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Effectiveness of *Bacillus* sp. to increase the body resistance of common carp (*Cyprinus carpio* Linnaeus, 1758) against the attack of *Aeromonas hydrophila*

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ABSTRACT

This study aims to determine the best density of *Bacillus* sp. which is given through feed to increase the body resistance of carp that is infected by pathogenic bacteria, *Aeromonas hydrophila*. The research was conducted at the Aquaculture Laboratory and Laboratory of Microbiology and Biotechnology at FPIK UNPAD. This study used an experimental method with a completely randomized design consisting of 4 treatments and 3 replications, namely treatment A (control), treatment B (*Bacillus* sp. 10^6 cfu/mL feed), treatment C (*Bacillus* sp. 10^8 cfu/mL feed) and treatment D (*Bacillus* sp. 10^{10} cfu/mL feed) for two weeks later in *Aeromonas hydrophila* infection with an intramuscular density of 10^8 cfu/mL for seven days. Observation parameters were clinical symptoms, fish response to feed, fish response to shock and survival. The results of this study showed that treatment C (*Bacillus* sp. 10^8 cfu/mL feed) resulted in 89% survival and reduced clinical symptoms.

***Keywords*:** Common carp, Bacillus sp, *Aeromonas hydrophila,* Body resistance, *Cyprinus carpio*

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1. INTRODUCTION

Common carp (*Cyprinus carpio* Linnaeus, 1758) is one type of freshwater fish that has economically important value, and has a fairly high protein content, and has a fairly affordable price. Therefore carp is a freshwater fish that is widely cultivated by the community. Demand for common carp production in West Java in 2014 to 2016 has increased by 177,822.18 - 213,535.97 tons (West Java Province Marine and Fisheries Service 2018).

One obstacle that often occurs in goldfish cultivation is the onset of pathogenic bacteria that can inhibit fish growth and can cause death. In 1980 there were mass deaths caused by the disease of *Aeromonas hydrophila*, this bacterium can kill 125 tons of carp in West Java and its surroundings. Since the outbreak of this disease in MAS aquaculture often occurs MAS (*Motile Aeromonas septicemia*) or red spot disease, these bacteria infect many types of freshwater fish such as Catfish, Cyprinidae, Cichlidae, Rainbow trout, Salmonidae, and Shrimp. These bacteria can also cause high mortality rates of 80-100% within one to two weeks.

The *Aeromonas hydrophila* bacterium is an opportunistic bacterium. This infection from bacteria can occur if there are changes in environmental conditions, stress, changes in temperature of contaminated water. MAS disease caused by bacterial infection *Aeromonas hydrophila* can attack carp and other types of freshwater fish through water brokers, contact fish body parts, or aquaculture equipment contaminated or contaminated by pathogenic bacteria.

Therefore, to improve the body's defense system of carp that is susceptible to MAS disease, there is a need to control the body's immune system by using probiotic bacteria. Probiotic bacteria are capable of monitoring biological maintenance conditions without causing adverse effects on the microbial ecological balance system both in digestion and in fish maintenance. Probiotics also have the potential to increase body resistance, improve feed efficiency and improve water quality. One type of probiotic bacteria is *Bacillus* sp.

Bacillus sp. bacteria is a gram-positive bacterium that has been developed and can control pathogenic bacteria. These bacteria can form endospores and can survive longer in environmental conditions that are not favorable for their growth. *Bacillus* sp. bacteria have the ability to control pathogenic bacteria and can inhibit the growth of other bacteria. These bacteria can produce antibiotics that are toxic to other microbes. Therefore *Bacillus* sp. can improve the immune system non-specifically in fish. This is characterized by an increase in total leukocytes which act as non-specific immunity.

Some studies also show that the bacteria *Bacillus* sp. able to improve the goldfish immune system, improve feed efficiency in catfish, improve the immune system of catfish, and improve non-specific immune responses in tilapia. The purpose of this research is to determine the best density of *Bacillus* sp. which is given through feed to increase the body resistance of carp that is infected by pathogenic bacteria, *Aeromonas hydrophila*.

2. MATERIALS AND METHODS

The materials used in this research include carp seeds, *Bacillus* sp. Isolates, isolation from carp intestines, *Aeromonas hydrophila* isolates, and commercial feed. This study used an experimental method with Completely Randomized Design (CRD) consisting of 4 treatments and 3 replications. The treatment used is A (without the addition of *Bacillus* sp. on feed), B (addition of *Bacillus* sp. with a density of 10^6 CFU/mL into feed), C (addition of *Bacillus* sp. with a density of 10^8 CFU/mL into feed), and D (addition of *Bacillus* sp. with a density of 10^{10} CFU/mL into feed).

2. 1. Procedure

Culture of *Bacillus* sp. bacteria

Isolate of *Bacillus* sp. in isolation was done again to get a single colony. Bacterial culture was carried out on NA (*Nutrient Agar*) media using the scratch method. Sterile NA media was poured aseptically on a petri dish and left to cool. Inoculation of *Bacillus* sp. bacteria was done on the media and incubated at 30 ºC for 24 hours. Growing bacteria were taken by single colonies to determine the density of these bacteria. Bacterial culture was carried out on NB (*Nutrient Broth*) media. Sterile broth media was transferred into the centrifuge tube and added *Bacillus* sp. bacteria, then incubator shaker was used for 24 hours at 30 ºC at 150 rpm. The results of bacterial broth media were inserted into the cuvette and the density was calculated using a spectrophotometer with a wavelength of 620 nm.

Mixing of *Bacillus* sp. bacteria in feed

The calculation of bacterial density was carried out using a spectrophotometer. The density of bacteria produced by liquid culture is around 10^{10} CFU/mL. Then serial dilution was carried out to obtain a density of 108 CFU/mL and 10^6 CFU/mL. The dilution procedure was carried out by taking 1 mL of the bacterial suspension (10^{10} CFU/mL) and then inserting it into a medium of distilled water containing 9 mL and homogenized with a vortex (the density produced at this stage was 10^9 CFU/mL). The next step was to do the same thing to get a density of 10^8 CFU/mL and 10^6 CFU/mL.

The result of bacterial density uses 10 mL/kg of feed. Before being mixed into the feed, a binder (egg white) was added as much as 2% of the weight of the feed. Then mixing into a feed and stirring until evenly dried was done. Feeding per day was twice a day using the ad satiation method, or as little as 2 weeks.

Culture of *Aeromonas hydrophila* bacteria

The isolates of *Aeromonas hydrophila* were inoculated on NA (*Nutrient Agar*) media using the scratch method to obtain a single colony, then incubated at 30 ºC for 24 hours. The results of the bacteria that were grown were taken by using one needle as much as 1 ose and dissolved with NB (*Nutrient Broth*) media. After that, the bacteria were incubated in a shaker incubator for 24 hours at 30 ºC at a speed of 150 rpm. Then the density calculation was performed to obtain a density of 10^8 CFU/mL using a spectrophotometer with a wavelength of 540 nm and an absorbent value reaching 0.8-1 for a density of 10^8 CFU/mL.

Challenging Test with *Aeromonas hydrophila* Bacteria

Infection of *A. hydrophila* bacteria against carp was done by injecting common carp in the intramuscular section as much as 0.1 ml/head with a density of 10^8 CFU/mL. Observation of clinical symptoms and survival of test fish after A. *hydrophila* infection was carried out every day during the maintenance period of 7 days.

2. 2. Observation parameter

The clinical symptoms of carp are damage to the body, response of fish to feed, the response of fish to shock and survival.

The percentage of survival can be obtained using the Effendie formula (1997), namely:

$$SR = \frac{Nt}{No} \times 100$$

Note:

SR = Survival Rate (%)
Nt = the number of fish that live at the end of the research (fish)
No = the number of fish that live at the beginning of the research.

Supporting parameters of this study are water quality measured including temperature, DO, pH, and ammonia. Measurements are made three times, namely at the beginning, in the middle, and at the end.

2. 3. Data analysis

Data on fish survival rates were analyzed using variance analysis and if there were significant differences between treatments followed by a double distance Duncan test with a 5% error rate. Clinical symptom data, fish response to feed, fish response to leukocyte shock, and water quality were analyzed descriptively.

3. RESULT DISCUSSION

3. 1. Damage to carp body after *Aeromonas hydrophila* infection

Clinical symptoms caused after 7 hours after injection are marked by changes in behavior such as swimming movements of fish that become sluggish and the position of the body of the fish becomes tilted because the balance of the fish body begins to decrease, and appetite begins to decrease. Besides the morphological symptoms that occur in damage to the body surface of carp, namely the appearance of redness on the back of the fish body (Figure 1b), there is inflammation of the wound in the injection site area (Figure 1c) looking different compared to healthy fish (Figure 1a). This is in accordance with the statement of Lukistyowati and Kurniasih (2011) that fish infected with *A. hydrophila* bacteria can cause a reddish color at the injection site then followed by inflammation of the wounds of the dilated ulcer with injection.

On the second day until the 7th day after infection with *A. hydrophila*, the clinical symptoms that arise in each treatment (A, B, C and D) are similar, in the form of red spots, inflammation or ulcer wounds, and some fish damage to the fins on the part caudal fin (Figure 1d) and broken meat. On the seventh day, the symptoms that appear are only in the form of wounds that have begun to be covered by new tissues. Based on observations of the occurrence of body damage in each treatment each day is different; this is thought to be the bacterium *A. hydrophila* which attacks carp gradually. In addition, the symptoms caused can cause the death of carp caused by the accumulation of liquid and broken meat. Inflammation of the wound occurs because of the toxins that come out of the bacteria.

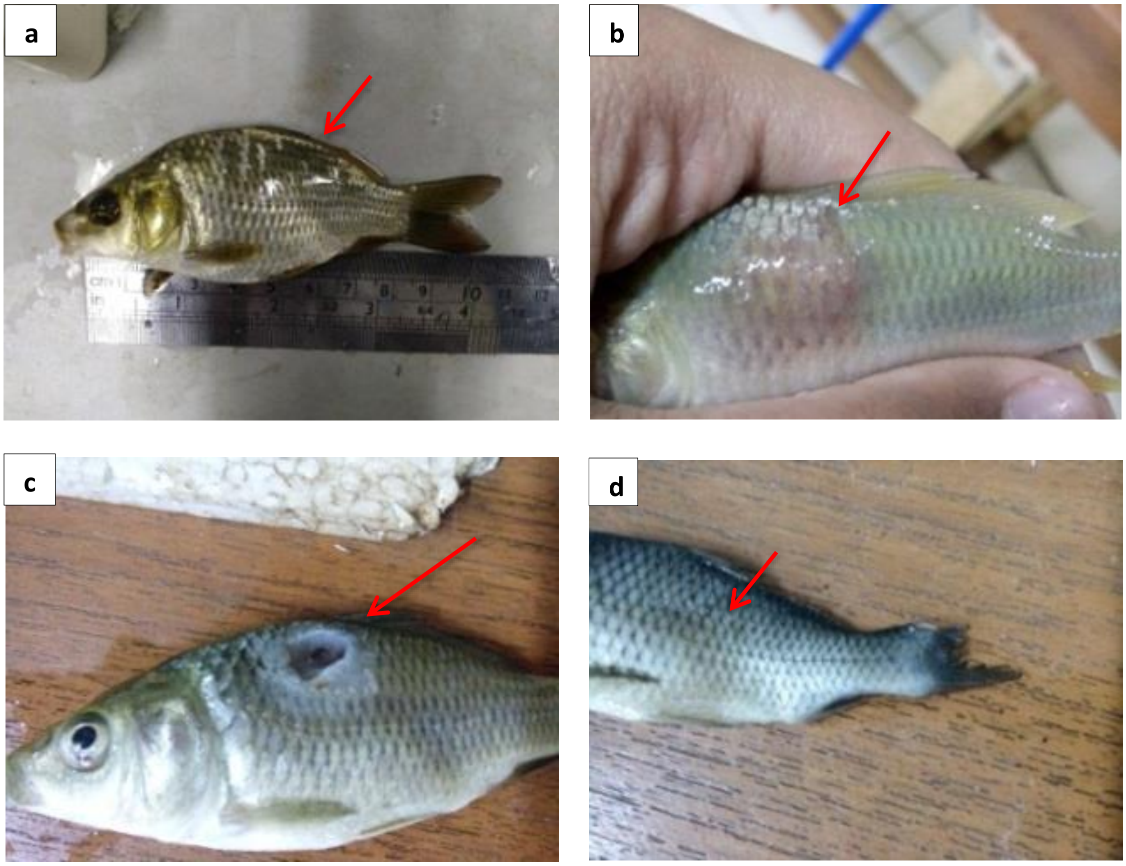

Figure 1. Clinical symptoms of common carp seeds.
Information: **(a)** healthy fish **(b)** haemorargik **(c)** inflammation of wounds **(d)** flaky fins

Damage to the surface of the body of the fish caused by *A. hydrophila* is thought to be due to a disruption in the muscle tissue and channels of fish blood vessels due to the effects of the bacterial toxin. Exotoxin enzymes such as hemolysin, proteases, and elastase are thought to cause damage to the body of infected fish, because in the muscle tissue and blood vessel channels there is a lot of protein content. Protease enzymes are enzymes that can fight the host's defenses to develop the disease and take up nutrients. The hemolysin enzyme that is dissolved in the blood is able to lyse red blood cells and free their hemoglobin so that much blood comes out through the wound on the surface of the infected body. This can cause hemorrhagic that occurs in the body of carp, caused by hemolysin toxins with the target of breaking down red blood cells so that the cells come out of the blood vessels and cause the reddish color on the skin surface. In addition, the effects of exotoxins can cause cells in the muscle tissue to die, so that it will cause necrosis, which is damaged and decaying meat.

The body damage caused by *A. hydrophila* can be reduced by the addition of probiotic bacteria *Bacillus* sp. into feed in each treatment except control. This is thought to be the addition of *Bacillus* sp. into feed can increase the immune system. *Bacillus* sp. bacteria produce antibiotics in the form of bacteriocin as an antibacterial and immunostimulant so as to reduce the pathogenic activity of *A. hydrophila*. These bacteriocins have a broad spectrum in inhibiting the growth of decomposing bacteria and pathogenic bacteria. This also will inhibit the process of growth and spread of *A. hydrophila* on infected body parts of the fish, so that there is a reduction in the clinical symptoms of fish.

3. 2. Fish response to feed

Based on Table 1, the results of observations of the test fish response to post-infectious feed, seen on the first day did not indicate a response of fish to feed. However, in the afternoon feeding, treatments B, C, and D showed an appetite response but still low. This is because the test fish experiences post-injection stress so that it decreases appetite and does not even want to be eaten.

Table 1. Observation of Fish Response to Feed.

P	Observation of the Day's Appetite Response													
	1		2		3		4		5		6		7	
	P	S	P	S	P	S	P	S	P	S	P	S	P	S
A1	-	-	-	-	+	+	+	+	+	+	+	+	+	+
A2	-	-	-	-	+	+	+	+	+	+	+	+	+	+
A3	-	-	-	-	+	+	+	+	+	+	+	+	+	+
B1	-	+	+	+	+	+	+	+	++	++	++	++	++	++
B2	-	+	+	+	+	+	+	+	++	++	++	++	++	++
B3	-	-	+	+	+	+	+	+	++	++	++	++	++	++
C1	-	+	+	+	+	+	+	+	++	++	++	++	++	++
C2	-	-	+	+	+	+	+	+	++	++	++	++	++	++
C3	-	+	+	+	+	+	+	+	++	++	++	++	++	++
D1	-	+	+	+	+	+	+	+	++	++	++	++	++	++
D2	-	-	+	+	+	+	+	+	++	++	++	++	++	++
D3	-	-	+	+	+	+	+	+	++	++	++	++	++	++

Note :

(-): No response to feed
(+): Response to low feed
(++): Response to normal feed
P: Morning
S: Afternoon

On the second day to the fourth day the feed response began to increase even though the response of the fish to the feed was still low with the rest of the feed on the maintenance media, but not with the control treatment which still did not show any response to the test fish from the first day to the second day. On the fifth day, the response of the test fish to the feed in each treatment had begun to show an increase in appetite until the seventh day. Except for the control of the 3rd day until the 7th day, the response of fish to the feed is still low with the remaining feed on the maintenance medium. This is thought to be the absence of response to test fish for feed due to fish experiencing stress due to post-infection. Rejection of food is often experienced in unhealthy fish. This also can disrupt the digestive system of fish so that it decreases fish appetite. Pathogenic bacteria that enter the body's organs, especially the digestive system can cause fish indigestion.

Provision of probiotics *Bacillus* sp. into the feed gives an effect on the appetite response of fish. This can be seen on the second day the fish given faster treatment increased appetite response compared to the control fish. It is suspected that the bacterium *Bacillus* sp. can restore the condition of the fish after infection with *A. hydrophila*, so that fish given probiotic bacteria can improve appetite response. This is also the bacterium *Bacillus* sp. produces several enzymes in the form of proteases, lipases, and amylases. These enzymes can convert complex molecules into the intestine, which can be converted into simple molecules, making it easier to absorb by the digestive tract of fish.

3. 3. Response of Fish to Surprise

Based on the observational data (Table 2), the response of the test fish to surprise was seen that on day 1 all treatments did not respond to surprises. This is suspected that the activity of *A. hydrophila* bacteria had attacked carp, causing fish stress and decreasing the shock response. It is also characterized by swimming movements of fish that begin to become unstable and fish that often approach aeration and appear to the surface to get oxygen. According to Olga (2014), fish infected with *A. hydrophila* can cause the body's balance to be disturbed and swim wear to the surface of the water and tend to be alone.

Table 2. Observation of the response of carp to surprises.

Observation day	**Treatment**											
	A (Control)			**B (10^6 CFU/mL)**			**C (10^8 CFU/mL)**			**D (10^{10} CFU/mL)**		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	+	+	+	+	+	+	+	+
3	-	-	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	++	++	++	+	++	++	+	+

5	+	+	+	++	++	++	++	++	++	++	++	++
6	+	+	+	++	++	++	++	++	++	++	++	++
7	+	+	+	++	++	++	++	++	++	++	++	++

Note :

(-): There is no response to surprises
(+): Response to weak surprises
(++): Response to normal surprises.

In treatment A (control) on the first day until the third day it has not addressed the response of the fish to the surprise. But on the fourth day until the seventh day the test fish has begun to show a shock response even though it is still weak. This is suspected in treatment A not given the treatment of probiotic bacteria *Bacillus* sp. so that after the infection of the test fish it is more susceptible to pathogenic bacteria, and can cause stress and decrease the shock response of the fish.

On the second day until the third day of treatment B, C, and D, the test fish began to give a surprising response even though it was still weak. However, on the fourth day to the seventh day the treatment was given by bacteria *Bacillus* sp. to start giving a good surprise response. It is seen that the fish has been stabilized even though there are still fish that have been attacked by the disease. It is suspected that in treatment B, C, and D, there was an addition of probiotic bacteria *Bacillus* sp. so that it can increase fish's body resistance.

3. 4. Survival Rate

Based on observations of common carp survival after infection with *A. hydrophila* (Figure 2), in treatments B (10^6 CFU/mL), C (10^8 CFU/mL), and D (10^{10} CFU/mL) which were added by probiotic bacteria *Bacillus* sp. into the feed, a higher survival rate was produced compared to treatment A. Based on the results of the statistical F test, the survival rate of carp after infection with *A. hydrophila* was not significantly different ($p > 0.05$). Although statistically, the survival rate between treatments was not significantly different, there was a tendency that treatment C had a higher survival rate of 89% compared to other treatments. It is suspected that the fish survival rate is higher due to the efforts to increase the body's immune system which is characterized by an increase in the total number of leukocytes which act as a non-specific defense.

Addition of probiotics *Bacillus* sp. into feed with a density of 10^{10} CFU/mL resulted with the survival rate lower than the treatment with a density of 10^6 CFU/mL and 10^8 CFU/mL. This is presumed that the addition of *Bacillus* sp. with a higher density does not provide better body resistance, causing more mortality. It is also suspected that the number of bacteria in the digestive tract that is getting denser can cause competition between bacteria so that bacterial activity in digesting nutrients will be hampered and cause the death of these bacteria, and disrupt the balance of the microflora in the media and host body. One can state that the factors that influence the host's response to probiotics, include the composition of the microflora, host intestine, dosage used, age and quality of probiotics. The criteria that need to be considered for obtaining an efficient probiotic with a positive influence on the host are the number of densities ranging from 10^7 CFU/mL to 10^9 CFU/mL.

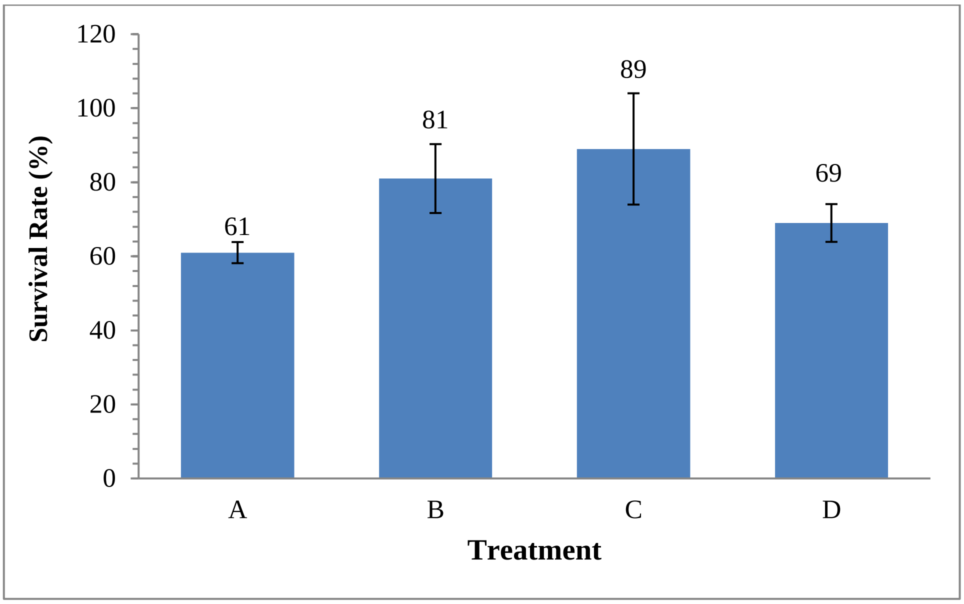

Figure 2. Survival rate of common carp.

Giving *Bacillus* sp. into the feed is thought to be able to improve the immune system so that it can fight the attack of pathogenic bacteria. These bacteria produce antibacterial compounds in the form of bacteriocins which are able to inhibit the growth of pathogenic bacteria, so they can improve survival. That *Bacillus* sp. can produce antibiotics to fight *Vibrio* sp. in groupers so that probiotics can improve survival.

3. 5. Water Quality

Table 3. Data on Water Quality Measurement Results.

Treatment	**Water Quality Parameters**			
	Temperature (°C)	**pH**	**DO (mg/L)**	**Ammonia**
A	26.8-30.6	6.97-7.79	5.8-8.9	0.007-0.012
B	26.8-30.8	7.09-7.96	5.9-8.6	0.027-0.029
C	28.6-29.5	7.09-7.81	5.5-8.7	0.013-0.016
D	28.7-30.4	7.15-7.85	5.9-8.5	0.016-0.017
Optimal	*25-30	*6.5-8.5	*>5	*8<0.1

Note:

*SNI 01-6133-1999; Carp production (*Cyprinus carpio* Linneaus 1758) strain Majalaya scattered seed class
** Boyd (1990)

Water quality parameters were measured three times, namely at the beginning, in the middle and at the end of the study. The parameters observed were temperature, DO, pH, and ammonia. Results of measurements of water quality are presented in Table 3. It can be seen that the average temperature in each treatment ranged from 26.8-30.8 ºC, the average pH ranged from 6.97 - 7.96, the average dissolved oxygen content (DO) ranged from 5.5 to 8.9 mg/L and the average ammonia content was 0.007-0.029 mg/L. The water conditions during the study period can be in the range of the optimum standard of carp fish according to the Indonesian National Standard SNI: 01-6133-1999. According to Boyd (1990), the ammonia content should not be more than 1 mg/L, then things correspond to measurement results.

4. CONCLUSION

Based on the results of research that has been done it can be concluded that the administration of *Bacillus* sp. into feed with a density of 10^8 CFU/mL was most effective for increasing carp body resistance from the attack of *Aeromonas hydrophila,* and it was seen from carp survival of 89% and reduced clinical symptoms.

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