BIODIVERSITAS
Volume 25, Number 11, November 2024
Pages: 4333-4339

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d251132

Analysis of two strain probiotics on digestive enzymes, liver function and antimicrobial activity of catfish (*Clarias gariepinus*) treated with *Aeromonas hydrophila*

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Manuscript received: 30 August 2024. Revision accepted: 19 November 2024.

Abstract. *Aini N, Wahyuningsih SPA, Uzakky FN, Betzy C, Fatimah, Andriyono S. 2024. Analysis of two strain probiotics on digestive enzymes, liver function and antimicrobial activity of catfish (*Clarias gariepinus*) treated with* Aeromonas hydrophila*. Biodiversitas 25: 4333-4339.* Catfish (*Clarias gariepinus* Burchell 1822) is one of the most consumed fish. Good fisheries management is needed to maintain fish stocks. One of the efforts to manage fisheries safely is by using probiotics in the form of Duo Strain Probiotics (DSP). DSP is a probiotic formula consisting of *Lactobacillus casei* and *Bacillus subtilis*. The purpose of this study was to determine the effect of DSP on digestive enzyme levels, liver health status, and the potential of catfish blood serum as an antimicrobial agent. The treatments included DSP to the feed with concentrations of 0%, 5%, 10% and 15% using *Aeromonas hydrophila* infection. The results showed no significant change in amylase enzyme level between DSP treatments of various concentrations and the control treatment. DSP treatment of 10% and 5% in the non-infected fish group was the best treatment for increasing the level of protease and lipase enzymes, respectively. For liver health status parameters, 15% DSP treatment was the best treatment that could suppress the production of AST and ALT enzymes in fish blood serum. Catfish blood serum infected with pathogens and treated with 15% DSP has the ability as an antibacterial agent against *A. hydrophila.*

Keywords: *Aeromonas hydrophila*, catfish, duo strain probiotics, fish stock, fisheries management

INTRODUCTION

Catfish (*Clarias gariepinus* Burchell 1822) is one of the species of freshwater aquaculture fish that is still ranked as the top consumption fish favored by the Indonesian people (Putra et al. 2020; Kusuma et al. 2022). However, the high market interest in catfish certainly also faces obstacles like diseases that can result in decreased production (Aini et al. 2020). Fish farming, which is generally carried out in limited space, high fish density, excessive feeding, and poor water quality, provides an entry point for pathogen infection (Dawood 2021). These conditions can disrupt environmental balance as a result of which fish experience stress and are easily attacked by diseases due to the weakening of the body's immune system (Hamid et al. 2016; Cavalcante et al. 2020). In addition, many diseases occur in fish due to their susceptibility to pathogens (Ehsannia et al. 2022). One of them is caused by *Aeromonas hydrophila* bacteria (Gobi et al. 2018; Febrianti et al. 2021; Sudrajat et al. 2023).

One of the prevention efforts against *A. hydrophila* bacteria made by catfish farmers is using synthetic antibiotics (Aini and Hariani 2019). The use of antibiotics can lead to resistance in pathogenic bacteria, and can cause pollution in the aquatic environment due to the chemical residues produced (Diwan et al. 2023). Therefore, in order to maintain fish stocks, an alternative is to administer probiotics (Trukhachev et al. 2021; Sumon et al. 2022; Abdul-Malik et al. 2023). Proven types of bacteria that are often used as probiotics are *Bacillus subtilis* and *Lactobacillus casei* (Aini et al. 2024a). According to research conducted by Lawal et al. (2019), the administration of *B. subtilis* bacteria to catfish can improve intestinal function in digesting food and nutrient absorption. Research by Baisakhi et al. (2024) using a combination of *B. subtilis* and *Saccharomyces cerevisiae* can increase the secretion of digestive enzymes in Indian major carp, *Labeo rohita* challenged with a lethal strain of *Aeromonas veronii* intraperitoneally at 1×10^8 CFU/mL. Meanwhile, the use of *L. casei* can improve growth, fish digestibility, and fish disease resistance and can change the composition of the gut microbiota (Adorian et al. 2019; Aini et al. 2024b). In some types of probiotics, it has been shown that the use of two strains of bacteria is more efficient than one strain of bacteria alone (Wang et al. 2021). The use of multistrain or multispecies probiotics can increase protection against pathogenic bacteria (Hai 2015; Kong et al. 2019). Luo et al. (2022) studied the use of probiotics from a mixture of *B. subtilis* and *L. plantarum*, which increased the levels of α-amylase, lipase, trypsin and total protease enzymes. Amenyogbe et al. (2024) stated that

probiotics that have the potential to improve health and physiological function of fish gastrointestinal tract come from the genus *Lactobacillus* and *Bacillus*. These two genera of bacteria can improve feed digestion and optimal utilization of nutrients by producing various digestive enzymes.

A. hydrophila is a pathogenic bacterium that infects catfish and is recognized as antigens that trigger antibody formation. The use of DSP helps the catfish to increase antibody production and the antibodies that are formed react specifically to *A. hydrophila* antigens (Vieco et al. 2019). The ability of fish to produce antibodies to recognize *A. hydrophila* antigens has been proven by Mulia et al. (2012), through antibody titer testing that showed the results of an agglutination reaction to *A. hydrophila* antigens in catfish serum.

Fish blood serum contains comprehensive information regarding the health status of fish (Docan et al. 2011). One of the research parameters using catfish blood serum is the enzyme ALT and AST. Most increases in ALT levels are caused by liver damage and the ALT test is usually conducted along with other tests that check for liver damage, including aspartate aminotransferase (AST) (Mollanourozi et al. 2021). The purpose of this study was to investigate the effectiveness of antibodies in catfish serum in inhibiting the growth of pathogenic bacteria *A. hydrophila*.

MATERIALS AND METHODS

Ethical statement

The study was conducted according to the ethical commission, Animal Care and Use Committee (ACUC) Faculty of Veterinary Medicine, Universitas Airlangga No: 2.KE.163.09.2018.

Time and place of research

The research was conducted from July to September 2023. The research was carried out at the Microbiology Laboratory and Molecular Genetics Laboratory, Faculty of Science and Technology, Universitas Airlangga, Surabaya, East Java, Indonesia, while the catfish rearing process took place at Kumis Lele Farm, Panjang Jiwo Surabaya, East Java, Indonesia.

Research design

The research was performed in a complete randomized design with 8 treatments and 3 replications each. The treatments were as follows A: 0% probiotics and infection with pathogens; B: 5% probiotics and infection with pathogens; C: 10% probiotics and infection with pathogens; D: 15% probiotics and infection with pathogens; E: 0% probiotics and no infection with pathogens; F: 5% probiotic and not infected with pathogens; G: 10% probiotic and not infected with pathogens; H: 15% probiotic and not infected with pathogens.

Re-culture of bacteria duo strain probiotic and *A. hydrophila*

The media used were NB (Nutrient Broth, HiMedia, USA) media for *B. subtilis* and *A. hydrophila* and MRSB (De Man Regosa Sharpe Broth, HiMedia, USA) for *L. casei*. Bacterial isolates of *A. hydrophila* were obtained from the Center for Brackish Water Fisheries Culture, Jepara, Central Java, Indonesia. Bacterial isolates of *L. casei* and *B. subtilis* were obtained from the Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. The re-cultured bacteria were incubated for ±48 hours at 35°C for *B. subtilis* and *L. casei* bacteria and at 30°C for *A. hydrophila* bacteria. The re-cultured bacteria were centrifuged at 5000 rpm for 5 minutes. Then, the pellet formed was separated from the supernatant. The pellet was then diluted with physiological NaCl solution to obtain bacterial suspension with an OD value equivalent to a cell density of 10^8 CFU/mL.

Catfish rearing

The catfish used was a fish measuring 20-25 cm in length and weighing 70-100 g. Each aquarium was filled with 8 catfish. Before treatment, the fish were acclimatized for 7 days. During acclimatization, the fish were routinely fed twice a day at 06.00 and 18.00. The feed used was PF 1000 MS commercial feed with protein content of 39-41% from PT Matahari Sakti. Before being given to the fish, the feed was first mixed with DSP according to the treatment. The concentration of DSP in the treatment was the total concentration of both bacteria with a ratio of *L. casei* and *B. subtilis* as much as 1:1. The feed was mixed with DSP and placed in a plastic box and then sprayed DSP, while stirring so that it was evenly distributed. Furthermore, the feed was allowed to stand for 24 hours at room temperature in a closed condition. To maintain feed quality, the feed was then stored in a refrigerator. The feed was then weighed as much as 3% of each total weight of catfish in each aquarium.

Pathogen infection process

Infection of *A. hydrophila* bacteria in catfish was carried out after 28 days of treatment. Infection was done by using a syringe loaded with bacteria at an appropriate dose for the LD_{50} test, which was 0.1 mL with a cell density of 10^8 CFU/mL. Then, the bacteria were injected into the catfish intraperitoneally. The treatment group that was not infected by *A. hydrophila* bacteria was also given an injection of distilled water with the same amount as the number of *A. hydrophila* bacteria in the intraperitoneal part of the fish.

Blood serum sample collection and preparation

Sampling was done after 7 days of catfish infection. Samples were collected by taking 3 fish in each aquarium. Catfish blood serum samples were taken from the caudal vein area as much as ±3 mL using a syringe. The blood sample was then inserted into a labeled falcon tube. Falcon tubes containing catfish blood in each treatment were left for 10-20 minutes at room temperature to allow the blood serum to coagulate and then centrifuge at 2000-3000 rpm for 20 minutes. After that, the supernatant obtained was used to measure the digestive enzyme levels of catfish. If the sample was not tested immediately, the supernatant obtained can be stored in a refrigerator at -80°C.

Digestive enzyme test

Digestive enzyme levels in fish were measured using ELISA kits produced by BT Lab (BT Lab, Zhejiang, China). Measurement of amylase enzyme using Fish α-amylase (E0061FI), lipase enzyme using Fish Gastric lipase (E0090FI), and protease enzyme using Fish Protease, Serine 1 (E0122FI). Measurement procedures were carried out in accordance with the usage protocol contained in the kit packaging instructions.

Liver enzyme testing

To determine the health status of the liver, ALT and AST were measured. These two enzymes were measured with catfish blood serum samples obtained at the end of the study. Measurements were made using ALT (GOT) FS* (IFCC mod) and AST (GOT) FS* (IFCC mod) kits manufactured by DiaSys Diagnostic Systems GmbH (Germany) according to the protocol.

Antimicrobial activity by Kirby-Bauer method

A. hydrophila bacteria used in this assay were re-cultured in Mueller Hinton Broth (MHB, HiMedia, USA) medium and incubated for 16-24 hours at 30°C. After re-culturing, the absorbance was measured using a spectrophotometer with a wavelength of 600 nm, and the results obtained were compared with a standard turbidity of 0.5 Mcfarland which was equivalent to a cell density of 1.5×10^8 CFU/mL or at an absorbance equivalent to 0.08 to 0.1 (Ene et al. 2020). Antimicrobial activity using blood serum samples was performed in vitro using disc paper in a petri dish containing MHA media divided into 4 quadrants. *A. hydrophila* was swabbed over the surface of media using a cotton swab. The paper discs in each treatment group were moistened with 20 µL serum. The test was carried out in duplicate with a positive control using Enrofloxacin antibiotic, and negative control using distilled water to compare the sample. The incubation time was 24 hours at 30°C in an incubator. The inhibition that appeared was measured using a caliper with mm units vertically and horizontally, then averaged until the final diameter result was obtained.

Data analysis

Data obtained were enzyme levels (amylase, lipase and protease), inhibition zone diameter, AST levels and ALT levels. The data were tested for normality and homogeneity, respectively, using the Kolmogorov Smirnov test and the Levene test. All data obtained were normally distributed and homogeneous, so the data were analyzed using Analysis of Variance (ANOVA) one way with a significance degree of 5% then continued with Duncan's Multiple Range Test (DMRT).

RESULTS AND DISCUSSION

Digestive enzyme levels

The difference in amylase enzyme levels in the infected and non-infected groups of *A. hydrophila* is presented in Figure 1. The level of amylase enzyme produced by the non-infected group was higher than the treatment group (infected). At 0%, 5% and 15% DSP concentrations, no significant differences were observed in amylase enzyme levels between *A. hydrophila*-infected and non-infected treatment groups.

The results obtained from the observation of lipase enzyme levels showed that the higher the concentration of a given DSP, the higher the lipase enzyme levels. The results of statistical analysis showed that the levels of lipase enzyme produced were significantly different between treatment groups infected and non-infected with *A. hydrophila* (Figure 2).

Figure 3 shows the difference in protease enzyme levels between the *A. hydrophila*-infected and non-infected groups. At 0%, 5%, 10%, and 15% concentrations, the levels of protease enzymes produced by the treatment groups that were not infected with *A. hydrophila* were higher than those of the infected treatment groups. However, 10% and 15% DSP concentrations showed insignificant differences in protease enzyme levels between the *A. hydrophila*-infected and non-infected treatment groups.

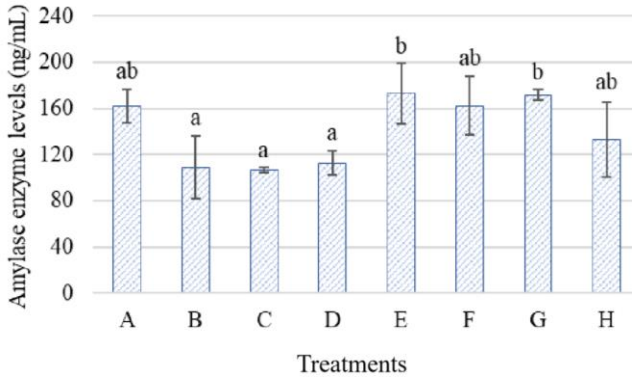

Figure 1. Results of amylase enzyme measurement in catfish blood serum. Different letters indicate significant differences between treatment groups

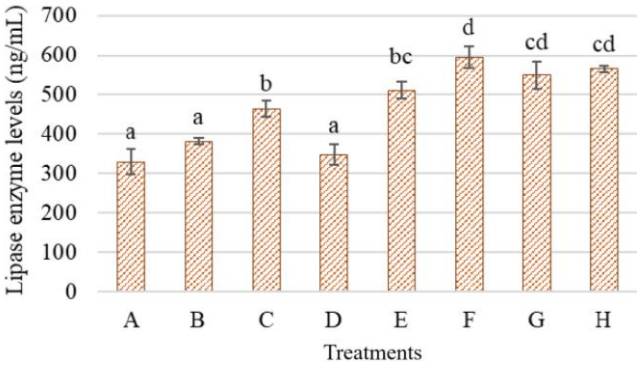

Figure 2. Measurement results of lipase enzyme in catfish blood serum. Different letters indicate significant differences between treatment groups

Liver function measurement

Measurement of liver function was seen from the AST and ALT enzyme levels (Table 1). The results showed that as the concentration of DSP increased, the levels of AST and ALT enzymes also decreased. Based on the results of statistical analysis in the infected fish group, the lowest AST and ALT levels were found in treatment D, with values of 24.21±7.70 U/L and 16.42±4.46 U/L, respectively. Similar to the non-infected fish group, ASAT and ALAT levels were noted in treatment H, each amounting to 9.26±1.91 U/L and 24.85±3.71 U/L.

Antimicrobial activity using disc diffusion method

The serum derived from catfish blood was tested to inhibit the growth of *A. hydrophila* using the Kirby-Bauer disc diffusion method. The samples used came from catfish blood serum that had been treated using different levels of DSP concentration and *A. hydrophila* infection. In this test also used control as a comparison, positive control using Enrofloxacin antibiotic and negative control using distilled water. Inhibition zone in fish blood serum as an antibacterial against *A. hydrophila* can be seen in Figures 4 and 5.

The results showed that there were significant differences among treatments with different concentrations of DSP 0%, 5%, 10% and 15%. In non-infectious group, it can be seen that 15% DSP concentration works optimally in increasing antibodies and inhibiting the growth of *A. hydrophila* bacteria, while in infectious group 5% DSP concentration works optimally in increasing antibodies and inhibiting the growth of bacteria.

Table 1. Measurement results of AST and ALT enzyme levels in catfish liver

Treatments	AST (U/L)	ALT (U/L)
A	102.41±9.62^{a}	86.43±12.37^{a}
B	89.01±5.03^{a}	82.14±9.89^{a}
C	32.64±6.40^{c}	17.85±10.57^{c}
D	24.21±7.70^{cd}	16.42 ±4.46^{c}
E	56.13±13.35^{b}	96.43±2.14^{a}
F	35.82±9.73^{c}	43.23±9.21^{b}
G	16.88±8.26^{d}	26.90±3.22^{c}
H	9.26±1.91^{d}	24.85±3.71^{c}

Notes: Different letters indicate significant differences between treatment groups

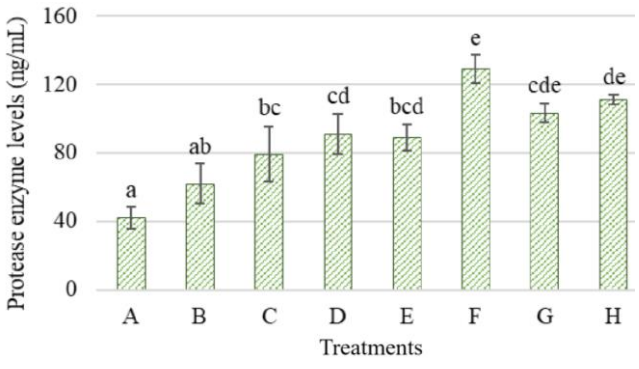

Figure 3. Measurement results of protease enzymes in catfish blood serum. Different letters indicate significant differences between treatment groups

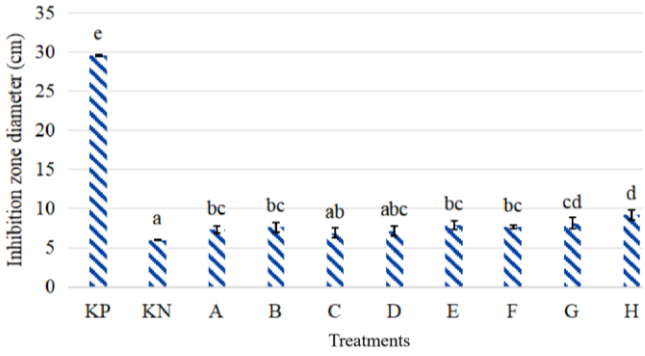

Figure 4. Inhibition zone in fish blood serum as an antibacterial against *A. hydrophila*

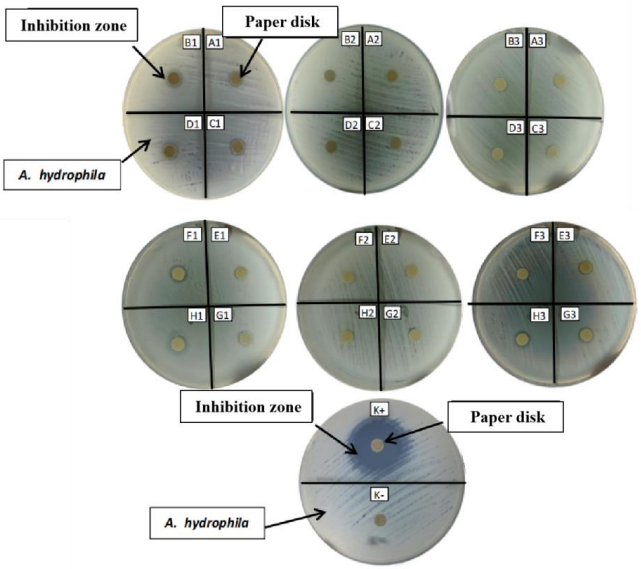

Figure 5. Antimicrobial activity of catfish blood serum against *A. hydrophila*

Discussion

In the process of catfish farming, quality feed becomes one of the determining factors for successful cultivation. Efforts that can be made to increase feed quality include adding probiotics that can help the process of feed digestibility (Apriliyanto et al. 2021). The addition of probiotics has been shown to increase feed digestibility with the help of enzyme secretion in the host's gastrointestinal tract (Arani et al. 2019). The results showed that treatment without DSP had higher levels of amylase enzyme in the blood serum of fish infected with *A. hydrophila*. These results contrast with several studies conducted, such as by Luo et al. (2022), who reported that amylase enzyme levels in koan fish (*Ctenopharyngodon idella* Valenciennes 1844) were higher in treated fish (*L. plantarum* and *B. subtilis*) than the control one. Similarly, research conducted by Adorian et al. (2019) also showed that amylase enzyme levels in treated white snapper (*Lates calcarifer* Bloch 1790) (*B. subtilis* and *B. licheniformis*) was higher than in untreated white snapper. The results obtained were also inversely

proportional to the observations of lipase and protease digestive enzyme levels carried out in the present study.

In the observation of other digestive enzyme levels, it was seen that the group treated with DSP- produced higher enzyme levels than the untreated group. It was also observed in amylase enzyme level that there was no significant difference in the results of enzyme levels produced by the treated fish and untreated. Amylase enzyme levels results show that fish given feed with DSP produce enzyme levels with almost the same value as fish that were not given DSP feed, thus indicating that there was no effect of adding DSP to fish feed on catfish amylase enzyme levels.

The difference in results obtained in the observation of amylase enzyme levels can be caused by the nutritional content of Prima Feed PF 1000 commercial fish feed with a protein content of 39-41% from PT Matahari Sakti, which is given to fish that does not contain carbohydrates. According to Assan et al. (2022), the level of digestive enzymes produced depends on the intake of nutrients that enter the body. The nutritional content of the feed used based on the composition of nutritional values contained in the feed label from PT Matahari Sakti is 6% fiber (maximum), 5% fat (minimum), 39% protein (minimum), 12% ash (maximum), and 10% moisture content (maximum). There was no carbohydrate content in the feed nutrient, which is why it could not trigger the non-production of amylase enzymes in the probiotic bacteria. Therefore, the result indicates that the levels of amylase enzyme produced in fish with the addition of DSP and without the addition of DSP did not differ significantly.

Based on the results obtained from the observation of lipase enzyme levels, it can be seen that the higher the concentration of DSP, the higher the lipase enzyme levels. Zhang et al. (2018) stated that if the concentration of probiotics is high, then the level of digestive enzymes is also higher. The results showed that the highest lipase enzyme level was produced by fish with 10% and 15% DSP. A study conducted by Adorian et al. (2019) also showed that lipase enzyme levels produced by white snapper (*L. calcarifer*) given probiotics (*B. subtilis* and *B. licheniformis*) with higher doses than fish with probiotic doses and controls. In addition, research conducted on shrimp (*Litopenaeus vannamei* Boone 1931) by Monier et al. (2023) also showed that the highest lipase enzyme levels were produced by treatments with probiotic doses (*B. subtilis* and *B. licheniformis*) 0.03 g/m^3 than treatments with probiotic doses of 0; 0.01 g/m^3; and 0.02 g/m^3.

Based on the results obtained from the observation of protease enzyme levels, the higher protease enzyme levels in the blood serum of fish infected with *A. hydrophila* were found in the treatment with the provision of DSP with high concentrations. This is in accordance with Wang et al. (2020), who stated that if the concentration of probiotics is high, then the level of digestive enzyme level is also higher. Meanwhile, the results of protease enzyme levels in the fish intestine showed the opposite result to Wang et al. (2020) who reported that the low DSP concentration actually produced high levels of protease enzymes. Monier et al. (2023) reported that the higher concentration of probiotics (*B. subtilis* and *B. licheniformis*) given to shrimp (*L. vannamei*) produces higher level of protease enzymes. It can be seen that the provision of 0.03 g/m^3 probiotics produced higher enzyme levels than the provision of 0; 0.01 g/m^3; and 0.02 g/m^3 probiotics.

Meanwhile, research conducted by Putra et al. (2021) showed that dumbo catfish (*C. gariepinus*) given 1.4% probiotics (*Bacillus* NP5) produced lower protease enzyme levels than fish given 1.1; 1.2; and 1.3% probiotics. According to Wilkins and Sequoia (2017), this may be because the effectiveness of probiotic use depends on the species, dose, and type of disease. The use of the right dose can provide maximum effect on the host. The use of higher doses does not necessarily increase the effectiveness of a probiotic or provide better results (Aini et al. 2024b). The use of high concentrations of probiotics can cause an imbalance between existing bacteria in the digestive tract and probiotic bacteria. With more probiotic bacteria entering the digestive tract of fish, there is increased competition between bacteria in taking nutrients that enter the body and make the activity of bacteria actually become inhibited (Zhu et al. 2019; Safari et al. 2022)

Based on all the results of the study, it can be seen that the group of catfish not infected with *A. hydrophila* gave higher and better results than the group of catfish infected with *A. hydrophila*. This can certainly occur due to the presence of *A. hydrophila*, which disrupts the metabolism of catfish. This may be due to the presence of these pathogenic bacteria can cause a decrease in the levels of digestive enzymes produced by catfish. The decrease can be caused by *A. hydrophila* which produces endotoxins and exotoxins, such as hemolysis, aerolysin, cytosine, gelatinase, and elastase (Kari et al. 2022; Semwal et al. 2023). With these various compounds, catfish can experience various diseases including damage to the fish pancreas. A decrease in the levels of digestive enzymes in catfish can be possible due to damage to the pancreatic organ of the fish which causes the performance of the pancreas in secreting digestive enzymes to be disrupted.

The results of ALT measurements showed that ALT levels were higher, especially in the group of infected fish without probiotics, whereas in fish treated with probiotics, liver function showed a better condition. Even in the treatment with a higher concentration of DSP, ALT levels were low and significantly different from the control treatment. The status of liver damage in fish infected by *A. hydrophila* can be known in various ways, one of which is by measuring the levels of AST and ALT enzymes (Hastuti and Subandiyono 2020). AST stands for alanine aminotransferase or has another name, Serum Glutamic Pyruvic Transaminase (SGPT). This enzyme is mostly found in the liver, but a small part can also be found in the kidneys, heart, muscles, and pancreas (Qiu et al. 2016). ALT enzyme levels indicate the status of liver function in fish. Normally, ALT is found in the blood in very small amounts, but if the fish's liver function decreases either due to environmental stress or due to pathogen infection, ALT will be secreted into the blood (Hastuti et al. 2019).

The liver function health parameter observed in this study was AST aspartate aminotransferase. In general, AST can be found in red blood cells, liver, heart, muscle tissue,

pancreas, and kidneys. However, if the liver is damaged, AST is secreted more into the bloodstream. The degree of tissue damage is directly related to AST levels in the blood (Dasgupta 2015). Research by Moghaddam and Purjaafari (2021) proves that by adding multistrain probiotics consisting of *Pediococcus pentosaceus*, *Weissella cibaria*, *Lactococcus lactis* and *Enterococcus faecalis* with the same dosage of each (10^8 CFU/kg) can improve the liver health status, growth and immune system of juvenile fish Persian sturgeon (*Acipenser persicus* Borodin 1897). Mollanourozi et al. (2021) reported that the addition of probiotic *L. casei* as much as (1.5×108 CFU/mL) for 24 days in Koi fish (*Cyprinus rubrofuscus*) infected by *Salmonella typhimurium* can reduce the value of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate transaminase (AST) in fish blood serum.

The results of antibacterial activity showed that catfish serum had the ability to inhibit the growth of *A. hydrophila*. There was a significant difference between infected and non-infected treatments in each treatment group with different levels of DSP concentration, where the non-infected group had a higher inhibition zone size compared to the infected group. Based on the results obtained, it can be stated that the catfish blood serum has compounds that can inhibit the growth of *A. hydrophila* bacteria. Fish serum already contains natural IgM antibodies before immunization, but exposure to antigens with T cells helps promote the release of specific tetrameric IgM. The antibody component in the form of immunoglobulins, as much as 10-20%, is thought to provide effectiveness in inhibiting the growth of pathogenic bacteria (Barrett et al. 2010). DSP, especially the *Lactobacillus* genus that has been consumed and accumulated in the body can increase the production of IgM which is one of the first types of antibodies in fish (Giri et al. 2013), while *B. subtilis* is antagonistic to *A. hydrophila* bacteria (Das et al. 2014). Thus, the combination of these two bacteria in the body can increase resistance to pathogens.

In conclusion, it was suggested that the best treatment could be the addition of DSP in catfish feed, which could increase digestive enzymes, such as amylase, lipase and protease enzymes. In addition, at a concentration of 15% DSP also improves the health status of catfish liver which can be seen from the decrease in AST and ALT enzymes when compared to the control treatment. The results showed that catfish blood serum treated with 15% DSP concentration had antimicrobial activity against *A. hydrophila*. In addition, further research should be conducted regarding the antibody titer in each serum, appropriate infection time for antibody formation, environmental conditions of sterile maintenance of experimental animals, and the effect of storage and optimal temperature on serum to be more precise.

ACKNOWLEDGEMENTS

The study was funded by Universitas Airlangga, Surabaya, Indonesia flagship research scheme (Grant no. 317/UN3.15/ PT/2023), Indonesia.

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