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Membrane vesicles from *Piscirickettsia salmonis* induce protective immunity and reduce development of salmonid rickettsial septicemia in an adult zebrafish model

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ABSTRACT

Infections caused by the facultative intracellular bacterial pathogen *Piscirickettsia salmonis* remains an unsolved problem for the aquaculture as no efficient treatments have been developed. As a result, substantial amounts of antibiotic have been used to limit salmonid rickettsial septicemia (SRS) disease outbreaks. The antibiotic usage has not reduced the occurrence, but lead to an increase in resistant strains, underlining the need for new treatment strategies. *P. salmonis* produce membrane vesicles (MVs); small spherical structures know to contain a variety of bacterial components, including proteins, lipopolysaccharides (LPS), DNA and RNA. MVs mimics' in many aspects their mother cell, and has been reported as alternative vaccine candidates. Here, MVs from *P. salmonis* was isolated and evaluated as a vaccine candidate against SRS in an adult zebrafish infection model. When zebrafish was immunized with MVs they were protected from subsequent challenge with a lethal dose of *P. salmonis*. Histological analysis showed a reduced bacterial load upon challenge in the MV immunized group, and the mRNA expression levels of several immune related genes altered, including *mpeg1.1*, *tnfα*, *il1b*, *il10* and *il6*. The MVs induced the secretion of IgM upon immunization, indicating an immunogenic effect of the vesicles. Taken together, the data demonstrate a vaccine potential of MVs against *P. salmonis*.
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1. Introduction

The Chilean salmon production is one of the largest aquaculture industries worldwide, with a production rate of 605.800 tons of Atlantic salmon in 2016 and a calculated exportation value of US$2.3 million [1,2]. The continuous expansion of the Chilean salmon industry has, however, not been without difficulties, as the introduction of new farming areas and species have led to the development of infectious diseases [3,4]. One of the most important pathogen found in seawater in Chile is the intracellular bacterial pathogen *Piscirickettsia salmonis*, the etiologic agent of salmonid rickettsial septicaemia (SRS), a chronic and often fatal disease in salmonid [5,6]. *P. salmonis* was isolated and characterized from Coho salmon (*Oncorhyncus kisutch*) in 1989 after a devastating epizootic in the Chilean aquaculture industry [5]. Since then, the bacteria have been recognized as an emerging problem with outbreaks of SRS reported across the world [7–9]. *P. salmonis* has been identified in salmon net-pens in Norway, Canada, Ireland and Scotland, but with a reduced virulence compared to the Chilean strains [10]. Continuous outbreaks of SRS have had a devastating impact on the Chilean aquaculture, with losses exceeding US$ 100 mill a year [11,12], despite the availability of several vaccine options on the marked [4].

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After the release of the first commercial vaccine against SRS in 1999, over 50% of the salmon in Chile was vaccinated against *P. salmonis*, but by 2003 the number had dropped to 17%, indicating that the initial vaccines provided unsatisfactory protection [11]. Nowadays, there are 33 different licensed vaccines against SRS available in Chile, where the majority are composed of *P. salmonis* pre-treated with either heat or formalin, known as bacterin based vaccines [4]. The use of bacterins for immunization of fish has provided substantial protection against a range of pathogens, including *Edwardsiella ictaluri*, *Flavobacterium columnare*, *Vibrio anguillarum* and *Yersinia ruckerii* [13,14]. There are, however, cases where the use of bacterins provides a limited protection against bacterial pathogens, which includes *P. salmonis* [10,13]. As a consequence, the Chilean aquaculture industry continues to use large amounts of antibiotics to control aquatic diseases, which in 2014 represented 70% of the total antibiotic use in the entire country [4]. The use of antibiotic treatment against SRS has, nonetheless, had little success in regards to disease control, but led to the emergence of antibiotic resistant strains of *P. salmonis* [15–17]. Thus, outbreaks of SRS are still an escalating problem for the aquaculture industry [4].

The development of new vaccines against *P. salmonis* is, however, challenging due to the intracellular nature of the bacteria. *P. salmonis* has been shown to infect, replicate and survive within macrophages as a part of its infection strategy. The infection process includes the formation of vacuoles within the host cells, enabling the bacterium to avoid the fish's primary immune defense [8,18–20]. Thus, vaccination against SRS depends on an activation of both the antibody- and cellular-mediated immune system to provide a sufficient protection [21]. Immunization activating both immune systems is, on the other hand, difficult as it require antigens to be represented through MHC receptors of specialized cells of the immune system [22]. Live attenuated vaccines have succeeded in activating both systems, as they in many ways mimic a natural infection upon immunization. There is, however, a risk of the attenuated bacterium reverting back to a virulent state, which can pose potential environmental, industrial and economical hazards [23]. This is particularly problematic for aquaculture, due to the potential release of a virulent strain into the fish's natural habitat [24]. An alternative would be a non-replicating version of the bacteria, like membrane vesicles (MVs), sharing many characteristics with live attenuated bacteria.

Membrane vesicles are 50–250 nm spherical structures, secreted from the surface of many bacteria during all stages of growth [25–27]. Bacterial MV secretion has been associated with several phenotypes including biofilm formation [28], bacterial survival [29], toxin delivery [30], cell-to-cell communication [31], and host-pathogen interactions [32]. Proteomic and biochemical characterization has revealed that the vesicles contain a variety of bacterial components, including proteins as well as lipopolysaccharides (LPS), DNA and RNA [33–36]. MVs have also been reported to contain several important immunogenic factors, such as toxins [37], chaperons [38], and active enzymes [34]. Together they represent several aspects of the bacteria, but in a non-replicative form. The mechanisms of the MV formation and their biological role have, however, yet to be clearly defined. Bacterial MVs have successfully been used for epidemic control against serogroup B meningococcal disease in Cuba, Norway, Brazil, and New Zealand [39–42]. MVs used in vaccination of fish have also been reported to provide protection against *Edwardsiella tarda* in olive flounder *(Paralichthys olivaceus)* [43], *Flavobacterium psychrophilum in* rainbow trout (*Oncorhynchus mykiss*) [44], and *Francisella noatunensis* in zebrafish *(Danio rerio)* [45]. MVs from *P. salmonis* have been shown to be internalized by fish cell cultures, express toxicity in adult zebrafish and contain several immunogenic proteins, such as TolC, GroEL and DnaK [46,47]. Thus, the main aim of this study was to evaluate the potential of MVs as a vaccine candidate against SRS using an adult zebrafish model.

2. Materials and methods

2.1. Bacteria, media and growth conditions

Cultivation of *P. salmonis* LF-89 (type-strain ATCC VR 1361) isolated from Coho salmon (*Oncorhyncus kisutch*) in Chile [5] were routinely grown at 20 °C on Eugon Chocolate Agar (ECA), containing 30.4 g/L BD Bacto TM Eugon Broth (Becton, Dickinson and Company), 15 g/L Agar Bacteriological (Thermo Fisher Scientific) and 5% bovine blood (Håtunalab AB) [48] or in EBFC containing BD Bacto TM Eugon Broth supplemented with 2 mM FeCl3 (Sigma-Aldrich) and 1% Casamino Acids (BD) with agitation (100 rpm) for 7–10 days. The bacterial stocks were frozen in autoclaved 10% skimmed milk (BD Difco) or in BD Bacto TM Eugon Broth supplemented with 20% glycerol (Sigma-Aldrich) and stored at −80 °C.

2.2. Isolation of membrane vesicles

10 mL of exponential-growth phase cultures of *P. salmonis* was used to inoculate 200 mL of EBFC. The cells were grown at 20 °C with agitation, and growth curves were measured by using optical density reading at 600 nm until the isolates reached late exponential-phase. OMVs were isolated as described [46]. Briefly, the bacterial cells were removed by centrifugation (10 min, 15 000 g, 4 °C), and the supernatant filtered sequentially through a 0.45- and 0.22 μm/pore filter in order to remove the remaining bacterial cells. The filtrate was then ultra-centrifuged sequentially at 125 000 g at 4 °C for 2 h and 125 000 g at 4 °C for 30 min to eliminate cell debris and aggregates. The MVs were resuspended in 100 μL 5 mM phosphate buffer (1:2 monobasic dihydrogen phosphate and dibasic monohydrogen phosphate) pH 6, and protein concentration determined by a Picodrop spectrophotometer (Picodrop Limited, UK). MV aliquots (10 μL) were spread onto ECA plates to check for sterility, and the remaining sample was stored at −80 °C until use.

2.3. Adult zebrafish rearing

10-11 months old male and female Zebrafish (*Danio rerio*) wild type strain AB was obtained from the model fish unit at the Norwegian University of Life Science. The fish were acclimatized to room temperature (20 ± 2 °C) two weeks prior to the experimental setup. The fish were fed every morning with brine shrimp (Scanbur AS) and SDS 400 Scientific Fish Food (Scanbur AS) in the afternoon. The water was provided by the model fish unit at the Norwegian University of Life Science and was supplemented with 0.55 g/L Instant Ocean sea salt, 0.053 g/L Sodium Bicarbonate and 0.015 g/L Calcium Chloride. The tanks were housed in a water-system with a controlled temperature (20 °C) and with a cycle consisting of 14 h of light and 10 h of darkness. The fish were closely monitored, and the animal's health recorded twice a day. Moribund fish that clearly showed deviant behavior and clinical symptoms not consistent with good animal welfare (greatly reduced level of activity, response to environment and appetite), were euthanized with an overdose of 250 mg/mL tricaine methanesulfonate (MS-222, Sigma Aldrich). Water parameters were monitored every third day using commercial test kits (TetraTest kit): pH, NO_2^-, NO_3^{2-}, NH_3/NH_4^+ and water hardness. All zebrafish experiment was approved by NARA (The Norwegian Animal Research Authority) and waste water decontaminated by chlorination and tested for sterility before disposal.

2.4. MV immunization and P. salmonis challenge in zebrafish

For the immunization experiment 65 fish per group were anesthetized by immersion in water containing 100 mg/mL tricaine methanesulfonate buffered with bicarbonate to pH 7–7.5 and immunized once with either 20 μg MV in phosphate buffer or phosphate buffer pH 6 by i.p. injection, using a 27 g needle [45,49]. After injection, the fish were immediately returned to recovery tanks. Immunized and control fish were held in static 15 L polycarbonate tanks (Pentair), with groups of up to 35 fish per tank, in which 50% of the water was manually changed daily. Fish that did not resume normal behavior after the injections were removed from the experiment and euthanized with an overdose of 250 mg/mL tricaine methanesulfonate. The fish were challenge by i.p. injection after an immunization period of 28 days with a lethal dose of 10^8 CFU *P. salmonis*. The challenge dose selected for the vaccine experiment was chosen according our dose-response results and as described in the literature [46,50]. Blood and organ sampling was performed at 24 h, 14 and 28 days' post immunization (dpi) and at 24 h, 3, 7 and 28 days' post challenge (dpc). Fish for histology was sampled at 28 days' post-immunization and 3 and 7 days' post-challenge.

2.5. Histology

For histological preparation, two randomly chosen fish from each experimental group were sacrificed by an overdose of tricaine methanesulfonate (250 mg/mL) after selected time points. The tail was removed to facilitate infiltration before the fish were transferred to glass bottles containing fixing solution (60% methanol, 30% chloroform and 10% acetic acid) and stored at 4 °C. The prefixed fish was dehydrated in a graded ethanol series at 70%, 80%, 90%, 95% and 3 × 100% for 60 min at room temperature with 100 rpm on a rotating table. The ethanol was then replaced with Preparation Solution (Technovit® 7100 with hardener I, according to manufacturer protocol Heraeus Kulzer Technique) and incubated on a rotating table at room temperature for two days. Fish were then transferred to separate silicone moulds and 50 μL hardener II/mL Preparation Solution was mixed and added to fill the moulds. The resin was left to harden at room temperature for 1–2 h before samples were incubated over night at 37 °C. Technovit Universal Liquid was mixed with Technovit 3040 (Heraeus Kulzer Technique) according to manufacturer protocol and poured into the Histoblock placed on top of each sample and allowed to harden for 15 min before the samples were taken out of the moulds. Sectioning to a section thickness of 3 μm was performed with a Leica RM2245 microtome before sections were transferred to a water bath and placed upon glass slides (TC 65 Leica disposal blades). The sections were dried at 50 °C on a HP-3 kunz instruments heating plate before staining using hematoxylin, Schiff's reagent and an Indirect Fluorescent Antibody Test (IFAT) (SRS-Fluorotest indirect, BiosChile S.A). For the hematoxylin and Schiff's reagent staining, the samples were washed in tap water for 1 min, incubated in 1% Periodic acid (Merck Millipore) for 10 min at room temperature. Washed 3 times in MQ water for 1 min, incubated in the dark at room temperature for 20 min with Shiffs's reagent (Merck Millipore) and washed in running tap water for 10 min. The samples were then stained with hematoxylin (Merck Millipore) for 14 min at room temperature, washed in running tap water for 10 min and in MQ water for 1 min in before left to dry at room temperature. The IFAT staining was preformed according to the manufactory's instructions. The sections were mounted with xylene and pertex before analysis using a Leica fluorescent Microscope DM2500 and a Leica DFC425C camera. Images were acquired using LAS version 4.1. Histological samples of non-infected fish were stained and used as a negative control and *P. salmonis* cells from a liquid culture used as a positive control. Selected histological samples from infected fish were also stained with only the secondary antibody to evaluate potential background noise. The number of IFAT stained bacteria was determined using Image J version 1.47 automatic particle counting of two images from each group.

2.6. RNA isolation and quantitative real-time PCR

For RNA isolation, four randomly chosen fish from each experimental group were sacrificed by an overdose of tricaine methanesulfonate (250 mg/mL) at selected time points, and kidney and spleen harvested. The organs were kept in RNAlater (Ambion) and stored at 4 °C until further processing. The tissue was homogenized in 600 μL with buffer RLT (supplemented in RNeasy Mini Kit, QIAGEN) using a mortar and pestle (Sigma-Aldrich), followed by passing the lysate through a blunt 20 gauge needle fitted to a small 1 mL syringe (BD). Total RNA was extracted using the QIAGEN RNeasy kit according to the manufactures instructions, including a 15 min on-column DNase treatment using an RNase-free DNase set (QIAGEN). The RNA was diluted in 30 μL RNase-free H_2O (QIAGEN). RNA quantity and quality was measured with a Picodrop spectrophotometer. Reverse transcription reaction was performed by using High Capacity RNA to cDNA kit (Applied Biosystems). Quantitative real-time PCR (RT-qPCR) was carried out for each of the sampling points for a defined set of genes. These included major histocompatibility complex II (*zgc:10370*), cluster of differentiation 40 (*cd40*), tumor necrosis factor alpha (*tnfα*), suppressors of cytokine signaling 3b (*socs3b*), immunoglobulin M (*ighm*), macrophage expressed gene 1, tandem duplicate 1 (*mpeg1.1*), myeloperoxidase expression (*mpx*) and the four interleukins: *il1b, il6, il8* and *il10.* QuantiTec bioinformatically validated primers were obtained from QIAGEN for most of the genes used; the remaining primers were obtained from Life Technologies Inc. Primers are listed in Table S1. RT-qPCR was performed in triplicates using a Lightcycler® 480 (Roche) as previously described [48]. 18S ribosomal RNA (*zgc:158463*) and Elongation factor-1 alpha (*eef1α111*) were used as reference genes for the normalization of the relative transcription levels of each gene. The normalized immune response data of MV injected fish was standardized against the transcription levels of phosphate buffer injected fish prior to challenge. After challenge the immune response data for both the MV and phosphate buffer group were standardized against the transcription levels of phosphate buffer injected fish the day before challenge.

2.7. Serum isolation

For serum isolation, four randomly chosen fish from each experimental group were sacrificed by an overdose of tricaine methanesulfonate (250 mg/mL) atselected time points, and blood harvested as previously described prior to organ harvest [51]. In short, the caudal fin was removed using a scalpel, and each fish transferred with the wound point down, to a 0.5 mL Eppendorf tube that had been perforated with a sharp needle. The 0.5 mL tube was then placed in a new 1.5 mL tube and centrifuged at 500 rpm for 3 min, followed by re-cutting the tail in order to remove coagulated blood and the sample centrifuged one more time. The blood was then left to coagulate at room temperature for 1 h, followed by a 10 min centrifugation at 3000 rpm in order to separate the cells and plasma. The serum was then collected and stored at - 20 °C until further processing.

2.8. Immunoblot analysis of zebrafish serum

Immunoblot analysis was used to detect the presence of the

heavy chain of zebrafish IgM in serum from both MV immunized and phosphate buffer injected fish. Prior to immunoblot analysis the protein concentration of the serum samples was measured using a Picodrop spectrophotometer. The samples where then diluted to ~5 μg of zebrafish serum protein before a standard SDS-PAGE procedure was used [52]. Briefly, 20 μL of diluted serum was separated on a 4–15% Mini-PROTEAN gel (Bio-Rad). Proteins were transferred to a nitrocellulose membrane and unbound sites were blocked with 5% dry skimmed milk in TBS-T (Tris buffered saline with 0.1% Tween-20) for a minimum of 1 h. The blots were then incubated at room temperature with 1000-fold diluted rabbit anti-CH4 zebrafish IgM (kindly given by the Dr. Julio Coll) for 24 h before three wash cycles with TBS-T. The membrane was then incubated with 5000-fold diluted anti-rabbit horseradish peroxidase-conjugated IgG (Santa Cruz) for 1 h at room temperature and washed three times with TBS-T. Finally, the bands were visualized by chemiluminescence with a Luminata Crescendo Western HRP substrate (Millipore) in a CHEMI Genius Bio Imaging System (SYNGENE). Control of protein load for western blot analysis was preformed using Ponceau S (Fig. S1).

2.9. Statistical analysis

Statistical analysis of the data sets was performed using Graphpad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). Kaplan Meier survival curves were used for analyzing percent survival and differences between groups were deemed statistically significant if p-value < 0.05 using Gehan-Breslow-Wilcoxon test and Log-rank test. Differences in transcription between groups were deemed statistically significant if $p < 0.05$ after using unpaired two-tailed Student's t-test assuming unequal variance.

3. Results

3.1. Outer membrane vesicles protect adult zebrafish challenged with Piscirickettsia salmonis

To evaluate the ability of the MVs isolated from *P. salmonis* to protect zebrafish from SRS, adult zebrafish was immunized with a dose of 20 μg MV or 20 μL phosphate buffer. Four weeks after immunization both groups were challenged with *P. salmonis* LF-89 of 1×10^8 CFU. In the MV immunized group a significantly reduced mortality was seen after challenge compared to the phosphate buffer injected group (Fig. 1A). The MV immunized group had an 84.2% survival at the end of the experiment (28 dpc), in contrast to 21.42% survival in the phosphate buffer group. However, the formation of granuloma-like structures was observed in both phosphate buffer injected and MV immunized fish at 3 and 7 dpc (Fig. 1B, III-VI). The granuloma-like structures were mainly found in the liver, located adjacent to the intestine. Pathologic processes were not found in control or immunized fish the day before challenge, 28 dpi, suggesting that the granulomas revealed in histology sections at 3 and 7 dpc are derived from the challenge dose. The anatomy of non-infected zebrafish is shown in Fig. 1B, I and II. The bacterial load after challenge was investigated by IFAT staining of histological sections (Fig. 2). Image analysis of IFAT stained sections showed that the *P. salmonis* was able to migrate from the initial injection site at the peritoneum and survive within the infected fish. Fish processed for histology 3 days after challenge (dpc) displayed positive staining for the bacterium in close proximity to the intestine, near the peritoneum (Fig. 2A, I and II). The bacterial load were similar in both MV immunized and phosphate buffer injected fish at 3 dpc (Fig. 2B). Analysis of histology section 7 dpc, did however, display a difference in bacterial load between the MV and phosphate buffer group, based on the IFAT staining (Fig. 2A, III and IV). The majority of the bacterium were in both cases still strongly associated with organs in close proximity to the intestine, but a larger number of bacterium were positive for the IFAT staining in the phosphate buffer injected fish compared to the MV immunized fish at 7 dpc (Fig. 2B).

3.2. Immune gene response upon vaccination with P. salmonis membrane vesicles

When investigating the immune gene response, the main alterations in the gene transcription levels were observed in kidney, while the transcription level was in general lower in spleen for the genes investigated. However, both kidney and spleen transcription levels of the pro-inflammatory cytokine *il6* were significantly higher in the phosphate buffer injected fish compared to the MVs immunized fish at 3 and 7 dpc (Fig. 3). The MV immunized fish, on the other hand, had a significant upregulation of *il6* at 1, 14 and 28 dpi in spleen, and at 1 dpi in kidney compared to the phosphate buffer injected fish. The anti-inflammatory cytokine *il10* was upregulated at 1 and 3 dpc in kidney of phosphate buffer injected fish, and at day 14 dpi and day 1 dpc in spleen of MV immunized fish. A similar *il1*b transcription response were shown in both phosphate buffer injected and MV immunized fish after challenge, but a significant upregulation was seen at 1dpi in kidney and at 3 dpi in spleen for the MV immunized fish. In spleen, only a minor but significant upregulation of *tnfa* was observed at 14 dpi for the MV immunized fish. The transcription level of *tnfa* was, on the other hand, increased in kidney for both groups. For the MV immunized fish *tnfa* was upregulated at all time points except 1 dpi, and significantly higher than the phosphate buffer injected fish at 14 and 28 dpi, in addition to 3 dpc. At 7 and 28 dpc the *tnfa* transcription level was significantly higher in phosphate buffer injected fish. The *mpeg1.1* transcription levels in the spleen were, as with *tnfa,* low for both groups through the experiment. Interestingly the MV immunized fish had a significantly higher transcription level of *mpeg1.1* in kidney at all time points compared to the phosphate buffer injected fish. The transcription level of *mpeg1.1* in the kidney of phosphate buffer injected fish was in general shown to be low through the experiment, and only a small upregulation was observed after infection. No significant difference was observed in either kidney or spleen for the remaining genes analyzed (*il8*, *ighm, mpx*, *socs3b, cd40* and *zgc:10370)*. The *t*-test results of immune gene transcription between phosphate injected and MV immunized fish before and after challenge are shown in Table S1.

3.3. Detection of zebrafish immunoglobulin M in serum

In order to study the humoral response against *P. salmonis,* a polyclonal rabbit antibody against the zebrafish IgM heavy chain was used to detect the corresponding IgM in serum from zebrafish at different time points (Fig. 4). The antibody confirmed the presence of IgM by immunoblot analysis in pooled serum from zebrafish ($n = 4$) both before or after challenge. Based on this analysis, there is an increased IgM secretion in the MV immunized fish compared to the phosphate buffer injected fish at 1, 14 and 28 dpi. After challenge the phosphate buffer injected fish did, on the other hand, display an increased secretion of IgM. Thus, ELISA analysis was preformed to detect the specific response against *P. salmonis* (Fig. S2). Based on the ELISA analysis, no difference were observed between the two groups after immunization, but for day 1, 3 and 7 after challenge the MV immunized fish displayed a higher degree of *P. salmonis* specific IgM compared to the phosphate buffer injected fish. The difference was, however, non-apparent at 28 days after challenge. Due to welfare reasons and a limited serum volume, it is important to notice that the IgM data only represent a pool of four

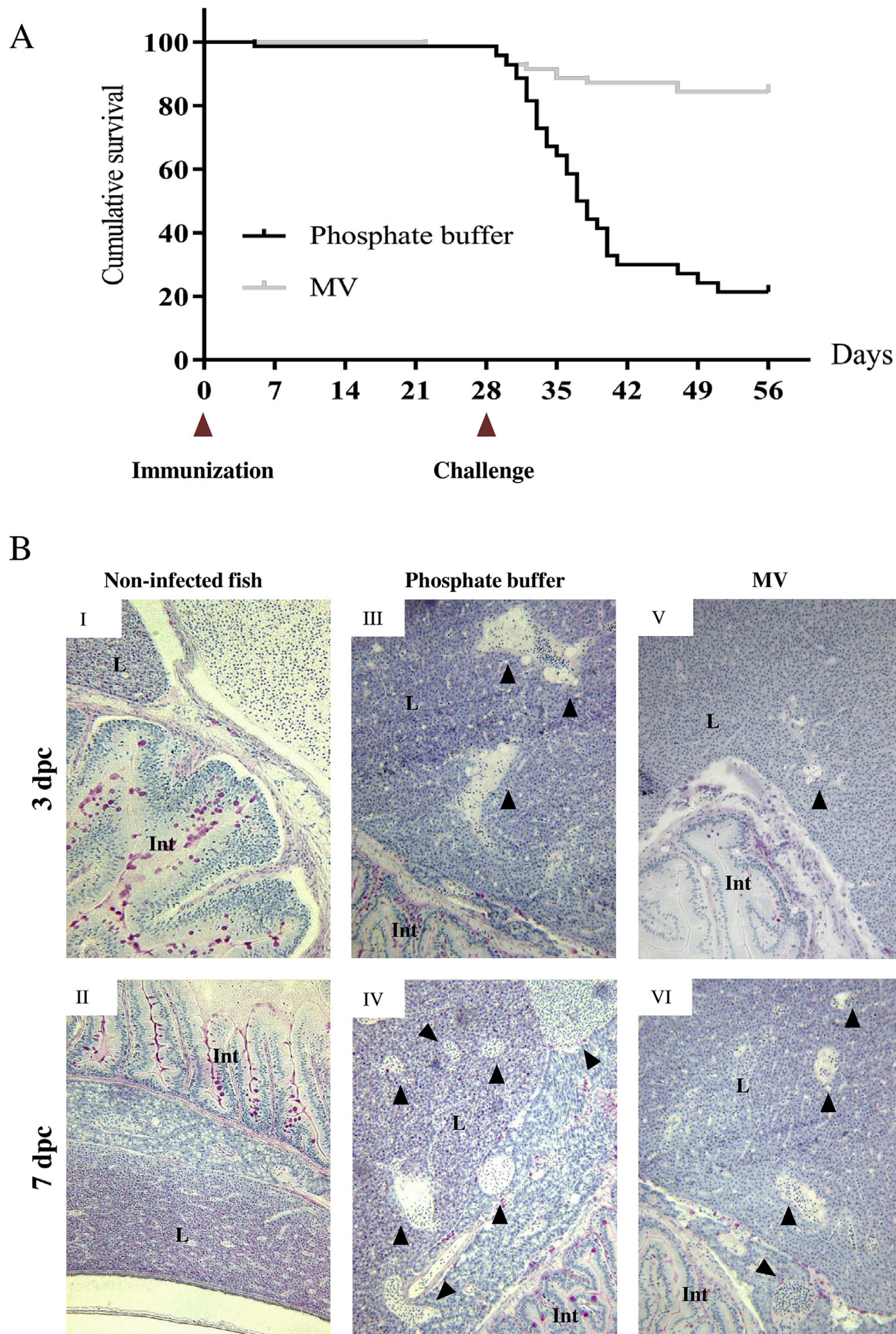

Fig. 1. Adult zebrafish immunized with membrane vesicles and subsequently challenged with *Piscirickettsia salmonis*. (A) Cumulative survival (%) of adult zebrafish immunized with 20 μg of MVs isolated from LF-89 or injected with phosphate buffer before challenged with *P. salmonis* 1×10^8 CFU (n = 65). (B) Histological sections from non-infected fish (I-II), fish injected with phosphate buffer (III-IV) and fish immunized with MVs (V-VI) at 3 and 7 days' post challenge (dpc), 10× magnifications, hematoxylin and Periodic acid Schiff's staining. Int: intestines, L. liver, arrows indicate the formation of granuloma-like structures.

fish for each time point and the analysis, is thus, restricted to a single replicate.

4. Discussion

The use of MVs for immunization against SRS has not previously been reported. In order to evaluate MVs as a potential vaccinate candidate against *P. salmonis* adult zebrafish was used as an infection model. Zebrafish has over the last decades become an important model for vertebrate development, and in recent years the model of choice for studies of both infectious diseases and immunology [53,54]. Due it's to short breeding time, small size and available genetic tools, the zebrafish offers an important bridge between cell lines and higher vertebrates [55]. In the present study,

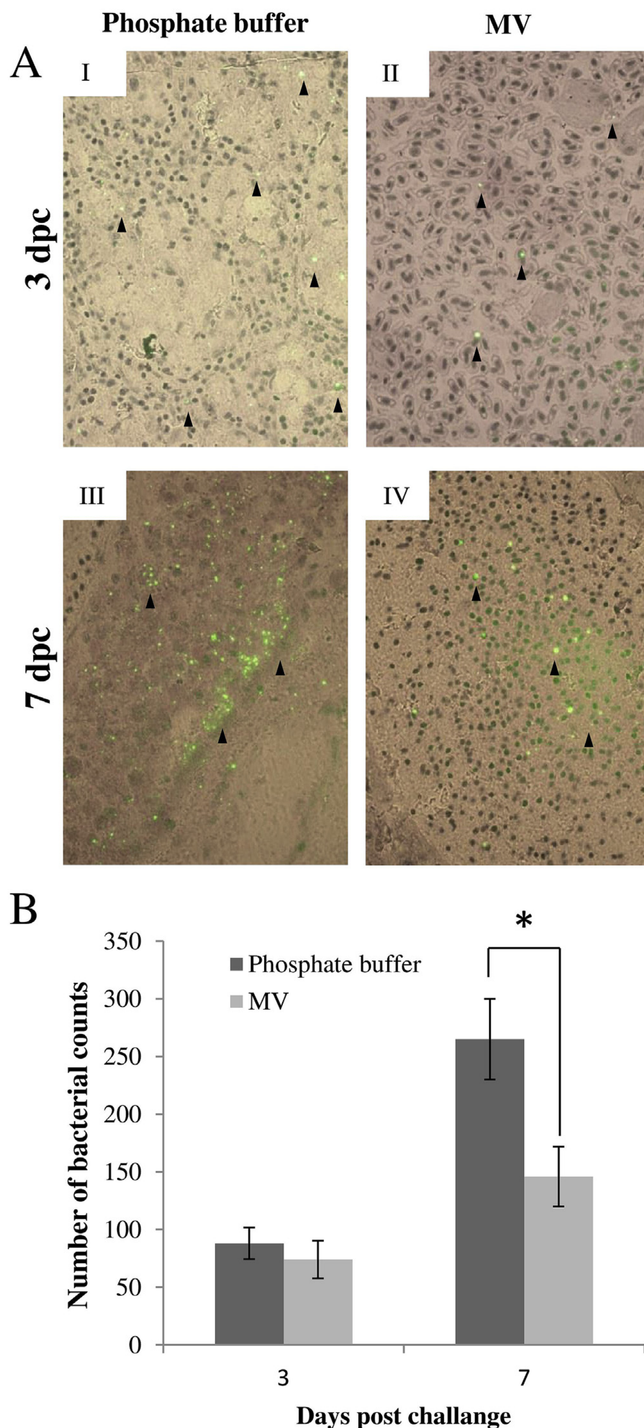

Fig. 2. Indirect Fluorescent Antibody Test of histological sections from adult zebrafish immunized with membrane vesicles and subsequently challenged with *Piscirickettsia salmonis*. (A) Identification of *P. salmonis* by Indirect Fluorescent Antibody Test (IFAT; green) at 3 (I-II) and 7 dpc (III-IV). Bacterial cells positive for IFAT staining are marked with arrowhead, 100× magnifications. (B) Image analysis using Image J automatic particle count of histological sections stained with IFAT at 3 and 7 dpc for quantification of the bacterial load. Results are presented as mean+/-SD. Asterisk indicate significant difference in particle count $p < 0.05$, two tailed unpaired Student's t-test ($n = 2$).

we show that MVs isolated from *P. salmonis* are able to protect zebrafish from subsequent challenge with a lethal dose of the bacterium. The overall survival of the zebrafish increased with 62.8% when fish were immunized with MVs compared to fish injected with phosphate buffer. Analysis of immune related genes in the fish, did however, display an increased il1*b* expression in kidney and spleen samples at 1 and 14 days post immunization with MVs, respectively. Interleukin 1β is one of the most powerful pro-inflammatory cytokines and its expression is regulated together with il18 though the activation of inflammasomes, including NLRP3 [56]. We have preciously shown that MVs from *P. salmonis* is associated with zebrafish leucocytes, thus they could potentially be taken up by macrophages during immunization of zebrafish, leading to the *il1b* gene expression [46]. The internalization of MVs can also trigger an immune response important for subset protection against SRS as il-1β is an important mediator of neutrophil recruitment, cytokines and chemokines induction, and the stimulation of adaptive immunity like the Th17 response [57]. However, no significant difference was observed between the *il1b* expression of MVs immunized and phosphate buffer injected fish after challenge. A similar upregulation was also shown for *tnfα*, having an increased gene expression at 14 and 28 dpi in the kidney of MV immunized fish, but only a limited difference between the MV immunized and the phosphate buffer injected fish were identified at 1, 3 and 7 dpc. TNFα is an important pro-inflammatory cytokine involved in both early and acquired immune response, and is secreted by activated immune cells [58]. The release of TNFa has been shown to promote increased respiratory activity, macrophage activity, phagocytosis and nitric oxide production in fish [59]. Thus, an upregulation of *tnfa* after immunization with MVs might promote increased macrophage activity resulting in a reduction of the bacterial infection [60].

Several of the genes investigated displayed significant difference between MV immunized and phosphate buffer injected fish, including *il10*, *il6* and *mpeg1.1.* The expression of *il10* was recently shown to be upregulated in a RTS-11 monocyte/macrophage cell line from *Oncorhynchus mykiss* upon *P. salmonis* infection, promoting the bacterial survival inside the cell through macrophage inactivation [61]. Furthermore, an upregulation of *il10* has been shown to promote the intracellular survival of several pathogens, including *Mycobacterium tuberculosis* and *Francisella tularensis* [62,63]. MV immunized fish displayed a significant lower *il10* gene expression in kidney at 1 and 3 dpc compared to fish injected with phosphate buffer, which could indicate a reduced survival of the bacterium in the MV immunized fish. A reduced bacterial load was also shown in MV immunized fish compared to phosphate buffer injected fish by histological analysis. Interleukin 10 is an important anti-inflammatory cytokine known to regulate the immune response by blocking chemokine receptors, minimizing damage caused by an excessive release of pro-inflammatory cytokines [64,65]. In the present study, *il10* were shown to be upregulated at 1 dpc in MV immunized fish, followed by a decrease of the pro-inflammatory genes *tnfa* and *il1b* from 3 to 28 dpc. This could indicate a quick protective response in immunized fish, leading to a reduction of the inflammatory response and the bacterial infection, as proposed by others [65,66]. In contrast, an increased gene expression was observed at the same time for *tnfa* and *il1b* in phosphate buffer injected fish, indicating reduced inflammatory regulation potentially caused by the bacterial infection. The gene expression of *il6* was shown to be significantly higher in the kidney of phosphate buffer injected fish at 3, 7 and 28 dpc compared to MV immunized fish. Increased gene expression of *il6* has, as with *il10*, been shown to promote bacterial survival inside cells, but though iron regulation [67]. Increased secretion of *il6* has been reported to recruit Hepcidin, a protein known to bind to the exporter ferroportin (Fpn), leading to the internalization and degradation of Fpn. The loss of cell surface Fpn also leads to increased intracellular iron, particularly in macrophages that are continuously obtaining iron

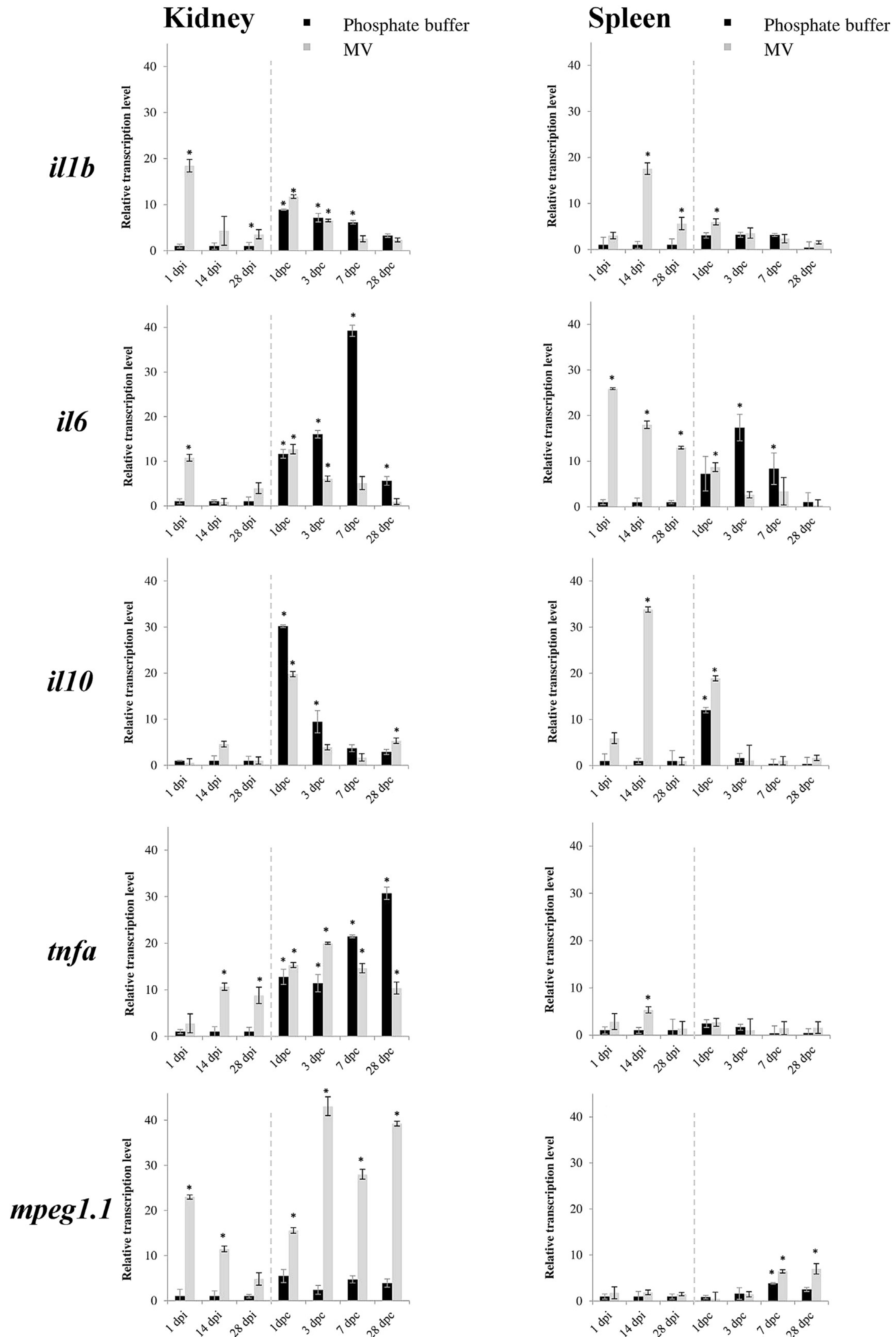

Fig. 3. Immune gene transcription in adult zebrafish immunized with membrane vesicles and subsequently challenged with *Piscirickettsia salmonis* analyzed by RT-qPCR. Immune gene expression of kidney and spleen isolated 1, 14 and 28 days' post immunization (dpi) and 1, 3, 7 and 28 days' post challenge (dpc) from fish immunized with either 20 µg OMVs isolated from LF-89 or injected with phosphate buffer (control) and challenged with *P. salmonis* 1×10^8 CFU. Results are presented as mean+/-SD. Asterisk indicate significantly upregulated genes compared to the non-challenged control $p < 0.05$, two tailed unpaired Student's t-test ($n = 4$).

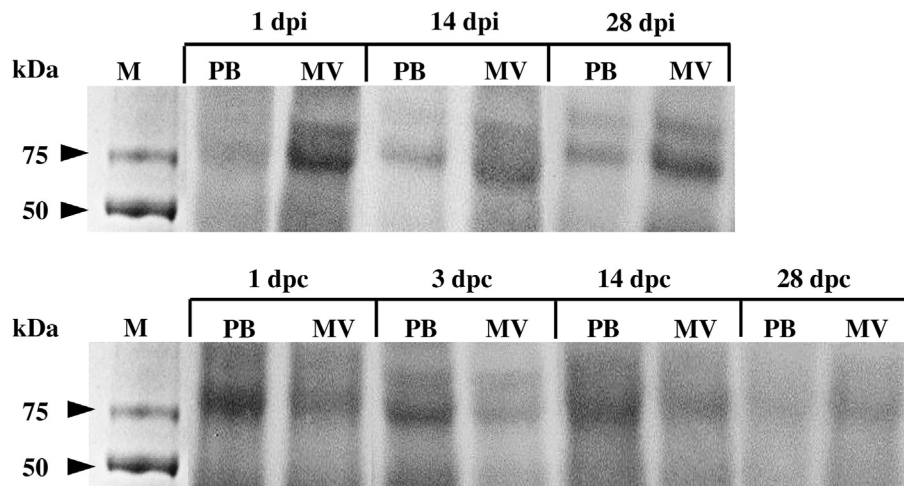

Fig. 4. IgM secretion in zebrafish immunized with membrane vesicles and subsequently challenged with *Piscirickettsia salmonis*. Detection of zebrafish IgM in serum of zebrafish immunized with 20 μg of MVs isolated from LF-89 or injected with phosphate buffer (PB) before challenged with *P. salmonis* 1×10^8 CFU at 1, 14 and 28 days' post immunization (dpi) and 1, 3, 7 and 28 days' post challenge (dpc).Immunoblot analysis of IgM heavy chain (84–86 kDa), M: molecular weight marker in kilo Daltons (kDa).

from senescent red blood cells [68]. Iron acquisition has, furthermore, been shown to be important for the intercellular growth and survival of *P. salmonis*, and studies of infected Atlantic salmon reported an increased resistance to SRS in fish able to limit iron availability to the bacterium [69,70]. Thus, a decreased gene expression of *il6* in MV immunized fish could limit the bacterium's iron availability, thereby also limiting the infection.

Interestingly the biggest difference in gene expression level between phosphate buffer injected and MV immunized fish was observed for *mpeg1.1*, being significantly higher in the MV immunized fish at all time points investigated. Mpeg is in general considered an important macrophage marker in zebrafish, used for both fluorescent labeling and gene expression studies [71,72]. The *mpeg1.1* gene encodes a perforin-like protein, suggested to be a pore-forming protein involved in the clearance of intracellular pathogens by promoting the phagosome-lysosome fusion [73]. The Mpeg1.1 protein has also been shown to have antimicrobial activity in zebrafish, and *mpeg1.1* knock down zebrafish mutants reported to display increased bacterial burden upon challenge with *Mycobacterium marinum* [74]. Thus, an upregulation of the *mpeg1.1* gene in MV immunized fish indicates an increased macrophage response to both the MVs and the bacterium. In contrast, a low expression of the *mpeg1.1* gene in phosphate buffer injected fish upon challenge might be a result of the bacterium's intracellular lifestyle, avoiding the phagosome-lysosome fusion. Moreover, zebrafish infected with *M. marinum* have been shown to display a decreased expression of *mpeg1.1*, indicating that the *mpeg1.1* gene expression levels could be affected by bacterial infections [74,75]. However, as the infection mechanism of *P. salmonis* is yet to be fully investigated, further studies are needed to confirm the indications given by the immune gene expression observed in zebrafish upon immunization and challenge. The immune gene expression in combination with the histology data, do however, indicate a reduced degree of *P. salmonis* infection in zebrafish after immunization with MVs as compared to fish injected with phosphate buffer.

Moreover, serum analysis from the fish did show an IgM response to both the MV immunization and the subsequent challenge. IgM is in general considered as the first line of defense during microbial infections as well as the first antibody produced upon immunization in mammals [76]. IgM has been recognized as an important antibody in the teleost immune system, being the most ancient and only isotype conserved in all jawed vertebrates. IgM is manly found in teleost blood and serum, and are in adult fish the dominant isotype expressed by both primary and secondary lymphoid organs [77–79]. A specific immune response upon vaccination or challenge can also be measured based on the IgM response in fish, and IgM antibody titers has been shown to increase significantly following immersion vaccination against enteric redmouth disease [79,80]. Thus, the specific IgM production against *P. salmonis* detected by ELISA analysis of serum from fish immunized with MVs might indicate a protective effect induced by the vesicles. However, as *P. salmonis* is an intracellular pathogen, and IgM a part of the humoral immune system, it can be discussed to what degree IgM promotes a protective effect [78]. Nonetheless, it has been shown that IgM might be an important factor in vaccination in mammals, and that the synergy between antibodies, cytokines and phagocytes are an important part in clearing bacterial infections [76,81]. Due to the miniscule amount of serum possible to obtain from zebrafish it is important to notice that the ELISA data is based on one replicate only and it would be of high interest to evaluate the response in a salmon host.

In the present study, several immunological components were shown to be activated upon immunization with MV derived from *P. salmonis*, indicating a potential use of bacterial derived vesicles for vaccination in aquaculture. It has previously been described that adult zebrafish is susceptible to *P. salmonis* outer membrane vesicles [46]. However, as *P. salmonis* is an intracellular pathogen, residing within the host's immune cells upon infection, the bacterium has a limited availability for antibody recognition by the immune system [82]. Thus, vaccination can be problematic as is relies on memory T-cells, which upon encounters with specific antigens will activate a defense system [83]. The activation of memory T-cells was not investigated in the present study, and will be interesting to examine in the future. However, as *P. salmonis* MVs has been shown to be internalized by leukocytes and in many ways is a small non-replicating copy of the bacteria, they could mimic a natural infection upon immunization [46]. Thus, MVs represents an interesting alternative for immunization against SRS, potentially activating the antibody- and cellular-mediated immune system. As *P. salmonis* has been shown to secret MVs when residing inside cells, the vesicles could potentially be broken down and represented by the cell though MHC class I. Thus, successful immunization using MVs could lead to a $CD8^+$ T cell mediated destruction of the *P. salmonis* infected cells [22, 84, 85]. However, further

studies are needed to evaluate the vaccine potential of the MVs and their mechanism of action. As *P. salmonis* mainly infect salmon, the protective effect should also be investigated using the bacterium's natural host.

5. Conclusion

In summary, MV isolated from *P. salmonis* was shown to induce a protective effect against SRS in an adult zebrafish infection model, and several immune related genes were upregulates after immunization. Thus, MVs for vaccination represents an interesting candidate for immunization against *P. salmonis*.

Ethics statement

All animal experiments were approved by the Norwegian Animal Research Authority, approval no. 16/36352, FOTS ID 8507 and treated according to institutional guidelines.

Contributors

JIT planned and preformed most of the experiments and participated in the writing of the paper. MG planned and preformed some of the experiments. CO and LL planned and preformed some of the experiments and participated in the writing of the paper. AJY, ER and HCWL planned some of the experiments and participated in writing of the paper. All authors have approved the final manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.06.015.

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