Efficacy and immune response of intraperitoneal vaccination of rainbow trout (*Oncorhynchus mykiss*) with a *Yersinia ruckeri* bacterin formulated with Montanide™ ISA 763 AVG adjuvant

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Abstract

Yersiniosis is an economically important bacterial disease affecting the aquaculture industry. Some immune response parameters and efficacy of intraperitoneal (IP) injection of *Yersinia ruckeri* bacterin (biotype I) formulated with Montanide ISA 763 AVG adjuvant were assessed in 100 g rainbow trout during 2-12 weeks post-vaccination. Fish were immunised by IP injection with the bacterin (ca 1 × 10⁷ cfu/fish) of a virulent strain of *Y. ruckeri* with or without adjuvant and kept at 14°C. Both cellular and humoral immune responses consisting of leukocyte count, lymphocyte population, lysozyme level, alternative hemolytic complement activity (ACH₅₀) and antibody titer in fish immunised with the bacterin containing Montanide were higher than in fish immunised with the bacterin alone. However, these differences were not consistently significant between the two groups. The relative percent survival (RPS) up to 12 weeks post-immunisation reached 85-100% in fish immunised with the adjuvanted formulation while that of fish immunised with the bacterin alone was in the range of 75-90%. Our data showed that Montanide ISA 763 AVG used as an adjuvant in injectable *Y. ruckeri* vaccines can enhance both survival and immune response of rainbow trout.

Introduction

Vaccination is the most adequate method to prevent infectious diseases that threaten the aquaculture industry worldwide (Cossarini-Dunier, 1986; Sudheesh et al., 2012). Protection of aquatic animals via vaccination from contagious diseases is nowadays a well-established method for sustainable aquaculture development. Although most vaccines stimulate the immune system of fish to the infectious agents (Newman, 1993; Stevenson, 1997; Ellis, 2001), some vaccines such as whole inactivated cells and vaccines based on recombinant antigens

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alone are usually not able to confer a complete protection in aquatic animals.

Enteric red mouth disease is a systemic bacterial infection caused by *Yersinia ruckeri* that can cause heavy economic losses in salmonid and non-salmonid species (Tobback et al., 2007; Shaowu et al., 2013). Also, recently this bacterium was recovered from a human patient whose leg was hit with a stone prior to paddling in a river in Belgium (Keukeleire et al., 2014).

Yersiniosis was the first infectious disease of fish for which an effective vaccine was made; and inactivated whole cell suspensions of *Y. ruckeri* bacterin is now available that can be used by ways of bath, injection and oral (as booster) but with different duration of efficacy (Kumar et al., 2015). Also, more recently new types of vaccines such as lipopolysaccharide, live aroA gene mutants or extracellular products have provided good efficacy against *Yersinia septicemia* in fish (Temprano et al., 2005; Ispir and Dorucu, 2010, 2014). However, the use of adjuvants and immunostimulants is often necessary to increase the efficacy and especially the duration of protection of inactivated fish vaccine antigens (Aucouturier et al., 2001; Anderson and Jeney, 1992; Siwicki et al., 1994).

Oil-based adjuvants work on the basis of inducing deposition of antigen, improving antigen delivery to antigen-presenting cells or by attracting effector cells to injection sites (Thim et al., 2014). We previously found a positive effect of Montanide ISM1312 VG adjuvant formulated in anti-*Yersinia ruckeri* bacterin as a bath immersion vaccine in rainbow trout for up to 10 weeks post-vaccination (Soltani et al., 2014). In the current study we aimed to evaluate the effect of Montanide™ ISA 763 AVG as adjuvant for an injectable *Y. ruckeri* vaccine in trout.

**Materials and methods**

**Experimental animals**

Twelve hundred rainbow trout (*Oncorhynchus mykiss*) weighing 100±10 g obtained from a commercial fish farm in Iran were used in this study. Fish were acclimatised for 15 days prior to the vaccination trial in 4 separate fiberglass tanks each containing 300 fish. Fish were fed at 2% body weight daily using a commercial feed (Fara Daneh fish feed company, Iran). During the trials well water was used. The water quality parameters were monitored daily and water temperature, pH, dissolved oxygen, NH₃ and NO₂ were 14±0.5°C, 7.8 and 8.2 mg/L, <0.01 mg/L and <0.1 mg/L, respectively.

**Antigen preparation**

A virulent strain of *Y. ruckeri* (KC291153) obtained from the Department of Aquatic Animal Health; University of Tehran was used for antigen preparation. This bacterial strain was previously isolated from diseased rainbow trout in north Iran and has been characterised as *Y. ruckeri* biotype I (Soltani et al., 2014). A lyophilised ampoule of the bacterium was first grown in tryptic soya broth (TSB) at 25°C for 48 h. The bacterial cells were then re-suspended in sterile phosphate buffered saline (PBS) after centrifuging (1400 x g) of the culture at 10°C for 30 min. After purity examination, the cell concentration was determined by spreading of a serial dilution (colony forming unit/mL) on blood agar, and adjusted so that the final concentration of the stock antigen was 1 × 10⁸ cfu/mL. The inactivated bacterin was made as previously described (Soltani et al. 2014). The sterility of the bacterin was confirmed using
intraperitoneal (IP) injection of the antigen into healthy trout plus its inoculation on blood agar at 25°C for 72 h.

**Adjuvanted vaccine formulation**
Montanide ISA 763 AVG (Seppic, France) was used to formulate the vaccine. The adjuvant was mixed with the antigen at low temperature (vortexing for 10 min) at a ratio of 66 parts of adjuvant: 33 parts of antigen, as recommended by Seppic (vortexing the mixture for 10 min).

**Immunisation protocol**
A total of 300 fish were used for each group. The first group was IP immunised with the antigen without the adjuvant at 0.3 mL/fish (1 × 10⁷ cfu/fish; 0.1 mL of stock antigen diluted in 0.2 mL sterile PBS). The second group was vaccinated by IP injection with 0.3 mL/fish of the adjuvanted vaccine formulation (i.e. 0.1 mL of the bacterin antigen). The third and fourth groups were IP injected with sterile PBS or the Montanide adjuvant at 0.3 mL/fish to serve as a saline or adjuvant control, respectively. Fish were not fed for 24 h prior to immunisation. Also, fish were anesthetised with MS₂₂₂ (Merck, Germany) at 25 mg/L under aeration before the injection.

**Immunocompetent cell population**
The Klontz procedure was used for measuring the leukocyte population sizes (white blood cells, lymphocyte and heterophil cells) (Sommerset et al., 2005). Fish from the immunisation tanks were anesthetized with MS₂₂₂ and blood samples were obtained from the caudal veins of 10 fish from each group for 10 weeks post-vaccination. The blood smears were fixed with methanol, stained with 5% Giemsa staining for 20 min and used for differential leukocyte count under a compound microscope with x 40 magnification.

**Serum lysozyme assay**
The Kumari and Sahoo (2006) procedure was used for measuring the lysozyme activity. *Micrococcus lysodeikticus* (20 mg) were diluted in 100 mL of 0.02 M citrate buffer at pH 5.5. A volume of 150 µL of the *Micrococcus* and 15 µL of the serum samples were added to micro plate wells and optical density of each well was read at 450 nm immediately and after 3 min. The serum lysozyme activity was assessed using a standard curve consisting of different concentrations of chicken egg white lysozyme (Sigma-Aldrich, USA).

**Alternative hemolytic complement activity (ACH₅₀)**
Alternative hemolytic complement activity (ACH₅₀) was undertaken following the method described by Matsuyama et al. (1988). Briefly, a volume of 200 µL of 2.5 × 10⁸ sheep RBC in EGTA-Mg-GVB buffer were added to 500 µL of the serum serially diluted in buffer EGTA-Mg-GVB (10 mM EGTA, 10 mM MgCl₂, 0.1% gelatin) incubated 90 min at 15°C. An aliquot of 2.8 mL of EDTA-GVB (10mM EDTA, 0.1% gelatin) were then added to the mixture and centrifuged. The optical density of the samples were read at 414 nm and Y value (percentage of hemolysis/100) was measured. The value Y/ (1-Y) and the reciprocal of the serum dilutions were inserted on semi-log graph paper and the ACH₅₀ (units / mL) was determined as the reciprocal dilution giving 50% hemolysis (Y/ (1-Y) = 1).

**ELISA**
Enzyme–linked immunosorbent assay (ELISA) described by Raida et al. (2011) and Deshmukh et al. (2012) with slight modifications was used to detect the anti-*Y. ruckeri* antibody titer in the
serum samples. Briefly, flat bottom 96-well plates were coated with 100 µL/well of coating buffer (Sigma-Aldrich cat no. 3041) containing 5 µg/mL of antigen (sonicated and diluted Y. ruckeri) and incubated overnight at 4°C. After discarding the coating solution, unbound antigen was removed by three washes with 400 µL washing buffer (0.1% Tween 20 in PBS, pH 7.2). Blocking buffer (2% bovine serum albumin (BSA) in washing buffer) was then added at 200 µL/well and incubated for 1 h at room temperature. Microplates were then sealed after three times washing with washing buffer and were stored at -20°C until used. The optimal ratio between specific reaction and background binding was obtained as a 100 fold dilution of serum in the pilot trial (data not shown), thus 100 fold dilutions of sera with assay diluent buffer (PBS pH 7.2 containing 0.1% Tween 20 and 0.1% BSA) were made in replicate from each fish serum sample. A volume of 100 µL of diluted serum sample was then added in each of duplicate wells, sealed and incubated at 4°C overnight. After three washings, a volume of 100 µL of a mouse anti-salmonid antibody solution (Ab Dserotec can no. MCA2182) was added to each well and incubated at room temperature for one h. The plates were then washed three times prior to adding a 100 µL Fab-HRP solution (AbD Serotec can no. Star 13B) to each well. After 1 h incubation at room temperature, the plates were washed three times again. The substrate (TMB cat no. BFC042A AbD Serotec) was then added at 100 µL/well followed by adding a stop solution (100 µL 1 N HCl/well) after 15 min. The optical density was then read at 450 nm. Wells with only dilution buffer were used as background reading as this value was subtracted from other values to adjust background. A positive reference control serum sample taken from hyperimmunised rainbow trout (trout immunised intraperitoneally three times with Y. ruckeri bacterin) was used. The test was run in duplicate for each plate.

**Efficacy of vaccination**

The setup for the evaluation of efficacy of the vaccine is given in Table 1. The LD<sub>50</sub> of the bac-

<table>
<thead>
<tr>
<th>Trial</th>
<th>Number of fish</th>
<th>Dosage of injection</th>
<th>Number of fish per challenge</th>
<th>Challenge time (weeks post immunisation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunised with Y. ruckeri bacterin</td>
<td>300</td>
<td>0.3 mL/fish (1 × 10&lt;sup&gt;7&lt;/sup&gt; cfu/fish) (0.1 mL antigen: 0.2 mL PBS)</td>
<td>20 × 2</td>
<td>2, 4, 6, 8, 10</td>
</tr>
<tr>
<td>Immunised with adjuvanted Y. ruckeri vaccine formulation</td>
<td>300</td>
<td>0.3 mL/fish (1 × 10&lt;sup&gt;7&lt;/sup&gt; cfu/fish) (0.2 mL adjuvant: 0.1 mL antigen)</td>
<td>20 × 2</td>
<td>2, 4, 6, 8, 10</td>
</tr>
<tr>
<td>Adjuvant control (Montanide ISA 763 AVG)</td>
<td>300</td>
<td>0.3 mL Montanide ISA 763 AVG)</td>
<td>20 × 2</td>
<td>2, 4, 6, 8, 10</td>
</tr>
<tr>
<td>PBS control</td>
<td>300</td>
<td>0.3 mL PBS</td>
<td>20 × 2</td>
<td>2, 4, 6, 8, 10</td>
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</table>

Table 1. Setup for evaluation of the effect of Montanide<sup>™</sup> ISA 763 A VG ISA adjuvant on the efficacy of a Y. ruckeri bacterin in rainbow trout. From two to 10 weeks post immunisations, challenge experiments were carried out in 40 fish per group every 2 weeks.
Y. ruckeri bacterin was obtained by intraperitoneal injection (IP) of serial dilutions of Y. ruckeri into groups of 10 healthy rainbow trout at 14°C for 2 weeks. Two replicates of twenty fish from each group were randomly used for each challenge test, for which each fish was IP injected with 0.1 mL/fish (1×10⁶ cfu/fish; equal to the LD₅₀) of a live Y. ruckeri culture prepared from the same strain used for vaccine preparation (homologous challenge). The fish were maintained (fish of each replicate in a separate tank) at similar water quality for 3 weeks, and kidney tissues of the daily mortality were inoculated on TSA, incubated at 20°C for 72 h to confirm the cause of death. The RPS of each challenge experiment was determined using the below equation (Ellis, 2001).

\[
RPS = \left(1 - \frac{\% \text{mortality of vaccinated fish}}{\% \text{mortality of unvaccinated fish}}\right) \times 100
\]

The saline control group was considered as unvaccinated group in this calculation.

**Statistical analysis**

One-way ANOVA analysis followed by Tukey post-hoc test was used for data analysis and differences were considered at P<0.05. Protection data were also compared between groups using achi-square statistical tests.

**Results**

**Leukocyte count**

Leucocyte and lymphocyte population sizes in fish immunised with the adjuvanted *Yersinia ruckeri* vaccine formulation were not significantly higher than fish immunised with *Yersinia ruckeri* vaccine throughout the trial (P >0.05). However, the leukocyte and lymphocyte counts in these two vaccinated groups were significant-

<table>
<thead>
<tr>
<th>Trial</th>
<th>Leukocyte count (10⁴/mm³) (mean±SD)</th>
<th>Lymphocyte count (%)(mean±SD)</th>
<th>Heterophil count (%)(mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time post vaccination (week)</td>
<td>Time post vaccination (week)</td>
<td>Time post vaccination (week)</td>
</tr>
<tr>
<td></td>
<td>2 4 6 8 10</td>
<td>2 4 6 8 10</td>
<td>2 4 6 8 10</td>
</tr>
<tr>
<td>A</td>
<td>6.2±0.9</td>
<td>5.9±0.9</td>
<td>87±0.6</td>
</tr>
<tr>
<td></td>
<td>5.9±0.8</td>
<td>5.7±0.8</td>
<td>87±0.6</td>
</tr>
<tr>
<td></td>
<td>5.6±0.7</td>
<td>5.3±0.5</td>
<td>87±0.6</td>
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<tr>
<td></td>
<td>5.3±0.5</td>
<td>5.1±0.3</td>
<td>87±0.6</td>
</tr>
<tr>
<td></td>
<td>5.1±0.3</td>
<td>4.9±0.8</td>
<td>87±0.6</td>
</tr>
<tr>
<td>B</td>
<td>6.8±1</td>
<td>6.4±0.8</td>
<td>92±0.3</td>
</tr>
<tr>
<td></td>
<td>6.4±0.8</td>
<td>6.1±0.1</td>
<td>92±0.3</td>
</tr>
<tr>
<td></td>
<td>5.8±0.9</td>
<td>5.4±0.7</td>
<td>92±0.3</td>
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<tr>
<td></td>
<td>5.4±0.7</td>
<td>5.1±0.5</td>
<td>92±0.3</td>
</tr>
<tr>
<td></td>
<td>5.0±0.5</td>
<td>4.9±0.8</td>
<td>92±0.3</td>
</tr>
<tr>
<td>C</td>
<td>8.7±0.8</td>
<td>8.3±0.8</td>
<td>95±0.7</td>
</tr>
<tr>
<td></td>
<td>8.3±0.8</td>
<td>8.0±0.7</td>
<td>95±0.7</td>
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<td></td>
<td>7.6±0.8</td>
<td>7.1±0.5</td>
<td>95±0.7</td>
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<td></td>
<td>7.1±0.5</td>
<td>6.6±0.9</td>
<td>95±0.7</td>
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<tr>
<td></td>
<td>6.6±0.9</td>
<td>6.1±0.7</td>
<td>95±0.7</td>
</tr>
<tr>
<td>D</td>
<td>9.8±0.9</td>
<td>9.4±0.5</td>
<td>99±0.8</td>
</tr>
<tr>
<td></td>
<td>9.4±0.5</td>
<td>9.1±0.5</td>
<td>99±0.8</td>
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<td></td>
<td>9.1±0.5</td>
<td>8.6±0.9</td>
<td>99±0.8</td>
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<td></td>
<td>8.6±0.9</td>
<td>8.1±0.8</td>
<td>99±0.8</td>
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<tr>
<td></td>
<td>8.1±0.8</td>
<td>7.6±0.8</td>
<td>99±0.8</td>
</tr>
</tbody>
</table>

A=PBS control, B=Adjuvant control (Montanide ISA 732 AVG), C=Immunised with *Yersinia ruckeri* bacterin, D=Immunised with adjuvanted *Yersinia ruckeri* vaccine formulation. Different letters (a,b,c) depict significant differences.
ly higher than both PBS control and adjuvant control groups (P <0.05) (Table 2). In addition, the adjuvant control group showed insignificantly higher levels of leukocyte and lymphocyte counts than the PBS control throughout the trial (P >0.05). In comparing the heterophil count, both vaccinated groups showed a significantly lower value than the PBS control and adjuvant control groups (P <0.05), while this immunocompetent cell value in fish vaccinated with *Yersinia ruckeri* bacterin was almost significantly higher than fish immunised with the adjuvanted *Yersinia ruckeri* vaccine formulation (P <0.05) (Table 2).

**Lysozyme level**
Lysozyme activity in fish immunised with bacterin containing Montanide was significantly higher than control groups throughout the trial (P <0.05) (Figure 1). Also, lysozyme levels in fish immunised with the vaccine containing Montanide was higher than fish immunised with the vaccine without Montanide throughout the trial but it was only significant for up to 6 weeks post-vaccination (P <0.05). The vaccinated fish without Montanide also showed a significantly higher level of lysozyme than adjuvant-only control (fish that received Montanide without antigen) during weeks 4, 8 and 10 post-vaccination. In addition, lysozyme activity in the Montanide group was not significantly higher than the PBS group (P>0.05).

**Complement activity (ACH<sub>50</sub>)**
The ACH<sub>50</sub> activity in both immunised groups was significantly higher than both control groups (P <0.05) (Figure 2). Also, fish immunised with vaccine containing Montanide showed no significantly higher level of ACH<sub>50</sub> than fish immunised with vaccine without the adjuvant (P>0.05). In addition, fish immunised

![Figure 1](image-url). Lysozyme activity in biweekly drawn sera from rainbow trout vaccinated with *Y. ruckeri* bacterin (1 × 10<sup>7</sup> cells/fish) with or without Montanide<sup>TM</sup> ISA 763 A VG (66:33; Montanide/antigen) adjuvant. Immunisation was via intraperitoneal injection, and at 14±0.5°C. Mean±SE, n= 10 fish; different letters indicating differences between groups.
with Montanide gave no significantly higher ACH$_{50}$ activity compared to the PBS group (P >0.05).

**Antibody titer by ELISA**

The anti-\textit{Y. ruckeri} antibody titers in both immunised groups were significantly higher than both control groups up to 10 weeks post-vaccination (P <0.05) (Figure 3). The antibody titer in fish immunised with antigen plus Montanide was higher than fish receiving the antigen alone throughout the trial, but the difference was only significant 4 weeks post-immunisation (P <0.05).

**Relative survival percent (RPS)**

No survival was seen in the PBS group (fish injected with PBS), while a maximum survival of 10\% was obtained in fish injected with Montanide for up to 4 weeks post-initiation of the experiment. Fish immunised with the vaccine with and without Montanide gave survivals of 90-100\% and 80-90\% during 12 weeks post-vaccination, respectively. The RPSs in these two immunised groups relative to PBS control group were in the range of 85-100\% and 75-90\%, respectively (Figure 4).

**Discussion**

In line with the developing aquaculture sector, economic burdens by ERM are becoming more remarkable in both fresh and marine environments. This is because of a wide spread of the presence of both carriers and vectors of \textit{Yersinia ruckeri} in aquatic ecosystems. Therefore, such broad spreading of the pathogen can cause frequent disease outbreaks in commercial species.
Figure 3. Antibody titer measured by ELISA in biweekly drawn sera from rainbow trout vaccinated with *Y. ruckeri* bacterin (1 × 10⁷ cells/fish) with or without Montanide™ ISA 763 AVG (66:33; Montanide/antigen) adjuvant. Immunisation was via intraperitoneal injection, and at 14±0.5°C. Mean±SE, n= 10 fish.

Figure 4. Relative percentage survival of rainbow trout vaccinated with *Y. ruckeri* bacterin (1 × 10⁷ cells/fish) with or without Montanide™ ISA 763 AVG (66:33; Montanide/antigen) as adjuvant. Immunisation was via intraperitoneal injection, and at 14±0.5°C. Control groups were IP injected with sterile PBS and Montanide. Mean±SE, n= 40 fish.
including rainbow trout (Kumar et al., 2015). Despite the availability of some commercial vaccines to this bacterial disease, it is still inevitable that there will remain a necessity for improving and prolonging the protection against this bacterial disease (Ispir and Dorucu, 2010; Raida et al., 2011; Deshmukh et al., 2012; Kumar et al., 2015).

The use of some adjuvants and booster vaccinations are ways to improve both specific and non-specific immune responses of fish to ERM (Aucouturier et al., 2001; Plant and LaPatra, 2011). For instance, in a study by Chettri et al. (2015) a longer protective immunity was seen in trout farming by providing a booster vaccination. These authors demonstrated that a booster vaccination using different dilutions of \textit{Y. ruckeri} bacterin (1:100, 1:1000 and 1:2000) with increased exposure time (1 h, 2 h) conferred a higher and longer lasting immunity while, a short term (30 s) booster in 1:10 of the bacterin proved to be superior. Deshmukh et al. (2013) also showed evidence that protection in the early phase of infection was mainly due to the innate immune responses including complement factors, lysozyme levels and acute phase proteins.

In our previous work the evaluation of immersion vaccination of \textit{Yersinia ruckeri} bacterin (1 x 10^8 cells/mL) containing Montanide IMS 1312 VG (1:1; Montanide/antigen) for 2 min at 12-14°C in rainbow trout resulted in a higher levels of immunocompetent cell sizes, lysozyme level and ACH\textsubscript{50} activity compared with fish immunised with the vaccine without the adjuvant for up to 10 weeks post-immunisation (Soltani et al., 2014). In this study similar results were obtained when trout were IP immunised with the antigen containing Montanide ISA 763 AVG (recommended for injection route), showing similar results made by both adjuvants. Also, similar results were seen in antibody titer and the RPS level in trout immunised either with bath immersion of \textit{Y. ruckeri} bacterin containing Montanide IMS 1312 VG (Soltani et al., 2014) or IP injection of the antigen containing Montanide ISA 763 AVG for up to 10 weeks post-vaccination. It is notable that the Montanide IMS 1312 VG is made for bath immersion vaccination while, the Montanide ISA 763 AVG is designed just for injection route. Therefore, in aquaculture practice use of Montanide IMS 1312 VG as bath immersion is preferred to Montanide ISA 763 AVG that is recommended for injection route. Jaafar et al. (2015) recently showed that the use of Montanide ISA763 AVG with yersiniosis vaccine improved efficacy against \textit{Yersinia} infection.

The higher levels of the efficacy and immune responses of antibody titer, lysozyme, ACH\textsubscript{50} and leukocyte population size seen in the trout immunised with the vaccine formulation containing Montanide ISA 763 AVG supports a positive effect of this adjuvant on the fish innate immune factors. However, these differences were not consistently significant between fish vaccinated with the bacterin plus Montanide and fish immunised with the bacterin without Montanide during 10 weeks post-vaccination. Also, higher levels of immunocompetent cell populations (leukocyte and lymphocyte counts), lysozyme and ACH\textsubscript{50} activities measured in fish injected with Montanide ISA 763 AVG alone compared to the PBS group showed a stimulating effect by the Montanide (Table 2). Furthermore, when the fish were injected with Montanide ISA 763 AVG alone, a 10% RPS was seen for up to 4 weeks, indicating a positive immune protective effect by this adjuvant. This was also supported by
a positive correlation between antibody titers in fish immunised with the *Y. ruckeri* bacterin with and without Montanide ISA 763 AVG. Such enhancement in the efficacy and immune parameters of the immunised trout with *Y. ruckeri* bacterin containing Montanide ISA 763 AVG could be due to the improving and prolonging of the antigen up-take by fish internal tissues as also mentioned by Khimmakthong et al. (2013) who showed the uptake following bath immersion of *Y. ruckeri* bacterin by some trout external and internal tissues. In other words, use of such adjuvant can delay the releasing of the absorbed antigen particles causing a prolongation of the antigen exposure as also mentioned by Petrovsky and Aguilar (2004).

Also a continued enhancement in the antibody titer during 2-10 weeks post-vaccination particularly in fish immunised with the bacterin plus Montanide ISA 763 AVG could be due in part to a delay in delivering of antigen particles gradually causing a maintained stimulation of the animal’s humoral immune responses as was also demonstrated in our previous work (Soltani et al., 2014). However, the exact function of such positive effects by Montanide ISA 763 AVG requires further studies.

In conclusion, the data obtained in the present work shows that the use of Montanide ISA 763 AVG adjuvant is able to enhance the efficacy of injection vaccination to *Y. ruckeri*, the cause of yersiniaosis in trout. An improvement in the leukocyte counts, lysozyme and complement activities, a rise in antibody titer as well as a higher obtained RPS are all supportive of such a positive effect made by this adjuvant.

### Acknowledgment

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