Efficacy and safety of a non-mineral oil adjuvanted injectable vaccine for the protection of Atlantic salmon (*Salmo salar* L.) against *Flavobacterium psychrophilum*

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Abstract

Flavobacterium psychrophilum is the causative agent of Rainbow Trout Fry Syndrome which has had a major impact on global salmonid aquaculture. Recent outbreaks in Atlantic salmon in Scotland and Chile have added to the need for a vaccine to protect both salmon and trout. At present no licensed vaccines are available in Europe, leaving antibiotics as the only course of action to contain disease outbreaks. Outbreaks generally occur in fry at temperatures between 10-15 °C. Recently outbreaks in larger fish have given added impetus to the development of a vaccine which can provide long term protection from this highly heterogeneous pathogen. Most fish injectable vaccines are formulated with oil emulsion adjuvants to induce strong and long lasting immunity, but which are known to cause side effects. Alternative adjuvants are currently sought to minimise these adverse effects.

The current study was performed to assess the efficacy of a polyvalent, whole cell vaccine containing formalin-inactivated *F. psychrophilum* to induce protective immunity in Atlantic salmon. The vaccine was formulated with an adjuvant containing squalene and aluminium hydroxide, and was compared to a vaccine formulated with a traditional oil adjuvant, Montanide ISA 760VG, and a non-adjuvanted vaccine. Duplicate groups of salmon (23.5 ± 6.8 g) were vaccinated with each of the vaccine formulations or phosphate buffered saline by intraperitoneal injection. Fish were challenged by intramuscular injection with *F. psychrophilum* six weeks post-vaccination to test the efficacy of the vaccines. Cumulative mortality reached 70% in the control salmon, while the groups of salmon that received vaccine had significantly lower mortality than the controls (p = 0.0001), with no significant difference in survival between vaccinated groups. The squalene/alum adjuvant was safe, more readily metabolised by the fish and induced less histopathological changes than the traditional oil adjuvant.

Keywords: *Flavobacterium psychrophilum,* RTFS, vaccine, salmon, adjuvant

1 Introduction

Rainbow trout fry syndrome (RTFS), caused by *Flavobacterium psychrophilum,* is one of the most significant disease problems facing the salmonid aquaculture industry worldwide [1]. Rainbow trout (*Oncorhynchus mykiss*) are the species most affected although there are increasing problems in Atlantic salmon (*Salmo salar*) hatcheries in Scotland and Chile. Disease episodes tend to occur between 10-15 ºC, with necrotic lesions often seen on the skin surrounding the dorsal fin and tail, while in very small fish no clinical signs are apparent and death occurs due to septicaemia. *F. psychrophilum* is a highly heterogeneous pathogen, which makes development of cross-protective vaccines to control this devastating disease problematic [2]. Antibiotic treatment is relied on to treat outbreaks, which has led to increased levels of antibiotic resistance in *F. psychrophilum* isolates [3-5], highlighting the urgent need for prophylactic treatments for RTFS.

The majority of inactivated whole cell or sub-unit vaccines available to the aquaculture industry are formulated in oil emulsions [6]. Adjuvanted vaccines are injected intraperitoneally, and provide protection via a prolonged release of antigen from the oil component stimulating primarily local inflammatory reactions followed by a systemic immune response [7]. While oil-based adjuvants have provided increased efficacy of vaccines for aquaculture, problems with side-effects at injection sites have resulted in the down grading of fish at harvest due to adhesions between the body wall and abdominal organs and spinal deformities [8-10] . Therefore, there is a need to develop adjuvants for use in injectable vaccines for salmonids, which balance the efficacy-safety profile. A previous study using an adjuvant containing squalene and aluminium hydroxide to formulate a vaccine for treatment

of viral haemorrhagic septicaemia (VHS) in Olive flounder (*Paralichthys olivaceus*), resulted in an efficacious vaccine inducing long term protection without injection site reactions, adhesions or pigmentation [11].

The current study was performed to assess the efficacy of a polyvalent, whole cell vaccine containing formalin-inactivated *F. psychrophilum*, with and without different adjuvants, to induce protective immunity in Atlantic salmon fry. A mixture of squalene/aluminium hydroxide was tested as an alternative adjuvant to the traditional oil adjuvant (Montanide) and compared to protection achieved by vaccine without adjuvant. Immune responses were investigated post-vaccination/pre-challenge by ELISA and western blot in addition to immune gene expression and histological investigation of the injection site.

2 Materials and Methods

2.1 Atlantic Salmon Fry

Atlantic salmon eggs were supplied by AquaGen (Norway) and transported on ice to the aquarium at the Institute of Aquaculture, Stirling. On arrival eggs were subjected to an iodophor surface disinfectant treatment according to the manufacturer's instructions (Buffodine, Evans Vanodine, UK). Five replicates of 10 eggs were removed and confirmed to be *F. psychrophilum* free using a nested PCR for the 16S rRNA gene with modifications [12,13]. The eggs were maintained in flow-through de-chlorinated tap water at 10 ºC until hatch, and thereafter maintained in a 100 L flow-through tank (5 L min^{-1}). The fry were fed to satiation daily (Inicio feed, 1.1 mm, BioMar, UK). All experimental procedures with live fish were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments and were approved by the Ethics Committee of the Institute of Aquaculture, University of Stirling, UK.

2.2 Preparation of formalin inactivated bacteria

Two isolates of *F. psychrophilum* recovered from trout and one recovered from salmon in the UK in 2013 were used to make the whole cell vaccine (Table 1) as described in [14].

Table 1. Details of the isolates of *F. psychrophilum* included in the experimental vaccine: species, geographical source and serotype.

ISOLATE	FISH SPECIES	SOURCE	SEROTYPE [15]
AVU-1T/13	Rainbow Trout	England	Th
AVU-2T/13	Rainbow Trout	Scotland	Fd
AVU-3S/13	Atlantic salmon	Scotland	FpT

2.3 Preparation of Vaccine formulations and Vaccination

The formalin-inactivated vaccine (formalin killed cells: FKC) was emulsified with squalene/alum adjuvant [(5 (v/v) % squalene (Sigma, Australia), 20 (v/v) % glycerol (Ameresco, USA), 0.5 (v/v) % Tween 80 (Sigma-Aldrich, USA) and 0.5 (w/v) % aluminium hydroxide (Sigma, USA)] or with Montanide ISA760VG (Seppic, France) (Montanide 70: FKC 30). The vaccine formulations were stored at 4 ºC and the stability of the emulsion was examined macro and microscopically following a period of 7 days.

Fish (23.5 ± 6.8 g) were randomly separated into 100 L flow-through tanks with aeration at 15 ºC. The experimental design of the vaccination trial is summarised in Table 2. Fish were anaesthetised with benzocaine (Sigma, 0.004%) and given one of the vaccine formulations by intra-peritoneal injection (50 µl per fish). Control groups were injected i.p. with 50 µl of sterile PBS. Fish were euthanized by an over-dose of benzocaine and sampling carried out at various time points (Table 2). Tissues (spleen, liver, kidney, intestine and heart) from three fish per replicate were collected and immediately fixed in formaldehyde in PBS

(100 mL 35% formaldehyde and 900 mL DW) for histology. Head-kidney from three fish per duplicate group (n=6) was placed immediately in RNA-later (Sigma) and stored at 4 ºC overnight. RNA-later was removed and tissues stored at -70 ºC until RNA extraction. Blood was sampled from the caudal vein using a 23 G needle and syringe from three fish per duplicate group (n=6) stored overnight at 4 ºC, centrifuged at 3000 x g 5 min for collection of serum which was stored at -20 ºC until analysis.

Table 2. Experimental design of vaccination trial and sampling.

Groups	No. Fish/ replicate	Innoculum (50 µl i.p.)	Challenge (no.CFU/fish)	Sampling point (samples taken)
Control (unvaccinated)	21 x 2	PBS	Homologous (4.0×10^7)	Day 2 pv (tissues qPCR)
Vaccine (FKC)	21 x 2	FKC		6 wpv (Blood, tissues)
Vaccine + squalene/alum	21 x 2	FKC: Squalene/alum		
Vaccine + Montanide	21 x 2	FKC: Montanide		

2.4 Experimental infection of vaccinated fish

Vaccinated and control fish were experimentally infected with a homologous isolate of *F. psychrophilum* (AVU-3S/13) at 4.0×10^7 CFU/fish six weeks post-vaccination (wpv) by intramuscular injection. The fish were maintained as above and monitored for 21 days post infection (dpi). Moribund fish or mortalities were removed and sampled by streaking head kidney, spleen and any lesions on Modified Veggietone (MV) medium [veggitones GMO-free soya peptone (Oxoid, UK), 5 g L^{-1}; yeast extract (Oxoid, UK), 0.5 g L^{-1}; magnesium sulphate heptahydrate (Fisher chemicals, UK), 0.5 g L^{-1}; anhydrous calcium chloride (BHD), 0.2 g L^{-1}; dextrose (Oxoid, UK), 2 g L^{-1}; agar (solid medium; Oxoid, UK), 15 g L^{-1}; pH 7.3] to confirm

specific mortality. A sub-sample of colonies recovered was examined for the presence of *F. psychrophilum* using a nested PCR method [12,13].

2.5 ELISA for detection of specific IgM in serum

Enzyme-linked immunosorbent assay (ELISA) was used to assess specific IgM titre to *F. psychrophilum* in serum according to [16] with some modifications. *F. psychrophilum* vaccine isolates and a heterologous isolate were used to coat the plates at 1 x 10^8/mL in PBS and incubated overnight at 4 °C. The dilution of fish serum used was optimised by first titrating sera from each group (1:32 to 1:1024). Fish serum samples at the optimised dilution of 1:64 in PBS were added to the wells (100 µl/well) in duplicate and incubated overnight at 4 °C. Specific IgM was detected using anti-trout IgM monoclonal antibody (Aquatic Diagnostics Ltd., 1/33 in PBS, 1h) followed by incubation with anti-mouse-HRP (1/4000, Sigma, 1h). The absorbance was read on a BioTek HT Synergy spectrophotometer at 450 nm.

2.6 SDS-PAGE and Western blotting

2.6.1 Sodium dodecyl sulphate polyacramide gel electrophoresis (SDS-PAGE)

Suspensions of the three vaccine isolates and a heterologous isolate of *F. psychrophilum* were aliquoted into 1.5 ml microcentrifuge tubes (1 mL of 2 x 10^8 cfu/mL), and centrifuged for 15 min at 3000 × g. Bacterial pellets were resuspended in 100 µl of DW and 30 µl of 5 X sample buffer (250mM Tris-HCl, 30% glycerol, 10% SDS, 0.5M dithiothreitol, 0.2% bromophenol blue) and boiled for 15 min. Finally, the samples were centrifuged at 10,000 × g for 10 min prior to analysis of the supernatants. A preparation of broad-range molecular weight markers (5 µl) (Bio-Rad) were added to the first well of a 12% polyacrylamide gel (Bio-Rad) and 15 µl of each sample were added to the remaining wells. The gel was run at 130 V for

approximately 90 min. The gel was stained in 50 mL of Coomassie (QC Colloidal Coomassie Stain, Bio-Rad) according to the manufacturer's instructions.

2.6.2 Western blot analysis

Bacterial components separated by SDS-PAGE as described above were transferred onto nitrocellulose membranes by semi-dry transfer (Pierce™ Power Blotter, ThermoFischer Scientific) applying 25 V (1.3A) for 7 min. The nitrocellulose membranes were then incubated overnight at 4 °C in 5 % (w/v) casein in distilled water (DW). After washing 3 times with Tris buffered saline with Tween (TBS: 10 mM Tris base, 0.5 M NaCl pH 7.5 with 0.1% [v/v] Tween 20) for 5 min at each wash, the membranes were incubated for 3 h at 22 °C with a 1/20 dilution of fish serum in TBS (serum was a pool from 2 fish from each treatment group, with a titre of 1/512, taken six wpv as described in Section 2.6). The membranes were washed as previously described and incubated for 1 h at 22 °C with a 1/20 dilution of anti-trout IgM monoclonal antibody in TBS (ADL). The membranes were again washed and incubated for 1 h at 22 °C with a 1/200 dilution of anti-mouse horse radish peroxidase (Sigma) in TBS. After washing, bands were visualised by adding chromogen and substrate (ImmPACT™ DAB Peroxidase substrate kit). The reaction was stopped by soaking the membranes in DW for 5 min.

2.7 Histology

Formalin fixed tissues were embedded in paraffin and sectioned using a Microtome (Shandon Finesse). Tissue sections were de-waxed and dehydrated in xylene (2 x 3 min), 100% ethanol (2 min), methylated spirit (1.5 min) and stained with haematoxylin and eosin. Slides were

examined using an Olympus BX40 microscope for signs of inflammation or adverse reactions to the vaccine/adjuvants and scored for inflammation and lipid droplets at the injection site.

2.8 Isolation of total RNA and cDNA synthesis

RNA was extracted from 30 - 40 mg of each head-kidney sample using TRI Reagent (Applied Biosystems) following the manufacturer's protocol. The resultant RNA pellet was re-suspended in 30 µL of nuclease-free water. Following spectrophotometric quantification (Nanodrop ND-1000, Thermo Fisher, Leicestershire, UK) and quality checking by gel electrophoresis (1% agarose gel stained with ethidium bromide), samples were stored at -70 ºC until required. RNA was reverse transcribed to construct cDNA using a high-capacity cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions. Briefly, 10 µl of RNA was added to 10 µl of 2X RT master mix (10X RT buffer, 25X dNTP Mix 100 mM, 10XRT Random Primers and oligo-dT mix, Reverse Transcriptase, RNase Inhibitor, nuclease-free water). The thermal cycle conditions consisted of 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min. The cDNA was aliquoted and stored at -20 °C prior to use.

2.9 Quantitative Real Time PCR (qRT-PCR)

Head-kidney samples were analysed by qRT-PCR for the expression of cytokines (*IL-1β, IL-8, IL-10, IFN-γ*) and immune genes (*CD4, CD8*). Real time PCR was performed on first strand cDNA using the Eppendorf® RealPlex^2 Mastercycler gradient S instrument with SYBR® Green I (Thermo Scientific) master mix and primers as shown in Table 3.

Table 3. Primers used for qPCR including product size and sequences.

Gene	Primers	Product size	Reference	Efficiency (E)
IL-1β	F: GCTGGAGAGTGCTGTGGAAGA R: TGCTTCCCTCCTGCTCGTAG	73	[17]	0.90
IL-10	F: CTGTTGGACGAAGGCATTCTAC R: GTGGTTGTTCTGCGTTCTGTTG	129	[18]	0.95
IFNγ	F: CTAAAGAAGGACAACCGCAG R: CACCGTTAGAGGGAGAAATG	159	[19]	0.96
CD8α	F: AATCAATGGTAACGCGCTTG R: TGGCTGTGGTCATTGGTGTA	101	[20]	0.97
CD4	F: GAGTACACCTGCGCTGTGGAAT R:GGTTGACCTCCTGACCTACAAAGG	121	[19]	0.85
IL-8	F: ATTGAGACGGAAAGCAGACG R: CGCTGACATCCAGACAAATCT	136	[21]	0.85
Elongation factor 1α	F:CGGCAAGTCCACCACCAC R:GTAGTACCTGCCAGTCTCAAAC	205	[21]	0.94
B-actin	F: ACTGGGACGACATGGAGAAG R: GGGGTGTTGAAGGTCTCAAA	157	[21]	0.91

Briefly the 20 µl reaction consisted of 5 µl of cDNA and 15 µl of master mix prepared using 1 µl of the forward and reverse primers (0.3 µM), 10 µl SYBR® Green I and 3 µl of nuclease free water. The cycling conditions consisted of 95°C initial denaturing for 15 s, followed by 40 cycles of 15 s denaturing at 95 °C, 30 s annealing at 58 °C and 30 s extension at 72 °C. RT-minus and non-template controls were included on every plate. Melting curve analysis was performed from 60 °C to 95 °C in 0.1 °C/s increments to assess the specificity of the RT-PCR products. Serial 10-fold dilutions of the cDNA were prepared in nuclease free water starting and the Ct values were used to generate a standard curve plot of cycle number versus log concentration in the *realplex* software V2.2 (Eppendorf). The quality of the standard curve was judged by the slope of the curve and the correlation coefficient (r). The slope of the line was used to estimate the estimate the efficiency of the target amplification using the equation $E= (10^{-1/slope})-1$. Elongation factor-α and β-actin were used as reference genes to correlate for potentially different loading amounts of RNA and for variation in cDNA synthesis efficiencies [22]. The threshold cycle (Ct) was determined at the linear slope in a log fluorescence/Ct plot. The expression results were analysed using the $2^{-\Delta\Delta}$ Ct method [23].

The gene expression data were normalised to the reference genes and expressed as a comparison of vaccinated fish compared to control fish using REST 2009™ software [24].

2.10 Statistical Analysis

Minitab software version 16 (Minitab Inc., Pennsylvania) was used to perform basic descriptive statistics and SPSS™ for survival analysis. Relative percentage survival (RPS) was calculated at the time point corresponding to when mortality had ceased in the control group (3 consecutive days of no mortality). Kaplan-Meier survival curves were generated and the log-rank test was used to compare the survival curves for the vaccinated fish and unvaccinated fish [25,26]. The relative percent survival (RPS) of this trial was calculated using the following equation [27]:

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

Specific antibody levels were analysed by one-way ANOVA followed by Welch's test.

3. Results

3.1 Vaccine Efficacy

All vaccinated groups showed significant protection to disease challenge compared with the controls when average percentage survival was calculated (p = 0.0001, Fig 1). Pairwise comparisons of individual tanks are given in supplementary table 1. Average cumulative mortality reached 70% in the control salmon. The vaccine formulation of formalin-killed cells (FKC) combined with Montanide ISA 760VG gave the highest protection (RPS of 95.2%), vaccine (FKC) without adjuvant and vaccine formulated with squalene/alum adjuvant also

induced good protection with RPS values of 85.71% and 75.17% respectively. No significant difference in survival was found between vaccinated groups. DNA samples extracted from selected bacterial colonies recovered from fish that had died post-challenge were positive for *F. psychrophilum* by nested PCR.

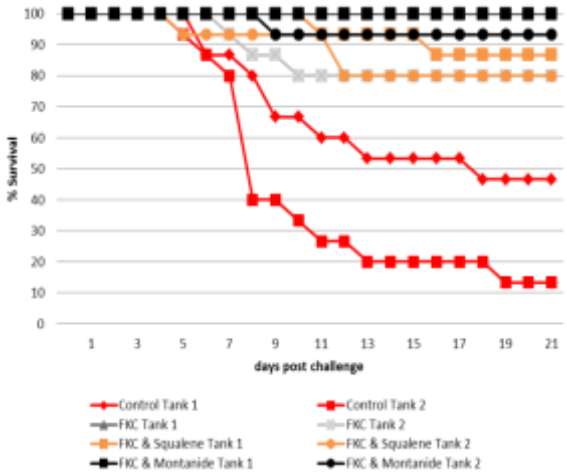

Figure 1. Cumulative percentage survival of salmon vaccinated by intraperitoneal injection with *Flavobacterium psychrophilum* formalin killed bacterin with and without adjuvant and challenged 630 degree days post-vaccination by intramuscular injection with one of *F. psychrophilum* vaccine strains (AVU-3S/13). Survival of each duplicate tank is shown. Average Relative percent survival (RPS): FKC: formalin-killed cells (85.71%); FKC & Squalene: formalin-killed cells emulsified with squalene and alum adjuvant (RPS 75.17%); FKC & Montanide: formalin-killed cells emulsified with Montanide ISA 760VG (RPS 95.24%). Controls were given sterile phosphate buffered saline by intraperitoneal injection.

3.2 Nested PCR for detection of *F. psychrophilum*

The eggs were free of *F. psychrophilum* (Fig. S1a) and *F. psychrophilum* was detected in moribund and dead fish sampled during the challenge (Fig. S1b).

3.3 Specific antibody response

Antibody levels (IgM) were measured at a 1:64 dilution as this gave the best resolution between groups. Antibody levels of vaccinated fish screened against a *F. psychrophilum* vaccine isolate (AVU-3S/13, serotype FpT) 6 wpv were significantly elevated in the group

which received the Montanide adjuvanted vaccine (p = 0.002) when compared to fish that received either PBS, unadjuvanted vaccine or vaccine emulsified with squalene/alum (Fig. 2 A). The levels of IgM to a heterologous isolate of *F. psychrophilum* (AVU-1T/07, serotype Th) were also significantly elevated in both groups of fish given the vaccine emulsified with adjuvants compared to fish injected with PBS or the unadjuvanted vaccine (p = 0.010) (Fig. 2 B).

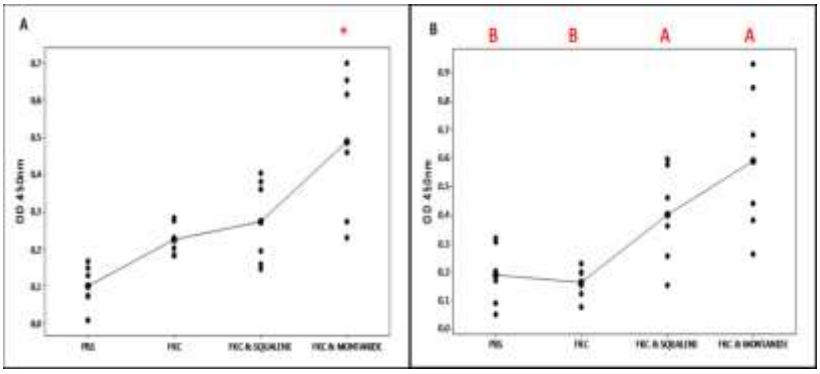

Figure 2. Specific antibody (IgM) levels to *F. psychrophilum* in vaccinated salmon 6 weeks post vaccination (A: to homologous isolate *F. psychrophilum* *denotes significantly different to other groups p=0.002; B: to a heterologous isolate *F. psychrophilum*; groups that do not share a letter are significantly different (p=0.01). The line denotes the mean antibody level of each group, n=6, 1:64 serum dilution.

3.4 SDS-PAGE and Western blot

Distinct bands ranging from 10-250 kDa were evident in the SDS-PAGE profiles of the *F. psychrophilum* isolates used to prepare the polyvalent vaccine (and a heterologous isolate AVU-1T/07) following staining with Coomassie (Fig 3A). Banding profiles of the isolates were similar, with the exception of a slight difference in the band between 20-25kDa in the heterologous isolate. When blots of these isolates were incubated with immune sera sampled 6 wpv (pooled sera with a titre of 512 by ELISA), the strongest staining was seen with serum from fish vaccinated with the Montanide and squalene/alum vaccine preparations (Fig. 3B) reflecting the results obtained by ELISA. Bands ranging from 15 to 250 kDa were recognised

by the Montanide group, whereas the serum from fish given unadjuvanted vaccine recognised bands between 37-250 kDa with much weaker staining. This was also the case with serum from fish given vaccine emulsified with squalene/alum adjuvant, with bands recognised between 37-75 kDa. These bands also stained weakly in control fish administered PBS.

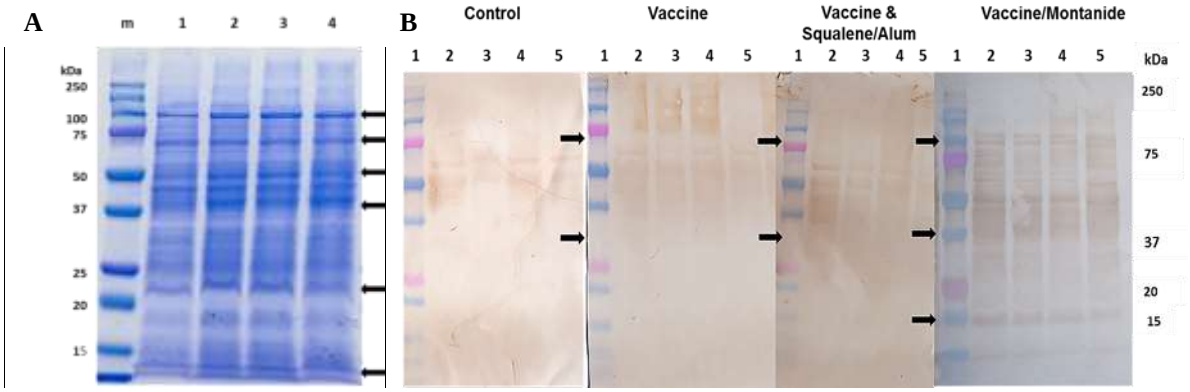

Figure 3. SDS-PAGE and western blotting of *F. psychrophilum* isolates. (A) Whole cell lysates from a heterologous isolate and vaccine isolates Lanes: (1) molecular weight markers (2) AVU171/07, (3) AVU-1T/13, (4) AVU-2T/13, (5) AVU-3S/13) were separated by SDS-PAGE and stained with Coomassie stain. Arrows indicate high intensity bands at 10-15, 20, 37-50, 75, 100 kDa. (B) Western blot analysis of the whole cell lysates (as shown in A) with serum from vaccinated or unvaccinated (control) fish. Serum used was a pool from 2 fish from each treatment group (titre 1/512, six wpv). Arrows indicate high intensity bands at 15kDa and between 37-75kDa. Molecular mass standards (kDa) are indicated.

3.5 Histology

Internal organs of spleen, kidney, liver, heart and digestive tract were examined histologically for signs of inflammation or adverse reactions to the vaccine/adjuvants six weeks post-vaccination. No histological changes were observed in the PBS injected fish. In fish administered the unadjuvanted vaccine, inflammatory cell accumulation was observed at the injection site and around the spleen, intestine and pancreatic tissue in one of the six fish sampled (Fig. 4 A, B). Another two fish had very few inflammatory cells in normal adipose

tissue around the pancreas. Vaccine emulsified with squalene and alum induced inflammatory cell infiltration higher than the FKC group but distantly less than the groups given vaccine formulated with Montanide ISA760VG adjuvant (Table 4). Lipid droplets were observed among the inflammatory cells, which originated from the squalene component of the adjuvant (Fig. 4 C, D). All six fish vaccinated with Montanide ISA760VG adjuvant showed inflammatory cell responses (Fig. 4 E, F). Three fish had severe inflammatory cell accumulations in a wide area of injection site around pancreas, intestine, liver and spleen. In 2 fish, the capsule of the spleen and liver was not obvious due to infiltrated inflammatory cells accompanied by newly produced fibrous tissue in the capsule area, and these changes may lead to adhesions of internal organs. Scoring of histological changes in the different groups is shown in Table 4.

Table 4. Scoring of histological reactions to injection with PBS: Phosphate buffered saline, FKC: Group vaccinated with formalin-killed cells of *F. psychrophilum*; FKC & squalene/alum: group vaccinated with formalin killed cells of *F. psychrophilum* mixed with squalene and alum adjuvant and FKC and Montanide: group vaccinated with formalin killed cells of *F. psychrophilum* mixed with Montanide ISA760VG adjuvant; (n=6).

Treatment Group	Injection site inflammation	Injection site lipid droplets	Tissue adhesion
PBS	- (0/6)	-	0/6
FKC	++ (1/6) ± (2/6)	-	0/6
FKC & Squalene/alum	++ (1/6) + (2/6) ± (1/6)	+	0/6
FKC & Montanide	+++ (3/6) + (2/6) ± (1/6)	+	2/6

-Absent, + Minimal, ++ Mild, +++ Moderate

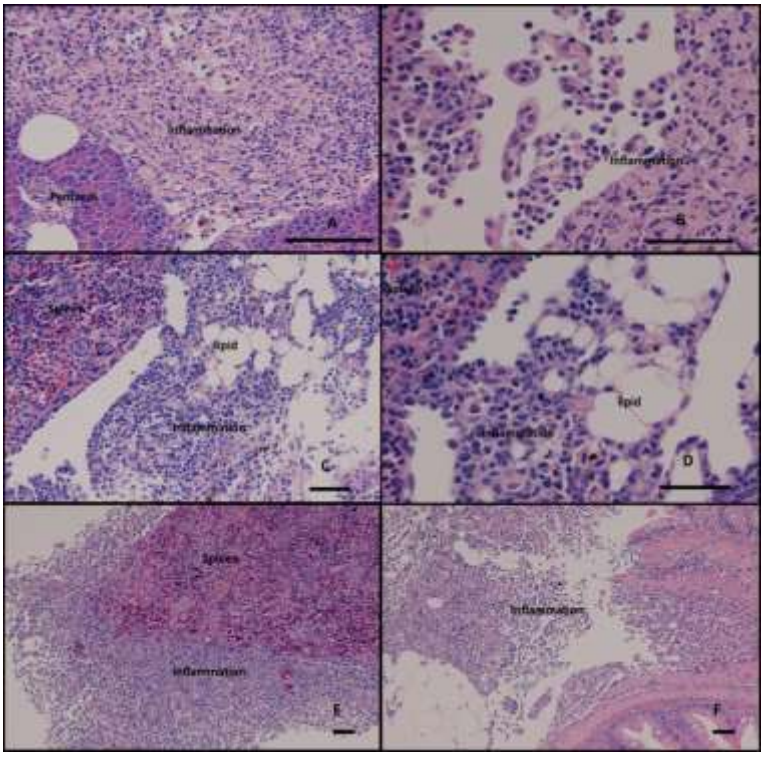

Figure 4. Atlantic salmon vaccinated with *F. psychrophilum* formalin killed cells (FKC) (A) inflammatory cell infiltrations near outer pancreas (B) Basophilic and polymorphic inflammatory cells outer spleen (bar = 50 μm). Atlantic salmon vaccinated with formalin killed cell (FKC) of *F. psychrophilum* mixed with squalene and alum adjuvant. (C) Inflammatory cell infiltrations near outer spleen (D) Basophilic and polymorphic inflammatory cells outer spleen (bar = 50 μm). Atlantic salmon vaccinated with formalin killed *F. psychrophilum* mixed with Montanide adjuvant. (E) Inflammatory cell infiltrations in injection site near outer spleen (E) and intestine (F) were observed from all 6 fish observed (bar = 50 μm).

3.6 Gene Expression (RT-qPCR)

The expression of cytokine genes (*IL-1β, IL-8, IL-10, IFN-γ*) and cell marker genes (*CD4, CD8*) was examined in the head-kidney 2 dpv. There was a significant up-regulation of the cytokines *IFN-γ* and *IL-10* in fish vaccinated with FKC alone or with FKC in combination with squalene and aluminium hydroxide adjuvant when compared to control fish injected with PBS ($p < 0.01$) Table 5. There were no significant differences in any of the genes examined in fish vaccinated with FKC and Montanide when compared to control fish.

Table 5. Quantitative PCR (qPCR) expression of genes in the head kidney of salmon day 2 post-vaccination with the *F. psychrophilum* vaccines.

Fold change of genes in vaccinated groups compared to controls ± SE. (n=6). Expression was compared to controls injected with PBS, and * indicates significant up-regulation relative to control ($p<0.05$), **($p<0.01$). FKC: Group vaccinated with formalin-killed cells of *F. psychrophilum*; FKC & squalene/alum: group vaccinated with formalin killed cells of *F. psychrophilum* mixed with squalene/alum adjuvant and FKC and Montanide: group vaccinated with formalin killed cells of *F. psychrophilum* mixed with Montanide ISA760VG adjuvant.

Gene	FKC	FKC & Squalene/Alum	FKC & Montanide
IL 10	**22.22**** ± 4.05	**24.25**** ± 6.22	1.90 ±0.35
IFNγ	**6.02*** ± 2.39	**4.10**** ±1.84	0.90 ±0.54
IL 1b	3.82 ± 0.93	2.31 ±0.69	1.29 ±0.31
IL 8	1.41 ± 0.61	1.21 ±0.56	1.06 ±0.44
CD4	1.86 ± 0.68	1.37 ±0.85	1.61 ± 0.80
CD8	1.87 ± 0.74	1.28 ±0.57	1.13 ±0.53

4. Discussion

The success of many injectable vaccines for aquaculture has been attributed to the inclusion of adjuvants [6]. Five modes of action of vaccine adjuvants have been proposed: (1) immunomodulation: the ability of many adjuvants to modify the cytokine network. (2) Presentation: the ability of an adjuvant to preserve the conformational integrity of an antigen and to present the antigen to appropriate immune effector cells. (3) CTL induction: induction of CD8+ cytotoxic T-lymphocyte (CTL) responses. (4) Targeting: the ability of an adjuvant to deliver an immunogen to immune effector cells, generally via antigen presentation cells (APCs). (5) Depot generation: generation of a short-term or long-term depot to give a

continuous or pulsed release [28]. The use of vaccine adjuvants allows for a reduction in the number of immunisations or the amount of antigen needed for immunisation.

Adjuvants are substances which enhance the immune response to an antigen [29] and one of the most effective used in aquaculture is mineral oil [30,31]. However, the traditional oil based adjuvants, such as Montanide, can cause adverse effects [8,32,33]. Therefore, there is a need to develop adjuvants for use in injectable vaccines for salmonids, which balance the efficacy-safety profile. This study compared the efficacy and safety of a novel adjuvant for salmonid aquaculture (Squalene/aluminium hydroxide) with that of the traditional water in polymer emulsion adjuvant Montanide ISA 760VG. Alum salts have a depot effect allowing the antigen to persist and the immune system to react and facilitate uptake into antigen-presenting cells (APCs)[34]. MF59, an adjuvant used for humans for over 14 years, is safe and contains a low content of squalene (4.3% w/w), a biodegradable oil naturally found in plants and animals including humans. MF59 induces low injection site reactions and is able to induce fast priming of antigen-specific CD4+ T-cell responses to induce strong and long-lasting memory T- and B-cell responses [35].

The polyvalent vaccine formulated with squalene/aluminium hydroxide against *F. psychrophilum* in this study provided significant protection to Atlantic salmon fry when administered by intraperitoneal injection with less severe side effects observed histologically as to those observed with a traditional oil-based adjuvant. The un-adjuvanted vaccine has previously been shown to provide cross-protection to trout fry against a heterologous isolate of *F. psychrophilum* by immersion vaccination [14].

The vaccine formulated without adjuvant resulted in a high level of protection (RPS 85.7%), second only to the group given vaccine combined with the traditional water in

polymer emulsion adjuvant Montanide ISA 760VG (RPS of 95.2%). The vaccine formulated with the novel squalene/alum adjuvant also gave good protection with an RPS of 75.2%. The group administered vaccine with Montanide had significantly higher specific antibody (IgM) levels (by ELISA and western blotting) to a homologous vaccine isolate six weeks post-vaccination compared with the other vaccine groups. This finding was in agreement with previous studies whereby the inclusion of oil-based adjuvants in vaccines developed for bacterial diseases of salmonids have been shown to stimulate a strong humoral response probably due to the retention of the antigen in the oil component of the vaccine and its subsequent slow release [7,32,36-38]. In the present study, specific antibody levels of the other vaccinated groups to this isolate were not significantly different to those of the control fish. These groups still had relatively high levels of protection perhaps due to even low levels of specific antibodies that are highly potent in conferring protection against *F. psychrophilum*. Future studies should include a group given adjuvant alone to further dissect the protective mechanisms behind these vaccines.

Recent studies have revealed the importance of the link between induction of the innate and adaptive immune response [39]. The type and strength of the signals recognised by the innate receptors, such as PRRs and cytokines, following vaccination affect the type of adaptive immune response induced [40]. When specific antibody was measured to an isolate of *F. psychrophilum* (AVU-1T/07) that was not present in the vaccine (a heterologous isolate) significant antibody levels were induced in both the groups given adjuvanted vaccines compared with controls or vaccine alone. The cross reaction was also observed by western blot with the strongest staining observed in the groups vaccinated with adjuvants (Squalene/Alum; Montanide). The capacity of the adjuvanted vaccine to produce a specific humoral response to a heterologous isolate is a promising indication that the combination of

all three serotypes and genetic variants in the vaccine may provide cross protection against other strains of *F. psychrophilum* in Atlantic salmon. Further studies using a number of heterologous isolates for challenge and adjuvant alone groups are warranted to further determine the cross-protective capacity of the vaccine for salmon.

Immune gene expression in head-kidney measured in the current study revealed a significant up-regulation of interferon gamma and interleukin-10 cytokines in all the vaccinated groups, except for those administered vaccine with Montanide. A similar pattern was observed when Atlantic salmon fry were experimentally infected with Salmonid alphavirus (SAV) with up-regulation of IFN-γ and IL-10 two to four weeks post-infection in head-kidney indicating a pro-inflammatory response [19]. IFN-γ is a type II IFN and has regulatory roles in both innate and adaptive immunity, including activating macrophages, enhancing antigen presentation and promoting the Th1 T cell responses. The involvement of IFN-γ at such an early stage post-vaccination (day two) suggests the stimulation of antigen presenting cells such as macrophages. IFN-γ is a powerful immunopotentiator and therefore needs to be under tight control (IL-10) as shown in studies of higher vertebrates [41]. Similar responses were seen in Atlantic salmon given oil-adjuvanted vaccines i.p. for *Aeromonas salmonicida* and infectious pancreatic necrosis virus, where gene expression profiling was used to investigate the T cell mediated immune response in spleen and head kidney from 1 to 28 dpv [18]. Expression of IFN-γ and IL-10 increased 2 dpv in spleen and head kidney in the group vaccinated with the bacterial vaccine (*A. salmonicida*), suggesting the importance of these cytokines and their interaction following vaccine delivery. In contrast to these studies the group given the *F. psychrophilum* vaccine formulated with Montanide adjuvant in the present study had no significant up-regulation of gene expression 2 dpv. Gene expression may have been delayed in this group due to the retention of the antigen compared with the other

groups as indicated by the inflammatory response observed histologically six wpv in this group.

Moderate inflammatory reactions were observed histologically in the fish administered the vaccine in conjunction with Montanide, whereas fish administered vaccine without adjuvant or the novel squalene/alum adjuvant had less inflammatory cell accumulations at the injection site as was observed when squalene based vaccines were used in humans[35]. This could be an indication of the differing mode of action of the adjuvants as the squalene/alum adjuvant (oil in water adjuvant) may have been more readily metabolised by the fish resulting in less chronic inflammation.

Squalene/alum adjuvants have seldom been incorporated into vaccines for aquaculture. Where it has been used the results have been impressive. Squalene/alum adjuvant was used in a vaccine for prevention of *F. psychrophilum* in Ayu (*Plecoglossus altivelis*) where it induced specific antibody titres and protection similar to that achieved with Montanide [42]. In addition it has been used to produce an effective vaccine with minimal side effects against VHS in Olive flounder [11]. The inclusion of this adjuvant in the present study produced significant protection in salmon against RTFS with less severe side effects observed histologically as to those observed with a traditional oil-based adjuvant and as such may hold promise for developing future vaccines for aquaculture, although length of protection still needs to be established. Future trials incorporating this adjuvant should therefore include long term efficacy studies and studies on protection in rainbow trout. Alternative methods of vaccine administration should also be tested (*e.g.* immersion vaccination) to enable vaccination of Atlantic salmon fry.

Competing interests

Conflicts of interest: the authors declare no conflict of interest.

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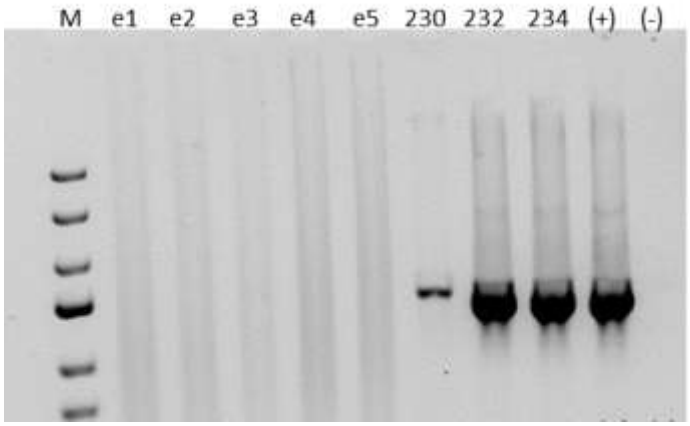

Figure S1 a. Nested PCR for detection of *F. psychrophilum* in DNA extracted from eggs. 1% agarose gel showing second round PCR products. Lane 1: (M) Ladder, Lane 2-6: Trout egg DNA, Lane 7-10: positive *F. psychrophilum* DNA, Lane 11: negative water.

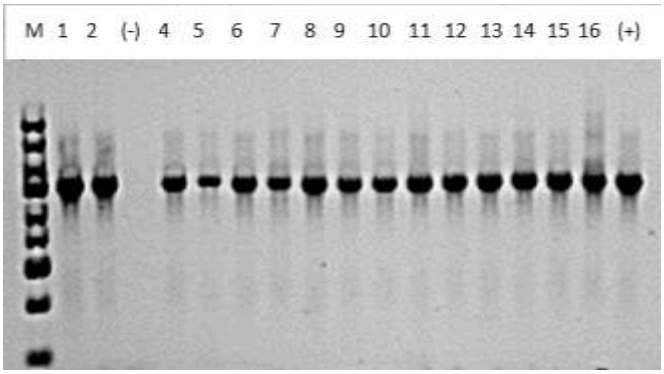

Figure S1b. Nested PCR for detection of *F. psychrophilum* in colonies recovered from moribund/mortalities post-challenge. 1% agarose gel showing second round PCR products. M: Ladder, Lane1-16: bacterial DNA recovered from fish, (-) negative control, (+): positive control.

Table S1. Survival analysis of different treatment groups showing results for individual tanks. **Treatment 1**: Control tank 1; **2**: Control tank 2; **3**: FKC tank 1; **4**: FKC tank 2; **5**: FKC & squalene tank 1; **6**: FKC & Squalene tank 2; **7**: FKC & Montanide tank 1; **8:** FKC & Montanide tank 2.

Overall Comparisons[a]

Wilcoxon (Gehan) Statistic	df	Sig.

37.930	7	.000

a. Comparisons are exact.

596
597

Pairwise Comparisons[a]

(I) treatment	(J) treatment	Wilcoxon (Gehan) Statistic	df	Sig.
1	2	2.770	1	.096
	3	8.339	1	.004
	4	1.493	1	.222
	5	3.973	1	.046
	6	2.633	1	.105
	7	8.339	1	.004
	8	5.299	1	.021
2	1	2.770	1	.096
	3	16.143	1	.000
	4	7.067	1	.008
	5	11.654	1	.001
	6	8.468	1	.004
	7	16.143	1	.000
	8	12.604	1	.000
3	1	8.339	1	.004
	2	16.143	1	.000
	4	3.212	1	.073
	5	2.069	1	.150
	6	2.219	1	.136
	8	1.000	1	.317
4	1	1.493	1	.222
	2	7.067	1	.008
	3	3.212	1	.073
	5	.450	1	.502
	6	.158	1	.691
	7	3.212	1	.073
	8	1.183	1	.277
5	1	3.973	1	.046
	2	11.654	1	.001
	3	2.069	1	.150
	4	.450	1	.502

	6	.021	1	.884
	7	2.069	1	.150
	8	.268	1	.605
6	1	2.633	1	.105
	2	8.468	1	.004
	3	2.219	1	.136
	4	.158	1	.691
	5	.021	1	.884
	7	2.219	1	.136
	8	.436	1	.509
7	1	8.339	1	.004
	2	16.143	1	.000
	4	3.212	1	.073
	5	2.069	1	.150
	6	2.219	1	.136
	8	1.000	1	.317
8	1	5.299	1	.021
	2	12.604	1	.000
	3	1.000	1	.317
	4	1.183	1	.277
	5	.268	1	.605
	6	.436	1	.509
	7	1.000	1	.317

a. Comparisons are exact.

598
599
600