Universal Journal of Agricultural Research 11(6): 1125-1132, 2023 http://www.hrpub.org
DOI: 10.13189/ujar.2023.110620

The Effects of Environmental Enrichment on The Immune Response of Vaccinated *Tor soro*

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Received August 21, 2023; Revised November 7, 2023; Accepted November 27, 2023

Cite This Paper in the Following Citation Styles

(a): *[1] Amalia Putri Firdausi, Bahtera Daffa Prakarsa Hakim, Sukenda, Widanarni, Rahman, Alimuddin, Muhammad Arif Mulya, Annisa Hakim, Sarah Sabilla Fauziah, Dya Nur Rezha , "The Effects of Environmental Enrichment On The Immune Response of Vaccinated Tor soro," Universal Journal of Agricultural Research, Vol. 11, No. 6, pp. 1125 - 1132, 2023. DOI: 10.13189/ujar.2023.110620.*

(b): *Amalia Putri Firdausi, Bahtera Daffa Prakarsa Hakim, Sukenda, Widanarni, Rahman, Alimuddin, Muhammad Arif Mulya, Annisa Hakim, Sarah Sabilla Fauziah, Dya Nur Rezha (2023). The Effects of Environmental Enrichment On The Immune Response of Vaccinated Tor soro. Universal Journal of Agricultural Research, 11(6), 1125 - 1132. DOI: 10.13189/ujar.2023.110620.*

Abstract This study aims to evaluate the effectiveness of plants and silica in *Tor soro* fish populations vaccinated with *Aeromonas hydrophila* antigen. *Tor soro* fingerlings with a size of 3.4 ± 0.15 cm were stocked in an aquarium measuring 50×30×30 cm with a density of 50 fish per aquarium. The study used a completely randomized design with 4 treatments, i.e. positive control (C+), negative control (C-), vaccinated fish without enrichment (A), and vaccinated fish with environmental enrichment of plants and silica sand (B). Four groups were repeated by three replications. Group C+, C-, A, and B were challenged with *A. hydrophila* by immersion method on day 21st with a concentration of 10^6 CFU per mL for 30 minutes, and the fish were reared in their original container until day 30. The results showed that environmental enrichment in the vaccinated fish group gave an RPS value of 67.22 ±6.14%, significantly different than vaccinated fish without enrichment with an RPS of 50.28 ±5.02%. Environmental enrichment did not provide significant differences for antibody parameters, lysozyme activity, respiratory burst and growth in *Tor soro*.

Keywords *Aeromonas hydrophila*, Environmental Enrichment, Fish Welfare, Immunity, *Tor soro*

1. Introduction

Mahseer fish is a freshwater commodity with a high market price [1]. One type of mahseer fish that has become a leading commodity is *Tor soro*. Mahseer fish spread in several areas in Indonesia, such as Sumatra, Java, and Kalimantan. This fish usually lives in clear upstream, rocky, sandy, and has fast currents [2]. Mahseer fish have prolonged growth. The growth cycle in mahseer fish from larvae to brood stock requires at least four years. Indonesia has at least three species: *Tor tambra, Tor tambroides,* and *Tor douronensis*. There is an increasing public interest in making this fish an ornamental fish because of its attractive shape and colour [3].

Overfishing in nature, water pollution, and the conversion of riverbank ecosystems by settlements and agriculture have put pressure on mahseer populations in the wild [4]. Several researchers report that mahseer in Asia has a very significant population decline, such as in Turkey, Pakistan, India, Bangladesh, Malaysia and Indonesia. One of the efforts to reduce pressure on mahseer fish populations in nature is to conserve and increase mahseer fish production through aquaculture.

Tor soro fish nursery technology has been well developed. Seed quality is one of the determining factors for the success of this farming. Good seed quality can be seen from the nursery unit that has good water quality maintenance and disease prevention management. One of the bacteria that can infect consumption fish or ornamental fish in freshwater is *Aeromonas hydrophila*. These bacteria are pathogenic and can cause mass death in cultured fish. Clinical symptoms caused by *Aeromonas hydrophila* bacteria are the appearance of wounds on the fish's body and can cause 100% of deaths in cultured fish. These bacteria can attack all ages and stages of fish [5]. One of the efforts to prevent this bacterial infection is to increase the fish's immune system, both specific and non-specific.

Many factors influence the performance of the fish's immune system. Some of them are the presence of stressors and environmental conditions of cultivation. The incidence of fish disease in fish farming tanks is incomplete compared to aquaculture systems with high-stress levels due to high stocking densities, bad handling, or predators in aquaculture tanks [6]. Meanwhile, the quality of the rearing water and the conditions of the cultivation tank that are not suitable for fish's biological needs often suppress the performance of the fish's immune system so that the fish are unable to survive during disease outbreaks [7]. Several researchers have tried to manipulate the physiological performance of fish by conditioning the culture environment to be more comfortable and resemble its original condition in nature, known as environmental enrichment in cultivation media [8].

Many environmental enrichment studies have been carried out on terrestrial cultivated animals, and even some findings have been adopted in the livestock industry. The same thing opens up the same opportunities for aquaculture.

Research by Raiha [7] proved that salmon (*Salmo salar*) and trout (*Salmo trutta*) reared in a culture environment enriched with submerged objects had a better survival rate when challenged with the pathogenic bacteria *Flavobacterium columnare* compared to the same fish, but maintained in a non-enriched culture environment. These findings indicate that genes associated with encoding the immune response will be expressed much better and work optimally in fish reared in a culture environment similar to their natural conditions.

Raiha [7] revealed the alternative opportunities in aquaculture animal health management practices to reduce fish mortality caused by disease outbreaks by increasing fish immunity performance. So far, improving the performance of the fish immunity is usually achieved by immuno-stimulation [9] and fish vaccination [10]. Vaccine effectiveness is usually done by giving immunostimulants before vaccination [11] or using adjuvants during vaccination [12]. The use of adjuvants for tiny seeds is not practical because adjuvants are usually given by injection, so efforts are needed to find other alternatives to increase the effectiveness of vaccines in fish at the seed stage. Environmental enrichment in mahseer fish rearing media is expected to increase the effectiveness of vaccines and minimize the impact of diseases caused by *Aeromonas hydrophila* bacteria in the vaccinated seed population.

2. Materials and Methods

2.1. Materials

The fish used in this research activity was *Tor soro* fish obtained from Cijeruk Germplasm Installation. Fish were reared as stock populations in fiber tanks with a water volume of 300 L and given vigorous aeration. Feeding was conducted three times a day at 08.00 am, 12.00 am, and 4.00 pm. The water quality was maintained according to SNI 01-747.2-2009, including temperature 29-30ºC, pH 6.5-8.5, DO 4.1-5.6 mg/L, TAN 0.06-0.2 mg/L. The plants that used were *Asplenium scolopendrium* and silica sand were obtained from commercial suppliers in the Sukabumi area. Meanwhile, the isolates of *Aeromonas hydrophila* were obtained from the culture collection of the Aquaculture Health Laboratory, Department of Aquaculture, IPB University.

2.2. Methods

2.2.1. Aeromonas Hydrophila Preparation

The culture of *A. hydrophila* that has been identified with API 20 E Kit (94% similarity) and has recovered its virulence in the Fish Health Laboratory, Department Aquaculture, IPB University was grown in trypticase soy broth (TSB, Sigma Aldrich) modification as much as 10 ml and incubated at 37°C for 24 hours. The culture obtained was then estimated for density by total plate count (TPC) on trypticase soy agar (TSA) media and incubated for 24 hours in an incubator at 37°C. Then, the pathogenic cultures were centrifuged for 10 minutes at 3000 rpm to separate the bacteria from the media. Pathogenic cells are then used for the vaccine manufacturing process.

2.2.2. Vaccine Preparation and Administration

The procedure for vaccine preparation followed the method described by Nur [13]. The fish to be vaccinated are placed in a 1-litre aquarium. The vaccine was soaked for 30 minutes in the aquarium, and then the fish were transferred to a rearing tank.

2.2.3. Challenge Test

The challenge test of fish was carried out on the 21st day since fish were stocked in the rearing aquarium by immersing the fish seeds for 15 minutes into a suspension of pathogenic bacteria *A. hydrophila*, carried out in a 1-litre volume. The concentration of the challenge test used the LC50 value obtained from the bioassay test. The fish that have been challenged are transferred back to maintenance according to their respective treatments. The chronology of tested and measured parameters is presented in Figure 1. below.

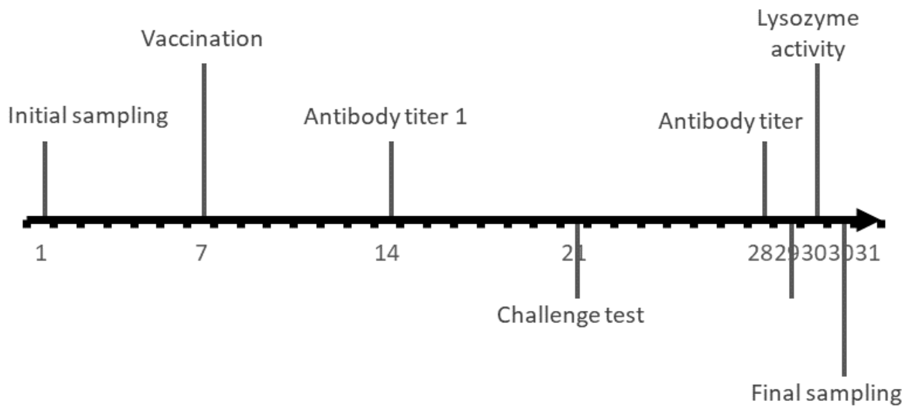

Figure 1. Timeline experiment and measurements in this study for 31 days. Initial sampling and final sampling were measurements of body weight, body length, and survival rate

2.2.4. Experimental Design

This research was conducted from October to December 2020 at Field Laboratory, Production Technology and Management of Aquaculture Program Study, College of Vocational Studies, Bogor Agriculture University (IPB). This study used a completely randomized design with four treatments and three replications, i.e. positive control treatment, without vaccinated with challenged test (C+); negative control, without vaccinated and without challenging test (C-); vaccinated fish without enrichment (A); vaccinated fish with environmental enrichment of *Asplenium scolopendrium* and silica sand (B). Especially for the treatment with environmental enrichment, the aquarium was given a substrate in the form of 1 kg silica sand and *Asplenium scolopendrium* with a total of six plants spread across the bottom of the aquarium.

2.2.5. Relative Percent Survival (RPS)

To determine the effect of treatment on the survival of the test animals, a comparison was made relative to the survival of the positive control, known as relative percent survival (RPS). Relative percent survival was calculated using the following formula:

$$\text{RPS}(\%) = 1 - \left(\frac{death\ on\ treatment}{death\ in\ positive\ control}\right) \times 100$$

2.2.6. Immune Response

The immune parameters included lysozyme activity, respiratory burst, and antibody titer. Lysozyme activity and respiratory burst performance were measured at the end of the observation for all treatments. Antibody titer was carried out twice on D-7 and D+7 of the challenge test. Lysozyme activity was determined using the microplate reader following the method [14]. Respiratory burst assay was evaluated by NBT assay by using a spectrophotometer UV-VIS according to [15]. Antibody titers were carried out of agglutination test using a 96-well microdilution plate following the method described [13].

2.2.7. Growth Performance

Evaluation of growth performance was measured after 31 days of maintenance. Feed utilization were conducted by determining survival rate (SR), feed conversion ratio (FCR), total feed intake (TFI), average daily growth (ADG), weight gain, length gain, and survival rate. Growth parameters were calculated with a formula from Efendi [16].

2.2.8. Data Analysis

Data obtained was tabulated and presented in a table and graph using Microsoft Excel 2017. The research parameters were analyzed statistically with the ANOVA test to examine the treatment effect. It was further tested with the Least Significance Different test to determine the difference between the treatments.

3. Results and Discussion

3.1. Results

3.1.1. Relative Percent Survival (RPS)

The RPS value describes how much a treatment improves the survival of the test animals if compared relatively with the control treatment test animals (C+). In this study, the addition of external stimuli in the form of aquatic plants and silica sand significantly affected the RPS value ($P<0.05$). The graph of fish seeds RPS is presented in Figure 2 below.

3.1.2. Lysozyme Activity

Lysozyme activity and respiratory burst are parameters of non-specific immunity in fish seeds. The performance of these parameters at the end of the observation for all treatments is presented in Figure 3 below. The highest increase in the lysozyme activity value in treatment B was 8,667 units/mL compared to all vaccinated and control treatments.

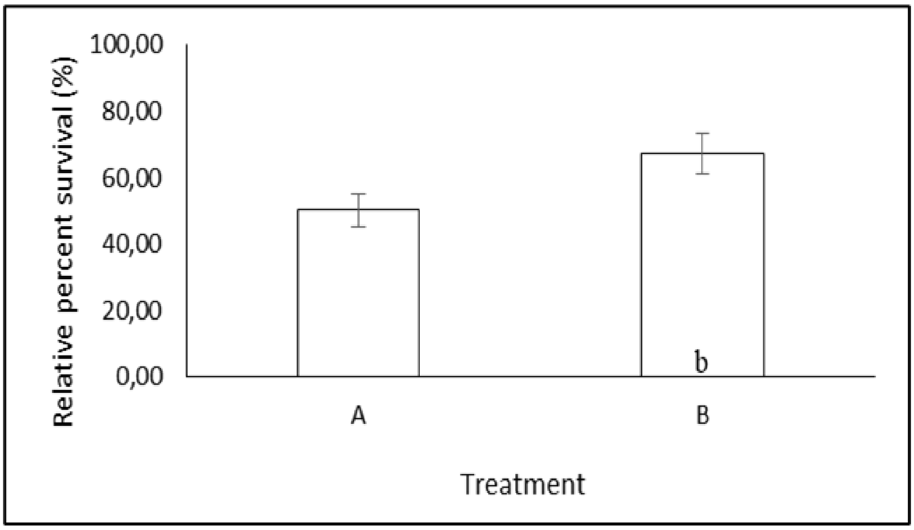

Figure 2. RPS value of fish seeds on the 31st day of observation in fish populations vaccinated with treatment without enrichment (A) and enrichment (B). Different letters on the bar graph indicate a significant difference (P>0.05) between treatments

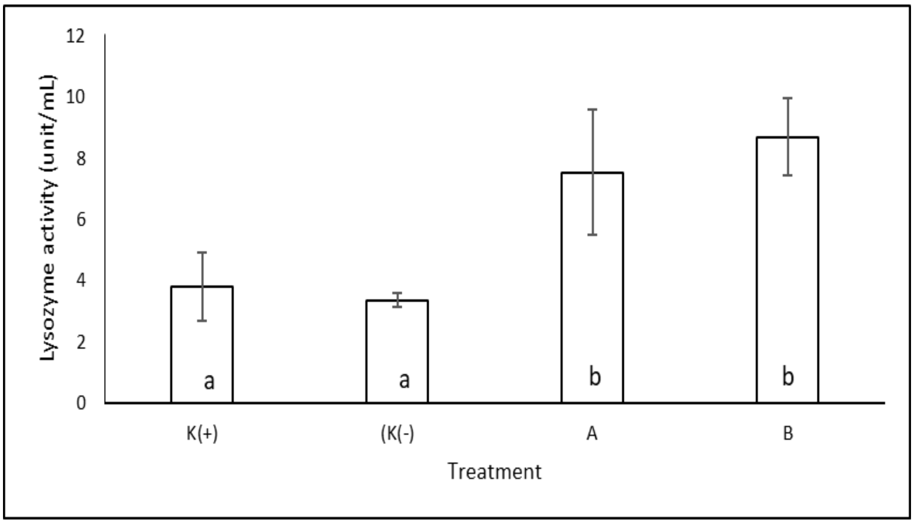

Figure 3. Lysozyme activity in fish seeds on the last day of observation on positive control (C+), negative control (C-), without enrichment (A) and with enrichment (B). Different letters on the bar graph indicate a significant difference (P>0.05) between treatments

3.1.3. Respiratory Burst

The performance of these parameters at the end of the observation for all treatments was not significant in all treatment groups presented in Figure 4 below. Vaccinated fish with enrichment (treatment B) had the highest increase in respiratory burst at 0.607.

3.1.4. Antibody Titer

Antibody concentrations in titer values (semi-quantitative) in fish seeds measured on the day before (H-7) and on the day after (H+7) the day of the challenge test was not significant in all treatment groups. Antibody titer after the challenge test on treatments A and B were higher than pre-challenge test, presented in Figure 5.

3.1.5. Growth Performance

After one-month cultivation, the environmental enrichment (treatment B) had the highest survival rate of about 88% (P<0.05) among the treatments, but had no effect (P>0.05) on weight and length gain, average daily growth, total feed intake, and feed conversion rate. The performance is presented in Table 1 below.

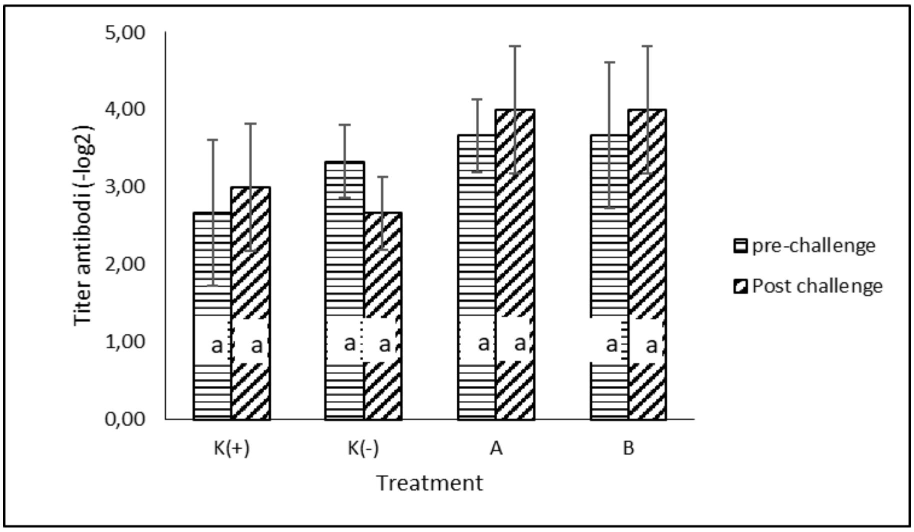

Figure 4. Respiratory burst activity in fish seeds on the last day of observation on positive control (K+), negative control (K-), without enrichment (A) and with enrichment (B). The same letter on the bar graph indicates no significant difference (P>0.05) between treatment

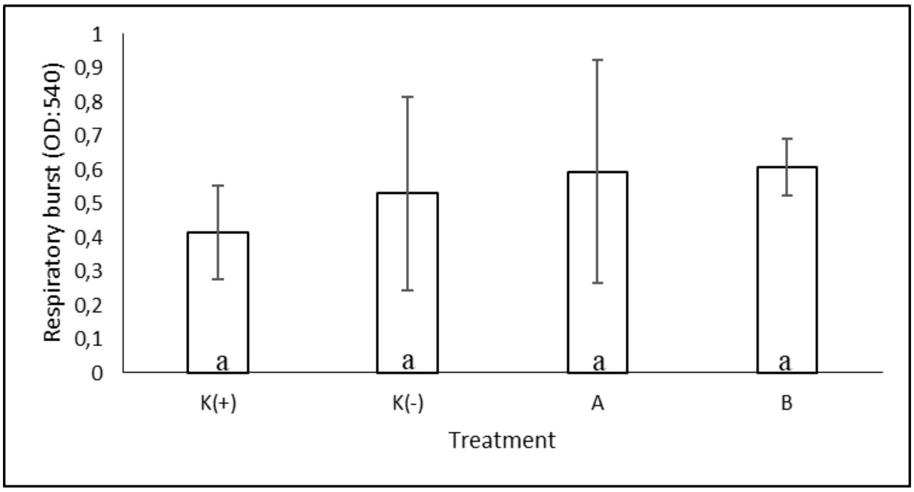

Figure 5. Antibody titer values in fish seeds on the last day of observation in the positive control (K+), negative control (K-), without enrichment (A) and with enrichment (B). The same letters on the bar graph indicate no significant difference (P>0.05) between antibody titer values in the same treatment

Table 1. Growth Performance of *T. soro* Fish Seeds Reared in Different Treatments

Parameters	Treatment			
	C (+)	C (-)	A	B
Weight gain (g)	6.85 ±4.06[a]	8.03 ±3.25[a]	4.84 ±2.48[a]	6.29 ±1.77[a]
Length gain (mm)	0.74 ±0.07[a]	0.42 ±0.22[a]	0.93 ±0.17[a]	0.74 ±0.10[a]
Average daily growth (g/day)	0.23 ±0.14[a]	0.27 ±0.11[a]	0.16 ±0.08[a]	0.21 ±0.06[a]
Total food intake (g)	19.41 ±1.85[a]	20.52 ±1.03[a]	19.34 ±1.08[a]	19.04 ±1.02[a]
Feed conversion ratio (g/g)	0.68 ±0.06[a]	0.72 ±0.03[a]	0.64 ±0.07[a]	0.59 ±0.06[a]
Survival rate after challenge test (%)	63.33 ±5.77[a]	77.3 ±4.62[b]	82 ±1[b]	88 ±3.46[c]

Note: Values are mean ±standard deviation. Different superscript letters on the same line indicate a significant difference (P<0.05) between treatments. K(+) = positive control, K(-) = negative control, A = fish vaccinated without environmental enrichment, B = fish vaccinated with environmental enrichment.

3.2. Discussion

As a candidate for aquaculture commodities, attempts to artificially spawn and grow the masheer group have been successfully carried out on fish species *T. soro* and *T. Douronensis* [3,17]. In fish-rearing facilities, limiting factors such as water quality, feed availability, and the presence of pathogens should be managed carefully. However, several factors that reflect other biological needs are sometimes missed, resulting in the loss of natural stimuli that might be important for the survival and development of fish. Those factors could be in the form of substrates such as gravel and aquatic plants [18], submerged shelter or objects [19]. audio stimuli [20], or the presence of predators [21]. The research was conducted to evaluate the immunity and growth performance of *T. soro* vaccinated against *A. hydrophila* by adding the aquatic plants and silica sand in rearing tank.

The LC50 for A. *hydrophilla* in T. *soro* was 10^6 CFU ml^{-1}. This value was used in the challenge test of fish seed populations, both vaccinated and unvaccinated fish. All treatments showed *A. hydrophila* infection in fish populations, but showed higher infected fish in positive control treatment (C+). Clinical symptoms include the appearance of red blood spots on the fish's body, loss of scales, anorexia, and swelling of the fish's abdomen. Some of these clinical symptoms are consistent with the findings of a study conducted by Firdausi [22].

The administration of the vaccine to *T. soro* fish seeds was proven to suppress fish mortality due to infection with the pathogen *A. hydrophila* by producing an RPS value of 50.28 ± 5.02%. The addition of external stimuli in the form of aquatic plants and silica sand in fish rearing containers that had been vaccinated increased the RPS value becomes 67.22±6.14%. This 16,94% increase in RPS confirms that a more complex rearing environment will provide a better stimulus to fish resistance to pathogens than a homogeneous culture environment. However, this increase cannot be adequately explained because, in this study, measurements of supporting parameters such as respiratory burst (RB) and antibody titer did not represent any natural effect from the enrichment. On the parameters of lysozyme activity, although the vaccinated fish populations (A and B) were significantly different from the control values, the addition of environmental enrichment (Treatment B) was not significantly different from those given only the vaccine (Treatment A). Thus, it is suspected that the increase in lysozyme activity was caused more by the administration of the vaccine and not by environmental enrichment. Research Brunet [19]. also found that humoral parameters such as lysozyme activity and complement activity in trout populations reared with PVC shelters, rocks, and plastic plants were not significantly different from the control treatment. It was suspected that the effect of environmental enrichment was not significant enough to be detectable at the level of molecules involved in humoral immunity.

Previously, some research has shown that salmon and trout reared in rocky containers have a high survival rate when exposed to columnar disease outbreaks [7] or during infection with the protozoan parasites *Ichthyopthirius multifiliis* and *Ichthyobodo necator* [23] and suspect that it was related to the performance of immune coding genes which were better expressed when it gets enough external stimuli. However, another possibility states that the high survival of fish 10^6 CFU mL^{-1} that reared in an enriched container environment is due to the emergence of a more complex microbial community on the surface of the submerged object. Those microbes suppress the growth of pathogenic bacteria through several competitive scenarios [24]. This may also explain why some humoral parameters, both specific (antibody titer) and non-specific (lysozyme and RB), in this study did not show significantly different values between treatments.

In this study, vaccination was proven to provide 82±1% fish survival, which was significantly different (P<0.05) from the K(+) treatment, which was only 63.33±5.77%. Furthermore, the addition of *Asplenium scolopendrium* and silica sand also had a significant effect (P<0.05) in increasing the survival of the *T. soro* that had been vaccinated to 88±3.46%. However, there was no significant difference in values (P>0.05) for other growth parameters such as average weight, absolute weight growth, absolute length growth, daily growth rate, biomass, and FCR. This finding is in line with the results of the study [25], which also found no significant difference in the growth of seabream fish reared in an environment enriched with ropes made of plant fibres.

The effect of environmental enrichment on fish growth has been reported to be inconsistent with each other by several researchers. Environmental enrichment provides better growth in trout [26], does not affect black rockfish [27] or even has a negative impact when overfeeding salmon [28]. According to Brunet [19], this difference may be due to differences in fish species, type of enrichment provided, amount, and the size of the enrichment coverage area in the culture container.

The natural environment is believed to be more complex than the aquaculture environment, and this difference can trigger stress in farm animals which has an impact on the welfare of farmed animals (animal welfare) which in turn will suppress physical performance such as growth and health [29]. Environmental enrichment is intended to restore the complexity of the cultivation environment so that it is expected to improve the welfare of farmed animals [30]. Although there are currently many inconsistent findings about the effect of environmental enrichment on fish growth, there have been reports of consistent positive effects of environmental enrichment, especially on behavior, aggressiveness, and predator avoidance (by hiding) in some fish species.

Environmental enrichment in *Tor putitora* increases fish exploration and hiding activities [18] and suppresses black rockfish's aggressiveness and stress levels [27]. Therefore, many researchers recommend enriching the aquaculture environment to support the welfare of aquatic animals, both in the fish-rearing industry [25] and to prepare fish seeds for restocking activities in public waters, so that fish are more alert to predation by other fish or reduce the incidence of aggression by other aquatic animals. Thus, the survival rate of fish restocked in natural waters is expected to be better.

4. Conclusions

Environmental enrichment treatment by using *Asplenium scolopendrium* and silica sand in *T. soro* fish-rearing container can increase the resistance of fish when challenged with *A. hydrophila* pathogens, especially in populations that have been vaccinated. Environmental enrichment in vaccinated fish populations gave an RPS value of 67.22 ± 6.14% and was significantly different from fish that were only vaccinated with an RPS of 50.28 ± 5.02%. Environmental enrichment did not provide significant differences for antibody parameters, lysozyme activity, respiratory burst, and growth in *T. soro*. It may be suggested that to increase the resistance of *T. soro* fish seeds, vaccination and environmental enrichment in the rearing container need to be given. If the size of the treated fish could be also big ie, also larger than that it would be better to justify the environmental and effect of the size too. Further research can focus on applying different substrates, fish size and scopes to the immunity and behavior of *T. soro* fish seeds.

Acknowledgements

This work has been funded by the College of Vocational Studies under the programme Research Grants 2020 with grant number (10225/IT3.S3/KS/2020)

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