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Developing vaccination strategies for the prevention of atypical furunculosis in sablefish *Anoplopoma fimbria*

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Abstract: Sablefish (also known as black cod) represent a promising and high-value species for marine aquaculture. Research efforts to optimize culture strategies and methods have been ongoing for more than two decades. This species is commercially produced using a combination of land-based tank and marine net-pen aquaculture in British Columbia, Canada, and Washington, USA. To set the stage for expanded production of farmed sablefish, NOAA Fisheries has prioritized research projects and partnerships to address remaining challenges. One such challenge that impacts the production of this species is the disease furunculosis. This is currently the primary disease threat for farmed sablefish and is caused by atypical strains of *Aeromonas salmonicida*, a gram-negative bacterium. Although antibiotic treatments can be administered to reduce mortality following an outbreak, disease prevention through vaccination is desirable and has been identified as a high-priority need for further development of sablefish aquaculture. Recent vaccination projects have explored practical mass vaccination strategies along with more traditional approaches to prevent furunculosis. These include an oral vaccination study that assessed the efficacy of a killed whole-cell *A. salmonicida* vaccine administered orally via feeding alginate/gelatin micro-particles to juvenile sablefish. Another study that took a more traditional approach, assessed the efficacy of a primary immersion immunization and injection booster with and without adjuvant inclusion. Although more labor intensive, results indicated that this approach currently represents the most practical strategy to achieve long-term protection of sablefish against furunculosis. A final ongoing project that is discussed involves the development and testing of attenuated atypical *A. salmonicida* strains that could be administered via immersion during early juvenile stages. To produce such attenuated vaccine candidates, known virulent *A. salmonicida* strains were repeatedly passaged on tryptic soy agar (TSA) plates containing increasing concentrations of the antibiotics rifampicin and novobiocin. To achieve further attenuation, strains were acclimated to high incubation temperatures outside of their optimal growth range. Four isolates that grew under these conditions and showed resistance to these antibiotics have been confirmed as fully attenuated. These four strains now serve as potential vaccine candidates and are being assessed for their ability to protect sablefish from disease following immersion or injection immunization.

Key words: sablefish, furunculosis, vaccination, immunity

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Introduction

Disease management in commercial aquaculture is critical to the success of most operations. Sablefish *Anoplopoma fimbria* represent an economically valuable marine species native to the Pacific Northwest of the United States and Canada. They are harvested commercially and have been farmed in net pens for nearly 20 years in Canada and more recently in the United States. Disease outbreaks do occur regularly in sablefish, resulting in direct mortality and reduced economic returns for farmed sablefish operations. The primary disease affecting this species is furunculosis, caused by an atypical strain of the bacterium *Aeromonas salmonicida* (Arkoosh *et al*. 2017; Goetz *et al*. 2021). Outbreaks of this disease can result in high mortality of fish during early juvenile stages and also reduce the marketability of later adult life stages due to clinical signs of the disease. Furunculosis is one of the oldest described bacterial diseases of fish and historically, *A. salmonicida* ssp. *salmonicida* was referred to as the 'typical' strain because of its early discovery and description in farmed salmonids (Wiklund and Dalsgaard 1998). However, 'atypical' strains and subspecies of *A. salmonicida* have since been isolated from non-salmonid hosts in freshwater and marine environments (Austin 2015). *Aeromonas salmonicida* can be identified phenotypically as a catalase and oxidase positive gram-negative, non-motile, non-encapsulated coccobacilli, that grows best between 22 to 25ºC (Austin *et al*. 1998; Gulla *et al*. 2016; Lian *et al*. 2020). Typical strains are generally diagnosed by the presence of brown pigmentation when cultured in the presence of tyrosine, and atypical strains are non-pigmented or exhibit reduced pigmentation (Donlon *et al*. 1983). Further genetic differences that separate typical and atypical strains based on gene expression and host affinity have been described (Vasquez *et al*. 2022).

In the 1980s, furunculosis vaccines were developed and implemented heavily in the Atlantic salmon industry. Midtlyng (2014) noted that in Scotland, salmon smolt survival to harvest increased from 65% to over 90% following the incorporation of injectable adjuvant-based bacterins. This shows that vaccination can be effective and protect against typical *A. salmonicida* strains in salmonids, but vaccines against atypical strains in non-salmonid fish species are less common. Early work to assess the potential of vaccinating sablefish has shown that certain injectable furunculosis vaccines can be effective against atypical *A. salmonicida* (Arkoosh *et al*. 2017). This is promising, but injection vaccination is not ideal due, in part, to the minimum size required for vaccination, labor costs, stress on the animal, and the inability to protect fish during susceptible early life stages. Therefore, alternative strategies that allow for mass vaccination of fish are desirable and include oral, immersion, or a combination of oral and immersion vaccinations with injection booster vaccinations when fish reach a larger size. This minipaper provides an overview of three studies aimed at developing new vaccines and practical delivery strategies to protect sablefish from disease caused by atypical strains of *A. salmonicida*.

In the first study, an oral vaccine platform that incorporated a formalin-killed *A. salmonicida* vaccine into liposome-containing complex alginate particles was developed and tested for its ability to elicit a protective immune response in sablefish. The primary objectives were to 1) encapsulate killed whole-cell *A. salmonicida* within alginate particles, and 2) evaluate experimental vaccines for protective immunity following pathogen challenge of sablefish.

The next study was aimed at the need to implement a practical vaccination strategy into production trials that are part of ongoing collaborations between NOAA Fisheries and the company Jamestown Seafood (operated by the Jamestown S'Klallam Tribe, Sequim, Washington, USA). To address this, a two-part study was conducted at NOAA's Manchester Research Station in Port Orchard, Washington (USA). This involved the semi-commercial scale grow-out of sablefish to market size in net pens or land-based tanks [flow through seawater or recirculated seawater (RAS)]. Previous production studies with sablefish at this site have resulted in furunculosis outbreaks and the need to treat fish periodically with antibiotics during the grow-out cycle. Vaccination of fish to prevent this disease has previously utilized commercial vaccines designed for salmonids and has met with limited success. Therefore, we evaluated a killed atypical *A. salmonicida* vaccine (utilizing strain KJ-1 – recently isolated from clinically diseased sablefish) by administering it to fish with or without adjuvant via an initial bath vaccination followed by an injection booster immunization. We hypothesized that bath immunization followed by an injection booster would provide long-term protection against furunculosis, and that vaccine efficacy would be enhanced by incorporating adjuvants. The primary objectives were to (1) vaccinate juvenile sablefish via immersion followed by a booster immunization at six weeks (approximately 50 g/fish), and (2) evaluate whether the inclusion of oil-based adjuvants during both immersion and booster injection administration enhanced the level of protection conferred.

The last project is ongoing and focuses on the development

of a live attenuated vaccine for sablefish with the goal of producing an efficacious immersion vaccine against atypical furunculosis. It was hypothesized that a live attenuated (non-virulent) atypical *A. salmonicida* vaccine could be developed and provide enhanced protection as a vaccine when compared to traditional killed whole-cell vaccine formulations. The primary objectives were therefore to 1) produce attenuated strains of atypical *A. salmonicida* using an antibiotic selection strategy, and 2) test live attenuated strains for their efficacy as vaccine candidates when delivered to sablefish by immersion or injection.

Overview of studies (objectives and methods)

1. Oral vaccination study

There were two trials conducted in this study to assess the potential of orally vaccinating sablefish using complex alginate particles (Fig.1). Alginate particles were produced using medium viscosity alginate and fish gelatin (Hawkyard *et al*. 2019). Liposomes containing compounds known to elicit feeding by marine finfish (betaine, alanine, and glycine) were included with these large alginate-gelatin particles resulting in 'complex particles'. In addition, the atypical *A. salmonicida* strain (T30), originally isolated from sablefish showing clinical signs of furunculosis (Arkoosh *et al*. 2017) was incorporated into alginate particles to achieve a high antigen dose. Several treatments were tested and included: 1) fish fed a sham control treatment consisting of alginate particles without killed-*A. salmonicida* cells (Sham_1); 2) fish fed a commercial diet (no particles) control (CC); 3) fish treated by injection vaccination (IV); 4) fish fed an oral vaccine particle (Oral_1) containing killed *A. salmonicida* and produced with the basic recipe described above; and 5) fish fed an oral vaccine particle (Oral_2) that integrated milled fish feed along with killed-*A. salmonicida* cells. All orally vaccinated treatments, including the sham, received the vaccine or sham particles as both primary and booster vaccinations. At 11 weeks post - primary vaccination, sablefish were challenged with atypical *A. salmonicida* strain T30, and mortality was monitored for 25 days to determine if vaccine treatments conferred protection. Relative percent survival (RPS) for each treatment was calculated and compared to the appropriate control/unvaccinated group using the following formula (Amend 1981):

RPS= 1 - [(% mortality in vaccinated fish) / (% mortality in unvaccinated reference fish)]*100

Following the initial trial, a second challenge trial was conducted using the atypical *A. salmonicida* strain with juvenile sablefish. This trial compared the protective efficacy of the oral vaccination methods described above with bath vaccination and combined oral–bath strategies.

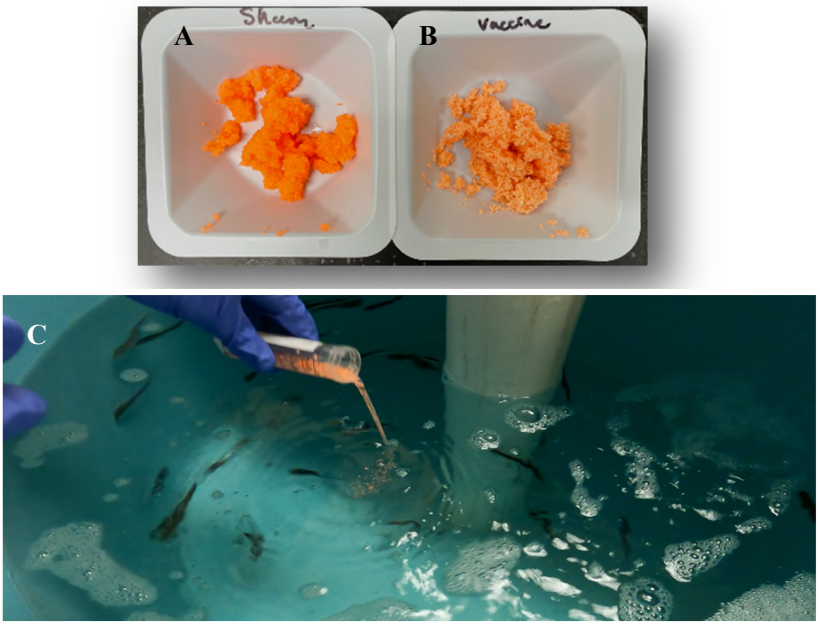

Fig.1 Complex alginate particles with or without killed whole-cell *Aeromonas salmonicida* incorporated as an oral vaccine

A, Sham particle (no vaccine); B, Alginate particle (with vaccine); C, Particles suspended in water and administered via feeding to fish (Photo Credit: Evan Jones).

2. Production scale fish vaccination study (adjuvant and booster assessment)

An initial small-scale experiment was designed that consisted of 1,500 fish (average 2.3 g/fish) split into three groups of 500 fish. **Group one** was immersion immunized for 30 minutes with a formalin-killed vaccine containing the KJ-1 *A. salmonicida* isolate diluted in seawater; **group two** was immersion immunized under identical conditions but with half the dose as the vaccine was mixed (50:50) with an adjuvant supplied by the company Seppic, Inc. (Fairfield, CT, USA); and **group three** was immersed in an equal volume of PBS diluted in seawater for 30 minutes and served as the sham control. At approximately six weeks post-primary immersion vaccination, 200 fish per group were injection-vaccinated with 100 µl of the killed KJ-1 isolate, with or without adjuvant. The negative control group received an injection of 100 µl of PBS. Groups were held in tanks receiving filtered seawater, and at 335 days post-vaccination, triplicate groups of fish from each treatment were challenged with the virulent KJ-1 strain of atypical *A. salmonicida*. Blood was collected from fish in each treatment throughout the study to determine specific serum anti-*A. salmonicida* antibody titers using an enzyme-linked immunosorbent assay (ELISA) recently developed by Jones *et al*. (2022).

In addition to the experiment described above, a production-level vaccination was implemented and 12,000 fish were immunized using the same vaccine treatments as described above, but an unvaccinated group was not included. Briefly, 6,000 fish received the primary bath and injection booster vaccinations with adjuvant and 6,000 fish received the vaccine treatments without adjuvant. These treatment groups were stocked (replicate pens or tanks/treatment) and grown to market size in either marine net pens (four pens with 2,000 fish/pen) or land-based tanks [four tanks (two flow through and two RAS) with 1,000 fish/tank] and monitored for mortality throughout the production cycle.

3. Development of a live attenuated atypical *A. salmonicida* vaccine

The methods for creating attenuated bacteria for use as vaccines are diverse (Ma *et al*. 2019). This study utilized an antibiotic-thermal selection strategy to induce random mutations, but did not involve genetic engineering. Briefly, four virulent parent strains of atypical *A. salmonicida* (T30, KJ-1, Spen-3, and K2-W), originally isolated from diseased sablefish, were selected and cultured in the presence of increasing concentrations of the antibiotics (rifampicin and novobiocin) as described by Pridgeon and Klesius (2011) with modifications. To achieve further attenuation, the same antibiotic-resistant strains were acclimated to incubation temperatures up to 30°C, which has been associated with the loss of virulence factors (Ishiguro *et al*. 1981). These thermal tolerant "resistant" strains were then tested to determine which were non-pathogenic (i.e. attenuated). Briefly, this was accomplished through *in vivo* challenge studies whereby, groups of juvenile sablefish were challenged by injection with either high doses of the mutant *A. salmonicida* strains or their corresponding virulent parent strain. Mortality was monitored for 14 days and mutant strains were considered completely attenuated if no infection-related mortality or clinical signs of disease occurred. Strains showing complete attenuation represent potential vaccine candidates and will be incorporated into future vaccination trials.

Results and Discussion

1. Oral vaccination in sablefish

To address the potential of an oral vaccine to be administered to sablefish and protect against atypical furunculosis, treatments were prepared and consisted of killed whole-cell *A. salmonicida* (T30 strain) incorporated into complex alginate particles. These particles were administered orally to fish as primary and booster immunizations in an initial trial. A second trial also combined alternating booster or primary bath immunization with the oral vaccine. Oral vaccines are challenging to develop and commercialize but are preferred because they are easy to apply and reduce handling-related stress to the fish. A key consideration for these vaccines is that for an effective immune response to be elicited in fish following oral vaccination, antigen degradation in the stomach must be minimized. Therefore, encapsulation methods using many materials have been described that can protect antigens until they are delivered to gut-associated lymphoid tissues (GALT) where antigen uptake and processing can occur (Ahmadivand *et al*. 2017; Jia *et al*. 2020). Results from the current study showed that fish fed a complex alginate particle vaccine had significant protection compared to negative control (unvaccinated) groups (Fig.2). However, the level of protection was significantly lower than that conferred by injection vaccination. The hybrid administration of the oral vaccine with a bath vaccine, either as a primary or booster vaccination, in a second trial, did not enhance protection compared to the oral primary and booster (data not shown). As expected, injection vaccination was highly protective

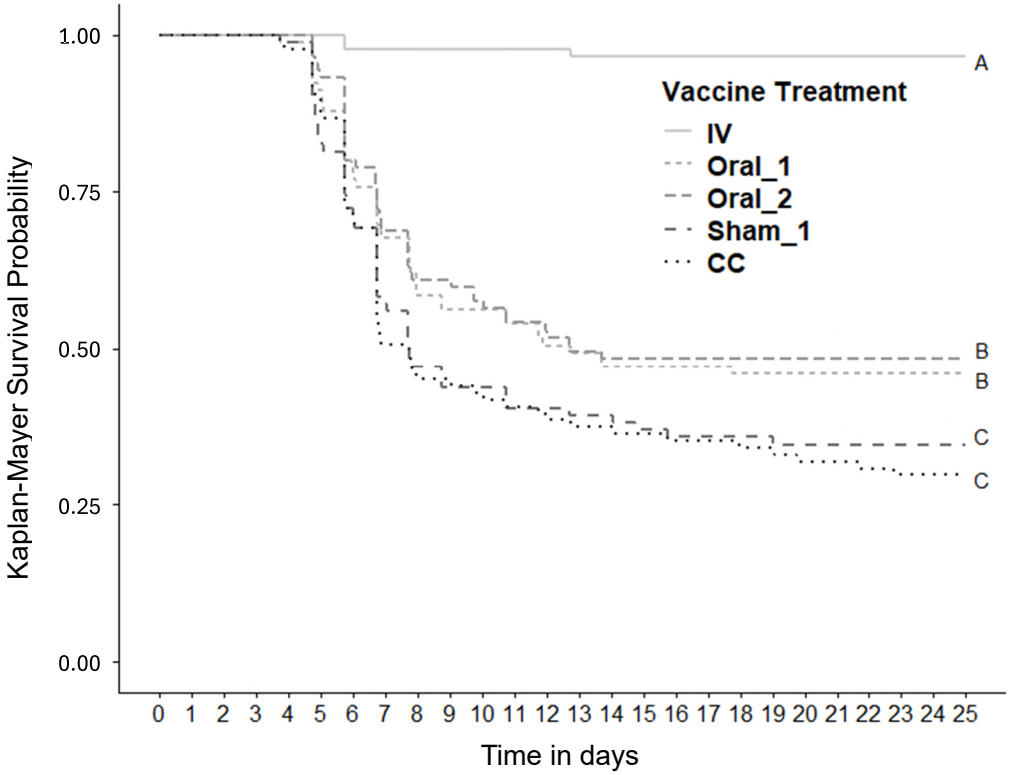

Fig. 2. Kaplan-Mayer survival curves following pathogen challenge for juvenile sablefish vaccinated by intraperitoneal injection (IV), orally using alginate particles (Oral_1, Oral_2), or unvaccinated (CC, Sham_1)

Different letters (A, B, or C) indicate significant differences between treatments.

with an RPS of 95%, whereas RPS values for oral vaccine treatments in these trials ranged from 17-21 % and were comparable to a bath vaccination with no booster, RPS 17% (data not shown). The study shows that oral vaccines can offer some protection against atypical furunculosis. The protection observed was similar to a bath-administered killed vaccine but was low compared to injection-based methods. Alternating the type of primary and booster administration between oral or bath vaccines did not enhance protection. The low RPS values in comparison to the injection vaccine indicate that additional research is required before such an oral vaccine would be commercially viable.

2. Production scale vaccination strategy

This study was designed to incorporate a bath vaccination when fish were small followed by an injection booster vaccination prior to stocking out into net pens or land-based grow-out tanks. Experiments were set up to test this approach in combination with or without the incorporation of newer adjuvants (Seppic, Inc) into the vaccine to determine if long-term disease protection could be conferred. Results from the experimental trials showed that sablefish vaccinated with or without adjuvant developed significant antibody responses by day 45 post-immersion vaccination. By day 90 (45 days post-booster) fish vaccinated with adjuvant showed significantly higher titers compared to fish not receiving adjuvant or fish in the control groups. In general, this trend continued out until day 335 (pre-challenge) and also held true for survivors of the pathogen challenge (Fig.3). Cumulative percent mortality following pathogen challenge showed that fish in groups vaccinated with or without adjuvant were highly protected (Fig.4) with RPS values of 77% and 85%, respectively. Interestingly, antibody titers were shown to be significantly enhanced in fish receiving the vaccine containing adjuvants; however, this was not reflected in higher protection for fish in these groups, at least when tested at nearly one year post-primary vaccination (Figs.3, 4). Furthermore, in the production-scale experiment, fish in the vaccine treatment groups never exhibited clinical signs of atypical furunculosis, and no *A. salmonicida* related mortality occurred in fish produced and grown to market size in marine net pens or land-based tanks. Overall, this strategy of vaccination proved highly effective at the both the experimental lab and field/production scale.

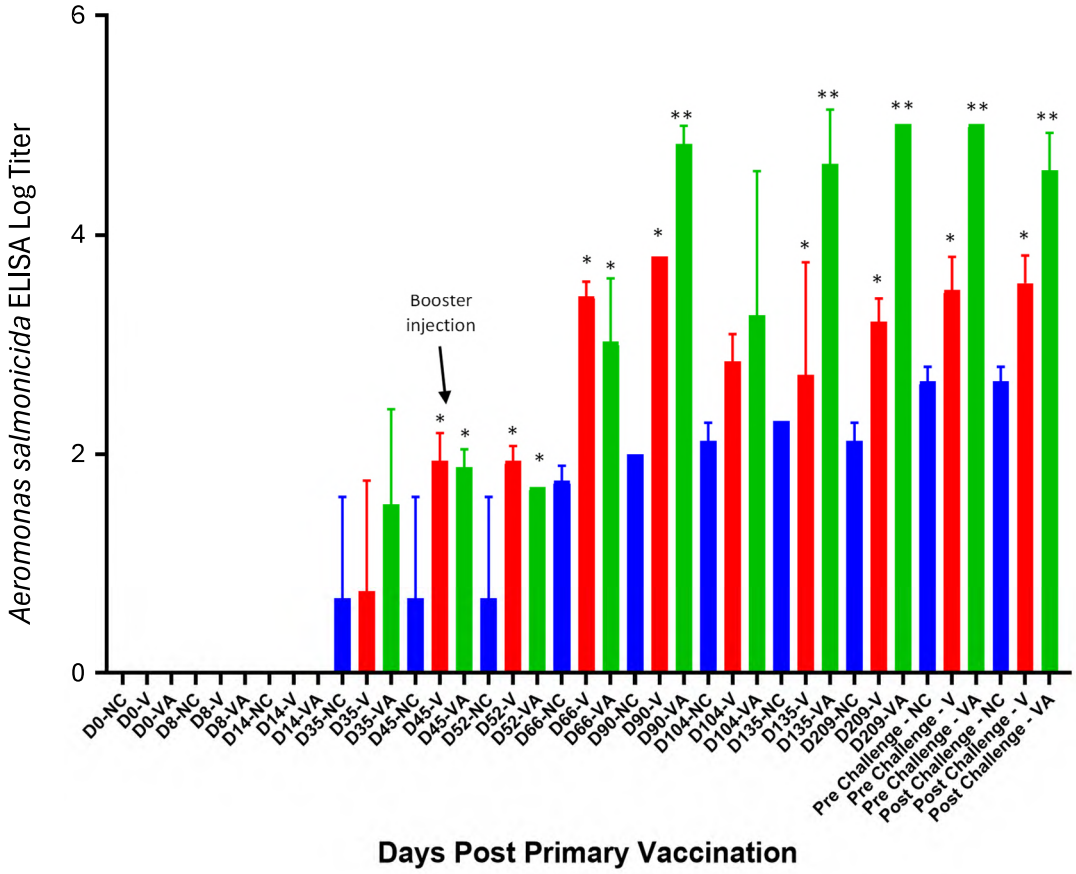

Fig.3 Specific serum anti-*Aeromonas salmonicida* antibody titers in sablefish following immersion vaccination and injection booster (day 45)

Antibody titers (as determined by ELISA) were determined from day 0 to day 335 (pre-challenge) and in surviving fish following pathogen challenge (post-challenge). Treatment groups included NC (unvaccinated groups receiving only PBS), V (vaccine only), VA (vaccine plus adjuvant). Different asterisks indicate significant differences from the control NC groups (*) or both the V and NC groups (**) at specific time points.

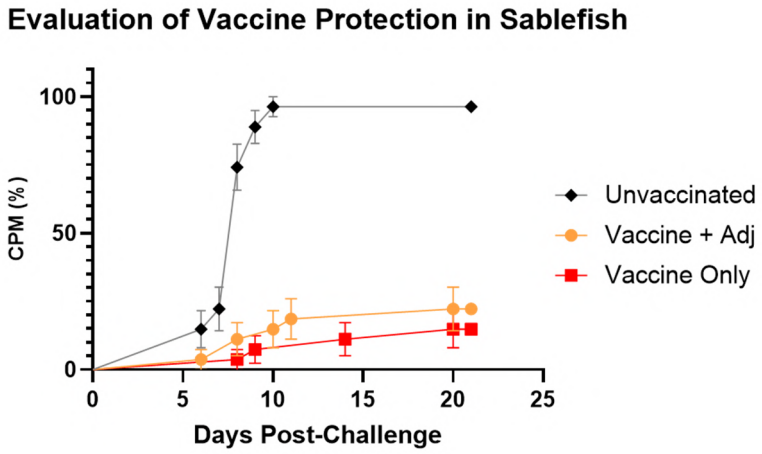

Fig.4 Cumulative percent mortality (CPM) of unvaccinated fish or fish vaccinated with a formalin killed-*Aeromonas salmonicida* vaccine with or without adjuvant

Fish were challenged at 335 days post primary vaccination and CPM calculated (triplicate groups) 21 days post-challenge with a virulent *A. salmonicida* strain.

3. Live attenuated *A. salmonicida* vaccine candidates

The final study described in this minipaper addresses the continued need for an effective vaccination strategy that does not require injecting large numbers of fish. The development of a live attenuated vaccine for atypical furunculosis in sablefish is being explored given the minimal protection previously observed with immersion vaccines comprised of killed whole-cell *A. salmonicida*. The use of live attenuated vaccines in the aquaculture industry can be effective and is not a new concept (Shoemaker *et al*. 2009). Since a live (non-pathogenic) pathogen is administered to fish, attenuated vaccines tend to stimulate a stronger immune response than killed vaccines, resulting in greater efficacy when delivered by immersion (LaFrentz *et al*. 2008; Ma *et al*. 2019). Here, an antibiotic-thermal selection method was applied to four virulent parent strains of atypical *A. salmonicida*. After multiple passages in the presence of rifampicin, novobiocin, and increased temperatures, 12 resistant strains were produced. These 12 strains were then used in a challenge study to determine if any had been partially or completely attenuated. Results from this challenge trial demonstrated that four of these resistant strains were completely attenuated; two stains produced from the T30 parent strain, one from the KJ-1 strain, and one from a Spen3 strain (Fig.5). These four strains now serve as potential vaccine candidates and will be further tested in upcoming vaccine trials to determine if long-term protection can be elicited in sablefish following immersion and injection vaccination. Preliminary trials with the KJ-1 resistant strain showed high protection following injection delivery and moderate protection via immersion vaccination (data not shown). Ongoing and future work will focus on evaluating all strains, quantifying dose requirements, duration of immunity, and developing alternative culture conditions to enhance vaccine efficacy.

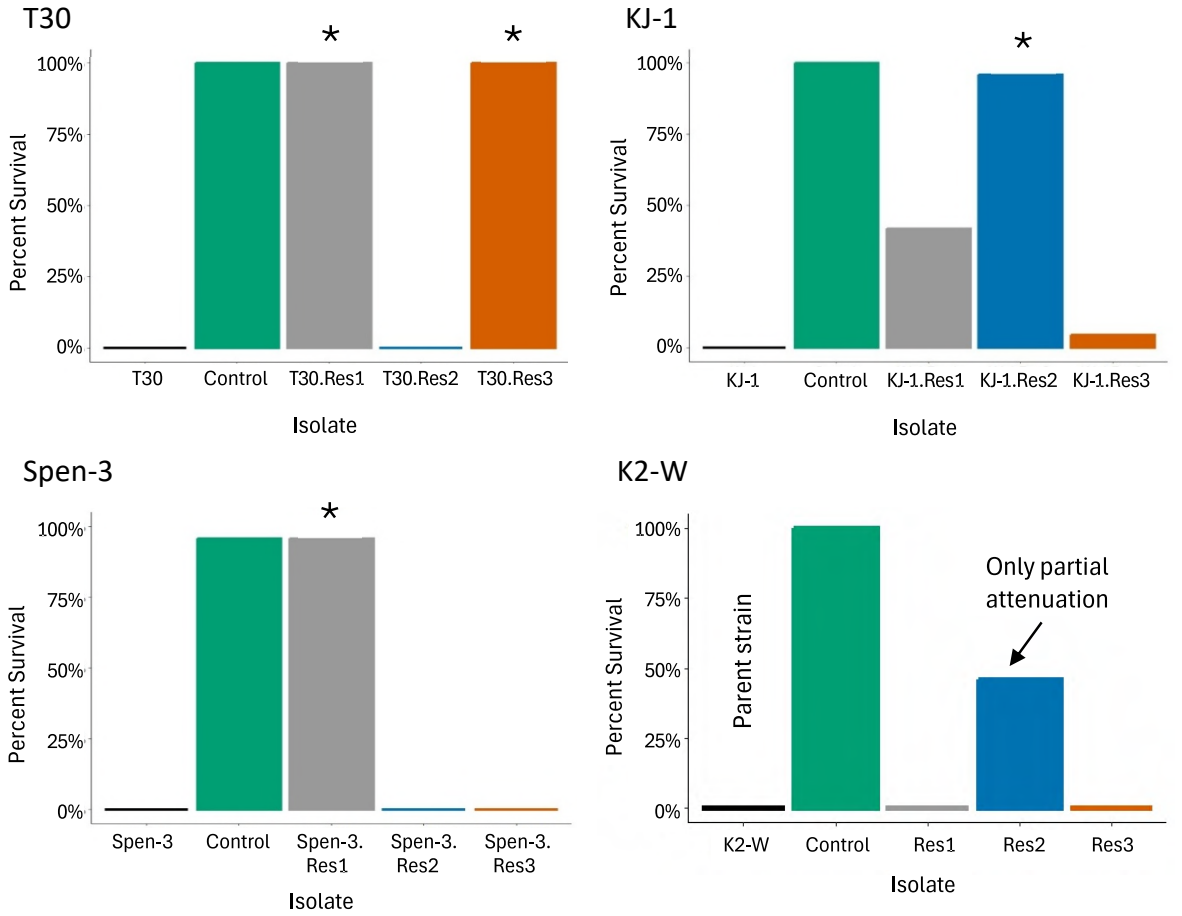

Fig.5 Challenge results showing percent survival of fish following challenge with four selected virulent atypical *Aeromonas salmonicida* parent strains (T30, KJ-1, Spen3, and K2-W) and "resistant" strains derived from each parent strain

Those strains that exhibited 100% survival are considered completely attenuated (*) and represent potential vaccine candidates for future evaluation.

Conclusion

The three studies described in this minipaper represent ongoing work that is aimed at developing novel approaches that can minimize the incidence of disease in sablefish caused by atypical strains of *A. salmonicida*. The goal is to provide a cost-effective and low-stress vaccine that can be easily administered to fish at both early and later life stages. The work here lays a foundation for this but also demonstrates that long-term immunity can be achieved in the interim with more traditional approaches if relevant atypical *A. salmonicida* strains are incorporated into killed immersion and injection vaccines. Newly developed attenuated *A. salmonicida* strains may enhance the effectiveness of immersion vaccines against atypical furunculosis in sablefish; however, further studies are needed to confirm their potential as vaccine candidates.

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Annotated Bibliography of Key Works

(1) Arkoosh MR, Dietrich JP, Rew MB, Olson W, Young G, Goetz FW (2017) Exploring the efficacy of vaccine techniques in juvenile sablefish, *Anoplopoma fimbria*. *Aquac Res*., **49**, 205-216. https://doi.org/10.1111/are.13449

This paper evaluates delivery strategies (immersion and injection) and tests multivalent vaccines for their ability to protect against atypical furunculosis for sablefish. 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 *Aeromonas salmonicida*. However, the relative percent 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.

(2) Goetz FW, Anulacion BF, Arkoosh MR, Cook MA, Dickhoff WW, and 10 other authors (2021) Status of sablefish, *Anoplopoma fimbria*, aquaculture. *J World Aquac Soc*., **52**, 607-646. https://doi.org/10.1111/jwas.12769

This is a key review article on sablefish, *Anoplopoma fimbria* (also called black cod), which is a long-lived marine species that is found in the Pacific from Baja California to Alaska, the Bering Sea, and through to the eastern coast of Japan. The value and feasibility of commercial aquaculture development along with important research needs are discussed. Advances in many research areas have been significant over the last 20 years and there are a few companies producing sablefish. Research advances include early life stage rearing along with production of all-female monosex offspring that grow faster than male or mixed sex populations.

Econometric models suggest that internal rates of return are 11-15% higher for monosex relative to mix-sex stocks over a 10-year period under typical cage culture conditions. Work showing that sablefish are susceptible to diseases (furunculosis and vibriosis) brought on by atypical *Aeromonas salmonicida* and *Vibrio anguillarum* is highlighted, but commercial vaccines (developed for salmonids) are only protective when given by injection. Long-term protection offered by vaccination has not been defined. Key takeaways from this paper are that more research is needed in relation to effective vaccine development and that improvements in methods for vaccine delivery would be beneficial.

(3) Jones EM, Oliver LP, Ma J, Leeuwis RHJ, Myrsell V, Arkoosh MR, Dietrick JP, Schuster CM, Hawkyard M, Gamperl KA, Cain KD (2022) Production of a monoclonal antibody specific to sablefish (*Anoplopoma fimbria*) IgM and its application in ELISA, western blotting, and immunofluorescent staining. *Fish Shellfish Immunol.*, **130**, 479–489. https://doi.org/10.1016/j.fsi.2022.09.038

This work addresses the issues with polyclonal antibodies and their limitations for important assays designed to monitor specific antibody kinetics or identify important immune cells in tissues. Sablefish (*Anoplopoma fimbria*) are an emerging aquaculture species and such new tools are needed to determine antibody response following vaccination or disease outbreaks. In this paper, a monoclonal antibody, UI-25A, specific to sablefish IgM was produced in mice. Western blotting confirmed that UI-25A recognizes the heavy chain of IgM and does not cross-react to proteins or carbohydrates in serum of four other teleost species. An ELISA was developed to measure *Aeromonas salmonicida* specific IgM in the plasma of sablefish, and UI-25A was used in Western blot analyses to identify immunogenic regions of *A. salmonicida* recognized by IgM from vaccinated sablefish. Immunofluorescent staining also demonstrated the ability of UI-25A to recognize membrane-bound IgM and identify IgM + cells (presumably B cells) in sablefish head kidney. Results demonstrate the usefulness of UI-25A as a tool to improve the understanding of antibody-mediated immunity in sablefish. This product will be valuable for vaccine development and the expansion of sablefish aquaculture efforts.

(4) Vasquez I, Cao T, Hossain A, Valderrama K, Gnanagobal H, Dang M, Leeuwis RMJ, Ness M, Campbell B, Gendron R, Kao K, Westcott J, Gamperl AK, Santander, J (2020) *Aeromonas salmonicida* infection kinetics and protective immune response to vaccination in sablefish (*Anoplopoma fimbria*). *Fish Shellfish Immunol*., **104**, 557-566. https://doi.org/10.1016/j.fsi.2020.06.005

This study addresses the need for effective vaccine programs against Aeromonas salmonicida, which have been identified as a high priority area for sablefish (*Anoplopoma fimbria*) aquaculture. This study established an *A. salmonicida* infection model to evaluate commercial vaccines and an autogenous vaccine preparation.

Using a clinical isolate of *A. salmonicida* (J410) they estimated a median lethal dose (LD50) of ~3 × 105 CFU/dose, and determined that the relative percent survival (RPS) for the autogenous bacterin mix was 65.22%, for commercial Forte Micro 4® vaccine it was 56.52%, and for Alpha Ject Micro 4® it was 30.43%. The RPS trends agreed with *A. salmonicida* tissue colonization levels at 10 days post-challenge. They measured total IgM titers, which peaked at 6-8 weeks post-immunization, but determined that the *A. salmonicida* A-layer binds to immunoglobulins F(ab)' in a non-specific fashion and affects immune assays and potentially vaccine efficacy. These results show that vaccine design influences sablefish immunity and provides a guide for sablefish vaccine programs.