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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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26 **ABSTRACT**

27 Atypical *Aeromonas salmonicida* (aAs) and *Vibrionaceae* related species are bacteria
28 routinely recovered from diseased ballan wrasse used as cleaner fish in Atlantic salmon
29 farming. Autogenous (i.e. farm specific inactivated) multivalent vaccines formulated from
30 these microorganisms are widely used by the industry to protect farmed wrasse despite
31 limited experimental proof that they are primary pathogens. In this study, the components of
32 a commercial multivalent injection vaccine containing four strains of *Aeromonas salmonicida*
33 and one strain of *Vibrio splendidus* previously isolated from ballan wrasse in Scotland, were
34 tested for infectivity, pathogenicity and virulence via intra peritoneal injection at pre-
35 deployment size (25-50g) and the efficacy of the vaccine for protection against aAs assessed.
36 Injection with 3.5×10^9 , 8×10^9 , 1.8×10^9 and 5×10^9 cfu/fish of *Vibrio splendidus*, *V.*
37 *ichthyenteri*, *Aliivibrio logeii* and *A. salmonicida*, respectively, did not cause significant
38 mortalities, lesions or clinical signs after a period of 14 days. IP injection with both aAs and
39 *Photobacterium indicum* successfully reproduced the clinical signs and internal lesions
40 observed during natural outbreaks of the disease. Differences in virulence (LD_{50} at day 8-post
41 infection of 3.6×10^6 cfu/fish and 1.6×10^7 cfu/fish) were observed for two aAs *vapA* type V
42 isolates. In addition, the LD_{50} for *Photobacterium indicum* was 2.2×10^7 cfu/fish. The
43 autogenous vaccine was highly protective against the two aAs *vapA* type V isolates after 700-
44 degree days of immunisation. The RPS_{FINAL} values for the first isolate were 95 and 91% at
45 1×10^6 cfu/fish and 1×10^7 cfu/fish, respectively, and 79% at 1×10^7 cfu/fish for the second
46 isolate tested. In addition, significantly higher anti aAs serral antibodies (IgM), were detected
47 by ELISA in vaccinated fish in contrast with control (mock vaccinated) fish. These results
48 suggest wrasse can be effectively immunised and protected against aAs infection by injection
49 with oil adjuvanted vaccines prepared with inactivated homologous isolates.

51 **Key Words:** wrasse vaccines, bacterial diseases, atypical *Aeromonas salmonicida*, cleaner
52 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.), corkscrew 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 physoclastic (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* (aAs) vapA type V and VI, *Vibrio splendidus*, *V. ichthyenteri*, *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 wolffish (*Anarhichas minor*, L) succumbed to disease when i.p. injected with aAs 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 lump sucker also experienced high morbidities (> 70%) when challenged with Norwegian aAs isolates at doses of 2×10^3 cfu / mL (bath) and 2×10^6 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, aAs 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 aAs *vapA* types V and VI, *Vibrio splendidus*, *V. ichthyenteri*, *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 aAs 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, aAs 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 (aAs *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 aAs

[6, 22], amoebic gill disease [23] and *Vibrionaceae* related bacteria [20, 21]. After confirming they were free of these pathogens, fish were transferred to the Centre for Environment, Fisheries and Aquaculture Science (Cefas) Weymouth Laboratory in February 2017.

Fish were acclimated and quarantined for 3 weeks after arrival in 6 aerated aquaria (approx. 900 L, tanks enriched with artificial plastic kelp and sections of plastic pipes to provide hides to the fish) at 12.0 ± 0.5 °C with a 20:4 h light:dark photoperiod, water flow of 4.0 L / min, salinity 34 ± 2 ‰, dissolved oxygen (DO) at 8 ± 0.5 mg / L and pH 7.8 ± 0.4 . During this period, fish were further screened for bacteriology (swabs from head kidney plated onto SWA), histopathology (fixed in 10% neutral buffered formalin) and molecular methods as described before. In addition, virology diagnostic tests were performed to discard the presence of notifiable viral diseases as per the protocols in the OIE manual of diagnostic tests for aquatic animals [24].

2.3 Vaccine

A commercial (oil-based, inactivated) multivalent autogenous (emergency) vaccine, containing aAs isolates TW3/14, TW4/14, TW187/14, TW164/15 and *Vibrio splendidus* isolate TW130/16 (Table 1) previously isolated from ballan wrasse [6] was provided by Ridgeway Biologicals Ltd. The vaccine was shipped to Cefas and stored at 4 ± 1 °C prior to use.

2.4 Confirmation of infectivity of components of multivalent autogenous vaccine

The virulence of bacterial isolates, representative of strains commonly used as components of the multivalent autogenous vaccines used in the industry, was assessed in a series of infection experiments performed in 30 L tanks enriched as for acclimation tanks (Table 2). For all the 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 μ L ($10^4 - 10^9$ cfu/fish) of the relevant bacterial suspension. Where included, control fish were injected with 100 μ L 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 $OD_{600} \sim 1.57$ bacterial suspension of different isolates representing 4 different bacterial species (aAs including two type V and one type VI isolates, *Vibrio splendidus*, *Aliivibrio salmonicida* and *Vibrio ichthyenteri*) (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 aAs *vapA* type VI (isolate TW164/15) recovered from lump sucker 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 aAs *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 passed 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.

Additional infection experiments 4 and 5 were also undertaken. These were to better determine both the relevant virulence of the different aAs isolates *vapA* type V and identify doses that would ideally result in high, but not excessive (50-75% mortality), suitable for use in vaccine efficacy testing. In infection experiment 4, 4x different doses of each pathogen were tested (n= 15 fish per dose) with a control treatment (PBS) included. Initial results generated by infection experiment 4 were confirmed in a second set of pre-tests with a longer observation period post injection (4 weeks) without PBS controls (Table 2). The isolates used were passaged (recovered from moribund fish) from infection experiments 1 and 2 (Table 2). For the isolates where the use of lower and higher doses caused a mortality response below and above 50% respectively, the median lethal dose (LD₅₀) was calculated according to [25] to define and compare their virulence at the time point of occurrence. Results obtained from both experiments 4 and 5 were used to select isolates for vaccine testing and determine the doses for the main challenge infection in the vaccine efficacy trial (Table 2). In addition, differences within the aAs *vapA* type V isolates TW4/14, TW187/14 and TW3/14 were investigated with macrorestriction analysis using pulsed field gel electrophoresis (PFGE) as described previously [26] with the following modifications. Bacteria were grown on TSA at 15 °C for 72 – 96 h, *SpeI* restriction enzyme (5U per 150 µL, New England Biolabs) was used [27] and the electrophoresis conditions comprised switch times of 2 – 6 s at 15 °C and 200 V for 37 h.

2.5 Vaccination

Two groups of 150 fish were tagged and i.p. injected with 0.05 mL of either the test vaccine or sterile PBS (control group / **mock vaccinated**). For this, fish were randomly transferred from their stock tank with a net into a bucket containing tank water at 12 ± 2 °C. Thereafter, groups of 2-5 fish were transferred at a time to a further bucket with MS-222 for anaesthesia 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 (aAs *vapA* type V) and TW3/14 (aAs *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

258 Animals (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039) Schedule 1
259 approved method (S1-M). All euthanised and dead fish were recorded throughout the
260 experiments and accounted for posterior statistical analyses. To confirm specific mortalities,
261 all moribund fish were necropsied, checked for gross pathology and sampled for bacteriology
262 and histopathology as previously described. The challenge experiments were typically
263 concluded when there was a period of at least five days with no mortalities. At the end of the
264 vaccine efficacy trial, all surviving fish were killed by S1- M and blood sampled and
265 processed as described before to measure specific antibody levels in the serum by ELISA.
266 All the experimental infections and vaccine efficacy tests were performed at 15 °C. Water
267 temperatures were gradually increased over an acclimation period of 5-7 days prior to
268 challenge.

269

270 **2.8 Specific IgM response**

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

275 Antibody titres were determined according to the protocols outlined by [28] with
276 modifications. Briefly, 96 – well ELISA plates (Immulon 4HBX, Thermo Scientific) were
277 coated with 50 µL of 0.05% w/v poly – L– lysine in carbonate – bicarbonate buffer (0.05 M
278 carbonate-bicarbonate pH 7.4, Sigma-Aldrich, St.Louis, UK) and incubated for 60 min at
279 room temperature (RT). Plates were then washed 2 times with a low salt wash buffer (LSWB)
280 (0.02 M Trizma base, 0.38 M NaCl, 0.05% Tween-20, pH 7.3). Bacteria i.e. aAs type V
281 isolate TW4/14, 100 µL at 10^8 cfu/mL (OD_{600} 1.0), were then added to each well and plates
282 were incubated overnight at 4 °C. The bacteria were previously prepared by growing them on
283 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 μ L, 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 LSBW.
287 The plates were then post-coated with 3% w/v casein in distilled water (250 μ L) 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. LSBW was used to wash the plate 3 times and
290 100 μ L 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 μ L 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 μ L), 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 LSBW was then added to the plates.
302 Chromogen in substrate buffer (prepared by adding 150 μ L of chromogen 42 mM trimethyl-
303 benzidine, TMB to 15 mL of substrate buffer containing 5 μ L H_2O_2 in 6 mL of 50% acetic
304 acid) was then added (100 μ L / well) for assay development.
305 The plates were incubated for 3 – 5 min at RT and the reaction stopped by adding 50 μ L
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].

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

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**

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

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

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

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

360 The *Photobacterium indicum* isolates TW138/16 and TW181/16 both caused 11 (92%)
361 overnight mortalities when administered at high doses of 3.2×10^9 and 9×10^9 cfu/fish
362 respectively. The remaining fish injected with a high dose of TW138/16 was removed on day
363 3 post infection while the last fish injected with TW181/16 were euthanised on welfare
364 grounds at day 6 post infection (Supplementary File 4). For the *Photobacterium indicum*
365 challenges with medium doses (3.2×10^7 and 9×10^7 cfu/fish), of isolates TW181/16 and
366 TW138/16 resulted in 8/12 and 6/12 mortalities respectively by day 3 post challenge
367 (Supplementary File 4). The remaining fish (n= 4) in the tank challenged with TW181/16
368 were terminated at day 9 post infection as no morbidities occurred for 3 days and all
369 presented lesions at the injection site during the daily observations. In the tank infected with
370 medium dose of isolate TW138/16 a single dead fish with a large lesion around the injection
371 site was removed on day 9, while monitoring of the remaining fish (n= 6) continued. On day
372 16 post infection all surviving fish (n=6) presented severe ventral lesions at the i.p. injection
373 site (Supplementary File 5) and some of these lesions extended into the cavity and for this
374 reason the experiment was terminated. Morbid fish infected with *Photobacterium indicum*
375 showed a reduced feeding response often followed by imbalance, lethargy and full loss of
376 equilibrium with a very rapid progression (< 24 h) of the signs. During the necropsies, the
377 majority of the fish had ascites, liquefaction of organs and swelling coelomic cavity due to
378 ascites. Internally, haemorrhages or lesions were noted and the severity of these progressed
379 over time.

380 Additional testing of isolate aAs type V (TW4/14) in infection experiments 4 and 5 confirmed
381 this organism was virulent. Morbidities were recorded within 2 dpi with the high dose (10^8
382 cfu/fish) and 100% mortalities were reached by day 4. A dose of 10^7 cfu/fish reached a
383 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 LD₆₀ 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 aAs 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

LD₅₀ 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 aAs *vapA* type V isolate TW3/14, *Photobacterium indicum* and aAs *vapA* type VI. The aAs *vapA* type V isolates (TW3/14, TW4/14 and TW187/14) were chosen for macrorestriction analysis using PFGE to select a replacement isolate for aAs *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 ichthyenteri* were not pathogenic. The average of the 3 estimations for aAs *vapA* type V (TW4/14) was 3.6×10^6 cfu/fish. The average of the 2 estimations for *Photobacterium indicum* was 2.2×10^7 cfu/fish. A comparison of all the LD₅₀ 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 aAs 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 1×10^8 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.

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

440 **4- DISCUSSION**

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

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

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

462 vaccine indeed triggered a specific protective humoral response against aAs. Similar
463 responses have been induced in other species immunised with typical or atypical strains of As,
464 such as lumpsucker [30, 31], Atlantic salmon [32, 33], rainbow trout [34] and spotted wolffish
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 aAs 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 aAs.

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 aAs *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 aAs type V and VI isolates [17]. In that study aAs
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 2×10^3 cfu/mL (bath) and 4×10^4 cfu
487 /fish (i.p. injection) after exposure [18]. Other fish species like spotted wolffish also

488 experienced high mortalities with low doses of aAs (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 aAs 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 aAs type V and VI [17].

498 The aAs *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 aAs 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 aAs 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 ichthyenteri* 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 5×10^9 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 aAs *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 aAs 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

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

541

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545

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551

552 **CONFLICTS OF INTEREST**

553 The authors declare no conflicts of interests

554

555

REFERENCES

- [1] J. Treasurer, Diseases of north European wrasse (*Labridae*) and possible interactions with cohabited farmed salmon, *Salmo salar* L, *J Fish Dis*, 35 (2012) pp. 555-562.
- [2] E. Leclercq, A. Davie, H. Migaud, Delousing efficiency of farmed ballan wrasse (*Labrus bergylta*) against *Lepeophtheirus salmonis* infecting Atlantic salmon (*Salmo salar*) post-smolts, *Pest Manag Sci*, 70 (2014) pp. 1274-1282.
- [3] A.J. Brooker, A. Papadopoulou, C. Gutierrez, S. Rey, A. Davie, H. Migaud, Sustainable production and use of cleaner fish for the biological control of sea lice: recent advances and current challenges, *Vet Rec*, 183 (2018) p. 383.
- [4] B. Austin, D.A. Austin, Aeromonadaceae representative (*Aeromonas salmonicida*), *Bacterial fish pathogens*, Springer, 2016, pp. 215-321.
- [5] B. Hjeltne, B. Bang-Jensen, G. Bornø, A. Haukaas, C.S. Walde, The Health Situation in Norwegian Aquaculture 2018. 2019.
- [6] A. Papadopoulou, T. Wallis, J.G. Ramirez-Paredes, S.J. Monaghan, A. Davie, H. Migaud, A. Adams, Atypical *Aeromonas salmonicida* *vapA* type V and *Vibrio* spp. are predominant bacteria recovered from ballan wrasse (*Labrus bergylta*) in Scotland, *Dis Aquat Org*, 140 (2020) pp. 47-54.
- [7] P.J. Midtlyng, Vaccination against furunculosis, in: R. Gudding, A. Lillehaug, Ø. Evensen (Eds.), *Fish Vaccination*, John Wiley & Sons, Ltd, Chichester, 2014, pp. 185-199.
- [8] B. Björnsdóttir, S. Gudmundsdóttir, S. Bambir, B. Gudmundsdóttir, Experimental infection of turbot, *Scophthalmus maximus* (L.), by *Aeromonas salmonicida* subsp. *achromogenes* and evaluation of cross protection induced by a furunculosis vaccine, *J Fish Dis*, 28 (2005) pp. 181-188.
- [9] V. Lund, L.M. Jenssen, M.S. Wesmajervi, Assessment of genetic variability and relatedness among atypical *Aeromonas salmonicida* from marine fishes, using AFLP-fingerprinting, *Dis Aquat Org*, 50 (2002) pp. 119-126.
- [10] V. Lund, H. Mikkelsen, M.B. Schrøder, Comparison of atypical furunculosis vaccines in spotted wolffish (*Anarhicas minor* O.) and Atlantic halibut (*Hippoglossus hippoglossus* L.), *Vaccine*, 26 (2008) pp. 2833-2840.

585 [11] P. Pylkkö, Atypical *Aeromonas salmonicida*-infection as a threat to farming of arctic
586 charr (*Salvelinus alpinus* L.) and European grayling (*Thymallus thymallus* L.) and putative
587 means to prevent the infection, University of Jyväskylä, 2004.

588 [12] Y. Attia, I. Schmerold, A. Hönel, The legal foundation of the production and use of
589 herd-specific vaccines in Europe, *Vaccine*, 31 (2013) pp. 3651-3655.

590 [13] European Medicines Agency / Coordination Group for Mutual Recognition and
591 Decentralised Procedures - Veterinary Recommendations for the manufacture, control and
592 use of inactivated autogenous veterinary vaccines within the EEA, London (2017)
593 https://www.hma.eu/fileadmin/dateien/Veterinary_medicines/CMDv_Website/Procedural_guidance/Miscellaneous/Recommendations_manufacture_control_use_inact_autogenous_vaccines.pdf
594 https://www.hma.eu/fileadmin/dateien/Veterinary_medicines/CMDv_Website/Procedural_guidance/Miscellaneous/Recommendations_manufacture_control_use_inact_autogenous_vaccines.pdf
595 [nes.pdf](https://www.hma.eu/fileadmin/dateien/Veterinary_medicines/CMDv_Website/Procedural_guidance/Miscellaneous/Recommendations_manufacture_control_use_inact_autogenous_vaccines.pdf) (Accessed 14 September 2020).

596 [14] B.K. Gudmundsdóttir, B. Björnsdóttir, Vaccination against atypical furunculosis and
597 winter ulcer disease of fish, *Vaccine*, 25 (2007) pp. 5512-5523.

598 [15] M. Ingilæ, J.A. Arnesen, V. Lund, G. Eggset, Vaccination of Atlantic halibut
599 *Hippoglossus hippoglossus* L., and spotted wolffish *Anarhichas minor* L., against atypical
600 *Aeromonas salmonicida*, *Aquaculture*, 183 (2000) pp. 31-44.

601 [16] Y. Santos, S. García- Marquez, P. Pereira, F. Pazos, A. Riaza, R. Silva, A. El Morabit,
602 F. Ubeira, Efficacy of furunculosis vaccines in turbot, *Scophthalmus maximus* (L.):
603 evaluation of immersion, oral and injection delivery, *J Fish Dis*, 28 (2005) pp. 165-172.

604 [17] E. Biering, Ø. Vaagnes, B. Krossøy, S. Gulla, D. Colquhoun, Challenge models for
605 atypical *Aeromonas salmonicida* and *Vibrio anguillarum* in farmed Ballan wrasse (*Labrus*
606 *bergylta*) and preliminary testing of a trial vaccine against atypical *Aeromonas salmonicida*, *J*
607 *Fish Dis*, 39 (2016) pp. 1257-1261.

608 [18] A. Rønneseth, G.T. Haugland, D.J. Colquhoun, E. Brudal, H.I. Wergeland, Protection
609 and antibody reactivity following vaccination of lumpfish (*Cyclopterus lumpus* L.) against
610 atypical *Aeromonas salmonicida*, *Fish Shellfish Immunol*, 64 (2017) pp. 383-391.

611 [19] B.K. Gudmundsdóttir, S. Gudmundsdóttir, Evaluation of cross protection by vaccines
612 against atypical and typical furunculosis in Atlantic salmon, *Salmo salar* L., *J Fish Dis*, 20
613 (1997) pp. 343-350.

614 [20] A. Klindworth, E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, F.O. Glöckner,
615 Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-
616 generation sequencing-based diversity studies, *Nucleic Acids Res*, 41 (2013) p. e1.

617 [21] S. Yamamoto, H. Kasai, D.L. Arnold, R.W. Jackson, A. Vivian, S. Harayama,
618 Phylogeny of the genus *Pseudomonas*: intrageneric structure reconstructed from the
619 nucleotide sequences of *gyrB* and *rpoD* genes, *Microbiology*, 146 (2000) pp. 2385-2394.

620 [22] S. Gulla, V. Lund, A. Kristoffersen, H. Sørum, D. Colquhoun, *vapA* (A- layer) typing
621 differentiates *Aeromonas salmonicida* subspecies and identifies a number of previously
622 undescribed subtypes, *J Fish Dis*, 39 (2016a) pp. 329-342.

623 [23] N.D. Young, I. Dyková, B.F. Nowak, R.N. Morrison, Development of a diagnostic PCR
624 to detect *Neoparamoeba perurans*, agent of amoebic gill disease, *J Fish Dis*, 31 (2008) pp.
625 285-295.

626 [24] Office International des Epizooties (OIE) Manual of diagnostic tests for aquatic animals,
627 Chapter 2.3. <https://www.oie.int/en/standard-setting/aquatic-manual/access-online/>, 2018
628 (Accessed 08 September 2020).

629 [25] L.J. Reed, H. Muench, A simple method of estimating fifty per cent endpoints, *Am J*
630 *Epidemiol*, 27 (1938) pp. 493-497.

631 [26] K. Bartie, F.W. Austin, A. Diab, C. Dickson, T.T. Dung, M. Giacomini, M. Crumlish,
632 Intraspecific diversity of *Edwardsiella ictaluri* isolates from diseased freshwater catfish,
633 *Pangasianodon hypophthalmus* (Sauvage), cultured in the Mekong Delta, Vietnam, *J Fish*
634 *Dis*, 35 (2012) pp. 671-682.

635 [27] M.-L. Hänninen, V. Hirvelä-Koski, Genetic diversity of atypical *Aeromonas*
636 *salmonicida* studied by pulsed-field gel electrophoresis, *Epidemiology & Infection*, 123
637 (1999) pp. 299-307.

638 [28] A. Adams, K.D. Thompson, D. Morris, C. Farias, S.C. Chen, Development and use of
639 monoclonal antibody probes for immunohistochemistry, ELISA and IFAT to detect bacterial
640 and parasitic fish pathogens, *Fish Shellfish Immunol*, 5 (1995) pp. 537-547.

641 [29] D.F. Amend, Potency testing of fish vaccines, *Fish biologics: serodiagnostics and*
642 *vaccines*, 1981, pp. 447-454.

643 [30] T. Erkinharju, M.R. Lundberg, E. Isdal, I. Hordvik, R.A. Dalmo, T. Seternes, Studies on
644 the antibody response and side effects after intramuscular and intraperitoneal injection of
645 Atlantic lumpfish (*Cyclopterus lumpus* L.) with different oil-based vaccines, J Fish Dis, 40
646 (2017) pp. 1805-1813.

647 [31] A. Rønneseth, D.B. Ghebretsaie, H.I. Wergeland, G.T. Haugland, Functional
648 characterization of IgM+ B cells and adaptive immunity in lumpfish (*Cyclopterus lumpus* L.),
649 Dev Comp Immunol, 52 (2015) pp. 132-143.

650 [32] A.B. Romstad, L.J. Reitan, P. Midtlyng, K. Gravningen, Ø. Evensen, Development of an
651 antibody ELISA for potency testing of furunculosis (*Aeromonas salmonicida* subsp
652 *salmonicida*) vaccines in Atlantic salmon (*Salmo salar* L), Biologicals, 40 (2012) pp. 67-71.

653 [33] A.B. Romstad, L.J. Reitan, P. Midtlyng, K. Gravningen, Ø. Evensen, Antibody
654 responses correlate with antigen dose and in vivo protection for oil-adjuvanted, experimental
655 furunculosis (*Aeromonas salmonicida* subsp. *salmonicida*) vaccines in Atlantic salmon
656 (*Salmo salar* L.) and can be used for batch potency testing of vaccines, Vaccine, 31 (2013)
657 pp. 791-796.

658 [34] K.R. Villumsen, I. Dalsgaard, L. Holten-Andersen, M.K. Raida, Potential role of specific
659 antibodies as important vaccine induced protective mechanism against *Aeromonas*
660 *salmonicida* in rainbow trout, PLoS ONE, 7 (2012).

661 [35] R.N. Grøntvedt, S. Espelid, Vaccination and immune responses against atypical
662 *Aeromonas salmonicida* in spotted wolffish (*Anarhichas minor* Olafsen) juveniles, Fish
663 Shellfish Immunol, 16 (2004) pp. 271-285.

664 [36] P. Vanden Bergh, M. Heller, S. Braga-Lagache, J. Frey, The *Aeromonas salmonicida*
665 subsp. *salmonicida* exoproteome: global analysis, moonlighting proteins and putative
666 antigens for vaccination against furunculosis, Proteome Science, 11 (2013) p. 44.

667 [37] S. Gulla, S. Bayliss, B. Björnsdóttir, I. Dalsgaard, O. Haenen, E. Jansson, U. McCarthy,
668 F. Scholz, M. Vercauteren, D. Verner-Jeffreys, Biogeography of the fish pathogen
669 *Aeromonas salmonicida* inferred by *vapA* genotyping, FEMS microbiology letters, 366
670 (2019) p. fnz074.

671 [38] R.C. Cipriano, J. Bertolini, Selection for virulence in the fish pathogen *Aeromonas*
672 *salmonicida*, using coomassie brilliant blue agar, J Wildl Dis, 24 (1988) pp. 672-678.

673 [39] R.A. Garduño, A.R. Moore, G. Olivier, A.L. Lizama, E. Garduño, W.W. Kay, Host cell
674 invasion and intracellular residence by *Aeromonas salmonicida*: role of the S-layer, Can J
675 Microbiol, 46 (2000) pp. 660-8.

676 [40] G. Olivier, Virulence of *Aeromonas salmonicida*: Lack of Relationship with Phenotypic
677 Characteristics, J Aquat Anim Health, 2 (1990) pp. 119-127.

678 [41] E.E. Ishiguro, W.W. Kay, T. Ainsworth, J.B. Chamberlain, R.A. Austen, J.T. Buckley,
679 T.J. Trust, Loss of virulence during culture of *Aeromonas salmonicida* at high temperature, J
680 Bacteriol, 148 (1981) pp. 333-340.

681 [42] A.E. Ellis, A.S. Burrows, K.J. Stapleton, Lack of relationship between virulence of
682 *Aeromonas salmonicida* and the putative virulence factors: A-layer, extracellular proteases
683 and extracellular haemolysins, J Fish Dis, 11 (1988) pp. 309-323.

684 [43] D. Basti, D. Bouchard, A. Lichtenwalner, Safety of Florfenicol in the Adult Lobster
685 (*Homarus americanus*), J Zoo Wildl Med, 42 (2011) pp. 131-133.

686 [44] D.A. Bouchard, Investigating Present-day Health Issues of the American Lobster
687 (*Homarus americanus*), 2018. *Electronic Theses and Dissertations*, 2890
688 <https://digitalcommons.library.umaine.edu/etd/2890> (Accessed 10 September 2020).

689 [45] J. Treasurer, T. Birkbeck, *Pseudomonas anguilliseptica* associated with mortalities in
690 lumpfish (*Cyclopterus lumpus* L.) reared in Scotland, Bull Eur Assoc Fish Pathol, 38 (2018)
691 pp. 222-224.

692 [46] A. Adams, Progress, challenges and opportunities in fish vaccine development, Fish
693 Shellfish Immunol, 90 (2019) pp. 210-214.

694 [47] C.W.E. Embregts, M. Forlenza, Oral vaccination of fish: Lessons from humans and
695 veterinary species, Dev Comp Immunol, 64 (2016) pp. 118-137.

696 [48] S. Gudmundsdóttir, S. Lange, B. Magnadóttir, B.K. Gudmundsdóttir, Protection against
697 atypical furunculosis in Atlantic halibut, *Hippoglossus hippoglossus* (L.); comparison of a
698 commercial furunculosis vaccine and an autogenous vaccine, J Fish Dis, 26 (2003) pp. 331-
699 338.

700 [49] R. Nordmo, A. Ramstad, Variables affecting the challenge pressure of *Aeromonas*
701 *salmonicida* and *Vibrio salmonicida* in Atlantic salmon (*Salmo salar* L.), Aquaculture, 171
702 (1999) pp. 1-12.

703

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 from 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 feel 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 (*Aeromonas 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 Juvakskyia 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.

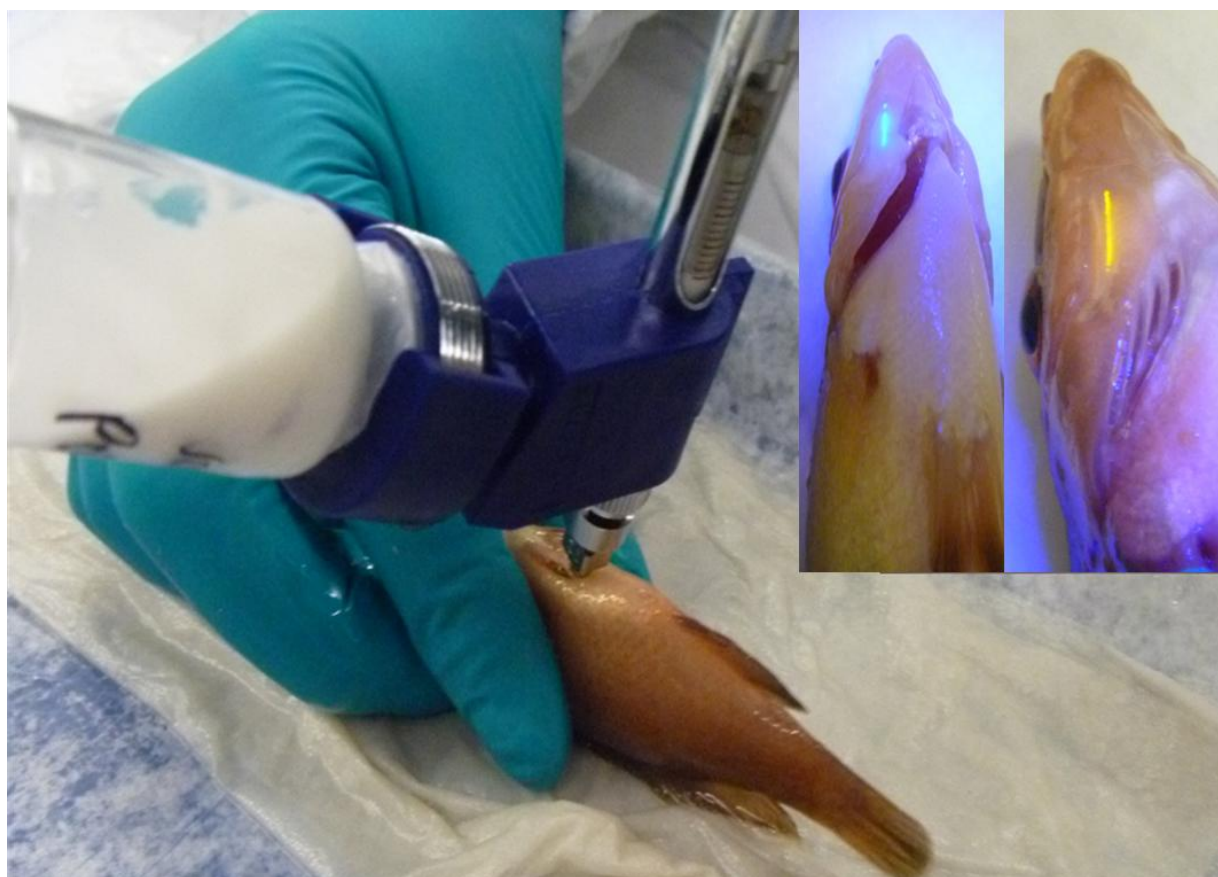


Figure 1. Intraperitoneal injection vaccination of ballan wrasse with oil-adjuvanted autogenous vaccine. Inset: wrasse tagged with Visible Implant Elastomer tagging system. Blue for the vaccinated fish and orange for mock vaccinated.

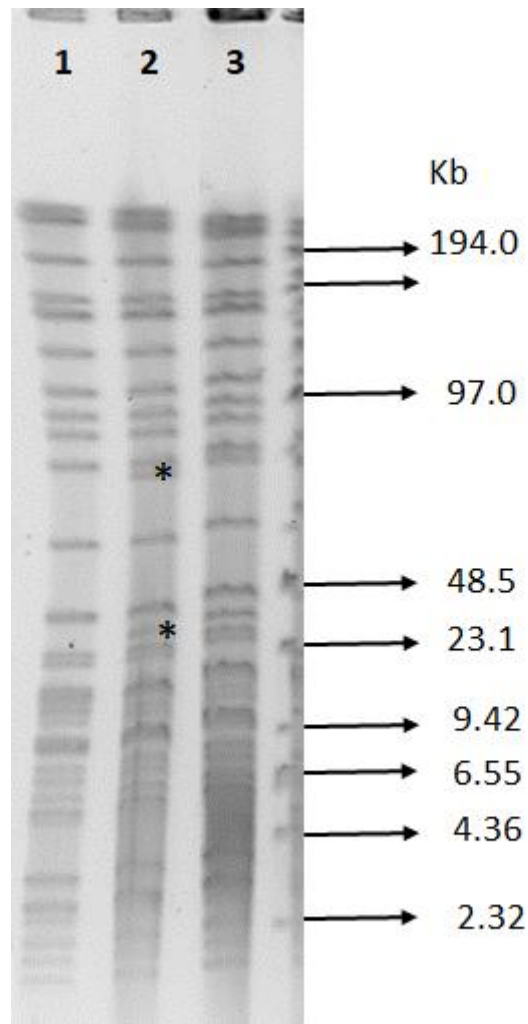


Figure 2. Pulsed-field gel electrophoresis patterns of *Aeromonas salmonicida* *vapA* type V isolates TW4/14, TW 187/14 and TW 3/14 restricted with *SpeI* enzyme (New England Labs, UK). From left to right, TW 3/14 (position 1), TW 4/14 (position 2) and TW 187/14 (position 3). Molecular marker mixture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

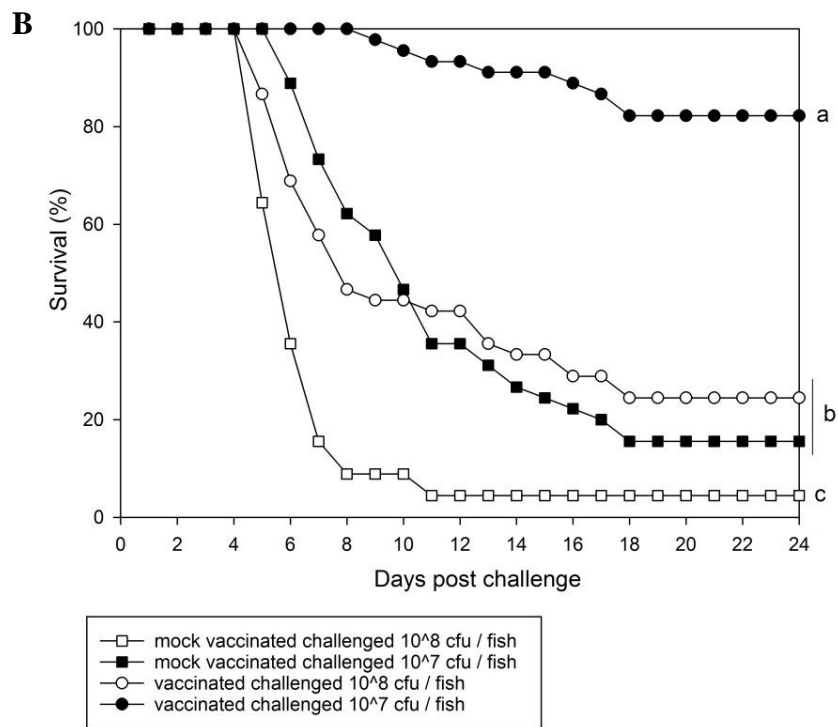
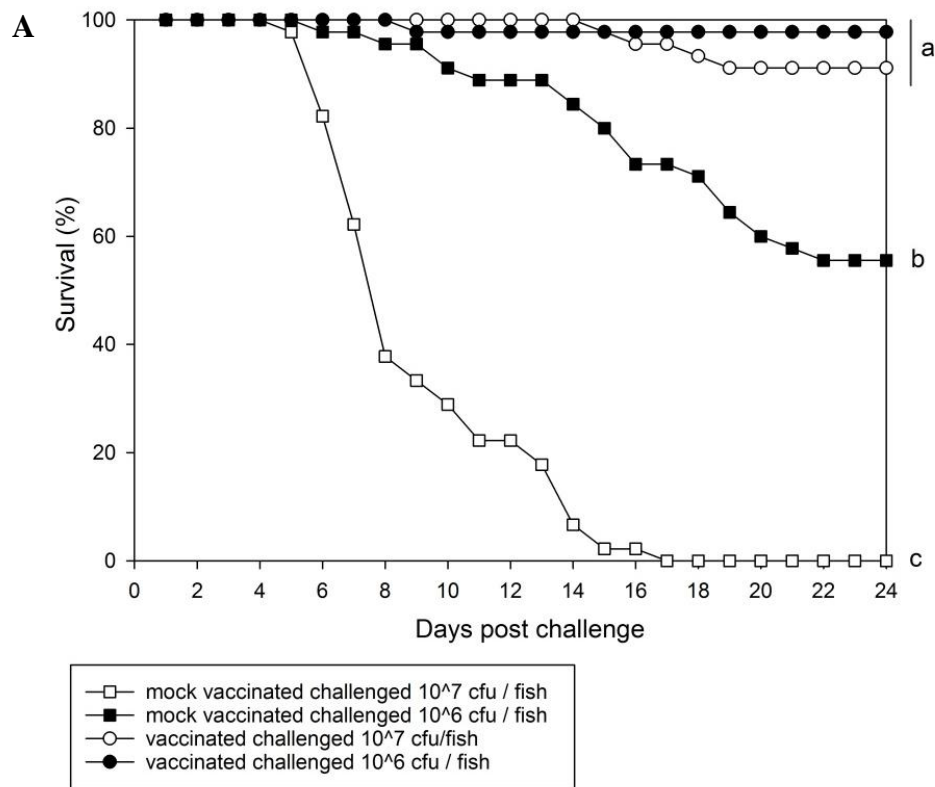


Figure 3. Survival (%) of i.p. injected vaccinated and non – vaccinated (mock vaccinated) ballan wrasse challenged with a *Aeromonas salmonicida vapA* type V (isolate TW4/14 – A

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 ($p < 0.05$).

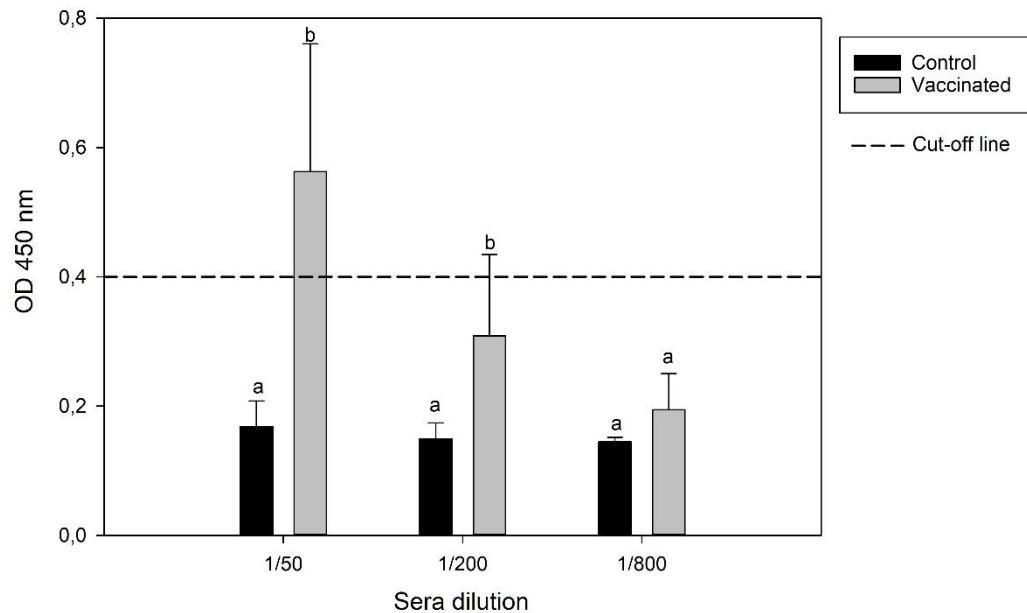


Figure 4. Ballan wrasse specific antibody (IgM) response to aAs pre-vaccination (control, n= 6 samples x 2 replicates) and after immunisation period was completed (780 DD after i.p. vaccination, n= 6 replicates x 2 replicates). Letters represents statistical significance ($p < 0.05$).

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

Isolate ID	Bacterial species	Year of isolation	<i>vapA</i> type and /or isolate #
TW4/14** ^v	<i>Aeromonas salmonicida</i>	2014	V
TW187/14 ^v	<i>Aeromonas salmonicida</i>	2014	V
TW3/14** ^v	<i>Aeromonas salmonicida</i>	2014	V
TW164/15 ^v	<i>Aeromonas salmonicida</i>	2015	VI
TW184/16*	<i>Aeromonas salmonicida</i>	2016	VI
TW242/16	<i>Aliivibrio logei</i>	2016	Isolate 1
TW186/16	<i>Aliivibrio logei</i>	2016	Isolate 2
TW322/16	<i>Aliivibrio salmonicida</i>	2016	Isolate 1
TW130/16 ^v	<i>Vibrio splendidus</i>	2016	Isolate 1
TW319/16	<i>Vibrio ichthyenteri</i>	2016	Isolate 1
TW138/16	<i>Photobacterium indicum</i>	2016	Isolate 1
TW181/16	<i>Photobacterium indicum</i>	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	aAs type V – TW4/14	1.46	9.5 x 10 ⁷	6 (100%)
	6	6	aAs type V – TW187/14	1.52	8.5 x 10 ⁷	6 (100%)
	6	6	aAs type V – TW3/14	1.49	1.0 x 10 ⁸	6 (100%)
	6	6	<i>Aliivibrio salmonicida</i> TW322/16	1.51	5.0 x 10 ⁴	0
	6	6	<i>Vibrio splendidus</i> TW130/16	1.45	2.0 x 10 ⁵	0
	6	6	<i>Vibrio ichthyenteri</i> TW319/16	1.48	1.0 x 10 ⁹	0
Experiment 2	15	7	<i>Aliivibrio salmonicida</i> TW322/16	>2.5	5.0 x 10 ⁹	2 (13%)
	15	7	<i>Vibrio splendidus</i> TW130/16	>2.5	3.5 x 10 ⁹	0
	15	7	<i>Vibrio ichthyenteri</i> TW319/16	>2.5	8.0 x 10 ⁹	0
	15	7	Control - 1x PBS	-	-	0
	15	8	aAs type VI – TW164/15	2	3.0 x 10 ⁹	15 (100%)
Experiment 3	12	15	aAs type VI – TW164/15	2	3.0 x 10 ⁹	12 (100%)
	12	15	aAs type VI – TW164/15	10 ⁻²	3.0 x 10 ⁷	4 (33%)
	12	15	aAs type VI – TW184/16	2	1.6 x 10 ⁹	12 (100%)
	12	15	aAs type VI – TW184/16	10 ⁻²	1.6 x 10 ⁷	3 (25%)
	12	12	<i>Photobacterium indicum</i> TW138/16	1.75	3.2 x 10 ⁹	12 (100%)
	12	12	<i>Photobacterium indicum</i> TW138/16	10 ⁻²	3.2 x 10 ⁷	6 (50%)
	12	12	<i>Photobacterium indicum</i> TW181/16	1.89	9.0 x 10 ⁹	12 (100%)
	12	12	<i>Photobacterium indicum</i> TW181/16	10 ⁻²	9.0 x 10 ⁷	8 (66%)
	12	25	<i>Aliivibrio logei</i> TW242/16	2	1.5 x 10 ⁹	0
	12	25	<i>Aliivibrio logei</i> TW242/16	10 ⁻²	1.5 x 10 ⁷	0
	12	25	<i>Aliivibrio logei</i> TW186/16	2	1.8 x 10 ⁹	0
	12	25	<i>Aliivibrio logei</i> TW186/16	10 ⁻²	1.8 x 10 ⁷	0
Experiment 4	15	16	aAs type V – TW4/14	1.9	1.0 x 10 ⁸	15 (100%)
	15	16	aAs type V – TW4/14	10 ⁻¹	1.0 x 10 ⁷	13 (87%)
	15	16	aAs type V – TW4/14	10 ⁻²	1.0 x 10 ⁶	8 (53%)
	15	16	aAs type V – TW4/14	10 ⁻⁴	1.0 x 10 ⁴	0
	15	16	Control - 1x PBS	-	-	0
	15	19	aAs type VI – TW164/15	2	2.5 x 10 ⁸	1 (7%)
	15	19	aAs type VI – TW164/15	10 ⁻¹	2.5 x 10 ⁷	0
	15	19	aAs type VI – TW164/15	10 ⁻²	2.5x 10 ⁶	0
	15	19	aAs type VI – TW164/15	10 ⁻⁴	2.5 x 10 ⁴	0
	15	19	Control - 1x PBS	-	n/a	0
Experiment 5	15	19	aAs type V – TW4/14	1.95 dil. 10 ⁻¹	1.0 x 10 ⁷	15 (100%)
	15	19	aAs type V – TW4/14	10 ⁻²	1.0 x 10 ⁶	8 (53%)
	15	19	aAs type V – TW4/14	10 ⁻³	1.0 x 10 ⁵	7 (47%)
	15	19	aAs type V – TW4/14	10 ⁻⁴	1.0 x 10 ⁴	1 (7%)
	15	19	aAs type V – TW4/14	10 ⁻⁵	1.0 x 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 (%)
<i>aAs vapA</i> type V (TW4/14)	T03-10; 90 fish (45v +45c)	Medium	1.0×10^6	95
<i>aAs vapA</i> type V (TW4/14)	T03-09; 90 fish (45v +45c)	High	1.0×10^7	91
<i>aAs vapA</i> type V (TW3/14)	T03-08; 90 fish (45v +45c)	High	1.0×10^7	79
<i>aAs vapA</i> 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% (LD₅₀) of 6 bacterial species used in the trials by day 8 post infection.

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

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

Supplementary material

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