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Effect of Maggot on the IL-6 and IFN-ɣ Expression of Koi Fish (*Cyprinus carpio*) Infected with *Edwardsiella tarda*

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Abstract

The increase in koi fish cultivation can lead to increase risks and cause problems. The main problem is high disease attacks caused by the bacteria *Edwardsiella tarda*. One of the alternatives to control the disease is immunostimulant treatment with the use of maggot, *Hermetia illucens* larvae, known as Black Soldier Fly (BSF). This study aims to report the effect of maggots as a substitute for koi fish feed formulations on the immune system of koi fish infected with *E. tarda*. This study used a descriptive method, by quantitative approach with the IL-6 and IFN-ɣ expression through RT-PCR analysis and fish survival rate (%). Identification of *E. tarda* was carried out by the PCR test method at a molecular weight of 200 bp. The density of bacteria that caused the death of the 50% koi fish population (LD_{50}) for 72 hours was 10^7 cells/ml. The highest IL-6 expression in the spleen was found in sample C at 28,844 and the lowest in-sample K (+) at 26,096. The highest IL-6 expression in the liver was in sample K (+) at 29,060 and the lowest was in sample D at 25,428. The highest IFN-ɣ in the spleen was in treatment C, 28,844, and the lowest was in treatment D, 27,219. The highest IFN-ɣ in the liver was sample K (+) 26,795, and the lowest in-sample C of 25,615. The survival rate of fish in this study reached 30%-100%. The optimal amount of substitution of maggot treatment is 75% (Treatment C) of fish feed.

INTRODUCTION

Koi fish (*Cyprinus carpio*) is an economically important freshwater ornamental fish cultivated in Indonesia (Sunarto *et al*., 2005). Koi fish (*C. carpio*) is an economically important freshwater ornamental fish cultivated in Indonesia. The Koi fish farming business is increasing along with the high market demand. This has also led to an increase in the development of the koi fish rearing business in a more intensive direction and caused problems faced by cultivators, keepers, and entrepreneurs (Manoppo and Kolopita, 2016). The main problems that occur in the maintenance of koi fish are high disease attacks and slow growth. Diseases that often attack koi fish are infectious diseases caused by bacteria, parasites, fungi, and viruses, as well as stress levels, intoxication, and decreased environmental quality (Adam and Maftuch, 2017). The bacteria that most

frequently infect koi fish are the motile bacteria *Aeromonas* and *Edwardsiella* spp. *E. tarda* is a bacterium that belongs to the Quarantined Fish Disease Pest group II, which means that this type of disease already exists in Indonesia but there is no technology to overcome it. *E. tarda* is a pathogenic bacterium that causes Edwardsiellosis disease and mostly attacks koi fish. This bacterium causes mass mortality in all age groups of fish (Rajapakshe *et al*., 2012).

One of the alternatives to control the disease is immunostimulant treatment. Immunostimulants work simultaneously to prevent attacks of various pathogens by increasing non-specific immune responses in fish and shrimp (Manoppo and Kolopita, 2016). Immunostimulants can be made from natural ingredients so that they do not cause residues in the fish's body and are not harmful to the environment and human health. Immunostimulants work by activating non-specific defense mechanisms to increase phagocytic activity, complement activity, lysozyme, and resistance to disease (Divyagnaneswari *et al*., 2007). One of the natural ingredients to increase the fish's immune system, as well as a high protein source for fish, is *Hermetia illucens* larvae, known as Black Soldier Fly (BSF) Larvae. Larvae from BSF are called maggots (Tippayadara *et al*., 2021). Maggot (*H. illucens* Linnaeus) is a BSF larva with a chewy texture, has a high protein content, and can secrete natural enzymes to help improve the digestive system of fish (Fauzi and Sari, 2018). The high protein in BSF maggots and the amount of chitin involved in the abundance of microbial communities in the fish gut can act as prebiotic substances that can cause immunostimulant effects in fish (Tippayadara *et al*., 2021).

Immunostimulants in infected fish work on complement activation by not involving antibodies. By the non-specific immune response, macrophages produce cytokines that function to stimulate non-specific inflammation and increase the activation of specific lymphocytes by bacterial antigens. Cytokines then induce the adhesion of neutrophils and monocytes to the vascular endothelium at the site of infection, along with the activation of inflammatory cells (Munasir, 2001). In this case, cytokines such as interleukin 6 (IL-6) and interferon-gamma (IFN-ɣ) have the opportunity to be used as indicators in assessing the level of inflammation experienced by endothelial cells due to bacterial infection. Inflammation is a non-specific immune response that arises due to a bacterial attack (Yuniarti, 2014). This study focused on the effect of the high protein content of maggots as a substitute for koi fish feed formulations on the immune system or immunity of koi fish infected with *E. tarda*.

METHODOLOGY

Place and Time

This research was carried out in January – August 2021 at the Laboratory of Fish Disease and Health, Faculty of Fisheries and Marine Science, Universitas Brawijaya, Chemistry Laboratory, State Islamic University of Malang, UPT Materia Medica Batu Malang, and Bioscience Laboratory, Universitas Brawijaya.

Research Materials

The materials used in this study include fish-rearing equipment, sample preparation, and cytokine analysis in fish. Aquarium 40x40x30 cm as many as 16 pieces, aerator, and aeration stones were used as a fish rearing tool. Feed-making tools include blenders, scales, and ovens, feed machines. In addition, the materials used consist of a tray, section set, DO meter (Lutron Electronic Enterprise Co., Ltd., Taiwan) dropper, ose needle, petri dish, and centrifuge (Thermo Fisher Scientific Inc., USA). The materials to be used in this study include maggot, koi fish seeds measuring 10-12 cm, pellet feed, 75% alcohol, tissue, *Edwardsiella tarda* isolate obtained from BUSKIPM (Test Center for Fish Quarantine Standards, Quality Control and Safety of Fishery Products), Indonesia Ministry of Marine

Affairs and Fisheries, NA media (nutrient broth), TSB media (tryptone soy broth), aquades, label paper, koi fish organ samples, as well as materials for cytokine expression analysis of koi fish including DNA isolation kits and Real-time PCR kits.

Research Design

The research design used in this study was a completely randomized design (CRD). This study used *H. illucens* maggot as a material for in vitro and in vivo testing. This study used 2 comparison controls, namely normal control, and infection control. The treatments used in this study are based on Azizah (2019). Treatment K (-) is treatment without bacterial infection and maggot, treatment K (+) is treatment with infection and without maggot administration, treatment (A) is the administration of maggot 25% of fish meal in feed, treatment (B) is the administration of maggot 50% of fish meal in the feed, treatment (C) is the administration of maggot 75% of fish meal in the feed, and treatment (D) is the administration of maggot 100% of fish meal in the feed. Treatments A, B, C, D, and K (+) were infected with *E. tarda* bacteria with a density according to the results of LD_{50} at week 4. The feed used in the control treatment was commercial pellet feed and did not use formulated feed without the substitution of maggot flour.

Work Procedure

Maggot Flour Production and Formulation

Harvested maggot was separated from the media and washed, then put in the freezer for 2 days. Maggot is dried in the sun until the ice melts for a day. Then it was dried in an oven at 150 °C for 3 hours. The dried maggots were then blended and sifted to make flour and tested for proximate content. After that, the formulation of maggot flour was by mixing it in pellet feed with a protein content of 33%. Mixing the ingredients until homogeneous, then printed and dried in an oven at a temperature of 30 °C and then aerated.

Identification of *E. tarda* with Polymerase Chain Reaction (PCR)

A sampling of fish organs was carried out surgically using a sectio set. The organs used as samples are the liver and spleen. Organ samples were stored in 90% alcohol for laboratory testing (Koesharyani *et al*., 2018). DNA isolation was carried out using the Dneasy Blood & Tissue Kit (Qiagen). The organs were weighed at 50 mg, ground using a mortar and pestle, then added with 10 μl of lysis buffer (ATL buffer) and 20 μl of proteinase K and homogenized until smooth. After that, the sample was vortexed for 10 seconds and incubated at 56 °C to lysis for 25 minutes. Then the sample was vortexed again for 15 seconds by adding 200 μl of buffer AL and again incubated at 56 °C for 10 minutes.

The sample was then added with 200 μl of absolute ethanol, pipetted, and put into a mini-column that had been placed in a 2 ml collection tube, then centrifuged at 8000 rpm for 1 minute, then the mini-column was transferred to a new 2 ml tube. The sample was added with 500 μl of Buffer AW1, centrifuged at 8000 rpm for 1 minute, and the mini-column was transferred to a new 2 ml tube. Then 500 μl of Buffer AW2 was added to the sample, centrifuged at 14000 rpm for 3 minutes, and the mini-column was transferred to a new 2 ml tube. Then, centrifuged at 8000 rpm for 2 minutes to air dry. The mini-column was transferred to a new 1.5 ml tube then added 200 μl of buffer AE, was incubated for 1 minute at room temperature, centrifuged at 8000 rpm for 2 minutes, and then the DNA was stored.

Bacterial Culture of *E. tarda*

This bacterial culture was initiated by preparing 6 g of TSB solution in a 200 ml Erlenmeyer tube. The ose needle is heated on a Bunsen and cooled. After cooling, the ose needle was touched to a

pure culture of *E. tarda* and dipped in TSB 2 times. Then the TSB solution was left for 12-24 hours in an incubator at 37 °C. After the TSB was cloudy, the ose needle was dipped into the TSB and scratched in a zigzag manner onto the surface of the TSA. Incubate on TSA media in an incubator at 37 °C for 24 hours.

E. tarda Pathogenicity Test with LD_{50}

The LD_{50} test aims to determine the density and time required for *E. tarda* to kill 50% of the fish tested. In general, bacteria will be cultured on TSB media as much as 10^{10} cells/ml, then graded dilutions with a density of 10^9 cells/ml, 10^8 cells/ml, 10^7 cells/ml, and so on (Lukistyowati and Kurnianingsih, 2011). The LD_{50} test was carried out by calculating the bacterial suspension using the following formula:

$$N_1 \times V_1 = N_2 \times V_2$$

Where:

N_1 = The density of bacteria in the media (cell/ml)
N_2 = Bacterial density (cell/ml)
V_1 = The volume of bacterial suspension in the required medium
V_2 = The volume of media water in fish-rearing containers

Maintenance and Treatment of Maggot Feed on Sample Fish

Koi fish were acclimatized in an aquarium container for 3 days. Then, the fish samples were transferred to a treatment container. The fish were fasted for a day and weighed to determine their initial weight of the fish. The fish stocked had sizes ranging from 7-10 cm and a density of 10 fish per treatment. The treatment was conducted by adding maggot flour to commercial feed flour which is then made into pellets. The feed was given as much as 5% of the weight of the fish biomass with the frequency of feeding 3 times, at 08.00, 15.00, and 21.00 WIB. Water quality measurements were carried out every day in the morning and evening including temperature and pH measurements. Maintenance is carried out for 30 days. The challenge test with *E. tarda* was carried out at week 4 with the appropriate bacterial density from the LD_{50} results.

Fish Infection Test

The fish were infected with *E. tarda* by immersion method. Immersion will be carried out for 72 hours using the density of *E. tarda* bacteria according to the LD_{50} results. The immersion method was generally carried out because it was efficient and more applicable (Amanu *et al.*, 2014).

Analysis of IL-6 and IFN responses with Real-Time PCR

The Real-Time PCR method was used to analyze koi fish cytokine gene expression. mRNA expression analysis was performed using SensiFAST SYBR No-ROX (Bioline). Positive control amplification using -actin primer and target gene using IL-6 and IFN-ɣ cytokine gene primers. For the detection of *E. tarda* bacteria, amplification of target genes using *E. tarda* bacteria primers. The primary sequences for detecting *E. tarda* infection are shown in table 1, and the primary sequences for -actin and IL-6 and IFN-ɣ cytokine genes are shown in table 2. Real-time reagent components consist of SensiFAST SYBR No-ROX Supermix 2x, 10μM forward primer, 10μM reverse primer, nuclease-free water, and DNA template. Real-time reagent components were run using Bio-Rad CFX96 Real-Time PCR Systems. The cycles used are as follows: Polymerase activation at 95 °C for 2 minutes, denaturation at 95 °C for 5 seconds, annealing at 60 °C for 10 seconds, Extension at 72 °C for 20 seconds, a cycle for 40 cycles.

Table 1. Primary sequences for detecting *E. tarda*.

Primary Name	Primary sequence (length)	Source
16S rDNA (forward)	5'-TAGGGAGGAAGGTGTGAA-3' (19 mer)	Maiti *et al.*(2011)
16S rDNA (reverse)	5'-CTCTAGCTTGCCAGTCTT-3' (18 mer)	Maiti *et al.*(2011)

Table 2. Primary sequences for gene expression analysis.

Gene	Forward Primer	Reverse Primer	Bp	Source
IL-6	5'-GCGAGACCAGCAGTTTGAGAGG-3'	5'-TGGACAGACAGCCGTCAGAGG-3'	110	Liu *et al.* (2021)
IFN-ɣ	5'-TCTTGAGGAACCTGAGCAGAA-3'	5'-TGTGCAAGTCTTTCCTTTGTAG-3'	211	Dawood *et al.* (2021)
B-actin	5'-CACCTCCCTTGCTCCCTCCAC-3'	5'-CTCCTGCTTGCTGATCCACATCTG-3'	133	Liu *et al.* (2021)

Survival Rate

The survival rate was calculated before and after bacterial infection in the fish. Asma *et al*. (2016) suggested that SR can be calculated by the following formula.

$$SR = \frac{N_0 - N_t}{N_0} \times 100\%$$

Where:
SR = survival rate (%)
No = initial number of fish (tails)
Nt = number of dead fish (tails)

Data Analysis

The results of the research were analyzed descriptively with the type of correlation where the approach used was a quantitative approach (Atmowardoyo, 2018). The choice of descriptive method in this study was based on the purpose of studying and examining the relationship and effect of feeding maggot flour on the expression of cytokine genes IL-6 and IFN-ɣ in koi fish infected with *E. tarda*.

RESULTS AND DISCUSSION

Identification of *E. tarda*

Identification of bacterial infections was carried out by the PCR test method. The quality and quantity of isolated DNA were tested qualitatively by agarose gel electrophoresis. DNA purity was assessed from the ratio of absorbance if the two values were in the range of 1.8-2.0. The number and quality of extraction of DNA from koi fish samples can be seen in Table 3.

Table 3. The amount and quality of the extracted DNA of Koi Fish samples were measured by nanodrops at a wavelength (OD 260/280 = 1.8 – 2.0).

Samples	Quantity (μg/ml)	Quality (OD 260/280)
Spleen (K+)	131	1,770
Liver (K+)	406	1,860
Spleen (A)	211	1,361
Liver (A)	357	1,765
Spleen (B)	139	1,866
Liver (B)	97,3	1,783
Spleen (C)	110	1,883
Liver (C)	45,4	1,096
Spleen (D)	350	1,975
Liver (D)	70,4	1,108

The extraction results are in table 3. It shows that the DNA extraction results have met the requirements for further PCR testing. The DNA quality was conducted by DNA electrophoresis on 1.5% agarose with a target molecular weight of 200 bp. According to Indriasari *et al*. (2020), fish infected with *E. tarda* show clinical

symptoms such as swelling of the liver and kidneys and there are grains filled with water in the liver. The liver is an organ that has an important role in the body's metabolism and secretory tool in the process of detoxifying foreign materials that enter the body so that the liver is susceptible to toxins produced by bacteria.

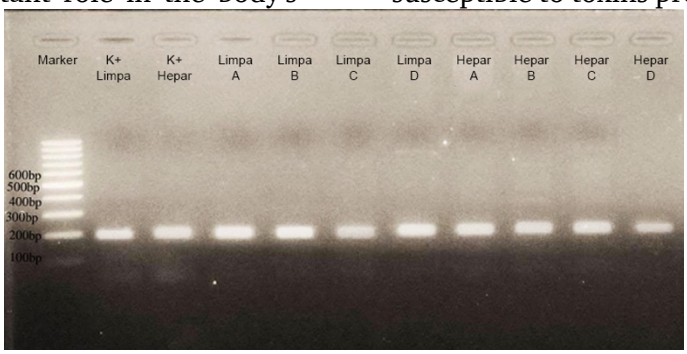

Figure 1. *E. tarda* bacterial infection in koi fish organs.

LD_{50} Test Results of *E. tarda*

Colonies of *E. tarda* have a flat or smooth surface, are circular with a diameter of 1 mm, slightly convex and the development of colonies is white or transparent. Based on the LD_{50} test, it was found that the density of bacteria that caused the death of the koi fish population by 50% for 72 hours was *E. tarda* with a density of 10^7 cells/ml. The results of the lethal dose of *E. tarda* are shown in Table 4.

Table 4. Lethal dosage 50% test of *E. tarda*.

Hour-	Number of Live Koi Fish			
	10^6	10^7	10^8	10^9
24	10	10	9	9
48	10	9	8	7
72	8	5	4	2
Percentage	80%	50%	30%	20%

Based on the results of the lethal dose test in table 4, the infection of koi fish was carried out through immersion with a bacterial density of 10^7 cells/ml *E. tarda* for 72 hours or 3 days. This is following the opinion of Narwiyani and Kurniasih (2011) where the mortality rate of the test fish is correlated with the increasing concentration of bacteria infected with the fish. Clinical symptoms that arise in fish infected with *E. tarda* are reddish lesions on the tail and fins, reduced fish appetite, sluggish movement, and reduced response to given stimuli.

IL-6 and IFNγ Responses

Figure 2 shows the expression of the IL-6 cytokine gene in the spleen and liver. The highest IL-6 expression in the spleen was found in sample C at 28,844 and the lowest in-sample K (+) at 26,096. Meanwhile, in the liver, the highest IL-6 expression was found in sample K(+) at 29,060 and the lowest was in sample D at 25,428. Thus, fish in treatment C with the administration of 75% maggot immunostimulant from fish meal showed a better immune response than in other treatments. The IL-6 cytokine gene that was expressed in the spleen organ of the K (+) sample had the lowest expression level of the other treatments. This is following the statement of Wei *et al.* (2018), that under normal conditions, the expression level of IL-6 is low, but IL-6 is overexpressed after stimulation, in this case, the administration of immunostimulants.

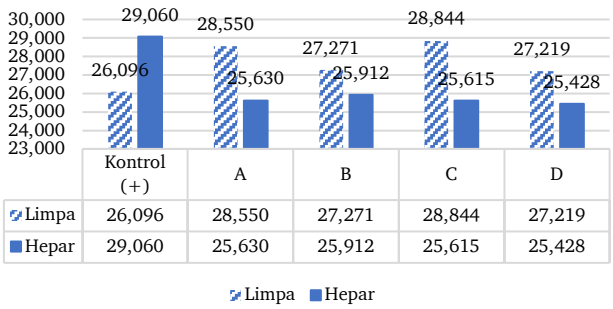

Figure 2. IL-6 response to *E. tarda* bacterial infection in koi fish organs

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The immune response appears when *E. tarda* enters the body. Extracellular antigens that enter the body are then captured by the Antigen-presenting cell (APC) and then the antigen is broken down into smaller molecules. The small molecule is then expressed by MHC II to the surface of the APC which then activates Th2 cells. Activated Th2 cells then produce pro-inflammatory cytokines, one of which is IL-6, to be used as a signal to B cells. B cells proliferate and differentiate into plasma B cells which will secrete antibodies against incoming antigens.

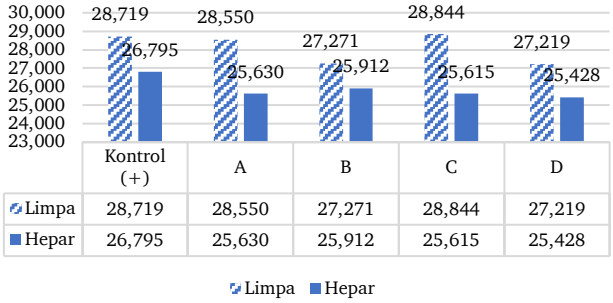

Figure 3. IFN-ɣ response to *E. tarda* bacterial infection in koi fish organs.

Meanwhile, the IFN-ɣ gene in koi organs was expressed and appeared on day 7 after infection with *E. tarda* (Santibañez *et al*., 2021). This indicates the activation of macrophages, induction of MHC peptides, and polarization of T cells which then stimulates apoptosis thereby increasing the antimicrobial mechanism against *E. tarda*. Figure 3 shows that the spleen samples in treatment C showed the highest IFN-ɣ gene expression of 28,844, while the lowest was shown in the spleen samples in treatment D, which was 27,219. In liver samples, the highest IFN- gene expression was found in sample K (+) with a value of 26,795, and the lowest IFN-ɣ gene expression was found in sample C of 25,615. The high expression of IFN-ɣ may be related to the immune response mechanism of the fish body that detects the entry of antigens so that they produce more IFN-ɣ genes (Junirahma *et al*., 2021).

The IFN-ɣ cytokine gene in the koi fish spleen is expressed more than in the liver. This is because the spleen acts as a secondary lymphoid organ which is also the site of the main immune response. The spleen is the main place for phagocytes to eat bacteria bound by antibodies, which is called the opsonization process. Phagocytic activity then removes particles, and cell residues and finally destroys erythrocytes. All activities of IFN-ɣ as the main activator of macrophages have an impact on increasing the inflammatory reaction filled with macrophages. From the graph shown, the fish in treatment C, with the administration of 75% maggot

feed, showed the best immune response compared to other treatments. Caused the result showed the highest activity of IFN-ɣ compared to other treatments. Thus, maggot contains antimicrobial compounds such as hexanoic acid (Choi and Jiang, 2014). The result of its mechanism is, the administration of a 75% maggot formulation can increase anti-bacterial activity which can be expressed from the high IFN-ɣ response that appears. These results were also supported by a related study that recombinant IFN-ɣ, namely rIFN-ɣ, is effective in strengthening the immune response and preventing edwardsiellosis caused by infection with *E. tarda* (Jung *et al*., 2012; Sakai *et al.*, 2021).

Survival Rate

Survival rate (SR) is the ability of fish to survive for a certain period. SR is determined by several factors including the condition of the water quality of the culture media, fish stocking density, inadequate nutrition, or inadequate treatment, causing stress to the fish which will affect the survival rate of the cultured fish. The SR of koi fish during the study is presented in Table 5.

Table 5. Survival rate (SR) of koi fish during research.

Treatment	Initial Number of Fish (N_o) (fish)	Final Number of Fish (N_t) (fish)	SR (%)
A	10	5	50
B	10	6	60
C	10	9	90
D	10	8	80
K (+)	10	3	30
K (-)	10	10	100

The survival rate of koi fish in treatment C is 90%, and treatment D is 80% is the treatment with the closest survival rate to the control treatment (-) which is 100%. This was due to the non-specific immune system acting on fish in treatment C, which showed the best immune response among all treatments, seen from the high expression of IL-6 and IFN-ɣ cytokine genes in fish spleen organs. Based on the results, the variable survival rate of koi fish is caused by stress factors in fish. Fish handling during quarantine before being kept in the rearing tank is quite short. In addition, the traceability factor or quality traceability and condition of the seeds must be ensured in healthy condition. Based on the factor of water quality conditions, the maintenance media still meets the threshold for maintaining koi fish.

Yurisman and Heltonika (2010) suggested that the factors that affect the survival rate of fish consist of biotic and abiotic factors. Biotic factors include stocking density or population density, age, competitors, and adaptability of fish to the aquatic environment. The abiotic factors that affect the survival rate of fish include water quality (dissolved oxygen, temperature, pH, and so on). Research on immunostimulants in aquaculture continues to be studied. The results of the study showed a positive significance that immunostimulants derived from natural ingredients were able to increase immunity, and fish resistance to pathogens including bacteria, and ultimately improve the quality of life of fish. Nugroho and Nur (2018) reported that the use of peptidoglycan was proven to trigger the immune response of tiger grouper. The administration of peptidoglycan of 20 mg kg-1 fish bodyweight was able to increase SR by 72% compared to 18.67% control without immunostimulants. Another study stated that the SR of tiger prawn seeds infected with WSSV was significant when given immunostimulants.

CONCLUSION

The amount of substitution of maggot meal in the fish meal which is suitable for increasing the non-specific immune response of koi fish (*C. carpio*) is 75% of fish meal seen from the immune response expressed through the cytokine genes IL-6 and IFN-ɣ, as well as the survival rate of fish. koi by 90%. The substitution of immunostimulants in the form of maggot flour into koi fish feed which was then challenged by *E. tarda* bacteria affected the non-specific immune response of koi fish. This is evidenced by the expression of pro-inflammatory cytokine genes in the form of IL-6 and IFN-ɣ with the highest number of each in the spleen organ in treatment C (substitution of maggot flour 75% from fish meal and infected with *E. tarda*). The total expression of each cytokine IL-6 and IFN-ɣ was 28.844%.

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