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Immunostimulant effect of *Chaetomorpha* sp in Tilapia infected with *Aeromonas hydrophila*

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ABSTRACT

Chaetomorpha sp is a type of green marcoalgae that is rich in bioactive compound that function as antibacterial, anti-inflammatory and can increase fish immunity. This study aims to determine the immunostimulating effect of Chaetomorpha sp macroalgae extract on the leukocyte profile of Tilapia infected with A. hydrophila. The experimental method used a completely randomized design (CRD) with four extract dose treatments (0, 25, 50, 75 mL/kg feed) and three replicates. The test fish used were 2.63 ± 0.26g, reared in black tanks with a size of 60x30x30 cm, a volume of 80 L with a stocking density of 1 fish/4 L, and a recirculation system. Maintenance was carried out for 60 days with a frequency of feeding three times a day, namely 08.00 AM, 01.00 PM, and 05.00 PM, as much as 5% of body weight. The parameters observed were total leukocytes, leukocyte differentiation, and phagocytosis index. The results showed that the administration of Chaetomorpha sp extract significantly increased total leukocytes, lymphocyte differentiation, and phagocytosis index ($p<0.05$). The dose of 50 mL/kg feed is the optimal dose that affects the leukocyte profile of Tilapia. T0 (without extract) experienced 100% mortality, while the treatment group showed increased resistance to infection. Bioactive compounds such as sulfated polysaccharides and flavonoids in Chaetomorpha sp are thought to play a role in stimulating the innate immune system. This study highlights the potential of Chaetomorpha sp as an alternative to antibiotics in sustainable tilapia aquaculture.

Introduction

Tilapia (*Oreochromis niloticus*) is a highly profitable species due to its adaptability, high economic value, and nutritional benefits. Tilapia can adapt to various environmental conditions, including various salinity levels and low oxygen environments, making it suitable for diverse aquaculture systems, from extensive to intensive settings (Ashouri *et al.*, 2023; Kiener, 2022). Tilapia aquaculture faces significant challenges due to infection by *Aeromonas hydrophila*, causing *Motile Aeromonas Septicemia* (MAS) disease. The disease is characterized by symptoms such as hemorrhage, ulceration (Laltlanmawia *et al.*, 2023), necrosis, degeneration in internal organs such as liver and kidney (Mohamad *et al.*, 2022), and death in fish (Austin and Austin, 2016). According to Al-Shammari *et al.* (2024), *A. hydrophila* can cause up to 80% of fish mortality seven days post-infection. This infection significantly impacts the aquaculture industry as it reduces productivity and causes economic losses. Using synthetic antibiotics to control infections often leads to bacterial resistance and harmful environmental residues, so safer and more sustainable alternative treatments are needed, such as immunostimulants from macroalgae.

Chaetomorpha sp is a type of green macroalgae (*Chlorophyta*) that is rich in bioactive compounds such as polysaccharides (including sulfated polysaccharides), flavonoids, alkaloids, and pigments that have immunomodulatory properties. These

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compounds stimulate the non-specific immune system in fish, including increasing leukocyte proliferation and activity (Sutili *et al.*, 2018). Leukocytes, such as lymphocytes, monocytes, and neutrophils, play a key role in fish body defense against bacterial infections. Optimal leukocyte differentiation can enhance phagocytosis ability and antibody production, thereby accelerating the recovery of infected fish.

According to Yengkhom *et al.* (2018), the administration of *Caulerpa scalpelliformis* increased leukocyte proliferation and the non-specific immune response of Tilapia after infection with *A. hydrophila* bacteria. In addition, the application of *Chaetomorpha* sp as an additive to feed was able to improve the immune response of fish, including catfish (*Clarias batrachus*) infected with *A. hydrophila* (Sattanathan *et al.*, 2024b), and tilapia infected with *Edwardsiella tarda* (Sattanathan *et al.*, 2024a). This suggests the activation of the innate immune system. These findings indicate that algal bioactive compounds, such as sulfated polysaccharides and fucoidan, can activate the innate immune system by stimulating phagocytic cells and antibody production (Assef *et al.*, 2023). However, data regarding the effects of *Chaetomorpha* on the leukocyte profile of Tilapia infected with *A. hydrophila* are still very limited. Therefore, this study aimed to determine the effect of *Chaetomorpha* extract on leukocyte differentiation of Tilapia infected with *A. hydrophila.*

Materials and Methods

Time and place

This research was conducted from August to December 2023 at the Marine Microbiology Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Riau.

Method

The method used is experimental by applying a complete randomized design (CRD) with four treatments and three replicates. The treatment dose refers to Wahyuni *et al.* (2024), namely:

T0 : Commercial pellets
T1 : *Chaetomorpha* sp extract dose of 25 mL/kg feed
T2 : *Chaetomorpha* sp extract dose of 50 mL/kg feed
T3 : *Chaetomorpha* sp extract dose of 75 mL/kg feed

Preparation of *Chaemtomorpha* sp Extract and Feed

Chaetomorpha sp macroalgae was obtained from Lhok Bubon Beach, Samatiga District, West Aceh Regency. A total of 20 g samples were washed with running water to remove impurities, then extracted using warm water (60°C) in a ratio of 1:5 (b/v) for 30 minutes. The extract obtained (brown in color) was filtered using filter paper and then used for further analysis according to the treatment.

The commercial pellets were FF-999 (PT. Central Proteina Prima Tbk, Deli Serdang, Indonesia). Commercial pellets were weighed as much as 1 kg for each treatment, and extract solution was provided using the spray method, according to the treatment of 25 mL, 50 mL, and 75 mL/kg feed. Then, the feed was dried and ready for use.

Fish Adaptation and Maintenance

Tilapia were sourced from farmers in Kampar Regency, Riau, Indonesia. The test fish weighed 2.63±0.26g and 5.87±0.07 cm in length. Before use, the fish were adapted for seven days in 2x1 m fiber tanks^2 with water quality parameters, such as temperature 28-30°C, pH 5.5-6.8, and DO 4-5 mg/L. In the rearing stage, fish were reared in black tanks with a size of 60x30x30 cm, a volume of 80 L with a stocking density of 1 fish/4 L, and provided with a water filter for water recirculation. Fish were randomly selected with clinical symptoms of agile movement, complete fins, and bright colors. Maintenance was carried out for 60 days with a frequency of feeding three times a day, namely 08.00 AM, 01.00 PM, and 05.00 PM, as much as 5% of body weight (Wahyuni *et al.*, 2024).

Challenge test with *A. hydrophila.*

The challenge test was carried out after 60 days of rearing. The bacteria used were obtained from the collection of the Marine Microbiology Laboratory, Faculty of Fisheries and Marine Sciences, Universitas Riau. The bacterial density used was 10^8 CFU/mL, which infected the fish via an intramuscular injection of 0.1 mL. Fish were reared after the challenge test for 14 days, and the clinical symptoms and mortality rate were observed.

Collecting fish blood

Fish blood samples were taken 3 times, namely at the beginning (Day 1), day 60 (Day 60), and after the challenge test (Day 75). Blood collection is carried out by anesthetizing the fish first using cold temperatures (±8°C) (Witeska *et al.*, 2022; Effendi *et al.*, 2025). After that, blood was drawn from the caudal vein using a syringe (1 mL). Next, the blood was stored in an Eppendorf tube to observe the total leukocytes, leukocyte differentiation, and phagocytosis index.

Then, fish that have been blooded are transferred to a container that has been given an aerator until they recover and move actively, and then transferred back to the research container.

Total leukocyte observation

The procedure for calculating total leukocytes is by sucking blood samples from microtubes using a leukocyte pipette up to a scale of 0.5 and adding Turk's solution to line 11, after which it is homogenized by shaking the leukocyte pipette to form a figure eight for five minutes. After homogenization, two drops of blood were removed to remove air, and then the blood was dripped on the hemocytometer box and covered with cover glass. Furthermore, it was observed under a microscope with a magnification of 10x40. The total number of leukocytes was counted using a microscope on four large hemocytometer boxes with the following formula (Anderson and Siwicki, 1995):

$\sum$leukocytes =$\sum$ total number of luekocytes x 50 cells/mm^3

Leukocyte Differentiation

Calculation of leukocyte types based on the method of Blaxhall and Daisley (1973), namely by taking fish blood, then making blood screw preparations on a glass object and then dried, then fixed with 95% methanol solution, after which it was rinsed with distilled water and then dried, and stained using 50% Giemsa for 2 minutes. After that, it was washed with running water and dried, then observed under a microscope with a magnification of 1000X. The types of leukocytes observed were lymphocytes, monocytes, neutrophils, and platelets. Then counted until a total of 100 cells and calculated with the following formula:

Cell % = $\sum$ number of cells counted x 100%

Data analysis

Data from growth rate and hematological measurements were collected and tabulated in Table. Next, it was analyzed statistically using the SPSS version 26 application and One-Way ANOVA. If the analysis results showed an influence, a further test was carried out using Student Newman Keuls (SNK).

Results

Molecular characteristics

The results showed that the provision of *Chaemtomorpha* sp extract in feed gives an effect between treatments ($p<0.05$) on tilapia leukocyte profile. More details can be seen in Table 1.

Table 1. Leukocyte profile of Tilapia (*O.niloticus*)

Parameters	Treatment (mL/kg feed)			
	T0 (control)	T1 (25)	T2 (50)	T3 (75)
60 days				
Total leukocytes (x10^4 cells/mm^3)	3.63±0.06^a	3.77±0.03^b	3.93±0.02^c	3.83±0.01^b
Lymphocytes (%)	76.67±0.58^a	78.67±0.58^b	81.00±1.00^c	78.33±0.58^b
Monocytes (%)	7.67±0.58	7.00±1.00	6.00±1.00	6.67±0.58
Neutrophil (%)	7.33±0.58	6.67±0.58	6.33±0.58	7.00±1.00
Platelets (%)	8.33±0.58	7.67±0.58	7.00±1.00	8.00±1.00
Phagocytosis index (%)	21.67±1.15^a	25.67±0.58^{bc}	26.67±0.58^c	24.33±0.58^b
Post challenge test				
Total leukocytes (x10^4 cells/mm^3)	-	3.81±0.02^a	4.10±0.02^c	3.86±0.02^b
Lymphocytes (%)	-	78.33±0.58^a	79.67±0.58^b	77.67±0.58^a
Monocytes (%)	-	7.67±0.58	7.33±0.58	7.67±0.58
Neutrophil (%)	-	7.67±0.58	6.33±0.58	5.33±1.15
Platelets (%)	-	6.33±0.58	6.67±0.58	7.33±1.53
Phagocytosis index (%)	-	28.00±0.00^a	30.33±0.58^b	27.00±1.00^a

Description: Superscript on the same line indicates there is an influence between treats ($P<0.05$)

Table 1 shows an increase in total leukocytes and phagocytosis index in Tilapia, given the addition of extracts to post-test feed with *A. hydrophila* bacteria. While in the control treatment, no observation could be made because mortality reached 100% post-test. Fish mortality during 60 days of rearing ranges from 80-92%. Mortality occurs at the beginning of maintenance, and this is suspected to be caused by fish not yet trying to adapt to the new environment (Figure 1).

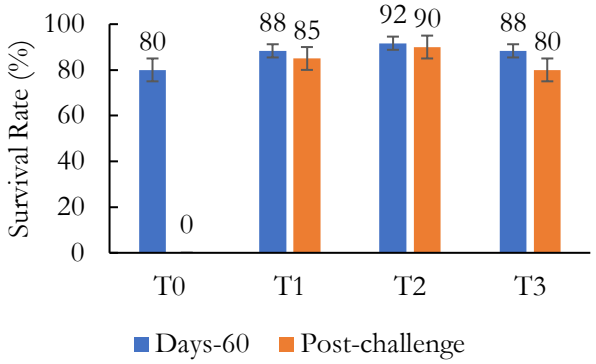

Figure 1. Survival rate of Tilapia

Discussion

The addition of *Chaemtomorpha* sp extract to the feed gave fluctuations in the total leukocyte values in each treatment, which ranged from 2.77-3.93 x10^4 cells/mm^3 higher than the control treatment (T0), which was 3.63 x10^4 cells/mm^3. This indicates that the administration of macroalgae extract can increase Tilapia's immunity. The increase in fish immunity can be seen from the number of leucocyte cells in fish and is also characterized by an increase in the number of lymphocyte cells, where lymphocyte cells function in the formation of antibodies (Kurniawan *et al.*, 2020). After the challenge test, the total fish leukocytes increased, ranging from 3.81-4.10 x10^4 cells/mm^3. Meanwhile, at T0, no observations could be made because mortality reached 100%. The range of total tilapia leukocytes during maintenance and post-challenge testing is still in the normal range. The normal range of total tilapia leukocytes is≥ 2.43 x10^4 cells/mm^3 (Yoswaty *et al.*, 2025) and≤ 4.51x10^4 cells/mm^3 (Gunanti *et al.*, 2019); this indicates that the provision of macroalgae into the feed can increase the immune response of fish.

Giving *Chaemtomorpha* sp extract as much as 20 mL/kg feed gave the highest total leukocyte results of Tilapia infected with *A. hydrophila* bacteria. This indicates that this dose provides the best increase in immunity in Tilapia compared to other treatments, including the control treatment, which reached 100% mortality.

Fluctuations in total leukocytes can be influenced by stress factors, age, weight, physiological activity, and the addition of immunostimulants (Kurniawan *et al.*, 2020). Sattanathan *et al.* (2024a) stated that supplementing 5 g/kg green algae in the diet increased hematological parameters, including increased leukocyte counts, contributing to a strong immune response and higher survival rates after bacterial challenge. This enhanced immunity is thought to be due to secondary metabolite compounds such as halogenated alkanes, alkenes, hydroquinones, polyphenols, oligomeric phlorotannins (Sattanathan *et al.*, 2024b), terpenoids (Tziveleka *et al.*, 2021), and flavonoids (Kamat *et al.*, 2020) which have been identified as potent antimicrobial agents, antioxidants, and anti-inflammatories.

An increase in lymphocytes also indicates an increase in the immune response of Tilapia. The dose of 20 mL/kg feed gives the highest value, which is 81%, and is effective in stimulating the formation of lymphocytes in fish blood. After the challenge test with *A. hydrophila* bacteria, lymphocyte levels decreased to 79.67% as an action to defend the body from bacterial infection. According to Firly *et al.* (2015), Lymphocytes function to provide immune substances or defense systems from the attack of foreign objects that enter the body. The number of lymphocytes will decrease if there has been an infection from microbes because most of the lymphocytes move from blood circulation and compete in body tissues where there is inflammation.

After the challenge test, the number of monocytes and neutrophils in Tilapia increased. This is an attempt by the fish body to consume antigens that enter the fish body, which can also be seen in the increase in the phagocytosis index value after being infected with *A. hydropila*. Neutrophils and monocytes in fish play an important role in the innate immune system, especially by destroying foreign bodies (Riswan *et al.*, 2021; Buchmann, 2022). According to Haugland *et al.* (2012), neutrophils and monocytes are professional phagocytes responsible for the consumption and destruction of 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 (Birkert, 2023). Speirs *et al.* (2024) State's that fish's innate immune response is sophisticated, with neutrophils and macrophages playing important roles in pathogen recognition, destruction, and initiation of adaptive immune responses.

Furthermore, the platelet count of post-challenge Tilapia also fluctuates. This is also related to the function of monocytes and neutrophils in inflammation and wound healing. According to Speirs *et al.* (2024), neutrophils and monocytes play a role in inflammation and wound healing beyond pathogen destruction, producing bioactive molecules critical for cellular communication, activation, and resolution of the inflammatory response. They resolve inflammation and can influence the adaptive immune response by presenting antigens to lymphocytes (Birket, 2023). Neutrophils and monocytes exhibit a high degree of plasticity, allowing them to adapt to various immune challenges

and environmental conditions (Havixbeck and Barreda, 2015).

The phagocytic ability of fish against bacterial infections is an important component of their innate immune response, which involves various cell types such as macrophages and neutrophils. These cells are responsible for engulfing and destroying pathogens, thus preventing the spread of infection. Phagocytosis efficiency in fish can be affected by several factors, including vaccines, metabolic changes, and probiotics. The secondary metabolite compound content of *Chaemtomorpha* sp extract can stimulate and enhance phagocytic performance, suggesting a beneficial role in inhibiting bacterial infection. Polysaccharide compounds can modulate immune functions such as phagocytosis to combat pathogens (Kamarul-Haniya *et al*., 2015). According to Aluta *et al*. (2022), Polysaccharides from *C. antennina* have been found to stimulate phagocytic activity in fish leukocytes and increase antioxidant activity, which helps maintain cell health during the immune response.

Conclusions

Chaetomorpha sp extract can increase Tilapia's immune response, as indicated by an increase in total leukocytes, lymphocytes, and phagocytosis activity, especially at a 50 mL/kg feed dose.

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