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INCREASING NUTRITIONAL CONTENT OF ARTIFICIAL FEED WITH WHOLE SPORE PROTEIN OF MYXOBOLUS KOI AS AN IMMUNOSTIMULTANT ON GOLDFISH (CYPRINUS CARPIO L.)

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ABSTRACT
The production of goldfish in Indonesia in 2010-2013 has increased by 7.09%, the lowest average production increase compared with other main commodities such as shrimp, tilapia, catfish, and others. One of the primary causes of the low increase in production is the presence of disease and high price of feed in some central goldfish productions. The purpose of this study was to analyze the effect of whole spore protein of Myxobolus koi on goldfish (Cyprinus carpio L.) through feed to immune response, growth rate, feed efficiency and survival rate. This research was conducted with complete randomized design with 5 replications. This study used two types of treatment, control (100% artificial feed) and artificial feed + immunostimulant (whole spore protein of Myxobolus koi + Boster® Progol adhesive spores). The results showed that whole spore protein of Myxobolus koi given to the feed as immunostimulant can cause response of the immune through the increase of monocytes and lymphocytes in white blood cells on days 7, 14 and 28 observations, daily growth rate of 5.55% compared without immunostimulant with the rate of 1.13%; feed efficiency of 36.57% compared with no immunostimulant, which is only 23.84%, and the treatment gives a 99% survival rate.

KEY WORDS
Myxobolus koi, Cyprinus carpio, growth rate, feed efficiency, survival rate.

The production of goldfish in Indonesia in 2013 reached 340,863 tons with an average increase of 7.09% from 2010. Despite of the average production increase, it is the lowest average compared with other main commodities such as shrimp, tilapia, catfish, and others that have an increase on average of 10-95%. Some of the causes to the low increase in the average production, among others, are due to the general capacity of goldfish businesses are still categorized in small business scale, and the high price of manufactured feeds also become influencial factor; the emergence of disease in some central production has become the main reason of low increase in production (Directorate General of Aquaculture Ministry of Marine Affairs and Fisheries, 2013).

The success of goldfish aquaculture is mainly influenced by the accuracy of feed management, water quality management and the accuracy in disease control. One of the obstacles in the aquaculture is that it is very susceptible to poor environmental conditions, so it is easily infected by pathogens. Based on these conditions, one way to increase the production of goldfish is by increasing the immune system and suppress the spread of disease on the body (Suprayudi et al, 2006).

Controling the spread of disease in the body should be executed as early as possible to avoid any possibility of outbreaks that may cause bigger economic losses (Alifuddin, 2002). In fish farming activities, the most commonly used method for treating pathogen infection is the limited use of antibiotics and chemotherapeutics. However, in addition to having low effectiveness and expensive price, and the use of chemicals can lead to accumulation in the environment. An alternative strategy that can be applied is the regulation of nutrients by utilizing the immune response and disease resistance found in the fish body (Suprayudi et al, 2006).

The increasing immunity of the body against disease cannot only be done by feeding with a balanced nutrient composition, but can also be accompanied by immunostimulant in the feeds. Immunostimulant is directly related to immune system cells that make the cells more active (Ekawati et al, 2012).

The strategies of controling pathogens on fish aquaculture business in the last 20 years have been carried out with the use of antibiotics and chemicals, without any awareness that its long-term use of chemicals can lead to resistance to some pathogens. Vaccination is considered as a very effective treatment in overcoming the pathogen problem in cultivation, but the price is very expensive and, on the other hand, may cause stress for the fish. One promising alternative technique in strengthening the fish immune system to deal with pathogen attack is the application using immunostimulants (Labh & Shakya, 2014).

A material may be used as an immunostimulant if it has immunogenic properties. Yusuf (2016) has successfully characterized each of the protein sizes of the whole protein of *Myxobolus koi* using the SDS-PAGE method (sodium dodecyl sulfate polyacrilamide gel electrophoresis). Results of SDS-PAGE of the whole spore protein of *Myxobolus koi* obtained the presence of proteins depicted in ribbon form on SDS-PAGE gel and obtained 6 bands of proteins with molecular weight (BM) 68.1 kDa, 38.5 kDa, 25.6 kDa, 23 kDa, 21.7 kDa, and 18.9 kDa. These results indicate that the proteins found have a high molecular weight. According to Harlow and Lane (1998), immunogenic proteins are proteins that have a molecular weight of 20,000-100,000 Daltons. The whole spore protein of *Myxobolus koi* can also increase immune response and survival ability of the goldfish from 10% to 86%. In addition, it can also increase the immune system against *Myxobolus koi* infection (Mahasri, 2016).

Based on the elaboration, it is necessary to develop a method of preventing proper pathogens through research by analyzing the increase of artificial feed nutrition value with the addition of whole spore protein of *Myxobolus koi* on the goldfish (*Cyprinus carpio* L.) to increase the growth rate as well as the survival rate.

The objective of this study is to analyze the addition of whole spore protein of *Myxobolus koi* on the goldfish (*Cyprinus carpio* L.) as the immunostimulant development material to the immune response (leukocyte differentiation), growth rate, artificial feed efficiency and also survival rate.

MATERIALS AND METHODS OF RESEARCH

This research was conducted in August 2016 until February 2017 at Installation of Freshwater Aquaculture (IBAT), in Dlanggu, Mojokerto, and the test of feed proximat analysis was conducted at the Feed Laboratory, Faculty of Veterinary, Airlangga University Surabaya.

This research was conducted with completely randomized design with two treatments and sampling technique was done 5 times. This study used two types of treatments, controlling treatment (100% artificial feed) and artificial feed + immunostimulant (whole spore protein of *Myxobolus koi*) + Boster® Progol adhesive.

Test animals used in the study were 2,200 goldfish (*Cyprinus carpio* L.) measuring 3-5 cm, put in the 2 ponds containing 1100 fish for each for two different treatments. The goldfish were brought from Punten, Malang Regency.

The equipments used in this study include two 1x7x8 meter sized ponds, 2 plastic tubs, nets, strainer, sieve, hoses, milling, sieving, basin, pellet producer, oven, plastic, scissors, paper, baking sheet and digital scales, namely water quality gauges (pH pen, thermometer, DO meter, ammonia test kit), surgical tools (scissors and tweezers), hematological parameters using light microscope, capillary pipette, syringe, sahm hemometer, eppendorf tube, glass object, glass cover, sectio set, microplate, ose, petri dish, pipette, test tube, micropipet, beaker glass, tubes, microtube, centrifuge and haemositometer.

The immunostimulant used in this study was an immunostimulant extracted from the Myxobolus koi parasite protein that has been studied and developed by Joseph (2016).

The ingredients for protein isolation are the phosphate buffer saline/PBS (Bio-Rad) solution, protease inhibitor consisting of: 100 ml PBS, 100 µL 40 mM PMSF, 7.3 mg TLCK and 7.5 mg EDTA (Sigma-USA) nonidet P40 0.5% (Sigma-USA).

Materials used in protein analysis using the SDS-PAGE method were separating gel and stacking gel, PBS, T-Akril, ddH2O, tetra methyl diamine (TEMED) (Bio-Rad), ammonium persulphate, Tris (*hydroxymethyl*), HCL (*Merck*) pH = 8.8 and 6.5, detergent sodium dodecyl sulphate (SDS), aquadest and comassie brilliant blue dyes.

Spores that have been calculated were pured with PBS sufficiently then centrifuged at 5,000 rpm for 10 minutes. The pellet plus 500 µl lysis buffer was then synonymized in ice (1 minute sonication, ½ min rest), repeated for 10 times. The result of sonication was then vortexed (½ minute vortex, 1 minute break) in ice, repeated for 15 times. The result of vortex was centrifuged with 12,000 rpm for 5 minutes, the supernatant formed was collected and then analyzed by SDS-PAGE (Aulanni'am, 2004).

The determination of the concentration of the whole spore protein of *Myxobolus koi* was conducted using Bio-Rad Protein Assay method and interpreted using UV-Visible Spectrophotometer with 600 nm wavelength.

The activity is intended to know the closely molecular pattern of each protein fraction. Analysis of the protein was done by electrophoresis method of SDS-PAGE with the composition of separating gel of 12.5 and stacking gel 5%. This method of electrophoresis was carried out by making running gel inserted into a glass plate. After the gel was hard, stacking gel was applied at the top of it (Osborne & Brooks, 2006).

A 10 µg sample added with Laemly buffers with 2: 1 ratio was boiled at 1000C for 5 minutes and put into a well located on the stacking gel. As the marker, protein was used with molecular weight in the range of 10-180 kDa (New England Bio-Labs), and running was performed on a chamber that had been filled with Electrode Buffers IX with 100 volts, 40 mA. The running process was stopped after the blue marker reached the lower limit of the plate gel. The gel was then introduced into a washing solution consisting of 25 ml methanol, 3.7 ml acetic acid and 100 ml aquadest. The sample was shaken on top of shaker for 30 minutes. Reprocessing was carried out with the same solution as the reduction of the ethanol composition and the addition of half acetic acid from the previous for 30 minutes. After washing the gel stained with silver nitrate ($AgNO_3$) for 15 minutes, then it was washed with aquadest 2 times for 2 minutes respectively. Given a color development solution consisting of 3.7% formaldehyde, 5% zintronsauce and aquadest. After the tape was seen, the reaction was stopped by adding 10% acetic acid. The resulting gel yielded of the protein bands were ready to be documented (Laemmli, 1970).

The result of SDS-PAGE electrophoresis in the form of bands can be determined by molecular weight by calculating Rf (Retardation Factor) score from each band with the following formula (Rantam, 2003):

$$Rf = \frac{\text{Distance of protein movement from starting point}}{\text{Distance of color movement from starting point}}$$

Then the Rf score is inserted to the linear regression equation as the following formula:

$$Y = a + bX$$

Where: Y = molecule weight, X = Rf score of sample.

Feeding was done in the feeding room of the IBAT Feed Laboratory, the feed was prepared at each treatment as much as 3% of the total weight of the fish of each fishpond for a single feeding. Artificial feed was prepared on treatment P0 as controlling treatment and artificial feed + immunostimulant made of whole spore protein of *Myxobolus koi* as the treatment P1 with dose of 1 µg protein /gram fish + progol adhesive with the volume of 5 ml/kg feed referring to research conducted by Yusuf (2016).

The two types of feeds were firstly tested for proximat analysis in Inspection Unit of Laboratory, Consultation and Training, Ministry of National Education, Faculty of Veterinary Medicine, Airlangga University.

Immunostimulatory mixing with the feed was done by spraying on the stirred feed to make it homogeneous, then it was dried to avoid moisture.

Object glass was cleaned with alcohol. The blood of the test fish was dropped about 1 cm from the left end of the object glass, shifted towards the right glass so that the blood will spread along the side of the shining glass. The blood was dried and ready for coloring. The smear preparation was fixed with methanol for 3-5 minutes and allowed to dry. The preparation was then stained with giemza solution for 30 minutes. The preparation was then washed with aquadest and allowed to dry on the shelf. When it was dry, the preparation was examined under a microscope with 400x magnification and calculated for each type of leukocyte using a blood counter. Cells counted were at least 100 cells and calculated in terms of the percentage of leukocyte types.

The calculation of specific growth rate (SGR) was calculated in daily bases for the growth rate of each individual. Specific growth rate of the fish was calculated using the formula proposed by Zonneveld et al. (1991) as follows:

$$SGR = \frac{Wt - Wo}{Wo \times t} \times 100\ \%$$

Where: SGR = Specific growth rate (% / day), W_t = Biomass of test fish at the end of the study (g), W_0 = Biomass of test fish at the beginning of the study (g), t = duration (days).

Feed efficiency is the value of the ratio between weight gain and the consumed feed in percentage. The feed efficiency can be calculated using the NRC Formula (1977), as follows:

$$EP\ (\%) = \frac{[(Wt + Wd) - W0]}{F} \times 100\%$$

Where: Wt = total weight of fish at the end of cultivation (gram), W0 = total weight of fish at the beginning of cultivation (gram), W_d = total weight of fish that are died during cultivation (gram), F = total feed given (gram).

Determining the survival rate (SR) was performed as a supporting parameter to analyze the goldfish immune response in each treatment. The survival rate is calculated as a percentage of the number of the goldfish living up to the 30th day after the experimental treatment of the total number of fish being cultivated. The survival of fish can be calculated using the following formula:

$$SR = \frac{Nt}{No} x\ 100\%$$

Where: SR = Survival rate, Nt = number of fish living at the end of the observation, N0 = number of fish living at the beginning of the test

RESULTS AND DISCUSSION

Based on its cytoplasmic granulation, leukocytes are differentiated into granular including basophils, eosinophils, and neutrophils, as well as agranular namely lymphocytes and monocytes. Leukocyte differential observations were calculated in 100 white blood cells observed under a microscope with 400x magnification. Leukocyte Differential can be seen in Table 1. On the 7th day of observation, lymphocytes and monocytes of treatment P1 (artificial feed + immunostimulant) increased in number from the day before the treatment, while the number of neutrophils, eosinophils and basofil of treatment P1 decreased. The highest lymphocytes and monocytes us shown in treatment P1. On day 14 observation, lymphocytes of treatment P1 (artificial feed + immunostimulant) increased from the day before treatment and the day 7 observation, while the number of monocytes, neutrophils, eosinophils and

basophils of treatment P1 decreased. On day 28 observation, lymphocytes and monocytes of P1 treatment decreased from the day 14 observation. The number of treatment lymphocytes P1 was higher than the treatment P0. The number of eosinophils and basophils in treatment P1 also increased.

Measuring the specific or daily growth rate was performed on the 28th day of the observation. Figure 1 shows the daily growth rate data on both treatments during the research, of which show the daily growth rate at treatment P1 (5.55%) is higher than the treatment of P0 (1.13%).

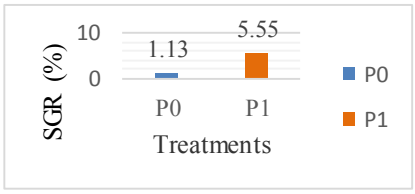

Figure 1 – Daily growth rate of both treatments on the goldfish during the research

The measurement of feed efficiency was conducted on the 28th day of the research. Figure 2 shows the data on feed efficiency in the two treatments during the research. From the data show that the feed efficiency at treatment P1 (36.57%) is higher than treatment P0 (23.84%).

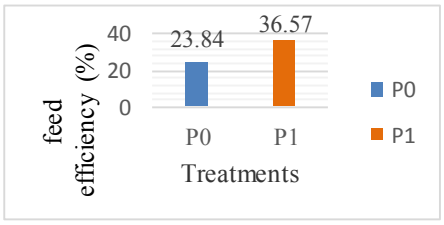

Figure 2 – Feed efficiency of the two treatments on the goldfish during the research

The survival rate of the goldfish during the cultivation process becomes crucial parameter of the success in the cultivation. Figure 3 below is the the graph showing the survival rate of the goldfish during the study.

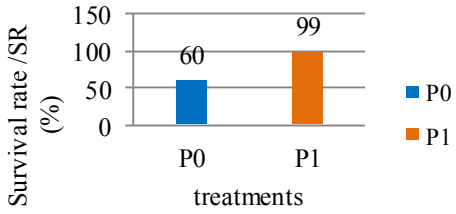

Figure 3 – Diagram of survival rate percentage of the goldfish of the two treatments

The results of the calculation of survival rate of the goldfish (*Cyprinus carpio* L.) had been conducted for 28 days of observation, showing the survival rate of treatment P1 (99%) is higher than the treatment P0 (60%).

The quality of the water in the fishpond during the study is an important factor in successful cultivation. The average result of the examination on the quality parameter of the water during the study is presented in Table 1.

During the research conducted, the water was changed every day as much as 25% of the total water volume. The quality of the water in this research was categorized into normal condition for the cultivation of goldfish.

DISCUSSION OF RESULTS

Yusuf (2016) has successfully characterized each of the protein size of the whole spore protein of *Myxobolus koi* using SDS-PAGE method (sodium dodecyl sulfate polyacrilamide gel electrophoresis). The results of SDS-PAGE of the whole Myxobolus koi spore protein indicated the presence of proteins depicted in ribbon form on SDS-PAGE gel and obtained 6 bands of proteins with molecular weight (BM) of 68.1, 38.5, 25.6, 23, 21.7 and 18.9 kDa. These results indicate that the proteins found have a high molecular weight. According to Harlow and Lane (1998), immunogenic proteins are the proteins that have a molecular weight ranging from 20,000-100,000 Daltons. Good immunogens mostly have the molecular size of <100 kDa, while for the proteins with molecular size of <5-10 kDa are categorized as poor immunogens (Mayer, 2011). Abbas et al. (2000) suggest that a molecule has immunogenic properties when its molecule weighs more than 10 kDa. Myxobolus koi spore protein can also increase the immune response and koifish life from 10% to 86% and can increase their immune against Myxobolus koi infection (Mahasri, 2016).

Table 1 – Average types of leukocytes (%) of goldfish during research

Duration	Treatments	Types of Leukocytes (%)				
		Limfocyte	Monocyte	Neutrophil	Eosinophil	Basophil
Day 0	P_0	56	19	17	7	1
	P_1	50	17	22	9	2
Day 7	P_0	55	20	17	7	1
	P_1	63	22	10	4	1
Day 14	P_0	56	19	17	6	2
	P_1	72	20	5	2	1
Day 28	P_0	51	24	19	6	1
	P_1	62	18	13	6	1
Normal (Yusuf, 2016)		52	18	20	8	2

Table 2 – Average results of parameter testing of the quality of water for goldfish cultivation during the research

Parameters	Average score for the quality of water in 30 day cultivation of the goldfish		Normal score range (Flajšhans and Hulata, 2007)
	P_0 (control)	P_1	
Temperature (^oC)	30	29	27-30
pH	7.8	7.5	6.5-9
DO (mg/l)	6.6	5.9	>3-7
Ammonia (mg/l)	0.5	0.5	0.5-0.52

The immunostimulatory mechanism is if the immunostimulants enter into the body, the immunostimulants will stimulate monocytes to produce cytokines such as interleukins which will activate lymphocyte cells, which then divide into B lymphocytes and T lymphocytes. T lymphocytes in non-specific responses will produce interferons which are capable of activating performance of macrophages through the mechanism of phagocytosis in the face of parasites, bacteria, viruses, and foreign particles that are considered as antigens (Raa, 2000).

Blood is one medium as a diagnosis of health status in an organism including fish. Blood will undergo serious changes when exposed to infectious diseases. Blood tests can also be an indicator of the degree of severity of a disease experienced by the fish (Bastiawan et al, 2001). White blood cells (leukocytes) fish are part of the fish's body immune system. Factors that affect the number of leukocytes are the condition and health of the fish (Chinabut et al., 1991). According to Effendi (2003), leukocytes consist of agranulocytes (monocytes and lymphocytes) and granulocytes (heterophils, eosinophils, and basophils). Total changes and types of leukocytes can be used as indicators of certain infections that

occur in fish's body. Lymphocyte is one form of leukocytes. The percentage of lymphocytes of P1 on Days 7, 14 and 28 increased from the day before the treatment.

The increasing number of lymphocytes in the goldfish that were fed with the artificial feed + immunostimulant with whole spore protein of *Myxobolus koi* is the response of the fish's immune system as the addition of pathogens. This is in accordance with the opinion of Bastiawan et al. (2001) that the lymphocytes serve as antibodies to the body's immune system from any disease. Monocytes migrate from the blood circulation to tissues when receiving stimuli that are compatible with their receptors (Feldman et al., 2000). The results of the two treatments showed that on Days 7 and 14 of treatment P1 were the highest (artificial feed + immunostimulant) with the percentage of 22% and 20% respectively. Percentage of monocytes Days 7 and 14 of treatment P1 increased from the day before the treatment, and decreased on the Day 28. The increase is due to the existence of monocytes phagocytes pathogens (bacteria and parasites) that attack the goldfish. The condition is similar as the assertion by Ardelli and Woo (2006), that monocytes together with macrophages will phagocytate those agents that cause disease into the body. Observations of P1 (artificial feed + immunostimulant) on Days 7, 14 and 28 show that the treatment has an effect on the immune response, with the percentage of lymphocyte and monocyte was 63%, 72% and 62% respectively.

Based on the results of the growth rate of the two treatments, the treatment P1 with the whole spore protein of *Myxobolus koi* has higher daily growth rate of 5.55% compared with treatment P0 without immunotimulant which is only 1.13%. The addition of whole spore protein of *Myxobolus koi* as the immunostimulants, besides as additional nutrients for the fish, it is beneficial as the enhancer of the immune response which is able to increase the growth rate of the goldfish. This is in accordance with the study carried out by Hidayat et al (2013) that one of factors affecting the growth rate of fish is the protein content in the feed as the function of protein is to form new tissue for growth and also replace the damaged tissue.

Based on the results of the feed efficiency test of the two treatments, it showed that feed efficiency on the treatment P1 with immunostimulatory whole spore protein of *Myxobolus koi* (36.57%) is higher than the treatment P0 (23.84%). This suggests that the addition of immunostimulatory can utilize the feed consumed for growth so as to increase the value of feeding efficiency and the digestibility of the fish. This is in accordance with the opinion of Djadjasewaka (1985) who states that the efficiency of feeding is directly proportional to the increase in body weight, so the higher the value of feeding efficiency means the more efficient the fish can use the feed consumed for the growth.

In terms of survival rate (SR), the results show the highest survival rate is on the treatment P1 of 99%, while the lowest in the treatment P0 with only 60%. This is in accordance with the statement of Mahasri (2016) that whole spore protein of *Myxobolus koi* can increase immune response and survival ability of the life from 10% to 86%, and it can increase the immune system against *Myxobolus koi* infection. In addition, according to Mudjiman (2000), feed that has good nutrition is instrumental in maintaining the survival ability and accelerating the growth of the fish.

The quality of the water during the study was still suitable for the survival of the golfish. The other environmental factors that support the survival of the goldfish are namely water temperature, pH, ammonia and the amount of oxygen dissolved in the water.

Conclusion. The conclusion of this research is that the whole spore protein of *Myxobolus koi* can be used as the immunostimulants.

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