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Research Article

Adjuvant effect of Licorice (*Glycyrrhiza glabra*) extract on the efficacy of lactococcosis vaccine in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

This study aimed to assess the adjuvant effect of Montanide ™ ISA 763 AVG and Licorice extract (*Glycyrrhiza glabra*) on the immune responses and disease resistance of rainbow trout (*Oncorhynchus mykiss*) immunized with Formalin inactivated whole cells of Lactococcus garvieae vaccine. Rainbow trout with an average initial weight of 72±3 g were randomLy divided into four experimental treatments: non-vaccinated fish (C), fish immunized with the vaccine without adjuvant (V), fish immunized with vaccine plus Montanide^{TM} ISA 763 A VG (VM), and fish immunized with containing Licorice extract (VL). Sixty fish were used for each treatment group in three replicates each with 20 fish. Fish were immunized via intraperitoneal (IP) injection after being anesthetized with clove oil. The results showed that the highest lysozyme activity, antibody titer and hemolytic activity of the alternative complement pathway were obtained in the VM group followed by VL and V groups compared to the control fish at 8 weeks post-vaccination. After challenge through IP route with *Lactococcus garvieae*, the highest survival rate (96.7%) was observed in VM group, followed by VL (76.7%) and V (76.7%) compared to 20% survival in the control group. In conclusion, the results of this study showed that inclusion of Montanide and lactococcosis vaccine can enhance the potency and efficacy of the vaccine in immunized rainbow trout, but fish immunized with vaccine plus Montanide was superior to fish immunized containing licorice extract.

Keywords: Rainbow trout, Lactococcosis, Vaccine, Montanide, licorice (*Glycyrrhiza glabra)*, Extract

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Introduction

Lactococcosis caused by *Lactococcus garvieae* is an economically worldwide bacterial disease of many aquaculture fish species including salmonids and tilapia (Vendrell *et al*., 2006; Meyburgh *et al*., 2017). The prevalence of Lactococcosis in Iranian rainbow trout farms has been reported by different researchers causing high morbidity and mortality (Soltani *et al*., 2008; Soltani *et al*., 2012; Soltani *et al*., 2015; Haghighi-Karsidani *et al*., 2010; Sharifiyazdi *et al*. 2010; Taherimirghaed *et al*., 2013). The disease prevention by vaccination is one of the major topics interested by the researchers and despite the presence of some commercial vaccines available in the market more research is required to enhance the efficacy and potency of the vaccines. Effect of adjuvants as enhancer of the vaccine efficacy is an important topic associated with fish vaccinology (Valdez *et al*., 2014). Adjuvants can improve the efficacy of vaccines by improving fish immune responses and survival towards the pathogens. Adjuvants may be, however, ineffective to fish immunological functions, or even sometimes with suppressive effects, depending on several variables including host species, age, and adjuvant characteristics (Sivakumar *et al*., 2011). It is, therefore, important to evaluate the clinical efficacy of the adjuvants under *in vivo* works (Soltani *et al*., 2018).

It has been shown that the application of many herbal medicines in form of either extract or essence can improve the fish immune functions (e.g., Soltanti *et al*. 2018; Elumalai *et al*., 2020). Thus, some of the bioactive products of herbs may be good adjuvants for fish vaccines. Montanide™ ISA 763 AVG is a well-known commercially available synthetic adjuvant (Seppic, France) containing an optimum mixture of mannitol and pure oleic acid. It is applicable through injection, combined with an emulsifier (Tepparin *et al*., 2018), and its adjuvant effect for fish vaccines against bacterial diseases such as yersiniosis in rainbow trout (Soltani *et al*., 2016), sterptococcosis in Nile tilapia (*Oreochromis niloticus*) (Tepparin *et al*., 2018) and vibriosis in turbot (*Scophthalmus maximus*) (Xu *et al*., 2019) have been demonstrated with positive efficacy. Licorice or Liquorice (*Glycyrrhiza glabra*) is considered as one of the most extensively used medicinal plants because of its innumerable pharmacological functions. *Licorice* root contains over 300 compounds such as glycyrrhizin/glycyrrhizic acid that exhibit potent antibacterial, antiviral and anti-inflammatory effects (Wang *et al*., 2015; Wang *et al*., 2017; Yang *et al*., 2017). Licorice is one of the most important native herbs in Iran with a large amount of annual export (Bahmani *et al*., 2014). Application of Licorice in form of either powder or extract in aquaculture is promising due to its immune-stimulatory effects. For instance, in a recent study by Abdel-Tawwab and El-Araby (2021), feeding Nile tilapia with Licorice root powder

at the level of 10–20 g/kg feed for 60 days significantly enhanced immunological responses and increased fish survival against *Aeromonas hydrophila* infection. However, there is no data on the Licorice extract as an adjuvtant of fish vaccine. This study aimed to assess a comparative adjuvant effect of Licorice extract as natural herb adjuvant with Montanide as a well-known synthetic adjuvant on efficacy and potency of lactococcosis vaccine against *L. garvieae* infection in rainbow trout.

Materials and methods

Fish and water quality

This study was conducted in the autumn of 2019 in the Faculty of Veterinary Medicine, Shahrekord University, Chaharmahal and Bakhtiari province, Iran. Healthy French strains of rainbow trout weighing 72±3 g were obtained from a trout farm and transported to the experimental tanks. The fish were adapted to new condition for two weeks and were fed with commercial extruded diet (Faradaneh Comp. Iran) with approximate feed composition of 38% crude protein, 9% carbohydrate, 14% crude fat, 3% fiber, 9% ash, and 8% moisture. Feeding was proportional to the size of the fish and the feeding table based on body weight percentage. Water quality parameters including temperature, pH, dissolved oxygen, NH_3 and NO_2 were 14±1ºC, 7.5±0.2, 8.5±0.5, <0.01 mg/mL, and <0.1 mg/mL, respectively.

Adjuvants

To prepare the alcoholic extract of Licorice (*Glycyrrhiza glabra*), the root cuts of the herb were thoroughly washed, dried and powdered before being soaked in high-purity alcohol at 2-8ºC and stirred every 8 hours by a glass mixer. After 32 hours, the obtained extract was filtered using Whatman No. 1 paper. Then the solvent (Ethanol) was used in a rotary vacuum distillation at 23ºC and the resulting mixture was sterilized using 0.2 µm Millipore filter before being stored at 4ºC (Gupta *et al.*, 2008). The sterile extract was mixed with the vaccine in equal volume (v/v: extract/vaccine) before being used in fish.

Commercial synthetic Montanide™ ISA 763 AVG (Seppic, Courbevoie, France) recommended for injection was used in the ratio of 3:7 according to the manufacturer's instruction.

Vaccine preparation

Formalin inactivated whole cells of *Lactococcus garvieae* (Department of Aquatic Animal Health, University of Tehran) obtained from diseased rainbow trout in a trout farm with sever lactococcosis outbreak in Iran was used for antigen preparation (Soltani *et al.*, 2014). The isolate was previously characterized by phenotyping and molecular studies by Soltani *et al.* (2008). A lyophilized ampoule of the fourth passage of bacterium was grown in tryptic soya broth agar (TSA) incubated at 30^{o}C for 48 h, before the cells being harvested in sterile

phosphate buffered saline (PBS) by centrifugation (7500 rpm) of the culture at 8°C for 25 min. The purity of the cells was undertaken by a subculture of the cells on blood agar at 30°C for 48 followed by Gram staining. The cell density was then measured using a serial dilution (CFU/mL) cultured on blood agar before inactivating the bacterial cells by formalin (Merck). The formalin-inactivated cells were kept at 4°C overnight before further centrifugation (8000 rpm) at 4°C for 15 min. After twice washing of inactivated pellet cells in sterile PBS, the safety of the antigens was examined by culturing of the cells on blood agar at 25°C for 72h and an intraperitoneal injection of 0.6 mL of the antigens into 10 healthy trout weighing about 200 g. Inactivated bacterial suspension was packed in a sterile bottle, kept at 4°C until used for immunization. Based on the viable cell count, the final concentration of inactivated whole cells used for the vaccination was 1×0^9 cells/mL.

Vaccination protocol

The fish were randomLy divided into four experimental groups: control, non-adjuvanted vaccine, Montanide-adjuvanted vaccine and licorice extract-adjuvanted vaccine (Table 1).

Table 1: Protocol of vaccination in rainbow trout towards *L. garvieae.*

Treatment	No. of fish	Vaccine dosage
Vaccine	180	200 µL vaccine (2×10^8 CFU/fish)
Vaccine+Montanide	180	200 µL vaccine (2×10^8 CFU/fish)+200 µL Montanide
Vaccine+Licorice extract	180	200 µL vaccine (2×10^8 CFU/fish) + 200 µL sterile Licorice extract
Control	180	PBS at 200 µL /fish

Each treatment contained 120 fish in three replicates, 10 fish in each replicate. Formalin-inactivated whole-cell bacterial vaccine was mixed with either Montanide™ ISA 763 AVG adjuvant or Licorice extract. Fish were vaccinated via IP injection after fish being anesthetized with clove oil at 150 mg/L. The control fish received sterile PBS at 200 µL/fish, and groups of vaccinated fish were given 200 µL vaccine (2×10^8 CFU/fish) (V), 200 µL vaccine (2×10^8 CFU/fish) containing 200 µL Montanide (VM), and 200 µL vaccine (2×10^8 CFU/fish) containing 200 µL of the sterile Licorice extract (VL). The final concentration of vaccine with or without the adjuvants was 2×10^8 CFU/fish. Each group was vaccinated separately and returned to their original tanks after vaccination. Fish feeding was stopped 24 h before vaccination. The vaccinated fish were kept for 56 days, before sampling and efficacy assessment. The safety of vaccines was tested by IP injection of double dose [400 µL (2×10^9 CFU/fish)] of the intended dose of the vaccine.

Sampling

Blood samples were taken from the caudal vein of 15 fish per treatment (five fish per replicate) at each sampling time (at weeks 2, 4, 6 and 8 post-vaccination) after fish been anesthetized with clove oil and sera samples were separated by centrifugation of blood samples at 5000 rpm for 15 min before been stored at 4 ^{o}C until used for the following assays.

Lysozyme assay

The level of lysozyme activity was detected according to Ellis (1990). Briefly, a twenty mg of *Micrococcus lysodeikticus* was diluted in 100 mL of 0.02 M citrate buffer at pH 5.5, and 15 µl of the serum samples and 150 µl of the *Micrococcus* were added to microplate wells, before the optical density of each well been measured at 450 nm wavelength immediately and after 3 min, and the results were expressed in µL/mL. The serum lysozyme activity was assessed using standard curve by different concentrations of chicken egg white lysozyme (Sigmae Aldrich, USA).

Alternative hemolytic complement (ACH_{50}) assay

Analysis of the alternative complement activity was achieved by evaluating the ability of serum samples to lyse rabbit red blood cells, following the method of Amar *et al*. (2000). In breif, rabbit red blood cells were washed three times with gelatin veronal buffer and EGTA Mg^{2+} and the number of cells was adjusted to 2×10^{8} cells per mL of buffer, using a hemocytometer. For obtaining a 100% lysis, 100 µL of the above suspension was added to 3.4 mL of distilled water; the sera samples were then diluted 100 times with the above buffer and different volumes were prepared in sterile test tubes and the volume of all tubes was increased to 250 µL, using the working buffer. Finally, 100 µL of rabbit red blood cells were added to all tubes and the mixtures were incubated at 20ºC for 90 minutes before adding 3.15 mL of 0.85% sodium chloride solution to each tube. The mixtures were centrifuged at 1600×g for 10 min at 4°C before measuring the optical density of the reaction supernatant at 414 nm. The reciprocal of the sample dilution inducing 50% lysis of RBCs was designated as the ACH50 titer. ACH_{50} was measured using the following equation:

$$ACH_{50}\ (U/mL)=K\times\text{Dilution factor}\times0.5$$

where K is the reciprocal of the sample dilution inducing 50% lysis and, dilution factor is 0.01, as serum was diluted 100 times.

Antibody titer

The antibody titer in 96-well plates was measured by agglutination test according to Eldar *et al*. (1997). A total volume of 100 µL of serum sample was used in each well and the plates were incubated at 37°C overnight. The formation of a round precipitate with sharp contours was considered as a negative reaction, while the appearance

of a good button with fuzzy edges was considered as a positive reaction.

LD_{50} determination

Lactococcus garvieae was cultured in TSB medium at 30°C for 24 h before been harvested by centrifugation at 3500 rpm for 10 min. The harvested cells were washed three times in sterile PBS before resuspending in normal saline solution to obtain turbidity equal to McFarland Standard No. 7 (2.1×10^9) (Sun *et al.*, 2011). A serial dilution of this suspension was prepared for LD_{50} determination. The anesthetized fish (12 fish per dilution) were injected IP with the serial dilutions of the prepared bacterial dilutions from 10^4 to 10^9 cells/mL. Fish in each dilution received 0.1 mL/fish. The fish mortality in each dilution was recorded for 14 days, and the cause of mortality was confirmed by re-isolation of the *L. garvieae* from the internal organs (head kidney or spleen) on blood agar. The LD_{50} was calculated using Probit analysis.

Efficacy of vaccination

The relative percent survival (RPS) in the vaccinated fish was determined at 2, 4, 6, and 8weeks post-immunization. Ten fish in three replicates from each trial were used randomLy for each challenge test. The fish were starved for 48 h before immunization. The anesthetized fish were then IP injected with 0.1 mL of the virulent strain of *L. garvieae* (the same strain used for vaccine preparation) based on the previous LD_{50} determination (2.1×10^6 CFU/fish). The fish were maintained at the similar water quality for two weeks, and daily mortality of each group was collected for disease confirmation by culturing of the kidney samples on blood agar. The RPS of each trial was determined using the below equation (Ispir and Dorucu., 2010):

RPS = 1 - (% mortality of vaccinated fish / % mortality of unvaccinated fish) × 100

Statistical analysis

Shapiro–Wilk test was used to examine the normal distribution of data. One-way analysis of variance (ANOVA) was carried out followed by Duncan and Tukey tests to compare the significant differences of means (Mean±SD). Differences were considered significant at $p<0.05$. All statistical tests were carried out with SPSS ver.22.

Results

Lysozyme activity

Serum lysozyme activity at 8 weeks post-vaccination is shown in Fig. 1. All vaccinated groups significantly exhibited a higher level of lysozyme activity than the control fish ($p<0.05$). The highest level of serum lysozyme activity was observed in fish vaccinated with vaccine + Montanide (169.24±6.85 µg/mL) followed by Licorice extract-adjuvanted vaccine group (153.21±5.27 µg/mL) and non-adjuvanted vaccine

group (147.12±7.12 µg/mL) at 8 weeks post-immunization. No significant difference was also observed among the vaccinated groups (p>0.05).

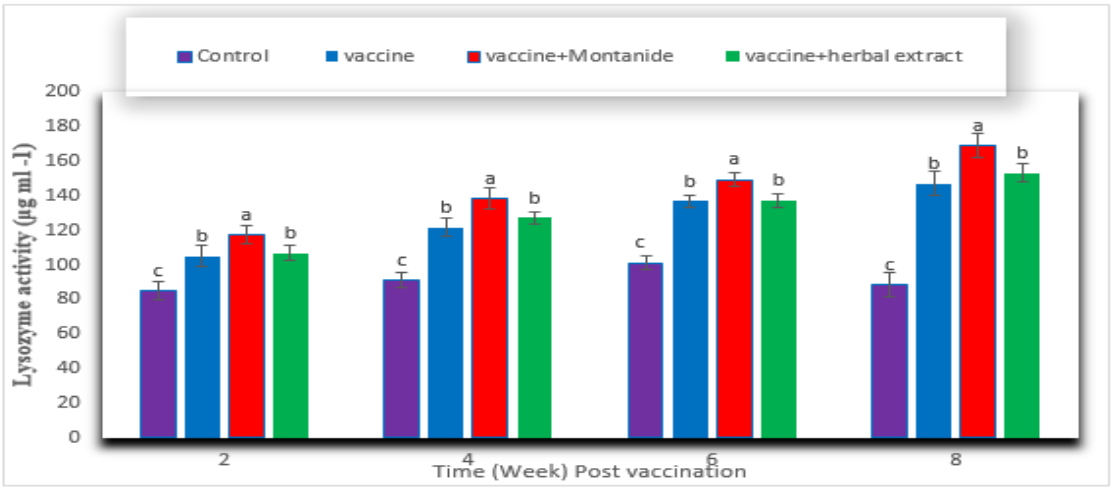

Figure 1: Serum lysozyme activity in rainbow trout vaccinated with lactococcosis vaccine contained synthetic and natural adjuvants at 15ºC (Mean ± SD). Non-identical letters in each column indicate a significant difference at the level of 5%.

ACH_{50} activity

All vaccinated groups revealed significantly higher ACH_{50} activity than the control fish (p<0.05), and the highest ACH_{50} activity was measured in Montanide-adjuvanted vaccine (267±4.33 mg/dl) and non-adjuvanted vaccine (260.67±6.8 mg/dl) groups, respectively, but no significant difference was seen between non-adjuvanted vaccine and Licorice extract adjuvanted vaccine (248.32 ±4.6 mg/dl) at 8-week post-vaccination (p>0.05) (Fig. 2).

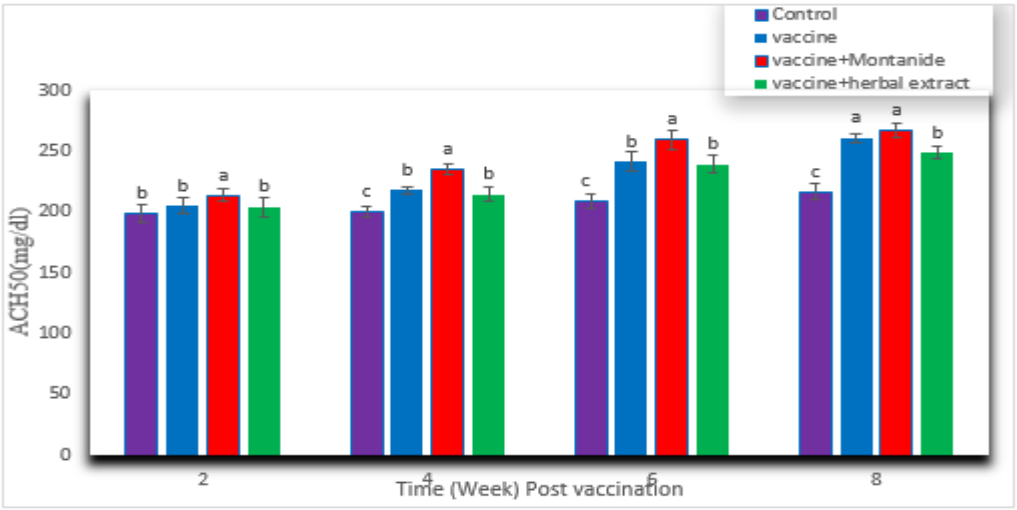

Figure 2: ACH_{50} activity in rainbow trout vaccinated with lactococcosis vaccine contained synthetic and natural adjuvants at 15ºC (Mean±SD). Non-identical letters in each column indicate a significant difference at the level of 5%.

Serum agglutination titer

Vaccinated fish significantly exhibited a higher level of agglutination titers than the control fish (p<0.05) at 8 weeks post-vaccination (Fig. 3). The highest level of antibody titers was observed in fish vaccinated with vaccine+Montanide (2.35±0.03) followed by Licorice extract-adjuvanted vaccine group (2.09±0.04) and non-adjuvanted vaccine group (1.45±0.01) at 8 weeks post-immunization. No significant difference was also observed between Licorice extract-adjuvanted vaccine group and non-adjuvanted vaccine group at 8 weeks post-vaccination (p>0.05).

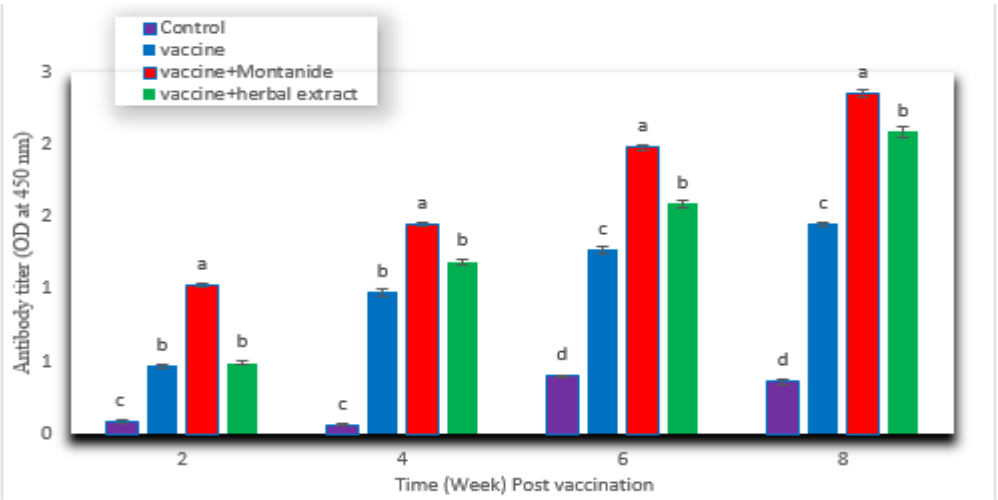

Figure 3: The antibody titer in rainbow trout vaccinated with lactococcosis vaccine contained synthetic and natural adjuvants at 15ºC (Mean ± SD). Non-identical letters in each column indicate a significant difference at the level of 5%.

Vaccine efficacy

The survival rate of immunized fish challenged with *L. garvieae* at different times post-vaccination are shown in Fig. 4. All vaccinated groups significantly presented a higher survival rate than the control fish (p<0.05). The highest survival rate was obtained in fish Montanide-adjuvanted vaccine group (96.66±3.33%), while the lowest survival was seen in the control group (20%) at 8 weeks post-immunization. An identical survival rate of 76.66% was obtained in both vaccines without adjuvant and Licorice extract adjuvanted-vaccine groups (p>0.05) but was significantly lower than Montanide-adjuvanted vaccine group (p<0.05).The relative survival percentage (RPS) at the end of the eight-week post-vaccination is shown in Fig. 5. The RPS in Montanide-adjuvanted fish was significantly higher (95.83±3.31) than the other vaccinated groups (p<0.05) at 8 weeks post-vaccination. No significant difference was seen between fish immunized with the vaccine without adjuvant (70.83±3.33) and Licorice extract-adjuvanted vaccine group (70.82±1.09) (p>0.05).

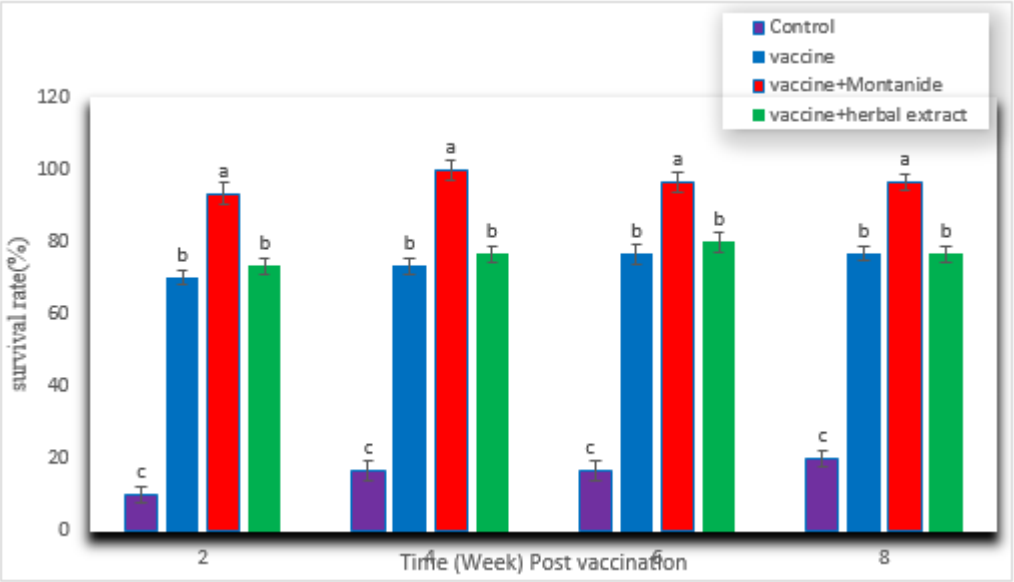

Figure 4: Survival rates in rainbow trout vaccinated with lactococcosis vaccine contained synthetic and natural adjuvants at 15ºC (Mean±SD). Non-identical letters in each column indicate a significant difference at the level of 5%.

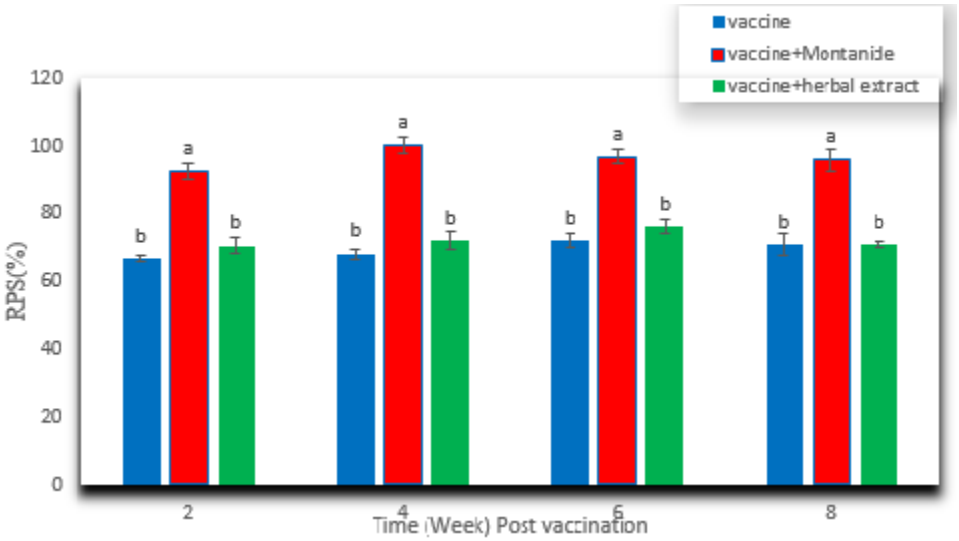

Figure 5: Relative percentage survival of rainbow trout vaccinated with lactococcosis vaccine contained synthetic and natural adjuvants at 15ºC (Mean ± SD). Non-identical letters in each column indicate a significant difference at the level of 5%.

Discussion

The widespread environmental problems posed by the overuse of drugs and antibiotics have threatened the health of aquatic consumers in many regions, particularly in Asian countries. Frequent outbreaks by virulent strains of *L. garvieae* are the cause of acute or hyperacute septicaemia with mass mortality in many commercial fish species worldwide which results in mass mortality (Vendrell *et al*., 2006; Soltani *et al*. 2008; Meyburgh *et al*., 2017). Protection via immunization is, therefore, an efficacious procedure for preventing the disease outbreaks in

aquaculture systems. At present, inactivated vaccines are the most common vaccines used in the aquaculture sector with a relatively acceptable efficacy (Munang'andu *et al.*, 2016). Bacterial pathogenicity, antigen characterization and host immune responses are primarily important criteria to produce efficacious vaccines. Results of some studies exhibited that the capsulated strains of *L. garvieae* are more virulent than the non-capsulated strains (Yoshida *et al.*, 1997). It seems that some European and Japanese strains of the bacterium are non-typeable for cross-reaction tests, indicating the diversity in isolates from different regions (Barnes and Ellis, 2004; Kawanishi *et al.*, 2006; Tsai *et al.*, 2012). In a study conducted by Taherimirghaed *et al.* (2013), the Iranian isolates of *L. garvieae* from rainbow trout are genetically diverse. These data reveal the diversity of *L. garvieae* isolates from a different host and geographic areas.

Although vaccination of fish against infectious diseases including bacterial pathogens has a long history in developed countries, this history is limited to the recent years in Iran (Soltani *et al.*, 2007, 2014, 2016, 2018). Extensive studies have been conducted to prove the role of various adjuvants on the specific and innate immune response and vaccine efficacy in aquaculture fish species (Tafalla *et al.*, 2013; Soltani *et al.*, 2014, 2018). In the present study, we demonstrated the mechanism of protection by evaluation of the ACH_{50} and lysozyme activities of serum, specific antibody response, and survival rate in vaccinated and non-vaccinated trout. The activity of lysozyme is an important factor to assess the animal innate immune response by activating the complement cascade and to improve chemotactic activity as an opsonin factor (Saurabh and Sahoo, 2008). At 8 weeks post-vaccination all vaccinated trout significantly demonstrated a higher level of lysozyme activity than the control fish, but the highest activity was found in trout vaccinated with vaccine+Montanide followed by *Licorice* extract-adjuvanted vaccine group and non-adjuvanted vaccine group. However, there was no significant difference in lysozyme activity among all vaccinated groups. Similarly, all vaccinated trout revealed significantly higher ACH_{50} activity than the control fish, and ACH_{50} activity in Montanide-adjuvanted vaccine was significantly higher than licorice extract adjuvanted vaccine at 8-week post-vaccination. The enhancement in lysozyme activity in all vaccinated fish may be in part due to the introduction of bacterial antigens and lipopolysaccharide exposure to macrophages causing an enhancement in the lysozyme production (Soltani *et al.*, 2008). The increase in lysozyme activity, respiratory burst and total IgM in tilapia (Rao *et al.*, 2020), yellow perch (*Perca flavescens*) (Elabd *et al.*, 2016) and yellow catfish (*Pelteobagrus fulvidraco*) (Wang *et al.*, 2020) have

been demonstrated after fish being fed with either powder or extract of licorice root extract indicating the immune-stimulating effects of licorice (Pastorino *et al*., 2018; Jiang *et al*., 2020), that could be in part due to the presence of the phenolic compounds especially glycyrrhizin (Mazumder *et al*., 2012). However, more studies are required to precisely show the role of lysozyme protection in the vaccinated fish. Moreover, the adjuvant assistance for a delayed release of the bacterial antigens may help a continuous activation of the complement cascade via the alternative pathway.

All vaccinated trout revealed significantly higher antibody titers than the control fish at 8 weeks post-vaccination, but fish immunized with Montanide-adjuvanted vaccine demonstrated the highest antibody titers followed by Licorice extract-adjuvanted vaccine group. In studies conducted by other researchers, vaccination of fish with *L. garvieae* containing different adjuvants such as Montainde ISA763 showed higher antibody titers, which was positively correlated with the protective efficacy after challenge with *L. garvieae* infection (Ravelo *et al*., 2006; ; Kubilay *et al*., 2008; Bastardo *et al*., 2012; Soltani *et al*., 2016; Bwalya *et al*., 2020; Rao *et al*., 2020). However, use of Licorice extract as a natural herb adjuvant was not superior to the synthetic adjuvant (Montanide ISA763) due to a lower RPS in the trout immunized with vaccine+Licorice extract (RPS=70.82±1.09) than the fish vaccinated with vaccine + Montanide (RPS=95.83±3.31). In a study by Raissy *et al*. (2018), using *Nigella sativa* and *Satureja bachtiarica* essential oils as possible adjuvants for lactococcosis vaccine exhibited that *N. sativa* was more effective than *S. bachtiarica* inducing higher protection (73.3% vs. 67.3% RPS) against *L. garvieae* infection at six weeks post-vaccination. Use of adjuvanted vaccines has been suggested to be considered as a validation tool for protection against *L. garvieae* infection (Rao *et al*., 2020), because the re-isolation of *L. garvieae* was possible from grey mullet which previously immunized with inactivated whole cells of the bacterium after challenging, but the bacterial re-isolation from immunized fish with Montanide-adjuvanted vaccine was nothing. The potential of the vaccine to protect against periodic heterologous isolates of *L. garvaiae* should also be considered.

In conclusion, this study exhibited that trout IP vaccinated by formalin inactivated whole cells of *L. garvieae* was able to enhance fish immune responses and caused significantly higher protection compared to control fish. However, the use of adjuvant vaccines was superior inducing higher protection compared to vaccine alone. The synthetic adjuvant (Montanide ISA763) was also superior in assisting a higher immune response and higher survival rate than natural herb (*Licorice* extract) at 8 weeks post-vaccination.

Acknowledgements

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Ethical approval

This article does not contain any studies with human participants by any of the authors. All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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