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Fish Waste Alchemy: Innate and Acquired Immunity Assessment Followed Vaccinated against Kocuriasis and Investigate the Function of Low Molecular Weight Peptides of Kilka Fish Stick-water As Both Immunostimulant and Adjuvant in Farmed Rainbow Trout

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Abstract

Investigation of the immunogenicity of Caspian sea sprat stick-water, rich in hydrolysate protein was set as the aim of this study. Therefore, peptides below 3 kilo Daltons were isolated, and then the concentration of low molecular peptides (µg/µl) was measured. *Kocuria rhisophila* bacterium, which is known as one of the pathogenic agents of rainbow trout reared, was formalin- killed to make its vaccine with concentration of 2×10^9 CFU/ml in Tris-Buffered Saline. Eight treatments with 3 repetitions, consist of 240 individuals were considered. Three treatments were injected by concentrations of 10, 25, and 50 mg/body weight of stick-water containing low molecular peptides. In one treatment, bacterin was injected alone; in the three treatments, bacterin was injected along with doses of 10, 25, and 50 mg/body weight of the stick-water. In addition, Tris-Buffered Saline was injected into the control treatment. Next, to measure immunity, innate immunity factors were examined. Finally, in order to check the specific immunity, the challenge was done by immersion with a concentration of 1.2×10^8 CFU/ml of *Kocuria rhizophila*, after 14 days, the blood bacteremia test of all treatments was performed. According to the results, stick-water containing low molecule peptides was immunogenic in low doses

Introduction

The world's population is growing every year, which leads to increasing demand for valuable proteins, so the available resources must be used efficiently to use protein for human consumption. More than half of the world's consumed fish is supplied by the aquaculture industry and plays an important role in the supply of cost-effective protein in developing countries (FAO, 2016). Therefore, the main goal in aquaculture is to maximize production efficiency, which is achieved in intensive and semi-intensive cultures to achieve this goal. However, increasing the density of fish is a stressful factor, which can lead to infectious diseases and mortality. So, it is one of the most important limiting factors of productivity in aquaculture (Meena *et al.,* 2013; Santos and Ramos, 2018; Wangkahart *et al.,* 2019).

On the other hand, improper use of antibiotics to treat fish diseases has led to an increase in antibiotic-resistant pathogenic bacteria, environmental pollution, accumulation in fish tissue, and endangering the general health of the community (Caipang *et al.,* 2011). Considering the side effects of antibiotics as a threat, their use should be reduced or replaced with new products. Immunostimulants and vaccination represent two important strategies and appropriate alternatives to antibiotics that use to prevent and control diseases in fish (Villumsen *et al.,* 2017; Wang *et al.,* 2017; Wangkahart *et al.,* 2019).

A vaccine is a bio-preparation that provides active immunity to a specific disease (Wang *et al.,* 2020). Vaccines are often made from weakened or killed forms of the germ, its toxins, or one of its surface proteins. This factor stimulates the immune system to kill the antigen as a threat, as well as to identify and destroy any microorganisms associated with the substance that it may encounter in the future. Vaccination is one of the ways to prevent the disease by stimulating the host immune response (Liu *et al.,* 2017). As yet, various types of vaccines have been proposed, including formalin-killed vaccine (Yamasaki *et al.,* 2015), live attenuated vaccine, and DNA subunit vaccine (Choi and Kim, 2012).

In recent years, immunostimulants in aquatic animals have received more attention one of the main purposes of using immunostimulants is to strengthen the innate immune system of fish against pathogens. Immunostimulants, both as oral additives and as an adjuvant in vaccines, are used to activate macrophages as well as to stimulate a group of B lymphocytes to produce cytokines. The innate and adaptive immune systems are interconnected, and the signals sent by innate immune cells following infection or vaccination form the adaptive immune response in the next stages (Vallejos-Vidal *et al.,* 2016). Adjuvants enhance specific immune responses in combination with specific antigens and actually act to aid the vaccine (Angosto *et al.,* 2018). One of the most important immunostimulants is peptides. In recent years, the study of the mechanism of action of bioactive peptides of plants and animals' origin has increased dramatically. As a basic immunological mechanism, when infected with a pathogen, the innate immune system is more important than the specific immune system in bony fish. Antimicrobial peptides (AMPs) are an integral part of the innate immune system and play an important role in pathogen infection (Zhou *et al.,* 2016). AMPs are short polypeptides that have multiple biological functions and are considered significant candidates for the prevention and treatment of diseases in teleost fish, especially in antibiotic-resistant strains and drug-resistant pathogens (Pasupuleti *et al.,* 2012). In other words, molecules represent innate immunity and can modulate the function of macrophages (Zhang *et al.,* 2017), and they form the host's first line of defense against infectious microorganisms before stimulating the specific immune system (Kościuczuk *et al.,* 2012). What it has been observed in, in vitro and in vivo studies is that many bioactive peptides have selective toxicity against a wide range of cancer cells, as well as molecular and antimicrobial immune effects (Cicero *et al.,* 2017).

So far, studies such as the effects of vaccines against *Flavobacterium psychrophilum* (Hoare *et al.,* 2017) and *Yersinia ruckeri* (Wangkahart *et al.,* 2017) in rainbow trout (*Onchoryhnchus mykiss*), as well as Lactococcus garvieae in *Pseudoplatystoma sp* (Fukushima *et al.,* 2017) have been done. Additionally, studies about the use of synthetic tetrapeptide FK-56 (Treves-Brown, 2013), KLP-602 peptide (Siwicki *et al.,* 1998) and ISK short chain polypeptide have been performed in order to immunogenicity in rainbow trout.

According to recent studies, fish waste, including stick-water, provides high-quality proteins and it is now a low-consumption source of protein. Given that large volumes of effluents are produced in seafood processing operations that contain high concentrations of soluble proteins (Martinez-Montano *et al.,* 2020).

It seems that, so far, no studies are available on the use of fish stick-water in order to fish immunization; also, this is the first report in which the role of the *K. rhizophila* bacterin immunogenicity in the development of acquired immunity has been investigated. In this study potential of being immunostimulant and adjuvant of Kilka stick-water containing low molecular weight of peptides (LMWPs) in different doses; and also, the production of an autogenous vaccine from the bacterium *K. rhizophila*, to protect rainbow trout (*Oncorhynchus mykiss*) against Kocuriasis disease (Yeganeh *et al.,* publishing), has been evaluated.

Material and Methods

Isolation of Low Molecular Weight Peptides

At first, some Caspian sea sprat stick-water was prepared from a fishmeal factory then separated the oil phase by a pipette, the Kilka stick-water was centrifuged (High Speed Universal Centrifuge, PIT320 R (Cooled)) at 20000 g (4°C) for 20 minutes to remove solid and suspended particles then filtrarted with 0.45 µm. In the next step, in 30 kDa Millipore Filter for 45 minutes at 3000 g (4°C), then respectively in 10 kDa and 3 kDa Millipore filters for 15 minutes at 3000 g (4°C) was centrifuged (Geirsdottir, 2009). Finally, that was passed a 0.22 µm filter and stored at -20°C.

Peptides Concentration

The concentration of peptides was measured by Bradford method**.** BSA (Bovine Serum Albumin, Merck, Germany) standard protein was made 1 mg/ml concentration by distilled water. Then 1x reagent was prepared from Bradford reagent (Bio Basic, Canada) solution 5x (considering the ratio of 1 reagent into 5 distilled-water). 200 µl of 1x Bradford reagent was poured into the wells, then 0, 2, 4, 6, 8, 10 µl of BSA protein solution was added to each of them, respectively (two replicate). After 10 minutes at room temperature, the protein was measured at OD_{595nm} by an ELISA reader (Epoch, USA). A regression diagram was drawn according to the numbers obtained in Excel software (Bradford, 1976).

Bacterin Preparation

At the first, *K. rhizophila* bacterium after isolation from rainbow trout, was examined by PCR and 16Sr RNA sequencing for definitive diagnosis. The desired strain

was registered under name of *K. rhizophila* TMU97910 in the NCBI (Yeganeh *et al.,* publishing). After that an overnight bacteria culture (24 h) was prepared in 100 ml nutrient broth medium (Ibresco, Iran) at 22°C (200 rpm). Growth was monitored by spectrophotometer (OD_{600nm}, JENWAY 6305 UV/VIS). The bacteria concentration (CFU/ml) was estimated by Miles & Misra method (Miles and Misra, 1938). Bacteria cells were inactivated by the addition of 10% formaldehyde (Sigma-aldrich, America) (v/v) at room temperature during 24 h. After inactivation, the solution was centrifuged to remove formaldehyde (4068 g, 5 min), followed three-time wash by TBS (Tris-Buffered Saline; Bio Basic, Canada). After that, triplicate 100 µl of that on blood agar plates (5% sheep blood-Ibresco) was cultured to confirm inactivation of bacteria (24h, 22°C). Finally, the concentration was adjusted to 2×10^9 CFU/ml in TBS (Villumsen et al., 2017).

Fish Rearing Management

The number of 240 rainbow trout (Villumsen et al., 2017) with an average weight of 30±0.6 g was transferred to 24, 100-liter aerated tanks with similar conditions in terms of light, water temperature (16°C), oxygen (8 mg / l) and pH (6.2). They were fed daily to 2% of body weight (Fukushima *et al.,* 2017) with the standard commercial extruder (Beyza, Iran) during the experiment period.

In order to evaluate the specific and innate immunity of *K. rhizophila* bacterium and peptides under 3 KDa (Kilo Daltons), Stick-water in 3 doses of 50, 25, 10 mg/kg body weight (Jeney and Anderson, 1993) in the presence of *K. rhizophila* bacterin and without it was evaluated in 8 treatments and 3 replications (Table 1).

Vaccination and Stick-water Injection

Fish were anesthetized by immersion in Clove solution (Barij essence-Iran) (0.2 g/L). All of Injections were given intraperitoneally. The injectable dose of bacterin was 0.1 ml and control fish were injected by 0.1 ml of TBS [Fukushima *et al.,* 2017). The stick-water containing LMWPs were injected 10, 25 and 50 mg /kg body weight, respectively (Jeney & Anderson, 1993). Each fish was transferred to a tank containing freshly aerated water after injection. The duration of the course was 30 days.

Blood Sampling

For the CBC (complete blood count) test, each sample syringe was immersed in lithium or sodium salt heparin (5000 injection, Alborz Darou, Iran). The heparin was drawn from the syringe and then drained until wetting was evident. 10 µl of heparin was poured into 2 ml vial, then some blood was taken from the caudal vein of the fish with a heparin-coated syringe, then the needle of the syringe was removed and 0.5 ml of blood was poured into the heparin vial, and then several times (up and down) mixed. Blood vials were prepared for CBC. In order to separate plasma separation, the rest of blood was transferred to a glass vial and centrifuged twice at 3500 rpm for 5 minutes, and then the supernatant was separated and poured into a vial (without heparin). It is observed that in sampling to prepare serum, heparin should not be used. The samples were transported to the laboratory by maintaining a cold chain (without direct contact with ice). The collected plasma was frozen at -20°C for further steps (Demaire *et al.,* 2020).

Blood Analyses

White Blood Cell Count

3.88 g of sodium chloride, 2.50 g of sodium sulfate, 1.74 g of sodium phosphate, 0.25 g of potassium phosphate, 7.50 ml of formalin (37%), and 0.10 g of methyl violet with distilled water Make up to 1000 ml and pass through an average 10-watt man paper filter. To dilute the blood 1:200, using a red blood cell diluting pipette, 0.5 marks of blood were drawn and 100 marks of Natt-Herrick dye was obtained to dilute 1:200. Then mix well and keep at room temperature for 5 minutes. A Neubauer hemocytometer was soaked in blood on both sides (cells precipitated over 5 minutes), all white blood cells (mm^3) were counted in four large squares on either side of the hemocytometer, and all eight numbers were counted together. This total was used to calculate the number of white blood cells (Natt and Herrick, 1952).

$$\text{Number of white blood cells } (mm^3) = \frac{\text{total white blood cell count}}{2000*8}$$

Table 1. The specifications of treatment for the empirical groups (**P_{10}, P_{25}, P_{50}, P_{10+}, P_{25+}, P_{50+}, Co**)

Number	Treatments	Description
1	**P_{10}**	mg / kg stick-water containing low molecular weight peptides 10
2	**P_{25}**	mg / kg stick-water containing low molecular weight peptides 25
3	**P_{50}**	mg / kg stick-water containing low molecular weight peptides 50
4	**B**	*K. rhizophila* TMU bacterin without stick-water
5	**P_{10+}**	10 mg / kg stick-water containing LMWPs + *K. rhizophila* TMU bacterin
6	**P_{25+}**	25 mg / kg stick-water containing LMWPs + *K. rhizophila* TMU bacterin
7	**P_{50+}**	50 mg / kg stick-water containing LMWPs + *K. rhizophila* TMU bacterin
8	**Co**	Control (no bacterin, no stick-water)

The stick-water containing low molecular weight peptides was used in treatments P_{10}, P_{25} and P_{50} as immunostimulant approach. The Stick-water containing low molecular weight peptides with the bacterin was used in treatments P_{10+}, P_{25+} and P_{50+} as adjuvant approach

Red Blood Cells Count and Hemoglobin

RBC (Red Blood Cells) count and hemoglobin were determined by the method recommended by Feldman (Feldman *et al.,* 2002).

Hematocrit Measurement

Two micro-hematocrit capillaries (75 ml × 1.1 ml, Globe Scientific NJ) were filled directly from the blood syringe for HCT (hematocrit) observations and then centrifuged at 5400 g. HCT values were measured in 1 minute. Then 0.1 ml of the sample was transferred to another tube containing 0.9 ml of Eme (Ecgonine methyl ester, Cerilliant Corporation, Texas) essential fluid with 2% bovine serum (Fetal Bovine Serum, DENAzist Asia, Iran). A hemocytometer was used to count the cells (Siwicki *et al.,* 1994).

Total Protein Measurement

Determination of total serum protein was performed by Total Protein Kit (Pars peyvand, Iran). 5 µl of serum, 25 µl of reagent A and 200 µl of reactor B were combined in a microtiter well. The mixture was briefly mixed with a pipette and read at 650 nm after a 15-minute measurement using a spectrophotometer (Siwicki *et al.,* 1994).

Total Immunoglobulin Assay

100 µl of Serum is mixed with an equal amount of 12% polyethylene glycol (Merck Germany) and placed in a shaker incubator at room temperature for 2 hours. After centrifugation at 3000 g for 15 minutes, the supernatant is removed and the remaining protein is then removed from the total serum protein concentration (Siwicki *et al.,* 1994).

Determination of Lysozyme Activity

First, the PBS solution (Denazist Asia, Iran) was poured into a glass tube and some Staphylococcus aureus bacteria were added to it. Then 200 µl of the solution was read at a wavelength of 450 nm. In the next step, 15 µl of serum was added to it and after 20 minutes of storage at room temperature, the absorbance was read and then the absorption was compared with the standard curve prepared from egg white lysozyme (Sigma-Aldrich) and the amount of lysozyme (Merck, Germany). The sample was calculated based on the turbidity method in mg/ml (Sankaran and Gurnani, 1972).

Determination Complement Activity

Complement activity (ACH_{50}) was performed based on Yano method (Yano, 1992) and rabbit red blood cell hemolysis (RaRBC). In summary, 10 ml of serum was first diluted to 25 ml using ethylene glycol tetra acetic acid-magnesium-gelatin vernal buffer (Sigma-aldrich) and then 10 ml of rabbit red blood cells (Sigma-aldrich) was added to the test tube and at room temperature. They were incubated at 22 ° C for 2 hours. After this time, 3.5 ml of 90% sodium chloride (Merck, Germany) was added to each of the test tubes. Samples were centrifuged at 836 g for 5 minutes at 4 ° C. Then the absorbance at 414 nm was measured by spectrophotometer. The volume of serum that causes 50% of hemolysis is the complement activity of the sample, which is calculated from below Equation.

$$ACH_{50}\ (u/ml) = (k \times \text{dilution factor}) \times 0.5$$

Challenge Assays

Kocuria rhizophila was inoculated from blood agar (5% sheep blood- Ibresco, Iran) into the nutrient broth medium and incubated (Jal Tajhiz, JTSL20, Iran) for 24 hours at 200 rpm. 30 days after vaccination, the fish in the tanks were transferred to infectious tanks and exposed to water contaminated with Bacteria suspension $1.2*10^8$ CFU/ml were aerated for one hour, during which all tanks were carefully monitored, after that all of fish were returned to the main tanks and mortality was recorded in 14 days. Finally, the vaccine efficacy in specific immunity was calculated and expressed with the relative percent survival (RPS) according to below Equation. At the end, moribund fish kidney and liver tissues were cultured in blood agar medium to confirm the cause of death by the *K. rhizophila* TMU bacterium (Villumsen *et al.,* 2017).

$$\text{RPS}\,\% = 1 - \frac{\text{mortality in treated group}}{\text{mortality in infected control group}}$$

Blood Bacterial Invasion and Clearance after Challenge

Two weeks after challenge, blood was collected aseptically 100 µl of blood per fish of each treatment was mixed with 900 µl sterile PBS (Denazist Asia, Iran) and homogenized with vortex. Then a ten-fold dilution was prepared from each treatment and 100 µl of each dilution were cultured in nutrient agar (Ibresco, Iran) plates and incubated at 22°C for 24 hours. In the end, the number of colonies (CFU/ml) was calculated (Nguyen *et al.,* 2017).

Histopathology

Two weeks after challenging, liver tissue samples collected from moribund fish in each treatment were placed in 10% formalin (Sigma-Aldrich) for 24 hours before processing. Then they were dewatered, clarified, and paraffinized in a Tissue processor (DS2080/H, Iran) and Tissue float (Labtron, Iran). Samples molded with a Microtome Accu-Cut (Accu-Cut® SRM™ 200 Rotary Microtome) were cut to a thickness of 5 microns.

Staining was performed by conventional hematoxylin and eosin methods. Tissue incisions were glued to the slide with antaline glue (Merck, Germany). They were then examined with a light microscope at up to 100× magnification equipped with a camera (Optika, B-293, Italy) in order to capturing images (Legario *et al.,* 2020).

Statistical Analysis

The sampling and analysis of this study were done completely randomly. One-way analysis of variance (ANOVA) was used to determine the effect of stick-water peptides on the proposed performance parameters of rainbow trout. Duncan's Test at a = 0.05 was used to determine the difference between the tests. All data were analyzed using SPSS statistical software (version 22). Excel software was used to draw charts and tables.

Results

Peptide Concentration Assay by Bradford Method

Accordingly, the concentration of micro molecular peptides in stick-water after ultrafiltration was evaluated at 1.92 ug/ul (Table 2). Concentration regression is shown in (Figure 1).

Humoral Factors

According to the results, the number of red blood cells (RBC), Hemoglobin (HB), and Hematocrit (HCT) in Co and P_{50} treatments were not significantly different ($p>0.05$). In most parameters, the low doses treatment was more significant than others ($p<0.05$) (Table 3).

The number of white blood cells and the percentage of Heterophils were significantly different between all treatments ($p<0.05$), the highest was in the P_{10} treatment and the lowest was in the P_{50} treatment. The percentage of monocytes was not significantly different between control and P_{50} treatments ($p>0.05$) but in P_{25} treatment it had the highest percentage with significant difference ($p<0.05$). The percentage of eosinophils was not significantly different between P_{10}, P_{25}, and P_{50} treatments ($p>0.05$), and also its amount was equal in the mentioned treatments, but a significant difference was observed compared to the control treatment ($p<0.05$). Lymphocyte count was significantly different in all treatments ($p<0.05$) so that the highest percentage of lymphocytes was observed in the P_{10} treatment and the lowest percentage of lymphocytes was observed in the P_{50} treatment (Figure 2).

Effect of *K. rhizophila* TMU bacterin injection without and with stickwater containing LMWPs at different levels (10, 25, 50 mg/kg) with an adjuvant approach in rainbow trout on blood factors in Table (4) shown. According to the results, the number of Red Blood Cells had the highest in P_{10+} treatment and the lowest in P_{50+} treatment ($p<0.05$). The amount of hemoglobin and hematocrit in B and control treatments were not significantly different ($p<0.05$) but these parameters were significantly higher in P_{10+} treatment and lowest in P_{50+} treatment ($p<0.05$).

The number of white blood cells was significantly higher in P_{10+} treatment and the lowest in P_{50+} treatment. It is noteworthy that in treatment B it increased significantly and after P_{10+} treatment showed the highest number of white blood cells ($p<0.05$). The amount of Heterophils with a significant difference showed the highest value in P_{10+} treatment and the lowest value in P_{50+} treatment ($p<0.05$). Monocyte content was not significantly different between treatments B and P_{10+} ($p>0.05$), while both showed the highest value compared to other treatments ($p<0.05$).

Table 2. The concentration of low molecular weight peptides of Caspian sea sprat stick-water

Sample Uptake Average	Total Sample Concentration	Final Concentration (ug/ul)
286/333	9/60	1/92

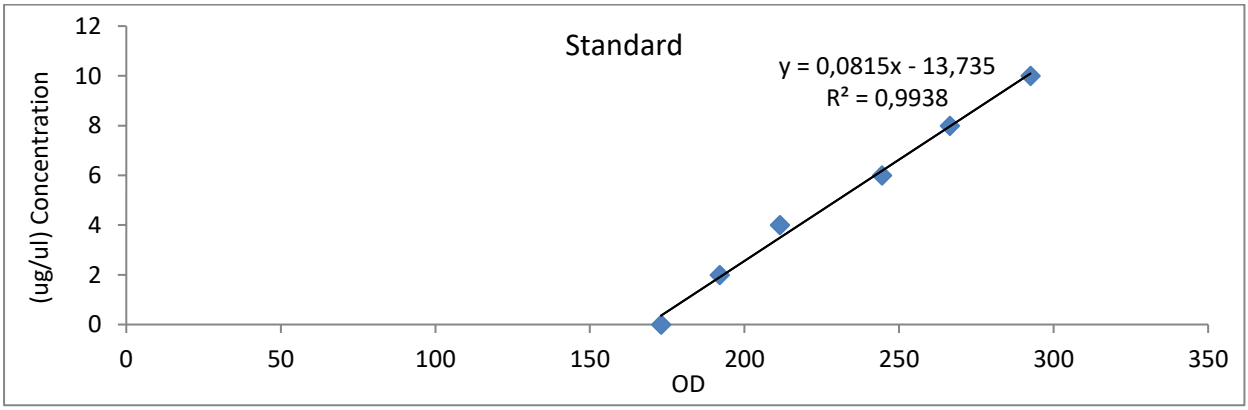

Figure 1. Concentration regression of low molecular weight peptides of Caspian sea sprat stick-water

Table 3. Blood factors of rainbow trout injected with stick-water containing Low molecular weight peptides at different levels (10, 25, 50 mg/kg) as immunostimulant (P_{10}, P_{25}, P_{50}) over a period of 30 days

Parameters	Treatments			
	P_{10}	P_{25}	P_{50}	Co
RBC 10^6 (Mm^3)	1.75 ± 0/02 [b]	1/87± 0/02 [a]	1/57± 0/03 [c]	1/55± 0/03 [c]
HB g/dL	8/6± 0/05 [a]	8/9± 0/17 [a]	7/50± 0/2 [b]	7/50± 0/26 [b]
HCT %	52± 0/1 [b]	55± 0/4 [a]	46± 0/26 [c]	45± 0/34 [c]

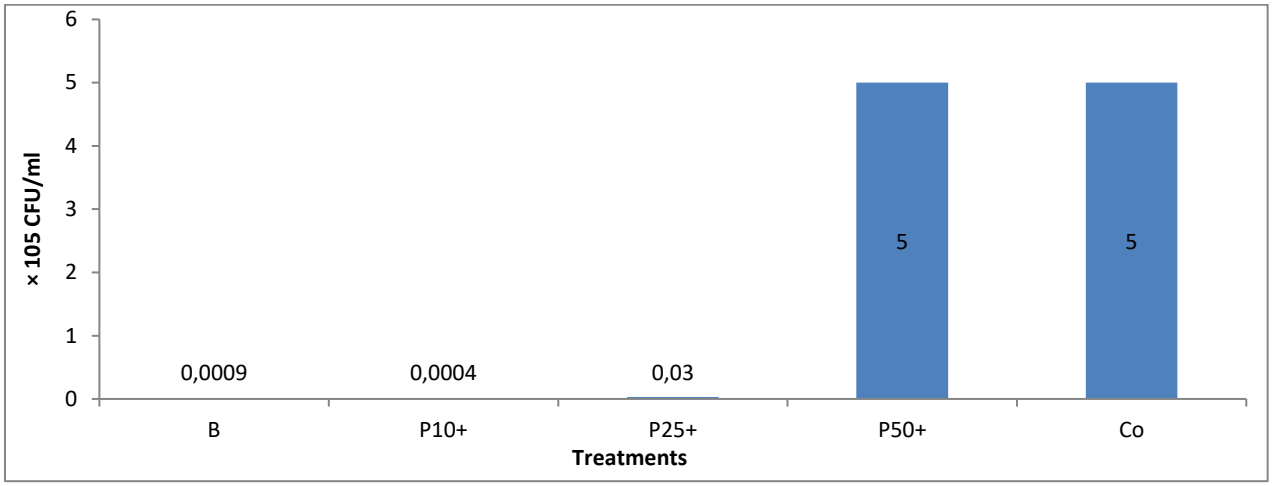

Figure 2. The number of the bacterium colonies in the blood of rainbow trout were injected with the *K. rhizophila* bacterin along with and without stick-water containing micro molecular peptides at different levels (10, 25, 50 mg/kg). Data are expressed in SD±ME. Different letters indicate a significant difference between the means ($p<0.05$). B: bacterin, P_{10+}: Injectable treatment with the bacterin along with stick water containing micro molecular peptides at a dose of 10 mg/kg, P_{25+}: Treatment injected with the bacterin along with stick water containing micro molecular peptides at a dose of 25 mg/kg, P_{50+}: Injected treatment with the bacterin along with stick Water contains micro molecular peptides at a dose of 50 mg/kg, Co: control treatment.

Table 4. Blood factors of rainbow trout injected with the *K.rhizophila* bacterin along with and without stick-water containing low molecular weight peptides at different levels (10, 25, 50 mg/kg) as adjuvant (**P_{10+}, P_{25+}, P_{50+}**) over a period of 30 days

Parameters	Treatments				
	B	P_{10+}	P_{25+}	P_{50+}	Co
RBC 10^6 (Mm^3)	1/57± 0/02[ab]	1.6±0.01[a]	1.45± 0.26[c]	1/43±0.02[c]	1/55± 0/03[b]
HB g/dL	7/5± 0/4[b]	8±0.26[a]	7.2± 0.05[bc]	6.9±0.17[c]	7/50± 0/26[b]
HCT %	45± 0/65[b]	48±0.6[a]	42± 0.3[c]	41± 0.43[d]	45± 0/34[b]

The lowest number of monocytes in P_{50+} treatment was observed. The highest number of eosinophils was observed with a significant difference in P_{10+} treatment ($p<0.05$). The highest lymphocyte count was observed with a significant difference in P_{10+} treatment and the lowest lymphocyte count was observed in the control treatment. It is noteworthy that the number of lymphocytes in treatment B with a significant difference after P_{10+} showed the highest value compared to others ($p<0.05$) (Figure 3).

Serum Immunity-Related Factors

The extent of changes in total protein activity, lysozyme activity, ACH_{50} activity, and total immunoglobulin in treatments injected with stick-water containing LMWPs at different levels (10, 25, 50 mg/kg) as an immunostimulant is shown in Figure 4. According to the results, a significant difference was observed between all treatments in all four parameters ($p<0.05$) so that total protein had the highest value in the P_{10} treatment and the lowest value in the control treatment. Lysozyme activity, ACH_{50} activity, and total immunoglobulin had the highest value in the P_{10} treatment and the lowest value in the P_{50} treatment.

The extent of changes in total protein activity, lysozyme activity, ACH_{50} activity, and total immunoglobulin in treatments with *K. rhizophila* TMU bacterin injection without and with stick-water containing micro molecular peptides at different levels (10, 25, 50 mg/kg) as an adjuvant is shown in Figure 5. Based on the results, the levels of total protein, complement, and immunoglobulin activities in P_{10+} and P_{50+} treatments showed the highest and lowest values, respectively ($p<0.05$). Lysozyme activity was highest in P_{10+} treatment and lowest in P_{50+} and Co treatments

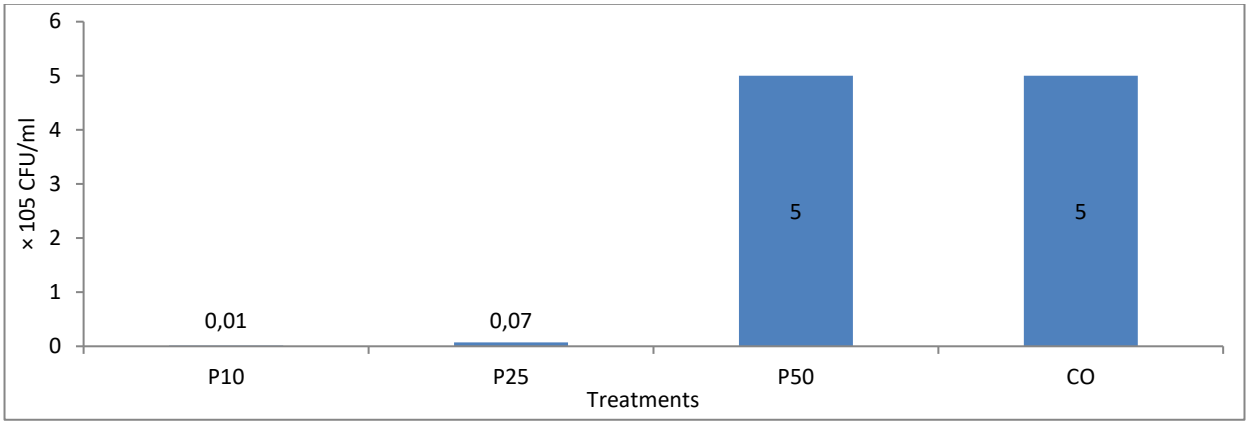

Figure 3. The number of the bacterium colonies in the blood of rainbow trout were injected with stick-water containing Low molecular weight peptides at different levels (10, 25, 50 mg/kg). injected with peptide 2 weeks after the challenge. Data are expressed in SD±ME. Different letters indicate a significant difference between the means ($p<0.05$). P_{10}: Injectable treatment with stick-water containing Low molecular weight peptides at a dose of 10 mg/kg, P_{25}: Treatment injected with stick-water containing Low molecular weight peptides at a dose of 25 mg/kg, P_{50}: Injected treatment with stick-water contains Low molecular weight peptides at a dose of 50 mg/kg Peptide, Co: control treatment.

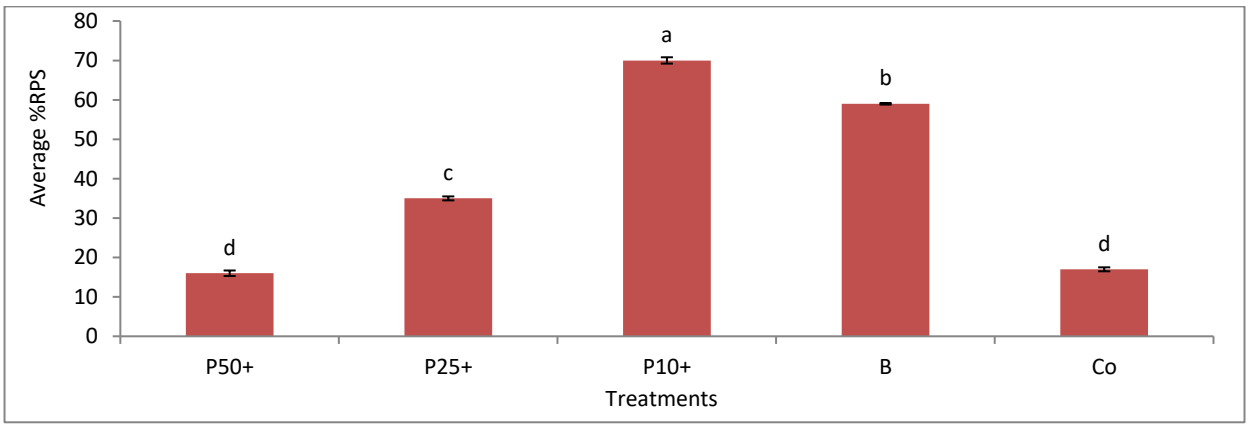

Figure 4. Relative percentage survival in rainbow trout were injected with the *K. rhizophila* bacterin along with and without stick-water containing micro molecular peptides at different levels (10, 25, 50 mg/kg). Data are expressed in SD±ME. Different letters indicate a significant difference between the means ($p<0.05$). B: bacterin, P_{10+}: Injectable treatment with the bacterin along with stick water containing micro molecular peptides at a dose of 10 mg/kg, P_{25+}: Treatment injected with the bacterin along with stick water containing micro molecular peptides at a dose of 25 mg/kg, P_{50+}: Injected treatment with the bacterin along with stick Water contains micro molecular peptides at a dose of 50.

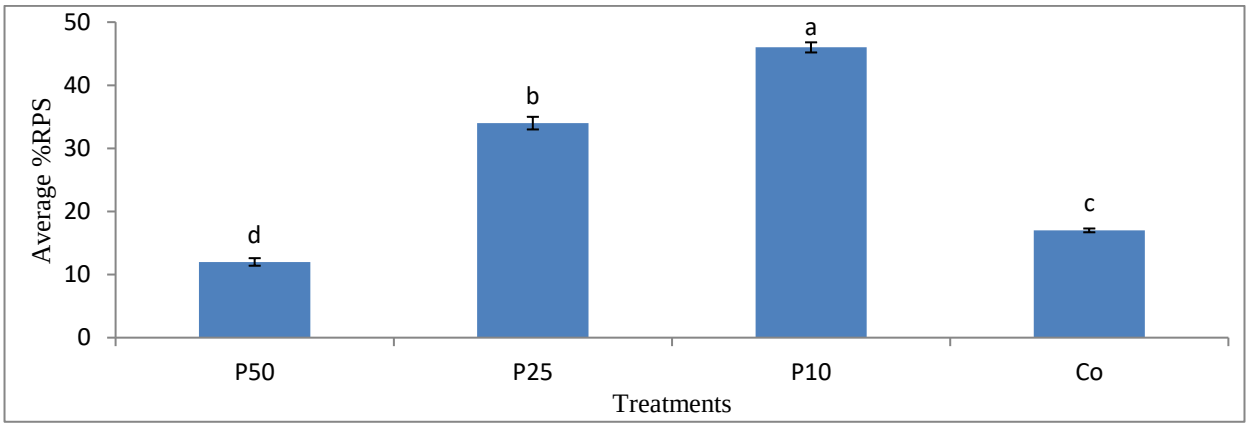

Figure 5. Relative percentage survival in rainbow trout were injected with stick-water containing Low molecular weight peptides at different levels (10, 25, 50 mg/kg). Data are expressed in SD±ME. Different letters indicate a significant difference between the means ($p<0.05$). P_{10}: Injectable treatment with stick-water containing Low molecular weight peptides at a dose of 10 mg/kg, P_{25}: Treatment injected with stick-water containing Low molecular weight peptides at a dose of 25 mg/kg, P_{50}: Injected treatment with stick-water contains Low molecular weight peptides at a dose of 50 mg/kg Peptide, Co: control treatment.

($p<0.05$). It is noteworthy that treatment B increased significantly and after P_{10+} treatment had the highest amount of lysozyme, complement, and total immunoglobulin ($p<0.05$).

Fish Immune Responses after Vaccination

The results of the relative percentage survival of treatments treated with stick-water containing micro molecular peptides at different levels (10, 25, 50 mg/kg) as an immunostimulant after challenge with the *K. rhizophila* TMU are shown in Figure 6. According to the results, relative percentage survival in P_{10} treatment was 46% ($p<0.05$), in P_{25} treatment, 34% in the control treatment, 17%, and in P_{50} treatment in the lowest value of 12% was observed after the challenge.

According to the results in bacterin injected group, P_{10+} treatment with RPS 70% significantly ($p<0.05$) showed the highest survival rate of rainbow trout infected with *K. rhizophila* TMU infection, in treatment B, 59 % in P_{25+} treatment, 35% in the control treatment, 17% and in P_{50+} treatment in the lowest amount of 16% was observed after challenge (Figure 7)

Blood Bacterial Invasion and Clearance after Challenge

In the stick-water injected group, the bacterial load in P_{10}, P_{25}, P_{50}, and Co was (1×10^3 CFU/ml), (7×10^3 CFU/ml), (5×10^5 CFU/ml), and (5×10^5 CFU/ml) respectively (Figure 8).

These results were as follows in the group injected with bacterin. the Number of the bacterium in B treatment (9×10^1 CFU/ml), P_{10+} treatment (4×10^1 CFU/ml), P_{25+} treatment (1×10^3 CFU/ml), P_{50+} treatment (5×10^5 CFU/ml), and in Co treatment (5×10^5 CFU/ml) was observed (Figure 9).

Histopathology

The results of histopathology in the group injected with bacterin and the control exposed after 14 days challenge with *K. rhizophila* TMU bacterium are shown in Figure 10. The histopathology of liver tissue from a healthy rainbow trout without exposure to bacterium is also shown for comparison in Figure 10f. In the liver tissue of P_{10+} treated fish, accumulation of melanomacrophages, sinusoid dilation, focal necrosis

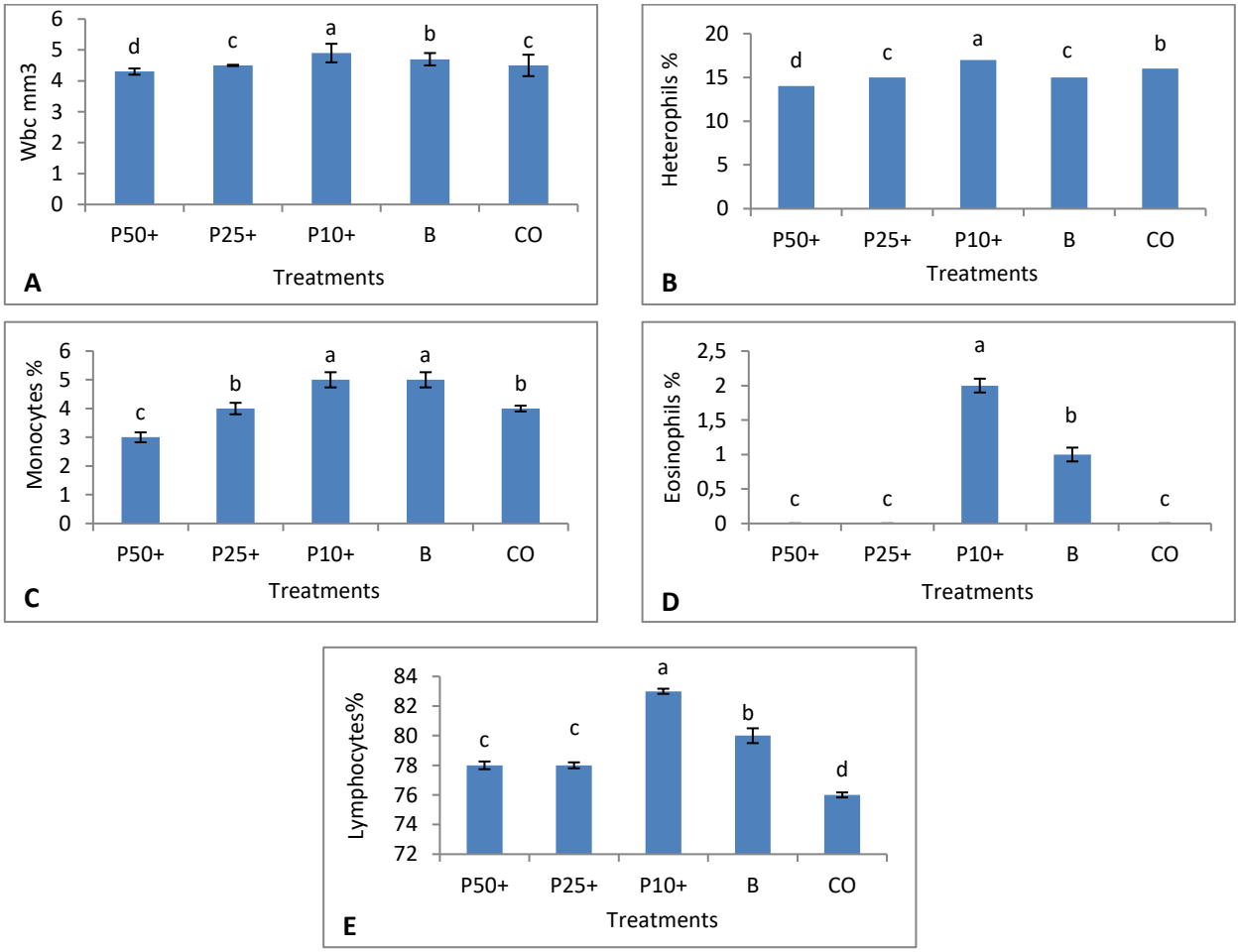

Figure 6. White blood cells in rainbow trout were injected with the *K.rhizophila* bacterin along with and without stick-water containing Low molecular weight peptides at different levels (10, 25, 50 mg/kg). A) White blood cells B) Heterophil C) Monocyte D) Eosinophil E) Lymphocytes. Data are expressed in SD±ME. Different letters indicate a significant difference between the means ($p<0.05$). B: bacterin, P_{10+}: Injectable treatment with the bacterin along with stick-water containing Low molecular weight peptides at a dose of 10 mg/kg, P_{25}+: Treatment injected with the bacterin along with stick-water containing Low molecular weight peptides at a dose of 25 mg/kg, P_{50}+: Injected treatment with the bacterin along with stick-water contains Low molecular weight peptides at a dose of 50 mg/kg, Co: control treatment.

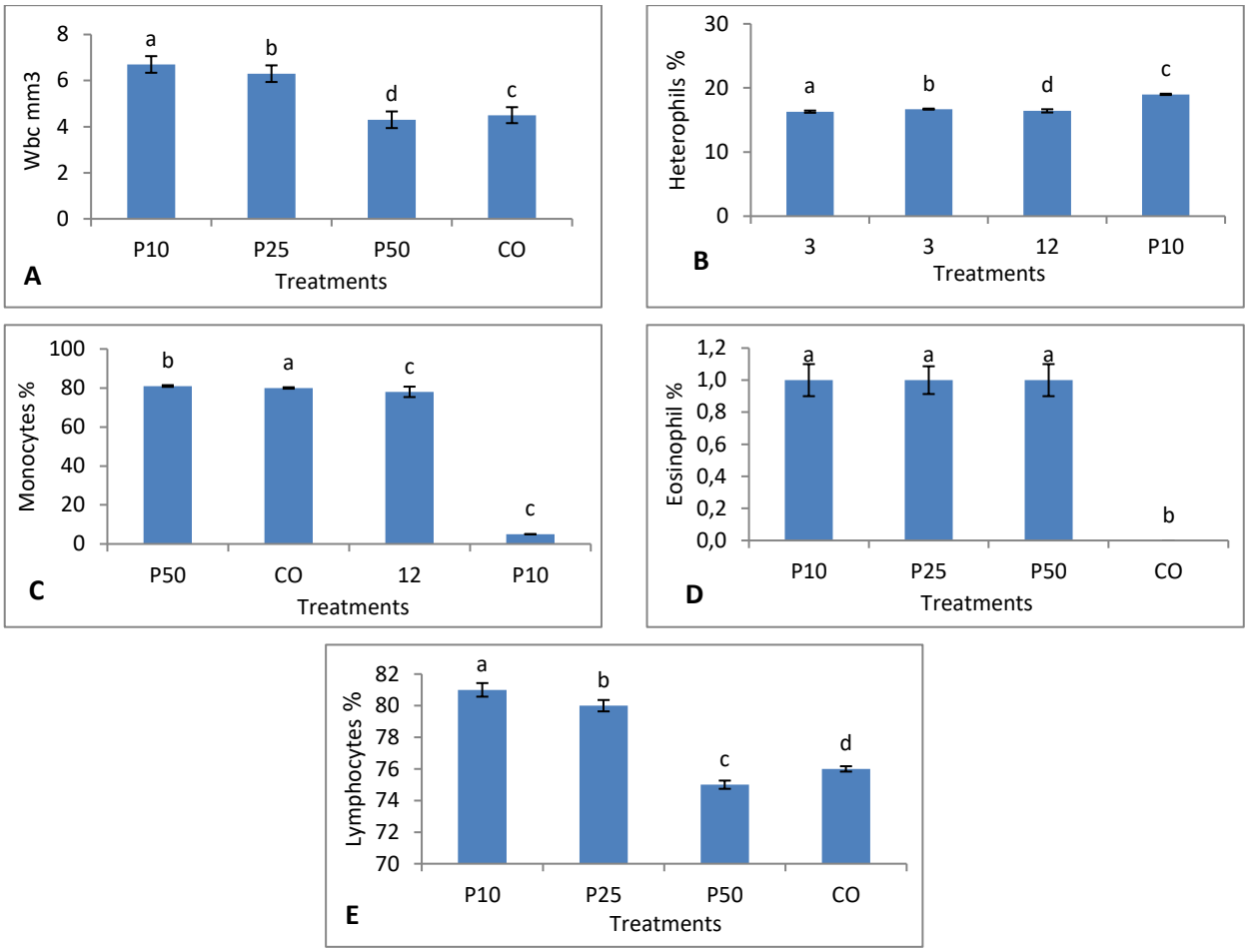

Figure 7. White blood cells in rainbow trout were injected with stick-water containing Low molecular weight peptides at different levels (10, 25, 50 mg/kg). A) White blood cells B) Heterophil C) Monocyte D) Eosinophil E) Lymphocytes. Data are expressed in SD±ME. Different letters indicate a significant difference between the means ($p<0.05$). P_{10}: Injectable treatment with stick-water containing Low molecular weight peptides at a dose of 10 mg/kg, P_{25}: Treatment injected with stick-water containing Low molecular weight peptides at a dose of 25 mg/kg, P_{50}: Injected treatment with stick-water contains Low molecular weight peptides at a dose of 50 mg/kg Peptide, Co: control treatment

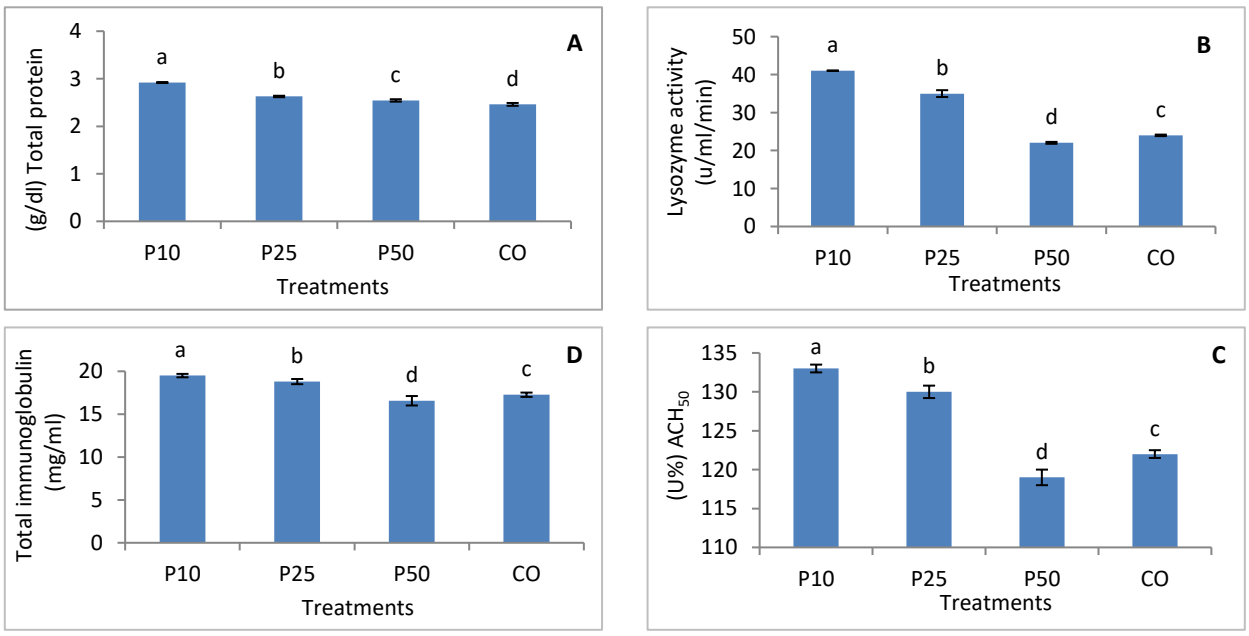

Figure 8. Serum immunity-related factors in rainbow trout were injected with stick-water containing Low molecular weight peptides at different levels (10, 25, 50 mg/kg). A) Total protein, B) Lysozyme activity C) ACH_{50} D) Total immunoglobulin. Data are expressed in SD±ME. Different letters indicate a significant difference between the means ($p<0.05$). P_{10}: Injectable treatment with stick-water containing Low molecular weight peptides at a dose of 10 mg/kg, P_{25}: Treatment injected with stick-water containing Low molecular weight peptides at a dose of 25 mg/kg, P_{50}: Injected treatment with stick-water contains Low molecular weight peptides at a dose of 50 mg/kg Peptide, Co: control treatment.

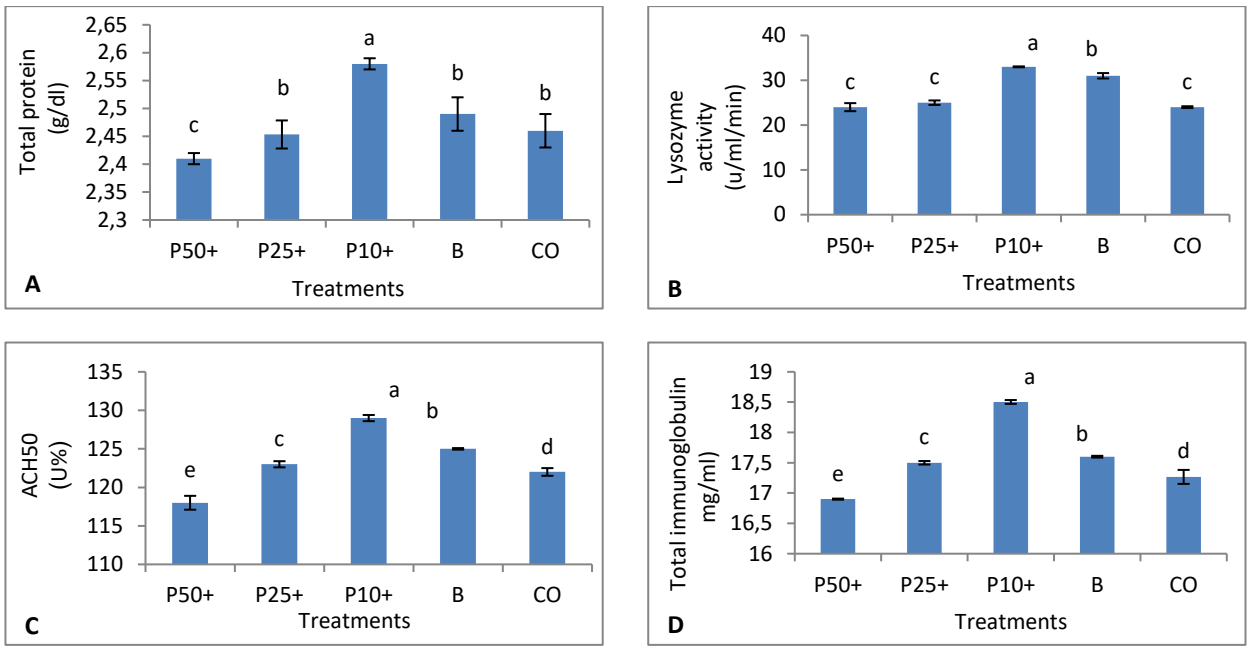

Figure 9. Serum immunity-related factors in rainbow trout were injected with the *K.rhizophila* TMU bacterin along with and without stick-water containing Low molecular weight peptides at different levels (10, 25, 50 mg/kg). A) Total protein, B) Lysozyme activity C) ACH_{50} D) Total immunoglobulin. Data are expressed in SD±ME. Different letters indicate a significant difference between the means ($p<0.05$). B: bacterin, P_{10+}: Injectable treatment with the bacterin along with stick-water containing Low molecular weight peptides at a dose of 10 mg/kg, P_{25+}: Treatment injected with the bacterin along with stick-water containing Low molecular weight peptides at a dose of 25 mg/kg, P_{50+}: Injected treatment with the bacterin along with stick-water contains Low molecular weight peptides at a dose of 50 mg/kg, Co: control treatment.

and lipid vacuole was observed (Figure 10a). In a large part of the liver tissue of P_{25+} treatment, sinusoid dilation, focal necrosis, hypertrophy, congestion and vacuolation were observed (Figure 10b). P_{50+} treatment, sinusoidal dilatation, focal necrosis, severe congestion and vacuolation, melanomacrophages and pycnotic nucleus were evident in all parts of liver tissue of fish (Figure 10c). In the liver of treatment B fish, sinusoid dilation, focal necrosis, congestion and melanomacrophages were observed (Figure 10d). Bleeding, severe congestion, sinusoidal dilatation, and accumulation of melanomacrophages were observed throughout the liver tissue of control fish (Figure 10e).

Discussion

Following the limitations of the use of antibiotics in aquaculture, alternative strategies such as the use of natural antimicrobials, probiotics, and immunostimulants have been proposed to control bacterial infections (Selvin *et al.,* 2004; Wang *et al.,* 2009; Peraza-Gomez *et al.,* 2014). In fish, innate immunity is very important due to the acquired immune limitations due to being cold-blooded, limited antibodies, and to some extent slowing down lymphocyte proliferation (Whyte, 2007). Immune-modulating peptides produced from natural sources usually have different types and play an important role in the immune response (Xing *et al.,* 2016). It has been reported that the molecular weight of most immune-modulating peptides is less than 3 kDa. Therefore, isolation and purification of immune-regulating peptides play an important role in analyzing the activity of immune-regulating peptides. As in the studies (Stuknyte *et al.,* 2011), peptides less than 3 kDa were obtained via ultrafiltration, which is consistent with the present study. Whereas the most prominent limiting factor in aquaculture is infectious diseases, which lead to great economic losses; Vaccination is the most effective way to prevent some infectious diseases because it boosts immunity. Despite the fact, researchers have begun to use immunostimulants as adjuvant to increase the effectiveness of vaccines in fish (Newaj-Fyzul and Austin, 2015).

Hematological parameters are often used as valuable indicators to assess the health status of fish (Prabu *et al.,* 2016). Injection of stick-water containing LMWPs caused a significant increase in RBC, HB, HCT parameters, while in treatment injected with high doses of it; all the mentioned parameters had the lowest values.

In the fish vaccinated group; RBC, HB, and HCT parameters was observed increase in P_{10+} fish, while in the P_{50+}, all the mentioned parameters showed lower values. The results showed that in B treatment, RBC values, HB, and HCT parameters were not significantly different from the fish control. As in the study to control the prevalence of streptococcosis in Brazil on Sorubim fish with pathogenicity of *Lactococcus garvieae*, no difference was observed in the amount of RBC and related parameters (Fukushima *et al.,* 2017). There is another conflicting report that the use of immunostimulant did not increase erythrocyte parameters to juvenile Nile tilapia (*Oreochromis*

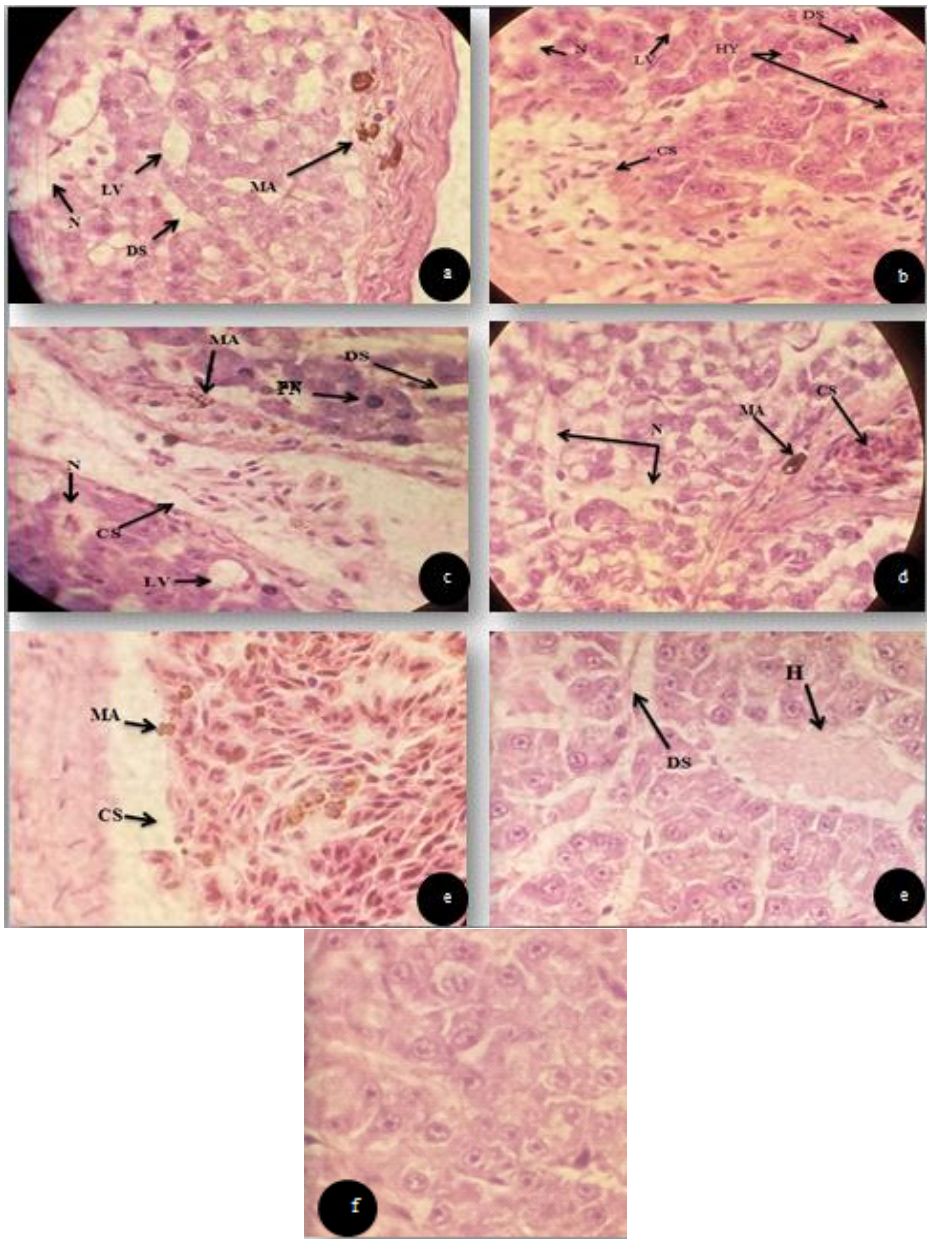

Figure 10. Liver tissue in P_{10+} treatment (Injectable treatment with the bacterin along with stick-water containing micro molecular peptides at a dose of 10 mg/kg), with melanomacrophage (MA) accumulation, sinusoidal dilatation (DS), focal necrosis (N) and lipid vacuole (LV). b) Liver tissue in P_{25+} treatment (Treatment injected with the bacterin along with stick-water containing micro molecular peptides at a dose of 25 mg/kg), with sinusoidal dilatation (DS), focal necrosis (N), hypertrophy (HY), congestion (CS) and lipid vacuolation (LV). c) Liver tissue in P_{50+} treatment (Injected treatment with the bacterin along with stick-water contains micro molecular peptides at a dose of 50 mg/kg), with sinusoidal dilatation (DS), focal necrosis (N), severe congestion (CS) and lipid vacuolation (LV), melanoma macrophages (MA) and pycnotic nuclei (PN). d) liver tissue in Treatment B (bacterin,), has sinusoidal dilatation (DS), focal necrosis (N), congestion (CS) and accumulation of melanoma macrophages (MA). e) 2 different parts of the liver tissue in Co treatment: control treatment (exposed to *K. rhizophila*) injected with TBS buffer, bleeding (H), severe congestion (CS), sinusoid dilation (DS) and melanomacophage accumulation (MA). F) Normal liver tissue without damage and exposure to pathogens in healthy rainbow trout.

niloticus), and was ineffective (Sado *et al.,* 2008). Reports on the use of immunostimulant and the increase in erythrocyte-related parameters in fish are available that are consistent with the results of the present study. Immunostimulants have the ability to improve head kidney function while increasing red blood cells and hematosynthesis (Nya and Austin, 2010; Prabu *et al.,* 2016). A significant increase in erythrocyte counts clearly demonstrates the effect of low doses of stick-water containing LMWPs because bioactive peptides directly contribute to the complex immune response by acting on erythrocytes (Nelson, 1953). This interaction of red blood cells with the immune system has been repeatedly demonstrated (Hess and Schifferli, 2003). Fish erythrocytes actively form rosettes to facilitate the clearance of pathogens by macrophages (Passantino *et al.,* 2002). They can produce specific signal molecules in response to the binding of cytokines (Passantino *et al.,* 2004; Passantino *et al.,* 2007). Red blood cells, hemoglobin, and the immune system play an

important role in the immune response to pathogens (Bishlawy, 1999). In several studies on the function of hemoglobin, a supporting argument is found. Hemoglobin is an important source of bioactive peptides that have been shown to be involved in the innate immune response. In addition, hemoglobin is the major protein in red blood cells and makes up 90% of its dry weight, therefore with an increase in red blood cells, an increase in hemoglobin is also expected (Liepke *et al.,* 2003; Jiang *et al.,* 2007). Total white blood cell count, percentage of lymphocytes, neutrophils, monocytes, and eosinophils significantly increased in P_{10} and P_{10+} treatments, while all the mentioned parameters in P_{50} and P_{50+} treatments were significantly less than other treatments was observed.

White blood cells are present in relatively stable numbers in the blood. However, these numbers may be temporarily less or more than what is happening in the body. Thus, relative evaluation of immune cells in fish blood can be a reliable indicator to ensure the activation of the immune system and its physiological function (Demaire *et al.,* 2020). In a study using immunostimulant in beluga fish in order to immunization, the number of white blood cells and the number of neutrophils increased significantly. This increase in white blood cell count is thought to be due to immune-stimulating compounds because they detect specific receptors on the surface of white blood cells and bind to receptors to stimulate phagocytosis activity in cells. The result is the production of cytokines. Cytokines themselves cause a further increase in new white blood cells. According to studies of bioactive peptides, peptide load, hydrophobicity, and peptide chain length have a significant effect on immune-modulating activity, and low-molecular-weight, positively charged peptides stimulate white blood cell proliferation at much lower doses. In explaining how innate and adaptive immunity during vaccination affects the outcome of protection, we can first mention TLRs (Toll-like receptors), which emerge as key components of the innate immune system and active the signals that Seriously play a role in initiating and maintaining consistent responses (Xing *et al.,* 2016).

Immunity parameters such as lysozyme activity, total immunoglobulin, and complement were significantly increased in P_{10} and P_{10+} treatments, while they were lowest in P_{50} and P_{50+} treatments. Total protein levels also increased with decreasing dose of the immunostimulant, and increasing the dose had the opposite effect. Lysozyme is an antibacterial enzyme produced by leukocytes, especially monocytes, macrophages, and neutrophils. Bioactive Peptides facilitate and accelerate lysozyme secretion by activating the white blood cell production pathway. Studies have shown an increase in serum lysozyme and globulins in relation to a stronger intrinsic response formed in fish and have stated that their levels in the serum of fish treated with immunostimulants it has always been higher than allowed (Rairakhwada *et al.,* 2007). Following the stimulation of white blood cells, immunoglobulins are produced depending on their specific function. The pathway of production of immunoglobulins in fish is due to the function of a set of reactions between antigen-providing cells, T helper cells, and interleukins that stimulate B lymphocytes. Upon stimulation, these lymphocytes produce plasma cells that are able to secrete immunoglobulins. Increased levels of immunoglobulin are easily effective in clearing toxins, pathogens, and other harmful molecules (Magnadottir *et al.,* 2004). Bioactive peptides activate the complement system through the classical pathway by activating the production pathway of immunoglobulin production (Pabst, 2012).

Some study about used an immunostimulants in fish, at low doses, showed a significant increase in the number of white and red blood cells in addition to increase lysozyme and immunoglobulins activity, complement and Total protein, in contrast, a higher level of immunostimulant, leading to suppression of the immune system in fish, that is consistent with the results of the present study (Morand *et al.,* 1999; Nayak *et al.,* 2008; Andrews *et al.,* 2009; Nya and Austin, 2010; Fukushima *et al.,* 2017; Sahoo *et al.,* 2017; He *et al.,* 2020).

What can be seen in the results of the present study is the effectiveness of low doses of stick-water containing LMWPs (10 mg/kg) and increasing innate and specific immunity in their low injection amount with and without bacterin in rainbow trout. In contrast, the high-dose injection did not increase immunity between treatments, so that the level of immunity at a dose of 50 mg/kg was even smaller than the control treatment. In other words, the high dose was an immunosuppressant. Due to the fact that the effects of immunostimulant vary based on the type of stimulants, fish species, route of administration, dose, duration, and relationship with other immunostimulants, therefore, the optimal level of immunostimulants in aquatic animals should be identified also care should be taken to investigate the beneficial effects and prevent the suppressive effects of the immune system (Dawood *et al.,* 2018). Immunostimulants increase immune responses and protection against pathogens, which creates the issue of dose-dependence (Kajita *et al.,* 1990; Robertsen, 1994; Kitao *et al.,* 1987). According to studies, the effects of immunostimulants are not directly dependent on the dose consumed, meaning that high doses may not only not be boosters but may also inhibit immune responses. Immunostimulants may be at low doses ineffective, at optimal doses immunogenic, and at high doses immunosuppressor (Sakai, 1999).

Economically and environmentally, vaccine prevention is the most appropriate way to control the growing diseases in the aquaculture industry (Tafalla *et al.,* 2013). The degree of immune response varies depending on the type of vaccine used (Plant and La Patra**,** 2011; Ye *et al.,* 2013). In the meantime, adjuvant vaccines increase the protection time and high titer of

specific antibodies (Tafalla *et al.,* 2013; Brudeseth *et al.,* 2013). Many studies have reported the role of adjuvants in the immune response in fish. Most studies show that adjuvants can increase the immune response by increasing the activity of leukocytes and plasmocytes as well as accelerating the production of specific antibodies, Serum phagocytic level, antibacterial activity and serum lysozyme (Fukushima *et al.,* 2017; Williams *et al.,* 1989; Thangaviji *et al.,* 2012).

The B treatment also had a significant increase in the number of white blood cells and related parameters as well as all immunity parameters, of course not more than P_{10+}. In a previous study, an increase in phagocytic activity of leukocytes was observed by oral administration of *Clostridium butyricum* bacterin to rainbow trout to increase fish resistance to vibrios. Unparalleled protection from *C. butyricum* bacterin showed that this substance itself was effective as an immunostimulant in controlling the disease (Sakai *et al.,* 1995) that this study is consistent with the results of the present study on the immunostimulant of *K. rhizophila* TMU.

For a challenge, intraperitoneal injection is the most common method for evaluating vaccine efficacy because it is a reliable and reproducible model in addition to ensuring equal challenge dose in all fish (Munangandu and Evensen, 2019). However, the injection method does not exactly mimic natural infection (Adams, 2019). Although in the immersion challenge method the challenge dose is unequal for each fish, it is closer to the reality of what happens in open or enclosed nature through cohabitation (Soto *et al.,* 2013). In the present study, after the challenge of rainbow trout with *K. rhizophila* TMU bacterium by immersion method, a relative survival percentage was observed within 14 days with a significant difference in P_{10+} treatment 70% and in B treatment 59%. As similar results can be seen in Thangaviji and Pourmozaffar's studies, in both studies following the challenge after vaccination, the relative survival rate in the adjuvant vaccine treatment was higher than in the non-adjuvant antigen group (Thangaviji *et al.,* 2015; Pourmozaffar *et al.,* 2015). According to the Pulpipat study, the immersion challenge was more effective in comparing the challenge of *Francisella noatunensis* with immersion and injection in tilapia due to its lower mortality and higher relative survival rate (Pulpipat *et al.,* 2020). Fish that were challenged by intraperitoneal injection showed higher mortality and lower relative survival rates because direct injection means that pathogens can bypass the first line of defense, including the mucosa, and the skin and gain direct access to the limbs, however in the immersion challenge, the bacteria must pass through the surface of the mucosa and skin before entering the host body (Nordmo and Ramstad, 1997).

Vaccines affect the activation of cytokine genes. Classic pro-inflammatory cytokine genes, including IL-1 and TNF, play a key role in regulating the inflammatory process in the early stages of infection in fish and provide the host's first line of defense (Zou *et al.,* 2016; Tsai *et al.,* 2014). The role of IL-1 as a chemical attraction for fish leukocytes is to activate lymphocytes, migrated leukocytes, and increase the phagocytic and lysozyme activity of macrophages. In addition, fish IL-1 can also induce TNF expression, modulate IL-17 family expression, which is important for antibacterial activity, increase antibody production, and express MHC II (major histocompatibility complex) induces chain. It has been reported that fish TNF overlaps with the IL-1 function. The role of fish TNF includes regulating leukocyte proliferation, migration, return to Origin cells and phagocytic granulocyte uptake, as well as induction of cellular apoptosis (Zou *et al.,* 2016). CXCL8 is a chemical cytokine produced by different cell types to stimulate the uptake and activation of neutrophils at the site of infection and inflammation (Harun *et al.,* 2008; Nguyen et al., 2018). Cytokines such as IL-1b and IL-6 induce the expression of APPs (Acute-phase protein), AMPs (Anti-microbial peptides), and complementary genes (Wangkahart *et al.,* 2017, 2019).

In the present study, the concentration of blood bacteria in P_{10+} and then B treatments was significantly lower than the others. This means that fish in these two treatments were able to reduce the bacterial load in the blood after challenge infection. What is certain lysozyme is a potent degrading enzyme found in the blood and lymph tissues of fish. This enzyme has many roles in fish immunity and is one of the most important factors in fish resistance (Magnadottir *et al.,* 2004). Lysozyme destroys and breaks down the cell wall of many bacteria, resulting in the release and death of protoplasts (Uygun *et al.,* 2014). This enzyme is very sensitive to gram-positive microorganisms but has no obvious bacteriostatic effect on gram-negative microorganisms (Callewaert *et al.,* 2012). In addition to the effect of lysozyme on serum bactericidal activity, it has been shown that most AMPs increase the permeability of the cytoplasmic membrane of microorganisms. This permeability increases sharply over time. The positively charged and hydrophobic structure of AMPs adapts them to interact with negatively charged lipid-rich cytoplasmic membranes to degrade them in the external environment (Shabir *et al.,* 2018).

After the fish is exposed to the pathogen, the bacteria in the fish cause systemic infection and affect various organs such as the liver. Improving histopathology in target organs is a visual parameter that indicates protection of fish against bacterial attack (Xu *et al.,* 2019). Histopathology has been widely used as a biomarker in assessing fish health. One of the great benefits of histopathology in assessing fish health is the ability to examine specific organs of the fish, including the gills, kidneys, and liver, which are responsible for vital functions. Like respiration, excretion, accumulation, and biological transmission, xenobiotics are present in fish (Gernhofer *et al.,* 2001). The results of histopathology in this study showed that in P_{10+} and B treatments after exposure to *K. rhizophila* TMU there

were not many pathological changes in the liver, while in other treatments abundant focal necrosis, congestion, and hemorrhage were observed, which could indicate the production of toxins. Bacteria and extracellular products such as hemolysin, protease, and elastase can cause severe liver necrosis (Rahayu *et al.,* 2018). In addition, all the findings of tissue pathology in this study are evidence of the occurrence of septicemia due to kocuriasis, which was detected in experimental and natural infections. Furthermore, due to the consequences earned from P_{10+}, LMWPs acted synergistically with bacterin and increased immunity compared to B treatment alone. Therefore, LMWPs may also have adjuvant potential, which requires more and more detailed research.

Conclusion

In conclusion, the Extraction of bioactive peptides from fish stick-water, which is part of the waste, is very effective and efficient, so the results of the present study show its immunogenic function in low doses, which can be used as an immunostimulant in the aquaculture industry. Additionally, in this study for the first time in the world (more likely), the role of *Kocuria rhizophila* TMU bacterium immunization in developing acquired immunity has been investigated and confirmed.

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Vaccine and the low-molecule peptides of Stick-water Kilka, both are led to an increase in innate and acquired immune in rainbow trout

Ethical Statement

All experimental protocols have been approved by TMU research Ethics Committee (51/7321)

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Author Contribution

All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version

Conflict of Interest

The author(s) declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper**.**

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Thanks to Pars Etehad Khazar Company for providing fresh Kilka fish steak-water. As well as full gratitude to Gharahboroun Company in order to provide facilities for keeping fish under the test. It is noteworthy that, the Tarbiat Modares University, Iran-Tehran provided all requirements funds for this study

References

Adams, A. (2019). Progress, challenges and opportunities in fish vaccine development. *Fish & shellfish immunology*, 90, 210-214; https://doi.org/10.1016/j.fsi.2019.04.066.

Andrews, S. R., Sahu, N. P., Pal, A. K., & Kumar, S. (2009). Haematological modulation and growth of *Labeo rohita* fingerlings: effect of dietary mannan oligosaccharide, yeast extract, protein hydrolysate and chlorella. *Aquaculture research*, *41*(1), 61-69; https://doi.org/10.1111/j.1365-2109.2009.02304.x.

Angosto, D., Lopez-Muñoz, A., Garcia-Alcazar, A., Meseguer, J., Sepulcre, M. P., & Mulero, V. (2018). Aluminum is a powerful adjuvant in teleost fish despite failing to induce interleukin-1β release. *Developmental & Comparative Immunology*, 85, 18-24; https://doi.org/10.1016/j.dci.2018.03.017.

Bishlawy, I. E. (1999). Red blood cells, hemoglobin and the immune system. *Medical hypotheses*, 53 (4), 345-346; https://doi.org/10.1054/mehy.1997.0778.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, 72(1-2), 248-254.

Brudeseth, B. E., Wiulsrød, R., Fredriksen, B. N., Lindmo, K., Løkling, K. E., Bordevik, M., & Gravningen, K. (2013). Status and future perspectives of vaccines for industrialised fin-fish farming. *Fish & shellfish immunology*, 35 (6), 1759-1768; https://doi.org/10.1016/j.fsi.2013.05.029.

Caipang, C. M. A., Lazado, C. C., Berg, I., Brinchmann, M. F., & Kiron, V. (2011). Influence of alginic acid and fucoidan on the immune responses of head kidney leukocytes in cod. *Fish physiology and biochemistry*, 37 (3), 603-612; https://doi.org/10.1007/s10695-010-9462-z.

Callewaert, L., Herreweghe, J. V., Vanderkelen, L., Leysen, S., Voet, A., & Michiels, C. W. (2012). Guards of the great wall: Bacterial lysozyme inhibitors. *trends in Microbiology*, 20, 501–510; https://doi.org/10.1016/j.tim.2012.06.005.

Choi, S. H., & Kim, K. H. (2012). Potential of auxotrophic *Edwardsiella tarda* double-knockout mutant as a delivery vector for DNA vaccine in olive flounder (*Paralichthys olivaceus*). *Veterinary immunology and immunopathology*, *145*(3-4), 625-630; https://doi.org/10.1016/j.vetimm.2012.01.014.

Cicero, A. F., Fogacci, F., & Colletti, A. (2017). Potential role of bioactive peptides in prevention and treatment of chronic diseases: a narrative review. *British journal of pharmacology*, *174* (11), 1378-1394; https://doi.org/10.1111/bph.13608.

Dawood, M. A., Koshio, S., & Esteban, M. Á. (2018). Beneficial roles of feed additives as immunostimulants in aquaculture: a review. *Reviews in Aquaculture*, 10(4), 950-974; https://doi.org/10.1111/raq.12209.

Demaire, C., Triki, Z., Binning, S. A., Glauser, G., Roche, D. G., & Bshary, R. (2020). Reduced access to cleaner fish negatively impacts the physiological state of two; https://doi.org/10.1007/s00227-020-3658-2.

FAO. (2016) The State of World Fisheries and Aquaculture 2016: Contributing to food security and nutrition for all. Rome: FAO. p. 200. Available online at: http://www.fao.org/3/a-i5555e.pdf

Fukushima, H. C. S., Leal, C. A. G., Cavalcante, R. B., Figueiredo, H. C. P., Arijo, S., Moriñigo, M. A & Ranzani-Paiva, M. J. T. (2017). *Lactococcus garvieae* outbreaks in Brazilian farms Lactococcosis in *Pseudoplatystoma sp*.– development of an autogenous vaccine as a control strategy. *Journal of fish diseases*, 40 (2), 263-272; https://doi.org/10.1111/jfd.12509.

Geirsdottir, M. (2009). Isolation, purification and investigation of peptides from fish proteins with blood pressure decreasing properties. *Food Research Innovation and Safety*, 36 (9), 1-28.

Gernhofer, M., Pawert, M., Schramm, M., Müller, E., & Triebskorn, R. (2001). Ultrastructural biomarkers as tools to characterize the health status of fish in contaminated streams. *Journal of Aquatic Ecosystem Stress and Recovery*, *8*(3), 241-260; https://doi.org/10.1023/A:1012958804442.

Harun, N. O., Zou, J., Zhang, Y. A., Nie, P., & Secombes, C. J. (2008). The biological effects of rainbow trout (*Oncorhynchus mykiss*) recombinant interleukin-8. *Developmental & Comparative Immunology*, 32(6), 673-681; https://doi.org/10.1016/j.dci.2007.10.005.

He, M., Liu, G., Liu, Y., Yang, K., Qi, X., Huang, A., & Wang, E. (2020). Effects of geniposide as immunostimulant on the innate immune response and disease resistance in crucian carp. *Aquaculture*, 529, 735713; https://doi.org/10.1016/j.aquaculture.2020.735713.

Hess, C., & Schifferli, J. A. (2003). Immune adherence revisited: novel players in an old game. *Physiology*, 18 (3), 104-108; https://doi.org/10.1152/nips.01425.2002.

Hoare, R., Ngo, T. P., Bartie, K. L., & Adams, A. (2017). Efficacy of a polyvalent immersion vaccine against *Flavobacterium psychrophilum* and evaluation of immune response to vaccination in rainbow trout fry (*Onchorynchus mykiss L*.). *Veterinary research*, 48 (1), 43; https://doi.org/10.1186/s13567-017-0448-z.

Jeney, G., & Anderson, D. P. (1993). Enhanced immune response and protection in rainbow trout to *Aeromonas salmonicida* bacterin following prior immersion in immunostimulants. *Fish & Shellfish Immunology*, 3 (1), 51-58; https://doi.org/10.1006/fsim.1993.1005.

Jiang, N., Tan, N. S., Ho, B., & Ding, J. L. (2007). Respiratory protein–generated reactive oxygen species as an antimicrobial strategy. *Nature immunology*, 8 (10), 1114-1122; https://doi.org/10.1038/ni1501.

Kajita, Y., Sakai, M., Atsuta, S., & Kobayashi, M. (1990). The immunomodulatory effects of levamisole on rainbow trout, *Oncorhynchus mykiss*. *Fish Pathology*, 25(2), 93-98.

Kitao, T., Yoshida, T., Anderson, D. P., Dixon, O. W., & Blanch, A. (1987). Immunostimulation of antibody-producing cells and humoral antibody to fish bacterin by a biological response modifier. *Journal of Fish Biology*, 31, 87-91; https://doi.org/10.1111/j.1095-8649.1987.tb05298.x.

Kościuczuk, E. M., Lisowski, P., Jarczak, J., Strzałkowska, N., Jóźwik, A., Horbańczuk, J., & Bagnicka, E. (2012). Cathelicidins: family of antimicrobial peptides. A review. *Molecular biology reports*, *39*(12), 10957-10970; https://doi.org/10.1007/s11033-012-1997-x.

Legario, F. S., Choresca Jr, C. H., Turnbull, J. F., & Crumlish, M. (2020). Isolation and molecular characterization of streptococcal species recovered from clinical infections in farmed Nile tilapia (*Oreochromis niloticus*) in the Philippines. *Journal of fish diseases*, *43*(11), 1431-1442; https://doi.org/10.1111/jfd.13247.

Liepke, C., Baxmann, S., Heine, C., Breithaupt, N., Ständker, L., & Forssmann, W. G. (2003). Human hemoglobin-derived peptides exhibit antimicrobial activity: a class of host defense peptides. *Journal of Chromatography B*, 791 (1-2), 345-356; https://doi.org/10.1016/S1570-0232(03)00245-9.

Liu, F., Tang, X., Sheng, X., Xing, J., & Zhan, W. (2017). Construction and evaluation of an *Edwardsiella tarda* DNA vaccine encoding outer membrane protein C. *Microbial pathogenesis*, 104, 238-247; https://doi.org/10.1016/j.micpath.2017.01.051.

Magnadottir, B., Lange, S., Steinarsson, A., & Gudmundsdóttir, S. (2004). The ontogenic development of innate immune parameters of cod (*Gadus morhua* L.). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 139(2), 217-224; https://doi.org/10.1016/j.cbpc.2004.07.009.

Martínez-Montaño, E., Osuna-Ruíz, I., Benítez-García, I., Osuna, C. O., Pacheco-Aguilar, R., Navarro-Peraza, R. S., & Salazar-Leyva, J. A. (2020). Biochemical and antioxidant properties of recovered solids with pH shift from fishery effluents (sardine stickwater and tuna cooking water). *Waste and Biomass Valorization*, p.1-13; https://doi.org/10.1007/s12649-020-01147-6.

Meena, D. K., Das, P., Kumar, S., Mandal, S. C., Prusty, A. K., Singh, S. K., & Mukherjee, S. C. (2013). Beta-glucan: an ideal immunostimulant in aquaculture (a review). *Fish physiology and biochemistry*, 39 (3), 431-457; https://doi.org/10.1007/s10695-012-9710-5.

Miles, A. A., Misra, S. S., & Irwin, J. O. (1938). The estimation of the bactericidal power of the blood. *Epidemiology & Infection*, *38*(6), 732-749.

Morand, M., Siwicki, A., Pozet, F., Klein, P., Vinaize, J. C., & Keck, N. (1999).Effects of dimerized lysozyme (KLP-602) on the cellular and humoral defence mechanisms in sheatfish (*Silurus glanis*): in vitro and in vivo study. *Veterinary research*, 30(4), 411-418.

Munangandu, H. M., & Evensen, Ø. (2019). Correlates of protective immunity for fish vaccines. *Fish & shellfish immunology*, 85, 132-140; https://doi.org/10.1016/j.fsi.2018.03.060.

Natt, M. P., & Herrick, C. A. (1952). A new blood diluent for counting the erythrocytes and leucocytes of the chicken. *Poultry Science*, 31 (4), 735-738.

Nayak, S. K., Swain, P., Nanda, P. K., Dash, S., Shukla, S., Meher, P. K., & Maiti, N. K. (2008). Effect of endotoxin on the

immunity of Indian major carp, *Labeo rohita*. *Fish & Shellfish Immunology*, 24(4), 394-399; https://doi.org/10.1016/j.fsi.2007.09.005.

Nelson, R. A. (1953). The immune-adherence phenomenon: an immunologically specific reaction between microorganisms and erythrocytes leading to enhanced phagocytosis. *Science*, 118 (3077), 733-737.

Newaj-Fyzul, A., & Austin, B. (2015). Probiotics, immunostimulants, plant products and oral vaccines, and their role as feed supplements in the control of bacterial fish diseases. *Journal of fish diseases*, 38(11), 937-955; https://doi.org/10.1111/jfd.12313.

Nguyen, H. T., Nguyen, T. T. T., Tsai, M. A., Ya-Zhen, E., Wang, P. C., & Chen, S. C. (2017). A formalin-inactivated vaccine provides good protection against Vibrio harveyi infection in orange-spotted grouper (*Epinephelus coioides*). *Fish & Shellfish Immunology*, *65*, 118-126; https://doi.org/10.1016/j.fsi.2017.04.008.

Nguyen, T., Nguyen, H. T., Vu-Khac, H., Wang, P. C., & Chen, S. C. (2018). Identification of protective protein antigens for vaccination against *Streptococcus dysgalactiae* in cobia (*Rachycentron canadum*). *Fish & shellfish immunology*, 80, 88-96; https://doi.org/10.1016/j.fsi.2018.05.052.

Nordmo, R., & Ramstad, A. (1997). Comparison of different challenge methods to evaluate the efficacy of furunculosis vaccines in Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases*, 20(2), 119-126; https://doi.org/10.1046/j.1365-2761.1997.d01-114.x.

Nya, E. J., & Austin, B. (2010). Use of bacterial lipopolysaccharide (LPS) as an immunostimulant for the control of *Aeromonas hydrophila* infections in rainbow trout *Oncorhynchus mykiss* (Walbaum). *Journal of applied microbiology*, 108(2), 686-694; https://doi.org/10.1111/j.1365-2672.2009.04464.x.

Pabst, O. (2012). New concepts in the generation and functions of IgA. *Nature Reviews Immunology*, 12(12), 821-832; https://doi.org/10.1038/nri3322.

Passantino, L., Altamura, M., Cianciotta, A., Jirillo, F., Ribaud, M. R., Jirillo, E., & Passantino, G. F. (2004). Maturation of fish erythrocytes coincides with changes in their morphology, enhanced ability to interact with *Candida albicans* and release of cytokine-like factors active upon autologous macrophages. *Immunopharmacology and immunotoxicology*, 26 (4), 573-585; https://doi.org/10.1081/IPH-200042323.

Passantino, L., Altamura, M., Cianciotta, A., Patruno, R., Tafaro, A., Jirillo, E., & Passantino, G. F. (2002). Fish immunology. I. Binding and engulfment of *Candida albicans* by erythrocytes of rainbow trout (*Salmo gairdneri* Richardson). *Immunopharmacology and immunotoxicology*, 24 (4), 665-678; https://doi.org/10.1081/IPH-120016050.

Passantino, L., Massaro, M. A., Jirillo, F., Di Modugno, D., Ribaud, M. R., Di Modugno, G., & Jirillo, E. (2007). Antigenically activated avian erythrocytes release cytokine-like factors: a conserved phylogenetic function discovered in fish. *Immunopharmacology and immunotoxicology*, 29 (1), 141-152; https://doi.org/10.1080/08923970701284664.

Pasupuleti, M., Schmidtchen, A., & Malmsten, M. (2012). Antimicrobial peptides: key components of the innate immune system. *Critical reviews in biotechnology*, 32(2), 143-171; https://doi.org/10.3109/07388551.2011.594423.

Peraza-Gomez, V., Luna-Gonza¡lez, A., Gonza¡lez-Prieto, J.M., Fierro-Coronado, A. and Gonz Ailez-Ocampo, H.A. (2014). Protective effect of microbial immunostimulants and antiviral plants against WSSV in *Litopenaeus vannamei* cultured under laboratory conditions. *Aquaculture* 420,160–164; https://doi.org/10.1016/J.AQUACULTURE.2013.10.044.

Plant, K. P., & LaPatra, S. E. (2011). Advances in fish vaccine delivery. *Developmental & Comparative Immunology*, 35 (12), 1256-1262; https://doi.org/10.1016/j.dci.2011.03.007.

Pourmozaffar, S. (2015). Study effect of Macrogard on efficacy of Anti-*streptococcus iniae* vaccine and some haematological and growth parameters in rainbow trout (*Oncorhynchus mykiss*). *Iranian Veterinary Journal*, 11(2), 44-53; https://doi.org/10.22055/ivj.2015.10111.

Prabu, D. L., Sahu, N. P., Pal, A. K., Dasgupta, S., & Narendra, A. (2016). Immunomodulation and interferon gamma gene expression in sutchi catfish, Pangasianodon hypophthalmus: effect of dietary fucoidan rich seaweed extract (FRSE) on pre and post challenge period. *Aquaculture research*, 47(1), 199-218; https://doi.org/10.1111/are.12482.

Pulpipat, T., Maekawa, S., Wang, P. C., & Chen, S. C. (2020). Immune responses and protective efficacy of a formalin-killed *Francisella noatunensis* subsp. orientalis vaccine evaluated through intraperitoneal and immersion challenge methods in *Oreochromis niloticus*. *Vaccines*, 8(2), 163; https://doi.org/10.3390/vaccines8020163.

Rahayu, K., Daruti, D. N., & Stella, M. S. P. (2018, April). Study on characterization, pathogenicity and histopathology of disease caused by *Aeromonas hydrophila* in gourami (*Osphronemus gouramy*). In *IOP Conference Series: Earth and Environmental Science* (Vol. 137, No. 1, p. 012003); https://doi.org/10.1088/1755-1315/137/1/012003.

Rairakhwada, D., Pal, A. K., Bhathena, Z. P., Sahu, N. P., Jha, A., & Mukherjee, S. C. (2007). Dietary microbial levan enhances cellular non-specific immunity and survival of common carp (*Cyprinus carpio*) juveniles. *Fish & Shellfish Immunology*, 22(5), 477-486; https://doi.org/10.1016/j.fsi.2006.06.005.

Robertsen, B. (1994). β-glucan as immunostimulants in fish. *Modulators of fish immune responses*, 1, 83-99.

Sado, R. Y., Bicudo, Á. J. D. A., & Cyrino, J. E. P. (2008). Feeding dietary mannan oligosaccharides to juvenile Nile tilapia, *Oreochromis niloticus*, has no effect on hematological parameters and showed decreased feed consumption. *Journal of the world Aquaculture Society*, 39(6), 821-826; https://doi.org/10.1111/j.1749-7345.2008.00219.x.

Sahoo, L., Parhi, J., Debnath, C., & Prasad, K. P. (2017). Effect of feeding Lipopolysaccharide as an immunostimulant on immune response and immune gene expression of *Labeo bata*. *Veterinary immunology and immunopathology*, 188, 48-58; https://doi.org/10.1016/j.vetimm.2017.04.012.

Sakai, M. (1999). Current research status of fish immunostimulants. *Aquaculture*, 172(1-2), 63-92; https://doi.org/10.1016/S0044-8486(98)00436-0.

Sakai, M., Yoshida, T., Atsuta, S., & Kobayashi, M. (1995). Enhancement of resistance to vibriosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum), by oral administration of *Clostridium butyricum* bacterin. *Journal of Fish Diseases*, 18(2), 187-190; https://doi.org/10.1111/j.1365-2761.1995.tb00276.x.

Sankaran, K., & Gurnani, S. (1972). On the variation in the catalytic activity of lysozyme in fishes. *Indian journal of biochemistry & biophysics*, 9 (2), 162.

Santos, L., & Ramos, F. (2018). Antimicrobial resistance in aquaculture: current knowledge and alternatives to tackle the problem. *International Journal of Antimicrobial Agents*, *52*(2), 135-143; https://doi.org/10.1016/j.ijantimicag.2018.03.010.

Selvin, J., Huxley, A. J., & Lipton, A. P. (2004). Immunomodulatory potential of marine secondary metabolites against bacterial diseases of shrimp. *Aquaculture*, 230 (1-4), 241-248; https://doi.org/10.1016/S0044-8486(03)00427-7.

Shabir, U., Ali, S., Magray, A. R., Ganai, B. A., Firdous, P., Hassan, T., & Nazir, R. (2018). Fish antimicrobial peptides (AMP's) as essential and promising molecular therapeutic agents: a review. *Microbial pathogenesis*, 114, 50-56; https://doi.org/10.1016/j.micpath.2017.11.039.

Siwicki, A. K., Anderson, D. P., & Rumsey, G. L. (1994). Dietary intake of immunostimulants by rainbow trout affects non-specific immunity and protection against furunculosis. *Veterinary immunology and immunopathology*, 41 (1-2), 125-139.

Siwicki, A. K., Klein, P., Morand, M., Kiczka, W., & Studnicka, M. (1998). Immunostimulatory effects of dimerized lysozyme (KLP-602) on the nonspecific defense mechanisms and protection against furunculosis in salmonids. *Veterinary immunology and immunopathology*, 61 (2-4), 369-378; https://doi.org/10.1016/S0165-2427(97)00140-2

Soto, E., Kidd, S., Mendez, S., Marancik, D., Revan, F., Hiltchie, D., & Camus, A. (2013). *Francisella noatunensis* subsp. orientalis pathogenesis analyzed by experimental immersion challenge in Nile tilapia, *Oreochromis niloticus* (L.). *Veterinary microbiology*, 164(1-2), 77-84; https://doi.org/10.1016/j.vetmic.2013.01.024.

Stuknyte, M., De Noni, I., Guglielmetti, S., Minuzzo, M., & Mora, D. (2011). Potential immunomodulatory activity of bovine casein hydrolysates produced after digestion with proteinases of lactic acid bacteria. *International dairy journal*, *21*(10), 763-769; https://doi.org/10.1016/j.idairyj.2011.04.011

Tafalla, C., Bøgwald, J., & Dalmo, R. A. (2013). Adjuvants and immunostimulants in fish vaccines: current knowledge and future perspectives. *Fish & Shellfish Immunology*, *35*(6), 1740-1750; https://doi.org/10.1016/j.fsi.2013.02.029

Thangaviji, V., Michaelbabu, M., Anand, S. B., Gunasekaran, P., & Citarasu, T. (2012). Immunization with the Aeromonas OMP provides protection against *Aeromonas hydrophila* in goldfish (*Carassius auratus*). *J Microbial Biochem technol*, *4*(2); http://dx.doi.org/10.4172/1948-5948.1000070.

Treves-Brown, K. M. (2013). Applied fish pharmacology (Vol. 3). *Springer Science & Business Media.* p.274; https://doi.org/10.1007/978-94-017-0761-9

Tsai, J. L., Priya, T. J., Hu, K. Y., Yan, H. Y., Shen, S. T., & Song, Y. L. (2014). Grouper interleukin-12, linked by an ancient disulfide-bond architecture, exhibits cytokine and chemokine activities. *Fish & shellfish immunology*, 36(1), 27-37; https://doi.org/10.1016/j.fsi.2013.10.009

Uygun, M., Uygun, D., Altunbas, C., Akgol, S., & Denizli, A. (2014). Dye attached nanoparticles for lysozyme adsorption. Separation *Science and technology*, 49, 1270–1278; https://doi.org/10.1080/01496395.2013.878723

Vallejos-Vidal, E., Reyes-López, F., Teles, M., & MacKenzie, S. (2016). The response of fish to immunostimulant diets. *Fish & shellfish immunology*, 56, 34-69; https://doi.org/10.1016/j.fsi.2016.06.028.

Villumsen, K. R., Koppang, E. O., Christensen, D., & Bojesen, A. M. (2017). Alternatives to mineral oil adjuvants in vaccines against *Aeromonas salmonicida subsp*. salmonicida in rainbow trout offer reductions in adverse effects. *Scientific reports*, 7 (1), 5930; https://doi.org/10.1038/s41598-017-06324-7.

Wang, Q., Ji, W., & Xu, Z. (2020). Current use and development of fish vaccines in China. *Fish & shellfish immunology*, *96*, 223-234; https://doi.org/10.1016/j.fsi.2019.12.010

Wang, T., Sun, Y., Jin, L., Xu, Y., Wang, L., Ren, T., & Wang, K. (2009). Enhancement of non-specific immune response in sea cucumber (*Apostichopus japonicus*) by *Astragalus membranaceus* and its polysaccharides. *Fish & shellfish immunology*, 27 (6), 757-762; https://doi.org/10.1016/j.fsi.2009.09.002

Wang, W., Sun, J., Liu, C., & Xue, Z. (2017). Application of immunostimulants in aquaculture: current knowledge and future perspectives. *Aquaculture Research*, 48 (1), 1-23; https://doi.org/10.1111/are.13161.

Wangkahart, E., Secombes, C. J., & Wang, T. (2017). Dissecting the immune pathways stimulated following injection vaccination of rainbow trout (*Oncorhynchus mykiss*) against enteric redmouth disease (ERM). *Fish & shellfish immunology*. p.71; https://doi.org/10.1016/j.fsi.2017.07.056

Wangkahart, E., Secombes, C. J., & Wang, T. (2019). Studies on the use of flagellin as an immunostimulant and vaccine adjuvant in fish aquaculture. *Frontiers in immunology*, 9, 3054; https://doi.org/10.3389/fimmu.2018.03054

Whyte, S. K. (2007). The innate immune response of finfish–a review of current knowledge. *Fish & shellfish immunology*, 23 (6), 1127-1151; https://doi.org/10.1016/j.fsi.2007.06.005.

Williams, D. L., Yaeger, R. G., Pretus, H. A., Browder, I. W., McNamee, R. B., & Jones, E. L. (1989). Immunization against *Trypanosoma cruzi*: adjuvant effect of glucan. *International journal of immunopharmacology*, *11*(4), 403-410.

Xing, J., Zhang, M., & Lin, S. (2016, March). The Research Progress of Immunomodulatory Peptides. In 2016 4th International Conference on Machinery, *Materials and Computing technology*. Atlantis Press.1479-1483; https://doi.org/10.2991/icmmct-16.2016.294

Xu, H., Xing, J., Tang, X., Sheng, X., & Zhan, W. (2019). Immune response and protective effect against *Vibrio anguillarum* induced by DNA vaccine encoding Hsp33 protein. *Microbial pathogenesis*, *137*, 103729; https://doi.org/10.1016/j.micpath.2019.103729

Yamasaki, M., Araki, K., Maruyoshi, K., Matsumoto, M., Nakayasu, C., Moritomo, T., & Yamamoto, A. (2015). Comparative analysis of adaptive immune response after vaccine trials using live attenuated and formalin-killed cells of *Edwardsiella tarda* in ginbuna crucian carp (*Carassius auratus* langsdorfii). *Fish & shellfish immunology*, 45(2), 437-442; https://doi.org/10.1016/j.fsi.2015.04.038

Yano, T. (1992). Assays of hemolytic complement activity. *Techniques in fish immunology*, 131-141..

Ye, J., Kaattari, I. M., Ma, C., & Kaattari, S. (2013). The teleost humoral immune response. *Fish & shellfish immunology*, 35 (6), 1719-1728; https://doi.org/10.1016/j.fsi.2013.10.015

Zhang, M., Zhu, H., Ding, Y., Liu, Z., Cai, Z., & Zou, M. H. (2017). AMP-activated protein kinase α1 promotes atherogenesis by increasing monocyte-to-macrophage differentiation. *Journal of Biological Chemistry*, 292 (19), 7888-7903; https://doi.org/10.1074/jbc.M117.779447.

Zhou, Q. J., Wang, J., Liu, M., Qiao, Y., Hong, W. S., Su, Y. Q., & Zheng, W. Q. (2016). Identification, expression and antibacterial activities of an antimicrobial peptide NK-lysin from a marine fish Larimichthys crocea. *Fish & shellfish immunology*, 55, 195-202; https://doi.org/10.1016/j.fsi.2016.05.035.

Zou, J., & Secombes, C. J. (2016). The function of fish cytokines. *Biology*, 5(2), 23 https://doi.org/10.3390/biology5020023