Development of autogenous vaccines for farmed European seabass against *Aeromonas veronii* using zebrafish as a model for efficacy assessment

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Abstract

Aeromonas veronii bv. *sobria* is an emerging pathogen for the European seabass cultured in the Aegean Sea (Mediterranean) causing significant problems in the Greek and Turkish aquaculture industry since no licensed vaccine is currently available for the disease. A bivalent vaccine was developed based on two phenotypically distinct strains of the pathogen, PDB (motile, pigment-producing strain) and NS (non-motile, non-pigment-producing). The two strains comprising the bivalent vaccine were evaluated as monovalent products in zebrafish before the seabass trials. Challenges using the homologous or the heterologous strain showed that both vaccines were protective with RPS values ranging between 66-100% in zebrafish. The bivalent vaccine was then tested in European seabass following dip or intraperitoneal administration. Efficacy was evaluated separately against both strains comprising the bivalent vaccine. Dip vaccination applied to juvenile seabass of 2.5 g average weight provided protection following challenge tests 30 days post vaccination only in one of the two strains tested (strain PDB, RPS: 88%). This was also the case in the injection vaccination of adult seabass of 60 g average weight where the vaccine was effective only against the PDB strain (RPS: 63%). High antibody titers against both strains were found at 30 and 60 days after intraperitoneal vaccination in the adult seabass. The use of zebrafish as a model for vaccine development for aquaculture species is discussed.

Keywords: European seabass, *Aeromonas*, zebrafish, vaccine

Introduction

European seabass (*Dicentrarchus labrax*) is a species of great economic importance for the Mediterranean aquaculture. Approximately 65% of the total production comes from sea cages located in the coasts of Turkey and Greece in the Aegean and the Black Sea [1,2]. Recently, a disease caused by a distinct group of *Aeromonas veronii* strains was described affecting farmed seabass in both sides of the Aegean Sea [3–5]. The same species has been reported again in diseased farmed seabass in Italy [6] and the Black Sea [7]. Despite rare, incidents of aeromonad infections in farmed seabass have been reported again including a case of furunculosis in Spain [8], an atypical infection caused by *A. salmonicida achromogenes* in the Black Sea [9] and an infection caused by *A. hydrophila* in the Aegean Sea [10]. At a global scale, *A. veronii* is gaining importance as a pathogen of a variety of freshwater, marine and ornamental fish species as summarized previously [4] including a case of a recently reported pathotype that affected farmed catfish in both USA and China [11].

Despite the progress in fish vaccines the last decades, there are still many bacterial diseases, especially for non-salmonids that lack preventive methods [12,13]. The few commercial vaccines available for farmed European seabass concern mostly photobacteriosis and vibriosis [14–16]. Experimental vaccines on seabass against *Tenacibaculum maritimum* and *Mycobacterium marinum* [17–20] have shown promising results but only one commercial vaccine is yet available in Spain against *T. maritimum* for turbot [21] and no vaccines are available for *M. marinum* while no vaccines are available against furunculosis for species other than salmonids. On the other hand, the infections caused by mesophilic aeromonads in accordance with their multifactorial virulence [22] are generally prevented with autogenous products [23].

Vaccine development for the Mediterranean species is hampered mainly by the fragmentation of the aquaculture industry in this area which is characterized by the culture of many different fish species making it small and therefore unappealing for serious investments by the pharmaceutical companies. Moreover, at a technical level, the limited availability of appropriate-sized fish throughout the year, and the scarce licensed experimental facilities are some of the obstacles for the development of species-specific vaccines. Meanwhile, zebrafish is increasingly used as a model organism in studies of infectious diseases of aquaculture fish species and has been

proposed as an alternative in aquatic biomedicine including vaccine efficacy studies for bacterial pathogens (e.g. *Vibrio anguillarum*) [24].

In the present study a vaccine against *A. veronii* infection in farmed European seabass was developed. Immune response to vaccination was assessed by specific antibody titer (ELISA) and efficacy by challenge tests. The seabass vaccine was also applied to zebrafish attempting to evaluate their applicability as a surrogate in screening for vaccine efficacy in Mediterranean fish species for the first time.

Materials and methods

Bacterial strains and vaccine development

Strains NS and PDB isolated from diseased European seabass farmed in the Aegean Sea [4] were cultured in Brain Heart Infusion broth of 0.5% NaCl (BHI 0.5) at 25^{o}C for 18-20 hours. Bacterial cultures were formalin (3% v/v) inactivated and pellet was collected by centrifugation and subsequently suspended in sterile saline. Bacterin concentration was determined by optical density (600nm) and confirmed by direct microscopic (optical) count on a Neubauer cytometry plate.

Two monovalent adjuvanted vaccines, **ZV1** and **ZV2** were prepared for the zebrafish trials using strains NS (ZV1) and PDB (ZV2), to be administered by intraperitoneal (IP) injection in a final bacterin concentration of 5 x 10^{10} cfu ml^{-1}. Montanide™ ISA 763 AVG (Seppic) was used as a non-mineral oil-based adjuvant, in a bacterin: adjuvant ratio of 30:70. Emulsification was conducted with a high shear mixer.

Two vaccines were prepared for the seabass trials; **SV1**, aqueous administered as a dip and **SV2**, adjuvanted to be administered by IP injection. The non-adjuvanted aqueous bivalent vaccine (SV1) was prepared using strains NS and PDB in a bacterin ratio of 1:1 and sterile saline at final titer 10^{9} cells ml^{-1}. The adjuvanted bivalent vaccine (SV2) was prepared using strains NS and PDB in a bacterin ratio of 1:1. Montanide™ ISA 763 AVG (Seppic) was used as adjuvant as described previously. The final bacterin dose was 10^{8} cells ml^{-1}.

Fish vaccination

Zebrafish vaccination experiment was performed at the licensed facilities of the Fish Physiology Laboratory of the Department of Biology, University of Crete (EC91-BIObr-09 and EL91-BIOexp-10. Adult zebrafish (*Danio rerio*) of 0.340-0.370 g and 3-4 cm length were used. The fish were placed in tanks (200 L) with a fixed internal filter (EHEIM - compact 600) and Electric heater (Juwel AquaHeat 200 – Automatic Heater, 200 Watt, 220V-240V and 50 Hz). Temperature was maintained at 26-28°C and photoperiod was set as 12-hour light. Fish were fed (4% body weight) twice daily with commercial flakes (tropical fish flakes – Prodac).

Zebrafish were IP injected (0.01 ml/fish) with each monovalent vaccine (5×10^8 cells/fish) with a BD Micro-Fine+ syringe (0.5 ml, 30 G). Control groups received an equal volume of adjuvanted saline. In total 200 zebrafish were used.

Seabass vaccination experiments were performed at the licensed facilities (EL91BIOexp04) of the Institute of Marine Biology, Biotechnology and Aquaculture, HCMR. Experiments were conducted in either 250 L (juvenile fish) or 500 L (adult fish) tanks with continuous flow of borehole seawater and aeration, with an average water temperature of 19-20^{o}C. Vaccinated fish were fed once per day in specified time with commercial dry food.

Juvenile seabass fish (2.5 g) were vaccinated by immersion for 1 min in a vaccine solution of 10^9 cells ml^{-1}. Control group received no treatment. In total 300 juvenile fish were used in this experiment.

Adult seabass fish (60 g) were IP injected (0.1 ml/fish) with the bivalent vaccine (10^7 cells/fish). Control group received equal volume of adjuvanted saline. Two samplings were conducted corresponding to 30- and 60-days post vaccination to assess antibody titer. Blood was collected by caudal vein puncture (10 fish/group/time point and 5 fish from the control group/time point). Blood was allowed to clot at 4°C overnight and serum was collected by centrifugation (4.000 rpm, 15 min). Serum samples were stored at -80^{o}C until further analysis. In total 200 fish were used in this experiment.

All procedures which involved fish handling were implemented following guidelines provided by the authorized personnel (FELASA accredited certificate). Fish were not fed the day before manipulation. Anesthesia was achieved using tricaine methanesulfonate (MS222).

142
143
144 *Challenge*

145 Challenge experiments for both European seabass and zebrafish were conducted
146 at the challenge facilities of the Fish Physiology Laboratory, University of Crete.

147 Challenge on zebrafish was conducted five weeks (34 days) post-vaccination.
148 Fish were maintained in 6 L tanks with ventilated freshwater at a temperature of 26-
149 28°C and were fasted during the experiments. Vaccinated and control fish were
150 challenged by injection (0.01 ml/fish) with a bacterial dose of 10^5 cfu/fish with strains
151 NS and PDB. Challenge tests were performed with the homologous and the
152 heterologous stain for each vaccine. In total 72 (36/vaccine type) fish were used (12
153 fish/group; one group was the control and the other two were challenged with the
154 homologous and the heterologous strain, respectively). Mortality was recorded daily,
155 and dead fish were removed from tanks at constant time for up to 7 days post challenge.
156 Macroscopic clinical condition of dead fish was recorded daily.

157 Challenge tests on seabass were conducted 30 days post-vaccination. Infections
158 were carried out in 70 L tanks with borehole seawater and aeration and then, fish were
159 transported in 250 L tanks with ventilated, borehole seawater at a temperature of 18-
160 20^{o}C. Fish were fasted during the experiments. Vaccinated and control fish were
161 challenged by immersion for 2.5 h in a bacterial solution of 10^5 cfu ml^{-1} [3] of either
162 strain NS or PDB. For the experiment with the juvenile fish, 80 individuals were equally
163 distributed in 4 tanks. Two of the tanks contained fish vaccinated with SV1 dip vaccine
164 and two unvaccinated control. One tank of vaccinated fish and one unvaccinated control
165 were challenged with *Aeromonas veronii* strain NS while the other tank of vaccinated
166 fish with its corresponding unvaccinated control were challenged with the strain PDB.
167 For the experiment with the adult fish, 60 individuals were equally distributed in 6
168 tanks. Four tanks contained vaccinated fish (SV2 IP administered vaccine) and two
169 contained fish that had received only the adjuvant. Two tanks (duplicate) of vaccinated
170 fish and one tank of unvaccinated control were challenged with *Aeromonas veronii*
171 strain NS while the other two tanks of vaccinated fish with its corresponding
172 unvaccinated control were challenged with the strain PDB. Mortality was recorded
173 daily, and dead fish were removed from tanks for up to 14 days post challenge.
174 Macroscopic clinical condition of dead fish was recorded daily. Bacteria were

aseptically isolated from all dead fish, from the kidney, in general (TSA 2%) and Aeromonas selective medium (AIAA), followed by incubation for 48 h at 25°C.

Antibody response - seabass

An indirect ELISA was used to assess the specific antibody production of seabass in response to vaccination with SV2. The 96-well microtiter plates were coated separately with one of the sonicated bacterial lysates (NS or PDB) in a protein concentration of 5 μg ml^{-1}. Indirect ELISA was conducted according to manufacturer instructions and [25] with minor modifications. Samples were analyzed in log_2 serial dilutions from 1/50 up to 1/12800.

To determine the non-specific interactions, three wells/plate containing all, but serum served as negative controls. The mean value of the optical density (OD_{450}) of these wells was subsequently subtracted from samples' values. One sample from the vaccinated fish group served as positive control and was further used to normalize the variation between plates. Each sample was normalized according to the plate specific regulation factor: the ratio of the mean absorbance from the entire pool of positive control wells to the mean absorbance of the positive control wells on each plate.

To distinguish the positive from negative for antibodies samples a cut-off limit was calculated between the two states using the absorbance of the control group's serums. The mean value and the standard deviation of the resulting absorptions were calculated, and cut-off value resulted from the average of the absorptions by adding 3 times the standard deviation between them. The antibody titer for each sample was determined at the highest dilution factor that still gives a positive signal above the cut-off value. The antibody titer is equal to the inverse of the dilution factor. The binary logarithm (log_2) of the titer used in the statistical analyses was then calculated.

Statistical analysis

Kaplan-Meier survival analysis was performed and the x^2 Log Rank (Mantel-Cox) tests was used to estimate the difference between curves. Vaccine efficacy was estimated by the Relative Percent Survival (RPS) at the end of the challenge calculated according to the formula: RPS = [1 - (% mortality of vaccinated fish / % mortality of control fish)] × 100 [26] and by RPS_{60} defined as the survival of the vaccinated fish when mortality of the control group is 60%.

Nonparametric Kruskal-Wallis Analysis of Variance on Ranks was conducted to compare the antibody titer a) among the two antigens for each sampling day and b) for each antigen according to time. Survival curves and graphs were generated in GraphPad Prism v.8.0.1. Statistical analyses were performed in IBM SPSS Statistics v.23.

Results

The clinical signs observed in zebrafish challenged with strains NS and PDB included lethargy, loss of appetite, hemorrhages, pale body, and yellowish coloration. Pale body, hemorrhage and yellowish coloration in the caudal region were recorded in the affected fish (both vaccinated and control-naive) in all strains used in the challenges.

Several white globules presumably adjuvant deposits were observed in the intraperitoneal cavity of survived fish (vaccinated and control-adjuvant groups) after dissection. Internal organs were found adhesive to the surface of peritoneal muscle which was clearly visible through the stickiness of internal organs to the cavity (**Figure 1**).

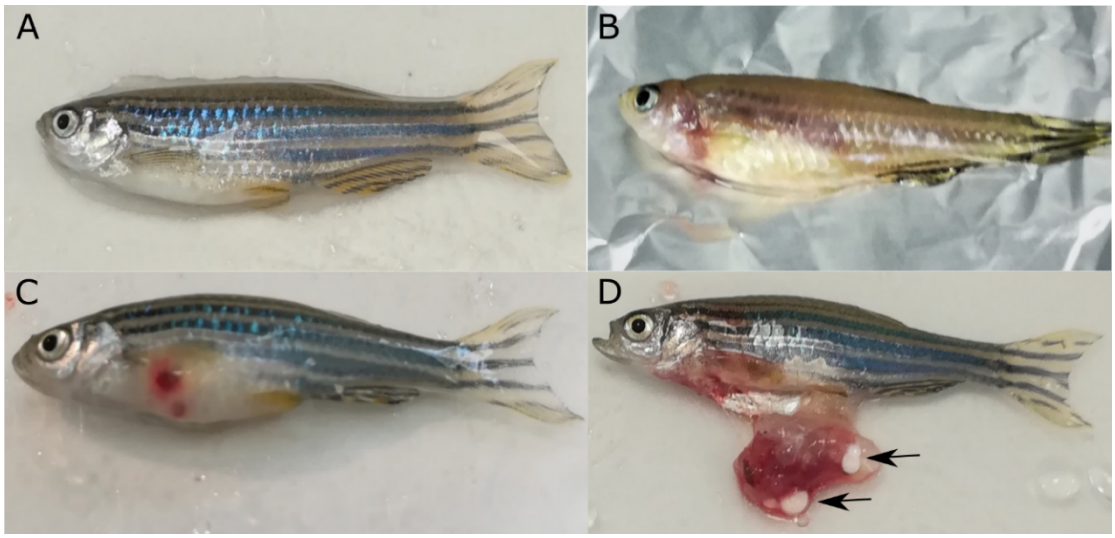

Figure 1. A) Zebrafish from the control group exhibiting normal appearance. B) Affected fish with pale body and yellowish coloration. C) External hemorrhages upon bacterial challenge. D) White vaccine deposits attached in the internal organs in some of the vaccinated fish.

The clinical signs observed in European seabass challenged with strains NS and PDB included lethargy, hemorrhages in skin, mouth, and fins and pale gills. Internally,

diffused hemorrhages were evident and ascitic fluid in the peritoneal cavity. Hemorrhages and abscesses were observed on the surface of the internal organs, mainly in the liver and spleen as well as splenomegaly. The clinical signs were similar in adult and juvenile fish (**Figure 2**), especially concerning the internal organs. Clinical signs developed in both vaccinated and control fish.

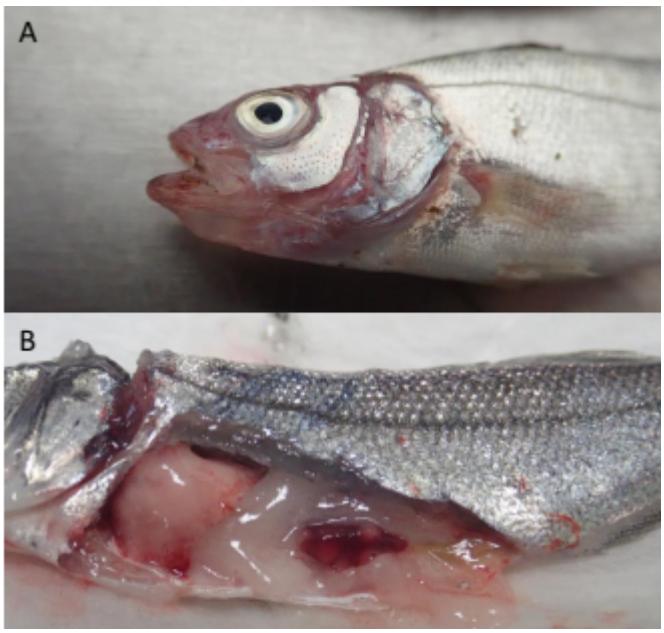

Figure 2. Juvenile European seabass exhibiting A) redness in the area of the head and the mouth and B) nodules in the spleen following challenge with *Aeromonas veronii* bv. *sobria.*

Vaccine efficacy

Zebrafish

Mortality when occurred, started on day 1 post-challenge in groups infected with strains NS or PDB (**Figure 3**). Mortality did not reach 100% of the population in any group during post-challenge observations (7 days). The highest mortality (50%) was observed in the control group challenged with strain NS followed by the one (33%) challenged with strain PDB. The lowest mortality (0%) was observed in ZV2 group challenged with the homologous (PDB) strain.

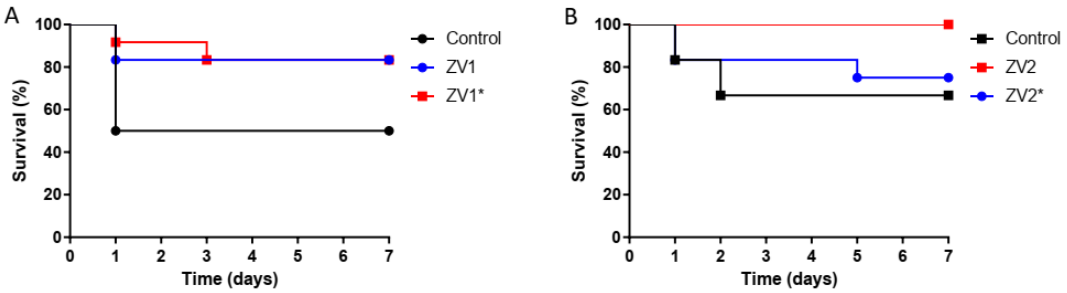

Figure 3. Cumulative daily survival (%) of A) zebrafish vaccinated with NS (ZV1) and B) vaccinated with strain PDB (ZV2) and challenged with the homologous and heterologous (with asterisk) strains for each vaccine.

In the Kaplan-Meier analysis, the PDB bacterin vaccinated (ZV2) group challenged with the homologous strain differed from the control group challenged with the same strain [χ^2 (1, N = 24) = 4.600, $p < 0.05$]. No differences were detected among homologous/heterologous challenges for both the vaccines, neither between the control groups [χ^2 (1, N = 24) = 0.854, p = 0.355]. The RPS value against homologous challenge at the end of the experiment (7 days) was 66% for ZV1 (NS) and 100% for ZV2.

Seabass – SV1

Mortality in groups challenged with strain NS, started on day 2 and 3 post-challenge for the control and the dip vaccinated (SV1) group, respectively (**Figure 4A**). Mortality in groups challenged with strain PDB started on day 3 and 5 post-challenge for the control and SV1 group, respectively (**Figure 4B**). Mortality did not reach 100% of the population in any group during the experiment (10 days). The highest mortality (95%) was observed in the control group challenged with strain PDB and the lowest (10%) in SV1 group challenged with the same strain.

In the Kaplan-Meier analysis, the group of vaccinated fish challenged with strain PDB differed from all groups. More specifically, the group of vaccinated fish challenged with PDB strain differed a) from control group challenged with strain PDB [χ^2 (1, N = 40) = 30.368, $p < 0.05$], b) from control group challenged with strain NS [χ^2 (1, N = 40) = 12.001, $p < 0.05$] and c) from the SV1 group challenged with strain NS [χ^2 (1, N = 40) = 15.343, $p < 0.05$]. No differences were detected among SV1 and control fish challenged with strain NS [χ^2 (1, N = 40) = 0.037, p = 0.847], nor among the two control groups each one challenged with either strain NS or PDB [χ^2 (1, N = 40) = 0.941, p = 0.332]. The RPS_{60} value for the bivalent vaccine was 27% against

strain NS and 87% against strain PDB. The RPS value at the end of the experiment (10 days) for the bivalent vaccine was 0% against strain NS and 88% against strain PDB.

Seabass – SV2

Mortality in control and IP vaccinated (SV2) groups challenged with strain NS started on day 6 post-challenge (**Figure 4C**). Mortality in fish challenged with strain PDB started on day 4 and 9 post-challenge in the control and SV2 group, respectively (**Figure 4D**). Mortality did not reach 100% of the population in any group during the experiment (14 days). The highest mortality (90%) was observed in the control group challenged with strain NS followed by the one challenged with PDB (80%) while the lowest (30%) was observed in the SV2 group challenged with strain PDB.

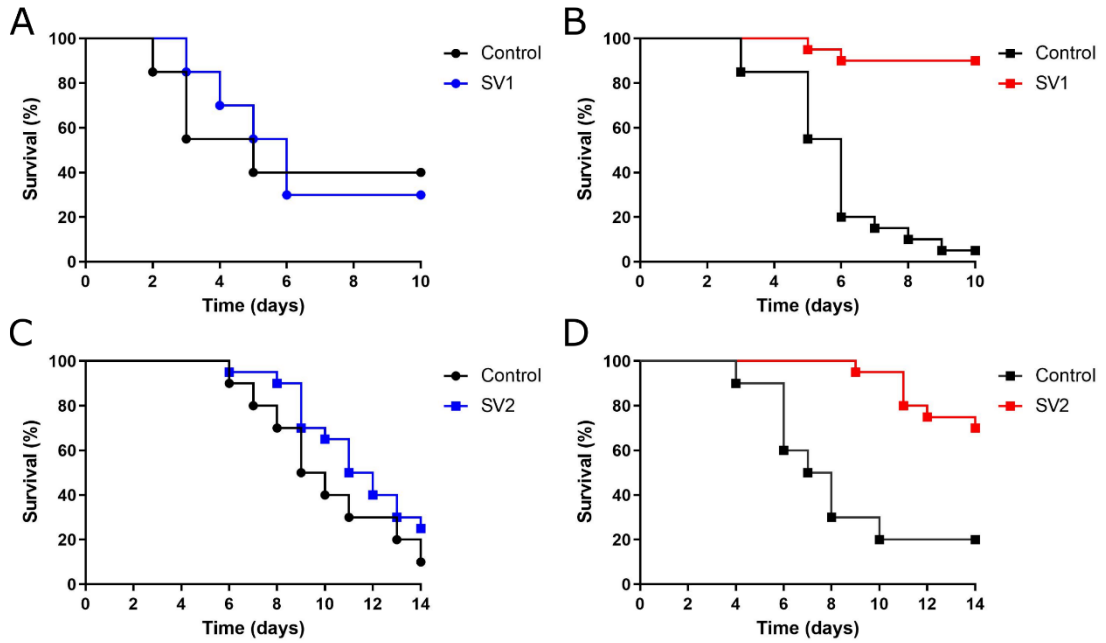

Figure 4. Cumulative daily survival (%) of dip-vaccinated (SV1) and control juvenile seabass challenged with A) strain NS and B) strain PDB. Cumulative daily survival (%) of vaccinated (SV2) and control adult seabass challenged with C) strain NS and D) strain PDB.

In the Kaplan-Meier analysis, the group of vaccinated fish challenged with strain PDB differed from all groups. In detail, the SV2 group challenged with strain PDB differed a) from control group challenged with strain PDB [χ^2 (1, N = 30) = 14.393, $p < 0.05$], b) from control group challenged with strain NS [χ^2 (1, N = 30) = 14.773, $p < 0.05$] and c) from the SV2 group challenged with strain NS [χ^2 (1, N = 40)

= 8.907, $p < 0.05$]. No differences were detected among SV2 and control fish challenged with strain NS [χ^2 (1, N = 30) = 1.522, p = 0.217], nor among the two control groups each one challenged with either strain NS or PDB [χ^2 (1, N = 20) = 0.316, p = 0.574]. The RPS_{60} value for the bivalent vaccine was 42% against strain NS and 100% against strain PDB. The RPS value at the end of the experiment (14 days) for the bivalent vaccine was 17% against strain NS and 63% against strain PDB.

Antibody titer – seabass – SV2

Specific antibodies for both antigens (NS, PDB) were detected on serum dilutions ranging between 1/50-1/6400 for day 30 post vaccination. On day 60 post vaccination antibodies for strains NS were detected in dilutions ranging between 1/50-1/1600 and for strain PDB between 1/100-1/6400. Mean antibody titers (log_2) for NS antigen were 9.8 ± 2.4 on day 30 and 8.2 ± 2.5 on day 60 post vaccination (**Figure 5**). Mean antibody titers (log_2) for PDB antigen were 10.8 ± 2.3 on day 30 and 10.4 ± 2.4 on day 60 post vaccination. No statistically significant differences were found in any of the tests performed to compare the antibody titer per antigen as a function of time as well as per time unit (day) between the two antigens.

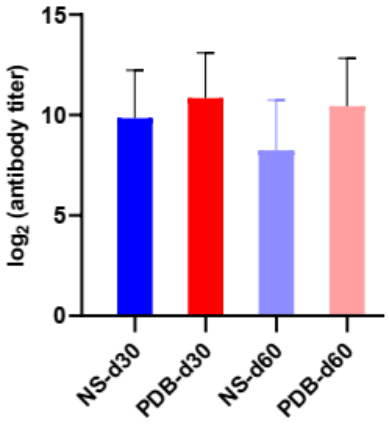

Figure 5. Antibody titer (log_2) of vaccinated (SV1) European seabass for each of the antigens (NS and PDB) on days 30 and 60 post vaccination.

Discussion

Autogenous vaccines can be considered only for the cases of absence of commercial vaccines for a specific pathogen. It has been suggested that autogenous vaccines can provide a viable and effective solution for aquaculture especially for the non-salmonid species for which licenced vaccines are scarce and can help to reduce drastically the dependency to antimicrobials [27]. Recent studies have shown that autogenous vaccines can be highly efficacious in zeroing mortalities even in challenging diseases like francisellosis in Nile tilapia [28]. Aeromonads are a typical example of such cases due to the multifactorial nature of their infectivity and the diversity of their antigenic proteins [22]. Autogenous products have been used for atypical *A. salmonicida* infections [29,30] and are also considered as a viable and promising solution for the well known mesophilic species *A. hydrophila* based on cost and effectiveness [23].

In the present study an autogenous bivalent vaccine for an *A. veronii* disease affecting farmed European seabass was tested for its efficacy considering the lack of commercial products for disease prevention for both the specific pathogen and the host species. The vaccine offered protection to European seabass against challenge with strain PDB (representing the dominant phenotype in the field) in adult and juvenile fish. Specific antibodies were detected for both antigens (NS and PDB) used, and titer remained stable from day 30 to 60 post vaccination. Furthermore, zebrafish served successfully as an alternative model to European seabass for vaccine efficacy studies.

Production of protective antibodies in European seabass is well known from vaccines for common pathogens such as for *V. anguillarum*, *P. damselae* subp. *piscicida*, *T. marinum*, and Betanodavirus [19,31–34]. Here, protection assessed in terms of RPS against challenge with strain PDB reached to 63% in adult IP and to 88% in juvenile dip-vaccinated European seabass. Although the antibody titer did not differ between the two antigens (NS and PDB), both SV1 and SV2 did not provide protection in adult or juvenile seabass against infection with the strain NS.

Both strains (NS and PDB) used here were isolated from the same farm in the same period and despite their high genetic, genomic, antigenic and virulence similarity they represent two different A. *veronii* phenotypic groups concerning their traits of motility and pigment production [4]. Strain PDB (motile, pigment producing)

represents the dominant phenotype in the field while strain NS (non-motile, non-pigment producing) has progressively become almost non-detectable in the area. Since European seabass produces antibodies to *A. veronii*, protective for some (PDB) strains, but not for others (NS), these should somehow differ in their antigenic and/or virulence properties. For example, the polar flagellum of *Aeromonas* spp. plays a critical role in biofilm formation and host cell adhesion, thus contributing to bacterial infectivity as suggested for *A. caviae* and *A. hydrophila* tested in human cell lines [35–37]. On the other hand, phagocytes can detect and respond to motility per se [38], and in that case, strain NS would have an advantage comparing to PDB in escaping immune defenses in the initial stage of infection.

As reported elsewhere, specific antibodies may be detected in response to vaccination but that does not necessarily imply protection against infection, at least not without booster vaccination as in the case of *A. salmonicida* in trout (*Salmo trutta*) [39]. Similarly, in European seabass fry, an immersion administered vaccine for *V. anguillarum* provided protection against injectable infection (at 298 dph) after booster vaccination while maximum protection (near 100%) was achieved after the third IP administration of the vaccine [32]. The immune response did not differ from the control group until 165 dph with the single vaccine administration. The necessity of booster vaccination against common pathogens such as *V. anguillarum* has been highlighted in other studies proposing the onset of vaccination of European seabass at 1 g by immersion and at least one more vaccination by injection or immersion (>5 g) [40,41]. The same has been suggested for larger seabass (40 g) in vaccines against *V. alginolyticus* and *V. parahaemolyticus* [42]. According to the results obtained here for strain PDB, the implementation of a vaccination strategy that includes booster vaccination presents high prospects of protection against the *A. veronii* disease and would shed light on the prospects of protection with NS bacterin as an antigen. In addition to this, the antigen dose that has been positively correlated with antibody response and protection levels in other studies e.g. salmon furunculosis vaccines [43] could also be elevated in the future in order to test for a higher vaccine efficacy.

Specific antibodies for both antigens (NS and PDB) were detected up to day 60 post-vaccination and the titer remained stable in the time frame studied between 30- and 60-days post-vaccination. On day 30 post-vaccination antibody titer ranged between 50-6400. Similarly, in adult European seabass (220 g) antibody titer ranged between 6.000 - 8.000, 14 days post-booster immunization with a commercial bacterin

inactivated, adjuvanted (Freund's complete adjuvant), intramuscularly administered vaccine for *V. anguillarum-ordalii* (10^{10} cells/fish) [44].

Comparing to other studies, *A. veronii* bacterin vaccines in different formulations resulted in promising results concerning protection especially concerning the injection challenges applied. Intraperitoneal (IP) injection of inactivated or ghost cells (2×10^7 cells/fish) of strain 7231 (isolated from ulcered *Cyprinus carpio*) with booster vaccination applied on day 14, offered protection to *Cyprinus carpio* (35 g) with an RPS of 43% and 74% respectively, after intramuscular injection challenge (3×10^6 cfu/fish) with the pathogen, 42 days post (first) vaccination [45]. In other study also in *C. carpio* (30 - 40 g), IP injectable, bacterin inactivated vaccine (10^8 cells/fish) supplemented with Freund's adjuvant, with booster vaccination applied on day 14, offered protection against IP injection challenge with *A. veronii* (4×10^5 cfu/fish) with an RPS of 70%, 28 days post (first) vaccination when antibody levels peaked [46]. The strain TH0426 used in the vaccine was originally isolated from *Pelteobagrus fulvidraco* [47]. In *Misgurnus anguillicaudatus* (6 g), injectable vaccine of live-attenuated *A. veronii* (TH0426) (2×10^6 cfu/fish) with booster vaccination applied on day 14, offered protection against IP injection challenge (equivalent to vaccine dose) 42 days post (first) vaccination with an RPS of 66% [48]. In the same study the same vaccine applied by immersion (2×10^7 cfu ml^{-1}) resulted in an RPS value of 51%.

It has already been demonstrated that zebrafish is susceptible to *A. veronii* infections [49,50] and LD50 values for strains NS and PDB used here have been reported as 4.2×10^5 and 2.4×10^6 cfu/fish respectively [4]. Furthermore, recent studies support the use of zebrafish in vaccine efficacy studies in aquaculture such as testing for cross-protection among *Streptococcus* spp. [51], antigen uptake mechanism among adult and juvenile fish of a *Yersinia ruckeri* immersion vaccine [52] and for screening of DNA-based vaccine candidates against *Mycobacterium* infections [53]. Here, the zebrafish model used for the efficacy study of the autogenous *A. veronii* vaccine for European seabass successfully predicted the potential of protection using adjuvanted bacterins. On the other hand, the model failed to predict the lack of cross-protection among the two strains as shown in ZV1 challenge. In terms of strain selection, no conclusion can be made as long as the challenge dose was adapted to the lowest one (10^5 cfu/fish) favoring that way the group infected with strain PDB.

One important difference between the vaccine efficacy assessment in the two fish species used in this study is the water temperature during experimentation. In

zebrafish vaccination efficacy was assessed at higher temperature (26-28^{o}C) whereas in European seabass in lower (18-20^{o}C). The selection of these temperature range was based on the physiological preferences of the two species but specifically for the European seabass was based also on the water temperature in which vaccination is usually performed in aquaculture. The difference in water temperature may affect both the virulence of the pathogen and the immunocompetence of the fish host [54]. In a previous study comparing antibody response of European seabass reared at temperatures ranging from 12 to 30^{o}C it showed that the optimum temperatures for high and prolonged antibody response was 24^{o}C followed by 18^{o}C [55]. On the other hand, *Aeromonas veronii* infections in European seabass are observed when water temperature increases above 18^{o}C and mortalities peak when temperature is above 24^{o}C [3]. Therefore, an efficacious vaccination program should be implemented considering these parameters and fish should be vaccinated at temperature that might not be optimum, but rather timely to result in a response synchronized with the expected season of the infection.

The results of the present study encourage the use of autogenous vaccines to prevent the *A. veronii* disease in farmed European seabass. The administration of a single vaccination gave protection against infection with strain PDB for at least 30 days post-vaccination in adult and juvenile fish. Further studies on vaccination strategy (e.g. booster administration), antigen dose and duration of protection are necessary in order to achieve higher efficacy and expand the time span of protection. Finally, results show the constraints that may appear in vaccine development especially in urgent circumstances and emerging pathologies when lacking the necessary availability of animal models and facilities for fast and reliable screening of strains/antigens.

Ethics statement

All experiments involving animals were conducted in licensed facilities by FELASA accredited staff. All protocols were approved by the competent authority with the license numbers: 255349 (29/11/2017) and 147115 (17-07-2017) according to the national and European legislation.

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