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Effect of Whole Protein Spore *Myxobolus koi* by Oral Treatment on Non-Specific Immune Response of Punten Carp (*Cyprinus carpio* L.) Infested with *Myxobolus koi*

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Abstract

Increased intensive cultivation of Punten Carp (*Cyprinus carpio* L.) is quite profitable, but on the other hand, it can result in a decrease in water quality due to uncontrolled leftover feed. Poor water quality can cause fish to become stressed and then susceptible to disease, one of which is *Myxobolus koi* parasite infestation. The purpose of this study was to determine the effect of immunostimulant treatment of whole protein *M. koi* spores on the blood profile of *Myxobolus*-infested punten carp. The method used was an experiment with two treatments, the treatment without *M. koi* spore whole protein (P0) and the treatment of commercial feed added with an immunostimulant from *M. koi* spore whole protein of 5 μg/kg feed (P1). The study was conducted for 28 days and observed on days 0, 7, 14, 21, and 28, including the calculation of the number of erythrocytes, leukocytes, leukocyte differential, and water quality as supporting data. The abundance of erythrocytes in fish treated with P1 ($1.16x10^6$ cells/mm^3-$2.44x10^6$ cells/mm^3) was higher than in fish treated with P0 ($1.09x10^6$ cells/mm^3-$1.55x10^6$ cells/mm^3). The abundance of leukocytes in fish treated with P1 ($3.79x10^4$ cells/mm^3 - $11.31x10^4$ cells/mm^3) was higher than in fish treated with P0 ($3.51x10^4$ cells/mm^3 - $6.58x10^4$ cells/mm^3). The results of differential observations of leukocytes in the P0 treatment found basophils (0.3-1.3%), neutrophils (12.3-21.5%), eosinophils (2.5-4.9%), lymphocytes (61.9-76.8%) and monocytes (3.8-6.3%). The results of differential observations of leukocytes in the P1 treatment found basophils (0.3-1.0%), neutrophils (10.7-19.9%), eosinophils (2.4-3.9%), lymphocytes (61.8-72.6%) and monocytes (3.9-5.4%).

INTRODUCTION

Carp (*Cyprinus carpio* L.) is a freshwater fish commodity that has the potential to develop and is widely consumed by the public. There are several strains of Carp in Indonesia, namely Sinyonya,

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Punten, Kumpay, Majalaya, Kancra Domas, Taiwan, and Merah (Supriatna, 2013). One of the seven stains in Indonesia is the Punten Carp strain (Susilo *et al*., 2018). The Punten Carp was first developed in 1933 in the village of Punten, Malang, East Java. Punten Carp are selected carp in Indonesia (Syafar *et al*., 2017). The Punten Carp has dark green scales; the shortest body cut; a flared high back; and slightly protruding eyes; the movements are agile; the ratio between body length and height is between 2.3:1 (KPPKP, 2011).

Carp production continues to increase by 8.92% annually from 2015 to 2019. Carp production in 2019 reached 785,800 tons (Directorate General of Aquaculture, 2015). The increase in carp cultivation is accompanied by dense aquaculture production; several aspects must be considered, including water quality (Sarimudin *et al*., 2016). The main problem with the production of carp aquaculture is poor water quality, such as high organic matter, unstable pH, and limited dissolved oxygen (Ojwala *et al*., 2018). Poor water quality can cause fish to become stressed and then susceptible to disease (Diansari *et al*., 2013; Yustiati *et al*., 2017).

Fish disease can be caused by interactions between pathogens, hosts, and environmental problems (Al Hasyimia *et al*., 2016). These interactions can cause stress to fish, then weaken their body's defense mechanisms. Generally, the diseases that are often found attacking Carp can be caused by parasites, bacteria, viruses, and fungi. One of the pathogens that can attack Carp fry is *M. koi*. This disease is usually found in Cyprinid fish seeds and can cause economic losses with a fish mortality rate of up to 100% (Mahasri *et al*., 2015). Clinical symptoms of carp infested with *Myxobolus* include swelling of the gills, which is characterized by reddish-white nodules on carp (*C. carpio*), which causes difficulty in breathing (Yanuhar *et al*., 2020). One of the prevention efforts to reduce mortality in aquaculture products is immunostimulant treatment. Immunostimulant is substances that can increase host resistance to pathogens by modulating non-specific defense mechanisms (Mastan, 2015). Immunostimulant applications have also been widely applied to various fish species by soaking, injection, or orally through food (Roza, 2017). One of the ingredients that have the potential as an immunostimulant for carp is whole protein spores from *M. koi*, which can be given through feed (Mahasri, 2017).

Immunogens are substances capable of stimulating an immune response or reacting with existing antibodies, regardless of their ability to stimulate antibody production (Syafar *et al*., 2017). Insariani *et al*. (2012) have succeeded in isolating, identifying, and characterizing the upper glycoprotein of *M. koi* as an antigen that is immunogenic for the production of antibodies. Based on research by Yusuf (2016), the proteins found have a high molecular weight. Most of the good immunogens have a molecular size of <100 kDa, while proteins with a molecular size of <5 to 10 kDa are weak immunogens (Mayer *et al*., 2011). Saad *et al*. (2017) stated that whole protein from *M. koi* spores can be used as an immunostimulant, as shown by the results of the highest IgM concentration on the seventh day of the study in the treatment of feed with immunostimulants of 1463.566 µg/ml. *M. koi* spore protein has also been reported to increase the immune response and survival of Koi fish from 10% to 86% and can increase resistance to *M. koi* infestations (Mahasri, 2017).

In fish, blood has an important physiological role. The use of the hematological method is quite effective for diagnosing fish diseases early on by analyzing blood parameter values such as the abundance of erythrocytes, leukocytes, and differential blood count (Yanto *et al*., 2015).

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Hematological abnormalities and fish immune responses describe changes in fish health from normal to abnormal. Changes in the amount of blood can determine the health condition of the fish (Zainun, 2017). When blood profile values are within the normal range, this indicates that giving immunostimulants as a treatment does not interfere with fish health but is thought to improve the health status of Carp (Maryani and Rosdiana, 2020). Besides being able to enhance the non-specific immune system, the addition of immunostimulants can also increase the growth rate of fish (Lu *et al*., 2019). When the immune system increases, fish are not susceptible to stress and are more resistant to disease, as a result, the energy formed from metabolic output can be used optimally in the growth rate (Rodnick and Planas, 2016).

Based on this background, research is needed to be related to non-specific immune response, parasite infestation, and growth of Punten carp to determine the potential of whole protein *M. koi* spores as an immunostimulant ingredient.

METHODOLOGY

Ethical Approval

This research has followed international principles regarding the use and care of laboratory test animals considerations from the American Fisheries Society (AFS, 2014) and the Canadian Council on Animal Care (CCAC, 2005) so this research attaches a statement letter with number 2030/UN.3.1.12/KP/2022 as a substitute Ethical Approval.

Place and Time

This research was conducted from December 2021 to January 2023. Carp-rearing activities were carried out at the Punten Batu Freshwater Cultivation Installation. Analysis of water quality and hematology of punten carp was carried out at the Laboratory of the Faculty of Marine Sciences and Fisheries, Universitas Brawijaya.

Research Materials

The tools used in fish rearing are nets, scoop fish, and digital scales (CAMRY ACS-30-JC33, Indonesia). Measuring water quality for pH using a pH pen (Bluelab, New Zealand), temperature using a thermometer, and dissolved oxygen with a DO meter (Lutron DO-5510, Taiwan). Hematology parameter measurement tools use a light microscope (Olympus CX23, Japan), syringe (Terumo, Philippines), hemocytometer (Sigma-Aldrich, America), Eppendorf tube (Thermo Fisher Scientific, America), glass object (Onemed, Indonesia), cover glass (Onemed, Indonesia), micropipette (Thermo Fisher Scientific, America), tubes (MonotaRO Co., Ltd., Japan), microtube (MonotaRO Co., Ltd., Japan), centrifuge (Thermo Fisher Scientific, America). The materials used are carp punten, water sample for NH3, NO3, and NO2 analysis, methanol solution, methylene blue, distilled water, Heyem's solution as a diluent for counting erythrocytes with a hemocytometer, and Turk's solution for leukocytes calculation.

Research Design

The fish used in this research were Punten Carp with a size of 10-12 cm (1000 fish) from the Punten Freshwater Cultivation Installation. There were two pond treatments, fish ponds without *Myxobolus* spore whole protein and ponds with *Myxobolus* whole protein spore treatment. In each pond, there are five units of net cages were installed, each unit filled with 100 fish.

In this study, there were two treatment factors, The first factor A treatment (P0) feeding without whole protein *M. koi* spores; The second factor A (P1) treatment with commercial feed was added with whole protein of *M. koi* spore 5 μg/kg feed. Sampling was carried out 5 times (within 28 days). Thus, factor B is the treatment of sampling time, namely (B0) 0th day, (B1) 7th day, (B2) 14th day, (B3) 21st day, and (B4) 28th day.

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Work Procedure

Production of Whole Protein *Myxobolus koi* Spores

Production of whole protein *M. koi* spores based on Mahasri (2017). PBS solution was added sufficiently to M. koi spores, and then centrifuged (5000 rpm, 10 minutes). The pellet was added 500 μl of lysis buffer and then sonicated on ice (1-minute sonication, 30 seconds rest) 10 times. Then mixed (30 seconds vortex, 1-minute rest) on ice 15 times. The vortex results were centrifuged (12000 rpm, 5 minutes). The supernatant formed was collected, and then SDS-PAGE analysis was carried out. The concentration of all *Myxobolus* spore proteins was determined using the Bio-Rad Protein Assay and read using a UV-visible spectrophotometer with a wavelength of 600 nm. Furthermore, *M. koi* spores can be stored in the refrigerator at a temperature of 2-4 ^{o}C (Mahasri *et al*., 2019).

Production of Treatment Feed

The feed used is artificial feed in the form of pellets. Production of feed by mixing whole protein *M. koi* spores in commercial feed. Take the whole protein of *M. koi* spores using a 1-10 μL micropipette as much as the calculated volume with a dose of 5 μg/kg of feed. The mixture of whole protein *M. koi* spore solution was transferred to a spray bottle, and then its application to commercial feed was carried out by spraying according to a predetermined dose and air drying. After that, the dry-treated feed is packed using plastic clips. Treatment feed that is ready to be given to fish.

Fish Treatments

Treatment includes the first pond (P0) as a treatment of 100% commercial feed (without giving whole protein *M. koi* spores). In the second pond (P1), the treatment of feed with an immunostimulant from whole protein *M. koi* spores of 5 μg/kg of feed. Sampling was carried out 5 times (day-0, 7, 14, 21, and 28), and each sampling in each treatment was 50 individuals.

Calculation of the Number of Erythrocytes and Leukocytes

The procedure for calculating the number of erythrocytes was measured according to Blaxhall and Daisley (1973). Blood is taken by injection. The blood sample was sucked with a pipette up to 0.5, then Heyem's solution was added to a scale of 11, while Turk's solution was added to leukocyte observations. The first two drops of blood solution in the pipette are discarded, then drip on the hemocytometer, and covered with a covered glass. Then count the number of red blood cells and white blood cells with a microscope with 400x magnification. The total number of erythrocytes and leukocytes was counted in as many as 4 small squares.

Leukocyte Differential Calculation

Leukocyte differential counting procedure according to Blaxhall dan Daisley (1973). Blood is dripped on the object glass, and placed the object glass is to the left of the blood drop by forming an angle of 300. Pull the object glass to the right until it touches the blood, after the blood has spread along the edge of the cover glass, push it to the left while still forming an angle of 300. After that, the review is dried and given with Giemsa stain. Fixation in methanol solution (5 minutes), and soak the smear in Giemsa solution (20 minutes). Then rinse under running water and dry.

Water Quality

Supporting data in this study are water quality including temperature, dissolved oxygen, ammonia, pH, nitrate, and nitrite. Temperature and dissolved oxygen were measured using a DO meter (Putriana *et al.*, 2015), ammonia, nitrate, and nitrite were measured in a laboratory (Azizah and Humairoh, 2015), nitrate (NO_3^-) using the phenol disulfonic acid spectral light degree method. Nitrite (NO_2^-) using

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naphthalene ethylene-diamine Spectrophotometry. Ammonia Nitrogen (NH_3) was measured by Nessler's reagent colorimetric method (Devi *et al*., 2015), and pH was measured using a pH meter (Aprilliyanti *et al*., 2015).

Data Analysis

Descriptive data analysis was used in fish blood profile parameters (Erythrocyte and Leukocyte Count, Leukocyte Differential). The abundance of erythrocytes and leukocytes was analyzed using the Two-Way ANOVA to determine the effect of the immunostimulant treatment given on days 0, 7, 14, 21, and 28. Data analysis used SPSS ver 2.4 software.

RESULTS AND DISCUSSION

Erythrocytes Abundance of Punten Carp

The results of erythrocyte abundance can be presented in Table 1. It was found that the abundance of erythrocytes in fish treated with P1 (1.16 x 10^6 cells/mm^3 - 2.44 x 10^6 cells/mm^3) was higher than those treated with P0 (1.09 x 10^6 cells/mm^3 - 1.55 x 10^6 cells/ mm^3). The normal number of erythrocytes in Carp ranges from 1.52-2.87 x 10^6 cells/ mm^3 (Yanto *et al*., 2015). When the erythrocyte value is within the normal range, this indicates that giving immunostimulants as a treatment does not interfere with fish health but is thought to improve the health status of Carp (Maryani and Rosdiana, 2020).

In this study, the number of erythrocytes was normal, especially on the 14^{th} to 28^{th} day of P1 treatment. This is in line with the research by Suryadi *et al*. (2021) that the number of erythrocytes of koi and tilapia seeds on the 10^{th} day after the challenge test began to recover. The fish body began to produce more erythrocytes to replace their previous cells that had been lysed by the hemolysin enzyme.

Table 1. Total erythrocytes of Punten Carp in each treatment.

Treatments	Total Erythrocytes (x 10^6 cells/mm^3)				
	B-0	B-7	B-14	B-21	B-28
Fish without Whole protein treatment (P0)	$1.09^{a}\pm0.16$	$1.2^{a}\pm0.09$	$1.37^{b}\pm0.10$	$1.47^{bc}\pm0.10$	$1.55^{c}\pm0.12$
Fish with Whole protein treatment (P1)	$1.16^{a}\pm0.09$	$1.35^{b}\pm0.13$	$2.03^{d}\pm0.15$	$2.31^{e}\pm0.19$	$2.44^{f}\pm0.13$

The results of the Two-Way ANOVA test show that the significance value is <0.05, meaning that the whole protein treatment on days 0, 7, 14, 21, and 28 significantly affects the abundance of erythrocytes. There is an interaction between treatment and days of observation.

Leukocytes Abundance of Punten Carp

The results of leukocyte abundance can be presented in Table 2. It was found that the abundance of leukocytes in fish treated with P1 (3.79 x 10^4 cells/mm^3 - 11.31 x 10^4 cells/mm^3) was higher than those treated with P0 (3.51 x 10^4 cells/ mm^3 - 6.58 x 10^4 cells/mm^3) The normal leukocyte count in carp according to research by Yustiati *et al*. (2020) ranges from 2-15 × 10^4 cells/mm^3 and the average leukocyte count of the healthy population was 12.55 × 10^4 cells/mm^3.

This indicated that the leukocytes of the punten carp were quite high in each treatment, which could be due to the response to antigens that entered the body. The increase in white blood cells is a reflection of the success of the fish's immune system. in developing a cellular (non-specific) immune response as a trigger for an immune response (Bhuvaneswari *et al*., 2018). It can be shown that the highest leukocyte abundance is best to respond to the treatment of *Myxobolus* whole protein immunostimulant.

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Table 2. Total leukocytes of Punten Carp in each treatment.

Treatments	Total Leukocytes (x 10^4 cells/mm^3)				
	B-0	B-7	B-14	B-21	B-28
Fish without Whole protein treatment (P0)	$3.51^{a}\pm0.56$	$4.60^{b}\pm0.70$	$6.07^{c}\pm1.29$	$6.21^{c}\pm0.71$	$6.58^{c}\pm0.74$
Fish with Whole protein treatment (P1)	$3.79^{a}\pm0.47$	$6.69^{c}\pm0.71$	$10.14^{d}\pm0.80$	$11.31^{e}\pm1.15$	$10.85^{de}\pm0.97$

The results of the Two-Way ANOVA test show that the significance value is <0.05, meaning that the whole protein treatment on days 0, 7, 14, 21, and 28 significantly affects the abundance of leukocytes. There is an interaction between treatment and days of observation.

Leukocyte Differential Percentage

Basophils

The results of differential leukocyte observations in treatment P0 found basophils (0.3-1.3%) and in P1 (0.3-1.0%) (Figure 1). Basophils at (P1) were highest on day 14 (1.0%) and started to decrease until day 28.

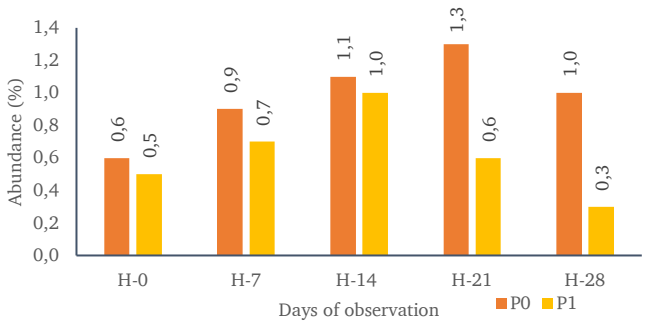

Figure 1. Percentage of basophils in carp infested with *Myxobolus* for 28 days with immunostimulant treatment (P1) and without immunostimulant treatment (P0).

Generally, basophils are rarely seen in normal fish blood circulation. Thus, the P1 fish group based on the percentage of basophils showed conditions that were close to normal. The normal range of basophil counts in goldfish (*C. carpio*) is reported to be 0-2% of the total leukocyte count (Kocan *et al.*, 2008). Basophils secrete histamine, which triggers an increase in inflammation, and heparin (a natural anticoagulant) prevents the formation of unnecessary blood clots (Aspinall and Cappello, 2015).

The results of the Two-Way ANOVA test show that the significance value is <0.05, meaning that the whole protein treatment on days 0, 7, 14, 21, and 28 significantly affects the percentage of basophils. There is an interaction between treatment and days of observation.

Neutrophils

Neutrophil results can be seen in Figure 2, P0 (12.3-21.5%) and P1 (10.7-19.9%).

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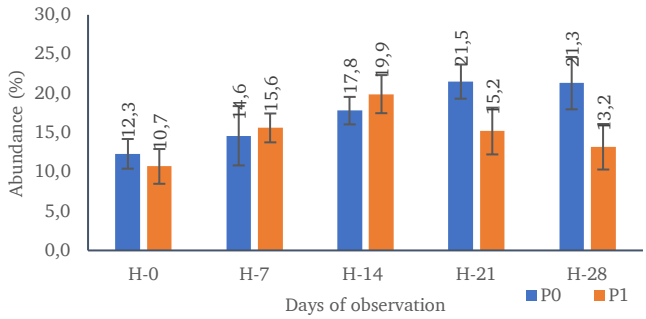

Figure 2. Percentage of neutrophils in Carp infested with *Myxobolus* for 28 days with immunostimulant treatment (P1) and without immunostimulant treatment (P0).

The normal range for several hematological parameters, including the percentage of neutrophils, in carp fish is 10–18.1% (Hardi, 2018). Neutrophils (P1) were highest on day 14 (19.9%) and decreased until day 28 (13.2%). The decrease in the percentage of neutrophils in the P1 treatment from the 14th to the 28th day indicated that there was a response to the treatment of the *Myxobolus* spore whole protein immunostimulant in Carp. A low decrease in the percentage of neutrophils indicates that there is no attack by microorganisms so neutrophils are not produced much by the fish's body (Hartika *et al*., 2014). This refers to the main function of neutrophils, namely destroying foreign substances through phagocytosis (Kurniawan *et al*., 2022).

The results of the Two-Way ANOVA test show that the significance value is <0.05, meaning that the whole protein treatment on days 0, 7, 14, 21, and 28 significantly affects the percentage of neutrophils. There is an interaction between treatment and days of observation.

Eosinophils

The results of differential leukocyte observations in treatment P0 found eosinophils (2.5-4.9%) and in P1 (2.4-3.9%) (Figure 3).

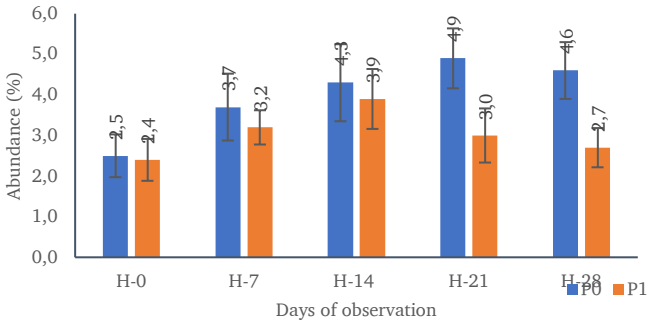

Figure 3. Percentage of eosinophils in Carp infested with *Myxobolus* for 28 days with immunostimulant treatment (P1) and without immunostimulant treatment (P0).

The research of Hrubec and Smith (2010), investigated the hematological parameters of carp fish (*C. carpio*) with normal eosinophil percentage values in the peripheral blood of healthy goldfish ranging from 2.4% to 8%. Eosinophils (P1) were highest on day 14 (3.9%). The increase in eosinophils on the 14th day of P1 treatment indicated that there was a response to the presence of antigens in the body. Then eosinophils decreased until the 28th day indicating infestation or the incidence of infection began to decrease. Eosinophils function against parasites that are often ingested or invade through the

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skin and move to the intestinal or respiratory mucosa. Eosinophils surround the parasite and release digestive enzymes onto the surface of the parasite (Akers and Denbow, 2008).

The results of the Two-Way ANOVA test show that the significance value is <0.05, meaning that the whole protein treatment on days 0, 7, 14, 21, and 28 significantly affects the percentage of eosinophils. There is an interaction between treatment and days of observation.

Lymphocytes

The percentage of lymphocytes in carp infested with *Myxobolus* can be seen in Figure 4.

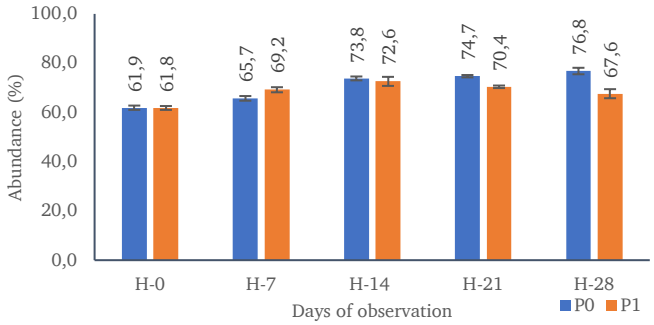

Figure 4. Percentage of lymphocytes in carp infested with *Myxobolus* for 28 days with immunostimulant treatment (P1) and without immunostimulant treatment (P0).

Lymphocyte results (Figure 4.) at P0 (61.9 - 76.8%) and P1 (61.8 - 72.6%). The percentage of normal lymphocytes in healthy goldfish ranges from 60.20 - 81% (Hrubec and Smith, 2010). Lymphocytes (P1) were highest on the 14th day (72.6%). The increase in lymphocytes on the 14th day of P1 treatment indicated that there was a response to the presence of antigens in the body. Then eosinophils decreased until the 28th day indicating infestation or the incidence of infection began to decrease. Lymphocytes act as agents of immunity against attacks by foreign bodies that enter the body (Colville and Bassert, 2016). The number of lymphocytes decreases when there is a parasitic infection because most lymphocytes migrate from the bloodstream and compete for body tissues in inflammation (Rahma *et al.*, 2015).

The results of the Two-Way ANOVA test show that the significance value is <0.05, meaning that the whole protein treatment on days 0, 7, 14, 21, and 28 significantly affects the percentage of lymphocytes. There is an interaction between treatment and days of observation.

Monocytes

Monocyte abundance (Figure 5.) at P0 (3.8 - 6.3%) and P1 (3.9 - 5.4%). Monocytes (P1) were highest on day 14 (5.4%) and lowest on day 28 (3.7%).

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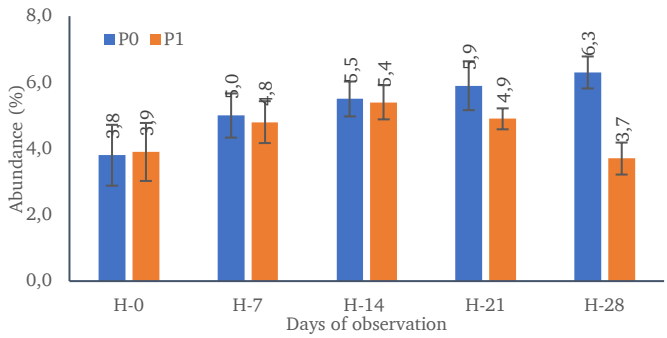

Figure 5. Percentage of monocytes in Carp infested with *Myxobolus* for 28 days with immunostimulant treatment (P1) and without immunostimulant treatment (P0).

The normal value of monocytes in fish is around 3.9-5.9% of the total number of leukocytes (Hardi *et al*., 2011). The increase in monocytes on the 14th day of P1 treatment indicated that there was a response to the presence of antigens in the body. Then eosinophils decreased until day 28 indicating that the treatment of immunostimulants was working and reduced parasitic infestation. A decrease in the value of monocytes indicates that the fish is in good health. Monocytic cells are not needed for phagocytosis because no infection enters the body or there is no stimulation of foreign substances to produce monocytes. The decrease in monocytes can also be caused by a blood balance response to an increase in the proportion of other types of leukocyte cells, namely lymphocytes (Utami *et al*., 2013; Hartika *et al*., 2014).

The results of the Two-Way ANOVA test show that the significance value is <0.05, meaning that the whole protein treatment on days 0, 7, 14, 21, and 28 significantly affects the percentage of monocytes. There is an interaction between treatment and days of observation.

Water Quality Analysis

Most of the water quality results are included in optimal conditions in the parameters of temperature, pH, and DO. However, the parameters of organic matter such as ammonia, nitrite, and nitrate in the waters are still quite high. High organic matter in water can be caused by the accumulation of organic matter (fish feed, feces) and lack of aeration in water bodies. In addition, excessive feeding of fish, infrequent water changes, and high stocking densities in ponds. High ammonia can cause oxidative stress in organisms, through increasing the concentration of reactive oxygen species (ROS).

Overproduction of ROS can damage important biomolecules, such as DNA, proteins, and lipids, and lead to impaired cellular function (Cheng *et al*., 2015). The deposition of organic matter in the water can affect the chemical and physical balance, thereby increasing the biological oxygen demand (BOD) (Ojwala *et al*., 2018). Poor water quality can cause fish to stress easily, making them susceptible to disease (Yustiati *et al*., 2017; Purbomartono *et al*., 2021). High ammonia can cause oxidative stress in organisms, through increasing the concentration of ROS. Overproduction of ROS can damage important biomolecules, such as DNA, proteins, and lipids, and lead to impaired cellular function (Cheng *et al*., 2015). One of the diseases that attack Carp larvae is *Myxobolus* ectoparasites which are reported to be capable of causing death in fish (Mahasri *et al*., 2015).

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Table 3. The results of the water quality analysis test for the pond of punten carp maintenance.

Parameters	Unit	Result	Optimal Rate
Temperature	°C	26 - 27	25 – 30*)
pH	-	6.62 – 7.81	6.5 – 8.5*)
Dissolved oxygen (DO)	mg/L	4 – 6	>3**)
Ammonia (NH_3)	mg/L	0.05 – 7.3	0.2***)
Nitrite (NO_2)	mg/L	1.2 – 6.9	0.06***)
Nitrate (NO_3)	mg/L	20 - 64	0.2 – 10***)

*) Pratama *et al*. (2020)
**) Yufika *et al*. (2019)
***) Government Regulation No. 22 (2021)

CONCLUSION

The abundance of erythrocytes in fish treated with P1 (1.16 - 2.44 x10^6 cells/mm^3) was higher than in fish treated with P0 (1.09 - 1.55 x10^6 cells/mm^3). The abundance of leukocytes in fish treated with P1 (3.79 - 11.31 x10^4 cells/mm^3) was higher than in fish treated with P0 (3.51 - 6.58 x10^4 cells/mm^3). The results of differential observations of leukocytes in the P0 treatment found basophils (0.3 -1.3%), neutrophils (12.3 - 21.5%), eosinophils (2.5 - 4.9%), lymphocytes (61.9 - 76.8%) and monocytes (3.8 -6.3%). The results of differential observations of leukocytes in the P1 treatment found basophils (0.3 - 1.0%), neutrophils (10.7 - 19.9%), eosinophils (2.4 - 3.9%), lymphocytes (61.8 - 72.6%) and monocytes (3.9 - 5.4%). Based on the results, it can be concluded that immunostimulatory whole protein *Myxobolus* treatment on days 0, 7, 14, 21, and 28 has a significant effect on the abundance of erythrocytes, the abundance of leukocytes, and differential leukocytes (basophils, neutrophils, eosinophils, lymphocytes, and monocytes). It can be concluded that treatment has a better effect than treatment without immunostimulants.

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTION

All authors have contributed to the final manuscript. Each author's contribution is WS, GM, and SS: Conception and study design. WS and GM: acquisition of data. WS and SS: Data analysis and interpretation. WS, GM, and SS: Drafting the manuscript. WS, GM, and SS: Revising the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

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