Addition of garlic (*Allium sativum*) extract in fry as immunostimulant on survival and blood profile of pomfret fish (*Colossoma macropomum*)

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Abstract. Pomfret fish (*Colossoma macropomum*) is a highly valuable freshwater fishery commodity. Bacterial diseases, particularly those caused by *Aeromonas hydrophila* bacteria, are a common issue in these fish. Using immunostimulants to control bacterial diseases can enhance the function of the immune system. Garlic is a natural ingredient that can serve as an effective immunostimulant. This study aims to assess the impact of adding garlic extract to feed on the survival and blood profile of *Colossoma macropomum*. The research employed an experimental method using a completely randomized design (CRD) with 6 treatments and 3 repetitions. The doses of garlic extract used were P1 (0 g/kg control +), P2 (0 g/kg control -), P3 (10 g/kg), P4 (15 g/kg), P5 (20 g/kg), and P6 (20 g/kg). A total of 180 pomfret fish were used in the study, and they were fed with additional garlic extract for 28 days. Subsequently, the fish were infected with *Aeromonas hydrophila* bacteria at a specific density. The results of the ANOVA test indicated that the provision of garlic extract had a significant effect on leukocytes (1,99±10388,73 $x10^5$ sel/mm^3) and erythrocytes (10,10±0,25$x10^6$ sel/mm^3) at H-15 (after bacterial infection) and leukocytes (1,43±9196,33$x10^6$ sel/mm^3)at H-28. Furthermore, the use of garlic extract in feed was shown to have a significant effect ($P<0.05$) on the survival of the fish, with the best survival rate at 83.33±5.77% in treatment P3 (10 g/kg). The study also identified the optimal dose of garlic extract for use as an immunostimulant, which was determined to be P3 (10g/kg).

1 Introduction

Pomfret fish (*Colossoma macropomum*) is one of the freshwater fishery commodities that has high economic value and a lot of market demand. [1] states that freshwater pomfret (*Colossoma macropomum*) is one of the freshwater fish that has a large demand in Indonesia. High demand for freshwater pomfret will be realized if there is an increase in fish farming. [2] states that increasing aquaculture requires the availability of sufficient, quality and

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sustainable seeds. The increase in aquaculture business occurs due to high production. High business production harms fish farming. A major factor hindering significant aquaculture production is disease outbreaks such as bacterial infections [3, 4].

One of the bacteria that often attack freshwater fish is *Aeromonas hydrophila*. [5] *Aeromonas hydrophila* bacteria are the cause of Motile Aeromonas Septicemia (MAS) disease, a major obstacle to the development and sustainability of the aquaculture industry, and limit productivity. [6] *Aeromonas hydrophila* is a pathogenic bacterial agent in freshwater that can cause great losses in aquaculture.7] Disease-stricken fish are characterized by changes in hematocrit values, hemoglobin levels, red blood cell counts and white blood cell counts. Blood picture is one indicator of the severity of a disease. Various efforts are made to control bacterial diseases such as the use of antibiotics. [5] Control that is often carried out is very expensive and excessive use can lead to the development of strains that are resistant to antibiotics and the accumulation of drug residues in fish tissue and water which can be harmful to the environment. Therefore, safe control of bacterial diseases is using natural ingredients that have immunostimulant properties.

Immunostimulants are substances or compounds that play a role in controlling disease by increasing the fish's immune system so that it can support growth and resistance to disease. [8]. Immunostimulants are an excellent alternative to chemicals or drugs in fish. In addition, the use of immunostimulants does not leave residues in the body of fish. It is safe for the surrounding environment and can increase the immune system of fish and shrimp against infectious diseases [9]. Immunostimulants are generally derived from natural ingredients that do not damage the environment [10]. One of the natural ingredients that can function as an immunostimulant is garlic.

[9] Garlic as an immunostimulant in aquaculture offers the potential to improve fish health and disease resistance. [11] The use of garlic can improve growth and increase non-specific immune responses such as total leukocytes and phagocytosis activity. [9] The use of garlic extract can increase non-specific immunity such as an increase in total hematocrit, total hemoglobin, total erythrocytes and phagocytosis activity. Garlic contains bioactive compounds, namely allisin. [12] Garlic has the ability as an antimicrobial due to the content of organosulfur compounds (such as Allicin). Allisin is an organosulfur compound whose presence is 70-80 percent of the total thiosulfinate in garlic. [13] Allicin in garlic can play a role in improving the immune system of fish. [14, 15] The content of allicin can activate non-specific immunity by increasing the expression of cytokine genes, so the addition of garlic extract to the feed will increase the defense system of the fish body. [16] Allicin compounds can effectively inhibit the growth and development of microorganisms by inhibiting the formation of essential cellular components and disrupting the process of synthesizing cellular macromolecules, such as DNA, RNA, and proteins, as well as inhibiting the proliferation of bacterial and fungal cells, and inducing cell death.

Previous research on garlic extract as an immunostimulant has been carried out in some freshwater fish, including [13] the use of garlic extract 30 g / kg of feed can increase the immune response and growth of goldfish; [17] a dose of garlic extract of 10 g / kg of feed can increase the survival of catfish; [18] a dose of garlic extract of 10 g / kg of feed can improve the performance of non-specific immune responses, survival and growth of tawes fish seeds. Based on this information, immunostimulant against pomfret survival has never been informed so researchers want to research the effect of garlic extract as an immunostimulant to improve the immune system and survival of freshwater pomfret.

2 Material and methods

2.1 Time and location of the research

The research was conducted for 28 days from February to March 2024. This research was conducted in the Laboratory of Hatchery and Breeding Faculty of Marine and Fisheries, Syiah Kuala University.

2.2 Design of the experiment

This experimental research uses a completely randomized design (CRD). The treatments carried out in this study were 6 treatments and 3 replications. The treatments were P1 Positive control (without the addition of garlic extract and bacterial soaking); P2 Negative control (without garlic extract and bacterial soaking); P3 (Addition of garlic extract at 10g/kg feed); P4 (Addition of garlic extract at 15g/kg feed); P5 (Addition of garlic extract at 20 g/kg feed); P6 (Addition of garlic extract at 25 g/kg feed).

2.3 Research procedures

2.3.1 Making of garlic extract

The making of garlic extract is done by thinly slicing the garlic and drying it. The dried garlic was then blended until smooth, then the garlic powder was macerated with 96% ethanol and allowed to stand for 3 days in a glass jar and stirred using a spatula every day. The dregs and filtrate of the bath were separated using filter paper. After that, the next step is to evaporate using a rotary evaporator with a temperature of < 50°C [19].

2.3.2 Preparation of fish feed, research containers, and test animals

First, the test material was weighed according to the dose used in the study, then the garlic extract was dissolved in 100 ml of water and mixed with egg white. Next, the solution was sprayed into 1 kg of feed evenly using a sprayer, then the feed was dried at room temperature.

The container used is a bucket of 24 units. Before use, the bucket was washed with fresh water and then sterilized with a 10ppm chlorine solution to kill bacteria and fungi attached to the bucket wall. After that the bucket is rinsed using fresh water until clean, then dried in the sun for 24 hours, then each bucket is filled with 10 L of water and equipped with aeration.

The 240 fish samples were obtained from an ornamental fish leader in Gampong Lambaro, Aceh Besar District. This study used pomfret fry as a test sample with an average length of 7-9 cm. The test fish that will be used are test fish that have been acclimatized for 24 hours and are not given feed. After acclimatization, the test fish were taken randomly and put in a bucket with a stocking density of 10 fish per container.

2.3.3 Maintenance and feeding

The test feeding was done three times a day, namely in the morning at 08:00, afternoon at 12:00 and afternoon at 16:00 WIB. The test fish were fed adlibitum for 28 days of maintenance. Pipetting was carried out every 2 days after 2 hours of feeding and water replacement was carried out as much as 30% every 10 days, length measurements and weighing of fish were carried out to determine the growth and adjustment of the daily ration

amount, measurement and weighing of fish were carried out after 2 hours of feeding in the afternoon or after pipetting in the third feeding in the afternoon. pomfret fry were reared based on the guidelines of the Institutional Animal Care and Use Committee (IACUC, 2018) and have ethical approval B/034SN/XII/2022 from the animal ethics committee of the School of Marine and Fisheries Faculty, Universitas Syiah Kuala.

2.3.4 Challenge test

The challenge test was conducted to determine the benefits of garlic extract immunostimulants. The test was conducted in vivo with *Aeromonas hydrophila* bacteria at a density of 108 CFU/ml. Isolation of bacteria using GSP (Glutamate Starch Phenol Red Agar) media. Infection of *Aeromonas hydrophila* bacteria was carried out using the immersion method on the 15th day of maintenance, for 24 hours with a bacterial density of 108 CFU/ml [20]. Bacteria were spread to the challenge test container and stirred using a stirring rod so that the bacteria were homogenized with water. Next, 10 fish of each treatment were put into the challenge test container and immersed and allowed the fish to be infected with bacteria for 24 hours. Fish that have been infected and have clinical symptoms are then changed and the fish are transferred to the maintenance container [21].

2.3.6 Sampling, hemoglobin count, and hematocrit count calculation

Observation of clinical symptoms was carried out from day H-15 to H-28 of maintenance. Blood collection for blood picture observation samples is taken at H-15 and H-28. Fish survival data were taken at H-0 and H-28. Water quality data were taken every day during the study.

The procedure for calculating hemoglobin usually refers to the Sahli method. First, a blood sample is taken using a Sahli pipette up to a scale of 20 mm^3 or on a scale of 0.2 ml. then the pipette tip is cleaned and the blood in the pipette is transferred into an Hb-meter tube that has been filled with 0.1 N HCl up to a scale of 10 (red color). After that, the blood was stirred with a stirring rod for 3 to 5 minutes. After that, distilled water was added to the tube until the color of the blood became like the color of the standard solution on the Hb-meter. Hemoglobin levels are expressed in g. The procedure for calculating hematocrit levels is that blood is taken in as much as 3/4 of the tube. After that, the tip of the tube containing blood was covered with crytoceal by poking the tip of the tube into the crytoceal approximately 1 mm deep so that a crytoceal plug was formed. After that, the microhematocrit tubes were centrifuged for 5 minutes at 5,000 rpm with the same volume of tubes facing each other to balance the centrifuge rotation. The length of the facing blood section (a) and the length of the total blood volume contained in the tube (b) were measured using a ruler. Hematocrit level expressed as % volume of blood cell solids [22].

2.4 Research parameters

The parameters in this study were observation of clinical symptoms, blood picture (total leukocytes, total erythrocytes, hemoglobin concentration, and total hematocrit), survival rate, and water quality. The calculation formula for survival (1) [23], total leukocytes (2) and total erythrocytes (3) [24] can be seen as under below.

$$SR = (Nt/No) \times 100\% \quad (1)$$

$$\sum leukocytes = \sum Leukocyte\ count\ average \times diluent/volume \quad (2)$$

$$\sum erythrocytes = erythrocytes\ count\ average \times diluent/volume \quad (3)$$

The description of the formula is as follows; SR = survival rate, Nt = number of fish at the end of the study, No = number of fish at the beginning of the study.

2.5 Analyze the data

Data on freshwater pomfret survival rate, leukocyte count, erythrocyte count, hemoglobin concentration, and hematocrit count were tested using ANOVA (Analysis of variance) with 95% reliability. Then, the follow-up test used was the Tukey test. Data on clinical symptoms and water quality were analyzed descriptively.

3 Results and discussion

Clinical symptoms of pomfret in all treatments that have not been infected with bacteria show normal symptoms. The observation of clinical symptoms after the challenge test showed no abnormal clinical symptoms in P3, P4, P5, and P6 (Fig. 1) because they had previously been fed with the addition of garlic extract. [25] The healing of clinical symptoms is due to the presence of active ingredients (Allisin) from garlic extract that enters the body to increase the body's resistance to pathogenic attacks of *A. hydrophilla* bacteria. [26] The antibacterial mechanism of garlic is believed to be related to the formation of allicin. This compound is formed when garlic cells are damaged by processes such as crushing or slicing. [27] Allicin, the active compound in garlic, can kill various types of bacteria. It works by damaging the structure of the bacterial cell wall, specifically the sulfhydryl and disulfide groups on the amino acid cysteine. This damage causes interference with the synthesis of enzymes that are essential for cell metabolism, as well as damaging the integrity of the cytoplasmic membrane. As a result, the metabolic processes of proteins and nucleic acids are inhibited, so the bacteria cannot replicate and die.

Fig. 1. The results of clinical symptom observation after the challenge test.

In the negative control P2 treatment (without garlic extract), the result showed physical and behavioral changes in pomfret. These clinical symptoms show a change in the color of the fish, red spots on the mouth of the fish, peeling scales, damage to the tail of the fish, and changes in behavior from a decrease in fish appetite and unstable swimming response. Clinical symptoms of treatment P2 negative control (without being given garlic extract) can be seen in Fig 2.

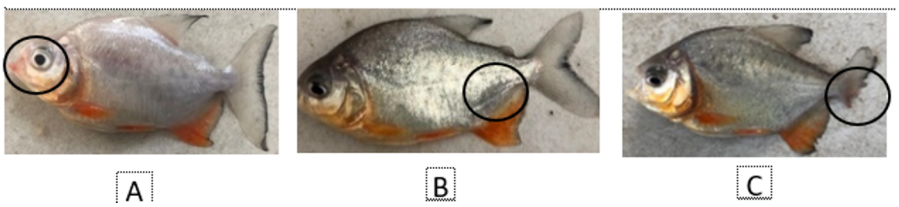

Fig. 2. Negative control P2 treatment (without garlic extract), A (red spots), B (scales peeling off), C (tail damage).

The results of clinical symptoms in pomfret attacked by Aeromonas hydrophyla bacteria are almost the same as the statement [28] that clinical symptoms in goldfish are the onset of hyperemia, inflammation, necrosis and ulcers in the former injection area, and the movement of fish becomes sluggish until death. [25] clinical symptoms of goldfish caused by *Aeromonas hydrophyla* bacterial infection such as inflammation, haemorrhage, ulcers and also death. In addition to clinical symptoms, blood picture parameters were also observed to see the effect of garlic extract on pomfret's blood picture.

The results of the blood picture at H-15 (Table 1) showed that garlic extract had no significant effect (P>0.05) on total hematocrit and haemoglobin concentration but had a significant effect (P<0.05) on total leukocytes and total erythrocytes. Based on BNT further test on leukocyte parameters and Duncan further test on erythrocyte parameters, P3 was not significantly different from P2, P4, P5, and P6, but significantly different from treatment P1.

Table 1. Blood picture results (total leukocytes, total erythrocytes, haemoglobin concentration, and hematocrit count) at H-15.

Treatment	Leukocytes ($x10^5$ Sel/mm^3)	Erythrocytes ($x10^6$ Sel/mm^3)	Hematocrit (%)	Haemoglobin (G/Dl)
P1	1,32±4945,45[a]	15,87±5,24[b]	14,00± 3,60	5,46±0,50
P2	2,18±7177,80[c]	10,03±0,25[a]	15,00± 5,19	5,73±0,30
P3	1,99±10388,73[bc]	10,10±0,25[a]	13,00± 2,64	4,70± 1,01
P4	1,83±7285,83[b]	10,08±0,92[a]	14,00± 6,08	5,50±0,50
P5	1,99±9961,46[bc]	10,11±0,29[a]	12,00± 6,24	4,40±0,52
P6	1,83±11429,16[b]	10,30±0,17[a]	13,67± 3,21	4,93± 1,25

The results of total leukocytes (Table 1) in the P3 treatment (10 g/kg feed) with a value of 1.83 ± 7285.83 x105 cells/mm3 is a leukocyte value above normal limits. [The normal range of white blood cell counts in normal fish generally ranges from 20,000 - 150,000 cells/mm^3. Leukocyte results above normal limits indicate that the immune system is increased due to garlic extract in pomfret feed. [30] The total number of leukocytes during the addition of prebiotics in tilapia feed has increased so it plays a significant role in increasing the immune response or body resistance of tilapia against disease and infection. [31] The addition of rabal probiotics in feed to the white blood cell picture of tilapia can increase the number of leukocyte values in tilapia.

In addition to the blood picture at H-15, blood picture observations were also made at H-28 (Table 2). The results of the blood picture at H-28 showed that garlic extract had no significant effect (P>0.05) on total erythrocytes, total hematocrit and hemoglobin concentration but had a significant effect (P<0.05) on total leukocytes. Based on the BNT further test on leukocyte parameters, treatment P3 was not significantly different from P1, P4, P5, and P6, but significantly different from treatment P2.

Tabel 2. Blood picture results (total leukocytes, total erythrocytes, hamoglobin concentration, and hematocrit count) at H-28.

Treatment	Leukocytes ($x10^5$ sel/mm^3)	Erythrocytes ($x10^6$ sel/mm^3)	Hematocrit (%)	Haemoglobin (g/dL)
P1	1,28±4150,00[a]	12,93±0,70	18,67± 1,52	6,40±0,52
P2	2,05±15071,69[b]	10,08±0,15	16,67± 1,52	6,50±0,50
P3	1,43±9196,33[a]	11,61±0,58	18,00 ± 2,0	6,73±0,68
P4	1,42±7086,78[a]	11,16±0,74	16,00± 5,29	6,50±0,50
P5	1,43±5445,71[a]	12,49±2,36	16,00± 2,64	6,43±0,40
P6	1,32± 4997,74[a]	13,47±3,37	19,33± 1,15	6,50±0,50

The best leukocyte result at H-28 was 1.43 ± 9196.33 x105 cells/mm3 (P3). The H-28 leukocyte value in the P3 treatment showed a difference from the H-15 leukocyte value. The H-28 leukocyte value decreased from the observation of the H-15 leukocyte value. This is because pomfret fed with the addition of garlic extract experienced healing. The healing that occurs is due to the Allicin compound in the pomfret feed. Allisin compounds are active compounds that can inhibit bacterial growth.

Another parameters observed in this study were the pomfret survival rate. The survival results obtained from ANOVA analysis show that the addition of garlic extract has a real effect ($P < 0.05$) on the survival of pomfret. Further tests conducted showed that the P4 treatment was significantly different from the P3 treatment, but not significantly different from the P1 treatment. The results of survival can be seen in Table 3 as follows.

Table 3. The result of the pomfret survival rate value.

Treatment	Survival rate (%)
P1	$73{,}33 \pm 5{,}77^{bc}$
P2	$63{,}33 \pm 5{,}77^{ab}$
P3	$83{,}33 \pm 5{,}77^{c}$
P4	$66{,}67 \pm 5{,}77^{ab}$
P5	$56{,}67 \pm 5{,}77^{a}$
P6	$50{,}00 \pm 10^{a}$

The best survival rate value of pomfret fish in the P3 treatment (10 g/kg feed) is 83.33 ± 5.77%. [25] the addition of garlic extract at a dose of 1.25% in tilapia feed can inhibit MAS disease infection and can increase the body resistance of the test fish so that survival rate (90.00 ± 10.00%) also increases. [30] The highest survival rate of tilapia was obtained in the 1% prebiotic treatment which amounted to 93.33 ± 5.77%. Based on the above statement, garlic extract affects the survival rate. The survival rate of pomfret fish is high because pomfret fish have a good immune system to fight bacterial attacks so fish remain alive until the end of maintenance.

Other parameters that need to be observed in this study are water quality such as pH, temperature and dissolved oxygen (DO). The results of observations of water quality parameters are presented in Table 5 as follows. Water quality observation data (Table 5) is good for pomfret rearing. [31] the water quality for pomfret rearing is 28.4-29.9°C, pH 7.59-8.40, DO 3.06-5.87 mg/L.

Table 4. Water quality observation results.

Treatment	End of maintenance		
	pH	DO (mg/L)	Suhu (°C)
P1	7,67	10,1	27,4
P2	7,71	11,2	28,1
P3	7,69	10,7	27,8
P4	7,61	11,3	27,9
P5	7,68	10,9	26,4
P6	7,89	11,2	27,1

4 Conclusion

The conclusion of this study is that garlic extract at a dose of 10 g/kg feed (P3) has a significant effect ($P<0.05$) on clinical symptoms, blood picture, and survival of pomfret. A dose of 10 g garlic extract/kg feed (P3) is the best dose to improve the immune system of pomfret. Garlic extract can be recommended as a natural antibiotic that is very environmentally friendly and can increase the immune system in pomfret.

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