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

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ABSTRACT

Atypical *Aeromonas salmonicida* (a*As*) and *Vibrionaceae* related species are bacteria routinely recovered from diseased ballan wrasse used as cleaner fish in Atlantic salmon farming. Autogenous (i.e. farm specific inactivated) multivalent vaccines formulated from these microorganisms are widely used by the industry to protect farmed wrasse despite limited experimental proof that they are primary pathogens. In this study, the components of a commercial multivalent injection vaccine containing four strains of *Aeromonas salmonicida* and one strain of *Vibrio splendidus* previously isolated from ballan wrasse in Scotland, were tested for infectivity, pathogenicity and virulence via intra peritoneal injection at pre-deployment size (25-50g) and the efficacy of the vaccine for protection against a*As* assessed. Injection with $3.5x10^9$, $8x10^9$ $1.8x10^9$ and $5x10^9$ cfu/fish of *Vibrio splendidus*, *V. ichthyoenteri*, *Aliivibrio logeii* and *A. salmonicida*, respectively, did not cause significant mortalities, lesions or clinical signs after a period of 14 days. IP injection with both a*As* and *Photobacterium indicum* successfully reproduced the clinical signs and internal lesions observed during natural outbreaks of the disease. Differences in virulence (LD_{50} at day 8-post infection of $3.6x10^6$ cfu/fish and $1.6x10^7$ cfu/fish) were observed for two a*As vapA* type V isolates. In addition, the LD_{50} for *Photobacterium indicum* was $2.2x10^7$ cfu/fish. The autogenous vaccine was highly protective against the two a*As vapA* type V isolates after 700-degree days of immunisation. The RPS_{FINAL} values for the first isolate were 95 and 91% at $1x10^6$ cfu/fish and $1x10^7$ cfu/fish, respectively, and 79% at $1x10^7$ cfu/fish for the second isolate tested. In addition, significantly higher anti a*As* seral antibodies (IgM), were detected by ELISA in vaccinated fish in contrast with control (mock vaccinated) fish. These results suggest wrasse can be effectively immunised and protected against a*As* infection by injection with oil adjuvanted vaccines prepared with inactivated homologous isolates.

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

1- INTRODUCTION

Sea lice, particularly *Lepeophtheirus salmonis* (Krøyer, 1837) have caused major economic losses to the Atlantic salmon industry. Parasitic infections have commonly been controlled with chemotherapeutants however alternative control methods were explored due to drug resistance. A successful alternative environmental friendly delousing approach was first tested in the Northern hemisphere in the mid-1990s where wild wrasse were deployed in salmon cages [1] including goldsinny (*Ctenolabrus rupestris*, L.), rock cook (*Centrolabrus exoletus*, L.), cuckoo wrasse (*Labrus mixtus*, L.), corkwing wrasse (*Crenilabrus melops*, L.) and ballan wrasse (*Labrus bergylta*, Ascanius). The latter was identified as the most desirable wrasse species as a cleaner fish for salmon mainly due to their size and delousing efficacy [2]. Ballan wrasse are distributed in-shore in rocky and algal (*e.g.* kelps) habitats [3] and they feed mainly on crustaceans and bryozoans, molluscs, jellyfish and other invertebrates [3]. Ballan wrasse are protogynous hermaphrodites, agastric (*i.e.* lacking a stomach) and physoclistic (*i.e.* closed swim bladder) hence they cannot rapidly regulate the gas pressure in the swim bladder [3]. Farmed ballan wrasse are currently being used for delousing salmon in sea cage sites, however, disease outbreaks in hatcheries and post-deployment in salmon pens are critical bottlenecks for the industry.

Bacterial pathogens are considered the major cause of infectious diseases and mortalities in farmed ballan wrasse produced for sea lice control in the salmon farming industry [3-5]. In Scotland, atypical *Aeromonas salmonicida (a*As*) vapA* type V and VI, *Vibrio splendidus*, *V. ichthyoenteri*, *Aliivibrio salmonicida*, *A. logeii*, and *Photobacterium indicum* are the bacterial pathogens most frequently isolated from ballan wrasse during outbreaks of disease in both hatcheries and post deployment in salmon sea sites [6]. Similar reports are available for the species in Norway [5].

77 Immunisation of farmed salmonids (Atlantic salmon and rainbow trout) against typical *As*
78 using fully licenced oil-adjuvanted injectable vaccines has historically proven successful and
79 is a standard practice [7]. However, immunisation of non–salmonid species against typical
80 and atypical *As* has been rather challenging [8-10]. For instance, an experimental vaccine
81 containing atypical strains protected Arctic charr (*Salvelinus alpinus*, L.) but not in European
82 grayling (*Thymallus thymallus*, L.) [11]. Furthermore, commercial furunculosis vaccines for
83 salmonids have induced protection in Atlantic halibut (*Hippoglossus hippoglossus*, L.) but
84 not in Atlantic cod (*Gadus morhua*, L.) or turbot (*Scophthalmus maximus*, L.) [8-10].
85 Currently no licenced or registered vaccines are commercially available in the UK for the
86 prevention and control of infectious diseases in ballan wrasse. Therefore, prophylactic
87 treatments in Scotland are mainly based on the use of autogenous vaccines, which are
88 formulated with antigens derived from pathogens recovered during episodes of elevated
89 mortality [3].
90 Autogenous or "herd specific" vaccines are farm specific immunological veterinary
91 medicinal products that have the potential to be rapidly developed and deployed when no off-
92 the shelf fully licensed vaccines exist or these have proven infective. In principle, autogenous
93 vaccines must be inactivated (killed), manufactured in licenced facilities, used only under
94 veterinary prescription and on the sites where the pathogens were isolated [12, 13].
95 In Scotland, autogenous vaccines for ballan wrasse were first developed from isolates
96 collected during disease outbreaks between 2013 and 2014 (Ridgeway Biologicals Ltd.) and
97 used in hatcheries and wild caught wrasse. The vaccine formulation later evolved and new
98 isolates were introduced following a health screening surveys [6]. However without
99 established challenge model for the Scottish bacteria and wrasse populations, the actual
100 virulence of the isolates and the efficacy/potency of the vaccine components remained
101 unknown.

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

Given that, a*As* and *Vibrionaceae* isolates are highly heterogenic and variable, and virulence is often strain and host dependant [4, 19], the establishment of similar experiments in other geographical areas such as Scotland is of high relevance for the local industry.

The objective of the present study was to develop *in vivo* challenge models via intraperitoneal (i.p.) injection in Scottish ballan wrasse (25-50 g) to investigate the infectivity, pathogenicity and virulence of bacterial isolates routinely recovered from diseased wrasse and used as antigens in commercial autogenous vaccines. These isolates included a*As vapA* types V and VI, *Vibrio splendidus*, *V. ichthyoenteri*, *Aliivibrio salmonicida*, *A. logeii*, and *Photobacterium indicum*. Furthermore, we aimed to assess survival rates in vaccinated and control fish which have been experimentally infected and vaccine potency expressed as relative percent survivals (RPS). Specific antibody (IgM) kinetics were also assessed in vaccinated fish.

2- MATERIALS AND METHODS

2.1 Bacterial identification and genotyping

The bacterial isolates used were recovered from diseased fish at commercial hatcheries and characterised on the basis of phenotypic and genotypic characteristics as part of a previous study [6]. In brief, bacterial DNA was extracted using Genesig® Easy DNA/RNA Extraction Kit (Primerdesign Ltd, Southampton UK) according to the manufacturer's instructions. Species confirmation was performed on the samples by targeting the V3-V4 hypervariable region of the *16S rRNA* gene [20] and the subunit B protein of DNA gyrase (topoisomerase type II) – *gyrB* gene [21]. The *Aeromonas salmonicida* isolates were then genotyped by sequencing the A-layer membrane as described previously [22].

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

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

2.2 Experimental fish

A population of naïve *i.e.* unvaccinated and non-diseased ballan wrasse (30 ± 5 g) was provided by a commercial cleaner fish hatchery on the west coast of Scotland. Prior to the study, the health status of the fish was checked by screening a subset of the population with standard histological, bacteriological and molecular methods to confirm the absence of a*As*

154 [6, 22], amoebic gill disease [23] and *Vibrionaceae* related bacteria [20, 21]. After
155 confirming they were free of these pathogens, fish were transferred to the Centre for
156 Environment, Fisheries and Aquaculture Science (Cefas) Weymouth Laboratory in February
157 2017.
158 Fish were acclimated and quarantined for 3 weeks after arrival in 6 aerated aquaria (approx.
159 900 L, tanks enriched with artificial plastic kelp and sections of plastic pipes to provide hides
160 to the fish) at 12.0 ± 0.5 °C with a 20:4 h light:dark photoperiod, water flow of 4.0 L / min,
161 salinity 34 ± 2 ‰, dissolved oxygen (DO) at 8 ± 0.5 mg / L and pH 7.8 ± 0.4. During this
162 period, fish were further screened for bacteriology (swabs from head kidney plated onto
163 SWA), histopathology (fixed in 10% neutral buffered formalin) and molecular methods as
164 described before. In addition, virology diagnostic tests were performed to discard the
165 presence of notifiable viral diseases as per the protocols in the OIE manual of diagnostic tests
166 for aquatic animals [24].
167
168 **2.3 Vaccine**
169 A commercial (oil-based, inactivated) multivalent autogenous (emergency) vaccine,
170 containing a*As* isolates TW3/14, TW4/14, TW187/14, TW164/15 and *Vibrio splendidus*
171 isolate TW130/16 (Table 1) previously isolated from ballan wrasse [6] was provided by
172 Ridgeway Biologicals Ltd. The vaccine was shipped to Cefas and stored at 4 ± 1 °C prior to
173 use.
174
175 **2.4 Confirmation of infectivity of components of multivalent autogenous vaccine**
176 The virulence of bacterial isolates, representative of strains commonly used as components of
177 the multivalent autogenous vaccines used in the industry, was assessed in a series of infection
178 experiments performed in 30 L tanks enriched as for acclimation tanks (Table 2). For all the
179 infection experiments, fish were transferred from a stock tank, anaesthetised with MS-222

(40 ppm; Tricaine methane sulphonate, Sigma) and i.p. injected with 100 µL (10^4 – 10^9 cfu/fish) of the relevant bacterial suspension. Where included, control fish were injected with 100 µL of sterile PBS. Fish were then allocated to respective 30 L aquaria each with water flow of 0.6 – 1.0 L / min, all other parameters remained the same as described above. Fish were observed at least twice a day for signs of disease for 7-14 days. The pathogens that caused mortalities, were recovered from the diseased fish, purified and stored at -80 °C.

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

In the second infection experiment, the pathogens that did not cause morbidities or signs of disease during the first infection experiment were i.p. injected in naïve ballan wrasse at higher doses ($3 – 8 \times 10^9$ cfu/fish; Table 2). To confirm that these isolates were not pathogenic via this exposure route, the number of fish tested was also increased to 15 per isolate, and the length of the experiment was prolonged to 16 days. In addition, an a*As vapA* type VI (isolate TW164/15) recovered from lumpsucker was also included (Table 2).

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

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

206 Additional infection experiments 4 and 5 were also undertaken. These were to better
207 determine both the relevant virulence of the different a*As* isolates *vapA* type V and identify
208 doses that would ideally result in high, but not excessive (50-75% mortality), suitable for use
209 in vaccine efficacy testing. In infection experiment 4, 4x different doses of each pathogen
210 were tested (n= 15 fish per dose) with a control treatment (PBS) included. Initial results
211 generated by infection experiment 4 were confirmed in a second set of pre-tests with a longer
212 observation period post injection (4 weeks) without PBS controls (Table 2). The isolates used
213 were passaged (recovered from moribund fish) from infection experiments 1 and 2 (Table 2).
214 For the isolates where the use of lower and higher doses caused a mortality response below
215 and above 50% respectively, the median lethal dose (LD_{50}) was calculated according to [25]
216 to define and compare their virulence at the time point of occurrence. Results obtained from
217 both experiments 4 and 5 were used to select isolates for vaccine testing and determine the
218 doses for the main challenge infection in the vaccine efficacy trial (Table 2). In addition,
219 differences within the a*As vapA* type V isolates TW4/14, TW187/14 and TW3/14 were
220 investigated with macrorestriction analysis using pulsed field gel electrophoresis (PFGE) as
221 described previously [26] with the following modifications. Bacteria were grown on TSA at
222 15 °C for 72 – 96 h, *SpeI* restriction enzyme (5U per 150 µL, New England Biolabs) was
223 used [27] and the electrophoresis conditions comprised switch times of 2 – 6 s at 15 °C and
224 200 V for 37 h.
225
226 **2.5 Vaccination**
227 Two groups of 150 fish were tagged and i.p. injected with 0.05 mL of either the test vaccine
228 or sterile PBS (control group / mock vaccinated). For this, fish were randomly transferred
229 from their stock tank with a net into a bucket containing tank water at 12 ± 2 °C. Thereafter,
230 groups of 2-5 fish were transferred at a time to a further bucket with MS-222 for anaesthesia
231 and tagging. On a clean worktable each fish was marked using the Visible Implant Elastomer

tagging system (VIE, Northwest marine technology, Inc). Mark colour was determined as orange for mock vaccinated and blue for the vaccinated fish (Figure 1).

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

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

2.6 Vaccine efficacy testing

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

2.7 Infection and vaccination experiments: observations and sampling

For all the infection experiments, fish were observed at least twice a day. Diseased fish were classified as moribund or near moribund (humane endpoints) based on clinical signs (typically extreme lethargy when approached with a hand net). They were then euthanised by overdose of anaesthetic followed by confirmation of death by brain destruction, a UK

Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039) Schedule 1 approved method (S1-M). All euthanised and dead fish were recorded throughout the experiments and accounted for posterior statistical analyses. To confirm specific mortalities, all moribund fish were necropsied, checked for gross pathology and sampled for bacteriology and histopathology as previously described. The challenge experiments were typically concluded when there was a period of at least five days with no mortalities. At the end of the vaccine efficacy trial, all surviving fish were killed by S1- M and blood sampled and processed as described before to measure specific antibody levels in the serum by ELISA.

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

2.8 Specific IgM response

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

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

284 resuspended and adjusted to an OD_{600} 1.0 prior to 96 – well plates inoculation.
285 Glutaraldehyde (50 µL, 0.05% (v/v)) diluted in PBS was added to the wells of the ELISA
286 plate to fix the antigen, incubated 20 min at RT and plates were washed 3 times with LSWB.
287 The plates were then post-coated with 3% w/v casein in distilled water (250 µL) to block non-
288 specific binding sites and incubated for 180 min at RT. The supernatant was decanted and
289 plates were stored at -20 °C for up to 3 weeks. LSWB was used to wash the plate 3 times and
290 100 µL of hydrogen peroxide (H_2O_2; 0.3% of 10% stock solution in 10% methanol) was
291 added to each well to quench endogenous peroxidase activity of the bacteria and incubated
292 for 30 min at RT.
293 Diluted serum (100 µL per well; from 1:50 to 1:800) in 0.5% casein (w/v) and in PBS, were
294 added to the plates and incubated for 1 h at RT. Plates were washed 5 times with high salt
295 wash buffer (HSWB) (0.02 M Trizma base, 0.5 M NaCl, 0.1% Tween-20, pH 7.4) and were
296 incubated with the last HSWB wash for 5 min at RT.
297 Anti – Asian sea bass IgM MAbs (ADL, Stirling, UK) (shown to cross react with ballan
298 wrasse IgM) diluted 1/50 with 0.01% Bovine Serum Albumin (BSA) in PBS was then added
299 to each well (100 µL), and incubated for 1 h at RT. The plates were then washed 5 times in
300 HSWB as described above. Goat anti – mouse – horseradish peroxidase (HRP) conjugate
301 (Sigma-Aldrich, UK) diluted 1/4000 with 0.01% BSA in LSWB was then added to the plates.
302 Chromogen in substrate buffer (prepared by adding 150 µL of chromogen 42 mM trimethyl-
303 benzidine, TMB to 15 mL of substrate buffer containing 5 µL H_2O_2 in 6 mL of 50% acetic
304 acid) was then added (100 µL / well) for assay development.
305 The plates were incubated for 3 – 5 min at RT and the reaction stopped by adding 50µL
306 sulfuric acid (2 M H_2SO_4). The absorbance was measured at OD_{450} using a 96 – well plate
307 spectrophotometer (Biotek Instruments, Friedrichshall, Germany). The sensitivity threshold
308 of the assay was determined as 3x the absorbance value of wells containing PBS (background
309 absorbance). Samples above this value were considered positive for specific antibodies.

310

311 **2.9 Statistical analyses**

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

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

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

323

324 **2.20 Ethical statement**

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

330

331 **3- RESULTS**

332 **3.1 Infection experiments**

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

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

In infection experiment 3 (Table 2), fish infected with a medium dose of a*As vapA* type VI isolates TW184/16 ($1.6x10^7$ cfu/fish) and TW164/15 ($3x10^7$ cfu/fish) resulted in mortalities of 25% and 33% respectively, while a high dose (10^9 cfu/fish) caused 100% mortalities for both isolates. The two a*As* type VI isolates presented brown pigment as described before. Signs of disease presented more rapidly for isolate TW184/16 for medium (6 dpi) and high (2 dpi) dose but similar to those of TW164/15 (7 and 3 dpi, respectively) (Supplementary File 2). Morbid fish showed some signs of reduced appetite often followed by imbalance, lethargy and full loss of equilibrium. Gross external pathology included ascites, and occasionally haemorrhaging at the injection site, internally liquefaction of organs and white deposits in the peritoneum (Supplementary File 3). More liquefaction was noted with TW184/16 than TW164/16. Interestingly the bacterium was not isolated from any survivor fish challenged

with medium dose at termination on 14 (TW184/16) and 16 (TW164/15) dpi and there were no any obvious external or internal signs of disease in them.

The *Photobacterium indicum* isolates TW138/16 and TW181/16 both caused 11 (92%) overnight mortalities when administered at high doses of $3.2x10^9$ and $9x10^9$ cfu/fish respectively. The remaining fish injected with a high dose of TW138/16 was removed on day 3 post infection while the last fish injected with TW181/16 were euthanised on welfare grounds at day 6 post infection (Supplementary File 4). For the *Photobacterium indicum* challenges with medium doses ($3.2x10^7$ and $9x10^7$ cfu/fish), of isolates TW181/16 and TW138/16 resulted in 8/12 and 6/12 mortalities respectively by day 3 post challenge (Supplementary File 4). The remaining fish (n= 4) in the tank challenged with TW181/16 were terminated at day 9 post infection as no morbidities occurred for 3 days and all presented lesions at the injection site during the daily observations. In the tank infected with medium dose of isolate TW138/16 a single dead fish with a large lesion around the injection site was removed on day 9, while monitoring of the remaining fish (n= 6) continued. On day 16 post infection all surviving fish (n=6) presented severe ventral lesions at the i.p. injection site (Supplementary File 5) and some of these lesions extended into the cavity and for this reason the experiment was terminated. Morbid fish infected with *Photobacterium indicum* showed a reduced feeding response often followed by imbalance, lethargy and full loss of equilibrium with a very rapid progression (< 24 h) of the signs. During the necropsies, the majority of the fish had ascites, liquefaction of organs and swelling coelomic cavity due to ascites. Internally, haemorrhages or lesions were noted and the severity of these progressed over time.

Additional testing of isolate a*As* type V (TW4/14) in infection experiments 4 and 5 confirmed this organism was virulent. Morbidities were recorded within 2 dpi with the high dose (10^8 cfu/fish) and 100% mortalities were reached by day 4. A dose of 10^7 cfu/fish reached a maximum of 87% mortality by day 6 post infection, while no signs of disease or morbidities

were noted for fish challenged with the lowest dose (10^4 cfu/fish) (Supplementary File 6). Similar results were obtained in the second pre-test, with 53% for group exposed to 10^6 cfu/fish and only 7% in the group exposed to 10^4 cfu/fish. (Supplementary File 7). The predicted LD_{60} based on these experiments was between 10^6 cfu/fish (53%) and 10^7 cfu/fish (87%), with 10^7 cfu/fish selected as one of the doses for the vaccine efficacy trials.

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

3.2 Virulence determination

LD_{50} values for the different isolates by day 8 post infection were calculated based on results from all the infection experiments. Atypical *Aeromonas salmonicida vapA* type V isolate TW4/14 was the most virulent followed by a*As vapA* type V isolate TW3/14, *Photobacterium indicum* and a*As vapA* type VI. The a*As vapA* type V isolates (TW3/14, TW4/14 and TW187/14) were chosen for macrorestriction analysis using PFGE to select a replacement isolate for a*As vapA* type VI (TW164/15) which was not virulent during experiment 3. Differences were observed in the restriction sites for isolate TW3/14 in comparison to TW4/14 and TW187/14 (Figure 2) which may explain the differences in virulence mentioned above. The *Aliivibrio logei*, *Vibrio splendidus*, *Aliivibrio salmonicida* and *Vibrio ichtyoenteri* were not pathogenic. The average of the 3 estimations for a*As vapA* type V (TW4/14) was $3.6x10^6$ cfu/fish. The average of the 2 estimations for *Photobacterium indicum* was $2.2x10^7$ cfu/fish. A comparison of all the LD_{50} values of the different isolates is presented in Table 4.

3.3 Vaccine efficacy

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

Mortalities were significantly higher for mock-vaccinated fish challenged with either of the two isolates over a period of 24 days (Figure 3A, B). Isolate TW3/14 at a very high dose of $1x10^8$ cfu/fish resulted in 96% mortality relative to the control fish and 34% mortality relative to the vaccinated group. With the same isolate, a dose of 10^7 cfu/fish caused 84% mortality relative to the control groups and only 18% relative to the vaccinated group. Isolate TW4/14, at a high dose of 10^7 cfu/fish, caused 100% mortalities relative to the control and only 9% relative to the vaccinated group while a medium dose of 10^6 cfu/fish caused 44% in the control groups and only 2% to the vaccinated fish.

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

3.4 Specific IgM response

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

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

4- DISCUSSION

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

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

A specific antibody response (IgM) to the vaccine was measured in vaccinated fish at 780 DD which was significantly higher compared to non-vaccinated fish. The high RPS levels in vaccinated fish and specific antibody response following vaccination are indicators that the

462 vaccine indeed triggered a specific protective humoral response against a*As*. Similar
463 responses have been induced in other species immunised with typical or atypical stains of *As*,
464 such as lumpsucker [30, 31], Atlantic salmon [32, 33], rainbow trout [34] and spotted wolfish
465 [35]. A high quantity of cytoplasmic peroxidases (*e.g.* thiol peroxidase) have previously been
466 reported in *A. salmonicida* cells [36] and high antigen endogenous peroxidase activity
467 appeared to cause substantial background during ELISA development. This background was
468 quenched using hydrogen peroxide prior to antibody-antigen complexing. However, a high
469 absorbance threshold of the ELISA could not be avoided using our cut-off criteria (3x
470 background OD = 0.4), thus a 1/50 test titre was the most preferable to use to determine
471 positive antibody responses to a*As* vaccination. Nonetheless, the titre of antibodies was
472 consistently higher in vaccinated fish up to and including a dilution of 1/800. These results
473 suggested that antibodies might be involved in protection against a*As*.
474 Interestingly, differences in virulence were observed for two atypical *Aeromonas salmonicida*
475 *vapA* type V isolates (TW3/14 and TW4/14), with the latter being the most virulent.
476 Microrestriction and PFGE analysis corroborated these results, indicating small but
477 potentially important genomic differences between isolates. Atypical *Aeromonas salmonicida*
478 isolates heterogeneity has been previously assessed with the same method [27].
479 Characterisation of all the available a*As vapA* types for ballan wrasse by PFGE will be
480 beneficial to select isolates for future vaccine formulations.
481 The atypical *Aeromonas salmonicida vapA* VI isolates appeared less virulent than type V and
482 similar results were reported for Norwegian a*As* type V and VI isolates [17]. In that study a*As*
483 type V induced high mortalities (75 – 89% morbidities) in 50 g ballan wrasse when i.p.
484 injected with 10^7 cfu /fish and also by cohabitation (51%). The type VI isolates were less
485 virulent, in particular by cohabitation (8%) than i.p. injection (70 – 85%). In previous reports
486 lumpsucker succumbed to disease at lower doses of 2 x10^3 cfu/mL (bath) and 4 x 10^4 cfu
487 /fish (i.p. injection) after exposure [18]. Other fish species like spotted wolfish also

488 experienced high mortalities with low doses of a*As* (10^3 and 10^4 cfu / mL) by i.p. injection
489 [10, 15]. In contrast, turbot required very high doses (10^8 and 10^{10} cfu / mL) for mortalities to
490 be induced by i.p. injection [16]. As suggested previously, there is a strong association
491 between host species and *vapA* type and it is possible that *vapA* type V is more strongly
492 associated with wrasse than lumpfish and vice versa for type IV [37]. Interestingly, in the
493 present study, survivor fish infected with a*As* type VI at a medium dose (10^7 cfu / fish) did
494 not show any obvious external or internal signs of the disease and no bacteria were recovered
495 from those fish, suggesting that ballan wrasse were able to clear the infection. This is in
496 agreement with a previous study that reported similar responses in survivors from groups
497 infected with a*As* type V and VI [17].
498 The a*As vapA* type VI isolates occasionally displayed a peculiar alternative morphology that
499 included the presence of large greyish and small transparent colonies (Supplementary File 9).
500 Previous reports have documented this phenomenon associated with variable expression of a
501 functional A-layer and consequently with variable virulence [22, 38-42]. Although in the
502 present study, the inclusion of isolates displaying such alternative morphology was generally
503 avoided, this should not be ruled out as a possible explanation behind the lack of virulence
504 observed in this experiment.
505 This study is also the first experimental confirmation that *Photobacterium indicum* can be
506 pathogenic towards wrasse, through fulfilment of Koch's postulates. *Photobacterium*
507 *indicum* was regularly isolated from diseased ballan wrasse during disease surveys in
508 Scotland and it was linked to histopathological lesions[6]. There are no previous reports on
509 fish susceptibility to *Photobacterium indicum* although it has been isolated from moribund
510 American lobster (*Homarus americanus*) associated with stress and has been reported as an
511 opportunistic pathogen for this crustacean species [43, 44]. In cleaner fish, *Photobacterium*
512 sp. was recently recovered from lumpsucker experiencing mortalities due to *Pseudomonas*
513 *anguilliseptica* under rearing conditions in Scotland [45]. The pathogenicity results for

514 *Photobacterium indicum* obtained in the present study need to be treated with caution as
515 disease was induced only via i.p. injection which bypasses the natural protective mucosal
516 barriers of the host *e.g.* skin, gills and gastrointestinal tract [46, 47]. Signs of disease and
517 gross pathology for *Photobacterium indicum* were similar to those seen with a*As* with
518 moribund fish showing reduced feeding response often followed by imbalance, lethargy and
519 full loss of equilibrium. The peritoneal cavity of the diseased fish was extended (ascites) and
520 internally, liquefaction was observed in all the organs. Granulomatous formations were seen
521 in livers of moribund fish infected with a*As* which concurred with previous reports [17]. This
522 needs to be considered when performing differential diagnosis based on gross pathology and
523 clinical signs. Ventral lesions at the i.p. injection site were observed on survivor fish, which
524 may be related to localised immune responses at the injection site [48, 49].
525 The *Aliivibrio logei*, *Vibrio splendidus* and *Vibrio ichthyoenteri* isolates were not pathogenic
526 to ballan wrasse by i.p. injection even when very high challenge doses were administered.
527 *Aliivibrio salmonicida* was the only pathogen that caused mortalities (13%) but only when
528 very high infection dose of 5 x 10^9 cfu/fish was administered.
529

530 **5- CONCLUSIONS**

531 Vaccination (by i.p.) can be used to control and potentially eliminate morbidities in ballan
532 wrasse hatcheries and cage sites caused by a*As vapA* type V and likely other *vapA* types (*e.g.*
533 VI). The pathogenicity and virulence of *Photobacterium indicum* to ballan wrasse was
534 reported for first time. Interestingly, *Vibrio* spp. and *Aliivibrio* spp. were not pathogenic by
535 i.p. injection to the ballan wrasse population tested herein. Vaccination efficacy tests are
536 required against *Photobacterium indicum* and a*As* type VI, as the species is susceptible to
537 them. Immersion vaccination strategies should be explored as the species encounters the
538 pathogens at earlier life stages (< 25 g) and this immunisation route is desirable for juvenile

ballan wrasse in the hatcheries. In addition, full characterisation of a*As* isolates should be performed within the same *vapA* types.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interests

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Editorial Office

Reviewer 1

Major comments:

- My main concern is about the vaccination procedure. It appears that the negative control was performed using phosphate buffered saline; while the actual vaccine was oil-adjuvanted. Under this condition; it would be impossible to differentiate between the effect of the vaccine proper and that of the adjuvant. This is a concern as the protective effect of oil adjuvants is well-demonstrated, especially for the infection with the high dose of isolate TW4/14 as the difference with the control is really small.

  Thank you for your comment. In this study, due to fish and facility limited availability and cost, we could not test for the effect of the adjuvant alone and decided to include a PBS negative control only to answer the most important "industry focused" question with the available resources at the time. As you commented below, the specific antibody detection is the evidence that immunity was achieved from antigens included in the vaccine. The oil adjuvant effect could be explored in a future experiment.

  This issue is mitigated by the that a specific antibody response was detected, as it is likely that this response would play a role but this issue should be addressed by the authors. Ideally, by testing the protective effect of the oil adjuvant. However, I do not believe that this has a significant effect on the conclusions of the experiment, at least for most challenges where a very high RPS was detected.

- In addition, more details should be given in section 2.3 regarding the preparation of the vaccine. How were the bacterial strain inactivated? I assume, the same quantity of each bacteria was added to the vaccine?
  This is a commercial product and therefore the partner company prefers not to disclose details related to the vaccine manufacturing. We would appreciate your understanding.

- The expression "containing antigen" on line 158 is a bit confusing, while not incorrect, it does sound a bit like a sub-unit vaccine, while my understanding is that inactivated whole cells were used.
  The use of "Antigen" has been removed in the sentence.

- The infection procedure on line 168 is lacking the volume and correspondence in CFU. I realise that the volume is presented on line 208, but I believe that the text would be easier to read if it was presented when the infection is described. Similarly, on line 173, the higher dose should be clarified.

  Authors agree and made changes accordingly. Volume and CFU are now given in line 176 together with the infection procedure and the higher dose is now specified in line 188.

Minor comments:

- On line 87: please correct to "challenge model". – Done.
- On line 145: please replace "confirmation" with "confirming". – Done.

- On line 521: please remove the unnecessary word "was" after "challenge model" and add the word "the" before "Scottish isolate". – Conclusion has been changed accordingly to Reviewer 2 comments.
- Highlights: I would suggest moving the last highli – Done.

Reviewer 2

1. *Abstract: Suggest delete the last two sentences (Lines 47-63).*

These two last sentences have been removed.

2. *Line 29: Suggest report clearly on autogenous multivalent vaccines.*

Line 29 now reads 'Autogenous (*i.e.* farm specific inactivated) multivalent vaccines…'.

3. *Line 30: What is wrasse vaccine? It is multivalent autogenous vaccines containing four strains of Aeromonas salmonicida and one strain of Vibrio splendidus.*

Text has been revised accordingly in lines 32 – 33.

4. *Lines 58-114: The authors introduced bacterial pathogen, farmed salmonids, immunization, vaccine, ballan wrasse in the second, third and fourth paragraph of Introduction, isolates of bacteria, specific antibody (IgM), and RPS. Suggest describe the distribution, habitat, biology, and importance of ballan wrasse, and its importance in farmed salmonids like sea lice clean in salmon. Suggest report the research objective.*

A paragraph has been included at the start of the introduction with all the above suggestions (Lines 59 – 73). Research objectives have also been made clearer in lines 116 – 123.

5. *Line 59: Suggest change to farmed ballan wrasse (Labrus bergylta, ascanius).*

The scientific name has been included at the first mention of the species (Line 60).

6. *Line 148-151: Suggest report source of water, and primary water quality parameters like total dissolved solids (TDS) or salinity, conductivity, hardness, and pH.*

More information has been added in line 159 however no data is available on conductivity and hardness of the water.

7. *Line 159: Suggest report bacterial isolates TW3/14, TW4/14, TW187/14, TW164/15 and TW130/16 (Table 1). They are the strains of Aeromonas salmonicida and Vibrio splendidus. Suggest report source of bacterial isolate, and bacterial identification.*

This has been addressed in lines 167 – 170.

8. *Lines 158-161: Suggest report culture of five isolates, preparation of bacterial solutions and concentration, and preparation of oil-based multiple autogenic vaccine and concentration. The statement "injectable (oil-based) autogenous vaccine" is not clear.*

The authors acknowledge your comment however given the vaccine is a commercial product, after discussion with the partner company, we are not allowed to disclose details related to the vaccine manufacturing. The word "injectable" has been removed and "emergency' and 'inactivated' added in bracket.

9. *Lines 158-161: The statement "oil-based" is not clear. Can you report what kind of oil?*

Please see response above. The authors can not disclose this information.

10. Lines 335-337: The statements did not match with the data (Table 2).

Data in Table 2 were corrected.

11. Line 448: The term "immunised fish" and "vaccinate fish" are used in the text. Suggest define the term. It is "vaccinated fish"?, not "immunized fish"?

Immunised changed to vaccinated (line 456).

12. This is lengthy manuscript with 40 pages, and 49 references including two form the same groups of authors [2, 4].

The manuscript is 29 pages long including references (without figures and table). The number of references in the original manuscript was <50 as recommended in the Journal guidelines. The authors feels that both references 2 and 4 (now 3 and 6 in the revised manuscript) are directly relevant to the work and would prefer to keep them in the manuscript.

13. Discussion is lengthy. Suggest delete the parts that are not related.

The discussion is only 3.5 pages which is thought to be appropriate for the manuscript. The discussion has been rearranged to avoid back and forth to the different topics, which may have caused confusion and led to the reviewer to question the relevance of some sections. The authors believe that the flow has been improved with the relevance of all sections reaffirmed and would prefer to keep all parts of the discussion.

14. Conclusion is lengthy. Suggest delete the last sentence (Lines 535-541), and rewrite the conclusion. Suggest write a solid conclusion about the pathogenicity of Photobacterium indicum, TW3/13, TW4/14 (Aeromonmas salmonicida), and suggest report the effect of ballan wrasse that vaccinated with autogenous vaccine, and then later encountered bacteria.

The authors agree with the reviewer comment. The conclusion has been shortened by half from 20 lines to only 10 now.

15. Fig. 3: Suggest describe clearly "mock vaccinated challenged" and "vaccinated challenged", and "vaccinated and non-vaccinated". Suggest also report M & M clearly in the text (Lines 214-239).

The manuscript has been revised accordingly.

Figure 3 description has been changed to *'Survival (%) of i.p. injected vaccinated and non – vaccinated (mock vaccinated) ballan wrasse challenged with atypical Aeromonas salmonicida* vapA *type V (isolate TW4/14 – A and isolate TW 3/14) at medium and high (106 and 107 cfu/fish) doses and high and very high doses (107 and 108 cfu/fish. Letters represents statistical significance (p < 0.05).'*

Changes have also been made in the M&M as follow:

Line 224 '…or sterile PBS (control group / mock vaccinated).'

Line 236 ' …2 tanks containing 75 control (mock vaccinated) fish each.'

line 243 '...vaccinated and control fish (mock vaccinated) were challenged with...'

16. Fig. 4: Suggest change "immunized" to "vaccinated" mock

Done.

17. References: Italicize scientific names. Suggest follow the Journal guide, and check the references.

References have been revised according to the Journal guidelines.

18. Line 564: Rewrite the reference.

Done.

19. Lines 567-568, Line 577: Suggest change to Dis Aquat Org.

Done.

20. Lines 573-574: Suggest change to J Fish Disease.

Done.

21. Line 583: What is "University of Juvakskyla 2004"?

Done.

22. Lines 586-588: Rewrite the reference.

Done.

23. Lines 591-592: Rewrite the reference.

Done.

24. Line 600, Line 605, Lines 610-611, Line 623, Lines 630-631, Lines 644-645, Lines 697-698 : Suggest change to J Fish Disease

Done.

25. Line 614: What is "e1-e1"?

This is the e-page of that the manuscript. Corrected to e1.

26. Line 627: Rewrite the reference.

Done.

27. Lines 635-636: Suggest change to Am J Epidemiology

Done.

28. Line 639: Suggest change to Fish Shellfish Immunol.

Done.

29. Line 648: Suggest change to Dev Comp Immunol.

Done.

30. Line 661-662: Change to Fish Shellfish Immunol.

Done.

31. Line 673: Change to J Aquat Animal Health

Done.

32. Line 676: Change to J Bacteriology

Done.

33. Line 679: Change to J Fish Disease

Done.

34. Line 683: Check "fnz074".

It is correct; It is the e-page of the manuscript.

35. Lines 684-687: Check the references.

Done.

36. Lines 689-690: Check abbreviation of journal ,'

Done.

37. Lines 691-692: Suggest change to Fish Shellfish Immunol.

Done.

38. Line 694: Suggest change to Dev Comp Immunol.

Done.

39. Line 697: Suggest change to J Fish Disease

Done.

40. Highlights: Highlights are lengthy and not clear. Suggest rewrite the highlights. What is autogenous vaccine? Not only the species Photobacterium indicum induced mortality to ballan wrasse.

Highlights have been revised to make them shorter and clearer. Highlight 4 has been removed according to reviewer 1 comment.

Credit author statement

J. Gustavo Ramirez-Paredes (principal post-doctoral research assistant): methodology, experimental design, data collection, formal analyses, writing original draft
D. Verner-Jeffreys (lead project scientist at cefas): experimental design, financial support and management, article review and editing
A. Papadopoulou (PhD student in project): supported data collection, analyses (PFGE), writing original draft
S. J Monaghan (post-doctoral research assistant): sample analyses for IgM responses, editing original draft
L. Smith (husbandry technician at Cefas): experimental design, fish husbandry
D. Haydon (Ridgeways): pathogens and isolates identification and characterisation by 16S and vapA genes prior to assignation of TW numbers, vaccine formulation, read and approved the final version of the MS.
T. S. Wallis (project lead at Ridgeway): research conceptualisation, coordination and management
A. Davie (Project co-PI**)**: experimental design, supervision (PhD and PDRA), article review
A. Adams (Project workpackage leader): experimental design, supervision (PhD and PDRA), article review
H. Migaud (Project coordinator): project conceptualisation, financial support, management, supervision (PhD and PDRA) and article review and editing.

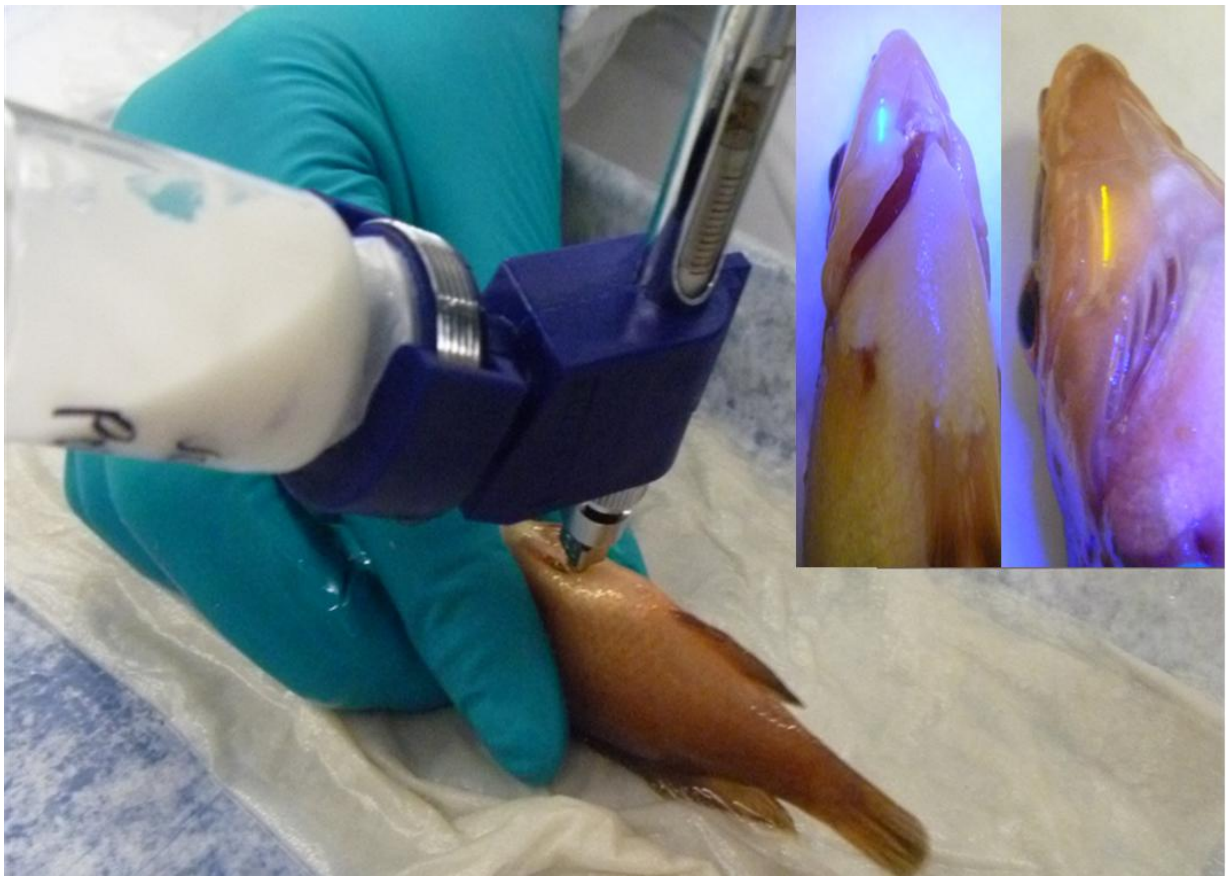


Figure 1. Intraperitoneal injection vaccination of ballan wrasse with oil-adjuvanted autogenous vaccine. Inset: wrasse tagged with Visible Implant Elastomer tagging system. Blue for the vaccinated fish and orange for mock vaccinated.

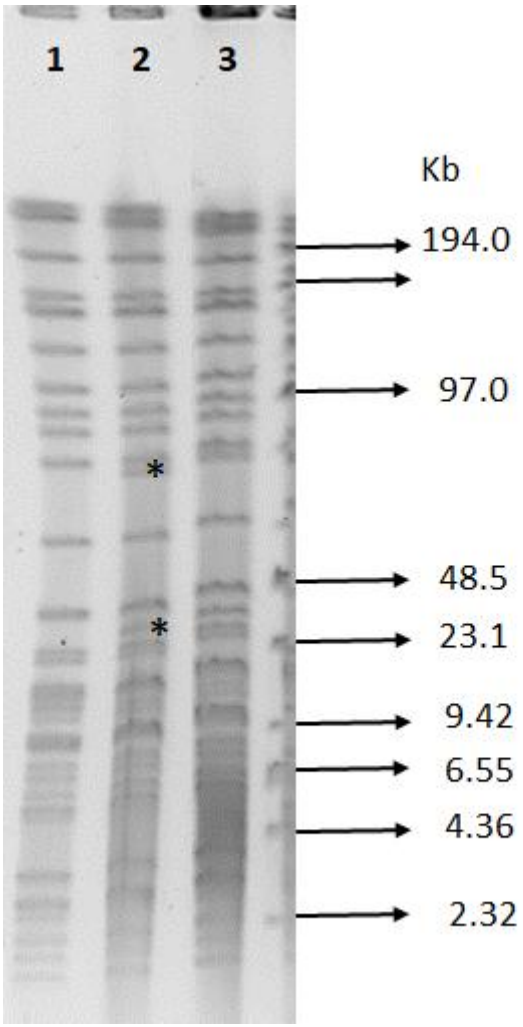
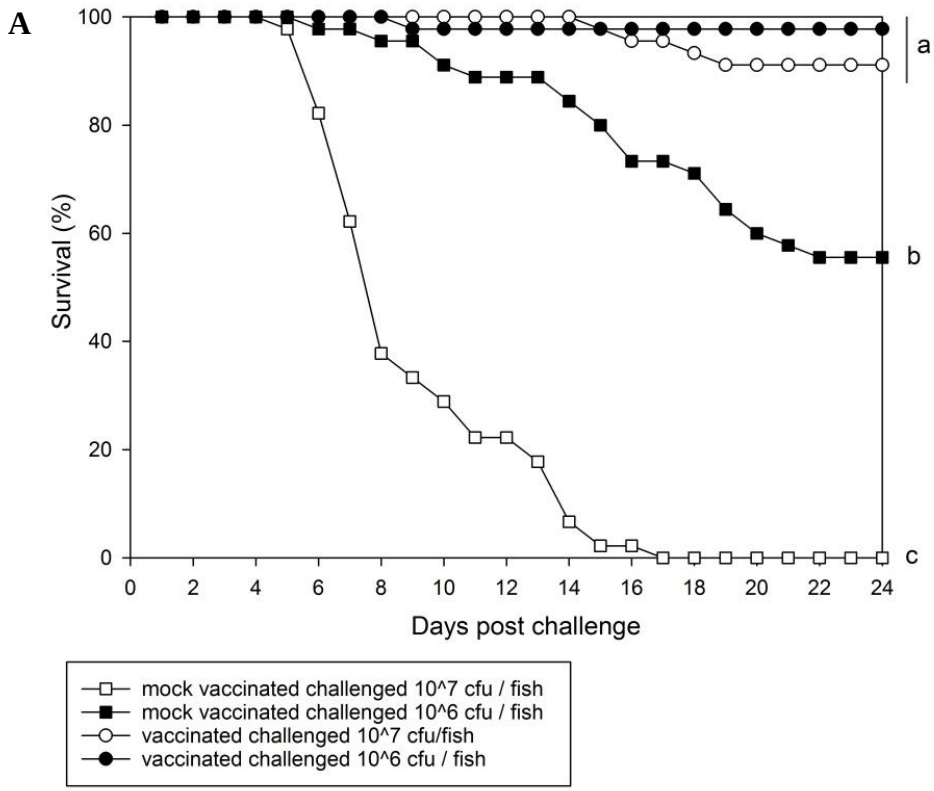


Figure 2. Pulsed-filed gel electrophoresis patterns of *Aeromonas salmonicida vapA* type V isolates TW4/14, TW 187/14 and TW 3/14 restricted with *SpeI* enzyme (New England Labs, UK). From left to right, TW 3/14 (position 1), TW 4/14 (position 2) and TW 187/14 (position 3). Molecular marker mixture of 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).



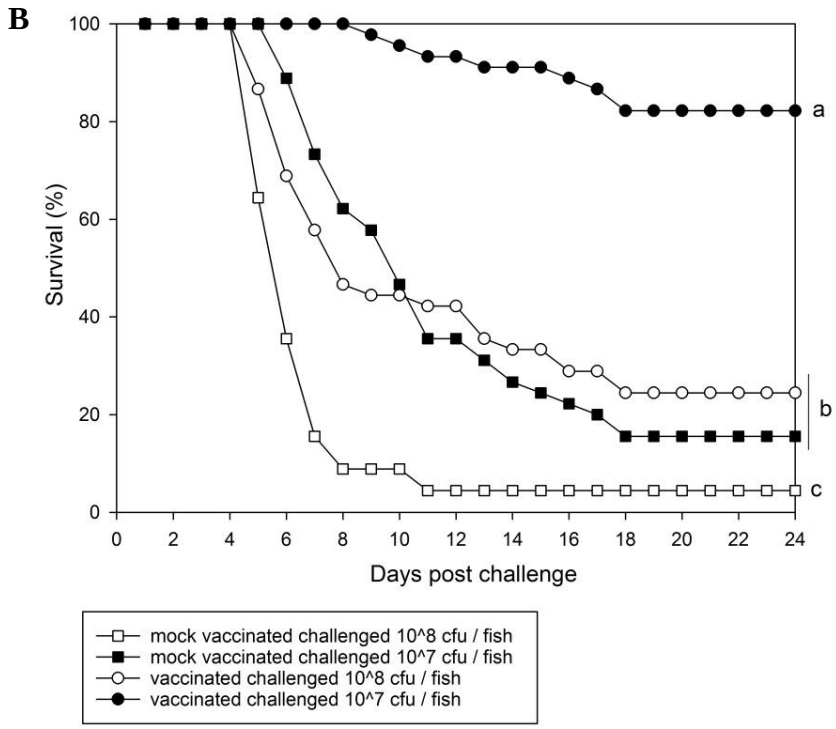


Figure 3. Survival (%) of i.p. injected vaccinated and non – vaccinated (mock vaccinated) ballan wrasse challenged with a *Aeromonas salmonicida vapA* type V (isolate TW4/14 – A

and isolate TW 3/14) at medium and high (10^6 and 10^7 cfu/fish) doses and high and very high doses (10^7 and 10^8 cfu/fish. Letters represents statistical significance ($p < 0.05$).

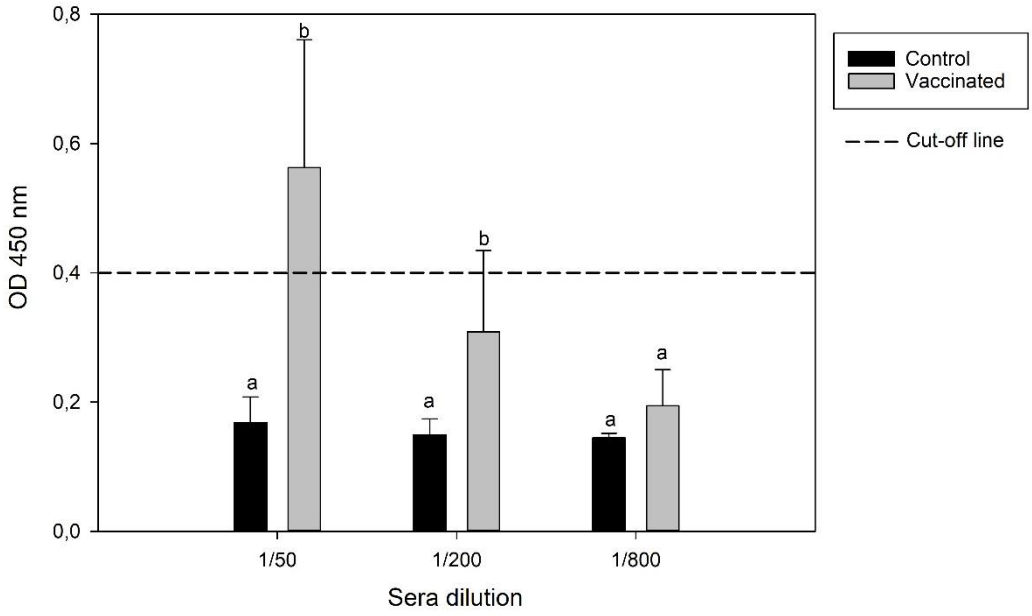


Figure 4. Ballan wrasse specific antibody (IgM) response to a*As* pre-vaccination (control, n= 6 samples x 2 replicates) and after immunisation period was completed (780 DD after i.p. vaccination, n= 6 replicates x 2 replicates). Letters represents statistical significance ($p < 0.05$).

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.

Table 2. Bacterial isolates, number of fish and doses used in the different infection experiments 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}	4 (33%)
	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}	3 (25%)
	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

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

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.

Supplementary material

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