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ORIGINAL ARTICLE

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Exploring the efficacy of vaccine techniques in juvenile sablefish, *Anoplopoma fimbria*

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Abstract

Wild sablefish, *Anoplopoma fimbria*, are a valuable commercial species whose populations are declining. Fortunately, sablefish are excellent species for commercial aquaculture. Sablefish raised under high-density conditions in the marine environment require the use of efficacious vaccines to control disease. Sablefish impacted by disease in net pens may have poor flesh quality and high mortality during grow-out. As a result, disease can cause financial hardship for sablefish aquaculture operators. The efficacy of a multivalent vaccine preparation for sablefish, administered either by intraperitoneal (i.p.) injection or by immersion, against atypical and typical *Aeromonas salmonicida*, the causative agents of atypical and typical furunculosis, respectively, was examined. *A. salmonicida* can affect sablefish at any age and size. Consequently, an efficacious vaccine that can be appropriately and optimally administered to all life stages is desirable. Sablefish vaccinated by immersion at ~1.5 or ~4.5 g with a whole-cell multivalent vaccine were not protected against either typical or atypical *A. salmonicida*. Factors that may have contributed to the ineffectiveness of the immersion vaccine are discussed. By contrast, the relative per cent survival (RPS) or potency of the whole-cell multivalent vaccine injected i.p. in juvenile sablefish at ~50 g against typical and atypical *A. salmonicida* was 94.3% and 81.7% respectively. The high RPS values indicated that the vaccine successfully initiated an immune response in sablefish upon a second encounter with the pathogen.

KEYWORDS
Aeromonas salmonicida, furunculosis, sablefish aquaculture, vaccine

1 | INTRODUCTION

Wild sablefish, *Anoplopoma fimbria*, are a valuable commercial marine species with U.S. landings in 2013 at 17,826 metric tons valued at $101.6 million. However, wild sablefish populations are declining. In 2013, there was a 5% decrease in commercial landings relative to 2012 (Van Voorhees, Lowther & Liddel, 2014) and the sablefish spawning biomass for 2015 is estimated to be only 33% of previously unexploited levels (Johnson et al., 2016). Raising sablefish in culture may reduce demand on wild sablefish populations.

Currently most sablefish found at the market are wild caught. However, sablefish have been identified as an excellent species for commercial marine aquaculture due to their high market value, rapid growth as juveniles (Shenker & Olla, 1986) and ability to grow well in net pens (Gores & Prentice, 1984). Culturing sablefish is currently occurring along the northwest coast of North America. The ability to

produce large and healthy fish is critical for the economic success of sablefish culture. Research based at the National Oceanic and Atmospheric Administration's (NOAA) Marine Research Station is directed towards optimizing commercial culture of sablefish (Cook et al., 2015; Immerman & Goetz, 2014). Once the juvenile sablefish are food trained in land-based tanks, they are moved to seawater net pens for grow-out. Environmental factors (e.g. water quality, temperature and pathogens) in the seawater net pens can influence the ability of these fish to survive or achieve optimal growth. Net pens permit the transfer of pathogens between domesticated and wild fish with the potential to alter survival in both populations (Krkosek et al., 2007; Lafferty et al., 2015). Disease transmission between wild and domesticated fish may be difficult to restrict in net pens and surrounding waters due to the fact that net pens are open to the aquatic environment and can be located near other shellfish and finfish aquaculture operations (Krokosek et al., 2012).

Disease has cost the aquaculture industry tens of billions of dollars over the last two decades (FAO 2016). Sablefish impacted by disease in net pens may have poor flesh quality and high mortality during grow-out. Accordingly, disease can cause financial hardship for sablefish aquaculture operations. Sumaila, Volpe and Liu (2005) listed a number of pathogens that have the potential to affect sablefish culture (e.g. *Anisakis* sp., *Flavobacterium branchiophila*, epitheliocystis, leeches, papillomatosis, *Pseudomonas* sp. *Dactlogyrus* sp., *Diplostomum* sp., *Trichoina* sp., *Listonella anguillarum*, *Renibacterium salmoninarum* and *Aeromonas salmonicida*). Bacteria that have been isolated from moribund sablefish or demonstrated to cause disease are *Vibrio logei*, *L. anguillarum*, *Enterobacter asburiae*, *R. salmoninarum* and *A. salmonicida* (Schulze, Alabi, Tattersall-Sheldrake & Miller, 2006; Evelyn, 1971; Bell, Hoffmann & Brown, 1990; Arkoosh & Dietrich, 2015, L. Rhodes, NMFS, personal communication).

Typical approaches used to manage bacterial infections in fish culture are antibiotics and vaccines. Antibiotic use in aquaculture is expensive, has the potential to contaminate the surrounding environs and can contribute to antibiotic resistance (Ringo, Olsen, Jensen, Romero & Lauzon, 2014). Antibiotic resistance can also be transferred to other bacteria present in the environment (Cabello, 2006). The use of antibiotics can also reduce gut microbiome in the fish (Ringo et al., 2014). By contrast, an effective vaccine strategy for fish diseases can be the most economically, environmentally, and ethically appropriate method for pathogen control (Brudeseth et al., 2013).

Vaccines are administered to fish through three routes, i.e. injection, oral and immersion, with each route having advantages and disadvantages (reviewed in the following: Sudheesh & Cain, 2017; Dadar et al., 2017). For example, immersion vaccination is the most appropriate method of mass vaccination of small fish. However, injectable vaccines have been demonstrated to generate greater protection against pathogens than immersion vaccinations but are not appropriate for mass vaccination of smaller fish. Larger fish are more easily injected with a vaccine than smaller fish but injecting vaccines is very labour intensive and lesions may result at the site of injection. Oral vaccination of fish is a very stress-free method of mass vaccinating both small and large fish but protection is regularly inconsistent due to the degradation of the antigen as it passes through the acidic environment of the foregut. Which delivery route is most effective for protecting the fish will depend upon a number of variables including fish size, the pathogen and the pathogen's route of infection, water temperature, safety, and ontological development of the host (Dadar et al., 2017; Sudheesh & Cain, 2017).

The pathogen, *A. salmonicida*, is devastating to fish due to the widespread distribution of the bacteria as well as its ability to affect a diversity of freshwater and marine fish. Five subspecies of *A. salmonicida* have been identified that can manifest the disease furunculosis (Han et al., 2011; Midtlyng, 2014). The term "furunculosis" is used to describe a number of disease presentations caused by the five subspecies of *A. salmonicida*. A further distinction is made between typical (classical) furunculosis and atypical furunculosis or ulcer disease. Typical furunculosis is considered to be a disease that occurs generally in salmonid species as a result of infection to *A. salmonicida* subsp. *salmonicida*. Atypical furunculosis arises from infection with the other *A. salmonicida* subspecies and includes subsp. *achromogenes*, subsp. *masoucida*, subsp. *smithia*, and subsp. *pectinolytica* (Midtlyng, 2014). Atypical furunculosis has been found to occur in over 20 species of both farmed and wild fish and is considered to be an "emerging disease" in Atlantic cod, *Gadus morhua*, Atlantic halibut, *Hippoglossus hippoglossus*, spotted wolfish, *Anarhichas minor*, common wolfish, *Anarhichas lupus* and turbot, *Scophthalmus maximus*, as well as a number of ornamental fish species (Gudmundsdottir & Bjornsdottir, 2007). In addition, atypical furunculosis has been isolated and identified in diseased sablefish reared in net pen culture at NOAA's Manchester Marine Research Station (Port Orchard, WA) during mortality events over a multi-year period.

The occurrence and severity of disease in fish is dependent upon the qualities of the host, the pathogen and the environment. For example, a total of 39 facilities are licensed to raise sablefish commercially in British Columbia. A number of these licenses are for locations within the Salish Sea (DFO 2016). Fish from the Salish Sea have been identified to host numerous bacterial and viral pathogens (Hershberger, Rhodes, Kurath & Winton, 2013). An environmental factor that contributes to the growth rate of a pathogen as well as an increase in a pathogen's range is temperature (Harvell et al., 2002). As a result, exposure to pathogenic bacteria including typical and atypical *A. salmonicida* as well as rising environmental water temperatures due to ocean coastal dynamics and global climate change (Leung & Bates, 2013) may contribute to disease in sablefish in the Salish Sea. The risk and cost associated with disease outbreaks may be a significant setback to sablefish aquaculture. However, vaccines have been found to reduce epidemics due to *A. salmonicida* in salmon farms, in addition to reducing the impact of high antibiotic expenses (Krkosek, 2010). Therefore, the objective of this study was to determine the efficacies of different vaccine strategies against atypical and typical *A. salmonicida* for sablefish using a whole-cell multivalent vaccine preparation. The vaccine was administered either by intraperitoneal (i.p.) injection or by immersion at two ages.

2 | MATERIALS AND METHODS

2.1 | Sablefish husbandry

All personnel, facilities and methods involved with the culturing and experimentation of sablefish were approved by the University of Washington's Institutional Animal Care and Use Committee (IACUC; #4078-05). For these experiments, sablefish were derived from two spawning pulses carried out at the NOAA's Manchester Research Station (Manchester, WA, USA) from gametes obtained from adult sablefish that were collected from the wild as described in Cook et al. (2015). Following embryo and yolk sac larval development in incubators (Cook et al., 2015), sablefish larvae in pulse 1 and 2 were stocked out into 2,000 L circular tanks on 3/3/15 and 5/12/15 respectively. Sablefish larvae were fed rotifers 18 days poststocking. Transition from rotifers to *Artemia* began on day 16 which overlapped with 2 days of rotifer feeding. The sablefish were fed *Artemia* to day 40 and then were transitioned to artificial Otohime diet (Reed Mariculture, Campbell, CA, USA) as described previously (Cook et al., 2015). After transitioning to the Otohime artificial diet, the sablefish fry were vaccinated and transported from Manchester, WA to NOAA's Fish Disease Laboratory (FDL) in Newport, OR, for disease challenge. The fish were held in circular tanks with flow-through seawater. The seawater was sand filtered and treated with ultraviolet light to remove particulates and potential bacterial and viral pathogens respectively. The sablefish were fed a commercial diet of dry pellets (Bio-Oregon, Longview, WA, USA).

2.2 | Vaccine preparation and administration

A proprietary vaccine was purchased from AquaTactics (Kirkland, WA, USA) and contained two isolates of formalin-killed *A. salmonicida* (a typical isolate of *A. salmonicida* provided by Hagerman National Fish Hatchery, and an atypical isolate of *A. salmonicida* provided by L. Rhodes (NWFSC, NOAA) isolated from a moribund sablefish undergoing an active infection). The vaccine preparation also contained three formalin-killed *Vibrio* species (*L. anguillarum*, *V. ordalii*, and *V. salmonicida*). The injectable vaccine was prepared in an oil-based emulsion.

A timeline is presented in Figure 1 identifying major activities associated with vaccination and disease challenges relative to stocking out the fish. Three groups of sablefish were vaccinated, either by: (i) immersion 58 days (≈8 weeks) post stock out (1.5 ± 0.7 g; mean ± *SD*) and referred to as the Immersion-1 treatment; (ii) immersion 72 days (≈10 weeks) post stock out (4.5 ± 1.1 g) and referred to as the Immersion-2 treatment or (iii) i.p. injected 121 days (≈17 weeks) post stock out (50 ± 5.5 g) and referred to as the Injected treatment (Figure 1).

Fish were immersion vaccinated as instructed by the manufacturer and followed vaccine immersion protocols used for concentrated vaccine suspensions (Brudeseth et al., 2013). In brief, for the Immersion-1 treatment, 500 sablefish were directly immersed in 5 L of a vaccine solution for 1 min at 15°C under constant aeration 8 weeks post stocking out (Figure 1). For the Immersion-2 treatment, sablefish were split into two batches for the immersion vaccination. In brief, two batches of 250 sablefish each were immersed in a 5 L solution of the vaccine for 1 min at 15°C under constant aeration 10 weeks post stocking out (Figure 1). The vaccine solution in both immersions consisted of one part vaccine to nine parts seawater. After immersion, fish from the two treatments were held at 13°C. In an attempt to increase the amount of vaccine the fish take up, they were immersed with a second treatment of the vaccine. Fish in both Immersion-1 and Immersion-2 treatments were given a second treatment of the vaccine 7 days after the initial immersion vaccine. Methods used to expose the sablefish to the second treatment of the vaccine were identical to the methods used in the initial vaccine treatment. A group of 500 sablefish referred to as the sham-treated sablefish were handled identically for each immersion vaccinate group, except they were not administered the vaccine. All treatment groups were delivered to the FDL approximately 2 weeks after their initial vaccination or sham vaccinations.

Fish were injected vaccinated as instructed by the manufacturer. For the Injected treatment, 250 sablefish were anesthetized with buffered tricaine methanesulfonate (30 mg/L) and i.p. injected with 150 μl of the vaccine preparation in an oil-based emulsion 17 weeks post stocking out. A group of sablefish, referred to as the sham-treated sablefish, were handled identically to the injected vaccinate group except injected with the oil-based emulsion without the bacteria. After vaccination, fish were held at 13°C and delivered the following week to the FDL.

2.3 | Disease challenges

2.3.1 | Bacteria

Atypical *A. salmonicida*, designated as T30, was provided by L. Rhodes (NOAA, NWFSC) and was originally isolated from sablefish with an active *A. salmonicida* infection. Typical *A. salmonicida* culture, designated as #51, was provided by Craig Banner (Oregon Department of Fish and Wildlife) and originally isolated from an adult Chinook salmon with an active *A. salmonicida* infection.

For the disease challenges, typical and atypical *A. salmonicida* were each incubated at 20°C in growth media (trypticase soy broth (TSB) supplemented with 1.5% NaCl) until an optical density (O.D.) near 1.0 at 540 nm was achieved. An aliquot was removed, serially diluted and spread on growth agar plates (trypticase soy agar (TSA) supplemented with 1.5% NaCl) in triplicate, in order to determine the colony forming units (CFU)/ml of bacteria in the stock culture and used in the disease challenges (Table 1).

2.3.2 | Lethal concentration response of the pathogen

Preliminary disease challenges with pulse 1 juvenile sablefish were performed in order to characterize the mortality curves produced

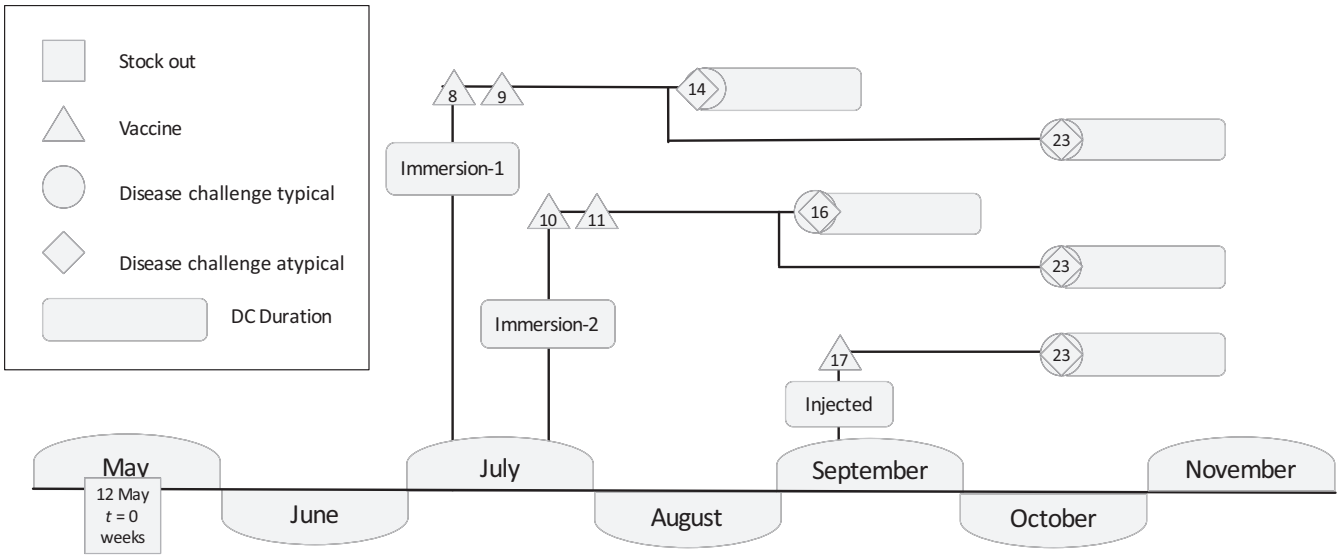

FIGURE 1 Timeline characterizing the sequence of events. A time line is presented outlining the chronology of the major activities of immersion and injection vaccination, transfer to the FDL in Newport Or. and the disease challenges relative to stocking out the sablefish to the 2,000 L tanks. Stocking out refers to the date at which yolk sac absorption was nearly complete. The number within the symbols represent the number of weeks the activity occurred post stocking out

by atypical and typical *A. salmonicida* (Arkoosh, Boylen, Stafford, Johnson & Collier, 2005; Arkoosh & Dietrich, 2015). The curves were used to select the bacterial dilutions required to test the efficacy of the vaccine treatments. Specific details including fish number and bacteria concentrations for generating the lethal concentration curves for both bacteria are provided in Table 1. Fish densities for all disease challenges were approximately 30 g/L. Sablefish were exposed to three dilutions of each pathogen (1:100, 1:1,000 and 1:10,000 volume stock bacteria:volume seawater) in triplicate treatments, for 40 min in seawater at water temperatures ranging from 12.5 to 13.0°C. No-pathogen controls of sablefish were also exposed to sterile growth media, in duplicate, for 40 min. Following the bath exposures, the groups of fish were transferred to separate flow-through seawater tanks. The fish were then monitored daily for up to 23 days. The mean water temperature during the monitoring period was 14°C. The fish were fed daily. All mortalities were collected, and their lengths, weights and times of discovery were recorded.

The three dilutions of atypical *A. salmonicida* resulted in 75%, 54% and 24% cumulative mortality, respectively, in sablefish 21 days post exposure (data not shown). For testing the efficacy of vaccines, Amend, Johnson, Croy and Mccarthy (1983) recommended that two concentrations of the bacteria are used that result in 50%–70% mortality and 70%–90% mortality. Therefore, the 1:100 and 1:1,000 dilutions of atypical *A. salmonicida* were selected to test vaccine potency. The three dilutions of typical *A. salmonicida* produced 45%, 29% and 14% cumulative mortality, respectively, in sablefish 23 days post exposure (data not shown). As only the lowest dilution, 1:100, of typical *A. salmonicida* approached the 50%–70% cumulative mortality recommend by Amend et al. (1983), it was the only dilution selected to test vaccine potency.

2.3.3 | Testing efficacy of vaccine

The efficacy of the vaccine against atypical and typical *A. salmonicida* in the Immersion-1, Immesion-2, and Injected treatments was tested by disease challenge as described above and in Table 1. In brief, juvenile sablefish were exposed to either a 1:100 or 1:1,000 dilution of atypical *A. salmonicida*, or to a 1:100 dilution of typical *A. salmonicida*. Disease challenges with atypical *A. salmonicida* were performed with the Immersion-1 treatment on 32 or 92 days post final vaccination, with the Immersion-2 treatment 36 or 78 days post final vaccination and with the Injection treatment 36 days post injection (Table 1). Disease challenges with typical *A. salmonicida* were performed with the Immersion-1 treatment on day 33 post final vaccination, with Immersion-2 treatment on day 35 post final vaccination and with the Injection treatment on day 36 post injection. Four to six replicate tanks of sablefish were exposed to each bacterial dilution during each disease challenge and two replicate tanks of sablefish were exposed to sterile growth media (Table 1). Verification of pathogen presence by polymerase chain reaction (PCR) was performed for each mortality collected during the disease challenge monitoring period. Across all of the disease challenges, only two fish in the no-pathogen control tanks died during the monitoring period, but *A. salmonicida* was not detected in either fish by PCR.

2.4 | Pathogen verification

PCR was used to confirm *A. salmonicida* infection in the kidneys of each dead sablefish. Using sterile technique, kidney tissue from sablefish disease challenge mortalities was streaked on growth agar, and allowed to incubate at 20°C until colonies appeared. Colonies were checked visually for presence of brown coloration, indicating

infection by typical *A. salmonicida*, while colonies remaining white after 96 hr of growth were determined to be atypical *A. salmonicida*. A single colony was harvested using a sterile loop and transferred into a sterile 1.5 ml tube containing Tris-EDTA (TE) buffer and stored at −20°C until PCR analysis. The bacterial sample was thawed at room temperature, and used as PCR template.

TABLE 1 Elements of the disease challenges to test the efficacy of the immersion and injectable vaccines against atypical and typical *Aeromonas salmonicida*

Disease challenge	Disease challenge date/days post final vaccination	Treatment	*A. salmonicida* and volume in exposure tanks	OD[a]	Stock concentration of bacteria (CFU/ml)	Dilution (volume bacteria:volume seawater)/final concentration of bacteria (CFU/ml)	Number of tanks/number of fish per tank	Relative per cent survival (RPS)[b]
Lethal concentration curve	6/3/15	Pulse 1	Typical 20 L	1.061	9.3×10^8	1:100/9.3×10^6	3/50	NA[c]
						1:1,000/9.3×10^5	3/50	NA
						1:10,000/9.3×10^4	3/50	NA
						Media only	2/50	NA
	6/5/15	Pulse 1	Atypical 20 L	0.970	1.33×10^9	1:100/1.33×10^7	3/50	NA
						1:1,000/1.33×10^6	3/50	NA
						1:10,000/1.33×10^5	3/50	NA
						Media only	2/50	NA
Immersion-1	8/17/15 32 days	Vaccinated	Atypical 16 L	0.923	7.6×10^7	1:100/7.6×10^5	6/30	−9.1
						1:1,000/7.6×10^4	6/30	−3.9
						Media only	2/30	NA
		Sham				1:100/7.6×10^5	6/30	
						1:1,000/7.6×10^4	6/30	
						Media only	2/30	
	10/16/15 92 days	Vaccinated	Atypical 59 L	0.997	8.4×10^8	1:1,000/8.4×10^5	4/20	8.5
						Media only	2/20	
		Sham				1:1,000/8.4×10^5	4/20	
						Media only	2/20	
	8/18/15 33 days	Vaccinated	Typical 16 L	0.897	4.9×10^8	1:100/4.9×10^6	6/30	7.3
						Media only	2/30	NA
		Sham				1:100/4.9×10^6	6/30	
						Media only	2/30	
Immersion-2	9/4/15 36 days	Vaccinated	Atypical 33 L	0.900	9.7×10^8	1:100/9.7×10^6	6/30	4.0
						1:1,000/9.7×10^5	6/30	−6.2
						Media only	2/30	
		Sham				1:100/9.7×10^6	6/30	
						1:1,000/9.7×10^5	6/30	
						Media only	2/30	
	10/16/15 78 days	Vaccinated	Atypical 59 L	0.997	8.4×10^8	1:1,000/8.4×10^5	4/20	−15.1
						Media only	2/20	
		Sham				1:1,000/8.4×10^5	4/20	
						Media only	2/20	
	9/3/15 35 days	Vaccinated	Typical 16 L	0.976	8.4×10^8	1:100/8.4×10^6	6/30	4.4
						Media only	2/30	NA
		Sham				1:100/8.4×10^6	6/30	
						Media only	2/30	

(Continues)

TABLE 1 (Continued)

Disease challenge	Disease challenge date/days post final vaccination	Treatment	*A. salmonicida* and volume in exposure tanks	OD[a]	Stock concentration of bacteria (CFU/ml)	Dilution (volume bacteria:volume seawater)/final concentration of bacteria (CFU/ml)	Number of tanks/number of fish per tank	Relative per cent survival (RPS)[b]
Injected	10/16/15 36 days	Vaccinated	Atypical	0.997	8.4×10^8	1:1,000/8.4×10^5	5/20	81.7
			NA			Media only	2/20	
		Sham				1:1,000/8.4×10^5	5/20	
						Media only	2/20	
	10/16/15 36 days	Vaccinated	Typical	0.882	2.39×10^8	1:1,000/2.4×10^6	5/20	94.3
			NA			Media only	2/20	
		Sham				1:1,000/2.4×10^6	5/20	
						Media only	2/20	

[a]Optical density (OD) measured at a wavelength of 540 nm.
[b]RPS; relative per cent survival. RPS is the vaccine potency, as per Amend (1980), and is the proportional survival between vaccinated and sham-treated fish: RPS = 1 − [% mortality in vaccinated fish/mortality in control fish] × 100.
[c]NA is not applicable.

PCR reactions were carried out in 10 μl volumes containing: 10× buffer; 2.0 mM $MgCl_2$; 800 μM dNTPs (deoxyribonucleotide triphosphates); 0.4 units of *Amplitaq Gold Taq* DNA polymerase (Applied Biosystems) and 250 nM of each primer (Integrated DNA Technologies). The bacterial slurry was diluted 1:10 (volume:volume), with 1 μl of diluted template added per 10 μl reaction well. Nuclease-free water was used to bring the final reaction volume to 10 μl. Novel primers: Asal 115 Forward (TGG CTG CCT CGA TAA GCA ATG G) and Asal 205 Reverse (AGA GAG TTG GCT AGC GGT GAG T) were designed based on a DNA probe for *A. salmonicida* (GenBank accession no. X64214; Hiney et al. 1992) and targeting a 91 base pair fragment. Amplification was performed on a thermal cycler (BioRad) under the following conditions: 95°C for 4 min, 35 cycles of 95°C for 10 s, 60°C for 10 s and 72°C for 20 s, followed by 72°C for 10 min. PCR products were verified using electrophoresis alongside positive and no-template negative controls. Bands appearing on the 1.5% agarose gel stained with SYBR Safe (Invitrogen) indicated successful amplification, and samples were scored as positive or negative for *A. salmonicida* infection based on the presence or absence of a 91 bp band.

2.5 | Data analysis

Non-parametric Kaplan–Meier (KM) survival analyses (Systat R13) were performed for each of the disease challenges to obtain survival probability estimates for sablefish exposed to each bacterial concentration. Significantly different survival estimates among bacterial concentrations were determined using the Mantel method for the log-rank chi-square test with the null hypothesis of a common survival curve and a significance level (α) set at .05 for all comparisons. Survival curves were generated across all replicate tanks for vaccinated and sham-treated fish in pathogen-exposed treatments.

The vaccine potency, as determined by relative per cent survival (RPS, Amend, 1980), is the proportional relationship between vaccinated and sham-treated fish (Gudmundsdottir & Bjornsdottir, 2007) and was calculated as:

$$\text{RPS} = 1 - [\%\,\text{mortality in vaccinated fish}/\text{mortality in control fish}] \times 100. \quad (1)$$

3 | RESULTS

The injected-vaccinated sablefish had 90% survival when challenged with an atypical *A. salmonicida* concentration, while the unvaccinated sham fish had 45% survival. The KM survival probability curves (Figure 2a) for vaccinated and sham-treated sablefish were significantly different ($p < .001$). The vaccine potency, as determined by RPS, of the injected vaccine was 81.7% (Table 1). The RPS suggests that 81.7% more fish would survive with the vaccine than the fish that were not vaccinated. The injection-vaccinated sablefish had 99.3% survival when challenged with typical *A. salmonicida*, while sham-treated fish had 87.8% survival (Figure 2b). The KM survival probability curves for vaccinated and sham-treated sablefish were significantly different ($p < .001$), and the RPS was 94.3% (Table 1).

By contrast, the immersion vaccine was not protective for sablefish against either atypical (Figure 3) or typical *A. salmonicida* (Figure 4) when the disease challenge was performed at approximately 5 weeks post final vaccination. The RPS values for the Immersion-1 and Immersion-2 treatments at the lowest atypical *A. salmonicida* exposure concentrations were negative, indicating greater mortality in the vaccinated group (Table 1). However, the KM survival probability curves between vaccinated and sham-treated sablefish were also not significantly different in either Immersion-1 or Immersion-2 treated sablefish challenged to these concentrations ($p = .507$ and .315 respectively). The RPS values at the greater atypical *A. salmonicida* concentrations for the Immersion-1 and Immersion-2 treatments were also low (−9.1% and 4.0% respectively; Table 1). However,

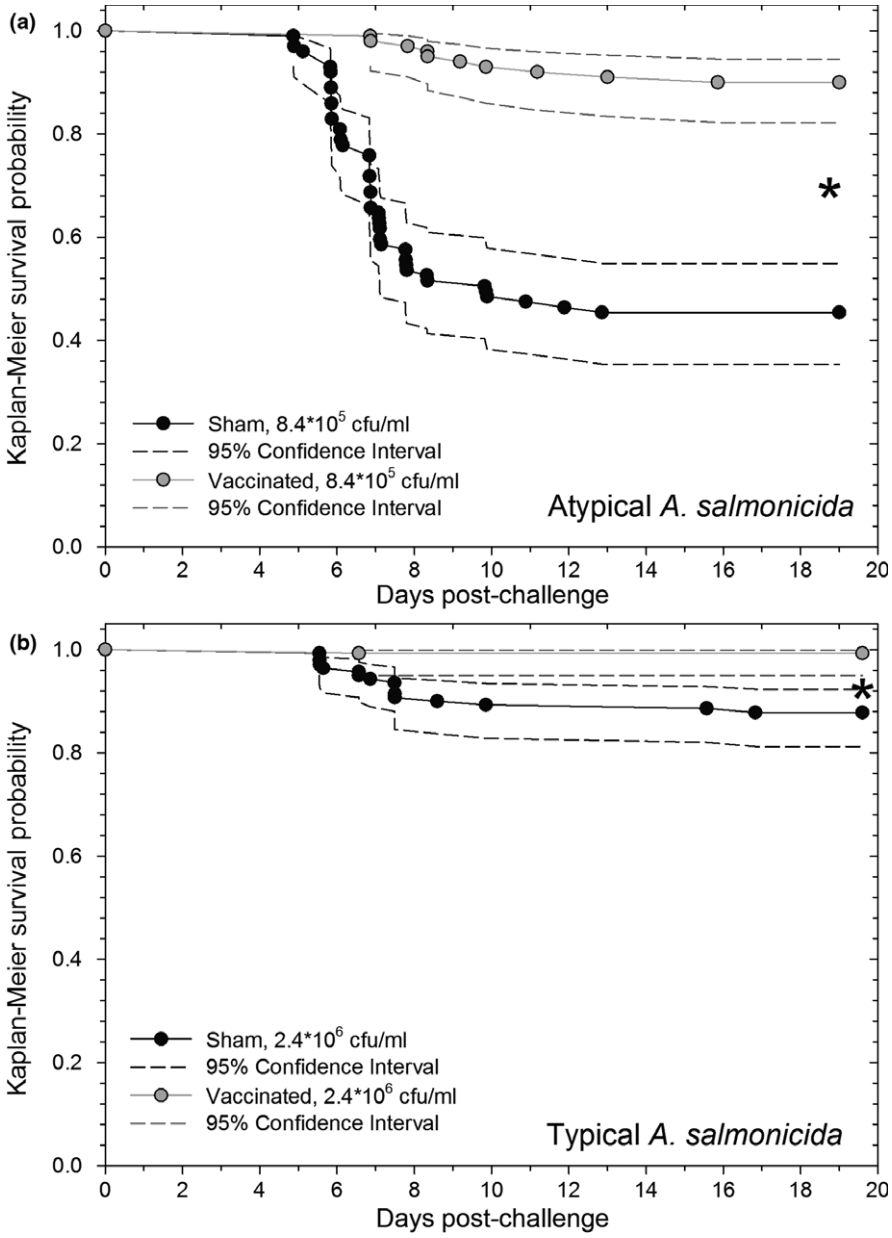

FIGURE 2 Injected vaccine results. Survival curves generated with the non-parametric Kaplan–Meier method for juvenile sablefish vaccinated or unvaccinated sham-treated fish by injection and challenged to either (a) atypical *Aeromonas salmonicida* (T30) or (b) typical *A. salmonicida* (#51) 121 days post stocking out. Specifically, sablefish were exposed to either 8.4×10^5 cfu/ml or 2.4×10^6 cfu/ml of atypical or typical *A. salmonicida*, respectively, 36 days post final vaccination. The asterisk (*) signifies that the curves generated between the vaccinated and sham fish are significantly different ($p < .001$)

significant differences in KM survival probability were found between vaccinated and sham treatments in both the Immersion-1 and Immersion-2 treatments (i.e. $p = .009$ and .025 respectively).

Similarly, the RPS values for the Immersion-1 and Immersion-2 treatments exposed to typical *A. salmonicida* were low (7.3% and 4.4% respectively; Table 1). In addition, the KM survival probability curves of the vaccinated and sham sablefish in the Immersion-1 (Figure 4a) and Immersion-2 (Figure 4b) treatments were not significantly different ($p = .550$ and .467 respectively).

The immersion vaccine was also not protective against atypical *A. salmonicida* when the disease challenge was performed with either Immersion-1- or Immersion-2-treated sablefish at 11 and 13 weeks post final vaccination respectively (Figure 5). Once again, the RPS values for the Immersion-1 or Immersion-2 treatments were low or negative (8.5% and −15.1% respectively). Likewise, the KM survival probability curves generated for the Immersion-1 or Immersion-2 treatments between vaccinated and sham-treated sablefish were not significantly different ($p = .401$ and .081 respectively). There was no difference in the KM survival probability curves of the Immersion-1 or Immersion-2 vaccinated sablefish ($p = .998$). Finally, the Injection vaccinated fish that were challenged with the same concentration of atypical *A. salmonicida* on the same day (Figure 2a) had significantly

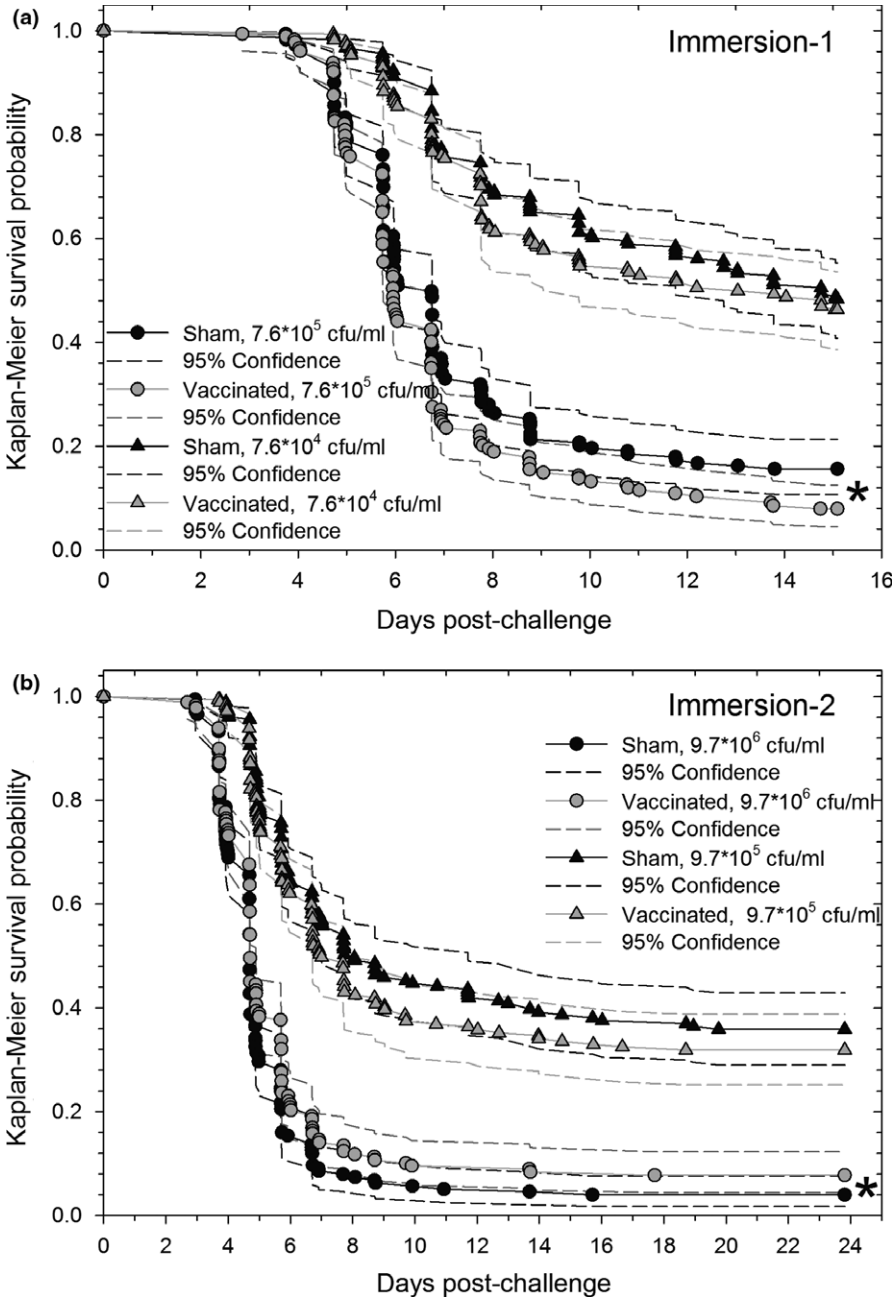

FIGURE 3 Immersion—ATYPICAL results. Survival curves generated after exposure to atypical *Aeromonas salmonicida* (T30) with the non-parametric Kaplan–Meier method for juvenile sablefish vaccinated or unvaccinated sham-treated fish by immersion either at (a) 58 days post stocking out (Immersion-1) or (b) 72 days post stocking out (Immersion-2). The Immersion-1 treated fish were challenged with either 7.6×10^5 or 7.6×10^4 cfu/ml of atypical *A. salmonicida* (T30) 32 days post final vaccination (a). The Immersion-2 treated fish were challenged with 9.7×10^6 or 9.7×10^5 cfu/ml of atypical *A. salmonicida* (T30) 36 days post final vaccination (b). The asterisk (*) signifies that the curves generated between the respective vaccinated and sham fish are significantly different ($p < .001$)

greater KM survival probability curves than either the Immersion-1 (Figure 5a) or Immersion-2 (Figure 5b) treatments ($p < .001$ and .001 respectively).

4 | DISCUSSION

This is the first study to demonstrate that vaccination can protect juvenile sablefish against losses due to furunculosis. Specifically, a whole-cell multivalent vaccine was found to be efficacious in juvenile sablefish against furunculosis caused by either typical or atypical *A. salmonicida* when injected. The RPS of the vaccine against atypical and typical *A. salmonicida* was 81.7% and 94.3%, respectively, when administered to juvenile sablefish by injection at 121 days post stocking out. Although sablefish were found to be less susceptible to typical *A. salmonicida* than to atypical *A. salmonicida*, the vaccine was still able to increase survival after exposure to typical *A. salmonicida*. The high RPS values indicated that the vaccine successfully initiated a greater

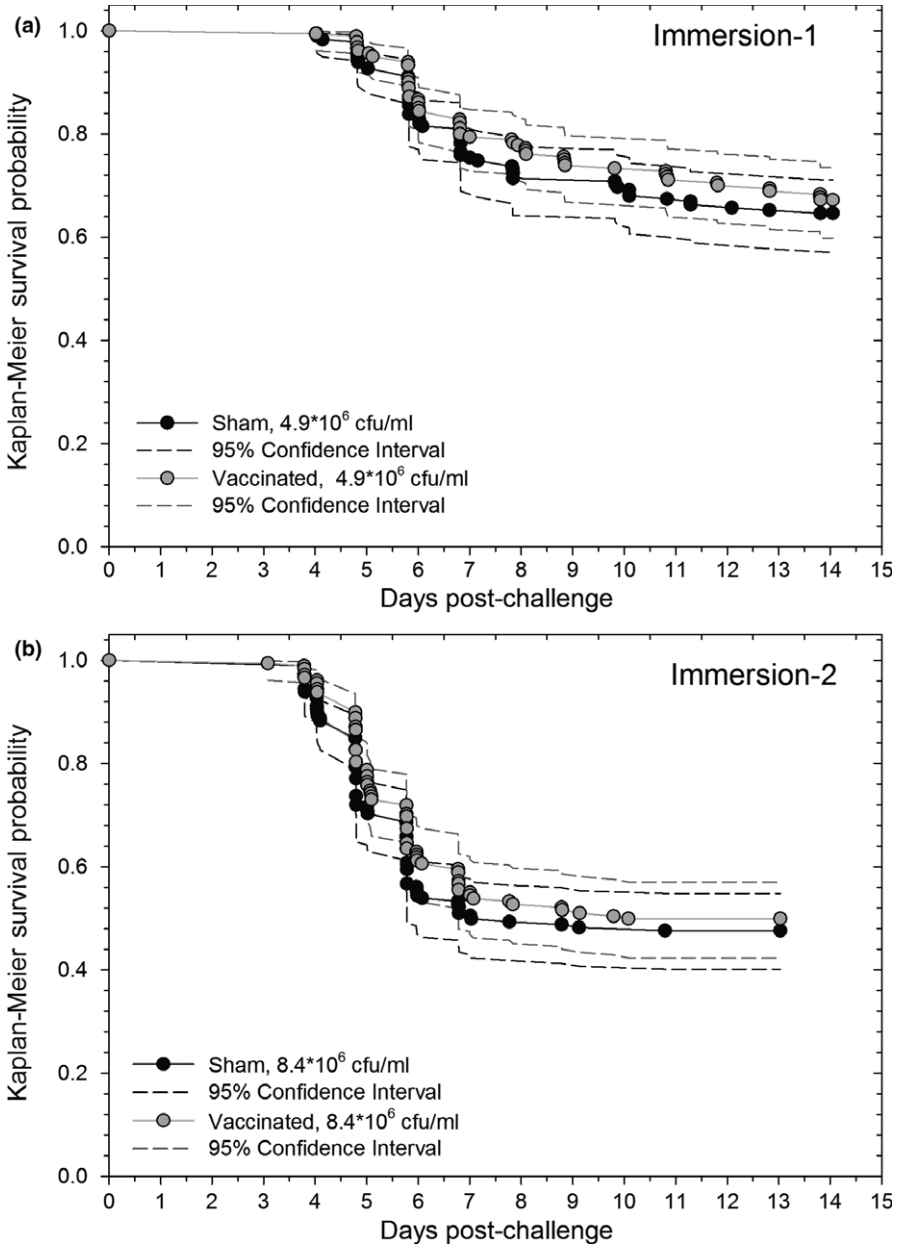

FIGURE 4 Typical immersion survival curves. Survival curves generated after exposure to typical *Aeromonas salmonicida* (#51) with the non-parametric Kaplan–Meier method for juvenile sablefish vaccinated or unvaccinated sham-treated fish by immersion either at (a) 58 days post stocking out (Immersion 1) or (b) 72 days post stocking out (Immersion 2). The Immersion-1 treated fish were challenged with 4.9×10^6 cfu/ml of typical *A. salmonicida* (#51) 33 days post final vaccination (a). The Immersion-2 fish were challenged with 8.4×10^6 cfu/ml of typical *A. salmonicida* (#51) 35 days post final vaccination (b). The survival curves were not significantly different ($p > .05$) between the vaccinated and sham-treated fish

immune response upon an encounter with the live pathogen. Prior studies demonstrated that Atlantic salmon, *Salmo salar* L., required 300–500 degree days or more to elicit immunity against *A. salmonicida* after receiving a vaccine via injection (Eggset, Mikkelsen & Killie, 1997; Midtlying, Reitan & Speilberg, 1996). In this study, juvenile sablefish challenged with either atypical or typical *A. salmonicida* at 36 days post-injection vaccination or approximately 533 degree days were also able to generate a protective response against the pathogens.

A multivalent vaccine containing both *Aeromonas* and *Vibrio* species was used in this study with sablefish as multivalent vaccines against furunculosis have been proven to be more efficacious than monovalent vaccines (Austin, 2012). For example, increased resistance in Atlantic salmon was developed against *A. salmonicida* with a multivalent injectable vaccine containing *A. salmonicida*, *V. salmonicida* and *L. anguillarum* (Midtlying et al., 1996). The enhanced protection was determined to be due to *V. salmonicida* (Hoel, Salonius & Lillehaug, 1997). Mechanistically, antibodies generated against *V. salmonicida* were able to cross react with *A. salmonicida* whole cells and LPS.

Conversely, sablefish administered the proprietary vaccine at either 58 (Immersion-1) or 72 (Immersion-2) days post stocking out

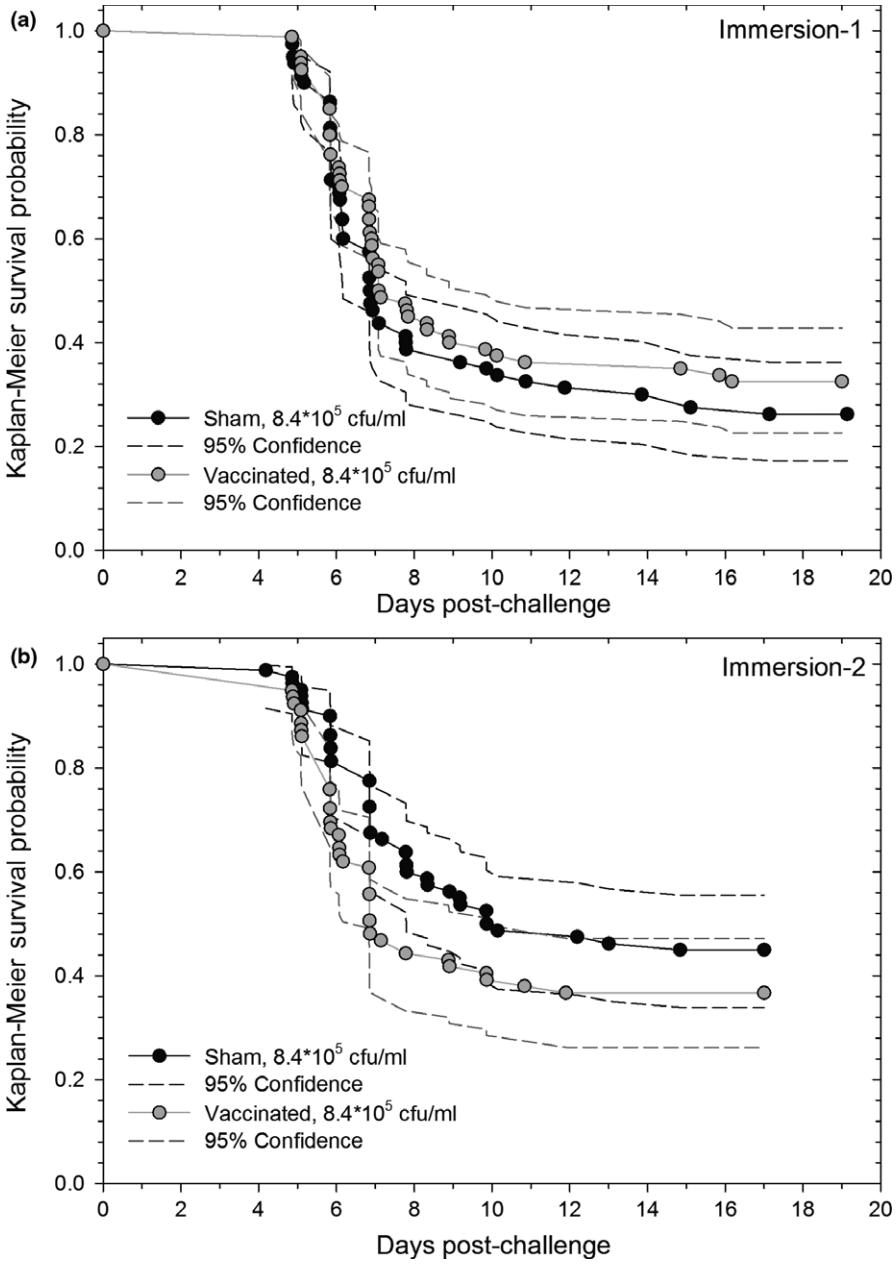

FIGURE 5 Atypical immersion survival curves at the time of the injection disease challenge. Survival curves generated after exposure to atypical *Aeromonas salmonicida* (T30) with the non-parametric Kaplan–Meier method for juvenile sablefish vaccinated or unvaccinated sham-treated fish by immersion either at (a) 58 days post stocking out (Immersion-1) or (b) 72 days post stocking out (Immersion-2). The Immersion-1 treated fish were challenged with 8.4 $\times$ 10^5 of atypical *A. salmonicida* (T30) 92 days post final vaccination (a). The Immersion-2 treated fish were challenged with 8.4 $\times$ 10^5 cfu/ml of atypical *A. salmonicida* (T30) 78 days post final vaccination (b). The survival curves were not significantly different ($p > .05$) between the vaccinated and sham-treated fish

via immersion were not protected from either atypical or typical *A. salmonicida* when challenged. The sablefish vaccinated at 58 days post stocking out were not protected from atypical *A. salmonicida* when tested at approximately 525 and 1,379 degree days. The sablefish vaccinated at 72 days post stocking out were also not protected from the atypical *A. salmonicida* at approximately 574 and 1,143 degree days. Similarly, neither Immersion-1 nor Immersion-2 treatments were protected against typical *A. salmonicida* at approximately 538 and 558 degree days respectively. The goal of an immersion vaccine is to induce mucosal immunity via the skin, gills and the gastrointestinal tract (Soto, Griffin & Tobar, 2015), in order to prevent pathogen invasion and colonization at portals of entry in the fish (Munang'andu, Mutoloki & Evensen, 2015).

A number of factors may have contributed to the ineffectiveness of the immersion vaccine (Lillehaug, 2014). The immersion vaccine may not have been efficacious due to the possibility that the fish were not of a size or age where their mucosal immunity was fully developed when they received the vaccine. The size or age that fish are exposed to the vaccine is a critical factor in developing a successful vaccine (Magnadottir, 2010). However, only a limited amount

of research exists that examines the size or age at which various fish species become fully immunocompetent (Lillehaug, 2014). For example, in marine species such as sea bass, *Dicentrarus labrax* L., spotted wolfish, *Anarhichas minor* O. and Atlantic cod, *Gadius morhua* L., the appearance of surface immunoglobulin can occur from 1 to 10 weeks post hatch (Magnadottir, Lange, Gudmundsdottir, Bogwald & Dalmo, 2005). At the time of the Immersion-1 and Immersion-2 vaccinations, the sablefish were 8–10 weeks post stock out (Figure 1) or a mean weight of 1.5 and 4.5 g, respectively, compared to 17 weeks post stock out or a mean weight of 50 g at the time of the Injection vaccination. In order for a vaccine to be successful, sablefish need to develop to the point where they have the ability to respond immunologically to the vaccine allowing them the potential to survive future encounters with a virulent pathogen.

Other potential issues may contribute to the inability of the immersion vaccine to protect sablefish against *A. salmonicida*. Direct immersion may not be an effective delivery system. Other immersion methods such as hyperosmotic infiltration, low-frequency sonophoresis and puncture may be more efficacious (reviewed in Soto et al., 2015; Rombout & Kiron, 2014; Plant & LaPatra, 2011; Sudheesh & Cain, 2017). In addition, variables to increase antigen uptake, such as vaccine concentration and incubation temperature, may need to be further optimized (Du, Tang, Sheng, Xing & Zhan, 2015). In this study, sablefish were immersed, as recommended by the manufacturer, for 1 min to the diluted vaccine which proved to be non-efficacious. However, Villumsen and Raida (2013) found that rainbow trout fry, *Oncorhynchus mykiss*, 9 g, immersed for a longer period, 5 min, to inactive *A. salmonicida* were protected against the pathogen (>90% RPS) 24 weeks later.

Other aspects to be considered to improve the efficacy of an immersion vaccine are the addition of adjuvants or immunostimulants (Soto et al., 2015). Adjuvants have historically been used to improve injectable fish vaccines (Tafalla, Bogwald & Dalmo, 2013). However, studies show that mucosal adjuvants are also helpful for the development of mucosal immunity (Soto et al., 2015). For example, studies with rainbow trout have shown that immersion vaccines against *A. salmonicida* are slightly more effective if the vaccine contains carrier molecules, such as liposomes, which may enhance uptake of the vaccine (Rodgers, 1990). Exposure to isolated virulence factors such as pathogen-associated molecular patterns (PAMPs) from the bacteria in liposomes may also act to elicit a protective response to the pathogen. For example, Ruyra et al. (2014) successfully encapsulated lipopolysaccharide (LPS) within a liposome to produce an efficacious immersion vaccine in zebrafish, *Danio rerio*, against a gram-negative bacteria *Pseudomonas aeruginosa*. Ruyra et al. (2014) also determined that empty liposomes and unassociated LPS were not efficacious against the pathogen.

In conclusion, we determined that a multivalent vaccine was efficacious against both atypical and typical *A. salmonicida* administered via injection but not efficacious when delivered by immersion. An advantage of an injectable vaccine relative to an immersion vaccine is that smaller volumes are required for immunization and an exact dose can be delivered to each fish. However, injectable vaccines are very difficult to administer to very small fish. As *A. salmonicida* can affect any age/size sablefish, efficacious immersion vaccines are also needed that can be efficiently administered to small fish. To further explore the development of an efficacious immersion vaccine to atypical and typical *A. salmonicida* in sablefish, variables such as size at initial vaccination, immersion delivery method, incubation temperature, vaccine concentration and the addition of immunostimulants should be examined.

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