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Aeromonas salmonicida infection kinetics and protective immune response to vaccination in sablefish (*Anoplopoma fimbria*)

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ABSTRACT

Effective vaccine programs against *Aeromonas salmonicida* have been identified as a high priority area for the sablefish (*Anoplopoma fimbria*) aquaculture. In this study, we established an *A. salmonicida* infection model in sablefish to evaluate the efficacy of commercial vaccines and an autogenous vaccine preparation. Groups of 40 fish were intraperitoneally (ip) injected with different doses of *A. salmonicida* J410 isolated from infected sablefish to calculate the median lethal dose (LD_{50}). Samples of blood, head kidney, spleen, brain, and liver were also collected at different time points to determine the infection kinetics. The LD_{50} was estimated as $\sim 3 \times 10^5$ CFU/dose. To evaluate the immune protection provided by an autogenous vaccine and two commercial vaccines in a common garden experimental design, 140 fish were PIT-tagged, vaccinated and distributed equally into 4 tanks (35 fish for each group, including a control group). Blood samples were taken every 2 weeks to evaluate IgM titers. At 10 weeks post-immunization, all groups were ip challenged with 100 times the calculated LD_{50} for *A. salmonicida* J410. *A. salmonicida* was detected after 5 days post-infection (dpi) in all collected tissues. At 30 days post-challenge the relative percentage survival (RPS) with respect to the control group was calculated for each vaccine. The RPS for the bacterin mix was 65.22%, for Forte Micro 4® vaccine was 56.52% and for Alpha Ject Micro 4® was 30.43%, and these RPS values were reflected by *A. salmonicida* tissue colonization levels at 10 days post-challenge. Total IgM titers peaked at 6–8 weeks post-immunization, where the autogenous vaccine group showed the highest IgM titers and these values were consistent with the RPS data. Also, we determined that the *A. salmonicida* A-layer binds to immunoglobulins F(ab)' in a non-specific fashion, interfering with immune assays and potentially vaccine efficacy. Our results indicate that vaccine design influences sablefish immunity and provide a guide for future sablefish vaccine programs.

1. Introduction

The sablefish (*Anoplopoma fimbria*), also referred to as “Black cod”, was originally described by Pallas in 1814 [1,2]. Wild sablefish are found in the Pacific Ocean as far north as the Bering Sea and as far south as Japan and California, with adult sablefish living on the continental shelf and slope at depths of approximately 1,500 m [2]. The sablefish is considered one of the most valuable fish species on Canada's west coast, with a value of US$12.25 per lb [3]. In addition, there is currently an emerging aquaculture for this species in the North Pacific region, with more than 74% of Canada's farmed sablefish exported to Japan. Other important markets include the United States and the United Kingdom [1,2,4]. Also, sablefish farming is attracting interest in South Korea [5] and the USA [3,6]. The decrease in wild sablefish stocks [7], increasing consumer demand [8] and market value [4], positioning sablefish farming as an important aquaculture.

The development of protocols and techniques for sablefish aquaculture have been on-going since 1984 [9] and this species has significant potential for future growth as an aquaculture species. For example, sablefish have one of the fastest industrial growth rates of all

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teleost species [10], and juveniles grown in marine net pens can reach commercial size (3–4 kg) in 2 years [9]. However, during the period in marine net pens the fish are exposed to infectious diseases [11]. *A. salmonicida* is the most common pathogen isolated from cultured sablefish in British Columbia, Canada [6], but *Vibrio anguillarum* outbreaks have also been reported [12], both having negative impacts on production costs.

A. salmonicida, one of the oldest marine pathogens known, is a Gram-negative pathogen that causes furunculosis in different cultured fish species [13,14], including sablefish [6]. Effective vaccine programs against *A. salmonicida* are a high priority area for the sablefish aquaculture. Vaccination is an important management strategy that reduces disease outbreaks and minimizes the use of antibiotics [15–17]. Currently, there are no effective commercial vaccines specifically developed for sablefish. Two commercial vaccines are beginning to be used in the sablefish industry, Alpha Ject Micro 4® (PHARMAQ, Norway) developed for Atlantic salmon of 15 g or larger, which has *A. salmonicida*, *Listonella* (*Vibrio*) *anguillarum* serotypes O1 and O2, and *Vibrio salmonicida* [18], and Forte Micro IV® (Elanco Canada Limited, Canada) developed for salmonids of 10 g or larger, which has inactivated Infectious Salmon Anaemia Virus, *A. salmonicida, V. anguillarum* serotypes I and II, *V. ordalii,* and *V. salmonicida* serotypes I and II in liquid emulsion with an oil based adjuvant [19]. Recently, an autogenous polyvalent vaccine was successfully evaluated against *A. salmonicida* in sablefish in the USA, and it was reported that an autogenous bacterin preparation injected intraperitoneally was more effective than bath vaccination [6]. However, Canadian regulations do not allow the importation of autogenous vaccines [20], and current vaccines available in Canada against *A. salmonicida* have not been evaluated in sablefish.

In this study, an *A. salmonicida* infection model was established in cultured sablefish, and we used this model to compare the immune protective response provided by an *A. salmonicida* autogenous monovalent vaccine preparation (bacterin mixture) and two polyvalent commercial vaccines (Alpha Ject Micro 4®, Pharmaq and Forte Micro®, Elanco) against an atypical *A. salmonicida* challenge.

2. Material and methods

2.1. Bacterial strains, media, and reagents

A. salmonicida J409, J410 and J411 strains were isolated from infected cultured sablefish and identified using standard phenotypic tests [21], 16S gene sequencing [22], and whole genome sequencing using established protocols [23]. Additionally, *A. salmonicida* typical isolates J223 (VapA$^+$) and J227 (VapA$^-$, A-layer mutant) were utilized as controls (lab collection). *A. salmonicida* strains were routinely grown in Trypticase Soy Broth (TSB) media (Difco, Franklin Lakes, NJ) from a single colony at 15 °C with aeration (180 rpm) in an orbital shaker, according to previous descriptions [24]. When required, TSB was supplemented with 1.5% bacto agar (Difco) and 0.02% Congo-red (Sigma-Aldrich, USA). Bacterial growth was monitored by spectrophotometer and by plating to count colony forming units (CFU/ml) [25]. Bacterial cells were harvested at mid log phase (OD_{600nm}–0.6–0.7) by centrifugation (4,200 × *g* for 10 min at 4 °C).

2.2. Bacterin preparation

The *A. salmonicida* J409 (Accession number: CP047374-75), J410 (Accession number: CP047376-77), and J411 (Accession number: SUB6785506) strains were grown independently in TSB media supplemented with 100 μM of 2, 2′-dipyridyl at 15 °C with aeration (180 rpm) to induce the expression of immunogenic outer membrane proteins (e.g., iron regulated outer membrane proteins) [16] up to an optical density (O.D. 600 nm) of 0.7 (~1 × 10^8 CFU/ml). The bacterial cells were washed three times by centrifugation (4,200 × *g* for 10 min at 4 °C) with phosphate buffered saline (PBS; 136 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4 (pH 7.2)) [21] and then fixed with 6% formalin for 3 days at room temperature with gentle agitation. Formalin was removed by centrifugation at 4,200 × *g* for 10 min at 4 °C and the bacterin mix was resuspended in PBS. Inactivated cells were dialyzed (Molecular weight cut off 3.5 kDa; Spectra/Por, CA) in 1 L of PBS three times at 4 °C with gentle stirring. Cell inactivation was determined by plating in TSB and TSA before and after dialysis. Finally, the three strains were mixed in equal quantities, and the bacterin mix was quantified using flow cytometry and the Bacteria Counting Kit (ThermoFisher, USA) according to the manufacturers' instructions. A BD FACS Aria II flow cytometer (BD Biosciences, CA) and BD FACS Diva v7.0 software were used for bacteria cell quantification. The number of bacterial cells/ml was calculated by dividing the number of signals in the bacterial frame by the number of signals in the microsphere frame (Fig. S1) as described previously [26]. The bacterin mix was stored at 4 °C at a concentration of 3.5 × 10^{10} CFU/ml until utilization.

2.3. Ethics statement

All animal protocols required for this research were approved by the Institutional Animal Care Committee and the Biosafety Committee at Memorial University of Newfoundland (MUN). Animals assays were conducted under protocols #16-92-KG, #18-01-JS #18-03-JS, and biohazard license L-01.

2.4. Fish origin and holding conditions

Cultured **s**ablefish juveniles (~1 g) were provided by Golden Eagle Sablefish, British Columbia, Canada. Upon arrival, the fish were acclimated to ~8–10 °C in 500 l tanks supplied with 95–110% air saturated and UV treated filtered flow-through seawater, and an ambient photoperiod, at the Cold-Ocean and Deep-Sea Research Facility (CDRF, MUN) for quarantine. Following veterinary clearance, the animals were transferred to the Dr. Joe Brown Aquatic Research Building (JBARB), Memorial University. Fish were subsequently reared under the previously described conditions. Tank biomass was maintained at < 30 kg m^{-3}, and the fish were fed daily using automatic feeders and a commercial diet (Skretting – Europa; 15 crude protein (55%), crude fat (15%), crude fiber (1.5%), calcium (3%), phosphorus (2%), sodium (1%), vitamin A (5000 IU/kg), vitamin D (3000 IU/kg) and vitamin E (200 IU/kg)) at 1.5% body weight per day.

2.5. Determination of the LD_{50} of A. salmonicida J410 in sablefish

Sablefish (215.5 ± 3.2 g) were transferred from the JBARB to the CDRF, separated into six 500 l tanks containing 10 and 30 fish per selected dose (see Fig. S2) and acclimated for 2 weeks under previously described conditions. The lethal dose 50 (LD_{50}) of *A. salmonicida* J410 was evaluated in these fish according to established protocols [27]. Briefly, the fish were anesthetized with 40 mg of MS222 (Syndel Laboratories, BC, Canada) per l of sea water and ip infected with 100 μl of 10^4, 10^6, or 10^7 CFU per dose. Three of the six tanks were utilized for monitoring mortality while the remaining three tanks were used to determine *A. salmonicida* tissue colonization and pathogen re-isolation (Fig. S2). The LD_{50} was calculated using the formula: $LD_{50} = 10^{a-(PD)}$, where a = $\log_{10}$ (dilution factor of mortality > 50%); Proportional Distance (PD) = $\frac{L\% - 50\%}{L\% - H\%}$; L% = Dilution point of mortality < 50%; H % = Dilution point of mortality > 50% [28].

2.6. Tissue sampling and analysis

Fish were netted and immediately euthanized with an overdose of MS222 (400 mg/l). Tissue samples from liver, spleen, head kidney and brain were aseptically removed from all groups at 5 and 10 dpi with *A.*

salmonicida J410 and placed into sterile homogenizer bags (Nasco whirl-pak®, USA). Thereafter, they were weighed and homogenized in PBS up to a final volume of 1 ml (weight: volume), serially diluted (1:10) and plate counted onto TSA-Congo red plates. The plates were incubated at 15 °C for 5 days to determine the CFU of *A. salmonicida* J410 per g of tissue. Total bacteria were normalized to 1 g of tissue according to the initial weight of the tissue using the following formula: $\mathrm{CFU{*}g^{-1}} = \frac{\text{colony forming units (CFU)} * \text{original tissue weight (g)} * 1\ \mathrm{ml^{-1}} * 1\ \mathrm{g^{-1}})}{\text{original tissue weight (g)}}$.

2.7. Histopathology

Tissue sections were fixed in 10% formalin diluted in PBS for three days at room temperature. The formalin was then removed, and the samples were stored in 100% ethanol at 4 °C until block processing according to established procedures [29]. Sectioned tissues for histology were stained with hematoxylin and eosin, and with Giemsa (Leica Biosystems, Canada), and visualized under the light microscope (Olympus CX21, USA).

2.8. Sablefish immunization using a common garden experiment

Sablefish (125.45 ± 0.5 g) were Passive Integrated Transponder (PIT)-tagged and acclimated for 2 weeks at ~8–10 °C before immunization. After this period, independent groups of 35 fish were starved for 24 h, and ip immunized with 100 μl of the *A. salmonicida* bacterin mix (10^9 CFU/dose; autogenous vaccine), Alpha Ject Micro 4®, Forte Micro® or PBS (control group). Alpha Ject Micro 4® (Pharmaq, Norway) is an oil-based vaccine containing *A. salmonicida*, *V. anguillarum* and *Vibrio salmonicida* formalin killed strains, whereas Forte Micro® (Elanco, Canada) is an oil-based vaccine containing formalin inactivated cultures of *A. salmonicida*, *V. anguillarum* serotypes I and II, *Vibrio ordalii*, and *V. salmonicida* serotype I and II. The immunization of fish with commercial vaccines was conducted following the manufacturer's instructions, and used to evaluate, and compare, the effectiveness of the bacterin mix. Fish were distributed randomly into four different tanks with an equal proportion of each group (Fig. S3). Fish length and weight were monitored every 2 weeks to determine the specific growth rate (SGR) [30] according to the formula: (ln(final weight (g)) − ln(initial weight (g)) × 100)/(time (days)). Non-lethal blood samples were taken every 2 weeks from a selected single tank. Each tank was sampled only once during the immunization assay (Fig. S3).

2.9. Challenge of immunized sablefish

The infection procedures were conducted in the AQ3 biocontainment facility at the CDRF. At 9 weeks post-immunization fish were transferred to the AQ3 biocontainment facility and acclimated for 1 week under previously described optimal conditions. After this period, sablefish (~200 ± 0.5 g) were ip challenged with 100 times the LD_{50} dose for *A. salmonicida* J410 (10^7 CFU/dose). Mortality was monitored daily. The relative percent survival (RPS) [31] of vaccinated fish was calculated according to the formula: $RPS = \left(1 - \frac{\%\ vaccinated\ mortality}{\%\ control\ mortality}\right) \times 100$.

2.10. Sablefish IgM purification

To produce anti-sablefish IgM and determine the IgM titers of sablefish post-immunization, IgM from sablefish was purified according to previously described protocols for other Teleostei with modifications [32]. Briefly, IgM was purified from fresh pooled sablefish serum using an immobilized mannan binding protein (MBP) column kit (Pierce™, ThermoFisher, USA) according to the manufacturer's instructions, except that 200 ml of serum were used instead of 1 ml. The integrity and purity of the sablefish IgM was evaluated by SDS-PAGE 10% [21], and quantified by DirectUV and the bicinchoninic acid (BCA) standard method (Pierce™, BCA Proteins Assay Kit, USA) using spectrophotometry (Genova-Nano spectrophotometer, Jenway, UK). High quality IgM fractions were pooled and dialyzed against 20 mM Tris-HCl (pH = 8.0) twice at 4 °C with gentle agitation using a dialysis cassette (10,000 Da cutoff, Thermo Scientific, USA). After dialysis, the purified sablefish IgM was lyophilized (EdwarDS-Super Modulyo, Boc Ltd, England). Sablefish IgM was resuspended in 20 mM Tris-HCl (pH = 8.0). The final IgM preparation was re-evaluated for integrity using SDS-PAGE 10%. Chicken IgY anti-sablefish IgM antibody was produced, purified, and biotinylated commercially at Somru BioScience Inc. (Charlottetown, PE, Canada).

2.11. Immunohistochemistry

Immunohistochemistry (IHC) for CD10 was performed using a Ventana Benchmark Ultra automated immunostainer (La Roche, Switzerland) in the Department of Anatomical Pathology, General Hospital, Eastern Health, St. John's NL, on paraffin sections of sablefish head kidney, spleen, brain and human tonsil applied to positively charged slides using rabbit monoclonal IgG antibody clone SP67 directed against human CD10. Sections were processed on the automated immunostainer using citrate-based (CC1, La Roche, Switzerland) antigen retrieval at 100 °C for 64 min followed by 32 min of incubation with either anti-CD10 antibody or a rabbit IgG as negative control (La Roche, Diagnostics 790–4795), and detected using Ultraview (La Roche, Switzerland) and counterstained with hematoxylin. For anti-*A. salmonicida* and anti-sablefish IgM IHC, we used an anti-VapA *A. salmonicida* rabbit IgG antibody pre-adsorbed with *A. salmonicida* $VapA^-$ outer membrane proteins, and an anti-sablefish-IgM chicken IgY antibody custom produced in collaboration with Somru BioScience. Anti-*A. salmonicida* and anti-sablefish-IgM antibodies were applied at a 1:500 dilution using previously described IHC procedures [33], except that an alkaline phosphatase-conjugated anti-IgY secondary antibody was used at 1:250 dilution to develop the IgM staining reaction.

2.12. Confocal microscopy and immune fluorescence visualization

It has been reported that *A. salmonicida* possesses an A-layer (extracellular membranal protein array) that binds to antibodies [34,35]. Confocal microscopy was used to evaluate the non-specific binding of different immunoglobulins to the *A. salmonicida* A-layer. *A. salmonicida* strains J409, J410, J411 and J223 expressing the A-layer ($VapA^+$) [36] and J227 (an A-layer mutant ($VapA^-$)) were utilized. The strains were grown until logarithmic phase and stained with 5-([4,6-dichlorotriazinyl] amino) fluorescein hydrochloride (DTAF) solution (100 μg in dimethyl sulfoxide (DMSO); Sigma, USA) according to established protocols [36] and 4′,6-diamidino-2-phenylindole (DAPI; ThermoFisher, USA) according to the manufacturer's instructions. Biotinylated IgY anti-sablefish IgM was labeled with conjugated avidin Texas-Red (Life Technologies, USA) and utilized to asses non-specific binding to the *A. salmonicida* A-layer. Additionally, goat anti-mouse F(ab)'2-FITC labeled IgG (CellLab, USA) was utilized. *A. salmonicida* strains labeled with DTAF, DAPI, and IgY-Texas red or *A. salmonicida* strains labeled with DAPI and IgG-F(ab)'-FITC were visualized with a Nikon AR1 laser scanning confocal microscope.

2.13. Direct enzyme linked immunosorbent assay (dELISA)

We verified that high affinity IgY anti-sablefish IgM binds strongly to the *A. salmonicida* A-layer in a non-specific fashion. Therefore, total sablefish IgM titers were evaluated after immunization using dELISA.

The sablefish serum samples were heat treated at 56 °C for 30 min to inactivate the complement and subsequently treated with 100 μl of chloroform (Sigma-Aldrich, USA) for 10 min at room temperature to remove fats. After this period, the samples were centrifuged at 4,000 g

for 10 min at room temperature, and the supernatant was transferred to a clean tube and stored at −80 °C until IgM titer determination. Two hundred μl of pre-treated sablefish serum was serially diluted (1:25) in coating buffer (0.015 mM Na_2CO_3; 0.035 mM $NaHCO_3$; pH 9.8) and added to 96 well plates (Ultra-High Binding Polystyrene Microtiter, ThermoFisher, USA). The plates were incubated at 4 °C overnight, washed 3 times with PBS-Tween (PBS-T; 0.1%) and blocked with 150 μl of ChonBlock™ (Chondrex, Inc., WA, USA) for 1 h at 37 °C. After this period the plates were washed 3 times with PBS-T, inoculated with 100 μl of the secondary antibody (IgY anti-sablefish IgM; 1:10,000), and incubated at 37 °C for 1 h. Following incubation and washing, 100 μl of streptavidin-HRP (Southern Biotech; 1:10,000) was added, and the plates were incubated at 37 °C for 1 h. For visualization and color development 120 μl of 1X TMB (Invitrogen, Austria) - H_2O_2 (ratio 1:5) was added and the plates were incubated at room temperature (20-22 °C) for 30 min in darkness. Optical density was determined at 450 nm after adding 50 μl of stop solution (2 M H_2SO_4). IgM titers were evaluated in naïve animals (10 fish) and after 2, 4, 6, and 8 weeks post-immunization (8–9 fish at each time point) (Fig. S3, Table S1).

The standard curve was developed using established protocols [21,37]. Briefly, purified sablefish IgM was serially diluted in coating buffer to 50, 25, 12.5, 6.25, 3.125, and 1.563 μg/ml and incubated overnight at 4 °C. Each concentration was evaluated in triplicate (Table S1). After incubation and washing, 100 μl of IgY anti-sablefish IgM (1:10,000) was added and incubated at 37 °C for 1 h. Following incubation and washing, 100 μl of Streptavidin-HRP (Southern Biotech, USA) (1:10,000) was added and incubated at 37 °C for 1 h. For visualization and color development 120 μl of 1X TMB (Invitrogen, Austria) - H_2O_2 (ratio 1:5) was added and incubated at room temperature (20-22 °C) for 30 min in darkness. Optical density was determined at 450 nm after adding 50 μl of stop solution (2 M H_2SO_4). The values were normalized using a natural logarithm standard curve of knowns concentrations.

2.14. Statistical analysis

All data are displayed as means ± standard error (SE). Assumptions of normality and homogeneity were tested for detected variances. For survival curves, one-way ANOVA analysis was used followed by Tukey's post-hoc tests to determine significant differences between treatment groups ($p < 0.05$). The Kaplan-Meier estimator was used to obtain survival fractions after the challenges, and the Log-rank test was used to identify differences between treatments groups. For ELISA IgM titers, a two-way ANOVA multi-comparison analysis was performed to determine significant differences between treatments. All statistical analysis was performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, California, USA).

3. Results

3.1. LD_{50} determination and A. salmonicida infection kinetics in sablefish

Three different groups of 30 fish were injected with three different doses of *A. salmonicida* J410 (10^4, 10^6 and 10^7 CFU/dose) (see Fig. S2) to determine the LD_{50}. After 5 dpi, symptoms of furunculosis and internal petechial hemorrhaging was observed (Fig. 1B). Mortality began within 7–10 dpi, reaching 97% in the fish infected with the 10^7 CFU/dose, and 94% in the fish infected with 10^6 CFU/dose after 30 days. In contrast, the fish infected with the lowest dose tested (10^4 CFU/dose) showed 7% mortality (Fig. 1A) in the absence of furunculosis symptoms. Using this data, the LD_{50} for *A. salmonicida* J410 in sablefish was determined as $\sim 3 \times 10^5$ CFU/dose (Table 1).

Three different groups of 10 fish each were injected with three different doses of *A. salmonicida* J410 (10^4, 10^6 and 10^7 CFU/dose) to determine the bacterial colonization at 5 and 10 dpi in different tissues (Fig. S2). We did not detect bacteria in the spleen at 5 dpi in sablefish injected with the lowest dose (10^4 CFU/dose) (Fig. 1C). However, one of five fish injected with the lowest dose of *A. salmonicida* presented bacteria in the head kidney, bacteremia was observed in three of five fish sampled, and all the fish sampled presented meninge-encephalitis and liver colonization (Fig. 1C). Fish injected with 10^6 and 10^7 CFU/dose showed bacterial colonization in all tissues sampled at 5 dpi (Fig. 1C). At 10 dpi all the tissues sampled showed bacterial colonization (Fig. 1D).

3.2. Expression of A. salmonicida, IgM and CD10 in A. salmonicida infected sablefish tissues

To explore the distribution of *A. salmonicida* IgM and CD10 in *A. salmonicida* infected tissues, we performed immunohistochemical analyses of sablefish tissue sections collected at 0 and 10 dpi. Giemsa histological staining revealed pathological changes in several tissues at 10 dpi including general tissue disorganization and cellular dysplasia in the head kidney, spleen and brain, and hemorrhaging in the head kidney and spleen (Fig. 2). Immunohistochemistry revealed that by 10 dpi the expression of *A. salmonicida* and IgM was in general increased in the head kidney, spleen and brain. Interestingly, the expression of CD10 appeared to decrease within infection in spleen and brain but increased slightly in the head kidney (Fig. 2).

3.3. Vaccine challenge

One hundred and forty fish were challenged 8 weeks post-immunization with 100 times the LD_{50} dose (1×10^7 CFU/dose) of the bacterin mix. Mortality began within 8–10 days post-challenge. PBS (mock immunized) fish showed 76.67% mortality (Fig. 3A). Based on these results, the relative percentage survival (RPS) for the three vaccine treatments was determined. Alpha Ject Micro 4® conferred an RPS of 30.43%, Forte Micro® conferred an RPS of 56.52% and the autogenous bacterin mix conferred an RPS of 65.22% (Fig. 3A and Table 1).

Bacterial loads were determined after 10 days post-challenge. *A. salmonicida* was detected in all tissues in mock immunized (control) fish, and in most of the tissue samples from Alpha Ject Micro 4® and Forte Micro® immunized fish (Fig. 3B). Fish immunized with Alpha Ject Micro 4® and Forte Micro® showed similar bacterial loads (Fig. 3B). In contrast, only one fish immunized with the *A. salmonicida* bacterin mix showed bacterial colonization in the liver, head kidney and brain (Fig. 3B), and only two fish showed *A. salmonicida* colonization in the spleen and blood (Fig. 3B).

3.4. IgM titers in immunized sablefish

Sablefish immunoglobulin M (IgM) was purified by an immobilized mannan binding protein (MBP) column from 200 ml of fresh serum. Following purification, the sablefish IgM was concentrated by lyophilization, and we obtained 3,573 μg/ml of IgM. The purified sablefish IgM was visualized by SDS-PAGE 10% under reducing conditions, and displayed a heavy (~75 kDa) and a light chain (~24 kDa) (Fig. 5A), similar to other fish IgM [38,39].

An indirect ELISA against the whole *A. salmonicida* cell was initially utilized to determine the IgM titers. However, we determined that *A. salmonicida* binds to the secondary IgY chicken antibody and additionally to goat IgG F(ab)' in a non-specific fashion (Fig. 4A). Using an *A. salmonicida* A-layer mutant strain we determined that this Ig non-specific binding is dependent on the A-layer. These results indicate that the *A. salmonicida* A-layer interferes with the determination of *A. salmonicida* specific IgM titers (Fig. 4A). Therefore, we determined the total plasma IgM titers using direct ELISA.

We developed a standard curve using different concentration (50, 25, 12.5, 6.25, 3.125, and 1.563 mg/ml) of purified sablefish IgM (Table S1). IgM concentrations were standardized using natural logarithm (ln). The linear regression equation was determined to be

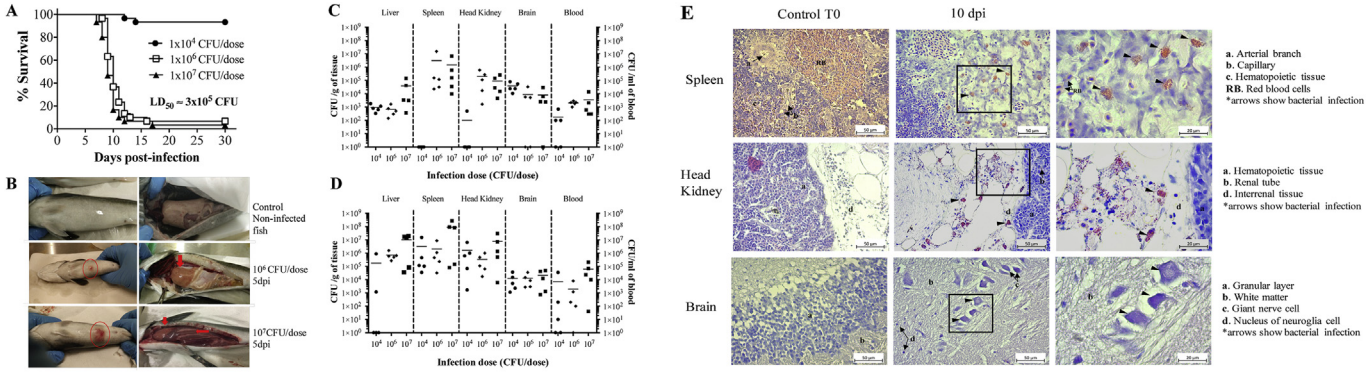

Fig. 1. *A. salmonicida* infection assay. A. Sablefish survival (%) after intraperitoneal infection with 10^4 CFU/dose (black circle), 10^6 CFU/dose (white square), and 10^7 CFU/dose (black triangle). The latter two groups were significantly different from the 10^4 CFU/dose group at $p < 0.0001$. **B.** *A. salmonicida* furunculosis symptoms were detected at 5 dpi. Circles and arrows show skin furunculosis, internal hemorrhage and liver as primary organ for infection at 10^6 and 10^7 CFU/dose. No signs of infection were detected in the group infected with 10^4 CFU/dose. **C.** *A. salmonicida* J410 tissue colonization (liver, spleen, head kidney, brain, and blood) after 5 dpi (n = 5). **D.** Tissue colonization after 10 dpi. **E.** Histopathology of spleen head kidney and brain after 10 dpi. In **C**, **D** and **E** the bar indicates the average.

$y = 0.03417 * \ln(x) + 0.04260$ with a $r^2 = 0.8756$ ($p > 0.0001$) (Fig. 5B). Total IgM was measured from non-lethal blood samples at 2, 4, 6, and 8 weeks post-immunization. Also, total IgM was quantified from non-immunized fish prior to vaccination (T0, n = 10). IgM titers in naïve sablefish (~125.45 g) were estimated to be ~0.097 mg/ml (Fig. 5C, Table S1). At 2 weeks post-immunization all the groups showed an increase in the IgM titer (control: 0.331 mg/ml; Alpha Ject Micro 4®: 0.270 mg/ml; Forte Micro®: 0.238 mg/ml; bacterin mix: 0.203 mg/ml) (Fig. 5C, Table S1). At 4 weeks post-immunization the PBS control (0.200 mg/ml), Alpha Ject Micro 4® (0.216 mg/ml), and Forte Micro® (0.255 mg/ml) immunized groups had similar IgM titers. However, the bacterin mix immunized group had a significantly higher IgM titer (0.423 mg/ml) (Fig. 5C, Table S1). At 6 weeks post-immunization all the immunized fish, with exception of the mock immunized group (0.068 mg/ml), showed an increase in the IgM titers (Fig. 5C). The Alpha Ject Micro 4® and Forte Micro® immunized groups had an increase on IgM titer up to 0.911 mg/ml and 0.606 mg/ml, respectively. The bacterin mix immunized group showed the highest IgM titers (1.734 mg/ml), and this difference was significant as compared to the Forte Micro®, the Alpha Ject Micro 4® and to the control group at 6 weeks post-immunization (Fig. 5C). At 8 weeks post-immunization we observed a reduction in the IgM titers in all treatments (Alpha Ject®: 0.415 mg/ml; Forte Micro®: 0.520 mg/ml; Bacterin mix: 0.827 mg/ml) but not in the control group (0.064 mg/ml), which showed stable IgM titers during the whole experiment, and only the he bacterin mix group had a significant difference compared to the control group (Fig. 5C).

4. Discussion

A. salmonicida has been described as causing chronic disease in different fish species like Atlantic salmon (*Salmo salar*) and common carp (*Cyprinus carpio*), and can be transmitted to other species or between wild and farmed fish in open sea net pens [11,40]. In this study, we evaluated the virulence of an atypical *A. salmonicida* J410 strain isolated from infected cultured sablefish at marine net pens in Kyuquot Sound, British Columbia. We selected *A. salmonicida* J410 as a model of study based on its phenotypical characteristics, genomic analysis, and its easy growth *in vitro* (Vasquez et al., manuscript under preparation). Although, we found that *A. salmonicida* J410 has low virulence in sablefish (LD_{50}: ~3 × 10^5 CFU/dose; Table 1), as compared to *A. salmonicida* J223 in rainbow trout (*Oncorhynchus mykiss*; LD_{50}: 5.7 × 10^2 CFU/dose) [24], it has a high morbidity rate. Further, *A. salmonicida* J410 did not kill 100% of infected sablefish (Figs. 1A and 3A) despite remaining in the internal tissues (Fig. 1E). Perhaps, the lower virulence of *A. salmonicida* J410 in contrast to other isolates is related to its persistence in the fish tissues.

We observed that *A. salmonicida* J410 first colonized the liver and brain at the lowest doses tested (Fig. 1C). These results suggest that the liver and the brain are the primary target organs of *A. salmonicida* J410 in sablefish. We also observed bacteremia at 5 dpi (Fig. 1C). Intracellular infection of erythrocytes by *A. salmonicida* has been described in Atlantic salmon *in vitro* and *in vivo* [36]. Also, colonization of the brain by *A. salmonicida* has been reported in rainbow trout [41]. It appears that *A. salmonicida* can cross the fish blood-brain barrier, and according to the current knowledge and our data, it is possible that infected erythrocytes could serve as a mechanism of transport for *A.*

Table 1
Summary table for the infection and vaccination model for sablefish (*Anoplopoma fimbria*).

Treatment	Fish N°	Weight (g)		SGR	IP infection (CFU/dose)	IP challenge (CFU/dose)	% Mortality	RPS %	Total IgM[a] (mg/ml)	
		Initial	Final						6 wpi	8 wpi
A. salmonicida J410 infection										
	30	215.5 ± 3.2		–	1 × 10^4	–	6.67	-	-	-
				–	1-x10^6	–	93.33	-	-	-
				–	1 × 10^7	–	96.67	-	-	-
Calculated LD_{50} dose					~3.5 × 10^5					
Vaccine trial for *A. salmonicida* J410 challenge										
Alpha Ject®	30	135.4	368.0	4.71	–	1 × 10^7	53.3	30.43	0.911*	0.415
Forte Micro®	30	134.3	307.8	4.71	–	1 × 10^7	33.3	56.52	0.605	0.520
Bacterin Mix	30	144.3	317.6	4.78	–	1 × 10^7	26.6	65.22	1.734*	0.827*

*Tukey's multiple comparison test significance ($p < 0.0001$) as compared to control group.
[a] Total IgM (mg/ml) average value at 6- and 8-weeks post-immunization.

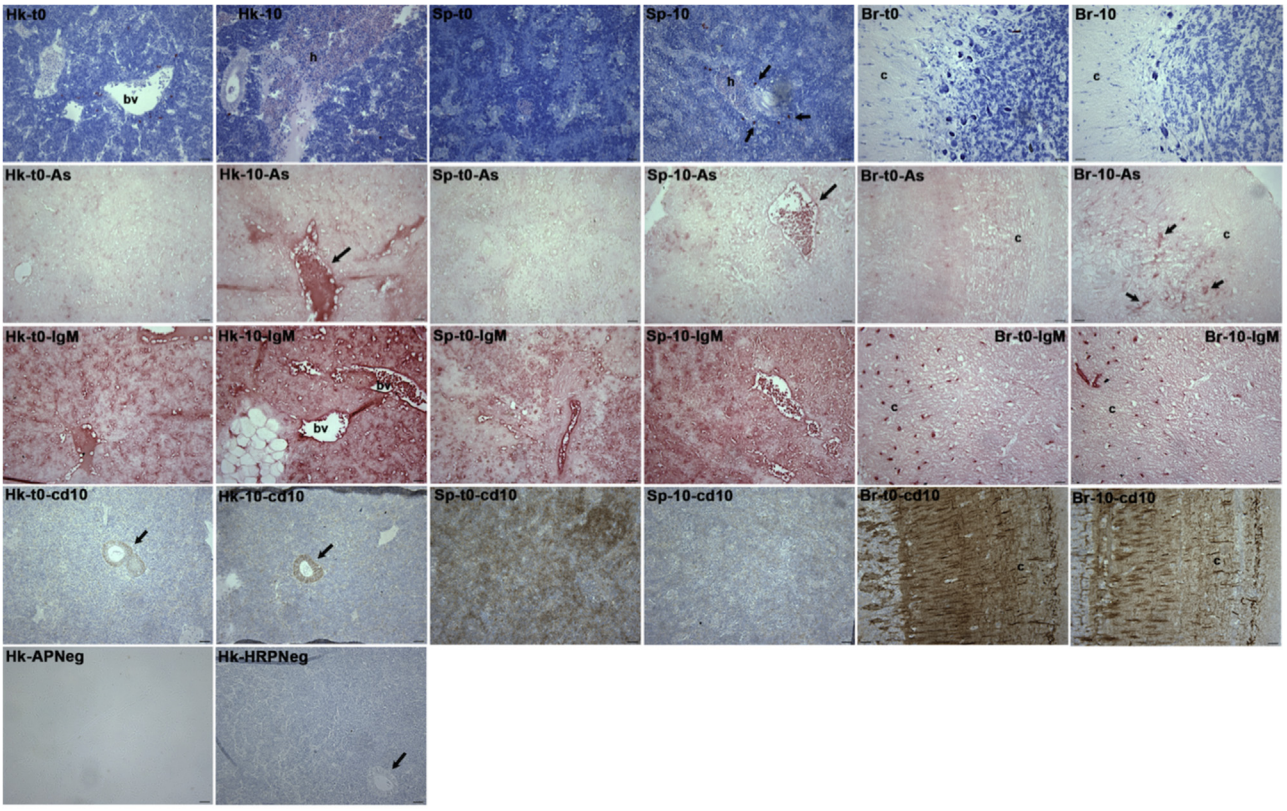

Fig. 2. Expression of *A. salmonicida*, IgM and CD10 in *A. salmonicida* infected sablefish tissues. Giemsa histological staining revealed pathological changes in several tissues by 10 dpi including general tissue disorganization and cellular dysplasia in the head kidney, spleen and brain, and hemorrhaging in the head kidney and spleen (top row). By 10 dpi (10), expression of (As) [see arrows indicating *A. salmonicida*-reactive vasculature] and IgM was in general increased in head kidney (Hk), spleen (Sp) and brain (Br) compared to time zero (T0) as shown in the figure. Interestingly, the expression of $CD10^+$ appeared to decrease with infection in spleen and brain, but increase slightly in head kidney. Expression of CD10 also appears to follow nerve body tracks in the cerebral cortical grey matter (c). Representative negative controls performed for the alkaline phosphatase-reacted antibodies (anti-*A. salmonicida* and anti-IgM; Hk-AP-Neg). The horseradish peroxidase reacted anti-CD10 (Hk-HRPNeg) is also shown at the bottom. Head kidney renal tubules are indicated by arrows in Hk-t0-cd10, Hk-10-cd10 and Hk-HRPNeg. Bv, blood vessel; h, hemorrhage; c, cerebral cortex. Magnification 200x. Scale bars are equal to 50 μm.

salmonicida to reach different organs, including the brain. The protection conferred by the blood-brain barrier against infectious diseases in fish is not well understood. Several bacterial pathogens have been reported to colonize the fish brain, and perhaps the blood-brain barrier does not confer protection against infectious agents in teleost since encephalitis seems common during bacterial infections [42–45].

Immunohistochemistry revealed that *A. salmonicida* detection increased in the head kidney, spleen and brain by 10 dpi. This is related to the *A. salmonicida* tissue colonization results (Fig. 1). CD10 is known as a marker of hematopoietic progenitor cells in mammalian bone marrow [46] and is present in sablefish (Fig. 2), and IgM is expressed by B lymphocytes in fish [47]. The general increase in IgM in the tissues, and decrease of CD10 expression in spleen and brain, could suggest that naïve B cells became plasma cells, and that the decrease of CD10 in the head kidney was related to B cell proliferation (Fig. 2) in response to the infection.

Symptoms of furunculosis were not evident at 5 dpi in fish inoculated with the lowest dose (10^4 CFU/dose) in contrast to fish infected with the higher doses (10^6 and -10^7 CFU/dose), which showed evident furunculosis symptoms (Fig. 1B). These results indicate that *A. salmonicida* infection in sablefish is dose-dependent and suggest that *A. salmonicida* strain J410 causes more of a chronic type of infection (Fig. 1A).

Enhanced expression of iron regulated outer membrane proteins (IROMPs) has been previously described as a strategy for vaccine development in teleosts [16,48]. Here, we developed an autogenous vaccine using three atypical *A. salmonicida* strains (J409, J410, and J411) grown under iron-limited conditions to up-regulate the expression of IROMPs and increase vaccine coverage. The RPS of the autogenous vaccine preparation was 64%, and although this was the highest RPS among the evaluated vaccines, this vaccine preparation did not reach the desired RPS of 70% [49]. Previously, an injectable autogenous vaccine against atypical *A. salmonicida* was evaluated by Arkoosh et al. (2018) in sablefish, and they obtained an RPS of 81.7% [6]. However, in contrast to our expreriments, the vaccinated animals were challenged with 8.4×10^5 CFU/dose, similar to our calculated LD_{50} for atypical *A. salmonicida*. Additionally, in the study of Arkoosh et al. (2018), the non-immunized control group had 45% survival, which is very close to the LD_{50} calculated in this study ($\sim 3 \times 10^5$ CFU/dose). This suggest that similar results would be obtained with a higher challenge dose, as we determined in our assays (Fig. 3A).

The RPS for Alpha Ject Micro 4® was previously determined to be 100% in Atlantic salmon [50], and 58.3% for Forte Micro® in Arctic charr (*Salvelinus alpinus*) [51]. However, the RPS in sablefish at 30 days post-challenge was only ~30% for Alpha Ject Micro 4®, which is almost 1/3 of the reported RPS in Atlantic salmon [50]. The RPS in sablefish was ~57% for Forte Micro® (Table 1), which is consistent with the observed RPS in Arctic charr [51].

Post-challenge bacterial load analysis showed that all non-immunized fish had high bacterial colonization in all tested tissues (Fig. 3B). Fish groups vaccinated with comercial vaccines showed high bacterial colonization, similar to the non-immunized fish group (Fig. 3B). Also, bacterial load was detected in a few fish vaccinated with the autogenous vaccine, suggesting that the vaccine design has room for

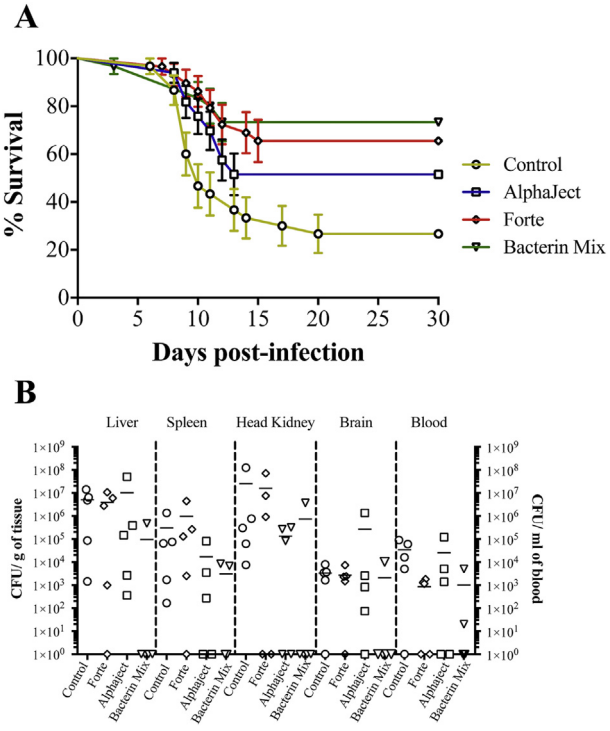

Fig. 3. Vaccinated sablefish challenge assay. A. Cumulative survival (%) of vaccinated sablefish after intraperitoneal (ip) challenge with 10^7 CFU/dose (100x LD_{50}) of *A. salmonicida* J410. Fish were immunized with phosphate saline buffer (PBS 1X) (yellow line); Alpha Ject Micro 4® (Pharmaq) (blue line); Forte Micro® (Elanco) (red line); or bacterin mix (autogenous vaccine; green line). Significant differences were calculated between control group and vaccinated groups ($p < 0.0001$). **B.** *A. salmonicida* J410 tissue colonization after 10 days post-challenge. White circle (Control); black diamond (Alpha Ject Micro 4®); white square (Forte Micro® micro IV) and black-down triangle (Bacterin mix). The bar indicates the average value. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

improvement (Fig. 3B).

Nevertheless, it is evident that bacterin-based vaccines against atypical *A. salmonicida* are not optimal. This could be due to the bacterin preparations containing several non-protective immune dominant antigens or immune suppressors, like VapA (A-layer unique component), which attract antibodies such as rabbit IgG and human IgM in a non-specific fashion [52]. Here, we showed that VapA binds to the antigen-binging fragment F(ab)' of the antibodies in a non-specific fashion (Fig. 4C). This suggests that *A. salmonicida* VapA might be used to enhance phagocytosis mediated opsonization [53] and that its efficacy as an immune protective antigen is controversial. For instance, VapA could be playing a role similar as the surface M protein in *Streptococcus pyogenes*, which is a well characterized immunoglobulin-binding protein with highly conserved domains among serotypes [54,55].

Previous studies have demonstrated that total IgM titers, as well as antigen-specific IgM titers, correlate with vaccine efficacy in rainbow trout (*Oncorhynchus mykiss*) [56], Atlantic salmon (*Salmo salar*) [57,58], brown trout (*Salmo trutta*) [57,59], coho salmon (*Oncorhynchus kisutch*) [59], chinook salmon (*Oncorhynchus tshwytscha*) [60], Artic charr (*Salvelinus alpinus*) [61], Nile tilapia (*Oreochromis niloticus*) [62], yellow catfish (*Pelteobagrus fulvidraco*) [63], Atlantic cod (*Gadus morhua*) [64] and lumpfish (*Cyclopterus lumpus*) [65] against bacteria *A. salmonicida* [61], *Vibrio anguillarum* [64] *Yersinia ruckeri* [66], *Streptococcus iniae* [56], *Streptococcus agalactiae* [62], *Flavobacteria columnare* [63] and viruses [61,67]. Here, we showed that total IgM levels reflected the vaccine's RPS in sablefish. Additionally, we determined that the serum of naïve sablefish of ~125 g fish contains ~75 μg/ml (Fig. 5C), which is low compared to other fish species. For instance, IgM titers in Atlantic salmon of 2–8 kg are 0.8–1.3 mg/ml [68], and in rainbow trout of 20 g are 0.67 ± 0.66 mg/ml [69]. To our current knowledge, IgM titers for fish of similar size to the sablefish used in this study are between 2.1 and 9.1 mg/ml of serum [59]. Nonetheless, the values for serum IgM that we obtained using the ELISA assay were comparable with the IgM titers that we obtained during purification, i.e. we obtained 3.5 mg/ml of sablefish IgM from 150 to 200 ml of serum.

Sablefish vaccinated with the autogenous vaccine (bacterin mix) preparation has higher IgM titers as compared to the commercial vaccines (Fig. 5C). Total IgM titers peaked at 6 weeks post-immunization (wpi) and were significantly higher (by 25 and 3 times) as compared to the control group and the Forte Micro® vaccinated group (Fig. 5C, Table 1). At 8 weeks post-vaccination, the bacterin mix immunized group again showed the highest IgM titers, these 12 times higher as compared to control fish (Fig. 5C, Table 1), which fits with their high RPS observed in this study. Forte Micro® showed higher IgM titers than Alpha Ject® at 8 wpi, however the difference was not significant.

The autogenous vaccine mix triggered superior immune protection as compared to currently available commercial vaccines, and our results agree with previous studies where IgM titer against *A. salmonicida* bacterin showed a peak between 6 and 12 weeks post-immunization in Atlantic salmon [70] and in wolfish (*Anarhichas minor*) immunized with atypical *A. salmonicida* bacterin mixed with an oil-adjuvant [71]. However, 100% immune protection was not achieved in our study, and this indicates that vaccine design plays an important role in efficacy. For instance, to improve the vaccine efficacy of the bacterin mix, immune dominant and not immunoprotected antigens could be removed.

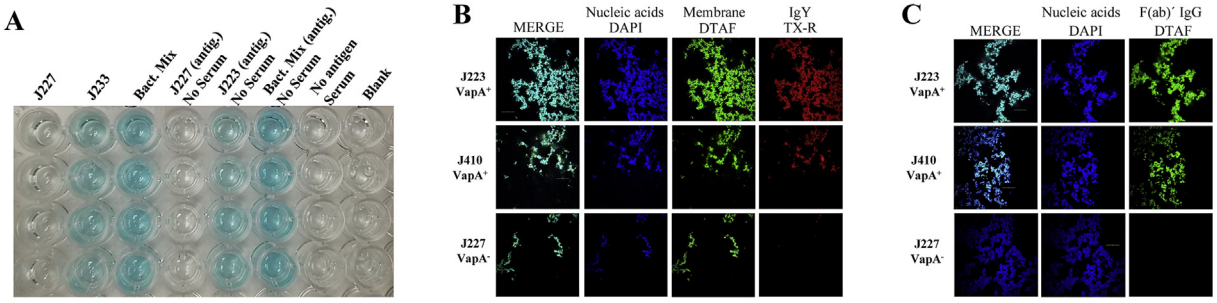

Fig. 4. Non-specific binding of immunoglobulins to *A. salmonicida* VapA protein. A. Indirect ELISA evaluation. Presence of *A. salmonicida* $VapA^+$ strains caused non-specific binding of the secondary IgY-biotinylated antibody. This was not influenced by pre-incubation with sablefish serum from immunized or non-immunized fish. **B.** Confocal microscopy of *A. salmonicida* J223 $VapA^+$; *A. salmonicida* J410 $VapA^+$ and *A. salmonicida* J227 $VapA^-$ strains labeled with 4′,6-diamidino-2-phenylindole (DAPI), 5-(4,6-dichlorotriazinyl) amino fluorescein (DTAF), and chicken IgY anti-sablefish IgM-avidin Texas-Red. **C.** Confocal Microscopy of *A. salmonicida* J223 $VapA^+$; *A. salmonicida* J410 $VapA^+$ and *A. salmonicida* J227 $VapA^-$ strains labeled with DAPI and goat F(ab')2 anti-rabbit IgG (H+L)-FITC. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

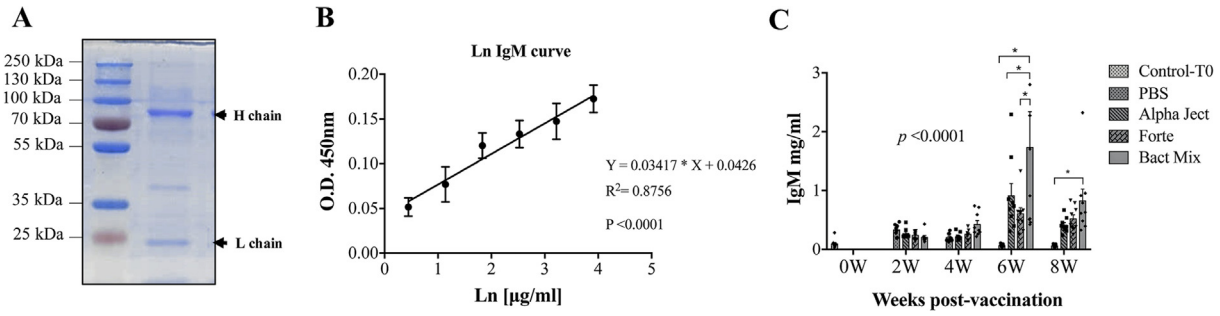

Fig. 5. Post-challenge IgM levels is sablefish as quantified by dELISA. A. SDS-PAGE 10% of purified sablefish IgM stained with Coomassie Blue (arrows show different chain structures). **B.** Standard curve for purified IgM of known concentrations (100; 50; 25, 12.5; 6.25; 3.125; 1.56 µg/ml); concentrations were standardized to natural logarithm (ln) using a linear regression [Y = 0.03417*ln(X) + 0.0426] that had an R^2 = 0.8756 and a *P* value < 0.0001. **C.** Total IgM quantification by dELISA in serum samples collected at 2, 4, 6, and 8 weeks post-vaccination from PBS (mock), Alpha Ject®, Forte Micro®, and bacterin mix immunized groups. Values from pre-immunization; time zero (T0) (n = 10) are also shown. An asterisk indicates significant differences as compared to all groups. Statistical differences were determined using GraphPad Prism 7.4. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Also, modern adjuvants can improve the immunogenicity of vaccines [72–74]. The commercial vaccines evaluated in this study are polyvalent. This adds a complexity to the antigenic balance between immune protective antigens and may impact vaccine efficacy in sablefish.

5. Conclusions

In conclusion, we developed an infection model for sablefish, including determining the LD_{50} dose for atypical *A. salmonicida*. Atypical *A. salmonicida* J410 does not cause an acute infection in sablefish. Rather it causes a chronic type of infection that involves initial colonization of the hematopoietic tissues and the brain. The monovalent autogenous vaccine mix using three *A. salmonicida* strains provided better protection than two polyvalent commercial vaccines. Sablefish IgM titers peaked at 6 weeks post-vaccination. Vaccine immune protection was associated with the IgM titers, where the autogenous vaccinated fish had the highest RPS and IgM titers. The *A. salmonicida* A-layer binds to immunoglobulins in a non-specific fashion, raising the question about its utility as an immune protective antigen. Although this study provides novel insights about sablefish vaccinology for the prevention of furunculosis, further research is required to develop an effective cross-protective vaccine for this species.

CRediT authorship contribution statement

Ignacio Vasquez: Conceptualization, Methodology, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Trung Cao:** Methodology, Investigation, Writing - review & editing. **Ahmed Hossain:** Methodology, Writing - review & editing. **Katherinne Valderrama:** Methodology, Writing - review & editing. **Hajarooba Gnanagobal:** Investigation, Writing - review & editing. **My Dang:** Investigation. **Robine H.J. Leeuwis:** Methodology, Writing - review & editing. **Michael Ness:** Conceptualization, Investigation, Writing - review & editing. **Briony Campbell:** Conceptualization, Resources, Writing - review & editing. **Robert Gendron:** Investigation, Writing - review & editing, Visualization. **Kenneth Kao:** Investigation, Writing - review & editing. **Jillian Westcott:** Resources, Writing - review & editing, Supervision, Funding acquisition. **A. Kurt Gamperl:** Conceptualization, Resources, Writing - review & editing, Funding acquisition. **Javier Santander:** Conceptualization, Methodology, Writing - original draft, Resources, Writing - review & editing, Visualization, Supervision, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.06.005.

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