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**Research Article**

# Effect of extracellular proteins (ECP) on protective efficacy of Yersiniosis vaccine in juvenile rainbow trout (*Oncorhynchus mykiss*)

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**Abstract**

Yersiniosis is the second important bacterial infections in coldwater fish culture with significant mortalities and economical losses in the Iranian fish farms. In the present study, the effect of extracellular proteins (ECP) on protective immunity of *Y. ruckeri* vaccine was evaluated in juvenile rainbow trout (7±1.2 g). For this purpose, 540 specimens of juvenile rainbow trout were randomly divided into 6 groups each in triplicates. Group 1 ($G_1$) and Group 2 ($G_2$) were orally administrated with formalin killed cells (FKC) and FKC+ECP, respectively. Groups 3 to 5 received ECP, FKC, ECP+FKC via intraperitoneal route, respectively. Group 6 received phosphate buffer saline as the control group. The humoral antibody responses to bacterial antigens were monitored by ELISA. $LD_{50}$ of *Y. ruckeri* was determined used probit method sixty days after vaccination. Then, fish in each treatment were challenged intraperitoneally (I.P) with $LD_{50}$ of *Y. ruckeri* virulent registered strain in Iran (KCW 291153). The ELISA results indicated that ECP could increase the serum ELISA antibody titer as the humoral immune response, but ECP with the FKC could increase antibody levels significantly in serum and intestine mucus. Also survival rates in $G_1$ to $G_6$ were 25, 31.25, 37.5, 56.25, 87.5 and 15 percent, respectively. Among I.P immunized fish the survival rate in $G_5$ was significantly higher than the other groups. Although in orally vaccinated fish with FKC (G1) survival rate did not show significant difference with the control group, the FKC /ECP group ($G_2$) showed a significant increase compared to control group ($p$=0.039). It was concluded that supplementing FKC with ECP increased protective immunity of Yersiniosis vaccine principally in I.P. route. Given the great benefits of the oral vaccine, whole cell/ECP can be considered as a protective antigen to design potential vaccines against this pathogenic micro-organism.

**Keywords:** *Yersinia ruckeri*, Immunity, Rainbow trout, Vaccination

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**Introduction**

The Gram-negative bacterium *Yersinia ruckeri* is the causative agent of an acute infection of rainbow trout which is typically characterized by hemorrhagic septicemia. It manifests as subcutaneous hemorrhages in and around the oral cavity, the latter giving rise to the name "redmouth" disease. Internally, petechial hemorrhages on the surfaces of the liver, pancreas, pyloric ceca, swim bladder, and lateral musculature, inflammation of the lower intestine and spleen, filling the lower intestine with an opaque yellowish fluid might occur.

Yersiniosis is the second important bacterial infections in coldwater fish culture with significant mortalities and economical losses in the Iranian fish farms. It was first isolated in Idaho, USA, from rainbow trout (*Oncorhynchus mykiss)* and has consequently been found in all trout producing countries including Iran (Soltani *et al.,* 1999; Soltani and Tarahomi, 2002; Behroozi and Soltani, 2003; Zorriehzahra *et al*., 2005; Tobback *et al.,* 2007; Soltani *et al.,* 2014,).

Currently, in the aquaculture industry prevention and control of disease outbreaks have become the biggest challenge. The use of antibiotics in the early stages of the development of intensive aquaculture is predominant. The lack of appropriate use of antibiotics to control bacterial diseases has led to public health and environmental concerns due to the potential development of antibiotic-resistant bacteria and antibiotic remains in aquaculture products or the environment. In the last decade increased immunity by vaccination is confirmed as one of the most cost-effective, practical and environmentally friendly methods that accessible to prevent or reduce losses due to infectious disease in aquaculture (Newaj-Fyzul and Austin, 2015)

Several investigations have described an extensive range of bacterial bioactivities for their immune-modulating efficacy on fish which can be classified into subcellular products such as Lipopolysaccharides (LPS), Peptidoglycan (PGN),outer membrane proteins (OMP); nucleotides, extracellular proteins (ECP); and cellular products. Furthermore, bacterial whole-cell products (WCP) and various bacterial secondary metabolites have also been reported to have adjuvant and immunostimulatory effects on fish (Sharifuzzaman and Austin**,** 2010; Giri *et al.,* 2018).

It has been found that extracellular products (ECP) produced by *Yersinia ruckeri* play an important role in the pathogenesis and, hence it can be considered to include ECP of this species as a potential vaccine (Romalde and Toranzo, 1993; Giri *et al.,* 2018). Among the variety of methods of vaccination, mucosal administration of antigens offers the most practical approach for immunization of small fish (Giri *et al.,* 2018). It also specifically targets stimulation of mucosal immunity in the fish, which provides the first line of defense against most pathogens. Oral immunoprophylaxis, in particular,

represents this an appropriate strategy for purpose as it has no fish-size limitation and requires minimal stress and skills for effective administration (Giri *et al.*, 2018).

In spite of the mentioned effect of bacterial extracellular products on the immune responses of fish, various researches focused on killed bacteria supplements because of their higher efficacy. In this study, the effectiveness of a newly developed vaccine (ECP enriched bacterin) was tested. Additionally, ECP enriched bacterin administered in both oral and injection routes to evaluate the protective efficacy of ECP-enriched *Y. ruckeri* in both routes in rainbow trout, because the determination of practical efficacy and potency are important to satisfy practical product considerations and government regulatory issues.

## Materials and methods

*Fish and water parameters*

Five hundred and forty pieces of juvenile rainbow trout (7±1.2 g) from a hatchery without any report of yersiniosis in past 4 years in Lorestan Province, Iran was transferred to the aquarium room in the Aquatic Animal Health Department of the Veterinary Faculty, Shahid Chamran University, Ahvaz, Iran. Fish were kept at 14±1.2°C in 200 L glass aquaria equipped with central aeration system, biofilters and thermostatic heaters. Fish were fed with commercial trout feed (BioMar, Denmark) based on 4% of their initial biomass, three times per day. During adaptation and experiment period chemo-physical parameters of water maintained in optimum condition for all groups. Daily water exchange were conducted in all treatments of experiment. Water parameters of experiment were as follow: temperature; 14±1.2°C, dissolved oxygen; 9±1 mg $L^{-1}$, pH; 7.9±0.53, $NH_3$<0.01 mg $L^{-1}$, $NO_2$<0.1 mg $L^{-1}$ and salinity 800 µSc $m^{-1}$. Fish were acclimatized to laboratory conditions for two weeks, and fed the control diet. Also, their health status was examined by sampling from brain and kidney.

*Formalin Killed Cell (FKC) preparation*

The Hagerman strain of *Y. ruckeri* (KCW 291153) were grown in trypticase soy broth medium (TSB; Difco) at 28°C for 48 h. Then bacteria were washed twice using sterile phosphate-buffered saline (PBS) The bacterial concentration adjusted to $10^{10}$ cfu/mL using pour plate count method. Formalin-killed *Y. ruckeri* (FKC) was produced according to Ghosh *et al*. (2016) with some modifications. The adjusted concentration of *Y. ruckeri* was inactivated by 1% formalin (in neutral buffered) for 24 h. Afterwards, the cells were centrifuged (8000 *g*; for 30 min) and washed twice in PBS. To ensure the bacterial inactivation, 100 µL of prepared FKC was inoculated in TSA plates and incubated at 28°C for 24h. The prepared bacterin was stored at 4°C until use.

*Extracting the extracellular proteins (ECPs)*

The bacterial cell was cultured using dialysis sac (Sigma D9777). 200 ml TSB

medium was added to five separate dialysis sacs, which was then clipped at both ends, placed in conical flasks (Fig. 1) and sterilized by autoclaving at 110°C for 20 min. *Y. ruckeri* inoculated to the flasks containing the dialysis sac and incubated at 28°C in an orbital shaker (150 rpm) for 24 h. The bacterial culture grown in the flasks around the dialysis sac were collected, pooled to make approximately 100 ml and centrifuged at 10000Ug for 20 min at 4°C. (Ladhani *et al.*, 2002).


**Figure 1: None Concentrated ECP of *Y. ruckeri* around the dialysis sac containing TSB media.**

The supernatant was separated from the whole cell bacteria then filtered and concentrated with a 0.22-µm filter and prepared for crude ECP vaccine (Zhang *et al.*, 2014) (Fig. 2). ECP was ten times concentrated by centrifuge protein purification filters of Sartorius Stedim Company (Vivaspin-6 Polyethersulfone, VS0601). Total protein of ECPs was measured by the method of Bradford (1976) with bovine serum albumin as a standard. The sterility of the final extracted ECP was confirmed by the absence of bacterial growth after the inoculation of the product in TSA and inoculation at 28$^{o}$ C for 2 days.


**Figure 2: Concentrated ECP of *Y. ruckeri* using centrifuge protein purification. Upper phase in filter refers to concentrated ECP.**

The safety of the ECP vaccine was evaluated by injecting 0.1 mL per fish to five rainbow trout through the intraperitoneal route, which was then observed for 4 days. When there had been no mortality or clinical signs of Yersiniosis by the 4 days, the ECP vaccine were declared safe to use (Sukenda *et al.*, 2018).

*Experimental design*

Fish were randomly divided into six experimental groups in triplicates (30 fish per each group). Experimental feed prepared according to Villumsen *et al*. (2014), briefly feed pellets (Biomar, Denmark, diameter=1 mm, protein=44%, Fat=14%, Ash=11%, Moisture=9%) were thoroughly spray coated with FKC ($1\times10^{10}$ cells $g^{-1}$) (G1) and FKC enriched with ECP (400 µg/kg/body weight daily) (G2). Another three groups of fish were immunized intraperitoneally with 0.2 mL $fish^{-1}$

extracted ECP (80 µg $mL^{-1}$) (G3), 0.1 mL of $1\times10^{10}$ bacterial cells $mL^{-1}$ FKC (G4), and FKC enriched with ECP (G5). The control group (G6) received just PBS. Fish in each group were fed up to 3% of average body weight three times a day every other week for 14 days (Villumsen *et al*., 2014).

*Enzyme-linked immunosorbent assay (ELISA)*

*Yersinia ruckeri*-specific antibody levels in plasma and intestine mucus were measured by ELISA with some modifications (Skov *et al*., 2018). Concisely, Microplates (Nunc, Denmark) were coated with 50 µL $well^{-1}$ of formalin-killed and sonically disrupted *Y. ruckeri* (100 µg/mL) antigen at a 1:15 dilution in bicarbonate coating buffer (pH=9.6) for 18 h at 4°C. Plates were washed with PBS containing 0.05% of tween-20 (PBS-T) and then non-specific binding sites were blocked with 2.5% skim milk (High media, India) for 1 h at 25°C. Rainbow trout plasma or mucus samples (100 µL) were then added at a 1:20 and 1:1 dilution respectively in PBS+0.05% Tween-20 (PBS-T) containing 0.1% skim milk. After 90 min incubation at 25°C, 100 µL of mouse monoclonal mouse anti-rainbow trout immunoglobulin (prepared and kindly donated by Prof. Seyfi in our faculty) at a 1:7500 dilution in PBS-T containing 0.1% skim milk was added to all wells and then shaken for 60 min. After washing in PBS-T (three times), 50 µL of goat anti-mouse IgG HRP conjugate (Sigma-Aldrich) at a 1:2500 dilution in PBS-T containing 0.1% skim milk was added and incubated for 60 min. Plates were washed as above and 50 µL TMB (3,3′, 5,5; -tetramethylbenzidine - $H_2O_2$) chromogen solution was added to each well for 10 min at 25°C. The reaction was stopped with 50 µL 2 N H2SO4. Lastly, serum and mucus antibody levels were read spectrophotometrically at 450 nm by an ELISA reader (Accu Reader, Taiwan).

*$LD_{50}$ determination of Y.ruckeri*

$LD_{50}$ of *Y. ruckeri* was determined using the correlation of bacterial concentration with fish mortality (Raida and Buchmann, 2008) Briefly 10 fold serial dilutions of *Y. ruckeri* ($10^5$ to $10^8$ CFU $mL^{-1}$) were prepared in PBS and 100 µL of each bacterial dilution injected intraperitoneally to 10 fish. The mortality rate recorded daily for 10 days, mortality rate analyzed and the $LD_{50}$ was determined using probit software (Table 1).

**Table 1: The lethal concentration of *Y. ruckeri* via injection challenge based on the probit analysis output.**

| lethal Dose (LD) | Bacterial concentration (CFU $mL^{-1}$) |
|---|---|
| $LD_{10}$ | $6.5\times10^5$ |
| $LD_{25}$ | $6.1\times10^6$ |
| $LD_{50}$ | $7.5\times10^7$ |
| $LD_{75}$ | $9.0\times10^8$ |
| $LD_{90}$ | $8.5\times10^9$ |

*Challenge experiment*

Thirty fish of each group (10 fish from each replicate) were challenged by I.P. route 60-day post-vaccination using live *Y. ruckeri*. Fish were anesthetized with 50 mg $L^{-1}$ MS-222 (Sigma—Aldrich, Denmark) and $1LD_{50}=7.5\times10^7$ CFU of *Y. ruckeri* were injected

intraperitoneally. Following challenge procedure fish were transferred to 100 L aquaria and mortality was checked several times daily for 10 days and dead fish were recorded and removed. Cause of mortality confirmed by re-isolating of *Y. ruckeri* from head kidney of dead fish. Standard PCR using *Y. ruckeri*-specific 16S ribosomal RNA gene primers were used for confirmation of *Y. ruckeri* (Villumsen *et al*., 2014; Ghosh *et al*., 2016). The cumulative percent mortality (CPM) was determined after 10 days, and the Relative Percentage of Survival (RPS) was calculated using the following equation: (RPS=1-(mortality in vaccinates / mortality in controls)×100. Two groups of 10 fish for each treatment were kept non-infected but under similar conditions and served as a negative control group.

*Statistical analysis*

$LD_{50}$ concentration of *Y. ruckeri* in rainbow trout was determined using the correlation of injected bacterial concentration with fish mortality through Probit Method. Comparison of differences in mortality between groups was analyzed using the Kaplan– Meier survival analysis test (Log-rank value) (SPSS 22.0 version).

**Results**

In injected groups, the serum ELISA antibody titer increased significantly in G5 and G4 month after immunization ($p$<0.05). On day 60, there was significant increase just in G5 (Fig. 3).

The ELISA antibody titer of intestine mucus show a significant increase on day 30 in FKC+ECP group compared to other groups ($p$<0.05). However, 60 days after immunization the antibody titer of G5 increased significantly just compared to G1, G3 and G6 (Fig. 4).

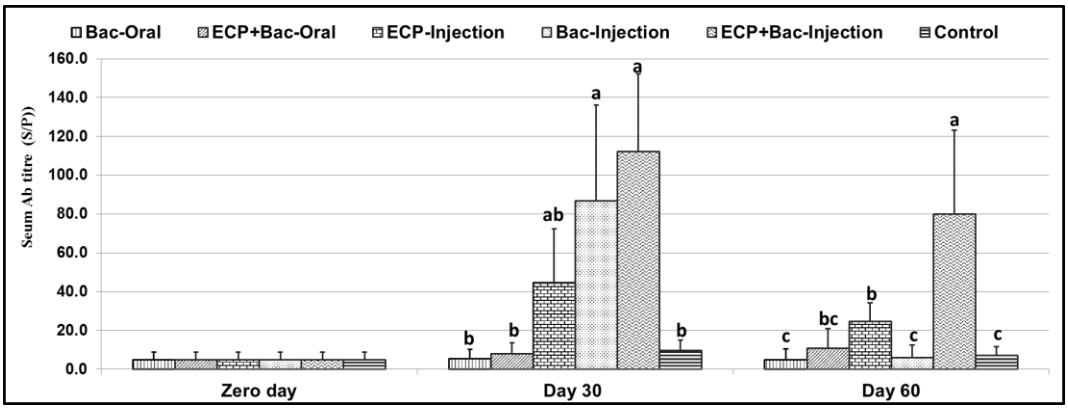


**Figure 3: Serum antibody titer *against Y. ruckeri* in experimental groups in different sampling point using ELISA method. Statistical differences between treatment groups are indicated by different letter notations.**

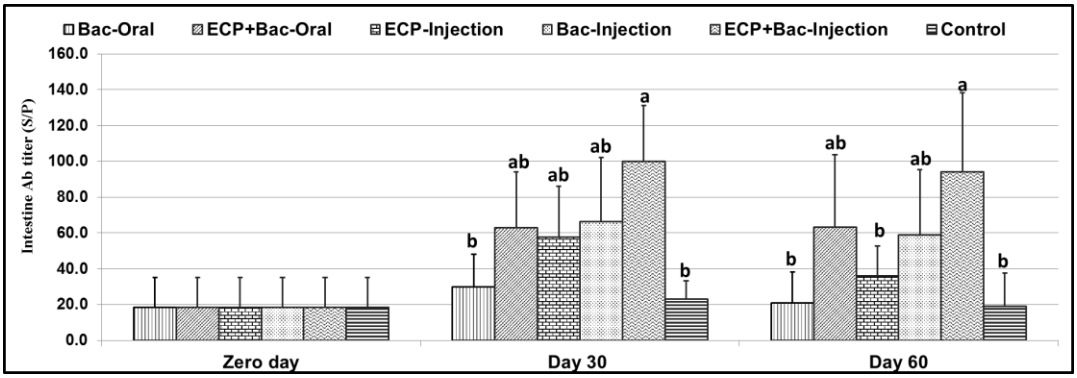


**Figure 4: Intestine mucus antibody titer against *Y. ruckeri* in experimental groups in different sampling point using ELISA method. Statistical differences between treatment groups are indicated by different letter notations.**

In the other word, the highest levels of antibody titers were measured in G5, G2, G4, G3, G1, G6, respectively. The percentage mortality rates of fish immunized with extracellular products of *Y. ruckeri* are summarized in Fig 6. The survival percentage rate in the experimental groups (G1-G5) was 25, 31.25, 37.5, 56.25 and 87.5, respectively. In this study, protection against the pathogen by ECP as indicated by percentage mortality rate was observed, which was significantly higher compared to control group fish. Although rainbow trout immunized by oral route with the FKC+ECP of *Y. ruckeri* were protected significantly ($p$<0.05) compared to the control group 60 days after immunization, there was not significant difference between groups 1 and 2 (Fig. 5). Among the injected groups, the mortality rate was 62.5%, 43.75% and 12.5% in groups 3, 4 and 5, respectively (Fig. 7), with the highest survival rate (87.5%), for group 5 which was significantly higher than all injection and oral groups ($p$<0.05).

The group that received FKC via the oral route ($G_1$) did not show significant immunogenicity and the difference with the control group, but the FKC /ECP ($G_2$) had a significant difference with the control group ($p$=0.039) (Fig. 5).

Accordingly, the results of the injection immunization trial with one injection at zero-day, gave the significant and prolonged protection especially in group 5.

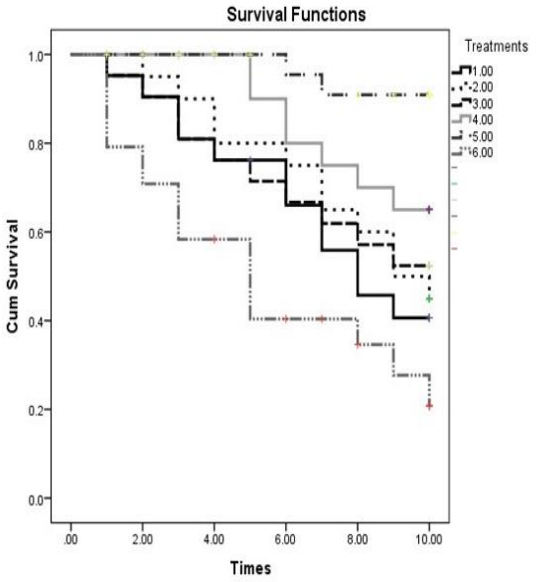


**Figure 5: Survival rate of immunized fish challenged intraperitoneally with *Y. ruckeri* (Kaplan–Meier test).**

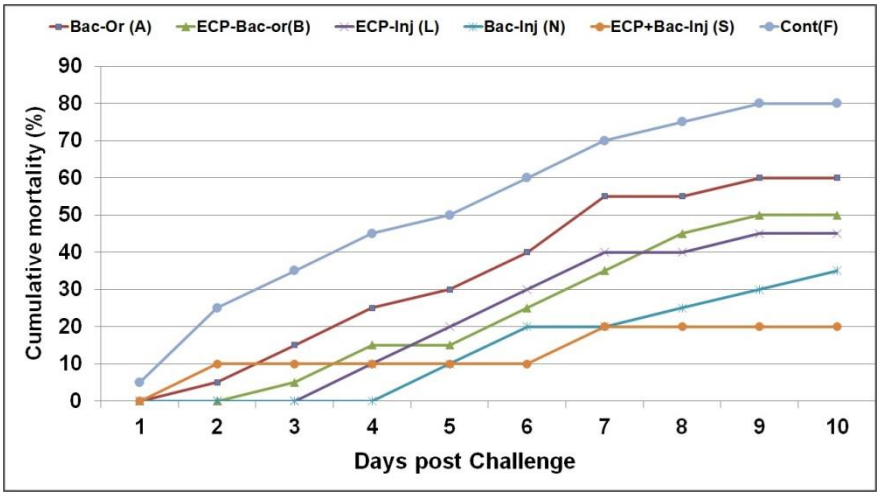


**Figure 6: The cumulative mortality pattern of vaccinated fish after intraperitoneal challenge with *Y. ruckeri*.**

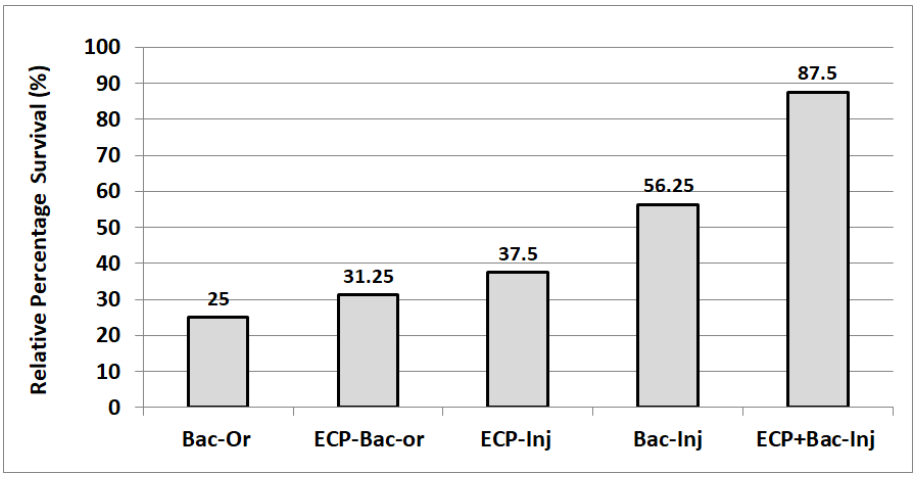


**Figure 7: The Relative Percentage survival (RPS) of vaccinated fish after intraperitoneal challenge with *Y. ruckeri*.**

## Discussion

Over the past decades, several vaccines have been reported to prevent Yersiniosis in rainbow trout. However, most of these studies have focused on killed vaccines in immersion and injection method. To the best of our knowledge, this work is the first to assess the protective efficacy of ECP as an immunogenic subunit for developing an innovative oral and injectable vaccine. In this study, a significant decrease in cumulative mortalities was noted in the vaccinating groups compared to the control group during the experimental period.

Extracellular products are important virulence factors of fish pathogens and are often sufficiently immunogenic to provide protection against challenge. Mass spectrometric analysis of ECP by Zhang *et al*. (2014) identified putative proteins such as chitinase, chitodextrinase, outer membrane protein85, putative metalloprotease, extracellular lipase, hemolysin and elastase that can attend as important immunogens (Zhang *et al*., 2014). Besides Romalde *et al*. (1992)

demonstrated the presence of permeability factors (PF) in the extracellular products (ECP) of 16 strains of the bacterial fish pathogen *Y. ruckeri* which may cause the higher immunogenicity of ECP enriched vaccine (Romalde *et al.*, 1992).

In numerous works the beneficial effects obtained after inoculation with different ECP of Gram-positive and Gram-negative bacteria have been reported by several authors, so that many researchers focused on introducing these products as complementary antigens and adjuvants for vaccine development (Magarinos *et al.*, 1994; Collado *et al.*, 2000; Zorrilla *et al.*, 2003; LaFrentz, *et al.*, 2004). ECP of *A. salmonicida* has also been reported to increase head-kidney macrophage activity in the rainbow trout, *Oncorhynchus mykiss*, in in-vitro situation (Francis and Ellis, 1994).

Non-specific immune responses activation of Nile tilapia *(Oreochromis niloticus)* following administration of some vaccine adjuvants including ECP from Mycobacterium (50 µg/fish) have been reported (Choi *et al.*, 2000). In current research significant correlation was obtained between the antibody titre to extracellular proteins with FKC and survival of vaccinated fish challenged by a virulent bacteria strain. Furthermore, the detection of antibodies in fish sera and intestine mucus was correlated with protection. The overall results indicate a longer duration of specific antibody response in fish vaccinated with the new experimental vaccine as compared with the other immunization groups.

It has been demonstrated that certain bacterial ECPs exert an immunomodulatory effect on hosts (Evenberg *et al.*, 1988; Gudmundsdottir and Magnadottir, 1997). Although most of the work focused on in vivo protective effect of both proteins in the cell wall, total protein or extracellular products of certain bacterial strains, Román *et al*. (2015) reported a potent immunomodulatory effect of the ECP of *V. fluvialis* L21 in the in-vitro situation on sea bass. These results were different somewhat from those obtained in vivo by Lund *et al*. (2003) with the ECPs of *Aeromonas salmonicida* in Atlantic salmon where no protection was obtained against furunculosis.

Results obtained in the present work are consistent with previous studies obtained by Evenberg *et al*. (1988) in carp (*Cyprinus carpio*) vaccinated against *A. salmonicida* with ECPs, where acceptable protective effect of ECP in the vaccinated group was reported.

The role of the ECP as a protective factor for the sole (*Solea solea*) against *V. harveyi* and *V. parahaemolyticus* was demonstrated. Interestingly results showed a high RPS in challenged fish with virulent *V. harveyi* strains despite ineligible RPS in the case of *V. parahaemolyticus* (Zorrilla *et al.*, 2003). Wangkahart *et al*. (2017) described that the level of protection can be used as an acceptable indicator for a successful fish vaccine against ERM.

According to various research, ECP antigens have been used to construct a number of efficacious vaccines against

piscine pathogens such as *Photobacterium damselae* spp. *piscicida* (Magarinos *et al.,* 1994), *Vibrio harveyi* (Zorrilla *et al.,* 2003) and *Flavobacterium psychrophilum* (LaFrentz *et al.,* 2004). The efficacy of ECP in fish immunomodulation highly dependent on bacterial species and rate of ECP concentrations.

The protection level in the group that received FKC via injection route was significantly higher than the control group ($p$=0.001), Meanwhile, a group that received ECP via injection route showed significantly improved protective immunity compared to control group ($p$=0.04). A novel finding of this work was significantly increased protection of fish received ECP enriched FKC in both oral and injection routes ($p$=0.0001). The results of this study demonstrate that however bacteria and ECP were able to produce immunity alone; a combination of the whole cell with ECP could increase efficacy and protective immunity significantly compared to other groups. Some studies have stated that the efficacy of the *S. agalactiae* whole-cell/ECP combination vaccine has been known to provide better protection in tilapia than single vaccines (whole-cell or ECP) (Pasnik *et al.,* 2005; Hardi *et al.,* 2013; Amrullah *et al.,* 2014). It seems that role of ECP in increasing the efficacy of the injection vaccine related to its either antigenic properties or adjuvant effects which induce a higher specific immune response against bacteria (Amrullah *et al.,* 2014).

The protection level after the challenge of immunized fish with ECP is dependent on the specific antibodies produced against ECP and the role of the antigenic properties of ECP which were confirmed in several types of research. The results of Pasnik *et al*. (2005) investigation about ECP of *S. agalactiae* on Nile tilapia revealed a direct correlation between the rate of protection against infection and antibody production upon ECP. They revealed that vaccination of fish with this ECP preparation apparently provided immune protection against the fatal challenge of *A. hydrophila* and 100% of RPS of vaccinated fish may be attributed to the representation of antigens in the ECP preparation group. In the other way, ECP contributed to the development of reliable humoral immune responses against the establishment of infection (Zhang *et al.,* 2014). Although the protein structure of ECP leading to destruction in the digestive tract of the fish, concentrated ECP in the method recommended in this study can preserve ECP and facilitate delivery of antigen to the absorption site in the intestine, which causes a better mucosal immune response to control group. Use of modern preservation methods such as microencapsulation will protect the antigens against gastrointestinal conditions so fish immune responses to oral vaccines will improve significantly (Halimi *et al*., 2018)

Findings revealed in this study suggest that ECP could have an important role in the Immunogenesis of *Y. ruckeri* and can be considered as a protective antigen to

design potential vaccines against this pathogenic micro-organism. Moreover, this work showed that injection administration of the whole cell enriched with ECP provides an extremely reliable immunity in trout, which can be used at the farm scale. Given the great benefits of the oral vaccine, it can be a good option for further investigation to produce oral vaccine in farm scale.

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