (RR)

$$\text{RR } (\%) = \frac{\text{number of fish recovered}}{\text{number of sick fish}} \times 100\%$$

Where: the number of fish recovered is a fish that shows signs of recovery, the clinical symptoms of sick fish have finished (Roberts, 2012).

c. Survival Rate (SR)

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

Where: Nt = number of live fish at the end of rearing (fish)
N0 = number of fish at the begining of rearing (fish) (Armin *et al*., 2023).

d. Calculation of hematocrit (He) was measured according to Anderson & Siwicki (1995). The blood is put into a microhematocrit tube until approximately ¾ of the tube. The end of the tube was closed with crytoceal to a depth of 1 mm. After that, the microhematocrit tube was centrifuged for 5 minutes at 5,000 rpm. The length of the sedimented blood (a) and the total length of the blood volume in the tube (b) were measured using a ruler. The hematocrit level is expressed as % of the volume of blood cell solids.

$$\text{He } (\%) = \frac{\text{a}}{\text{b}} \times 100\%$$

e. Leukocytes count was measured according to Blaxhall & Daisley (1973). The blood sample is sucked with a pipette containing a white stirrer up to a scale of 0.5 then Turk's solution is added up to a scale of 11. Stirring is done by swinging the hand in a figure 8 shape for 5 minutes until the blood is evenly mixed. Drops First, the blood in the pipette is discarded, then the blood sample is dropped into the hemocytometer and covered with a cover glass, then observed under a microscope. Leukocytes were counted in 4 counting chambers measuring 1 mm × 1 mm. The number of leukocytes is calculated using the following formula.

$$N = n \times 50 \text{ cell/mm}^3$$

Where: N = leukocytes count in 1 mm^3
n = number of leukocytes in 4 counting chambers, 50 = dilution factor.

f. Hemoglobin levels were measured according to Wedemeyer & Yasutake (1977). Suck fish blood with a Sahli pipette as much as 0.02 ml. Clean the tip of the pipette then insert the blood into a Sahli tube that has previously been filled with 0.1 N HCl solution on a scale of 10, stir and let stand for 3-5 minutes. Add distilled water with a dropper pipette little by little while stirring until the color is the same as the standard solution. Then read the scale by looking at the surface of the liquid and checking it with the Sahli tube scale, which means the amount of hemoglobin in grams per 100 ml of blood. Hemoglobin are expressed in grams per 100 ml of blood.

Data Analysis

The result of prevalence, recovery rate, survival rate, observations of blood profile including hematocrit, leukocyte count, and hemoglobin were analyzed using the ANOVA.

RESULTS

Prevalence, Recovery Rate, and Survival Rate

Observation of the prevalence revealed that feeding synbiotics to fish for 15 days revealed a significantly differences of prevalence (P<0.05) compared to the Ebp0 (synbiotic free feed). Ebp10 and Ebp12.5 were treatments that produced lower prevalence than the other treatments (Figure 2). Meanwhile, observations of the recovery rate revealed that the feeding synbiotics produced a significantly differences of the recovery rate (P<0.05) compared to the Ebp0 (synbiotic free feed), especially in Ebp10 and Ebp12.5 (Figure 3). In fish fed with synbiotic feed (Eb7.5, Ebp10 and Ebp12.5) revealed that significantly differences of the survival rate (P<0.05) compared to the Ebp0 (synbiotic free feed) (Figure 4).

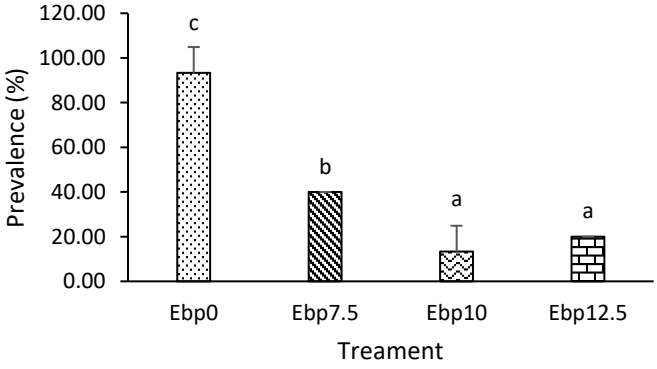

Figure 2. Prevalence of Fish Infected with MAS Disease

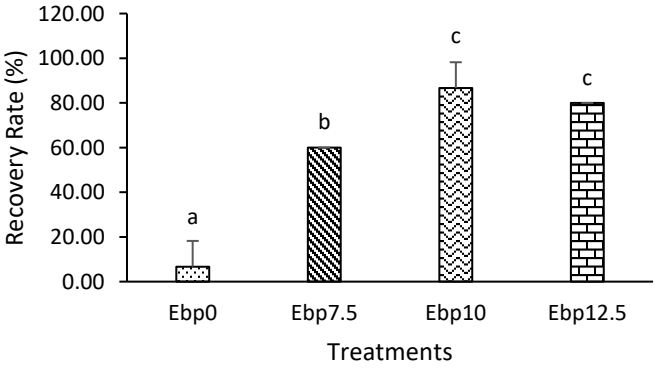

Figure 3. Recovery Rate of Fish After Infected with MAS Disease

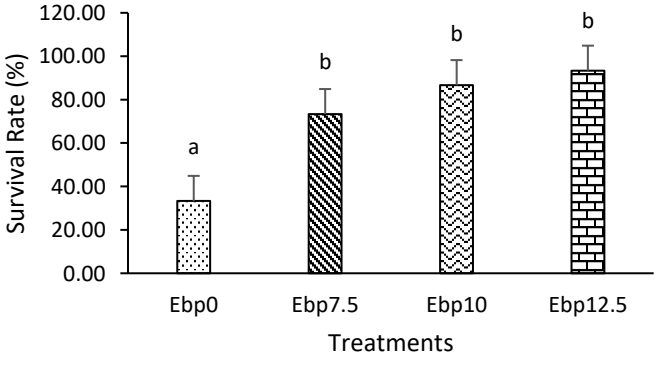

Figure 4. Survival Rate of Fish at the End of Rearing

Blood Profile

Observation of blood profiles showed that feeding synbiotics to fish revealed that a significantly differences of hematocrit and leukocyte count (P<0.05) compared to the Ebp0 (synbiotic free feed), especially at day 21. Hematocrit and leukocyte count at day 15 (before challenge test) and 18 (3 days post challenge test) did not revealed significant differences (P>0.05) between the treatments given (Figure 5 and 6). Hemoglobin in all treatments on day 15 were not significantly different (P>0.05) (Figure 8). However, feeding synbiotics to fish produced tilapia fish hemoglobin on days 18 and 21 (3 and 6 days after challenge) revealed that significantly differences of the hemoglobin levels (P<0.05) (Figure 7).

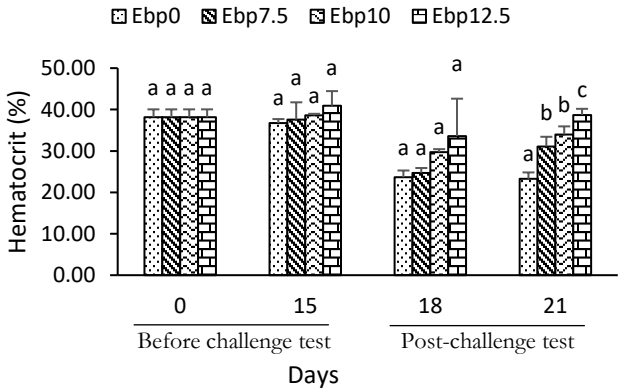

Figure 5. Hematocrit of Fish Before and After Challenge Test by *A. Hydrophilla*

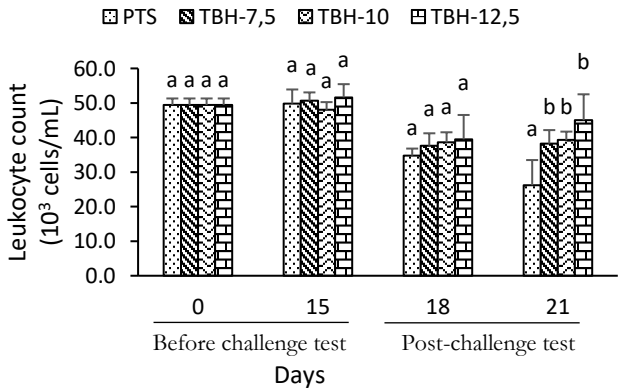

Figure 6. Leukocyte Count of Fish Before and After Challenge Test by *A. Hydrophilla*

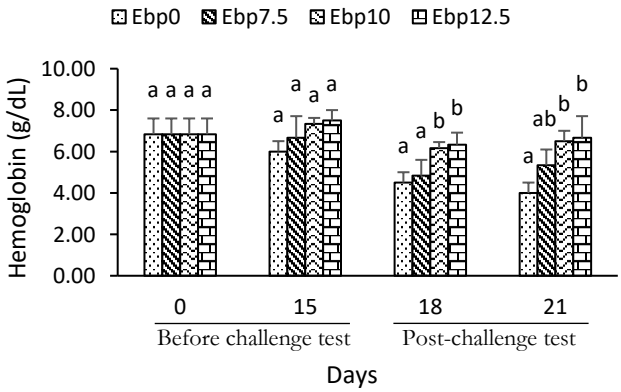

Figure 7. Hemoglobin of Fish Before and After Challenge Test by *A. hydrophilla*

DISCUSSION

Observation of clinical symptoms of test fish is the basis for determining prevalence and recovery rate. Tilapia infected with MAS diseases showed clinical symptoms scales coming off, fins being torn, wounds or ulcers on the surface of the body, hemorrhage in the area of infection, and exophthalmia (Hardi *et al*., 2014). Fish are categorized as having recovered from MAS disease if the disease symptoms have completely cleared up.

The provision of synbiotic feed, *E. bulbosa* powder and probiotics, in this research gave significant results on the prevalence and the recovery rate parameters of tilapia (Figure 2 and 3). Fish fed synbiotic feed became more resistant to *A. hydrophila* infection so that the number of fish showing clinical symptoms of MAS disease was less than fish fed without synbiotics. This shows that synbiotic feed can improve the immune system. In addition to reducing prevalence, providing this synbiotic feed can also improve fish recovery. Munaeni *et al*. (2020b) explained that *E. bulbosa* have active compounds including flavonoids, tannins, saponins, steroids, and triterpenoids. Behl *et al*. (2021) stated that flavonoids, alkaloids, tannins, saponins, and terpenoids in plants can act as immunomodulators for disease control. Munaeni *et al*. (2020c) explained that providing feed enriched with *E. bulbosa* powder has succeeded in increasing the expression of immune system genes in whiteleg shrimp and increasing shrimp resistance to pathogenic bacterial infections. In this research, fish were fed with synbiotic feed for 15 days, then it was not given after the challenge test was carried out. This aims to prevent the data obtained from being inaccurate. However, the active compounds contained in *E. bulbosa* also act as antibacterials (Munaeni *et al*., 2017), so it is feared that these active compounds contribute to the process of inhibiting bacteria directly.

The survival rate of fish in this research (Figure 4) is in line with the level of fish recovery. The ability of the test fish to defend themselves from the impact of pathogen infection and the ability to heal after infection greatly supports their survival. This proves that the provision of synbiotic feed has succeeded in increasing the survival of fish infected with pathogenic bacteria.

The results of observations in this research showed that the Ebp10 and Ebp12.5 treatments consistently showed better results than other treatments, especially Ebp0. Ebp10 and *Eb*p12.5 were able to improve the immune system of tilapia so that the prevalence of MAS disease was lower than other treatments. Likewise, the resulting recovery rate was higher, thus having an impact on increasing the survival of the test fish. This is likely due to the increase in the concentration of *E. bulbosa* powder (Ebp) correlating with the increase in active compounds, thereby improving the immune system of tilapia.

Hematocrit of tilapia showed an increase after being fed with synbiotics (Figure 5). The highest increase in hematocrit percentage after 14 days of maintenance was in the Ebp12.5 treatment. This is thought to be due to the role of active compounds that are antioxidants and anti-inflammatory contained in the forest onion feed (Munaeni *et al*., 2019). The results of observations on the 18 days, hematocrit levels in all treatments decreased compared to day 14. Hematocrit levels in Ebp0 showed values that were less than the normal range, namely 23.66%, while the normal range of hematocrit for tilapia is 27-37% (Hrubec & Smith, 2010). Hematocrit levels in Ebp0 indicate a condition of fish that is approaching anemia. On the 21 days of observation, all hematocrit of the test fish increased compared to the hematocrit percentage on the 18 days, except for the Ebp0 treatment. This indicates that the immune system has responded to the occurrence of pathogenic bacterial infection.

In the observation of the leukocytes count (Table 3), leukocytes increased after 14 days of maintenance with synbiotic feed. However, on the 18 days of observation, the leukocytes count in the test fish decreased in all treatments. In the synbiotic feed treatment, the leukocytes was higher compared to the Ebp0 treatment. This indicates that the synbiotic feed treatment is relatively better at improving the immune system compared to the Ebp0 treatment. According to Abidin *et al*. (2022), this decrease in leukocytes count can be caused by these blood cells being active and gushing out of the blood vessels due to hemorrhage and ulcers in the fish's body. Observations on the 18 days showed that leukocytes count in the synbiotic feed treatment was still within the normal range. Lagler *et al*. (1997) stated that the range of normal leukocyte in tilapia is 20,000-150,000 cells/mm^3. On the 21 days of observation, the leukocytes count in the test fish increased, especially in the three synbiotic fortified feed treatments. The leukocytes count in synbiotic feed was within the normal range except for Ebp0 treatment.

In the observation results of hemoglobin levels (Table 4), the hemoglobin levels of tilapia fish also increased on the 14 day of observation, after being fed with synbiotics. On the 14 days, the hemoglobin levels of the fish ranged from 6.00-7.50 g/dl. This value is still within the normal range. Hardi *et al*. (2011) explained that the normal hemoglobin levels of tilapia fish range from 6.00-11.01 g/dl. On the 18 days of observation, the test fish experienced a decrease in hemoglobin levels. Pathogenic bacterial infection has caused a decrease in hemoglobin levels in the blood of the fish. However, the hemoglobin levels in fish fed with synbiotics showed better values than the Ebp0 treatment. In Ebp10 and Ebp12.5, they even showed hemoglobin levels that were still within the normal range. This indicates that giving synbiotic feed to tilapia fish can prevent a decrease in hemoglobin levels when pathogen infection occurs. These results indicate that increasing the concentration of *E. bulbosa* powder in synbiotic feed has contributed to increasing hemoglobin levels after infection with pathogenic bacteria. This is thought to be the impact of the flavonoid compounds in *E. bulbosa* onions. Lesjak & Srai (2019) stated that flavonoids can affect iron status in the blood by regulating the expression and activity of proteins that involve systemic regulation of metabolism and iron absorption. Syahrial *et al*. (2013) explained that flavonoid compounds function as antioxidants, thereby protecting hemoglobin from oxidation. On the 21 days of observation, hemoglobin levels increased again. The percentage of hemoglobin in the Ebp12.5 treatment showed a better effect compared to other synbiotic feeding treatments. This is because the active compounds contained in the feed are higher than other treatments so that the resulting impact on improving fish health is also greater.

CONCLUSION

The conclusion of this research is that the provision of synbiotic feed, *E. bulbosa* powder and probiotics, containing *L. casei* and *S. cerevisiae*, can improve the immune system of nile tilapia to prevent *A. hydrophila* infections. Supplementation of Ebp12.5 treatment (12.5 g *E. bulbosa* powder and 15 ml probiotics in 1 kg of feed) is the treatment that gives the best results as an immunostimulant to prevent MAS disease in nile tilapia.

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