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1 **A COMMERCIAL AUTOGENOUS INJECTION VACCINE PROTECTS BALLAN**
2 **WRASSE (*LABRUS BERGYLTA*, ASCANIUS) AGAINST *AEROMONAS***
3 ***SALMONICIDA VAPA* TYPE V**

4

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22 **ABSTRACT**

23 Atypical *Aeromonas salmonicida* (a*As*) and *Vibrionaceae* related species are bacteria routinely
24 recovered from diseased ballan wrasse used as cleaner fish in Atlantic salmon farming.
25 Autogenous multivalent vaccines formulated from these microorganisms are widely used by
26 the industry to protect farmed wrasse despite limited experimental proof that they are primary
27 pathogens. In this study, the components of a commercial multivalent injection wrasse vaccine
28 were tested for infectivity, pathogenicity and virulence via intra peritoneal injection at pre-
29 deployment size (25-50g) and the efficacy of the vaccine for protection against a*As* assessed.
30 Injection with $3.5x10^9$, $8x10^9$ $1.8x10^9$ and $5x10^9$ cfu/fish of *Vibrio splendidus*, *V. ichthyoenteri*,
31 *Aliivibrio logeii* and *A. salmonicida,* respectively, did not cause significant mortalities, lesions
32 or clinical signs after a period of 14 days. IP injection with both a*As* and *Photobacterium*

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33 *indicum* successfully reproduced the clinical signs and internal lesions observed during natural
34 outbreaks of the disease. Differences in virulence (LD_{50} at day 8-post infection of $3.6x10^6$
35 cfu/fish and $1.6x10^7$ cfu/fish) were observed for two a*As vapA* type V isolates. In addition, the
36 LD_{50} for *Photobacterium indicum* was $2.2x10^7$ cfu/fish. The autogenous vaccine was highly
37 protective against the two a*As vapA* type V isolates after 700-degree days of immunisation.
38 The RPS_{FINAL} values for the first isolate were 95 and 91% at $1x10^6$ cfu/fish and $1x10^7$ cfu/fish,
39 respectively, and 79% at $1x10^7$ cfu/fish for the second isolate tested. In addition, significantly
40 higher anti a*As* seral antibodies (IgM), were detected by ELISA in vaccinated fish in contrast
41 with control (mock vaccinated) fish. These results suggest wrasse can be effectively immunised
42 and protected against a*As* infection by injection with oil adjuvanted vaccines prepared with
43 inactivated homologous isolates. Further work should assess the efficacy of vaccination against
44 other isolates that have proven to be pathogenic such as a*As* type VI and *Photobacterium*
45 *indicum* and explore the feasibility of immersion vaccination. In addition, a full
46 characterisation of a*As* isolates within the same *vapA* types should be performed as differences
47 in virulence between *vapA* type V isolates were observed and partial genome analysis indicated
48 small but potentially important genomic differences in these isolates.

49

50 **Key Words:** wrasse vaccines, bacterial diseases, atypical *Aeromonas salmonicida*, cleaner
51 fish diseases, vibriosis in wrasse, *Photobacterium indicum.*

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52 **INTRODUCTION**

53 Bacterial pathogens are considered the major cause of infectious diseases and mortalities in
54 farmed ballan wrasse produced for sea lice control in the salmon farming industry [1-3]. In
55 Scotland, atypical *Aeromonas salmonicida (*a*As) vapA* type V and VI*, Vibrio splendidus*, *V.*
56 *ichthyoenteri*, *Aliivibrio salmonicida*, *A. logeii*, and *Photobacterium indicum* are the bacterial
57 pathogens most frequently isolated from ballan wrasse during outbreaks of disease in both
58 hatcheries and post deployment in salmon sea sites [4]. Similar reports are available for the
59 species in Norway [3].

60 Immunisation of farmed salmonids (Atlantic salmon and rainbow trout) against typical *As*
61 using fully licenced oil-adjuvanted injectable vaccines has historically proven successful and
62 is a standard practice [5]. However, immunisation of non–salmonid species against typical and
63 atypical *As* has been rather challenging [6-8]. For instance, an experimental vaccine containing
64 atypical strains protected Arctic charr (*Salvelinus alpinus,* L.) but not in European grayling
65 (*Thymallus thymallus*, L.) [9]. Furthermore, commercial furunculosis vaccines for salmonids
66 have induced protection in Atlantic halibut (*Hippoglossus hippoglossus*, L.) but not in Atlantic
67 cod (*Gadus morhua*, L.) or turbot (*Scophthalmus maximus*, L.) [6-8].

68 Currently no licenced or registered vaccines are commercially available in the UK for the
69 prevention and control of infectious diseases in ballan wrasse. Therefore, prophylactic
70 treatments in Scotland are mainly based on the use of autogenous vaccines, which are
71 formulated with antigens derived from pathogens recovered during episodes of elevated
72 mortality [2].

73 Autogenous or "herd specific" vaccines are farm specific immunological veterinary medicinal
74 products that have the potential to be rapidly developed and deployed when no off-the shelf
75 fully licensed vaccines exist or these have proven infective. In principle, autogenous vaccines
76 must be inactivated (killed), manufactured in licenced facilities, used only under veterinary
77 prescription and on the sites where the pathogens were isolated [10-13].

78 In Scotland, autogenous vaccines for ballan wrasse were first developed from isolates collected
79 during disease outbreaks between 2013 and 2014 (Ridgeway Biologicals Ltd.) and used in
80 hatcheries and wild caught wrasse. The vaccine formulation later evolved and new isolates
81 were introduced following a health screening surveys [4]. However without established
82 challenged models for the Scottish bacteria and wrasse populations, the actual virulence of the
83 isolates and the efficacy/potency of the vaccine components remained unknown.

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84 Overall, i.p. injection challenges with atypical strains of *As* have been successful in several
85 species with a wide range of doses used [14]. For instance, juvenile spotted wolfish
86 (*Anarhichas minor,* L) succumbed to disease when i.p. injected with a*As* at 10^3 and 10^4 cfu /
87 mL [8, 15], while high morbidities in turbot were reported [16], but only in fish exposed with
88 the same method to 10^8 and 10^{10} cfu / mL. Experimentally infected ballan wrasse and
89 lumpsucker also experienced high morbidities (> 70%) when challenged with Norwegian a*As*
90 isolates at doses of 2 x10^3 cfu / mL (bath) and 2 x 10^6 cfu /mL (i.p. injection), and 10^8 cfu /mL
91 (i.p. injection), respectively [17, 18]. As for the *Vibrionaceae* pathogens in cleaner fish, in a
92 previous study in Norway, only *Vibrio anguillarum* originally isolated from Atlantic salmon
93 caused high mortalities (up to 60%) in ballan wrasse under experimental conditions, while
94 Norwegian ballan wrasse isolates of the same bacterial species caused < 20% mortalities when
95 challenged via bath, cohabitation and i.p. injection [17].

96 Given that, a*As* and *Vibrionaceae* isolates are highly heterogenic and variable, and virulence is
97 often strain and host dependant [1, 19], the establishment of similar experiments in other
98 geographical areas such as Scotland is of high relevance for the local industry.

99 In the present study, *in vivo* challenge models were developed via intraperitoneal (i.p.) injection
100 in Scottish ballan wrasse (25-50 g) to investigate the infectivity, pathogenicity and virulence
101 of isolates routinely recovered from diseased wrasse and used as antigens in commercial
102 autogenous vaccines. These isolates included a*As vapA* types V and VI, *Vibrio splendidus*, *V.*
103 *ichthyoenteri*, *Aliivibrio salmonicida*, *A.* l*ogeii*, and *Photobacterium indicum*. Furthermore, the
104 efficacy of the a*As vapA* type V components of the vaccine was assessed by measuring survival
105 rates after experimentally infecting vaccinated and control fish by i.p. injection with
106 homologous isolates at medium, high and very high doses and its potency expressed in terms
107 of RPS. Specific antibody (IgM) kinetics were assessed as a relevant correlate of protection.

108 **MATERIALS AND METHODS**

109 **Bacterial identification and genotyping**

110 The bacterial isolates used were recovered from diseased fish at commercial hatcheries and
111 characterised on the basis of phenotypic and genotypic characteristics as part of a previous
112 study [4]. In brief, bacterial DNA was extracted using Genesig® Easy DNA/RNA Extraction
113 Kit (Primerdesign Ltd, Southampton UK) according to the manufacturer's instructions. Species
114 confirmation was performed on the samples by targeting the V3-V4 hypervariable region of
115 the *16S rRNA* gene [20] and the subunit B protein of DNA gyrase (topoisomerase type II) –

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116 *gyrB* gene [21]. The *Aeromonas salmonicida* isolates were then genotyped by sequencing the
117 A-layer membrane as described previously [22].

118 For the experimental infections, the a*As* isolates were grown on tryptone soya agar (TSA,
119 Oxoid, UK) or blood agar (BA; TSA + 5% sheep blood Thermo Fisher) while the *Vibrionaceae*
120 isolates were on sea water agar (SWA, Oxoid, UK) and incubated at 22 °C for 48 and 24 h,
121 respectively. For growth in liquid media, a*As* isolates were inoculated onto trypticase soy broth
122 (TSB, Oxoid, UK) and *Vibrionaceae* isolates onto TSB + 2% NaCl (Oxoid, UK,) and incubated
123 at 22 °C for 18-24 h, with continuous shaking at 180 rpm. For harvesting, all bacteria were
124 centrifuged at 4 °C for 10 min at 2,000 x g, bacterial pellets were then washed with sterile 1x
125 phosphate-buffered saline (PBS) and resuspended in sterile PBS to the required concentration
126 (cfu/mL) for the experiments.

127 With the exception of isolate TW164/15 (a*As vapA* type VI) that was recovered from moribund
128 lumpsucker (*Cyclopterus lumpus*) the rest of the isolates were recovered from ballan wrasse.
129 A summary of the isolates used in this study is presented in Table 1.

Table 1. Bacterial isolates used in this study for pathogenicity, virulence and vaccine assessment.

Isolate ID	Bacterial species	Year of isolation	*vapA* type and /or isolate #
TW4/14**[v]	*Aeromonas salmonicida*	2014	V
TW187/14 [v]	*Aeromonas salmonicida*	2014	V
TW3/14** [v]	*Aeromonas salmonicida*	2014	V
TW164/15 [v]	*Aeromonas salmonicida*	2015	VI
TW184/16*	*Aeromonas salmonicida*	2016	VI
TW242/16	*Aliivibrio logei*	2016	Isolate 1
TW186/16	*Aliivibrio logei*	2016	Isolate 2
TW322/16	*Aliivibrio salmonicida*	2016	Isolate 1
TW130/16 [v]	*Vibrio splendidus*	2016	Isolate 1
TW319/16	*Vibrio ichthyoenteri*	2016	Isolate 1
TW138/16	*Photobacterium indicum*	2016	Isolate 1
TW181/16	*Photobacterium indicum*	2016	Isolate 2

(*) isolated from lumpsucker, (**) isolates used for vaccination efficacy trial, (v) isolates present in the commercial vaccine tested.

130 **Experimental fish**

131 A population of naïve *i.e.* unvaccinated and non-diseased ballan wrasse (30 ± 5 g) was provided
132 by a commercial cleaner fish hatchery on the west coast of Scotland. Prior to the study, the

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133 health status of the fish was checked by screening a subset of the population with standard
134 histological, bacteriological and molecular methods to confirm the absence of a*As* [4, 22],
135 amoebic gill disease [23] and *Vibrionaceae* related bacteria [20, 21]. After confirmation they
136 were free of these pathogens, fish were transferred to the Centre for Environment, Fisheries
137 and Aquaculture Science (Cefas) Weymouth Laboratory in February 2017.

138 Fish were acclimated and quarantined for 3 weeks after arrival in 6 aerated aquaria (approx.
139 900 L, tanks enriched with artificial plastic kelp and sections of plastic pipes to provide hides
140 to the fish) at 12.0 ± 0.5 °C with a 20:4 h light:dark photoperiod, water flow of 4.0 L / min and
141 dissolved oxygen (DO) at 8 ± 0.5 mg / L. During this period, fish were further screened for
142 bacteriology (swabs from head kidney plated onto SWA), histopathology (fixed in 10% neutral
143 buffered formalin) and molecular methods as described before. In addition, virology diagnostic
144 tests were performed to discard the presence of notifiable viral diseases as per the protocols in
145 the OIE manual of diagnostic tests for aquatic animals [24].

146 **Vaccine**

147 A commercial injectable (oil-based) multivalent autogenous vaccine, containing antigens from
148 isolates TW3/14, TW4/14, TW187/14, TW164/15 and TW130/16 (Table 1) was provided by
149 Ridgeway Biologicals Ltd. The vaccine was shipped to Cefas and stored at 4 ± 1 °C prior to
150 use.

151 **Confirmation of infectivity of components of multivalent autogenous vaccine**

152 The virulence of bacterial isolates, representative of strains commonly used as components of
153 the multivalent autogenous vaccines used in the industry, was assessed in a series of infection
154 experiments performed in 30 L tanks enriched as for acclimation tanks (Table 2).

155 For the first infection experiment, limited numbers of 30 ± 5 g fish (n= 6) were injected with
156 an OD_{600} ~1.57 bacterial suspension of different isolates representing 4 different bacterial
157 species (a*As* including two type V and one type VI isolates, *Vibrio splendidus*, *Aliivibrio*
158 *salmonicida* and *Vibrio ichthyoenteri*) (Table 2).

159 In the second infection experiment, the pathogens that did not cause morbidities or signs of
160 disease during the first infection experiment were i.p. injected in naïve ballan wrasse at a higher
161 dose. To confirm that these isolates were not pathogenic via this exposure route, the number of
162 fish tested was also increased to 15 per isolate, and the length of the experiment was prolonged
163 to 16 days. In addition, an a*As vapA* type VI (isolate TW164/15) recovered from lumpsucker
164 was also included (Table 2).

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165 In the third infection experiment, fish (n= 12) were i.p. injected with medium (10^7 cfu/fish) and
166 high (10^9 cfu/fish) doses of 2x isolates of *Aliivibrio logei* and *Photobacterium indicum* as well
167 2x isolates of a*As vapA* type VI and observed for at least 25 days (Table 2). The isolates used
168 were prepared directly from cryopreserved stocks and had not previously passaged in fish.

169 Moribund fish and mortalities from all experiments were removed from the tanks, their external
170 and internal condition assessed. Head kidney swabs were taken onto solid media for
171 bacteriological assessment. Isolates not recovered, despite being i.p injected into the fish at
172 high doses, were regarded as non-infectious. The bacteria recovered were subcultured to purity,
173 their identities confirmed and cryopreserved at – 80 °C until further use.

174 Additional infection experiments 4 and 5 were also undertaken. These were to better determine
175 both the relevant virulence of the different a*As* isolates *vapA* type V and identify doses that
176 would ideally result in high, but not excessive (50-75% mortality), suitable for use in vaccine
177 efficacy testing. In infection experiment 4, 4x different doses of each pathogen were tested (n=
178 15 fish per dose) with a control treatment (PBS) included. Initial results generated by infection
179 experiment 4 were confirmed in a second set of pre-tests with a longer observation period post
180 injection (4 weeks) without PBS controls (Table 2). The isolates used were passaged (recovered
181 from moribund fish) from infection experiments 1 and 2 (Table 2).

182 For the isolates where the use of lower and higher doses caused a mortality response below and
183 above 50% respectively, the median lethal dose (LD_{50}) was calculated according to [27] to
184 define and compare their virulence at the time point of occurrence. Results obtained from both
185 experiments 4 and 5 were used to select isolates for vaccine testing and determine the doses for
186 the main challenge infection in the vaccine efficacy trial (Table 2). In addition, differences
187 within the a*As vapA* type V isolates TW4/14, TW187/14 and TW3/14 were investigated with
188 macrorestriction analysis using pulsed field gel electrophoresis (PFGE) as described previously
189 [25] with the following modifications. Bacteria were grown on TSA at 15 °C for 72 – 96 h,
190 *SpeI* restriction enzyme (5U per 150 µL, New England Biolabs) was used [26] and the
191 electrophoresis conditions comprised switch times of 2 – 6 s at 15 °C and 200 V for 37 h.

192 For all the infection experiments, fish were transferred from a stock tank, anaesthetised with
193 MS-222 (40 ppm; Tricaine methane sulphonate, Sigma) and i.p. injected with 100 µL of the
194 relevant bacterial suspension. Where included, control fish were injected with 100 µL of sterile
195 PBS. Fish were then allocated to respective 30 L aquaria each with water flow of 0.6 – 1.0 L /
196 min, all other parameters remained the same as described above. Fish were observed at least

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197 twice a day for signs of disease for 7-14 days. The pathogens that caused mortalities, were
198 recovered from the diseased fish, purified and stored at -80 °C.

199

200 **Table 2**. Bacterial isolates, number of fish and doses used in the different infection experiments
201 to assess infectivity, pathogenicity and virulence.

Infection Experiment	Number of fish i.p. injected	Days observation post challenge	Bacterial species and isolate	OD_{600} / dilution factor	Dose (cfu/ fish)	No. dead/moribund by termination
Experiment 1	6	6	a*As* type V – TW4/14	1.46	9.5×10^7	6 (100%)
	6	6	a*As* type V – TW187/14	1.52	8.5×10^7	6 (100%)
	6	6	a*As* type V – TW3/14	1.49	1.0×10^8	6 (100%)
	6	6	*Aliivibrio salmonicida* TW322/16	1.51	5.0×10^4	0
	6	6	*Vibrio splendidus* TW130/16	1.45	2.0×10^5	0
	6	6	*Vibrio ichthyoenteri* TW319/16	1.48	1.0×10^9	0
Experiment 2	15	7	*Aliivibrio salmonicida* TW322/16	>2.5	5.0×10^9	2 (13%)
	15	7	*Vibrio splendidus* TW130/16	>2.5	3.5×10^9	0
	15	7	*Vibrio ichthyoenteri* TW319/16	>2.5	8.0×10^9	0
	15	7	Control - 1x PBS	-	-	0
	15	8	a*As* type VI – TW164/15	2	3.0×10^9	15 (100%)
Experiment 3	12	15	a*As* type VI – TW164/15	2	3.0×10^9	12 (100%)
	12	15	a*As* type VI – TW164/15	10^{-2}	3.0×10^7	12 (100%)
	12	15	a*As* type VI – TW184/16	2	1.6×10^9	12 (100%)
	12	15	a*As* type VI – TW184/16	10^{-2}	1.6×10^7	12 (100%)
	12	12	*Photobacterium indicum* TW138/16	1.75	3.2×10^9	12 (100%)
	12	12	*Photobacterium indicum* TW138/16	10^{-2}	3.2×10^7	6 (50%)
	12	12	*Photobacterium indicum* TW181/16	1.89	9.0×10^9	12 (100%)
	12	12	*Photobacterium indicum* TW181/16	10^{-2}	9.0×10^7	8 (66%)
	12	25	*Aliivibrio logei* TW242/16	2	1.5×10^9	0
	12	25	*Aliivibrio logei* TW242/16	10^{-2}	1.5×10^7	0
	12	25	*Aliivibrio logei* TW186/16	2	1.8×10^9	0
	12	25	*Aliivibrio logei* TW186/16	10^{-2}	1.8×10^7	0
Experiment 4	15	16	a*As* type V – TW4/14	1.9	1.0×10^8	15 (100%)
	15	16	a*As* type V – TW4/14	10^{-1}	1.0×10^7	13 (87%)
	15	16	a*As* type V – TW4/14	10^{-2}	1.0×10^6	8 (53%)
	15	16	a*As* type V – TW4/14	10^{-4}	1.0×10^4	0
	15	16	Control - 1x PBS	-	-	0
	15	19	a*As* type VI – TW164/15	2	2.5×10^8	1 (7%)
	15	19	a*As* type VI – TW164/15	10^{-1}	2.5×10^7	0
	15	19	a*As* type VI – TW164/15	10^{-2}	$2.5x 10^6$	0
	15	19	a*As* type VI – TW164/15	10^{-4}	2.5×10^4	0
	15	19	Control - 1x PBS	-	n/a	0
Experiment 5	15	19	a*As* type V – TW4/14	1.95 dil. 10^{-1}	1.0×10^7	15 (100%)
	15	19	a*As* type V – TW4/14	10^{-2}	1.0×10^6	8 (53%)
	15	19	a*As* type V – TW4/14	10^{-3}	1.0×10^5	7 (47%)
	15	19	a*As* type V – TW4/14	10^{-4}	1.0×10^4	1 (7%)
	15	19	a*As* type V – TW4/14	10^{-5}	1.0×10^3	0

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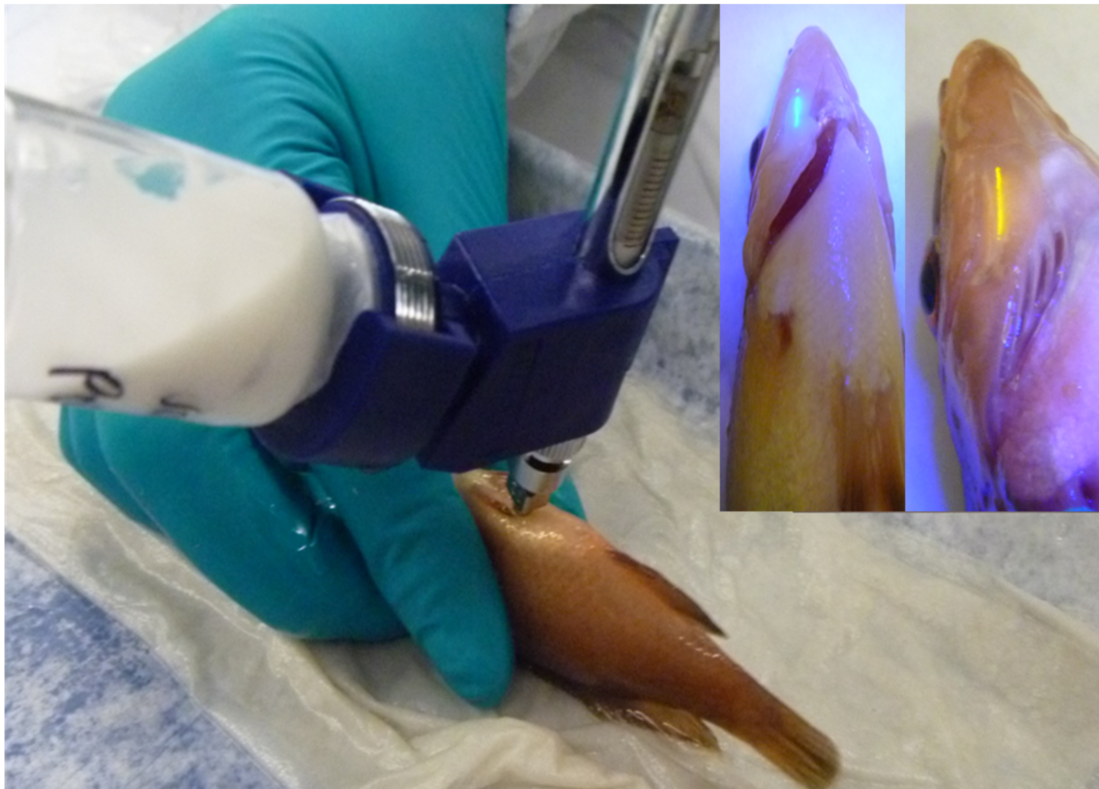
203 **Vaccination**

204 Two groups of 150 fish were tagged and i.p. injected with 0.05 mL of either the test vaccine or
205 sterile PBS (control group). For this, fish were randomly transferred from their stock tank with
206 a net into a bucket containing tank water at 12 ± 2 °C. Thereafter, groups of 2-5 fish were
207 transferred at a time to a further bucket with MS-222 for anaesthesia and tagging. On a clean
208 worktable each fish was marked using the Visible Implant Elastomer tagging system (VIE,
209 Northwest marine technology, Inc). Mark colour was determined as orange for mock
210 vaccinated and blue for the vaccinated fish (Figure 1).



211

212 **Figure 1.** Intraperitoneal injection vaccination of ballan wrasse with oil-adjuvanted autogenous
213 vaccine. Inset: wrasse tagged with Visible Implant Elastomer tagging system. Blue for the
214 vaccinated fish and orange for mock vaccinated.

215 Immediately after tagging each fish was injected with the appropriate treatment using an
216 automatic gun for the group of vaccinates and a sterile syringe for the mock vaccinates. For
217 this, fish were i.p. vaccinated through the ventral wall of the coelomic cavity, one pelvic fin
218 length anterior to the pelvic girdle and transferred directly into their holding tank at 12 ± 0.5
219 °C to recover (Figure 1). Vaccinated fish were then divided into 4 tanks (300 L with artificial
220 plastic kelp and sections of plastic pipes to provide hides to the fish), 2 containing 75 fish
221 vaccinated fish each and 2 tanks containing 75 mock vaccinated fish each.

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222 Fish were held for 65 days at 12 ± 0.5 °C (780 DD) and blood samples collected from the
223 caudal vein on days 31 and 65 post vaccination (prior to challenge) from 15 fish of each tank.
224 The blood samples were centrifuged immediately after collection at 3,000 x g for 10 min and
225 serum kept at -20 °C until used for serological analyses.

226 **Vaccine efficacy testing**

227 After the immunisation period was completed, vaccinated and control fish were challenged
228 with the two most virulent strains *i.e.* TW4/14 (a*As vapA* type V) and TW3/14 (a*As vapA* type
229 V) using a tag and mix model with two different doses (pseudo replicate tanks), here referred
230 as medium and high for isolate TW4/14 and high and very high for isolate TW3/14 as detailed
231 in Table 3.

Table 3. Experimental design of the vaccine efficacy trial and relative percent survival (RPS) results.

Species and Isolate	Tank and (n)	Dose type	cfu/fish	RPS (%)
a*As vapA* type V (TW4/14)	T03-10; 90 fish (45v +45c)	Medium	1.0×10^6	95
a*As vapA* type V (TW4/14)	T03-09; 90 fish (45v +45c)	High	1.0×10^7	91
a*As vapA* type V (TW3/14)	T03-08; 90 fish (45v +45c)	High	1.0×10^7	79
a*As vapA* type V (TW3/14)	T03-07; 90 fish (45v +45c)	Very high	1.0×10^8	20

V= vaccinated; C= control

232

233 **Infection and vaccination experiments: observations and sampling**

234 For all the infection experiments, fish were observed at least twice a day. Diseased fish were
235 classified as moribund or near moribund (humane endpoints) based on clinical signs (typically
236 extreme lethargy when approached with a hand net). They were then euthanised by overdose
237 of anaesthetic followed by confirmation of death by brain destruction, a UK Animals
238 (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039) Schedule 1 approved
239 method (S1-M). All euthanised and dead fish were recorded throughout the experiments and
240 accounted for posterior statistical analyses. To confirm specific mortalities, all moribund fish
241 were necropsied, checked for gross pathology and sampled for bacteriology and histopathology
242 as previously described. The challenge experiments were typically concluded when there was
243 a period of at least five days with no mortalities. At the end of the vaccine efficacy trial, all
244 surviving fish were killed by S1-M and blood sampled and processed as described before to
245 measure specific antibody levels in the serum by ELISA.

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246 All the experimental infections and vaccine efficacy tests were performed at 15 °C. Water
247 temperatures were gradually increased over an acclimation period of 5-7 days prior to
248 challenge.

249 **Specific IgM response**

250 An indirect enzyme-linked immunosorbent assay (ELISA) was developed to detect and
251 estimate the levels of specific anti-a*As* IgM in the ballan wrasse sera pre-vaccination and when
252 the immunisation period was completed. Six samples including 2 replicates from each group
253 were used for assessment of specific antibody responses by ELISA.

254 Antibody titres were determined according to the protocols outlined by [28] with modifications.
255 Briefly, 96 – well ELISA plates (Immulon 4HBX, Thermo Scientific) were coated with 50 µL
256 of 0.05% w/v poly – L– lysine in carbonate – bicarbonate buffer (0.05 M carbonate-bicarbonate
257 pH 7.4, Sigma-Aldrich, St.Louis, UK) and incubated for 60 min at room temperature (RT).
258 Plates were then washed 2 times with a low salt wash buffer (LSWB) (0.02 M Trizma base,
259 0.38 M NaCl, 0.05% Tween-20, pH 7.3). Bacteria i.e. a*As* type V isolate TW4/14, 100 µL at
260 10^8 cfu/mL (OD_{600} 1.0), were then added to each well and plates were incubated overnight at
261 4 °C. The bacteria were previously prepared by growing them on TSB at 22 °C for 48 h with
262 continuous shaking at 150 rpm and washed 2 times with PBS, resuspended and adjusted to an
263 OD_{600} 1.0 prior to 96 – well plates inoculation. Glutaraldehyde (50 µL, 0.05% (v/v)) diluted in
264 PBS was added to the wells of the ELISA plate to fix the antigen, incubated 20 min at RT and
265 plates were washed 3 times with LSWB.

266 The plates were then post-coated with 3% w/v casein in distilled water (250 µL) to block non-
267 specific binding sites and incubated for 180 min at RT. The supernatant was decanted and
268 plates were stored at -20 °C for up to 3 weeks. LSWB was used to wash the plate 3 times and
269 100 µL of hydrogen peroxide (H_2O_2; 0.3% of 10% stock solution in 10% methanol) was added
270 to each well to quench endogenous peroxidase activity of the bacteria and incubated for 30 min
271 at RT.

272 Diluted serum (100 µL per well; from 1:50 to 1:800) in 0.5% casein (w/v) and in PBS, were
273 added to the plates and incubated for 1 h at RT. Plates were washed 5 times with high salt
274 wash buffer (HSWB) (0.02 M Trizma base, 0.5 M NaCl, 0.1% Tween-20, pH 7.4) and were
275 incubated with the last HSWB wash for 5 min at RT.

276 Anti – Asian sea bass IgM MAbs (ADL, Stirling, UK) (shown to cross react with ballan wrasse
277 IgM) diluted 1/50 with 0.01% Bovine Serum Albumin (BSA) in PBS was then added to each

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278 well (100 µL), and incubated for 1 h at RT. The plates were then washed 5 times in HSWB as
279 described above. Goat anti – mouse – horseradish peroxidase (HRP) conjugate (Sigma-Aldrich,
280 UK) diluted 1/4000 with 0.01% BSA in LSWB was then added to the plates. Chromogen in
281 substrate buffer (prepared by adding 150 µL of chromogen 42 mM trimethyl-benzidine, TMB
282 to 15 mL of substrate buffer containing 5 µL H_2O_2 in 6 mL of 50% acetic acid) was then added
283 (100 µL / well) for assay development.

284 The plates were incubated for 3 – 5 min at RT and the reaction stopped by adding 50µL sulfuric
285 acid (2 M H_2SO_4). The absorbance was measured at OD_{450} using a 96 – well plate
286 spectrophotometer (Biotek Instruments, Friedrichshall, Germany). The sensitivity threshold of
287 the assay was determined as 3x the absorbance value of wells containing PBS (background
288 absorbance). Samples above this value were considered positive for specific antibodies.

289 **Statistical analyses**

290 The efficacy/potency of the vaccine was assessed by calculating the relative percent survival
291 (RPS) which indicates the proportional percentage between the cumulative (cm) morbidities
292 of vaccinated group and cumulative morbidities of mock vaccinated group using the equation
293 below [29].

294
$$RPS = \left[1 - \left(\frac{\%\ mortality\ in\ vaccinated\ fish}{\%\ mortality\ in\ non\text{–}vaccinated\ fish}\right)\right] \times 100\ \%$$

295 Minitab 18 was used to produce Kaplan – Meier survival curves and perform log-rank non-
296 parametric tests (significance level $p < 0.05$) for survival comparisons. Antibody responses in
297 serum samples of vaccinated and non – vaccinated ballan wrasse were tested for normality
298 (Anderson-Darling test) and homogeneity of variance (Levene's test). Kruskal-Wallis non –
299 parametric test was used for dose response assessment in relation to antibody titres while a
300 pairwise comparison (Mann Whitney-U test (CI = 95%) was conducted between the antibody
301 responses.

302 **Ethical statement**

303 Bacterial infection and vaccination procedures were performed under the authority of UK
304 Government Home Office project licences, following approval by the Animal Welfare and
305 Ethical Review Body (AWERB) at the Centre for Environment, Fisheries and Aquaculture
306 Science (Cefas) and University of Stirling. Ballan wrasse were treated in accordance with the
307 Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039).

308

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309 **RESULTS**

310 **Infection experiments**

311 In the first two infection experiments, injection with high doses of a*As* type V isolates TW3/14,
312 TW4/14 and TW187/14 and type VI isolate TW164/15 resulted in 100% moribundity/mortality
313 by 7 days post challenge (Table 2). Clinical signs were first recorded at 4 days post infection
314 (dpi) for both *vapA* types and 100% morbidities achieved by 4 and 8 dpi, for *vapA* types V and
315 VI respectively. In all experiments, the a*As* isolates were recovered from moribund fish as pure
316 cultures (punctate whitish to greyish colonies) from swabbed internal organs. The a*As* type VI
317 isolate tested produced a diffusible pigment, (brown on TSA and grey on BA) that became
318 more evident after five days incubation (Supplementary File1). A representative isolate from
319 each strain was stored at -80 °C under Cefas bacterial culture collection codes 17032, 17033
320 and 17034 after being in vivo passaged in fish.

321 The infections performed with *Aliivibrio salmonicida*, *Allivibrio logei*, *Vibrio splendidus* and
322 *Vibrio ichtyoenteri* did not cause any sign of disease or mortalities after 7 dpi in infection
323 experiment 1 (Table 2). In infection experiment 2, only *Aliivibrio salmonicida* caused 2
324 mortalities (13%) on day 3 and the other three were not pathogenic.

325 In infection experiment 3 (Table 2), fish infected with a medium dose of a*As vapA* type VI
326 isolates TW184/16 ($1.6x10^7$ cfu/fish) and TW164/15 ($3x10^7$ cfu/fish) resulted in mortalities of
327 25% and 33% respectively, while a high dose (10^9 cfu/fish) caused 100% mortalities for both
328 isolates. The two a*As* type VI isolates presented brown pigment as described before. Signs of
329 disease presented more rapidly for isolate TW184/16 for medium (6 dpi) and high (2 dpi) dose
330 but similar to those of TW164/15 (7 and 3 dpi, respectively) (Supplementary File 2). Morbid
331 fish showed some signs of reduced appetite often followed by imbalance, lethargy and full loss
332 of equilibrium. Gross external pathology included ascites, and occasionally haemorrhaging at
333 the injection site, internally liquefaction of organs and white deposits in the peritoneum
334 (Supplementary File 3). More liquefaction was noted with TW184/16 than TW164/16.
335 Interestingly the bacterium was not isolated from any survivor fish challenged with medium
336 dose at termination on 14 (TW184/16) and 16 (TW164/15) dpi and there were no any obvious
337 external or internal signs of disease in them.

338 The *Photobacterium indicum* isolates TW138/16 and TW181/16 both caused 11 (92%)
339 overnight mortalities when administered at high doses of $3.2x10^9$ and $9x10^9$ cfu/fish
340 respectively. The remaining fish injected with a high dose of TW138/16 was removed on day
341 3 post infection while the last fish injected with TW181/16 were euthanised on welfare grounds

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342 at day 6 post infection (Supplementary File 4). For the *Photobacterium indicum* challenges
343 with medium doses ($3.2x10^7$ and $9x10^7$ cfu/fish), of isolates TW181/16 and TW138/16 resulted
344 in 8/12 and 6/12 mortalities respectively by day 3 post challenge (Supplementary File 4). The
345 remaining fish (n= 4) in the tank challenged with TW181/16 were terminated at day 9 post
346 infection as no morbidities occurred for 3 days and all presented lesions at the injection site
347 during the daily observations. In the tank infected with medium dose of isolate TW138/16 a
348 single dead fish with a large lesion around the injection site was removed on day 9, while
349 monitoring of the remaining fish (n= 6) continued. On day 16 post infection all surviving fish
350 (n=6) presented severe ventral lesions at the i.p. injection site (Supplementary File 5) and some
351 of these lesions extended into the cavity and for this reason the experiment was terminated.
352 Morbid fish infected with *Photobacterium indicum* showed a reduced feeding response often
353 followed by imbalance, lethargy and full loss of equilibrium with a very rapid progression (<
354 24 h) of the signs. During the necropsies, the majority of the fish had ascites, liquefaction of
355 organs and swelling coelomic cavity due to ascites. Internally, haemorrhages or lesions were
356 noted and the severity of these progressed over time.

357 Additional testing of isolate a*As* type V (TW4/14) in infection experiments 4 and 5 confirmed
358 this organism was virulent. Morbidities were recorded within 2 dpi with the high dose (10^8
359 cfu/fish) and 100% mortalities were reached by day 4. A dose of 10^7 cfu/fish reached a
360 maximum of 87% mortality by day 6 post infection, while no signs of disease or morbidities
361 were noted for fish challenged with the lowest dose (10^4 cfu/fish) (Supplementary File 6).
362 Similar results were obtained in the second pre-test, with 53% for group exposed to 10^6 cfu/fish
363 and only 7% in the group exposed to 10^4 cfu/fish. (Supplementary File 7). The predicted LD_{60}
364 based on these experiments was between 10^6 cfu/fish (53%) and 10^7 cfu/fish (87%), with 10^7
365 cfu/fish selected as one of the doses for the vaccine efficacy trials.

366 For the additional testing undertaken with a*As* type VI (TW164/15) in infection experiment 4,
367 only a single morbidity occurred at 3 dpi, while the rest of the fish showed no visual signs of
368 disease or adverse behaviour. The trial was terminated at 20 dpi and fish (n= 6) sampled for
369 bacteriology. All inoculated plates were considered negative as no significant bacterial colonies
370 were observed. For these reasons this isolate was not used for the vaccine testing and a
371 replacement isolates was selected as described below.

372 **Virulence determination**

373 LD_{50} values for the different isolates by day 8 post infection were calculated based on results
374 from all the infection experiments. Atypical *Aeromonas salmonicida vapA* type V isolate

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375 TW4/14 was the most virulent followed by a*As vapA* type V isolate TW3/14, *Photobacterium*
376 *indicum* and a*As vapA* type VI. The a*As vapA* type V isolates (TW3/14, TW4/14 and
377 TW187/14) were chosen for macrorestriction analysis using PFGE to select a replacement
378 isolate for a*As vapA* type VI (TW164/15) which was not virulent during experiment 3.
379 Differences were observed in the restriction sites for isolate TW3/14 in comparison to TW4/14
380 and TW187/14 (Figure 2) which may explain the differences in virulence mentioned above.
381 The *Aliivibrio logei*, *Vibrio splendidus*, *Aliivibrio salmonicida* and *Vibrio ichtyoenteri* were
382 not pathogenic. The average of the 3 estimations for a*As vapA* type V (TW4/14) was $3.6x10^6$
383 cfu/fish. The average of the 2 estimations for *Photobacterium indicum* was $2.2x10^7$ cfu/fish. A
384 comparison of all the LD_{50} values of the different isolates is presented in Table 4.

385

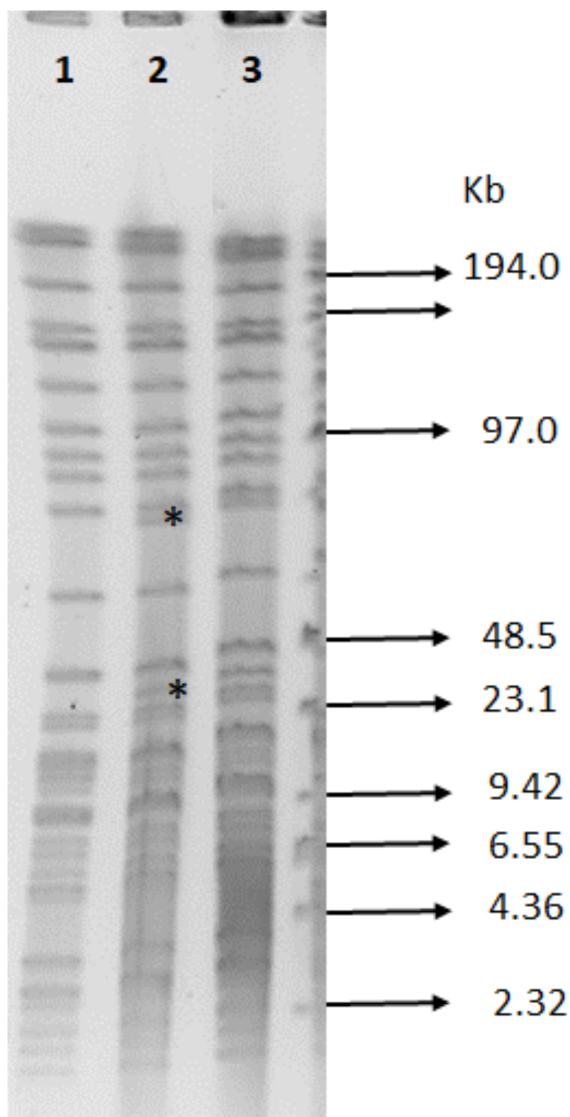

Figure 2. Pulsed-filed gel electrophoresis patterns of *Aeromonas salmonicida vapA* type V isolates TW4/14, TW 187/14 and TW 3/14 restricted with *SpeI* enzyme (New England Labs, UK). From left to right, TW 3/14 (position 1), TW 4/14 (position 2) and TW 187/14 (position 3). Molecular marker mixture of lambda DNA-Hind III fragments and lambda concatamer; 48±5 kb (Low Range PFG Marker, New England Labs, UK). Notice the difference between pulsotype profiles for isolates TW 3/14 and TW 4/14 and TW 187/14 (asterisk).

386

387

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Table 4. Lethal dose 50% (LD_{50}) of 6 bacterial species used in the trials by day 8 post infection.

Bacterial species	Isolate	LD_{50} (cfu/fish)
Atypical *Aeromonas salmonicida* type V	TW4/14*	2.0×10^5
Atypical *Aeromonas salmonicida* type V	TW4/14	2.8×10^6
Atypical *Aeromonas salmonicida* type V	TW4/14**	6.1×10^6
Atypical *Aeromonas salmonicida* type V	TW3/14**	1.6×10^7
Photobacterium indicum	TW181/16	$<3.2 \times 10^7$
Photobacterium indicum	TW138/16	1.3×10^8
Atypical *Aeromonas salmonicida* type VI	TW184/16	3.4×10^8
Atypical *Aeromonas salmonicida* type VI	TW164/15	5.3×10^8
Aliivibrio logei	TW242/16	$>1.5 \times 10^9$
Aliivibrio logei	TW186/16	$>1.8 \times 10^9$
Vibrio splendidus	TW130/16	$>3.0 \times 10^9$
Aliivibrio salmonicida	TW322/16	$>5.0 \times 10^9$
Vibrio ichthyoenteri	TW319/16	$>8.0 \times 10^9$

(*) passaged isolate used in dose trials; (**) data from mock vaccinated.

389 **Vaccine efficacy**

390 Significant protection was demonstrated with vaccinated fish experiencing significantly lower
391 mortalities than control fish when challenged with a*As* type V from isolates TW4/14 and
392 TW3/14. First morbidities were recorded at 5 dpi (TW3/14) and 6 dpi (TW4/14) in the mock-
393 vaccinated groups, and 7 dpi (TW3/14) and 15 dpi (TW4/14) in the vaccinated groups (Figure
394 3A, B).

395 Mortalities were significantly higher for mock-vaccinated fish challenged with either of the
396 two isolates over a period of 24 days (Figure 3A, B). Isolate TW3/14 at a very high dose of
397 1×10^8 cfu/fish resulted in 96% mortality relative to the control fish and 34% mortality relative
398 to the vaccinated group. With the same isolate, a dose of 10^7 cfu/fish caused 84% mortality
399 relative to the control groups and only 18% relative to the vaccinated group. Isolate TW4/14,
400 at a high dose of 10^7 cfu/fish, caused 100% mortalities relative to the control and only 9%
401 relative to the vaccinated group while a medium dose of 10^6 cfu/fish caused 44% in the control
402 groups and only 2% to the vaccinated fish.

403 The RPS values in the ballan wrasse challenged with isolate TW4/14 at medium and high doses
404 were 95% (10^6 cfu/fish) and 91% (10^7 cfu/fish), respectively. The group exposed to strain
405 TW3/14 had an RPS of 79% with the high dose of 10^7 cfu/fish but a low RPS (20%) was
406 recorded for the group injected with the very high challenge dose (10^8 cfu/fish) (Table 4).
407 Despite the low RPS, the survival of vaccinated fish in this group was still significantly higher
408 when compared with the mock vaccinated group (Figure 3B).

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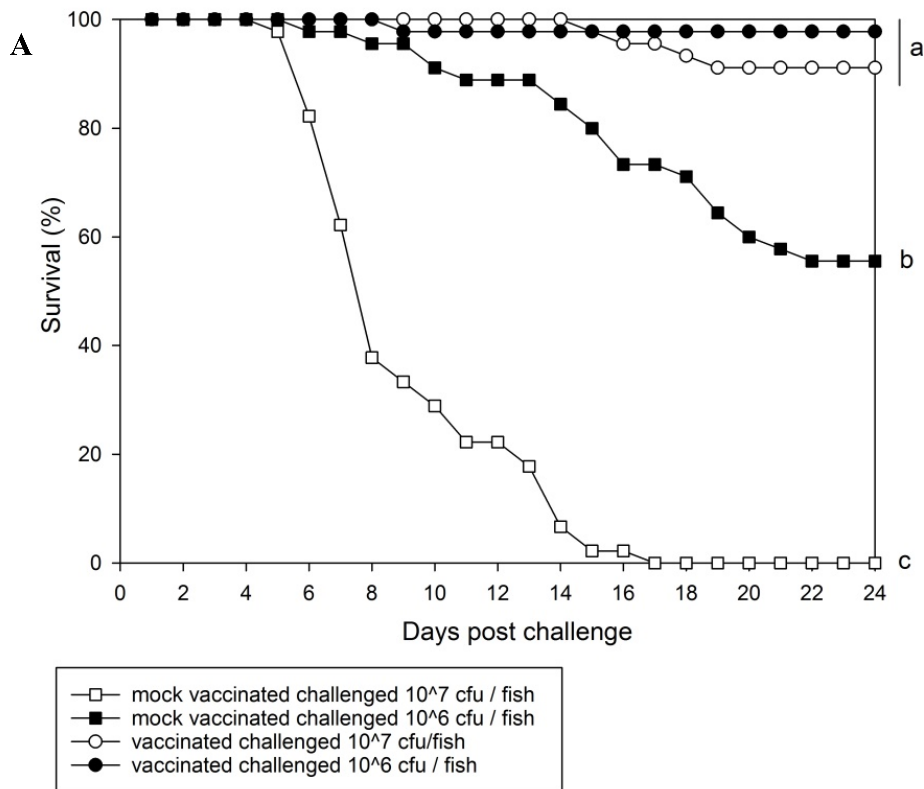

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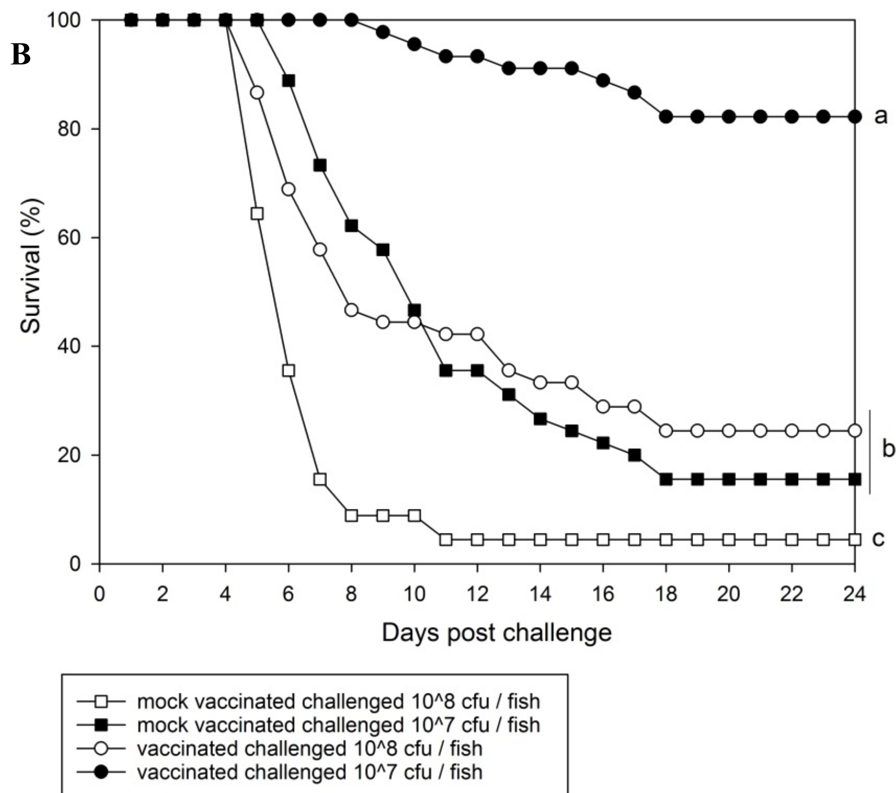

410

411 **Figure 3.** Survival (%) of i.p. injected vaccinated and non – vaccinated ballan wrasse
412 challenged with A) a*As* type V (isolate TW3/14) at high and very high doses and B) a*As* type
413 V (isolate TW4/14) at medium and high doses. Letters represents statistical significance ($p <$
414 0.05).

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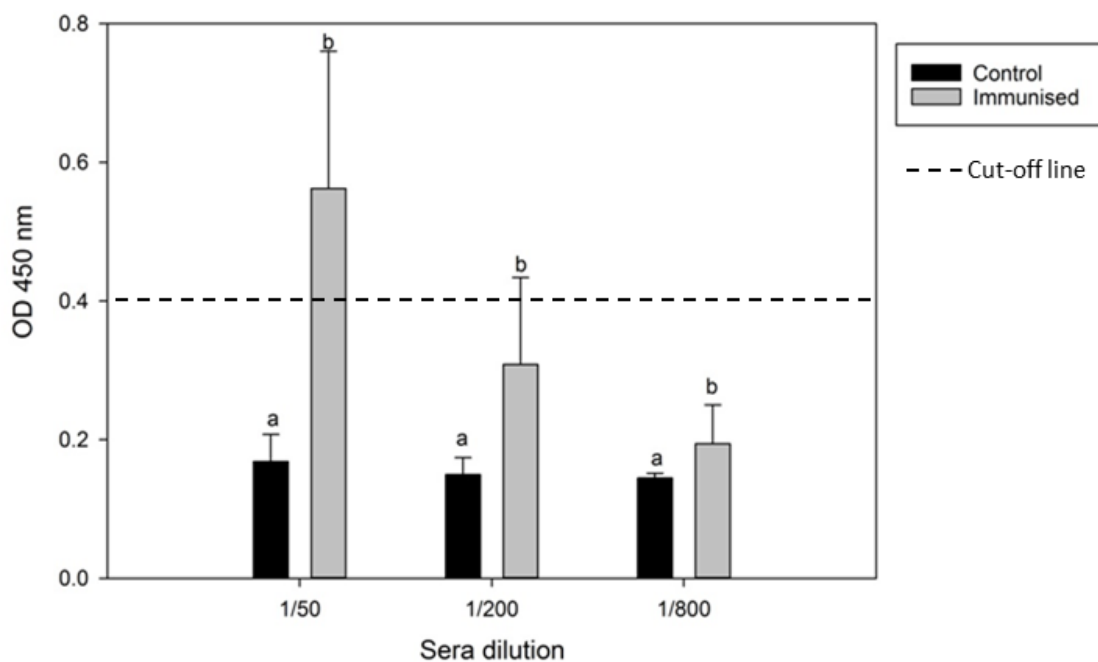
415 **Specific IgM response**

416 Non – specific binding was observed in the preliminary results (Supplementary File 8). This
417 was reduced when plates were treated with 0.3% hydrogen peroxide to quench endogenous
418 peroxidase activity of the bacteria and when 0.5% casein and 0.01% BSA were included in the
419 fish serum and Anti – Asian sea bass IgM MAbs, respectively.

420 A very weak antibody response was noted for serum samples collected from mock-vaccinated
421 fish (controls) prior to challenge and these were considered negative. The vaccinated fish had
422 significantly higher mean antibody titres at all sera dilutions in contrast to mock-vaccinated
423 fish (Figure 4).



424

425 **Figure 5.** Ballan wrasse specific antibody (IgM) response to a*As* pre-vaccination (control, n=
426 6 samples x 2 replicates) and after immunisation period was completed (780 DD after i.p.
427 vaccination, n= 6 replicates x 2 replicates). Letters represents statistical significance ($p < 0.05$).

428 **DISCUSSION**

429 In the present study, the virulence of a*As* type V and VI, *Aliivibrio logei*, *Aliivibrio*
430 *salmonicida*, *Vibrio splendidus*, *Vibrio ichthyoenteri* and *Photobacterium indicum* were
431 assessed. The results obtained confirmed that a*As vapA* type V was the most pathogenic of all
432 the bacterial species (followed by *Photobacterium indicum*, a*As* VI and the rest of the
433 *Vibrionaceae*). Importantly, the vaccine tested was highly protective against a*As* type V and
434 significantly higher titres of specific systemic IgM were detected in vaccinated fish when
435 compared to controls.

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436 The virulence studies confirmed that a*As vapA* type V (from both isolates tested) were highly
437 virulent in ballan wrasse when i.p. injected. The RPS obtained with medium and high doses
438 for *vapA* type V from isolate TW4/14 (95% and 91%, respectively) and high dose of *vapA* type
439 V from isolate TW3/14 (79%) strains, confirmed the effectiveness of the injection vaccine
440 against homologous strains of a*As* and were in agreement with previous results conforming
441 that ballan wrasse can be effectively immunised by i.p. injection against this pathogen [17].
442 When vaccinated ballan wrasse were challenged with a very high dose (10^8 cfu/fish) of the
443 strain TW3/14 RPS was only of 20% suggesting that high challenge dose may have suppressed
444 or overwhelmed protective memory responses. This highlights the relevance of biosecurity and
445 good farming practices to maintain the pathogens challenge pressure as low as possible during
446 the production cycle.

447 A specific antibody response (IgM) to the vaccine was measured in immunised fish at 780 DD
448 which was significantly higher compared to non-vaccinated fish. The high RPS levels in
449 vaccinated fish and specific antibody response following vaccination are indicators that the
450 vaccine indeed triggered a specific protective humoral response against a*As*. Similar responses
451 have been induced in other species immunised with typical or atypical stains of *As,* such as
452 lumpsucker [30, 31], Atlantic salmon [32, 33], rainbow trout [34] and spotted wolfish [35]. A
453 high quantity of cytoplasmic peroxidases (*e.g.* thiol peroxidase) have previously been reported
454 in *A. salmonicida* cells [36] and high antigen endogenous peroxidase activity appeared to cause
455 substantial background during ELISA development. This background was quenched using
456 hydrogen peroxide prior to antibody-antigen complexing. However, a high absorbance
457 threshold of the ELISA could not be avoided using our cut-off criteria (3x background OD =
458 0.4), thus a 1/50 test titre was the most preferable to use to determine positive antibody
459 responses to a*As* vaccination. Nonetheless, the titre of antibodies was consistently higher in
460 vaccinated fish up to and including a dilution of 1/800. These results suggested that antibodies
461 might be involved in protection against a*As*.

462 Interestingly, differences in virulence were observed for two atypical *Aeromonas salmonicida*
463 *vapA* type V isolates (TW3/14 and TW4/14), with the latter being the most virulent.
464 Microrestriction and PFGE analysis corroborated these results, indicating small but potentially
465 important genomic differences between isolates. Atypical *Aeromonas salmonicida* isolates
466 heterogeneity has been previously assessed with the same method [26]. Characterisation of all
467 the available a*As vapA* types for ballan wrasse by PFGE will be beneficial to select isolates for
468 future vaccine formulations.

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469 The *vapA* VI isolates appeared less virulent than a*As* type V and similar results were reported
470 for Norwegian a*As* type V and VI isolates [17]. In that study a*As* type V induced high
471 mortalities (75 – 89% morbidities) in 50 g ballan wrasse when i.p. injected with 10^7 cfu /fish
472 and also by cohabitation (51%). The type VI isolates were less virulent, in particular by
473 cohabitation (8%) than i.p. injection (70 – 85%). Interestingly, in the present study, survivor
474 fish infected with a*As* type VI at a medium dose (10^7 cfu / fish) did not show any obvious
475 external or internal signs of the disease and no bacteria were recovered from those fish
476 suggesting that ballan wrasse were able to clear the infection. This is in agreement with a
477 previous study that reported similar responses in survivors from groups infected with a*As* type
478 V and VI [17].

479 The *Aliivibrio logei*, *Vibrio splendidus* and *Vibrio ichthyoenteri* isolates were not pathogenic
480 to ballan wrasse by i.p. injection even when very high challenge doses were administered.
481 *Aliivibrio salmonicida* was the only pathogen that caused mortalities (13%) but only when very
482 high infection dose of 5 x 10^9 cfu/fish was administered.

483 The a*As vapA* type VI isolates occasionally displayed a peculiar alternative morphology that
484 included the presence of large greyish and small transparent colonies (Supplementary File 9).
485 Previous reports have documented this phenomenon associated with variable expression of a
486 functional A-layer and consequently with variable virulence [22, 37-41]. Although in the
487 present study, the inclusion of isolates displaying such alternative morphology was generally
488 avoided, this should not be ruled out as a possible explanation behind the lack of virulence
489 observed in this experiment.

490 In previous reports of experimental infections with a*As* type VI isolates in cleaner fish,
491 lumpsucker succumbed to disease at lower doses of 2 x10^3 cfu/mL (bath) and 4 x 10^4 cfu /fish
492 (i.p. injection) after exposure [18]. Other fish species like spotted wolfish also experienced
493 high mortalities with low doses of a*As* (10^3 and 10^4 cfu / mL) by i.p. injection [8, 15]. In
494 contrast, turbot required very high doses (10^8 and 10^{10} cfu / mL) for mortalities to be induced
495 by i.p. injection [16]. As suggested previously, there is a strong association between host
496 species and *vapA* type and it is possible that *vapA* type V is more strongly associated with
497 wrasse than lumpfish and vice versa for type IV [42].

498 This study is the first experimental confirmation that *Photobacterium indicum* can be
499 pathogenic towards wrasse, through fulfilment of Koch's postulates. *Photobacterium indicum*
500 was regularly isolated from diseased ballan wrasse during disease surveys in Scotland and it
501 was linked to histopathological lesions [4]. There are no previous reports on fish susceptibility

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502 to *Photobacterium indicum* although it has been isolated from moribund American lobster
503 (*Homarus americanus*) associated with stress and has been reported as an opportunistic
504 pathogen for this crustacean species [43, 44]. In cleaner fish, *Photobacterium* sp. was recently
505 recovered from lumpsucker experiencing mortalities due to *Pseudomonas anguilliseptica*
506 under rearing conditions in Scotland [45]. The pathogenicity results for *Photobacterium*
507 *indicum* obtained in the present study need to be treated with caution as disease was induced
508 only via i.p. injection which bypasses the natural protective mucosal barriers of the host *e.g.*
509 skin, gills and gastrointestinal tract [46, 47]. Signs of disease and gross pathology for
510 *Photobacterium indicum* were similar to those seen with a*As* with moribund fish showing
511 reduced feeding response often followed by imbalance, lethargy and full loss of equilibrium.
512 The peritoneal cavity of the diseased fish was extended (ascites) and internally, liquefaction
513 was observed in all the organs. Granulomatous formations were seen in livers of moribund fish
514 infected with a*As* which concurred with previous reports [17]. This needs to be considered
515 when performing differential diagnosis based on gross pathology and clinical signs. Ventral
516 lesions at the i.p. injection site were observed on survivor fish, which may be related to
517 localised immune responses at the injection site [48, 49].

518 **CONCLUSIONS**

519 This study developed an i.p. injection challenge model was for ballan wrasse against Scottish
520 a*As vapA* type V isolates and this was used to test the efficacy of an injectable autogenous
521 multivalent vaccine. The vaccination results obtained in the current study are very encouraging
522 as they confirmed i.p vaccination can be used as means to control and potentially eliminate
523 morbidities in ballan wrasse hatcheries and cage sites due to a*As vapA* type V and likely other
524 *vapA* types. The vaccine tested was highly protective against medium and high challenge doses
525 of a*As* type V from two different isolates (RPS 79-95%) and significantly higher titres of
526 specific systemic IgM were detected in vaccinated fish when compared to controls. In addition,
527 this study provided important new data on the pathogenicity and virulence of routinely
528 recovered bacterial species from diseased ballan wrasse. The pathogenicity and virulence of
529 *Photobacterium indicum* to ballan wrasse is reported for the first time. Atypical *Aeromonas*
530 *salmonicida* type V isolate TW4/14 was the most virulent pathogen followed by a*As* type V
531 isolate TW3/14, *Photobacterium indicum* and atypical a*As* type VI (including isolate from
532 lumpsucker). The *Vibrio* spp. and *Aliivibrio* spp. were not pathogenic by i.p. injection to the
533 ballan wrasse population tested. Further work is needed to assess the efficacy of vaccination
534 against other isolates that have proven to be pathogenic such as a*As* type VI and
535 *Photobacterium indicum* and to explore the feasibility of immersion vaccination strategies as

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536 the species encounters the pathogens at earlier life stage (< 25 g) and this immunisation route
537 is desirable for juvenile ballan wrasse in the hatcheries. In addition, full characterisation should
538 be performed on a*As* isolates within the same *vapA* types.

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547 **CONFLICTS OF INTEREST**

548 The authors declare no conflicts of interests

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