Jurnal Akuakultur Indonesia 21 (2), 93–108 (2022)

Original article

DOI: 10.19027/jai.21.2.93-108

The effectiveness of chitosan in increasing immunity against *Aeromonas hydrophila* in sangkuriang catfish (*Clarias gariepinus*)

Efektivitas kitosan dalam meningkatkan immunitas terhadap *Aeromonas hydrophila* pada ikan lele sangkuriang (*Clarias gariepinus*)

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ABSTRACT

The purpose of this research is to find out the optimal dose of chitosan that is appropriate to be added to commercial feed in increasing the immune system of sangkuriang catfish fry, the fish fry used are 6-8 cm in size. April – June 2021 was the time the research was carried out and the experimental method used in this study was a Completely Randomized Design (CRD) with five treatments and three replications. The treatment used was the addition of chitosan to 1 kg of feed with doses of 0 g (A), 2.5 g (B), 5 g (C), 7.5 g (D), and 10 g (E). Maintenance of test fish for 14 days. *Aeromonas hydrophila* bacteria were used to infect fish with a density of 108 CFU/ml by immersing 20 ml in 20 liters of water for 14 days. White blood cells, leukocyte differential, clinical symptoms in fish body, survival rate and water quality were used as parameters in this research. The results showed that the addition of chitosan at a dose of 10 g (E) per 1 kg of feed was effective for improving the immune system performance of catfish fry. During 14 days of chitosan administration, the number of white blood cells in treatments A, B, C, and D increased by 3.2%, 7.07%, 18.31%, and 27.71%, respectively, while in treatment E (10 g) experienced the largest increase with 41.60%, from the value of 13.96 × 104 cells/mm3 to 23.90 × 104 cells/mm3. The treatment of chitosan 10 g (E) per 1 kg of feed showed the largest increase so it was an effective dose to stimulate differential leukocytes of catfish to improve the immune system, it was also marked by the fastest healing period of clinical symptoms and after the challenge test had the highest survival rate of 96.6%.

Keyword: *Aeromonas hydrophila*, chitosan, immune system, sangkuriang catfish fry

ABSTRAK

Riset ini bertujuan untuk menentukan dosis optimum kitosan yang tepat untuk ditambahkan pada pakan komersil dalam meningkatkan sistem imun benih ikan lele sangkuriang, benih ikan yang digunakan berukuran 6-8 cm. April – Juni 2021 adalah waktu penelitian dilaksanakan dan metode eksperimen yang digunakan dalam penelitian ini adalah Rancangan Acak Lengkap (RAL) yang menggunakan lima perlakuan serta tiga ulangan.. Perlakuan yang digunakan adalah penambahan kitosan pada 1 kg pakan dengan dosis masing-masing 0 g (A), 2.5 g (B), 5 g (C), 7.5 g (D), dan 10 g (E). Pemeliharaan ikan uji selama 14 hari. Bakteri *Aeromonas hydrophila* digunakan untuk menginfeksi ikan dengan kepadatan 108 CFU/ml dengan cara merendam 20 ml dalam 20 liter air selama 14 hari. Parameter yang diamati meliputi sel darah putih, diferensial leukosit, gejala klinis, kelangsungan hidup dan kualitas air. Hasil riset menunjukkan bahwa penambahan kitosan dengan dosis 10 g (E) per 1 kg pakan efektif untuk meningkatkan performa sistem imun benih ikan lele. Selama pemberian kitosan 14 hari, jumlah sel darah putih pada perlakuan A, B, C, dan D berturut turut mengalami peningkatan sebesar 3.2%, 7.07%, 18.31%, dan 27.71%, sementara pada perlakuan E (10 g) mengalami peningkatan terbesar dengan 41.60 % yaitu dari nilai 13.96 × 104 sel/mm3 menjadi 23.90 × 104 sel/mm3. Perlakuan kitosan 10 g (E) per 1 kg pakan menunjukkan peningkatan terbesar sehingga merupakan dosis yang efektif untuk menstimulasi diferensial leukosit ikan lele untuk meningkatkan sistem imun, hal tersebut ditandai juga dengan masa penyembuhan gejala klinis tercepat dan tingkat kelangsungan hidup tertinggi setelah uji tantang yaitu sebesar 96.6%.

Kata kunci: *Aeromonas hydrophila*, kitosan, sistem imun, benih ikan lele sangkuriang

INTRODUCTION

Currently, Indonesia is increasing the fisCurrently, Indonesia is increasing the fishery sector, especially in the field of cultivation, this is motivated by the potential for the development of the fishery industry in the field of aquaculture. One of the fishery commodities that are suitable and much in demand for cultivation is sangkuriang catfish (*Clarias gariepinus*). In quarterly I-III Fish Production in 2017–2018, Catfish increased by 114.82% from 841.75 thousand tons to 1.81 million tons (BPS, 2018). Data on catfish production in Indonesia according to the KKP in 2020 is 993.768,29 tons (Statistik-KKP, 2020). This catfish species is widely cultivated because it has a wide market in Indonesia. In addition, catfish also has a high level of resistance to poor media so that farmers do not need to give special treatment in cultivating the catfish.

One of the most common obstacles in cultivation activities is the presence of disease. Infectious diseases are caused by viruses, bacteria, fungi and parasites. *Aeromonas hydrophila* is one of the bacteria that usually attacks freshwater fish, including catfish, as the cause of Motile *Aeromonad Septicemia* (MAS) disease, which is an acute or sub acute or chronic fish infection disease caused by *Aeromonas hydrophila* bacteria which is also known as red spot disease (Singha *et al*., 2018). According to Pramudita *et al*. (2013) a bacterial disease that often attacks catfish is *Aeromonas* sp. This disease is quite virulent, especially in catfish, because it can cause a death rate of more than 60% within 7 days.

There needs to be an effort to prevent MAS using chitosan in fish that can increase body resistance which is quite effective, inexpensive, safe for the environment and safe for consumer. Chitosan is one of the fishery wastes derived from the skin or shells of crustaceans that have undergone a process of demineralization, deproteination, and deacetylation (Sukenda and Jamal, 2008). Potential antibacterial action of chitosan showed that gram-positive bacteria are stronger than gram-negative bacteria which are more susceptible to chitosan, but there are some research results showing that gram-positive bacteria have a higher sensitivity level to chitosan than gram-negative bacteria. (Ke *et al*., 2021). The effect of chitosan as immunostimulants can also be seen from the number of lymphocytes, monocytes, and neutrophils increased significantly in all groups during the experiment (Mari *et al*., 2014).

The addition of 7.5 and 10 g/kg chitosan increased protection against *Aeromonas hydrophila* infection in Prussian carp (Chen *et al*., 2014). Chitosan with optimum concentration (5 g/kg and 7.5 g/kg) in feed can also increase lysozyme activity in the intestine so that it can withstand pathogenic bacteria in the intestine (Li *et al*., 2011). Meshkini *et al*. (2012) found that using chitosan at a concentration of 2.5 g/kg diet had a significant effect on hematological parameters and increased survival of rainbow trout (*Oncorhynchus mykiss*). The increase in leukocytes is thought to be due to the body's resistance response to pathogens by increasing the activity of phagocytic cells that function to destroy foreign objects that enter the fish body, phagocytosis is the initial stage in the body's defense mechanism (Sonida *et al*., 2014). Phagocytic cells will recognize and engulf antigenic particles, including bacteria and damaged host cells through three stages of the process, namely attachment, phagocytosis and digestion (Ode, 2013).

Based on the role of chitosan in increasing immunity, it is hoped that it can be an alternative solution for natural ingredients in an effort to prevent Motile *Aeromonad Septicemia* (MAS) disease, especially in sangkuriang catfish. The aim of this research is to observe the effectiveness of chitosan in increasing the body's resistance of sangkuriang catfish against *Aeromonas hydrophila*.

MATERIALS AND METHODS

Experimental design

This study used a completely randomized design (CRD) with 5 treatments and 3 replications each. The treatment used was the addition of chitosan into the feed with a concentration of:

A : Control (without giving chitosan to feed)
B : 2.5 g of chitosan per 1 kg of commercial feed
C : 5 g of chitosan per 1 kg of commercial feed
D : 7.5 g of chitosan per 1 kg of commercial feed
E : 10 g of chitosan per 1 kg of commercial feed

The Sangkuriang catfish used as test fish in size 5-8 cm were 300 fish originating from fish farmers in Cileunyi, Bandung and first acclimatized in a fiber tub for 7 days. The stocking density of fish in the aquarium (30 x 25 x 25) cm^3 is 20 fishes per aquaria. The amount of feed given is 5% of the fish's weight for 14 days, it is given twice a day, at 10.00 am and 18.00 pm. Siphoning was carried out every morning to remove metabolic waste and feed residue in the aquarium before feeding. During the research the water quality of the media was maintained to obtain an optimal environment such as temperature, dissolved oxygen (DO), and pH.

Research procedure

Aeromonas hydrophila obtained from the Laboratory of Biotechnology, Faculty of Fisheries and Marine Sciences, Padjadjaran University and The Research Station of Fish Disease Control Resarch Institute for Freshwater Aquaculture and Fisheries Extension, Bogor.

Commercial feed was mixed with chitosan in accordance with predetermined concentrations of 2.5, 5, 7.5, and 10 g/kg. *Carboxymethy cellulose* (CMC) added to the feed according to the specified formulation as binder. According to Azhari *et al.* (2016) the CMC dose used as a binder in feed is 2% - 3%. The chitosan feed is added with CMC in a ratio of 3:1 before being sprayed on feed.

The test fish after being treated for 14 days then challenged with *A. hydrophila* with a density of 10^8 CFU/ml. The challenge test was carried out using the immersion method (Kamiso and Triyanto 1992), 20 ml/20 L of a bacterial solution was added to each aquarium. The test fishes were observed at 4, 6, 12, 24 hours and 14 days. There is blood sampling for white blood cells and differential leukocytes (lymphocytes, monocytes, and neutrophils) for data collection.

Data collection

Fish blood was taken before starting the treatment, after feeding for 14 days, and after the challenge test. Blood samples from the three treated fish samples were taken every three, seven, ten, and fourteen days after the challenge test to see the leukocyte count and leukocyte differential. Observation of clinical symptoms including *hemorrhagic* (bleeding), inflammation, ulcers on the skin, *dropsy* (protruding stomach), *exophthalmia* (protruding eyes) was carried out after the test fish were challenged with *Aeromonas hydrophila*. Other observations were the response of fish to feed and movements due to shock. The water quality parameters observation were temperature, pH, dissolved oxygen (DO). The survival of the test fish was observed every day after injection until the end of the study. The survival rate of the test fish was calculated according to Effendie (1979).

Data analysis

Data of white blood cell count and survival rate were analyzed using variance with a 95% confidence level, if there were any difference in treatment, further tests were carried out with Duncan's test. Analysis of clinical symptoms, differential leukocytes and water quality were carried out descriptively.

RESULTS AND DISCUSSION

Results

White blood cells (leukocytes)

Observation of white blood cells aims to determine the effect of giving chitosan to increase the body's resistance of catfish, because white blood cells are an indicator of body resistance. Based on observations, the test fish in each treatment experienced an increase in the number of leukocytes after being given chitosan. The higher the addition of chitosan, the number of leukocytes will increase. The average value is 13.96×10^4 cells/mm^3 to 14.28×10^4 cells/mm^3 from the observation that the number of leukocytes from the test fish before chitosan treatment. After chitosan treatment, the mean leukocytes increased, ranging from 14.60×10^4 cells/mm^3 to 23.90×10^4 cells/mm^3 (Figure 1).

The test fish that were not given leukocyte chitosan after treatment showed an increase in leukocytes, the increase was due to the increase in age and weight of the fish after 14 days of rearing, from 14.13×10^4 cells/mm^3 to 14.60×10^4 cells/mm^3 (Figure 1). After a period of 14 days of rearing fish, a challenge test (CT) is carried out. There was an increase in the number of leukocytes during the challenge test period on day 3 (CT-3) in each treatment with the average number of leukocytes ranging from 18.86×10^4 cells/mm^3 to 24.50×10^4 cells/mm^3 (Figure 1). On the 7th day after the challenge test (CT-7) there was still an increase in the number of leukocytes in each treatment with the average number of leukocytes ranging from 22.19×10^4 cells/mm^3 to 24.68×10^4 cells/mm^3 (Figure 1). The test fish in the control treatment experienced the highest increase in the number of leukocytes from 18.86×10^4 cells/mm^3 to 24.00×10^4 cells/mm^3 (Figure 5). The test fish in treatment E experienced the lowest increase in the number of leukocytes from 24.50×10^4 cells/mm^3 to 24.68×10^4 cells/mm^3 (Figure 1).

In Figure 2, it can be seen that the test fish in the control treatment had an increase in the number of leukocytes, although only slightly (3.2%), when compared to fish treated with chitosan. The highest increase was found in treatment E, namely 41.6% (Figure 2). The change in the increase in leukocytes in the challenge test (CT-3) was the highest in treatment A (without chitosan) which increased by 22.60%, while the lowest increase in treatment B (2.5 g chitosan) was 22.21%. The number of leukocytes on day 7 (CT-7), treatment A remained the treatment with the highest increase in the number of leukocytes, which was 21.43%, and the treatment with the lowest increase in the number of leukocytes, namely in treatment E, which was only 0.71%. On the 10^{th} day (CT-10), and the 14^{th} day (CT-14) it decreased. Treatments A and B showed the highest number of leukocytes compared to still experiencing an increase, which was 9.02% in treatment A and 1.81% in treatment B.

Leukocyte differential

After the catfish fry were challenged, blood samples were taken to observe the leukocyte differential. The leukocyte differentials observed included cell types of lymphocytes, monocytes, and neutrophils (Suhermanto and Andayani, 2013).

Lymphocytes

Blood is checked by counting the number of leukocytes and differential leukocytes in fish blood to see and detect an increase in the body's immune response (Septiarini *et al.*, 2012). In the formation of antibodies, lymphocytes have an important role but are not phagostic (Baratawidjadja, 2012).

Based on the research conducted, it was shown that the percentage of lymphocytes before the challenge test ranged from 67.7%-74.3% (Figure 3), while the post-challenge test decreased in each

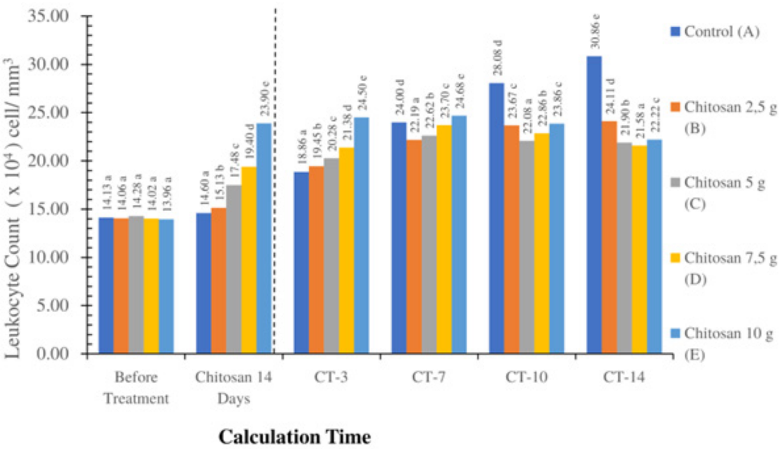


Figure 1. White blood cell (leukocyte) count during research.

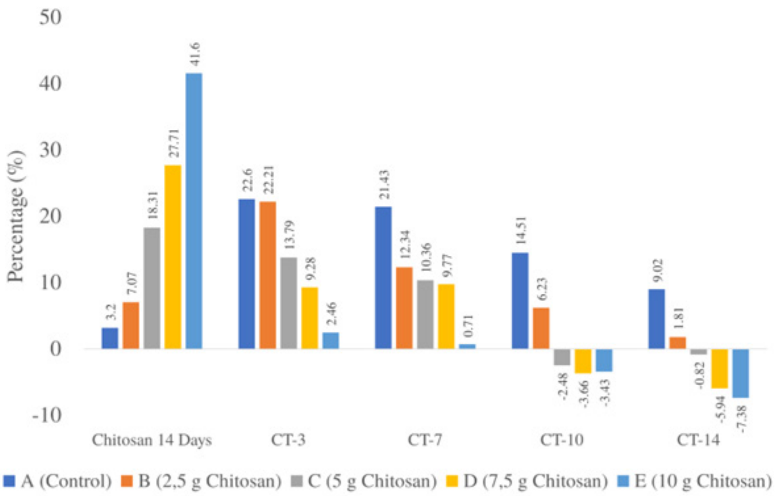


Figure 2. The percentage of increase/decrease in number of leukocytes in catfish during the study.

treatment ranging from 41.3%-59%. In Figure 3 it can be seen that the number of lymphocytes in fish blood has increased and decreased from before treatment to after treatment which has a percentage with a range of lymphocyte counts from 69.7% to 71.7%. After 14 days of challenge trial period, results of treatment E although decreased, but showed the highest value after the challenge test among the others, namely 59% followed by treatment D by 52%, treatment C by 47.7%, treatment B by 43.3% and the smallest treatment A by 41.3%.

Monocytes

The number of monocytes of sangkuriang catfish before treatment ranged from 15.3%-16.7%, after treatment ranged from 20%-22.7%. After being challenged with *A. hydrophila* bacteria, the increase in each treatment ranged from 30%-36.7% (Figure 4).

In Figure 4, the percentage of monocytes after feeding with chitosan for 14 days showed an increase in each treatment, with the highest percentage of monocytes in treatment E, from 16% to 22.7%. The lowest increase in the control treatment was from 16.3% to 20%. After a 14-day challenge test period, there were results, namely the lowest percentage was in treatment A (Control) with a value of 30% and the highest percentage was in treatment E (10 g) with a value of 36.7%.

Neutrophils

The number of sangkuriang catfish neutrophils before treatment ranged from 12%-13.7%, after treatment ranged from 3%-12.3%. After being

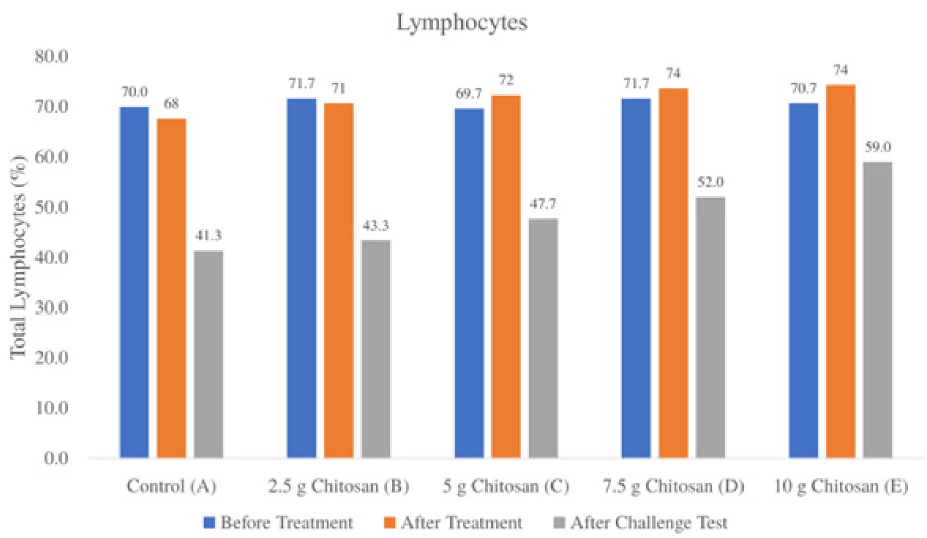


Figure 3. Total lymphocytes during the study.

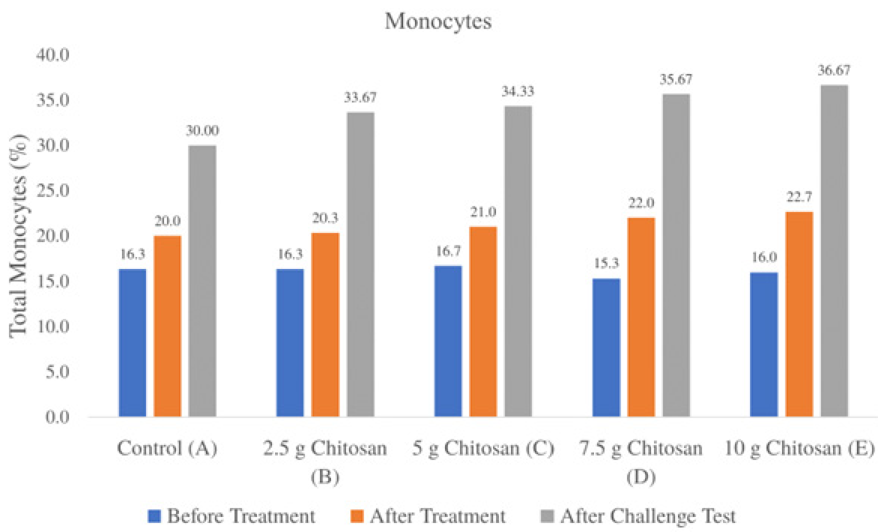


Figure 4. Total monocytes during the study.

challenged with *A. hydrophila* bacteria, the increase in each treatment ranged from 4.3%-28.7%. (Figure 5).

Based on the research conducted, the percentage of neutrophils from before treatment to after treatment decreased in each treatment, where the highest decrease occurred in treatment E with the number of neutrophils from 13.3% to 3%, and the lowest decrease was in the control treatment with the number of neutrophils from 13, 7% to 12.3% (Figure 5).

Clinical symptoms

The results of observations of damage to the surface of the fish body after the challenge test for 24 hours is in the form of inflammation characterized by red spots, thin fins and a distended belly (dropsy) (Figure 6). Body surface damage due to infection with *A. hydrophila* began to show after 24 hours of the challenge test. Treatments B and C showed that the body surface damage that appeared was almost the same as the other treatments, but some of the body surface damage did not have much impact on fish mortality compared to treatment A which experienced many deaths. Treatment without chitosan experienced fish mortality of 48% after the challenge test (Figure 7).

The results of observations for 24 hours after being infected with *A. hydrophila*, catfish fry did not experience clinical symptoms in the form of uniform body surface damage. Clinical symptoms began to appear at 24 hours after infection by bacteria in the form of a distended abdomen on the body (dropsy) (Figure 6 d) that occurred in catfish fry in treatment A, while in other treatments there

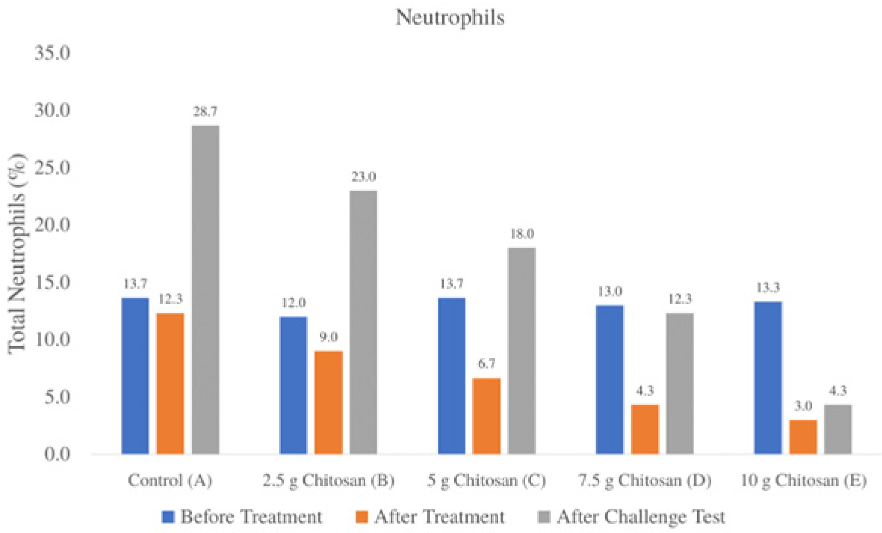
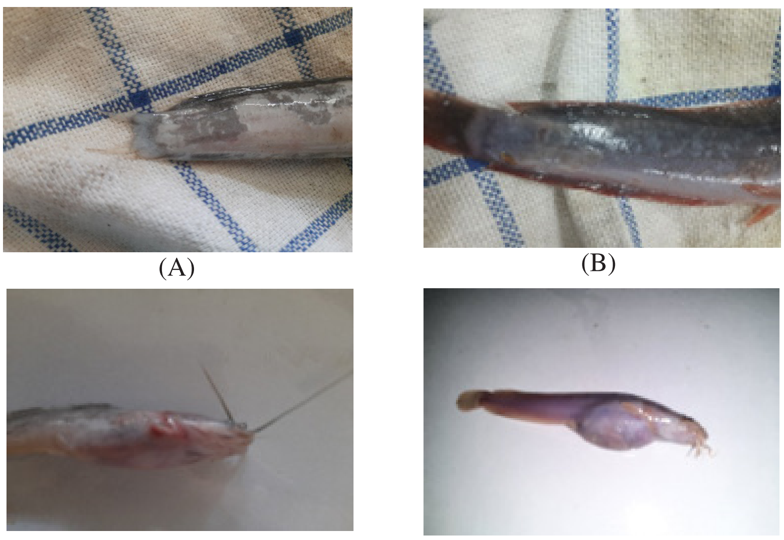


Figure 5. Total neutrophils during the study.



(A) (B) (C) (D)

Figure 6. Clinical symptoms in fish during research.
Description: (A) Thin fins, (B) Ulcers, (C) Bleeding (hemorrhagic), (D) Distended Stomach (Dropsy)

were no clinical symptoms. Until the 24th hour observation, catfish fry treated B, C, D and E did not show clinical symptoms.

Based on the observation of clinical symptoms in sangkuriang catfish, four clinical symptoms appeared on the fish's body, the clinical symptoms included thin fins, ulcers, bleeding (hemorrhagic), and distended stomach (dropsy) (Figure 6).

In Table 1 it can be seen that there was more severe body surface damage indicated by treatments A and B compared to treatments C. D and E. Treatment E (10 g) showed no visible body damage on the surface of the fish's body so that it showed better body resistance. compared to

Table 1. Fish body surface damage during research.

		Days													
Treatment	Repetition	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	1	-	c	cd	c	c	c	dc	adc	adc	bc	bc	bc	bc	bc
	2	c	bc	c	c	c	c	dc	adc	adc	dc	dc	c	c	c
	3	-	c	c	c	c	dc	adc	adc	adc	bcd	bcd	bc	c	c
B	1	-	-	bc	abcd	abcd	abcd	abc	c	c	c	c	c	-	-
	2	-	c	c	b	ab	abc	abc	c	c	bc	bc	b	b	-
	3	-	-	c	d	d	d	cd	c	c	dc	dc	c	-	-
C	1	-	-	-	c	c	c	c	c	c	ac	-	-	-	-
	2	-	-	c	c	c	c	c	c	c	-	-	-	-	-
	3	-	-	-	c	c	c	d	d	d	-	-	-	-	-
D	1	-	-	-	-	d	d	d	d	-	-	-	-	-	-
	2	-	-	-	-	-	d	c	c	-	-	-	-	-	-
	3	-	-	-	-	-	d	d	d	-	-	-	-	-	-
E	1	-	-	-	-	-	c	c	c	-	-	-	-	-	-
	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Description: (a) Thin fins, (b) Ulcers, (c) Bleeding (hemoragic), (d) Distended abdomen (Dropsy), and (e) Exopthalmia

Table 2. Response test to shock during research.

		Days													
Treatment	Repetition	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	1	++	+	+	+	+	+	+	+	+	+	+	+	+	-
	2	++	+	+	+	+	+	+	+	+	+	+	+	+	-
	3	++	+	+	+	+	+	+	+	+	+	+	+	+	-
B	1	++	++	++	++	++	++	++	++	++	+	+	+	+	+
	2	++	++	++	+	+	++	+	+	+	+	+	+	+	+
	3	++	++	++	++	++	++	++	++	++	+	+	+	+	+
C	1	++	++	++	++	++	++	++	++	++	++	++	+	+	+
	2	++	++	++	++	++	++	++	++	++	++	++	+	+	+
	3	++	++	++	++	++	++	++	++	++	++	++	++	+	+
D	1	++	++	++	++	++	++	++	++	++	++	++	++	+	+
	2	++	++	++	++	++	++	++	++	++	++	++	++	+	+
	3	++	++	++	++	++	++	++	++	++	++	++	++	++	+
E	1	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	2	++	++	++	++	++	++	++	++	++	++	++	++	++	++
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Description: (-) No response, (+) Low response to shock, (++) Response to normal shock other treatments. Treatment C showed almost the same recovery as treatment D, the wound started to shrink on the 9th day.

Response to Shock

The aquarium walls were tapped to test the fish's response to shock on each treatment. Fish responses to different shocks were shown by each treatment based on the results of observations that had been made after the challenge test (Table 2).

The shock response shown in treatments D and E on the first day after the *A. hydrophila* challenge test treatment was the best compared to other treatments (Table 2). Treatment E is the best treatment with the fastest recovery, because starting from the first day it has shown a normal response to shock. The addition of chitosan to the feed showed a better response to shock than the treatment without chitosan.

Response to feed

There was a decrease in fish appetite in each treatment due to infection from *A. hydrophila* based on the observations made (Table 3), the

Table 3. Test response to feed during research.

		Days													
Treatment	Repetition	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	1	1	++	+	+	+	+	+	+	+	+	+	+	+	+
	2	2	+	+	+	+	+	+	+	+	+	+	+	+	+
	3	3	++	+	+	+	+	+	+	+	+	+	+	+	+
B	1	1	++	++	+	+	+	+	+	++	+++	+++	+++	+++	+++
	2	2	++	++	+	+	+	+	+	++	+++	+++	+++	+++	+++
	3	3	++	++	+	+	++	+	+	++	+++	+++	+++	+++	+++
C	1	1	++	++	+	+	+	++	+++	+++	+++	+++	+++	+++	+++
	2	2	++	++	+	+	+	++	+++	+++	+++	+++	+++	+++	+++
	3	3	++	++	+	+	++	++	+++	+++	+++	+++	+++	+++	+++
D	1	1	++	++	+	+	++	++	+++	+++	+++	+++	+++	+++	+++
	2	2	++	++	+	+	++	+++	+++	+++	+++	+++	+++	+++	+++
	3	3	+++	++	++	+	++	+++	+++	+++	+++	+++	+++	+++	+++
E	1	1	+++	+++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++
	2	2	+++	+++	++	++	++	++	+++	+++	+++	+++	+++	+++	+++
	3	3	+++	+++	++	++	++	+++	+++	+++	+++	+++	+++	+++	+++

Description: (-) No response, (+) Low response to shock, (++) Response to normal shock

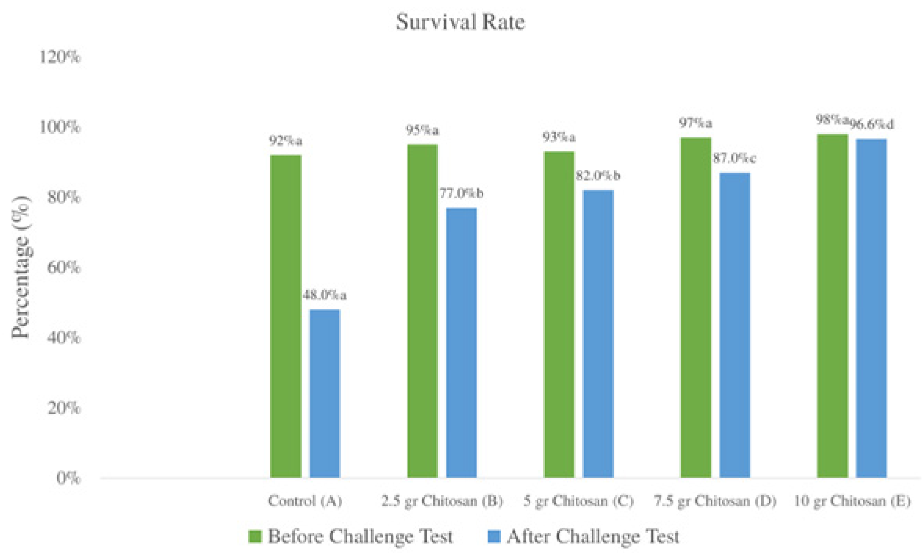


Figure 7. Survival rate of fish during research.

cause of the decreased fish appetite was metabolic disorders that occurred due to infection in the fish body.

Based on observations, on the first day the treatment that showed the best feed response in treatment E (10 g) because it showed a normal and good feeding response (Table 3), compared to treatments A (without chitosan), B (2.5 g), C (5 g), and D (7.5 g) which indicates a low feed response. Treatment A (without chitosan) showed the lowest feed response, it could be seen from the amount of uneaten feed residue. The feed response began to return to normal on the 9th day of treatment B, treatment C on the 7th day, treatment D on the 6th day while treatment A on the 12th day.

Survival rate

In each treatment, the survival rate of fish was observed during the rearing period before and after the fish were infected with *A. hydrophila* bacteria. Based on the results of observations, each treatment showed varying survival rates, the highest survival after the challenge test treatment was treatment E of 96.6%, then treatment D was 87%, treatment C was 82%, treatment B was 77%, while treatment A of 48% was the lowest survival rate (Figure 8).

In Figure 7 it can be seen that treatment A (without chitosan) resulted in the lowest survival among all other treatments, both before the challenge test and after the challenge test (Figure 7). Catfish fry that were given additional chitosan in the feed (treatment B 2.5 g, C 5g, D 7.5 g and E 10 g) showed higher survival, chitosan administration showed a good effect on the fish's body resistance to disease. Due to the immunostimulant effect of the chitosan. Based on the analysis of variance, there were significant differences between treatments. The results of Duncan's test at a significance level of 5% showed that survival after the challenge test of treatment E was significantly different from treatments A, B, C and D.

Water quality

Observations on the water quality of fish rearing media were also carried out, namely temperature, pH, and dissolved oxygen (DO). This measurement was carried out during the fish rearing period until it entered the challenge test period. In Table 4 it can be seen that the water quality values of each parameter measured during the maintenance period until the challenge test.

Water temperature during maintenance ranged from 26.5-29.5 ^{o}C, vulnerable water temperatures were almost the same in all treatments due to the use of a heater. The degree of acidity (pH) of water in the maintenance media ranged from 7.00 to 7.29 and was still in the optimal range. The content of dissolved oxygen (DO) in the maintenance medium ranged from 5.27 to 5.87 mg/L (Table 4).

Discussions

White blood cells (leukocytes)

Based on observations, it can be seen that the test fish treated with chitosan showed an increase number of leukocytes along with the increase in the dose of chitosan given, so that the highest number of leukocytes occurred in treatment E, which was 23.90 × 104 cells/mm3 (Figure 5). This shows that the active immunostimulant substance contained in chitosan is chitosan nanoparticle (ChNP) Wardani *et al*. (2018) at a dose of 10 g (treatment E) worked more effectively in increasing the fish's immune system which was indicated by an increase in the number of white blood cells. As the opinion of Shahbazi & Bolhassani (2017) that immunostimulants develop non-specific immunotherapy and immunoprevention by stimulating the main factors of the immune system including phagocytosis, T and B lymphocytes, specific synthetic antibodies that can trigger an increase in white blood cells. There is an influence from other factors in increasing the number of leukocytes, one of which is the addition of chitosan to the feed. Chitosan is

Table 4. Value of water quality range during the study.

	Water Quality Parameters		
Treatment	Suhu (^{o}C)	pH	DO (mg/l)
A	26.7 - 28.3	7.07 - 7.26	5.30 - 5.67
B	26.5 - 27.2	7.13 - 7.29	5.43 - 5.67
C	27.1 - 29	7.00 - 7.22	5.27 - 5.87
D	27.1 - 29.5	7.10 - 7.24	5.37 - 5.83
E	27 - 27.6	7.07 - 7.23	5.43 - 5.70
Optimal (SNI 2014)	25-30 (SNI)	6,5-8 (SNI)	>3 (SNI)

able to induce an increase in the number of white blood cells with immunodulation activity that optimizes the body's immune function, this is in line with the statement of Rosidah *et al*., (2019), namely increasing the number of white blood cells in fish given Moringa leaf extract which has immunomodulatory activity which plays a role in making the immune system more active in carrying out its function in strengthening the body's immune system.

The test fish that were not given chitosan also showed a slight increase in the number of leukocytes after treatment, the increase was due to the increase in age and weight of the fish after 14 days of rearing. As according to Sudirman *et al*. (2021) The increase in white blood cells is caused by several factors, namely the age and size of the fish, as well as nutrition feed. This is also in line with the opinion of Salasia *et al*., (2001) namely the number of leukocytes in fish is influenced by several factors, namely the type or species, age and muscle activity. Based on the results of the variance, it was shown that different doses of chitosan gave significantly different effects on the number of leukocytes. The results of Duncan's test at the 5% confidence level showed that the treatments were significantly different from each other (treatments A, B, C, D and E were significantly different).

The 3rd day after the challenge test (CT-3) showed an increase in the number of leukocytes in each treatment, this was due to the attack of the bacterium A. hydrophila which was also shown by the presence of clinical symptoms in the form of body damage (dropsy, hemorrhagic, ulcers, and thin fins) (Table 1). The bacterial attack that occurred caused the highest change in the number of leukocytes in treatment A, because the bacterial attack caused the need for a high number of leukocytes to be produced as body defense and against bacterial attack on the fish body. The total leukocyte count increases in response to infection, trauma, inflammation, and certain diseases (Chmielewski & Strzelec, 2018). This is in line with Sukenda and Jamal (2008) statement that the increase in white blood cells after the challenge test is the body's reaction or body's defense against antigens (pathogenic bacteria) that enter the body. Based on the results of the variance, it was shown that different doses of chitosan gave significantly different effects on the number of leukocytes. The results of Duncan's test at the 5% confidence level shows that there is a significantly different treatment in each treatment.

On the 7th day after the challenge test (CT-7) there was still an increase in the number of leukocytes in each treatment, this indicated that the body of catfish fry in all treatments was still in a condition against bacterial attack, so the number of leukocytes was still increasing. The test fish in treatment E experienced the lowest increase in leukocyte count from 24.50 × 104 cells/mm3 to 24.68 × 104 cells/mm3 (Figure 5). The number of leukocytes in treatment E after being given treatment with chitosan E showed the highest increase, but during the challenge test with bacteria the number of leukocytes decreased on day 7, because during treatment there was the highest increase in leukocyte levels in treatment E. The results of Duncan's test at the 5% confidence level showed that treatments B and C were not significantly different, while treatments A, D and E were significantly different from other treatments.

The number of leukocytes of the test fish in treatments A and B until day 14 still increased and the highest increase occurred in treatment A. Treatments C, D and E decreased with the lowest decrease occurred in treatments D, and E. This showed that the test fish in treatments A and B were still in a condition against the attack of A. hydrophila bacteria, while treatments C, D and E had experienced a period of recovery from infection with A. hydrophila bacteria. This indicated that the catfish fry in treatment A after the challenge test with A. hydrophila bacteria increased in attack resulting in an increase in the number of leukocytes to fight and phagocytize the bacterial cells. The results of Duncan's test at the 5% confidence level showed that on day 14 after the challenge test, there was a significant difference between treatments. Treatments B, C, D and E showed lower leukocyte counts, Treatment E experienced the highest decrease in leukocyte counts among other treatments, This increase is shown by the decrease in the intensity of bacterial attack and fish are starting to become resistant to the attack of A. hydrophila bacteria. The results of this study indicate that feeding chitosan as an immunostimulant can increase the total leukocytes. Immunostimulants pass through a group of biologics and synthetic compounds that increase both specific (antibody and agglutination titer) and non-specific immunity (lysozyme, complement, myeloperoxidase, phagocytosis, bactericidal activity, respiratory blast activity, nitric oxide, total haemocytes, phenoloxidase) against infectious disease in different species of fish and shellfish (Srivastava & Pandey, 2015). Treatment E was the most effective dose where this dose was immunogenic and protective in increasing the immunity of catfish against bacterial attack (Utami *et al*., 2013).

Leukocyte differential

Lymphocyte

The number of lymphocytes in fish blood increased and decreased from before treatment to after treatment. An increase in treatments C, D, and E indicates that chitosan can improve the performance of the body's immune system, as stated by Sukenda and Jamal (2008) that increasing the dose of chitosan increases the proliferation of lymphocyte cells. Treatments A and B showed a small decrease in lymphocytes, this happened because the control treatment was not fed chitosan so there was no additional protection in fish immunity. The number of lymphocytes in the blood decreased after the challenge test with A. hydrophila, this was presumably due to the resistance activity of leukocytes against A. hydrophila. Whereas in the control treatment and a dose of 2.5 g of chitosan, the number of lymphocytes produced was lower, this was presumably because the number of lymphocytes produced was not comparable to the lymphocytes sent to infected body tissues. After being challenged, the lymphocyte value decreased in all treatments. This decrease occurred because white blood cells were used to attack pathogens from A. hydrophila bacteria.

The increase in resistance activity causes an increase in the need for white blood cells, especially phagocytic cells and causes a reduction in the number of antibody-providing cells, namely lymphocytes. As stated by Rukyani and Silvia (1997) that an increase in the intensity of infection by certain pathogens will trigger the need for white blood cells and the increased need causes a reduction in the number of antibody cells (lymphocytes). Treatments A to E decreased, which showed a value that was below the normal range of lymphocytes after the challenge test, which meant that the fish were sick due to bacterial attack. This is in accordance with the statement of Pratiwi *et al*. (2019), the presence of infection in the body of the catfish so that the lymphocytes decrease because lymphocytes are the foremost defense against infection.

Monocyte

The percentage of monocytes after feeding chitosan for 14 days showed an increase in each treatment. After the fish were challenged, there was an increase in each treatment along with an increase in the dose of chitosan given, so that the highest total monocytes occurred in treatment E. The increase in the number of monocyte cells occurred due to bacterial infection in fish, so that monocyte cells would develop into macrophages towards the site of infection. To eliminate foreign bodies and carry out phagocytosis. This is in accordance with Pratiwi (2014) statement that an increase in the number of monocyte cells can be caused by the response of immune stimulation in killing pathogenic bacteria.

According to Rukyani and Silvia (1997) the increase in monocytes occurs because macrophages can phagocytize foreign objects that enter the body. Monocytes are short-lived in the blood, then enter the tissue to differentiate into macrophages so that the number of monocytes fluctuates in the blood. In the event of infection by bacteria or foreign objects that enter the body, monocytes will move to the infected area to carry out phagocytosis (Affandi and Tang, 2017). The highest percentage monocytes was in treatment E (10 g), the increase indicated the presence of non-specific defense activity of fish in the form of an increase in blood monocytes to eliminate foreign agents. Monocyte-macrophage system function is important to clean blood, lymph and interstitial spaces from foreign body, thus an important defense function (Bonardo *et al*., 2015).

Neutrophils

Based on the research conducted, the percentage of neutrophils from before treatment to after treatment decreased in each treatment, where the highest decrease occurred in treatment E. This decrease in the number of neutrophils indicates that the administration of chitosan can strengthen the fish's immune system, neutrophils which are an indicator of sick fish and attacked by pathogens can be an indication that the fish have a good immune system condition if their numbers decrease. Post-challenge test increased in each treatment. The increase in the number of neutrophil cells in all treatments indicated the activity of neutrophil cells in attacking antigens (foreign particles) and showing the process of phagocytosis by entering the body. In line with the statement Tizard (1988) that the increase in the number of neutrophils after the challenge test is associated with the response to infection, namely killing bacteria and cleaning tissue debris. These neutrophils are the first line of defense that move quickly towards foreign material and destroy it, but are not able to last long (Rukyani and Silvia, 1997). Usually neutrophils only completely destroy any foreign material ingested and do not process the antigen in preparation for presentation to antigen-sensitive cells.

The difference in the change in the percentage of neutrophils in each treatment is thought to be due to the response of blood balance to an increase in the proportion of other types of leukocytes, namely lymphocytes and monocytes. According to Delman & Brown (1989) when a bacterial infection occurs, the number of neutrophils in the blood usually increases, this is due to the need for lymphoid to release leukocytes to fight infection. The high number of neutrophils in treatment A was caused by a fairly low number of lymphocytes, the low number of lymphocytes indicates that the fish's immune system is decreasing due to the presence of foreign objects, so the body will automatically increase the production of neutrophils to kill and destroy these foreign objects. According to (Suhermanto and Andayani, 2013) neutrophils can be released during infection due to the influence of external chemical stimuli by immunostimulants. The decrease in the number of neutrophils is thought to be due to reduced infections that occur in fish (Rustikawati, 2012). The low percentage of neutrophils compared to other types of white blood cells is because neutrophils are more commonly found and accumulate in the area of infection, where neutrophils will move actively to the infected area when an injury occurs.

Based on the results of the differential leukocytes (lymphocytes, monocytes and neutrophils) showed that the addition of chitosan to the diet stimulated the immune system better than without the addition of chitosan, this was indicated by the higher percentage of lymphocytes (as the body's defense against antibodies) and monocytes (as phagocytic cells), antigen in the addition of chitosan. Treatment E (10 g of chitosan) was an effective dose to stimulate differential leukocytes in catfish to improve the immune system.

Clinical symptoms

These symptoms began to be observed on the body surface at 24 hours after the challenge test. This situation is in accordance with the statement (Haryani *et al*., 2012) that the clinical symptoms of fish are characterized by inflammation (inflammation) which is characterized by swelling and redness of the body, which then continues to become larger wounds. A. hydrophila infection shows damage to the body surface after a few hours after the challenge test. Clinical symptoms began to appear 24 hours after infection, in the form of a distended abdomen on the body (Figure 10 d) that occurred in catfish fry in treatment A, while in other treatments there were no clinical symptoms. As according to Rosidah *et al*. (2019), 24 hours after infection by the bacterium A. hydrophila, fish suffered damage to their bodies in the form of inflammation (inflammation), bleeding (hemorrhagic) and ulcers (ulcer). The occurrence of ulcers in some fish is thought to be due to the high density of bacteria in the area, so that the intensity of toxins released by bacteria is also high. There are also red spots and fin flakes that occur at the base of the fish fins, this is as stated Kartikaningsih *et al*. (2020) A. hydrophila infected catfish shows the following signs: body-color becomes dark, skin becomes rough, and bleeding occurs, which will then occur ulcers (haemorrhagic).

Based on Table 1, it shows that treatments A and B showed more severe body surface damage than treatments C. D and E. Treatment E (10 g) showed better body resistance than other treatments, it could be seen from the invisible body damage. On the surface of the fish body. Treatment C showed almost the same recovery as treatment D, the wound started to shrink on the 9th day. The damage to the body surface and the recovery period from each treatment of the test fish were different. Treatment A showed that some of the fish were getting worse, leading to death. Treatment A suffered the most severe body damage due to the absence of addition of chitosan in the feed, so that the fish did not get the immunostimulant effect which resulted in low fish body resistance. Meanwhile, treatment E showed that giving 10 g of chitosan showed the best clinical symptoms because chitosan works as an immunostimulant to prevent disease infection from A. hydrophila bacteria.

Response to shock

The results of post-challenge observations showed various responses to each treatment (Table 2). According to Handayani (2016), no response fish is thought to be due to inflammation in the fish's body, unhealthy fish can be slower or slower faster than usual. Treatments D and E gave the best response to shock, it could be seen by the response on day 1 after the challenge of A. hydrophila. Treatment A showed no response to shock on the 2nd day, this is because fish that become sick and experience a decrease in behavioral activity such as swimming by gathering around aeration and swimming in a hanging or standing position are the result of fish being attacked by bacteria. According to Haryani *et al*. (2012) stated that A. hydrophila infection causes stressed fish, swimming around aeration and in general fish swim sideways due to reduced body balance. Treatment E is the best treatment with the fastest recovery, because starting from day 1 it has shown a normal response to shock. Chitosan showed a better response to shock than the treatment without chitosan. This shows that chitosan has a good effect on increasing the immune system to fight the attack of A. hydrophila, so that fish can recover faster.

Response to feed

A decreased feeding response was shown by each treatment in observations that had been made after the fish were infected with A. hydrophila bacteria (Table 3), the disturbed metabolism of the fish as a result of bacterial attack was the cause of reduced fish appetite. According to Fitriyanti *et al*. (2020), changes in the behavior of catfish that swim abnormally or passively and have a high appetite decreased after infection with A. hydrophila bacteria. Treatment E (10 g) showed the best feed response, this was indicated by a normal feed response to fish on day 1. Treatment A (without chitosan) showed the lowest feed response, it could be seen from the amount of uneaten feed residue.

The appetite decreased is one of the behavior of catfish after infection with A. hydrophila bacteria (Fitriyanti *et al*., 2020). The low feed response could also be associated with low leukocytes on day 3 post challenge (Figure 6). The decrease in the number of leukocytes indicates that the fish are exposed to diseases that affect the fish's appetite to be low. The feed response began to return to normal on the 9th day of treatment B, treatment C on the 7th day, treatment D on the 6th day while treatment A on the 12th day. The treatment with the addition of chitosan showed the best results on the feed response compared to the treatment without chitosan, this is presumably due to the active ingredient of chitosan which can help stimulate the work of the fish's immune system so as to provide the fastest feed response.

Survival rate

Based on the results of observations, each treatment showed varying survival rates, the highest survival after the challenge test treatment was treatment E. Treatment A (without chitosan) experienced many deaths after bacterial infection, this was indicated by the continued increase in white blood cells in an effort to fight off attacks. bacteria and severe fish body surface damage with a longer recovery time than other treatments. Catfish fry that were given additional chitosan in the feed (treatment B 2.5 g, C 5g, D 7.5 g and E 10 g) showed higher survival, chitosan administration showed a good effect on the fish's body resistance to disease, due to the immunostimulant effect of the chitosan.

The research of Ranjan *et al*. (2012) showed an increase in serum lysozyme activity in fish given different doses of chitosan. Lysozyme is an enzyme that is hydrolytic and has a non-specific defense system and has an important role. The activity of the lysozyme enzyme and aminopolysaccharide groups contained in chitosan can damage the bacterial cell wall, the lysozyme enzyme is able to digest the bacterial cell wall which causes the loss of the protective ability of bacterial cells (Radhia & Jumaetri, 2015). There were significant differences between treatments. The results of Duncan's test at a significance level of 5% showed that survival after the challenge test of treatment E was significantly different from treatment A, B, C and D. Figure 7 shows that the control fish had the lowest percentage of survival rates. This is because the control treatment was not fed chitosan for 14 days, so the fish's immune system decreased and the fish only relied on their natural immune system and white blood cells to defend themselves from the attack of A. hydrophila. In addition, the bacterial activity was stronger and faster when compared to the activity of increasing the natural immune system of the control treated fish, so that the fish's body defenses became weak and unbalanced due to infection with A. hydrophila bacteria.

Treatment E showed the highest survival compared to other treatments, which means a dose of 10 g of chitosan can improve the immune system and survival of catfish fry, this is also indicated by macroscopic clinical symptoms that recover quickly. The treatment with chitosan showed a higher survival rate than the treatment without chitosan.

Water quality

The water temperature during maintenance ranged from 26.5 - 29.5 oC, the water temperature was almost the same in all treatments due to the use of a heater. The degree of acidity (pH) of water in the maintenance media ranged from 7.00 to 7.29 and was still in the optimal range. The content of dissolved oxygen (DO) in the maintenance medium ranged from 5.27 to 5.87 mg/L (Table 4). Dissolved oxygen needs have been met for fish life, this is because aeration is always available. Based on the results of observations of water quality during the research, it was shown that chitosan did not have a bad effect on water quality, it could be seen from the value of the water quality range which was not much different from the treatment without chitosan. The water quality during the maintenance period is included in the optimal range according to KKP (2014) about the Indonesian National Standard (SNI) for catfish fry, this is because water changes and siphoning are always carried out on the rearing media.

CONCLUSION

Based on the results of the research, it can be concluded that the addition of chitosan 10 g/1 kg of feed is the most effective dose to improve the immune system performance of sangkuriang catfish (*Clarias gariepinus*), with the highest percentage increase in white blood cells, After being challenged with *Aeromonas hydrophila* bacteria, sangkuriang catfish fed chitosan 10 g/1 kg of feed have the fastest recovery and showed the highest survival of 96.6%.

REFERENCES

Akhmad Rukyani, Evi Silvia AST. 1997. Peningkatan Respon Kebal Non-Spesifik Pada Ikan Lele Dumbo (*Clarias* sp.) Dengan Pemberian Imunostimulan (B-Glucan). Jurnal Penelitian Perikanan Indonesia 3: 1–10.

Badan Pusat Statistik. 2018. Refleksi Outlook. Refleksi 2018 & Outlook 2019 Kementrian Kelautan Dan Perikanan: 67.

Baratawidjadja KG. 2012. Immunologi Dasar (Edisi Kese). Fakultas Kedokteran Universitas Indonesia. https://library.ui.ac.id/detail?id=20417471.

Bonardo B, Christina H, Fransisca C, Kristin K, Sudiono J. 2015. Peran Monosit (Makrofag) Pada Proses Angiogenesis Dan Fibrosis. Seminar Nasional Cendekiawan 2015: 254–259.

Chen Y, Zhu X, Yang Y, Han D, Jin J, Xie S. 2014. Effect of Dietary Chitosan on Growth Performance, Haematology, Immune Response, Intestine Morphology, Intestine Microbiota and Disease Resistance in Gibel Carp (*Carassius auratus gibelio*). Aquaculture Nutrition 20: 532–546. https://doi.org/10.1111/anu.12106.

Chmielewski PP, Strzelec B. 2018. Elevated Leukocyte Count As a Harbinger of Systemic Inflammation, Disease Progression, and Poor Prognosis: A review. Folia Morphologica (Poland) 77: 171–178. https://doi.org/10.5603/FM.a2017.0101.

Delman H, Brown EM. 1989. Buku Teks Histologi Veteriner I. Hartono (penterjemah): Vol. Delman H.D. Penerbit Universitas Indonesia (UI-Press) 1992.

Effendie IM. 1979. Metode Biologi Perikanan. Yayasan Dewi Sri.

Fitriyanti PD, Desrina D, Prayitno SB. 2020. Pengaruh Perendaman Kombinasi Ekstrak Daun Binahong dan Bawang Putih pada Ikan Lele Dumbo (*Clarias gariepinus*) yang Diinfeksi *Aeromonas hydrophila*. Sains Akuakultur Tropis 4: 61–67. https://doi.org/10.14710/sat.v4i1.6912.

Handayani L. 2016. Pemanfaatan Ekstrak Buah Lambai-Lambai Untuk Mengobati. SEBATIK 2621-069X: 230–234.

Haryani A, Grandiosa R, Dwi Buwono I, Ayi Santika D, Fakultas Perikanan dan Ilmu Kelautan Unpad A, Dosen Fakultas Perikanan dan Ilmu Kelautan Unpad S, Pegawai Balai Besar Pengembangan Budidaya Air Tawar Sukabumi S. 2012. Uji Efektivitas Daun Pepaya (*Carica papaya*) Untuk Pengobatan Infeksi Bakteri *Aeromonas hydrophila* Pada Ikan Mas Koki (*Carassius auratus*). Jurnal Perikanan Dan Kelautan 3: 213–220.

KKP. 2014. Ikan lele dumbo (*Clarias* sp.) Baian 4 : Produksi Benih. SNI 6484.4.2014.

Kartikaningsih H, Yahya Rohman FZ, Jaziri, AA. 2020. Characteristics of *Aeromonas hydrophila*-Infected Catfish (*Clarias* sp.). IOP Conference Series: Earth and Environmental Science: 493. https://doi.org/10.1088/1755-1315/493/1/012036.

Ke CL, Deng FS, Chuang CY, Lin CH. 2021. Antimicrobial Actions and Applications of Chitosan. *Polymers*: 13. https://doi.org/10.3390/polym13060904.

Statistik.kkp.go.id. 2020. Produksi Perikanan. Diakses pada. 1 Juni 2020, dari https://statistik.kkp.go.id/home.php?m=prod_ikan_prov&i=2#panel-footer.

Li X, Yu Y, Xie S, Yan Q, Chen Y. 2011. Effect of Chitosan on Intestinal Bacteria of Allogynogenetic Crucian Carp, *Carassius auratus gibelio*, as Depicted by Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis. Journal of the World Aquaculture Society 42: 539–548. https://doi.org/10.1111/j.1749-7345.2011.00496.x.

Meshkini S, Tafy A, Tukmechi A, Farhang-pajuh F. 2012. Effects Of Chitosan On Hematological Parameters And Stress Resistance In Rainbow Trout *(Oncorhynchus mykiss)*. October.

Ode I. 2013. Kajian Sistem Imunitas Untuk Pengendalian Penyakit Pada Ikan dan Udang. *Agrikan:* Jurnal Ilmiah Agribisnis Dan Perikanan 6: 41. https://doi.org/10.29239/j.agrikan.6.2.41–43.

Pramudita, Sarjito, Budi PS. 2013. Identifikasi Bakteri Agensia Penyebab Motile Aeromonas Pada Ikan Lele Dumbo (*Clarias gariepinus*) Yang Berasal Dari Kecamatan Rowosari. Journal of Aquaculture Management and Technology 2: 1–9.

Pratiwi DAN. 2014. Aktivitas Antibakteri Ekstrak Etanol Daun Pacar Kuku *(Lawsonia inermis L.)* Dan Bioautografi Terhadap Bacillus subtilis dan Shigella sonnei. Universitas Muhammadiyah Surakarta.

Pratiwi Via Anggraini, Eddiwan E. 2019. Studi Kondisi Darah Ikan Lele Lokal (*Clarias batrachus*) Di Sungai Tapung Kiri Dan Sungai Sail Provinsi Riau. Fakultas Perikanan Dan Kelautan, Universitas Riau 0–8.

Radhia R, Fitriyanti Jumaetri, S. 2015. Formulasi Krim Anti Jerawat Dari Nanopartikel Kitosan Cangkang Udang Windu *(Penaeus monodon)* 3: 153–16. https://doi.org/10.3724/SP.J.1047.2015.01269.

Ranjan R, Prasad KP, Vani T, Kumar R. 2012. Effect of Dietary Chitosan on Haematology, Innate Immunity and Disease Resistance of Asian seabass *Lates calcarifer* (Bloch). *A*quaculture Research 45: 983–993. https://doi.org/10.1111/are.12050.

Ridwan Affandi, UMT. 2017. Fisiologi Hewan Air. UNRI Press.

Rosidah, Buwono ID, Lili W, Suryadi IB, Triandika AR. 2019. The Resistance of Sangkuriang catfish (*Clarias gariepinus* Burchell 1822) Against Aeromonas hydrophila Bacteria Given Moringa Leaf Extracts (*Moringa oleifera L.*) Through The Feed. Jurnal Iktiologi Indonesia 19. https://doi.org/10.32491/jii.v19i1.435.

Rustikawati I. 2012. Efektivitas Ekstrak *Sargassum* Sp. Terhadap Diferensiasi Leukosit Ikan Nila (*Oreochromis Niloticus*) Yang Diinfeksi *Streptococcus iniae*. Jurnal Akuatika Indonesia 3: 245375.

Salasia SIO, Sulanjari D, Ratnawati A. 2001. Studi Hematologi Ikan Air Tawar. In Jurnal Berkala Ilmiah Biologi (Vol. 2, Issue 12, pp. 710–723). http://i-lib.ugm.ac.id/jurnal/detail.php?dataId=238.

Septiarini, Esti Harpeni W. 2012. Pengaruh Waktu Pemberian Probiotik Yang Berbeda Terhadap Respon Imun Non-Spesifik Ikan Mas (*Cyprinus carpio L.*) Yang Diuji Tantang Dengan Bakteri *Aeromonas salmonicida*. Jurnal Rekasaya Dan Teknologi Budidaya Perairan I.

Shahbazi S, Bolhassani A. 2017. Immunostimulants : Types and Functions. J Med Microbiol Infec Dis 4: 45–51.

Shanthi Mari LS, Jagruthi C, Anbazahan SM, Yogeshwari G, Thirumurugan R, Arockiaraj J, Mariappan P, Balasundaram C, Harikrishnan R. 2014. Protective Effect of Chitin and Chitosan Enriched Diets on Immunity and Disease Resistance in Cirrhina mrigala Against *Aphanomyces invadans*. Fish and Shellfish Immunology 39: 378–385. https://doi.org/10.1016/j.fsi.2014.05.027.

Singha J, Sciences F, Priyadarsani L, Dash G, Sciences F, Adikesavalu H. 2018. Motile Aeromonas septicemia (MAS) Infection in Freshwater Finfish and Its Control. In Sustinable Management of Aquatic Resources (Issue December, pp. 507–512). Narendra Publishing House.

Sonida A, Esti H, Tarsim. 2014. Deskripsi Respon Imun Non Spesifik Kakap Putih (*Lates calcarifer*) Yang Diberi Jintan Hitam (*Nigella sativa*) Dan Uji Tantang Dengan Viral Nervous Necrosis. Jurnal Ilmu Perikanan Dan Sumberdaya Perairan 1.

Srivastava PK, Pandey AK. 2015. Role of Immunostimulants in Immune Responses of Fish and Shellfish. Biochemical and Cellular Archives 15: 47–73.

Sudirman I, Syawal H, Lukistyowati I. 2021. Profil Eritrosit Ikan Mas (*Cyprinus carpio L*) yang Diberi Pakan Mengandung Vaksin *Aeromonas hydrophila*. Ilmu Perairan (Aquatic Science) 9: 144. https://doi.org/10.31258/

jipas.9.2.p.144–151.

Suhermanto A, Andayani S, M. 2013. Pengaruh Total Fenol Teripang Pasir (*Holothuria scabra*) Terhadap Respon Imun Non Spesifik Ikan Mas (*Cyprinus carpio*). Jurnal Bumi Lestari 13: 225–233.

Sukenda L, Jamal DW, AH. 2008. Penggunaan Kitosan Untuk Pencegahan Infeksi *Aeromonas hydrophila* Pada Ikan Lele Dumbo *Clarias* sp. Jurnal Akuakultur Indonesia 7: 159–169.

Tarmizi Azhari, Muhammad Agus Suprayudi Nur Bambang Priyo Utomo JE. 2016. Evaluasi Dosis *Polymethylolcarbamide* Pada Leaching Out Nutrien Dan Pertumbuhan Udang Vaname *(Litopenaeus Vannamei)* [Bogor Agricultural University (IPB)]. http://repository.ipb.ac.id/handle/123456789/82555.

Tizard I. 1988. An Introduction to Veterinary Immunology (2nd ed.). Airlangga University Press.

Triyanto, KHN. 1992. Vaksinasi Monovalen dan Polivalen Vaksin Untuk Mengatasi Serangan *Aeromonas hydrophila* PadaIkan Lele (*Clarias* sp.). Ilmu Pertanian 1992 IV: 4.

Utami T, Prayitno SB, Hastuti S, Santika A. 2013. Gambaran Parameter Hematologis Pada Ikan Nila (*Oreochromis niloticus*) Yang Diberi Vaksin Dna *Streptococcus iniae* Dengan Dosis Yang Berbeda. Journal of Aquaculture Management and Technology 2: 7–20.

Wardani G, Mahmiah Sudjarwo SA. 2018. Immunostimulatory Activity of Chitosan Nanoparticles on Wistar Albino Rats. *Pharmacognosy* Journal 10: 892–898. https://doi.org/10.5530/pj.2018.5.150.