

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

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25 **Abstract**

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

36 The current study was performed to assess the efficacy of a polyvalent, whole cell vaccine
37 containing formalin-inactivated *F. psychrophilum* to induce protective immunity in Atlantic
38 salmon. The vaccine was formulated with an adjuvant containing squalene and aluminium
39 hydroxide, and was compared to a vaccine formulated with a traditional oil adjuvant,
40 Montanide ISA 760VG, and a non-adjuvanted vaccine. Duplicate groups of salmon ($23.5 \pm$
41 6.8 g) were vaccinated with each of the vaccine formulations or phosphate buffered saline by
42 intraperitoneal injection. Fish were challenged by intramuscular injection with *F.*
43 *psychrophilum* six weeks post-vaccination to test the efficacy of the vaccines. Cumulative
44 mortality reached 70% in the control salmon, while the groups of salmon that received
45 vaccine had significantly lower mortality than the controls ($p = 0.0001$), with no significant
46 difference in survival between vaccinated groups. The squalene/alum adjuvant was safe, more
47 readily metabolised by the fish and induced less histopathological changes than the traditional
48 oil adjuvant.

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

50

51 **1 Introduction**

52 Rainbow trout fry syndrome (RTFS), caused by *Flavobacterium psychrophilum*, is one of the
53 most significant disease problems facing the salmonid aquaculture industry worldwide [1].

54 Rainbow trout (*Oncorhynchus mykiss*) are the species most affected although there are
55 increasing problems in Atlantic salmon (*Salmo salar*) hatcheries in Scotland and Chile.

56 Disease episodes tend to occur between 10-15 °C, with necrotic lesions often seen on the skin
57 surrounding the dorsal fin and tail, while in very small fish no clinical signs are apparent and

58 death occurs due to septicemia. *F. psychrophilum* is a highly heterogeneous pathogen, which

59 makes development of cross-protective vaccines to control this devastating disease

60 problematic [2]. Antibiotic treatment is relied on to treat outbreaks, which has led to increased

61 levels of antibiotic resistance in *F. psychrophilum* isolates [3-5], highlighting the urgent need

62 for prophylactic treatments for RTFS.

63 The majority of inactivated whole cell or sub-unit vaccines available to the aquaculture

64 industry are formulated in oil emulsions [6]. Adjuvanted vaccines are injected

65 intraperitoneally, and provide protection via a prolonged release of antigen from the oil

66 component stimulating primarily local inflammatory reactions followed by a systemic

67 immune response [7]. While oil-based adjuvants have provided increased efficacy of vaccines

68 for aquaculture, problems with side-effects at injection sites have resulted in the down grading

69 of fish at harvest due to adhesions between the body wall and abdominal organs and spinal

70 deformities [8-10] . Therefore, there is a need to develop adjuvants for use in injectable

71 vaccines for salmonids, which balance the efficacy-safety profile. A previous study using an

72 adjuvant containing squalene and aluminium hydroxide to formulate a vaccine for treatment

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

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

83

84 **2 Materials and Methods**

85 **2.1 Atlantic Salmon Fry**

86 Atlantic salmon eggs were supplied by AquaGen (Norway) and transported on ice to the
87 aquarium at the Institute of Aquaculture, Stirling. On arrival eggs were subjected to an
88 iodophor surface disinfectant treatment according to the manufacturer's instructions
89 (Buffodine, Evans Vanodine, UK). Five replicates of 10 eggs were removed and confirmed to
90 be *F. psychrophilum* free using a nested PCR for the 16S rRNA gene with modifications
91 [12,13]. The eggs were maintained in flow-through de-chlorinated tap water at 10 °C until
92 hatch, and thereafter maintained in a 100 L flow-through tank (5 L min⁻¹). The fry were fed to
93 satiation daily (Inicio feed, 1.1 mm, BioMar, UK). All experimental procedures with live fish
94 were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and
95 associated guidelines, EU Directive 2010/63/EU for animal experiments and were approved
96 by the Ethics Committee of the Institute of Aquaculture, University of Stirling, UK.

97

98 **2.2 Preparation of formalin inactivated bacteria**

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

101

102 **Table 1.** Details of the isolates of *F. psychrophilum* included in the experimental vaccine: species,
103 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

104

105 **2.3 Preparation of Vaccine formulations and Vaccination**

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

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

119 (100 mL 35% formaldehyde and 900 mL DW) for histology. Head-kidney from three fish per
 120 duplicate group (n=6) was placed immediately in RNA-later (Sigma) and stored at 4 °C
 121 overnight. RNA-later was removed and tissues stored at -70 °C until RNA extraction. Blood
 122 was sampled from the caudal vein using a 23 G needle and syringe from three fish per
 123 duplicate group (n=6) stored overnight at 4 °C, centrifuged at 3000 x g 5 min for collection of
 124 serum which was stored at -20 °C until analysis.

125

126 **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 x 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		

127

128 **2.4 Experimental infection of vaccinated fish**

129 Vaccinated and control fish were experimentally infected with a homologous isolate of *F.*
 130 *psychrophilum* (AVU-3S/13) at 4.0 x 10⁷ CFU/fish six weeks post-vaccination (wpv) by
 131 intramuscular injection. The fish were maintained as above and monitored for 21 days post
 132 infection (dpi). Moribund fish or mortalities were removed and sampled by streaking head
 133 kidney, spleen and any lesions on Modified Veggietone (MV) medium [veggitones GMO-free
 134 soya peptone (Oxoid, UK), 5 g L⁻¹; yeast extract (Oxoid, UK), 0.5 g L⁻¹; magnesium sulphate
 135 heptahydrate (Fisher chemicals, UK), 0.5 g L⁻¹; anhydrous calcium chloride (BHD), 0.2 g L⁻¹;
 136 dextrose (Oxoid, UK), 2 g L⁻¹; agar (solid medium; Oxoid, UK), 15 g L⁻¹; pH 7.3] to confirm

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

139

140 **2.5 ELISA for detection of specific IgM in serum**

141 Enzyme-linked immunosorbent assay (ELISA) was used to assess specific IgM titre to *F.*
142 *psychrophilum* in serum according to [16] with some modifications. *F. psychrophilum*
143 vaccine isolates and a heterologous isolate were used to coat the plates at 1×10^8 /mL in PBS
144 and incubated overnight at 4 °C. The dilution of fish serum used was optimised by first
145 titrating sera from each group (1:32 to 1:1024). Fish serum samples at the optimised dilution
146 of 1:64 in PBS were added to the wells (100 µl/well) in duplicate and incubated overnight at 4
147 °C. Specific IgM was detected using anti-trout IgM monoclonal antibody (Aquatic
148 Diagnostics Ltd., 1/33 in PBS, 1h) followed by incubation with anti-mouse-HRP (1/4000,
149 Sigma, 1h). The absorbance was read on a BioTek HT Synergy spectrophotometer at 450 nm.

150

151 **2.6 SDS-PAGE and Western blotting**

152 **2.6.1 Sodium dodecyl sulphate polyacramide gel electrophoresis (SDS-PAGE)**

153 Suspensions of the three vaccine isolates and a heterologous isolate of *F. psychrophilum* were
154 aliquoted into 1.5 ml microcentrifuge tubes (1 mL of 2×10^8 cfu/mL), and centrifuged for 15
155 min at $3000 \times g$. Bacterial pellets were resuspended in 100 µl of DW and 30 µl of 5 X sample
156 buffer (250mM Tris-HCl, 30% glycerol, 10% SDS, 0.5M dithiothreitol, 0.2% bromophenol
157 blue) and boiled for 15 min. Finally, the samples were centrifuged at $10,000 \times g$ for 10 min
158 prior to analysis of the supernatants. A preparation of broad-range molecular weight markers
159 (5 µl) (Bio-Rad) were added to the first well of a 12% polyacrylamide gel (Bio-Rad) and 15
160 µl of each sample were added to the remaining wells. The gel was run at 130 V for

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

163

164 **2.6.2 Western blot analysis**

165 Bacterial components separated by SDS-PAGE as described above were transferred onto
166 nitrocellulose membranes by semi-dry transfer (Pierce™ Power Blotter, ThermoFischer
167 Scientific) applying 25 V (1.3A) for 7 min. The nitrocellulose membranes were then
168 incubated overnight at 4 °C in 5 % (w/v) casein in distilled water (DW). After washing 3
169 times with Tris buffered saline with Tween (TBS: 10 mM Tris base, 0.5 M NaCl pH 7.5 with
170 0.1% [v/v] Tween 20) for 5 min at each wash, the membranes were incubated for 3 h at 22 °C
171 with a 1/20 dilution of fish serum in TBS (serum was a pool from 2 fish from each treatment
172 group, with a titre of 1/512, taken six wpv as described in Section 2.6). The membranes were
173 washed as previously described and incubated for 1 h at 22 °C with a 1/20 dilution of anti-
174 trout IgM monoclonal antibody in TBS (ADL). The membranes were again washed and
175 incubated for 1 h at 22 °C with a 1/200 dilution of anti-mouse horse radish peroxidase
176 (Sigma) in TBS. After washing, bands were visualised by adding chromogen and substrate
177 (ImmPACT™ DAB Peroxidase substrate kit). The reaction was stopped by soaking the
178 membranes in DW for 5 min.

179

180 **2.7 Histology**

181 Formalin fixed tissues were embedded in paraffin and sectioned using a Microtome (Shandon
182 Finesse). Tissue sections were de-waxed and dehydrated in xylene (2 x 3 min), 100% ethanol
183 (2 min), methylated spirit (1.5 min) and stained with haematoxylin and eosin. Slides were

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

186

187 **2.8 Isolation of total RNA and cDNA synthesis**

188 RNA was extracted from 30 - 40 mg of each head-kidney sample using TRI Reagent (Applied
189 Biosystems) following the manufacturer's protocol. The resultant RNA pellet was re-
190 suspended in 30 µL of nuclease-free water. Following spectrophotometric quantification
191 (Nanodrop ND-1000, Thermo Fisher, Leicestershire, UK) and quality checking by gel
192 electrophoresis (1% agarose gel stained with ethidium bromide), samples were stored at -70
193 °C until required. RNA was reverse transcribed to construct cDNA using a high-capacity
194 cDNA Reverse Transcription kit (Applied Biosystems, USA) according to the manufacturer's
195 instructions. Briefly, 10 µl of RNA was added to 10 µl of 2X RT master mix (10X RT buffer,
196 25X dNTP Mix 100 mM, 10XRT Random Primers and oligo-dT mix, Reverse Transcriptase,
197 RNase Inhibitor, nuclease-free water). The thermal cycle conditions consisted of 25 °C for 10
198 min, 37 °C for 120 min and 85 °C for 5 min. The cDNA was aliquoted and stored at -20 °C
199 prior to use.

200

201 **2.9 Quantitative Real Time PCR (qRT-PCR)**

202 Head-kidney samples were analysed by qRT-PCR for the expression of cytokines (*IL-1β*, *IL-*
203 *8*, *IL-10*, *IFN-γ*) and immune genes (*CD4*, *CD8*). Real time PCR was performed on first
204 strand cDNA using the Eppendorf® RealPlex² Mastercycler gradient S instrument with
205 SYBR® Green I (Thermo Scientific) master mix and primers as shown in Table 3.

206

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

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215

Gene	Primers	Product size	Reference	Efficiency (E)
IL-1 β	F: GCTGGAGAGTGCTGTGGAAGA R: TGCTTCCCTCCTGCTCGTAG	73	[17]	0.90
IL-10	F: CTGTTGGACGAAGGCATTCTAC R: GTGGTTGTTCTGCGTTCTGTTG	129	[18]	0.95
IFN γ	F: CTAAAGAAGGACAACCGCAG R: CACCGTTAGAGGGAGAAATG	159	[19]	0.96
CD8 α	F: AATCAATGGTAACGCGCTTG R: TGGCTGTGGTCATTGGTGTA	101	[20]	0.97
CD4	F: GAGTACACCTGCGCTGTGGAAT R:GGTTGACCTCCTGACCTACAAAGG	121	[19]	0.85
IL-8	F: ATTGAGACGAAAAGCAGACG R: CGCTGACATCCAGACAAATCT	136	[21]	0.85
Elongation factor 1 α	F:CGGCAAGTCCACCACCAC R:GTAGTACCTGCCAGTCTCAAAC	205	[21]	0.94
B-actin	F: ACTGGGACGACATGGAGAAG R: GGGGTGTTGAAGGTCTCAA	157	[21]	0.91

216 Briefly the 20 μ l reaction consisted of 5 μ l of cDNA and 15 μ l of master mix prepared using 1
217 μ l of the forward and reverse primers (0.3 μ M), 10 μ l SYBR® Green I and 3 μ l of nuclease
218 free water. The cycling conditions consisted of 95°C initial denaturing for 15 s, followed by
219 40 cycles of 15 s denaturing at 95 °C, 30 s annealing at 58 °C and 30 s extension at 72 °C.
220 RT-minus and non-template controls were included on every plate. Melting curve analysis
221 was performed from 60 °C to 95 °C in 0.1 °C/s increments to assess the specificity of the RT-
222 PCR products. Serial 10-fold dilutions of the cDNA were prepared in nuclease free water
223 starting and the Ct values were used to generate a standard curve plot of cycle number versus
224 log concentration in the *realplex* software V2.2 (Eppendorf). The quality of the standard
225 curve was judged by the slope of the curve and the correlation coefficient (r). The slope of the
226 line was used to estimate the estimate the efficiency of the target amplification using the
227 equation $E = (10^{-1/\text{slope}}) - 1$. Elongation factor- α and β -actin were used as reference genes to
228 correlate for potentially different loading amounts of RNA and for variation in cDNA
229 synthesis efficiencies [22]. The threshold cycle (Ct) was determined at the linear slope in a
230 log fluorescence/Ct plot. The expression results were analysed using the $2^{-\Delta\Delta}$ Ct method [23].

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

233

234 **2.10 Statistical Analysis**

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

242

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

243

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

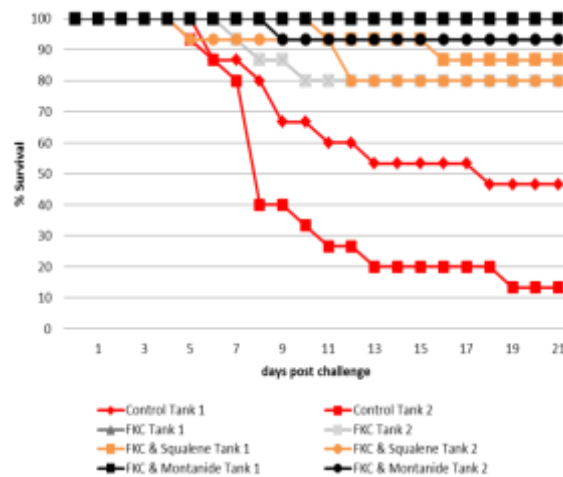
245

246 **3. Results**

247 **3.1 Vaccine Efficacy**

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

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



258

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

266

267 3.2 Nested PCR for detection of *F. psychrophilum*

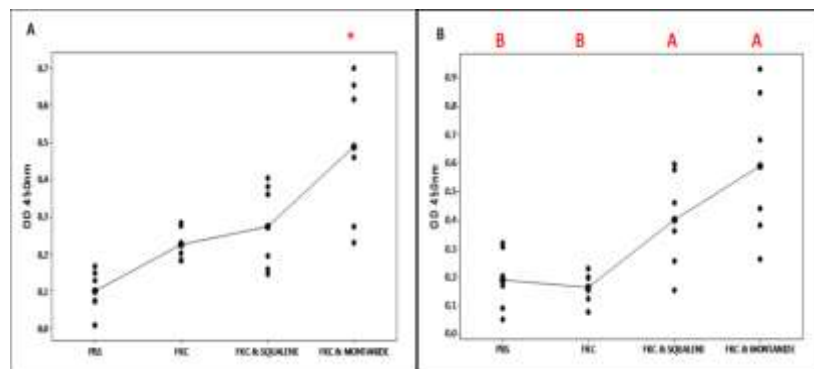
268 The eggs were free of *F. psychrophilum* (Fig. S1a) and *F. psychrophilum* was detected in
269 moribund and dead fish sampled during the challenge (Fig. S1b).

270

271 3.3 Specific antibody response

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

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

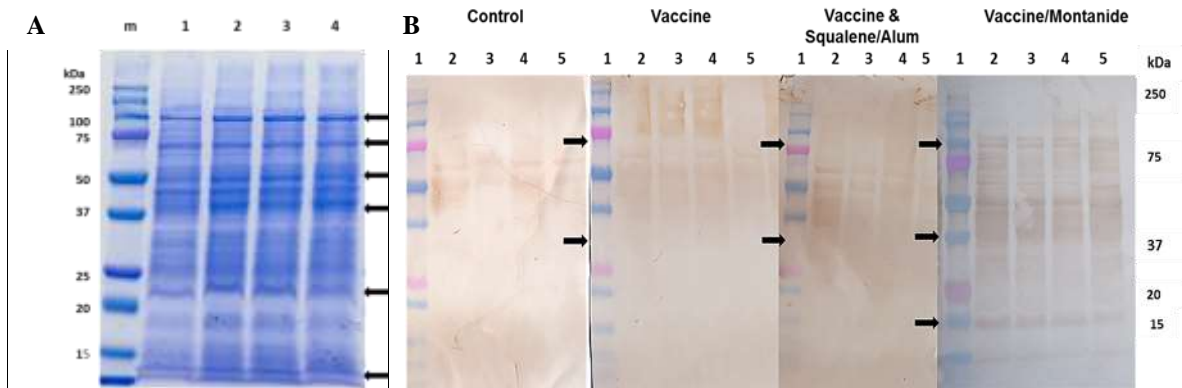


281
 282 **Figure 2.** Specific antibody (IgM) levels to *F. psychrophilum* in vaccinated salmon 6 weeks post vaccination (A:
 283 to homologous isolate *F. psychrophilum* *denotes significantly different to other groups $p=0.002$; B: to a
 284 heterologous isolate *F. psychrophilum*; groups that do not share a letter are significantly different ($p=0.01$). The
 285 line denotes the mean antibody level of each group, $n=6$, 1:64 serum dilution.

286
 287 **3.4 SDS-PAGE and Western blot**

288 Distinct bands ranging from 10-250 kDa were evident in the SDS-PAGE profiles of the *F.*
 289 *psychrophilum* isolates used to prepare the polyvalent vaccine (and a heterologous isolate
 290 AVU-1T/07) following staining with Coomassie (Fig 3A). Banding profiles of the isolates
 291 were similar, with the exception of a slight difference in the band between 20-25kDa in the
 292 heterologous isolate. When blots of these isolates were incubated with immune sera sampled
 293 6 wpv (pooled sera with a titre of 512 by ELISA), the strongest staining was seen with serum
 294 from fish vaccinated with the Montanide and squalene/alum vaccine preparations (Fig. 3B)
 295 reflecting the results obtained by ELISA. Bands ranging from 15 to 250 kDa were recognised

296 by the Montanide group, whereas the serum from fish given unadjuvanted vaccine recognised
 297 bands between 37-250 kDa with much weaker staining. This was also the case with serum
 298 from fish given vaccine emulsified with squalene/alum adjuvant, with bands recognised
 299 between 37-75 kDa. These bands also stained weakly in control fish administered PBS.
 300



301
 302
 303 **Figure 3.** SDS-PAGE and western blotting of *F. psychrophilum* isolates. (A) Whole cell lysates from a
 304 heterologous isolate and vaccine isolates. Lanes: (1) molecular weight markers (2) AVU171/07, (3) AVU-1T/13,
 305 (4) AVU-2T/13, (5) AVU-3S/13) were separated by SDS-PAGE and stained with Coomassie stain. Arrows
 306 indicate high intensity bands at 10-15, 20, 37-50, 75, 100 kDa. (B) Western blot analysis of the whole cell
 307 lysates (as shown in A) with serum from vaccinated or unvaccinated (control) fish. Serum used was a pool from
 308 2 fish from each treatment group (titre 1/512, six wpv). Arrows indicate high intensity bands at 15kDa and
 309 between 37-75kDa. Molecular mass standards (kDa) are indicated.

310
 311 **3.5 Histology**

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

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

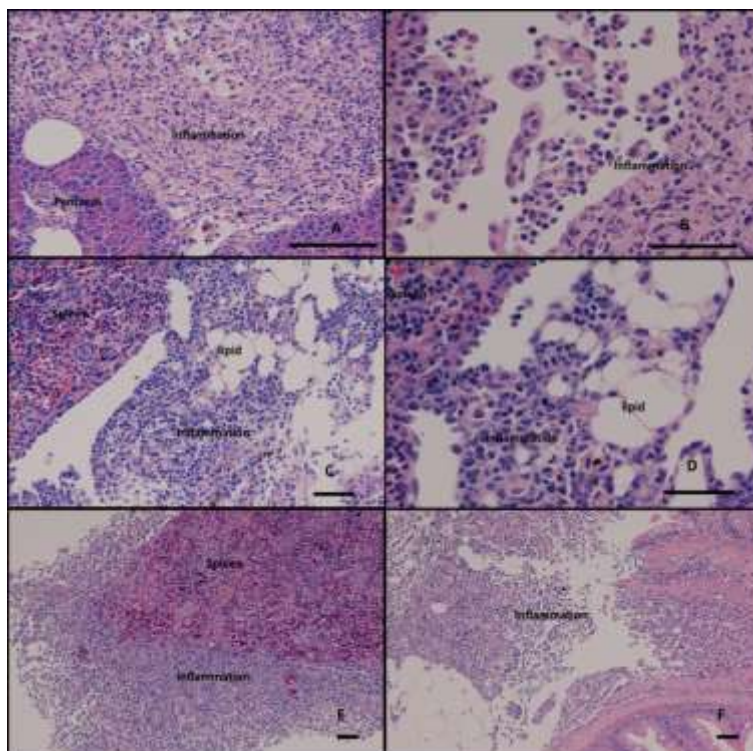
329 **Table 4.** Scoring of histological reactions to injection with PBS: Phosphate buffered saline, FKC: Group
 330 vaccinated with formalin-killed cells of *F. psychrophilum*; FKC & squalene/alum: group vaccinated with
 331 formalin killed cells of *F. psychrophilum* mixed with squalene and alum adjuvant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

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

334

335

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



336

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

344

345 **3.6 Gene Expression (RT-qPCR)**

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

352

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

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

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

360

361

362 4. Discussion

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

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

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

386

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

393 The vaccine formulated without adjuvant resulted in a high level of protection (RPS
394 85.7%), second only to the group given vaccine combined with the traditional water in

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

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

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

423 Immune gene expression in head-kidney measured in the current study revealed a
424 significant up-regulation of interferon gamma and interleukin-10 cytokines in all the
425 vaccinated groups, except for those administered vaccine with Montanide. A similar pattern
426 was observed when Atlantic salmon fry were experimentally infected with Salmonid
427 alphavirus (SAV) with up-regulation of IFN- γ and IL-10 two to four weeks post-infection in
428 head-kidney indicating a pro-inflammatory response [19]. IFN- γ is a type II IFN and has
429 regulatory roles in both innate and adaptive immunity, including activating macrophages,
430 enhancing antigen presentation and promoting the Th1 T cell responses. The involvement of
431 IFN- γ at such an early stage post-vaccination (day two) suggests the stimulation of antigen
432 presenting cells such as macrophages. IFN- γ is a powerful immunopotentiator and therefore
433 needs to be under tight control (IL-10) as shown in studies of higher vertebrates [41]. Similar
434 responses were seen in Atlantic salmon given oil-adjuvanted vaccines i.p. for *Aeromonas*
435 *salmonicida* and infectious pancreatic necrosis virus, where gene expression profiling was
436 used to investigate the T cell mediated immune response in spleen and head kidney from 1 to
437 28 dpv [18]. Expression of IFN- γ and IL-10 increased 2 dpv in spleen and head kidney in the
438 group vaccinated with the bacterial vaccine (*A. salmonicida*), suggesting the importance of
439 these cytokines and their interaction following vaccine delivery. In contrast to these studies
440 the group given the *F. psychrophilum* vaccine formulated with Montanide adjuvant in the
441 present study had no significant up-regulation of gene expression 2 dpv. Gene expression may
442 have been delayed in this group due to the retention of the antigen compared with the other

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

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

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

465

466 **Competing interests**

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

468

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472

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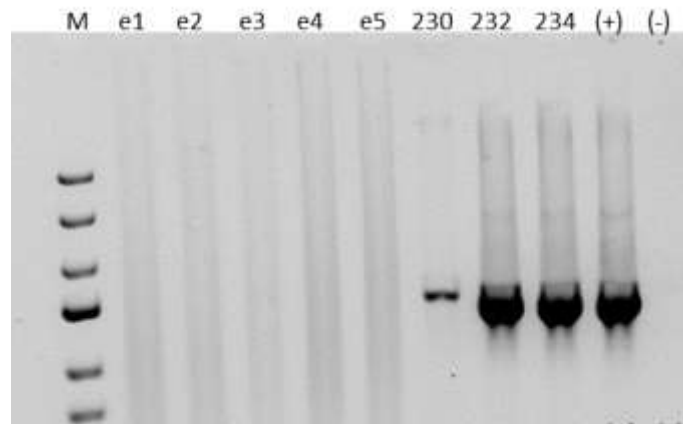
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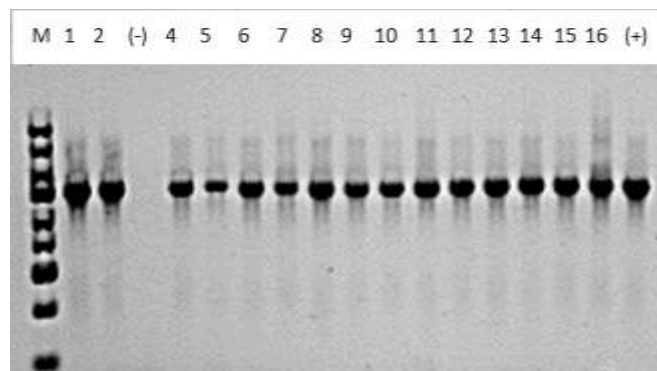
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 582
 583 Figure S1 a. Nested PCR for detection of *F. psychrophilum* in DNA extracted from eggs. 1% agarose gel
 584 showing second round PCR products. Lane 1: (M) Ladder, Lane 2-6: Trout egg DNA, Lane 7-10: positive *F.*
 585 *psychrophilum* DNA, Lane 11: negative water.



586
 587 Figure S1b. Nested PCR for detection of *F. psychrophilum* in colonies recovered from moribund/mortalities
 588 post-challenge. 1% agarose gel showing second round PCR products. M: Ladder, Lane1-16: bacterial DNA
 589 recovered from fish, (-) negative control, (+): positive control.

590
 591
 592 **Table S1.** Survival analysis of different treatment groups showing results for individual tanks. **Treatment 1:**
 593 Control tank 1; 2: Control tank 2; 3: FKC tank 1; 4: FKC tank 2; 5: FKC & squalene tank 1; 6: FKC & Squalene
 594 tank 2; 7: FKC & Montanide tank 1; 8: FKC & Montanide tank 2.

595

Overall Comparisons ^a		
Wilcoxon (Gehan) Statistic	df	Sig.

37.930	7	.000
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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