Int. J. Aquat. Biol. (2026) 14(4): 136-149
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Immunostimulatory effects of octopus ink extract on the hematological and immune responses of catfish (*Clarias* sp.) challenged with *Aeromonas hydrophila*

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Abstract: Catfish (*Clarias* sp.) cultivation is often challenged by bacterial infections caused by *Aeromonas hydrophila*, which can lead to Motile Aeromonas Septicemia (MAS). The excessive use of antibiotics can lead to antibiotic resistance and residues, creating a need for natural immunostimulants. Octopus ink, a byproduct of the fisheries industry rich in bioactive compounds, shows potential as a sustainable alternative. This study evaluated the effectiveness of octopus ink extract in enhancing the immune response and survival of catfish against *A. hydrophila*. The experiment used a Completely Randomized Design with four treatments: control (no extract) and feed supplemented with octopus ink extract at 50, 100, and 150 ml/kg, each replicated three times. A total of 240 catfish (6-7 cm) were reared for 28 days, fed according to treatment, and challenged with *A. hydrophila* (10^8 CFU/ml) by immersion. Measured parameters included erythrocyte and leukocyte counts, leukocyte differential, hematocrit, and survival rate (SR). The results showed that octopus ink extract significantly improved hematological parameters and survival. The 150 ml/kg dose yielded the best results: erythrocytes 2.4×10^6 cells/mm^3, leukocytes 5.78×10^4 cells/mm^3, lymphocytes 89%, hematocrit 33.8%, and SR 88.3%. Thus, octopus ink extract effectively enhances catfish immunity and survival against *A. hydrophila*, supporting its use in sustainable aquaculture.

Article history:
Received 25 February 2026
Accepted 21 May 2026
Available online 25 August 2026

Keywords:
Octopus ink extract
Catfish
Immune response
Aquaculture

Introduction

Aquaculture primarily involves the cultivation of aquatic organisms in controlled environments for various purposes, including commercial, recreational, and public use. In this system, fish are raised within enclosed artificial water bodies, such as tanks, where they live, feed, grow, and release waste (Tamim et al., 2022). One of the aquaculture organisms widely cultivated in Indonesia is catfish (*Clarias* sp.). Clarias catfish ranks as the second most important freshwater aquaculture commodity in Indonesia after tilapia (Gustiano et al., 2021). This species has high market demand, not only as a popular food fish but also as a widely cultivated commodity among Indonesian farmers. Strong consumer demand drives continuous, large-scale production to meet market needs (Salsabilla et al., 2021).

Although *Clarias* sp. is generally considered a disease-resistant species, recent studies have shown that it remains susceptible to several diseases (Mamun et al., 2022). *Aeromonas hydrophila* is a pathogenic bacterium that frequently infects catfish. This microorganism can lead to severe diseases, including Motile Aeromonas Septicemia (MAS), catfish enteric septicemia, and columnaris disease (Aini et al., 2024). In managing fish health, disease prevention can be achieved through several approaches, including chemical antibiotics and natural immunostimulants. However, the use of chemical antibiotics can lead to adverse effects, including residue accumulation in fish tissues and the emergence of antibiotic-resistant pathogens. Thus, the use of natural immunostimulants is essential as a safer alternative for disease prevention in fish (Affandi et al., 2019). The effective use of immunostimulants depends on determining the appropriate dosage and frequency of application. Excessively high doses may inhibit the immune defense system, whereas doses that are too low may

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DOI: https://doi.org/10.22034/ijab.v14i4.2829

fail to stimulate an adequate immune response. Therefore, proper frequency and consistent administration are important for strengthening immune function and ensuring optimal protection (Zhang et al., 2023). Immunostimulants may be delivered through oral feeding, immersion, or injection methods (Ching et al., 2020).

It is important to identify natural substances rich in bioactive compounds that can serve as effective immunostimulants to help prevent *A. hydrophila* infections in catfish. One of the wastes produced by fisheries is octopus' ink, which comes from the remains of octopuses consumed by humans and may cause environmental problems if not properly handled. Only limited attempts have been made to utilize this waste in aquaculture. Previous studies have revealed that octopus ink possesses antifungal activity against *Aspergillus fumigatus* and *Fusarium* sp. (Vennila et al., 2011). Additionally, it contains several bioactive compounds, including melanin (Besednova et al., 2017; Hossain et al., 2019; Moustafa and Awaad, 2016; Shazwani and Rabeta, 2020; Hernández-Zazueta et al., 2021), amino acids (such as taurine, aspartic acid, glutamic acid, alanine, and lysine) (Nair et al., 2011; Derby, 2014), and alkaloids (Kalor et al., 2019). Overall, research on the use of octopus ink in aquaculture, particularly as an immunostimulant, remains scarce.

Octopus ink contains a variety of bioactive compounds, making it a potential immunostimulant in aquaculture. Hence, this study aimed to evaluate the effect of octopus ink extract on the immune response of catfish infected with *A. hydrophila* and to identify the optimal extract dose to prevent MAS in catfish, thereby enhancing aquaculture productivity.

Materials and Methods

This research was conducted at the Fish Health Laboratory and Fish Production and Reproduction Laboratory, Department of Fisheries and Marine Science, Faculty of Agriculture, University of Mataram, from April to September 2025.

Octopus ink extraction: The preparation of the octopus ink extract began by removing and collecting the ink from the ink sac. The collected ink was placed in a sterile container and stored in a refrigerator to prevent degradation. The extraction process was conducted using maceration, which involves soaking the ink in a solvent. In this study, the ink was mixed with methanol at a 1:3 ratio, using 1500 ml of methanol. The mixture was measured with a graduated cylinder, transferred into an Erlenmeyer flask, and manually homogenized for 1.5 hours. The flask was then covered with aluminum foil, secured with a string, and stored in a refrigerator at 4°C for seven days (Affandi et al., 2019). After the maceration period, the extract was concentrated by evaporation on a rotary evaporator at 62 rpm for 5 hours (Girija et al., 2012).

***Aeromonas hydrophila* culture:** *Aeromonas hydrophila* used in this research was obtained from BBPBAP Jepara. Before conducting the challenge test, culture media for bacterial growth were prepared. A total of 1.5 g of TSB (Tryptic Soy Broth) powder was mixed with 50 ml of distilled water in an Erlenmeyer flask, heated on a hotplate until boiling, and then sterilized using an autoclave. Prior to inoculation into the growth medium, *A. hydrophila* culture was diluted to a concentration of 10^8 CFU/ml (Subhan et al., 2020).

Aeromonas hydrophila bacteria were prepared at a concentration of 10^8 CFU/ml (Mufidah et al., 2022) using Nutrient Agar (NA) as the growth medium. The bacteria were cultured by transferring a loopful of *A. hydrophila* from a slant agar culture, streaking it onto NA medium, and incubating it for 24 hours. After 21 days of treatment with octopus ink extract, the test fish were exposed to *A. hydrophila* at a density of 10^8 CFU/ml on the 22nd day via immersion (Aini et al., 2024). Observations of experimental parameters were conducted from the first to the seventh day following infection.

Research design: This study employed an experimental approach using a Completely Randomized Design (CRD), consisting of one control and three treatment groups, each replicated three times, for a total of 12 experimental units. Each treatment group contained 20 catfish. The treatment

dose used in this study is based on previous research (Affandi et al., 2025). The treatments involved the administration of octopus ink extract through feed at the following dosage levels: P1 (Control) = without administration of octopus ink extract + bacterial infection, P2 = administration of octopus ink extract of 50 ml/kg + bacterial infection, P3 = administration of octopus ink extract of 100 ml/kg + bacterial infection, P4 = administration of octopus ink extract of 150 ml/kg + bacterial infection.

Research procedures: The rearing medium in this study consisted of 12 containers. Prior to use, each container was cleaned with detergent, thoroughly rinsed with water, and left to air-dry for 24 hours. The experimental animals were catfish measuring 6-7 cm, sourced from the Batu Kumbung Fish Farm in Lingsar, West Lombok Regency. Fish preparation began with a 7-day acclimation period before the fish were placed into the containers. During acclimation, the fish were fed commercial feed three times daily at a feeding rate of 3% of their total biomass (Hartono and Barades, 2022).

This study utilized commercial feed, which was combined with the designated treatment doses in a tray. The mixing procedure followed a method from previous research (Affandi et al., 2025), in which the extract solution was evenly sprayed onto 1 kg of feed, then air-dried for 15 minutes at room temperature. Once dried, the feed was weighed at 3% of the total fish biomass for each treatment group or container (Hartono and Barades, 2022). The prepared feed was then stored in ziplock bags, labeled with the corresponding treatment dosage.

Twelve containers were each filled with 20 liters of water and equipped with aeration. The test fish were then stocked at a density of 20 fish per container (Rachmawati et al., 2023). The fish received feed mixed with octopus ink extract as treatment for 21 days. Pellet feed was administered at 3% of total fish biomass, with the rate adjusted every 10 days (Hartono and Barades, 2022). Feeding was carried out three times daily—morning, afternoon, and evening—throughout the rearing period. The parameters observed in this study included erythrocyte count, leukocyte count, leukocyte differential, hematocrit level, and survival rate.

Erythrocytes: Before blood collection, the fish were first anesthetized until they became unconscious. Blood was then drawn from the caudal artery using a syringe containing EDTA and prepared for hematological analysis. The red blood cell (erythrocyte) count was determined following the Klontz method. A blood sample from the Eppendorf tube was drawn into a capillary pipette containing a small red bead until it reached the 0.5 mL mark. Hayem's solution was then added up to the 101 mL mark, and the mixture was homogenized by gentle shaking. Two drops of the mixture were discarded to eliminate air bubbles before placing a drop into the counting chamber, which was covered with a glass slide. The sample was observed under a microscope at 10x10 magnification across five small squares of the hemocytometer chamber. The following formula can be used to calculate the number of red blood cells (erythrocytes). According to Blaxhall and Daisley (Yanuhar et al., 2021): The number of erythrocytes = Σfound erythrocytes x 10^4 cells/mm^3.

Leukocytes: White blood cell (leukocyte) counts were obtained by drawing a blood sample from the Eppendorf tube using a leukocyte pipette containing a small white bead until the volume reached the 0.5 ml mark. Turk's solution was then added up to the 11 mL mark, and the mixture was thoroughly homogenized by gentle shaking. Two drops were discarded to eliminate air bubbles before placing a drop into the hemocytometer chamber, which was covered with a glass slide. The sample was examined under a microscope at 10x40 magnification across four large squares of the hemocytometer chamber. The number of white blood cells (leukocytes) can be calculated using the following formula according to Blaxhall and Daisley (Lestari and Fatimatuzzahra, 2021): The number of leukocytes = Σfound leukocytes x 50 cells/mm^3.

Leukocyte differential: The procedure for determining the leukocyte differential count followed the methods described by Setyono et al. (2025). First, a clean glass slide was held between the index finger

and thumb, and a small drop of blood was placed on its right side. Another slide was then positioned to the left of the blood drop at a 30° angle and pulled to the right until it touched the blood. Once the blood spread along the edge, the slide was pushed back to the left—maintaining the same angle—to create a thin smear suitable for microscopic observation. The smear was then air-dried. To enhance visibility, the smear was stained using Giemsa stain. The staining procedure involved air-drying the blood smear, fixing it in methanol for 5 minutes, immersing it in diluted Giemsa solution (1:20) for 15 minutes, rinsing with distilled water, and air-drying again. The prepared slide was observed under a microscope at 400x magnification. The percentage of leukocyte types was determined by examining 10 fields of view and classifying each cell type (lymphocytes, monocytes, and neutrophils). The counts of lymphocytes, monocytes, and neutrophils were then calculated using the following formula: Lymphocytes = Σfound lymphocytes/100 x 100%; Monocytes = Σfound monocytes/100 x 100%; Neutrophils = Σfound neutrophils/100 x 100%.

Hematocrit: Blood samples were collected using a microhematocrit tube, which was filled up to three-quarters of its length. The tube was then sealed with approximately 1 mm of Crystoseal and centrifuged for 15 minutes, following the method of Nadiro et al. (2020). The hematocrit value was determined by comparing the length of the packed red blood cell layer at the bottom of the tube to the total length of the blood column. The hematocrit level was expressed as the percentage of solid blood cell volume and calculated using the following formula: Hematocrit level = blood sediment length volume/total length of blood volume in the tubes x 100%.

Survival rate: The survival rate (SR) was calculated at the end of the study using the following formula (Indriastuti et al., 2022): SR = final number of fish / initial number of fish × 100%.

Statistical analysis: The data obtained were then analyzed using analysis of variance (ANOVA) with SPSS. The significance level was set at 5% to determine the effect of treatment on the research. If the data show a real effect, then Duncan's further test is carried out at the 95% confidence level.

Results

Erythrocytes: Based on the results, the number of erythrocytes in catfish during the 21-day maintenance period, fed the addition of octopus ink extract immunostimulant at different doses, ranged from 2.31-2.61 x 10^6 cells/mm^3, and after *A. hydrophila* bacterial infection for 7 days, ranged from 1.61-2.40 x 10^6 cells/mm^3 (Fig. 1). The results also showed that feeding with the addition of octopus ink extract immunostimulants did not differ significantly (P>0.05) during the 21-day maintenance period. Different results were observed after 7 days of *A. hydrophila* infection, with a significant difference (P<0.05) in the number of erythrocytes in catfish fed immunostimulants containing octopus ink extract. The results showed that the number of erythrocytes in P1 differed significantly from those in P2, P3, and P4. This indicates that the addition of octopus ink extract immunostimulants affected the number of erythrocytes of catfish after *A. hydrophila* bacterial infection.

Leukocytes: Based on our findings, the leukocyte counts of catfish fed with octopus ink extract immunostimulants at different doses ranged from 2.80-2.89 x 10^4 cells/mm^3 during 21 days of rearing, and after 7 days of *A. hydrophila* infection, the count ranged from 3.07-5.78 x 10^4 cells/mm^3 (Fig. 2). The results showed that feeding with the addition of octopus ink extract immunostimulants did not differ significantly (P>0.05) in catfish leukocytes during the 21-day maintenance period. Different results were observed after 7 days of *A. hydrophila* infection, with a significant difference ($P<0.05$) in the number of catfish leukocytes in catfish fed feed supplemented with octopus ink extract immunostimulants. The Duncan test results showed that the number of catfish leukocytes (P1) differed significantly from P2, P3, and P4, indicating that the addition of octopus ink extract immunostimulants affected the number of catfish leukocytes after *A. hydrophila* bacterial infection.

Leukocyte differential: The leukocyte differential of

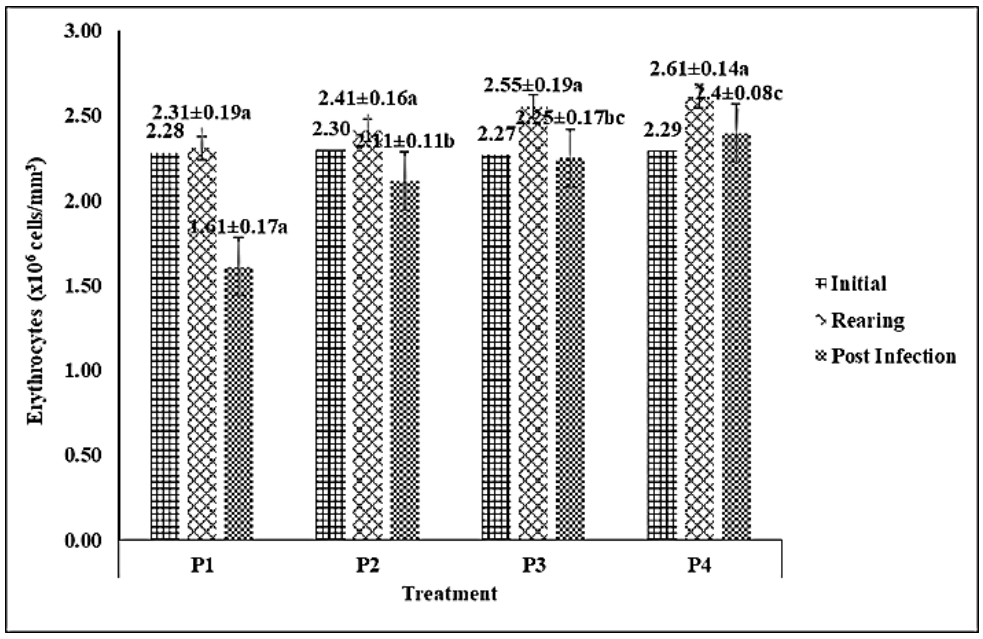

Figure 1. Erythrocytes in catfish (*Clarias* sp.) fed different levels of octopus ink extract.

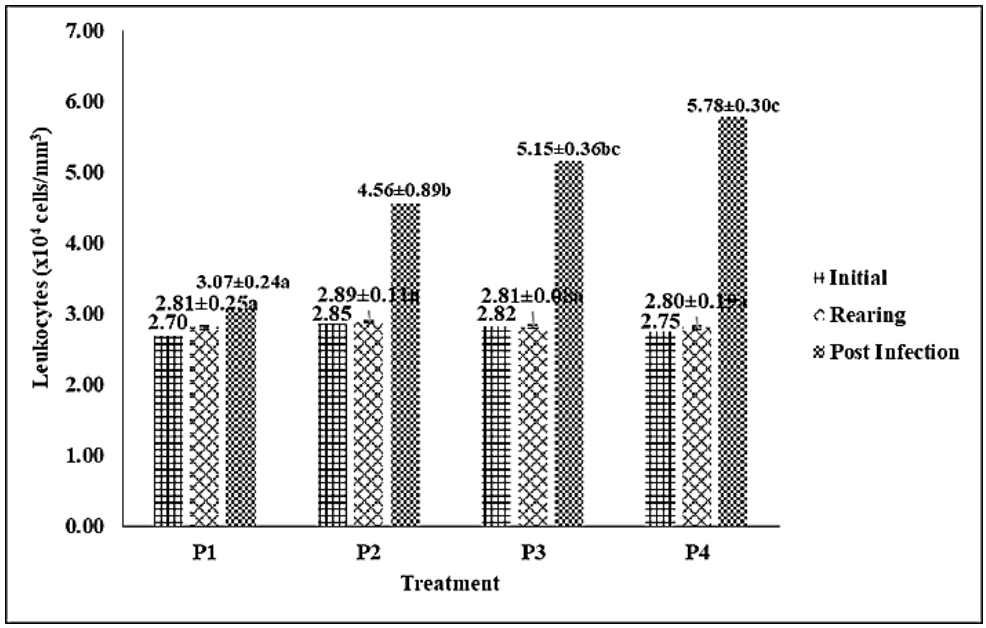

Figure 2. Leukocytes in catfish (*Clarias* sp.) fed different levels of octopus ink extract.

catfish fed feed supplemented with octopus ink extract at different doses during 21 days of culture showed lymphocytes counts ranging from 71-82%, monocytes 9.7-14.7%, and neutrophils 8.0-14.3% after 7 days of *A. hydrophila* infection, lymphocyte counts ranged from 77.7-80.0%, monocytes 12.7-15.3%, and neutrophils 6.0-8.0% (Table 1, Fig. 3). The results show that feeding with the addition of immunostimulant octopus ink extract is significantly different (P<0.05) in the differential leukocytes of catfish during the 21-day maintenance period and after *A. hydrophila* infection for 7 days. The Duncan Test results showed that catfish lymphocytes during the maintenance period and post-infection at P1 were significantly different from P2, P3, and P4, whereas P2 was not significantly different from P3 and P4. Catfish monocytes during the maintenance period and post-infection at P1 also showed significantly different results from P2, P3, and P4, but P2 was not significantly different from P3 and P4. Similar results

Table 1. Leukocyte differential of catfish (*Clarias* sp.) fed different levels of octopus ink extract.

Leukocyte Differential	Treatment	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)
Initial	P1	70.0±1.7	16.3±1.5	13.7±3.2
	P2	70.0±2.0	18.7±1.2	11.3±1.2
	P3	66.0±1.0	18.7±1.2	15.3±0.6
	P4	71.0±2.0	17.0±0.0	12.0±2.0
Rearing	P1	71.0±1.0^{a}	14.7±0.6^{b}	14.3±1.2^{c}
	P2	79.7±1.5^{b}	10.3±1.5^{a}	10.0±0.0^{b}
	P3	81.7±2.1^{b}	10.3±1.2^{a}	8.0±1.0^{a}
	P4	81.7±2.5^{b}	9.7±1.5^{a}	8.7±1.2^{ab}
Post Infection	P1	73.0±6.1^{a}	13.0±3.5^{b}	14.0±2.6^{c}
	P2	82.7±2.5^{b}	8.3±0.6^{a}	9.0±0.0^{b}
	P3	86.0±1.0^{b}	7.0±0.0^{a}	7.0±0.0^{ab}
	P4	89.0±2.0^{b}	5.7±1.2^{a}	5.3±0.6^{a}

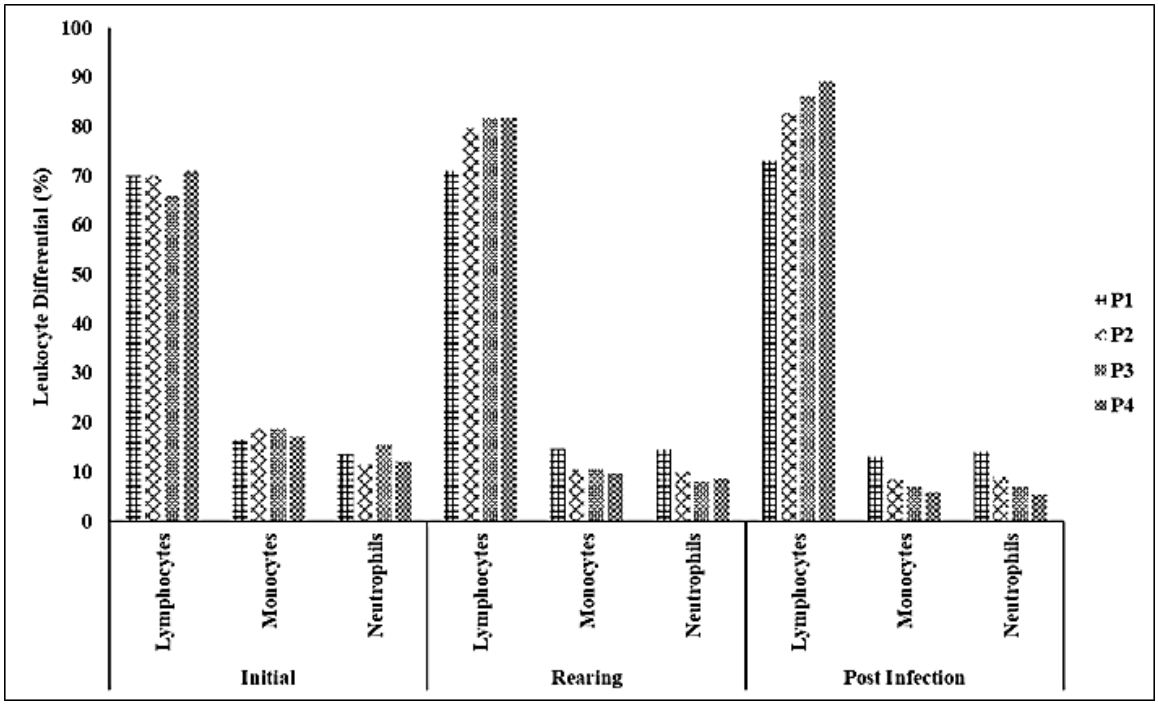

Figure 3. Leukocyte differential in catfish (*Clarias* sp.) fed different levels of octopus ink extract.

were also observed in neutrophils during the maintenance period and post-infection at P1, which differed significantly from those at P2, P3, and P4. This indicates that the addition of the immunostimulant octopus ink extract affects the differential leukocyte counts in catfish during the maintenance period and post-infection with *A. hydrophila*.

Hematocrit: The hematocrit levels of catfish fed octopus ink extract immunostimulants at different doses ranged from 32-36% during 21 days of rearing, and between 27.6-33.8% after 7 days of *A. hydrophila* bacterial infection (Fig. 4). The results showed that feeding with the immunostimulant octopus ink extract did not significantly affect catfish hematocrit during the 21-day rearing period (P>0.05). A different result was observed after 7 days of *A. hydrophila* infection, indicating a significant difference (P<0.05) in the hematocrit of catfish fed the immunostimulant octopus ink extract. The results showed that the hematocrit of catfish P1 differed significantly from those of P2 and P4, but not from P3, indicating that the addition of the immunostimulant octopus ink extract affected the hematocrit of catfish following *A. hydrophila* infection.

Survival rate: The survival rate (SR) of catfish during 21 days of rearing and 7 days after *A. hydrophila* infection, fed feed supplemented with the

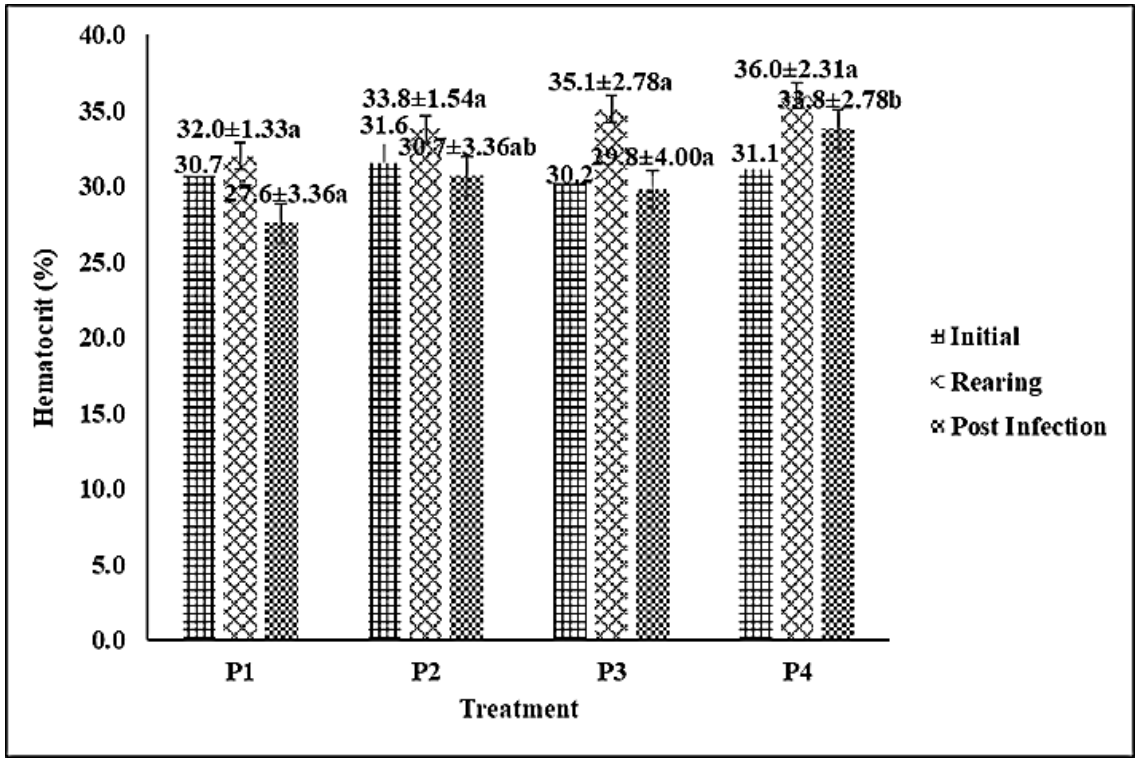

Figure 4. Hematocrit in catfish (*Clarias* sp.) fed different levels of octopus ink extract.

immunostimulant octopus ink extract at different doses, ranged from 73.3% to 88.3% (Fig. 5). The results showed that feeding with the addition of the immunostimulant octopus ink extract significantly (P<0.05) affected the survival rate (SR) of catfish during the 21-day rearing period and after 7 days of *A. hydrophila* infection. The results showed that the survival rate (SR) of catfish P1 was significantly different from that of P2, P3, and P4, indicating that the addition of the immunostimulant octopus ink extract affected the survival rate (SR) of catfish after *A. hydrophila* infection.

Discussions

Red blood cells (erythrocytes) are the most common type of blood cell in fish and are responsible for transporting oxygen (O_2). Erythrocytes absorb oxygen in the gills and release it into the tissues (Esmaeili, 2021). Based on the results, the average erythrocyte counts during the research ranged from 2.27-2.30 x 10^6 cells/mm^3 at the beginning (day 1), 2.31-2.61 x 10^6 cells/mm^3 after administration of the immunostimulant octopus ink extract (day 21), and 1.61-2.40 x 10^6 cells/mm^3 seven days after *A. hydrophila* bacterial infection (day 28). These results show that the total erythrocyte count across the three observations remained within the normal range. In healthy catfish, the range of erythrocyte counts in the blood ranges from 1-3 x 10^6 cells/mm^3 (Lestari and Fatimatuzzahra, 2021).

The number of red blood cells in catfish decreased after infection (day 28). This is due to infection by the bacteria *A. hydrophila*. Infection with *A. hydrophila* produces hemolysin, which can lyse red blood cells, decreasing the red blood cell count and preventing the blood-producing organs from producing more blood cells to replace blood lost from wounds in the infected area (Nurfitri et al., 2023). Red blood cells play a vital role in fish immunity. Low red blood cell counts indicate anemia, whereas high levels indicate stress in fish. Furthermore, low red blood cell counts prevent fish from absorbing sufficient oxygen even when oxygen is abundant in the water. As a result, fish experience anoxia (oxygen deficiency). Red blood cells are produced in the spleen and kidneys. Anemia affects fish growth by reducing nutrient supply to cells, tissues, and organs, thereby inhibiting fish metabolic processes (Yanuhar et al., 2021). The total

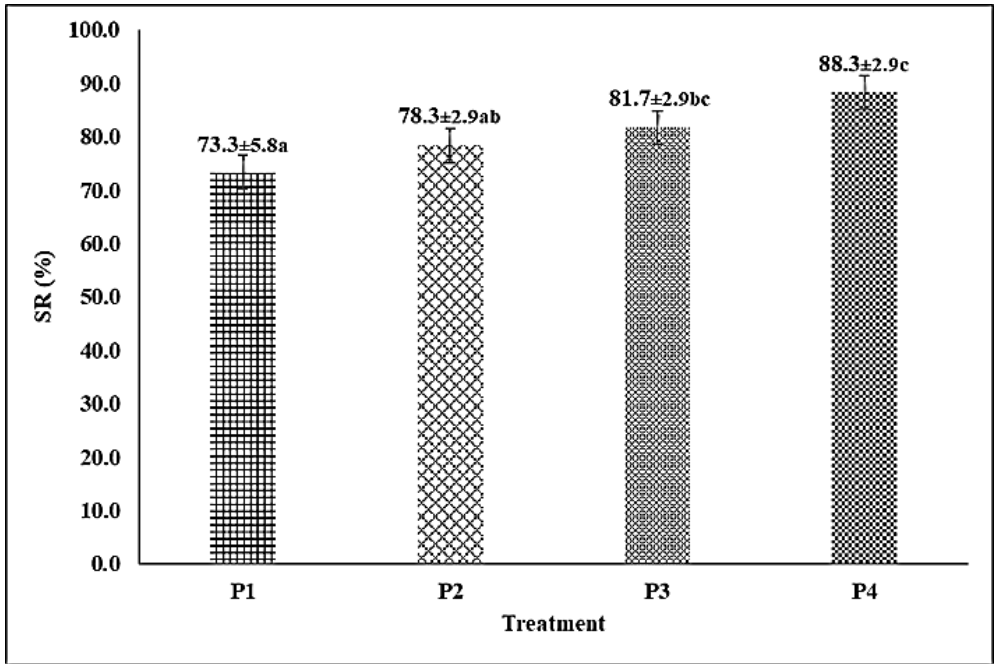

Figure 5. Survival rate in catfish (*Clarias* sp.) fed different levels of octopus ink extract.

erythrocyte counts of catfish post-infection in treatments 2, 3, and 4 were higher than the control and within the optimal range. This is due to bioactive compounds in octopus ink extract, which can enhance catfish immunity. Octopus ink contains alkaloid compounds that can function as antimicrobials, antioxidants, anticancer, anti-inflammatory, and antiviral agents. Alkaloids can increase nonspecific immune activity and are used as immunostimulants due to their role in preventing disease (Affandi et al., 2025). The remaining normal erythrocyte counts in fish infected with bacteria indicate that the fish's immune system is able to increase erythrocyte production to replace those lysed by bacterial infection (Dangeubun and Metungun, 2017).

Leukocytes are immune cells that help protect the body from disease and foreign invaders. Leukocytes are found throughout the body, including the blood and lymphatic system (Kiboneka, 2021). Based on our results, the average leukocyte counts obtained during the study ranged from 2.70-2.85 x 10^4 cells/mm^3 at the beginning of the study (day 1), 2.81-2.89 x 10^4 cells/mm^3 after administration of the immunostimulant octopus ink extract (day 21), and 3.07-5.78 x 10^4 cells/mm^3 seven days after *A. hydrophila* bacterial infection (day 28). Based on the graph in Figure 2, the calculation results indicate that the total leukocyte counts across the three observations remained within the normal range. In healthy catfish, the blood leukocyte counts range from 2-15 x 10^4 cells/mm^3 (Ekasari et al., 2023). The leukocyte counts in catfish after infection (day 21) increased in all treatments, including the control. This is suspected to be due to infection with *A. hydrophila*. The increase in white blood cells after a challenge test is the body's response to antigens (e.g., pathogenic bacteria) entering the body (Radityo et al., 2022). White blood cells (leukocytes) are part of the fish's innate immune system, acting as the first line of defense against invading pathogens (Adeyemi et al., 2022).

Treatment with octopus ink extract resulted in a significantly greater increase in leukocyte counts. This is suspected to be due to the bioactive compounds in the octopus ink extract, which can boost the catfish's immune system. Octopus ink contains alkaloids that can function as antimicrobials, antioxidants, anticancer, anti-inflammatory, and antiviral agents. Alkaloids can increase nonspecific immune activity and are used as immunostimulants due to their role in preventing disease (Affandi et al., 2025). Alkaloids are known to increase the number of white blood cells (leukocytes) (Bamidele et al., 2024). Alkaloids can act as immunostimulators, strengthening the fish's

defenses against antigens by promoting leukocyte production (Al-Ardawi et al., 2024).

Leukocyte differential is the total number of white blood cells (leukocytes) divided into percentages of each type of white blood cell (George-Gay and Parker, 2003). Leukocytes play a role in the immune system of fish. They are classified into lymphocytes, monocytes, and granulocytes. Granulocytes are further classified into: neutrophils, eosinophils, and basophils. These five types of cells have different functions as the body's defense mechanism (Balasubramanian et al., 2022). Based on the results of the current study, the average lymphocyte values during the research activities ranged from 66-71% on day 1, 71-81.7% after administration of the immunostimulant octopus ink extract (day 21), and 73-89% after seven days post-infection with *A. hydrophila* bacteria (day 28). The normal range of lymphocytes in catfish is between 74-86%; in this condition, the catfish group in the study had normal physiology (Nugraha et al., 2022). The increased percentage of lymphocytes in catfish is due to the alkaloid content in octopus ink extract, which can boost the fish's immune system. Alkaloids act as immunostimulants that can activate lymphocyte cells (Chakraborty and Hancz, 2011). Lymphocytes include T cells, B cells, and natural killer (NK) cells. T cells, or T lymphocytes, play a central role in cellular immunity. B cells, or B lymphocytes, function in the humoral immune response through antibody production. Natural killer (NK) cells, also known as Large Granular Lymphocytes (LGLs), are the third type of cell that produces T and B lymphocytes. Natural killer (NK) cells have been reported to provide a rapid response to virus-infected cells and respond to tumor formation (Orakpoghenor et al., 2019).

Based on our results, the average monocyte counts during the research ranged from 16.3-18.7% at the beginning of the study, 9.7-14.7% after administration of the immunostimulant octopus ink extract (day 21), and 5.7-13% seven days after *A. hydrophila* bacterial infection (day 28). The catfish monocyte counts ranged from 0.98% to 6.69%, which are still within normal limits (Mangkunegara et al., 2023). This decrease may be due to the short lifespan of these cells in the blood (only 14-36 hours), after which they migrate to other tissues and differentiate into macrophages (Nugraha et al., 2022). Octopus ink extract has been shown to inhibit the growth of *A. hydrophila*, thereby decreasing monocyte counts. Monocytes leave the bloodstream and migrate to the site of infection, where they phagocytose bacteria due to their superior phagocytic capacity compared to neutrophils. Monocytes function as macrophages and are involved in immune function (Syaifurrisal et al., 2024). Monocytes function in the fish body to block the invasion of incoming pathogens (Andayani et al., 2023). They are a central component of the innate immune system and play a crucial role in regulating inflammation. These cells play a crucial role not only in the formation of inflammatory mediators and the regulation of innate and adaptive immunity, but also contribute to the resolution of inflammation and the restoration of homeostasis. During the inflammatory process, monocytes are directed to the site of inflammation and ultimately differentiate into inflammatory macrophages or dendritic cells (Austermann et al., 2022).

Based on the current results, the average neutrophil counts ranged from 12-15.3% on day 1, 8-14.3% after administration of the immunostimulant octopus ink extract (day 21), and 5.3-14% after 7 days post-infection with *A. hydrophila* (day 28). Normal neutrophil levels in catfish are 26.2±4.9% (Kurniawati et al., 2025). Neutrophils, the first leukocytes to be activated at the site of infection, are responsible for neutralizing pathogens through mechanisms such as the production of reactive oxygen species, neutrophil extracellular traps (NETs), antimicrobial peptides, and proteolytic enzymes (Kurniawati et al., 2025). In early infection, neutrophils are the frontline of the innate immune system, and activated neutrophils provide signals that drive the activation and maturation of monocytes (Purbomartono et al., 2021). Neutrophils in fish play an important role in the innate immune system, especially by destroying foreign objects. Neutrophils are phagocytes that destroy pathogens through phagocytosis. They are involved in the

recognition and clearance of foreign entities, including pathogens and cellular debris, contributing to tissue integrity and homeostasis. Neutrophils play a role in inflammation and wound healing beyond pathogen destruction, producing bioactive molecules essential for cellular communication, activation, and resolution of inflammatory responses. They also counteract inflammation and can influence the adaptive immune response by presenting antigens to lymphocytes. Neutrophils exhibit a high degree of plasticity, allowing them to adapt to various environmental conditions. The phagocytic ability of fish to respond to bacterial infections is an important component of the innate immune response, involving various cell types, including neutrophils. These cells are responsible for engulfing and destroying pathogens, thus preventing the spread of infection (Kurniawan et al., 2025).

Hematocrit (Hct) is the percentage of red blood cells in a blood volume, or the ratio of red blood cell volume to total blood volume. Hct is used as a measure of the blood's capacity to carry oxygen. Theoretically, the higher the Hct, the greater the oxygen-carrying capacity (Ahmed et al., 2022). Based on our findings, the average hematocrit values obtained during the research ranged from 30.2-31.6% on day 1, 32-36% after administration of the immunostimulant octopus ink extract (day 21), and 27.6-33.8% seven days after *A. hydrophila* bacterial infection (day 28). Based on the results, the hematocrit values across the three observations remained within the normal range. In healthy catfish, the hematocrit value in the blood ranges from 30.8-45.5% (Yanuhar et al., 2021). Hct levels of the catfish decreased after infection (day 28) due to infection with *A. hydrophila*. If fish are sick or have a decreased appetite, their hematocrit will be abnormal, and if the hematocrit is low, their red blood cell count will also be low. Normal hematocrit levels in teleost fish range from 20-30%, whereas in marine fish they are around 42%. Fish with anemia have a hematocrit percentage of at least 10% (Yanuhar et al., 2021). Low hematocrit levels can affect fish immune systems (Rosidah et al., 2018). Fish infected with bacteria show a significant decrease in hematocrit levels, indicating erythropenia and hemolysis. These changes may be caused by inhibition of erythropoiesis and hemosynthesis, osmoregulatory dysfunction, or increased red blood cell destruction (Isla et al., 2022). A decrease in hematocrit is a sign of anemia in catfish (Docan et al., 2018). Anemia is one of the most common blood disorders. Symptoms of anemia can range from asymptomatic to weakness, shortness of breath, fatigue, and, in the most severe cases, organ damage and heart failure that can lead to death (Neves et al., 2016).

The hematocrit values of catfish after infection in treatments 2, 3, and 4 were higher than the control and within the optimal range. This is thought to be due to bioactive compounds in octopus ink extract that can improve catfish immunity. Octopus ink contains alkaloids that can act as antimicrobials, antioxidants, anticancer agents, anti-inflammatory agents, and antivirals. Alkaloids can increase nonspecific immune activity and are used as immunostimulants due to their role in preventing disease (Affandi et al., 2025). High hematocrit values in catfish fed octopus ink extract and infected with *A. hydrophila* were still within normal conditions. Feeding with octopus ink extract can increase the body's immune system because octopus ink extract contains antioxidants that can improve cell structure in the blood. Alkaloids act as antioxidants in neutralizing free radicals and play a role in the process of repairing blood cell structure (Wahyono et al., 2023).

Survival rate is defined as the number of live fish divided by the number of fish stocked during the research process (Hidayatullah et al., 2025). Based on our results, the average survival rate ranged from 73.3 to 88.3% after 28 days of rearing. Fish survival rates were classified as good if the value was >50%, moderate if the survival rate was between 30 and 50%, and poor if it was less than 30% (Latifah et al., 2022). The survival rate of catfish treated with octopus ink extract was higher than that of the control. This is due to bioactive compounds in octopus ink extract, which can enhance catfish immunity. Octopus ink contains alkaloids, and administration of alkaloids in feed significantly increased fish survival after exposure to

bacteria. These results indicate that alkaloids enhance fish immunity and exhibit positive effects against bacterial infections. Alkaloids improve growth parameters, amino acid and lipid deposition, and enhance the organism's immunity and resistance to disease (Ye et al., 2019; Affandi et al., 2025).

Conclusion

In conclusion, the addition of octopus ink extract to the feed significantly improves the immune system, as evidenced by hematological parameters (erythrocytes, leukocytes, leukocyte differentials, and hematocrit) and the survival rate in catfish infected with *A. hydrophila*. The best results were obtained in treatment P4 (150 ml/kg), which produced erythrocyte values of $2.4x10^6$ cells/mm^3, leukocytes $5.78x10^4$ cells/mm^3, lymphocytes 89%, monocytes 5.7%, neutrophils 5.3%, hematocrit 33.8%, and a survival rate of 88.3% in catfish infected with *A. hydrophila*.

Acknowledgments

The authors would like to thank the Institute for Research and Community Service, University of Mataram, for funding this research under the Beginner Lecturer Research Scheme and grant number 3724/UN18.L1/PP/2025. Special thanks are due to all parties who have contributed to this research activity. Thanks are also extended to the Aquaculture Study Program at the University of Mataram for its cooperation and participation in developing sustainable aquaculture.

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