Evaluating a Novel Oral Vaccine Particle for use in Disease Prevention in Rainbow Trout (*Oncorhynchus mykiss*) and Development of Important Tools for the Study of Immunity in Sablefish (*Anoplopoma fimbria*)

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Abstract

Vaccination is an important tool in preventing diseases in animals and humans. As the aquaculture industry has grown, so too has the demand for efficacious and efficient vaccination strategies. Vaccines in aquaculture are mainly administered by injection or immersion, with relatively few exceptions for orally administered vaccines against enteric pathogens. Oral vaccines encounter several problems associated with their delivery but most important may be the degradation during intestinal travel. A novel alginate-based oral vaccine delivery platform was developed by researchers at Oregon State University (OSU) that is designed to protect vaccine antigens during gastrointestinal travel. In cooperation with the National Oceanic and Atmospheric Administration (NOAA) and OSU, this project evaluated the ability of this oral vaccine platform to induce a specific immune response and provide protection against *Aeromonas salmonicida*, the causative agent of furunculosis, when fed to rainbow trout (*Oncorhynchus mykiss*).

The immune response and efficacy of a simple formalin killed *A. salmonicida* vaccine was tested by using comparing routes of immunization in rainbow trout: intraperitoneal injection, immersion, anal intubation, and the experimental oral vaccine particle. Fish were given a booster dose of vaccine using the same protocols two weeks after the initial dose. Rainbow trout were then challenged with the virulent *A. salmonicida* strain that was used for vaccine development. Results show that intraperitoneal injection induced the highest level of specific antibodies out of all treatments, and protection was significantly higher than other vaccination routes only after Freund's adjuvant was included with injections. Immersion and anal intubation treatments produced similar levels of both specific antibodies and protection against pathogen challenge. The novel oral vaccine particle successfully stimulated antibody production and provided significant short term protection, but insignificant long term protection during a pathogen challenge. Interestingly, fish fed the novel alginate particle without vaccine showed reduced disease related mortality, indicating a potential adjuvant effect of the particle formulation itself.

Beyond the vaccine study in rainbow trout, parallel studies in sablefish (*Anoplopoma fimbria*) at OSU and NOAA were underway. To assist in evaluation of an immune response in this species, a monoclonal antibody (mAb) specific to sablefish Immunoglobulin M (IgM) was also developed as a tool to measure the immune response when vaccinated with similar alginate particle vaccines. This mAb , known as UI-25A, was created using mice and recognizes the conserved heavy chain of sablefish IgM. UI-25A is highly specific to sablefish IgM and lacks any reactivity to blood antigens of rainbow trout, Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), or burbot

(*Lota lota*) as well as antigens found in lysed preparations of *A. salmonicida*. Using UI-25A, an enzyme linked immunosorbent assay (ELISA) was developed to measure specific antibodies in plasma of vaccinated sablefish. This ELISA was successful at differentiating between vaccinated and unvaccinated sablefish based on the level of antibody titers from each group. UI-25A was further characterized and used to visualize immunogenic antigens in western blot analysis for both whole cell protein profiles and isolated lipopolysaccharide of *A. salmonicida*. Further, immunofluorescent staining of head kidney tissue imprints showed that UI-25A could detect membrane bound IgM on the surface of B-cells. These applications of the UI-25A mAb demonstrate its broad applicability to aid research into sablefish immunology.

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Dedication

I dedicate this work to those close to me who have provided unwavering support as long as I have known them. To my parents, Carol and Bruce Jones, who have always believed in me no matter where my path has taken me. I am fortunate to be raised by them, I could not have asked for better examples of what I should grow up to be; caring, thoughtful, and loving. To my fiancé, Whitney Thiel, who grounded me during this process, and was always there for me no matter the circumstance. I may not spend as much time on my vows as I have on this, but I will spend more time reliving the memories we've shared. I can't wait to marry you in 3 weeks <3.

Table of Contents

List of Tables

Supplemental Tables

List of Figures

List of Supplemental Figures

Chapter 1: Literature Review

1.1 Oral Vaccination

The first study of disease prevention using vaccines in fish was likely in 1938, where common carp (*Cyprinus carpio*) were protected against disease after immunization with *Aeromonas caviae* [1]. This was followed closely by another study in 1942 that demonstrated protection in cutthroat trout (*Oncorhynchus clarkii*) against *Aeromonas salmonicida* infection [2]. In the 1980s, Norway's aquaculture industry experienced a devastating loss due to disease caused by *Vibrio salmonicida* [3]. Since 1988, most salmonids in Norway have been vaccinated against this pathogen, and as a result mortality and antibiotic use have been greatly reduced [4]. As aquaculture production has increased, so too has the research and availability of fish vaccines. New biotechnological tools and research has allowed for the development of a variety of vaccine types including killed, live attenuated, subunit, DNA, and RNA based vaccines [5]. Though the commercialization of new types of vaccines is an arduous process [6], they remain one of the most important tools in the aquaculture industry and are an essential component to any large scale intensive culture facility.

For oral vaccines, the major structures of interest are the gut associated lymphoid tissue (GALT), because it is the first immune system structure exposed to the vaccine and thus key for antigen absorption. Humoral, or antibody mediated, immunity remains the most common parameter measured to evaluate vaccination success. This is because of a lack of tools to identify surface markers related to T cells in many fish species, which are the foundation of cell mediated immunity. Monoclonal antibodies (mAbs) specific to fish immunoglobulin exist for a variety of species and are a necessary tool to measure the adaptive immune response, and this is discussed in Chapter 3. To understand oral vaccination, this section will first review humoral and mucosal immunity before reviewing oral vaccination specific studies.

1.1.1 Humoral Immunity

Immunology is an important discipline within vaccinology, with a special focus on the activation of the adaptive immune response. However, it is important to note that without the innate immune response's ability to recognize and signal potential infectious bodies within a host, the adaptive response would be dead in the water. The adaptive response is characterized by a slow response that can take days to weeks to manifest and is highly dependent on water temperature with lower temperatures associated with a slower response [7,8].The adaptive immune response can be broadly split into two categories; humoral and cell mediated responses [9]. The cell lineage vital to this process is the lymphocyte, divided into B (humoral) and T (cell mediated) cells [10]. In teleost fish,

both T and B cells originate in the head kidney; B cells mature here while T cells migrate to the thymus for maturation [11,12].

B cells are responsible for the humoral, or antibody mediated, immune response. To express and secrete antibodies, B cells are first exposed to antigens, with the help of the innate system and antigen presenting cells (APCs), before developing into plasmablasts and eventually mature plasma cells secreting specific antibodies [13]. Three distinct types of immunoglobulins have been described in teleost fish, each following the basic structure of 2 heavy chains and 2 light chains: IgM, IgD, and IgT/IgZ [14]. The most abundant was also the first discovered immunoglobulin in fish, IgM, which is also found in almost all vertebrates [15]. IgM can be found on B cell membranes and in its secreted, tetrameric form in blood circulation and mucus. Some amount of IgM can be detected in circulation without antigen stimulation, but levels are increased after exposure to an antigen and subsequent immune system activation [14]. Studies have also shown that the secreted tetrameric form of IgM can be transported into the skin mucus [16]. This antibody class assists with a wide range of pathogen defenses including complement activation, agglutination, and neutralization [14,17,18]. As the most abundant immunoglobulin, it is a popular parameter to measure in fish vaccination studies to evaluate immune stimulation and predict efficacy [19–21].

Other immunoglobulin classes are not nearly as well described in function or origin relative to IgM. IgD was first discovered in channel catfish (*Ictalurus punctatus*) in 1997 [22], and its functions remain somewhat of a mystery, even in mammalian species [14]. There is evidence of B cells expressing both IgM and IgD, as well as solely IgD [23]. IgD has been found in several other fish species [24–26] and the highest levels have been generally found within the head kidney [27]. A monoclonal antibody specific for rainbow trout IgD has been used to measured levels within sera, finding that levels were up to 400 times lower relative to sera IgM [28]. IgT (known as IgZ in zebrafish) has been the focus of several recent studies aimed at characterizing mucosal immunity of fish [23]. It was first discovered in rainbow trout and zebrafish in 2005, and no ortholog has been found in mammals or birds to date [29,30]. One study found that specific IgT antibodies were increased in the mucosal barriers of the gastrointestinal tract, but sera antibodies were dominated by IgM during a parasitic infection [23]. Bath and oral vaccinations, which act on the mucosal barriers of skin and intestinal tract, have also been shown to induce greater levels of IgT expression in tissues, for both bacterial and viral antigens [31,32]. To date, no B cells have been identified that jointly express IgT and IgM, indicating a separate development and lineage for each isotype [18]. These separate lineages are further supported by the current lack of evidence demonstrating class switching

capabilities of Igs in teleost fish [33]. Results from these studies are only possible because monoclonal antibodies (mAbs) have been developed that are specific to various Ig forms in teleosts.

1.1.2 Mucosal Immunology

Fish lack the traditional, organized structures of lymphoid tissues found in other animals such as lymph nodes and Peyer's Patches [34], except for some evidence of similar structures in the gills of Atlantic salmon (*Salmo salar*) [35]. Teleost do possess diffuse networks of leukocytes at their mucosal barriers, known as mucosal associated lymphoid tissues (MALT). These tissues have been characterized at several mucosal sites of fish including skin (SALT), gill (GiALT), gut (GALT), nasal (NALT), buccal, and pharyngeal [36,37]. Each of these lymphoid tissues are colonized with a diverse arrangement of commensal microbes, and any immune response must strike a balance between pathogen defense and over stimulation [38].

Immune function of the NALT has been the subject of several recent papers in rainbow trout, finding that vaccines can stimulate a local and systemic response through the nasal cavity [39,40], and in response to parasitic infection [41]. It is hypothesized that immersion vaccination also stimulates a local nasal response in fish, but further research is needed to determine the extent of local stimulation [42]. The epidermis of fish contains mucus secreting cells, making it the largest diffuse lymphoid tissue. IgM plays a major role in the SALT, but IgT levels are much higher in the skin mucus relative to sera [43,44]. The buccal and pharyngeal ALTs are relatively new discoveries in rainbow trout, with both being characterized by a strong IgT response after parasitic infection, with only limited IgM and IgD detection [45]. The GiALT is unique to fish, and the only tissue that also contains organized lymphoid structures, the interbranchial lymphoid tissue (ILT), thus far found only in salmonid species [46] and more recently zebrafish (*Danio rerio*) [47]. These structures are characterized by aggregations of T cells and are thought to be important in antigen encounter but not a site of maturation or development of immune cells.

1.1.3 Intestinal Immunology

In terms of oral vaccination, the gut associated lymphoid tissue (GALT), is the most important site of immune activation. This may also be the most well researched ALT in fish and was first reviewed in 1988 [48]. Gut B cells, both IgM+ and IgT+, are primarily found in the lamina propria, with IgT+ cells making up 54% of total resident B cells in rainbow trout [23]. A major role in the GALT is its ability to absorb antigens to stimulate an immune response, this was first reported in carp [49,50] and later sea bass (*Dicentrarchus labrax*) [51]. In most fish, the gastrointestinal tract can be divided into three segments; the first being dedicated to protein absorption [52], the second specializes in

macromolecule uptake [53], and the full function of the third segment is still debated but ostensibly is not involved with nutrient absorption due to limited presence of microvilli [54].

Absorption was closely characterized in carp with both soluble and particulate antigens delivered to the gastrointestinal tract in several studies. Researchers found that both antigen types were absorbed in the second gut segment by epithelial cells, then transported through vacuoles before being presented to interepithelial macrophages [55]. They also found specific antibodies in the skin mucus and sera for the particulate antigen, but the soluble antigen only provoked an antibody response in the sera [56]. This demonstrates a common mucosal system throughout the fish, since gut antigen exposure led to skin antibody production, and that the transportation of sera antibodies to mucosal sites is somewhat limited. Differences were also found in the processing time for the antigen types, with the receptor mediated uptake of particulate antigens transporting them to the blood in as little as 30 minutes in trout [57] and carp [58], while the soluble antigen presence in the blood was only observed 4 hours after exposure in carp [56]. These studies confirmed that the second gut segment was the primary site of immune activation, with large numbers of resident macrophages present in a stable state and smaller, mobile macrophages arriving after antigen exposure [56]. Though it should be noted, due to the diversity of teleosts, there are likely differences in the sites and structures important for antigen sampling among species [59].

Tolerance of antigens is an important part of mucosal and gut immunity because the mucosal barriers are permanently exposed to foreign substances as the host's first line of defense. Over exposure, or continuous stimulation of the GALT has been shown to limit the immune response over time in several species including trout [60], coho salmon [61], and carp [62]. Known as 'oral tolerance' due to the focus on oral vaccination, this effect has also been observed with anal delivery of antigens [56,63]. Mucosal surfaces of animals, including fish, are required to be somewhat tolerogenic due to the constant interaction with commensal microbes. The process of this tolerance and its effect on stimulation of the systemic immune system is not well understood, though as our understanding of the microbiome of animals increases it should help illuminate these unknowns [38].

Presence of immunoglobulins in the gut mucosa are difficult to measure in part due to the difficulty of obtaining high quality samples [64]. Research has demonstrated that sera IgM is quickly degraded in the presence of gut mucus, but not skin mucus, likely because of the high proteolytic activity [65]. Several authors have also hypothesized that there are subtle differences in the structure or specificity of sera and mucus IgM and that current mAbs used to identify Igs may not be sensitive to these [23,50,64]. One study found that a mAb could be specific to the heavy (H) chain of mucus IgM, but not sera IgM in carp [65]. There is still difficulty in using tools to measure specific IgT levels in

mucosal samples. Much of the research has focused on rainbow trout, where the isotype was originally discovered, and on mucosal surfaces other than the gut [41,66,67]. Recently, a mAb was developed for IgT in yellow croaker (*Larimichthys crocea*), which will likely provide a useful comparison as IgT and other mucosal immune responses are characterized in multiple fish species [68].

1.1.4 History and Present Status of Oral Vaccination

Oral vaccination refers to any formulated vaccine that is delivered through the buccal cavity into the gastrointestinal tract. The earliest oral vaccination experiment in the literature is by Duff in 1942, who used a killed *A. salmonicida* preparation to coat feed particles which successfully protected cutthroat trout (*Oncorhynchus clarkii*) in a pathogen challenge [2]. Since then, advancements in both preparation and administration of oral vaccines have been made for fish and there are currently several commercial products used to protect against the following pathogens: infectious pancreatic necrosis virus (IPNv), spring viremia of carp virus (SVCv), infectious salmon anemia virus (ISAv), *Piscirickettsia salmonis, Yersinia ruckeri*, *Vibrio spp.*, and *Lactococcus garviae* [5,69–71]. One of the main obstacles to developing efficacious oral vaccines is protecting the antigen during passage through acidic environment of the stomach until it reaches the second gut segment [72]. This destruction means that oral vaccines more often require higher doses of antigen, compared to anal vaccination, in order to stimulate similar levels of specific IgM. In one case, oral vaccination required a dose 50x higher than anal vaccination to achieve similar specific IgM levels in sera [73]. Though oral vaccination is proven to stimulate an immune response and provide protection against disease, this inefficiency currently makes it a less attractive commercial option in the industry.

Various strategies have been used for antigen encapsulation or delivery preparation for use in oral vaccines, and it remains an active area of study [74]. One common method is coating feed particles directly with vaccine antigens either by mixing with feed in a liquid form [75] or using an oil to bind other forms of vaccine (encapsulated particles or powdered) to the surface of the feed particle [76]. These methods are simple; however, they leave the vaccine exposed to potential degradation during gut transit. Vaccines can be incorporated into the feed during the formulation process, but only at later stages because temperatures and pressures during other production stages would destroy the vaccine [72].

Encapsulation of oral vaccines, to protect them prior to absorption, is achieved in several ways. Microalgae research is growing quickly and has great potential because of the wide availability and low costs. One study transformed algae to promote expression of an antigenic protein from *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease, and found that oral

vaccination with the algae promoted a specific antibody response [77]. Another study encapsulated green fluorescent protein (GFP) in microalgae and detected it in intestinal tissues of zebrafish after oral feeding [78]. Though these results are promising, no evidence of disease protection from a microalgal encapsulated oral vaccine has been published. Encapsulation within microparticles is another common method for oral vaccine delivery. Alginate microparticles (AM), a natural polysaccharide found in the cell wall of brown algae species, is an extremely promising drug delivery system in human and animal medicine [79]. AMs have been tested for oral vaccination in fish at least since 1994 [80], and include many vaccines against a variety of pathogens such as *V. anguillarum* [81], A. *hydrophila* [82], *L. garvieae* [83], IHNv [84], *S. iniae* [85], SVCv [86], IPNv [87], LCDv [88], and *A. salmonicida* [89]. All studies were successful at promoting a specific immune response, though the type of vaccine varied, with DNA vaccines being well represented [84–89] relative to whole cell or antigen preparations [81–83]. Nanoparticles are another promising method of encapsulation that have recently been the subject of several reviews [74,90–92]. Nanoparticles with inactivated virus reduced mortality in Atlantic salmon challenged with ISAv, though an adjuvant within the nanoparticles was required for significant protection [93]. In rohu (*Labeo rohita*) an outer membrane protein vaccine to *A. hydrophila* stimulated specific antibody production and reduced mortality during challenge after oral administration of nanoparticles [94]. Several studies have obtained relative percent survival (RPS) greater than 70% using nanoparticles or virus like particles (VLP) orally administered in a variety of species including black seabream (*Acanthopagrus schlegelii*) [95], Atlantic salmon [93], rohu [96], and grouper [97].

Some microorganisms are hearty enough to weather the digestive environment, and bacteria, yeast, and plant cells are all potential carriers for oral vaccines [71]. Oral vaccines that protect against enteric pathogens, or those that normally colonize and infect the gastrointestinal tract, have had the most widespread success. The first commercial oral vaccine, AquaVac ERM Oral [98], was developed after a long history of successful experimental oral vaccines for rainbow trout starting in 1965 [99]. This is an inactivated whole cell bacterium that ultimately offers better protection through injection and immersion routes. Therefore, the commercial product is offered strictly as a secondary booster to the more effective primary routes. More recently there has been progress in a live attenuated vaccine against *Edwardsiella ictaluri* for use in channel and hybrid catfish (*Ictaluris punctata*) [75,100]. This is a unique and simple preparation, with the live bacterial vaccine diluted in well-water before being sprayed onto feed immediately before feeding. Significant protection has been demonstrated with RPS ranging between 80-100% depending on the dose used. Other research into live attenuated oral vaccines is limited, partly because few live attenuated vaccines are available for any immunization route. Instead of coating feed directly, other studies have utilized oral gavage or

microparticle encapsulation to administer the vaccines. In Nile tilapia (*Oreochromis niloticus*), an avirulent *Streptococcus agalacitae* was delivered by oral gavage and provided an RPS of 71% during challenge 15 days after vaccination, but this declined to 53% at 30 days post vaccination and both were outperformed by the injection vaccination [101]. Another study tested alginate microparticles both unencapsulated and encapsulation with a live vaccine *Flavobacterium psychrophilum* [102]. Researchers demonstrated that the attenuated vaccine was still viable after encapsulation, though a stronger serum antibody response was observed in the nonencapsulated treatment that was equal to the antibody response of fish vaccinated by injection. There was evidence of protection during pathogen challenge; however, a high challenge dose overwhelmed protection even in injection vaccinated groups, muddling interpretation of the results. An older study encapsulated a live attenuated VHSv in lipid particles fed directly to fish which provided RPS values of 37% and 100% during challenge 28 days after vaccination [103]. However, this study did not report specific antibody responses or duration of protection.

Though their effectiveness is variable and generally lower than injection or immersion, orally administered vaccines can stimulate immune responses and, in some cases, provide acceptable levels of protection. In certain situations, like the live attenuated *E. ictaluri* vaccine [100], it is preferred over immersion, because fish are stocked into ponds prior to immunocompetence. Harvesting fish for vaccination is prohibitively expensive and stressful, reducing the economic returns of vaccination, so oral delivery is ideal for pond raised fish. The same argument can be made for fish stocked in net pens, since limited access to animals requires a much easier and efficient vaccination strategy for implementation. Enteric pathogens, such as *Y. ruckeri*, are good candidates for oral vaccination because of the reduced complexity of encapsulation requirements [99]. Though few oral vaccines have widespread use, the potential benefits provide incentive enough for continued research and development. To create more successful oral vaccines, research should aim to improve encapsulation techniques, develop more tools to evaluate mechanisms of mucosal immunity, and further characterize the relationship between commensals and the mucosal lymphoid tissues.

1.2 Aeromonas salmonicida

1.2.1 Background

Diseases caused by *Aeromonas salmonicida* are some of the oldest described in aquaculture facilities, dating back to 1890 in brown trout (*Salmo trutta*) [104]. According to the name '*salmonicida*' it was originally thought that the bacteria inhabited only cultured salmonid species, but epidemiological investigations into wild fish, both salmonid and other, have revealed the wide variety of species and aquatic environments where the bacteria is present. Many members of the Aeromonas are associated

with diseases in fish; however, relatively few are primary pathogens that cause disease in healthy fish populations, *A. salmonicida* being one of the most important in the aquaculture industry.

Within the species *A. salmonicida* there exist 5 currently described subspecies; *salmonicida, achromogenes* [105], *masoucida* [106], *smithia* [107], *pectinolytica* [108]. Subspecies *salmonicida* was the first discovered and most important fish pathogen in aquaculture for decades. The others, collectively known as 'atypical' strains, were discovered later, and are separated mainly because they lack the characteristic brown pigment developed on agar cultures of 'typical' *A. salmonicida* subsp. *salmonicida*. These atypical strains are relatively diverse in their physiological, genetic, and biochemical characteristics compared to the more homologous typical strains [107,109,110]. Even with modern genetic and biochemical assays to describe bacteria, grouping novel isolates into specific subspecies has been anything but straightforward [111], and new phenotypes are still being discovered [112]. Recent reports of ulcer diseases caused by atypical *A. salmonicida* infection in marine species include lumpfish (*Cyclopterus lumpus*) [112], common dab (*Limanda limanda*) [113], ballan wrasse (*Labrus bergylta*) [114], and black rockfish (*Sebastes schlegeli*) [115]. The occurrence of atypical infections in a variety of hosts has been reviewed extensively in recent years [116,117]. Reports of novel species infections and literature reviews emphasize to the diversity of the bacterium and its ability to infect a variety of hosts, causing similar diseases with a variety of names including trout ulcer disease, goldfish ulcer disease, carp erythrodermatitis, furunculosis, or simply ulcer disease [117,118].

Typical *A. salmonicida* subsp. *salmonicida* was the first characterized group and is the causative agent of furunculosis [119,120]. It is most often identified as a nonmotile, gram-negative rod bacterium that produces a brown diffuse pigment when grown aerobically in the presence of tyrosine, which is still the main characteristic to presumptively identify typical *A. salmonicida* growth [120]. Infections are grouped into several categories based on clinical signs of disease [121]. Latent infections, or carrier status, is quite common in fish farms and result in no mortality or signs of disease even though the bacterium may be isolated from apparently healthy fish. Chronic and subacute infections are characterized by the skin lesions, or furuncles, which the disease is named for, and are most often seen in older fish. Acute infections are associated with darkened skin, reduced feeding, petechial hemorrhaging, and internal pathology such as softened kidney, enlarged spleen, pale liver, and extensive visceral hemorrhaging [121].

Transmission of the pathogen is still not completely clear, though research continues to illuminate potential mechanisms. Currently, *A. salmonicida* is known to survive in fresh, brackish, and salt water; however, methods used for these studies are questioned due to unrealistic (i.e., sterilized)

preparation of the growth cultures [122]. These questions, combined with the difficulty of isolating the pathogen from mixed cultures obtained from water samples, indicate that there may be other environmental reservoirs of the pathogen other than covert (i.e., no clinical signs of disease) fish carriers of the bacteria [122,123]. It was recently shown that microplastics in the marine environment can harbor *A. salmonicida* and be a possible vector of transmission [124]. One study attempted to induce vertical transmission in rainbow trout, but the bacteria could not be isolated from offspring of either naturally or experimentally infected groups [125] and no other research has demonstrated its significance in spreading disease. Horizontal transmission through carrier fish, contaminated water, or contaminated equipment is viewed as the most significant contributor to disease spread. This mode has been proven by experiments where a small number of 'donor' fish were injected with large concentrations of virulent bacteria and placed into tanks with other fish that eventually succumbed to disease and mortality [126–129]. However, it should be stated that transmission of the pathogen does not necessarily equate to a disease outbreak and mortalities. In some cases, high water temperatures [129] and corticosteroid injections [123,130] have been successfully used to induce an outbreak in carrier fish.

The uptake of bacteria into fish has been experimentally demonstrated through the gill, lateral line, abraded skin, anus, and oral cavity [131]. In one study, *A. salmonicida* was found in the blood, kidney, and spleen of rainbow trout within 2 minutes of immersion in tank water containing 10^4 bacterial cells, though it did not induce disease and by 24 hours after immersion the pathogen could not be cultured from fish tissues unless a corticosteroid injection was administered [132]. There has been debate about the ability of *A. salmonicida* to infect fish through the gastrointestinal tract, several studies were unable to induce infection by coating feed with viable *A. salmonicida* [133,134], or by oral intubation [123]. However, at least one study was successful in causing disease in Atlantic salmon through oral intubation [135], and another recent experiment demonstrated the ability of *A. salmonicida* to be translocated across the intestinal epithelia of rainbow trout *in vitro* [136]. There is likely no single route of entry for the pathogen, and its ability to infect or cause disease is a result of a variety of abiotic and biotic factors influencing the host-pathogen relationship.

1.2.2 Virulence Factors

There are numerous factors that contribute to virulence of *A. salmonicida*, the main mechanisms defined in the literature include the A-layer, a Type III Secretion System, and various extracellular products [137]. Possibly the most studied is the presence of an additional layer on the outside of the cell membrane, found on 'rough' colonies that are traditionally more virulent than their smooth counterparts [138,139]. Known as the A-layer and encoded by the *vapA* gene, it is the main cell

surface protein and arranged in a repeating tetragonal patterned structure attached to a diazoidosulfanilic acid associated with the cell surface lipopolysaccharide (LPS) [140]. Garduno et al. [141] showed that colonies possessing this structured protein were able to adhere and survive within macrophages after engulfment, this was confirmed by Graham et al. [142] who found that isolates without the A-layer were destroyed by macrophages. It is now thought that the A-layer is not required for a virulent strain because isolates lacking it can still be moderately virulent [143] and virulence varies widely across isolates with similar phenotypes [144]. The A-layer is an important component of immune avoidance during early stages of infections, but the precise mechanisms of how it relates to virulence after initial phagocytosis remains unclear. The outer membrane LPS is an immunoreactive cell surface antigen; however, purified *A. salmonicida* LPS has not been demonstrated as pathogenic [145,146]. On the other hand, LPS has been shown to aggregate important toxins for virulence, most notably glycerophospholipid cholesterol acyltransferase (GCAT) [147]. This GCAT/LPS complex demonstrated higher leukocytolytic, cytotoxic, and hemolytic tendencies compared to GCAT in isolation when administered to Atlantic salmon, indicating that LPS is an important component of virulence for this toxin [147]. Originally, evidence of a capsule around *A. salmonicida* cells, grown under specific *in vitro* conditions or *in vivo* with intraperitoneal chambers, was attributed to the LPS [148] but is now recognized as a capsule [149]. Merino et al. [150,151] demonstrated its ability to increase invasion into cells and resist complement by decreasing the binding ability of C3b to the surface of bacteria.

A Type III secretion system (T3SS) was discovered in *A. salmonicida* in 2002 [152] and is likely necessary for virulence [153]. The T3SS has been extensively reviewed and diverse forms have been found in over 25 species of gram negative bacteria [154]. By using mutants of *A. salmonicida* with inhibited T3SS it has been demonstrated that T3SS is the primary virulence mechanism of *A. salmonicida* [152,155–157]. It has also been shown that growing *A. salmonicida* at high temperatures can inhibit virulence [158]. This occurs due to rearrangement of the relatively unstable pAsa5 plasmid, high temperatures can also disrupt A-layer formation further altering virulence [157]. The detailed structure, function, and effects on host cells of T3SS in *A. salmonicida* has been reviewed by Vanden Bergh and Frey [159], and more recently by Frey and Origgi [160]. The T3SS appears to be necessary to cause disease and mortality in fish, one study showed that A. salmonicida isolates lacking the T3SS were unable to suppress the immune system of infected rainbow trout and caused no mortality [161]. There has also been research into possible virulence mechanism of a type IV pili system [162]. Even though it exists in *A. salmonicida* subsp. *salmonicda*, its relationship to virulence has not been well characterized to date [122].

Extracellular products (ECP), primarily proteases and lipases, also aid in the virulence of *Aeromonas* spp. by overcoming host defenses and sourcing nutrients from the host for bacterial use [163]. There are a wide variety of proteases characterized in *A. salmonicida* [164], and without them some mutants become avirulent [165]. Recently it was found that expression of the enzyme lactoylglutathione lyase was significantly overexpressed in the bacteria during the early stages of infection in rainbow trout [166]. This enzyme is associated with resistance to oxidative stress and cellular detoxification in other bacteria [167].There is evidence that combinations of protease and lipases can form toxins that cause the traditional pathology of furunculosis [168,169], and the current list of known ECPs produced by *A. salmonicida* is well described [122].

1.2.3 Vaccines

Earlier sections of this chapter have described the importance of vaccines in aquaculture and the mechanisms of the adaptive immune response. Vaccines to protect against furunculosis, or any disease caused by typical or atypical *A. salmonicida* infection, are still an important area of research for aquaculture. The call for widespread vaccination came in the early 1980s, as Scotland's Atlantic salmon industry was experiencing heavy mortalities after asymptomatic carrier fish were transferred to marine net pens [170]. First attempts at formalin killed and purified cell surface antigen vaccines resulted in little to no protection against disease [171], and previously infected rainbow trout showed no protection during subsequent infections [172]. These early results suggest the difficulty of obtaining immunity against *A. salmonicida*, as well as providing some evidence that disease protection may rely heavily on non-specific factors which simple vaccine preparations cannot effectively stimulate. Addition of adjuvants, such as mineral oil [133,134] or Freund's complete adjuvant (FCA) [173] to these vaccines greatly increased efficacy and agglutinating antibody levels. It was also noted that primary protective antigens were the polysaccharide capsule and iron regulated outer membrane proteins [174]. It was initially thought that antibodies to the A-layer were not necessary for protection [175]; however, results from several more recent studies have shown the opposite [176–178] and it is now accepted that furunculosis vaccines should include some components of the A-layer.

As furunculosis expanded to the Norwegian aquaculture industry, interest in vaccine research increased. Intraperitoneal injection with adjuvant continued to demonstrate the greatest protection in experimental [179] and field studies [180]. The success of these experiments, and development of multivalent vaccines that protect against both furunculosis and vibriosis [181], eventually lead to mass vaccination and a significant decrease in antibiotic use for the industry [182]. Currently,

injection vaccines adjuvanted with an oil-based mixture are still the standard, and the lack of mortality events in areas with mass vaccination are evidence of their continued success [183].

Historic evidence has shown some ability of oral vaccines to provide protection against furunculosis in salmonids [2], but other studies have shown that protection was either short lived, or highly variable [184–186]. Other studies have attempted to use oral vaccines as a booster after the initial injection dose, this was done in turbot (*Scophthalmus maximus)* with no success at increasing protection or immune response for an atypical furunculosis vaccine [187]. Another study developed a recombinant vaccine with a modified A-layer antigen and encapsulated it within alginate microparticles fed directly to goldfish (*Carassius auratus*), they found that specific antibody titers were increased but they offered no protection during a pathogen challenge [89]. Another oral vaccine was developed by Irie et al. [188], which used extracellular supernatant as the vaccine antigen that was encapsulated within liposomes. They found an increase in specific antibodies and protection with this formulation in carp (*Cyprinus carpio*); however, sample sizes were 8 and 6 for the control and vaccinated groups, respectively. Only one study has been published since 2007 using oral vaccines against furunculosis [189].

Immersion vaccines have also proven somewhat successful for furunculosis in several recent studies. Villumsen and Raida [190] found high levels of antibody production and protection from a simple formalin killed immersion vaccine and found that even without a booster fish were protected for 24 weeks after immunization with a relative percent survival of 70%. Chettri et al. [128] also found significant protection against furunculosis using a multivalent mixture of commercial and autogenous formalin killed vaccines administered by immersion. Both studies were performed using rainbow trout as a model species, generally seen as the most resistant salmonid to *A. salmonicida* infection, but mortality was still induced in both challenges, with survival at 70% for Villumsen and Raida, and as low as 16% in Chettri's study.

Forms of vaccine other than formalin killed *A. salmonicida* isolates have also been investigated. One strain was attenuated by continuous culture in lab conditions and after injection or immersion it provided significant protection to both brook trout and Atlantic salmon [191]. The mechanisms of attenuation were not discussed, but it is likely that it was A-layer deficient due to a lack of autoagglutination. Other researchers have constructed *aroA* deficient isolates that worked well under certain conditions; specifically, the broth grown isolate outperformed the freeze-dried form during pathogen challenges [192,193]. Grove et al. [194] studied the persistence of *aroA* mutant DNA in head kidney and spleen, finding that 16s DNA was absent by 12 weeks post vaccination, while 75% of fish vaccinated with a commercial product were positive. Research into the importance of certain

antigens in vaccine production has created some conflicting results. Bergh et al. [195] tested two inactivated vaccines, one from an isolate lacking the T3SS and one wildtype isolate, they found that presence of the T3SS reduced protective ability in rainbow trout. However, another study found that a T3SS mutant producing a weak toxoid provided protection equal to commercial multivalent vaccine in Arctic char (*Salvelinus alpinus*) [196]. Varying results could be caused by other differences of the *A. salmonicida* mutants used, the different species used, or the challenge timing and method.

Antibody production specific to *A. salmonicida* is an important, but not absolute, indicator of vaccine success. Romstad et al. [177] showed that antibody levels and protection were related in Atlantic salmon. Villumsen et al. [197] found a similar relationship in rainbow trout, and interestingly that antibody levels decreased significantly within 3 days of challenge initiation. Previous passive immunization studies have demonstrated the abilities of specific antibodies to induce protection in challenged fish. Antibodies specific to proteases produced by bacteria were protective when rainbow trout were infected [198]. Antibodies developed against whole cell virulent isolates were also protective during *A. salmonicida* infection in brook trout (*Salvelinus fontinalis*) [191]. Other results suggest that non-antibody mediated defenses play a role in vaccine efficacy, even when specific antibodies are almost non-existent there remains some level of protection in turbot [187]. Chapter 2 will discuss more about the potential role and importance of specific antibodies induced by vaccination.

1.3 Sablefish (Anoplopoma fimbria)

1.3.1 Life History

Sablefish *(Anoplopoma fimbria*), also known as black cod, is the only member of its genus and one of two members of the family Anoplopomatidae, the other being skilfish (*Erilepis zonifer*) [199]. It is estimated that these species diverged ~450,000 years ago, based on cytochrome oxidase *c* and mitochondrial control region genes [200]. Sablefish have been considered a “classical marine species” because of their high fecundity and long planktonic larval stage [201]. Adults are demersal and long lived, with reports of fish over 100 years old [202]. Their native range is along the North American continental shelf from Baja California to Alaska and along the Bering Sea and down the eastern coast of Japan [203]. They are typically found at depths between 200 and 1500 meters, depending on seasonality, and tend to avoid environments with temperatures lower than 2 °C [204]. In the past, it was hypothesized that two distinct populations, northern and southern, of sablefish occurred based on differences of growth rate and size at maturity [205]. Surprisingly, recent genetic analysis found that populations across the habitable range were similar, indicating a substantial amount of mixing in sablefish stocks [206,207].

Sablefish reach sexual maturity and spawning at approximately 5 years of age, though this can vary based on geographic region [208,209]. Observations suggest sablefish produce and develop only one cohort of eggs for each spawning season [210] which runs from January through March for females, with a slightly longer window for males [211]. Females produce an average of 100 oocytes per gram of fish weight [212]. Hormonal and histological changes in both male and female adult sablefish were explored recently, confirming information about the reproductive cycle of sablefish [209]. Sablefish spawning has been observed along the continental shelf at average depths greater than 200 m, with embryos sinking further down the shelf mostly hatching at depths greater than 400 m [211]. Larvae remain in deep waters and slowly migrate upwards as their yolk sac is absorbed [203]. Sablefish are primarily surface oriented at this stage and planktivorous, but if resources are limited or as they continue to grow, they move deeper to find new prey sources [213]. It is hypothesized that currents and drift may account for significant distances of travel for eggs and larvae during these stages, which is supported by evidence of considerable genetic mixing in populations across their geographic range [207]. They typically reach near-shore environments as early juveniles in the Fall season [214]. Sablefish exhibit extremely rapid growth in sub-juvenile and early juvenile stages, with reports of as much as 2 mm growth in total length per day [214].

By age 3, most sablefish have migrated offshore to depths greater than 100 m and continue to move downward as they increase in age and size [215]. At these stages they are primarily piscivorous but also incorporate crustaceans in their diet [216]. Adults exhibit large diel vertical migrations of greater than 100 m for feeding [217], their lack of swim bladder makes this possible and instead sablefish rely on their high lipid content for buoyancy [218]. Sablefish are tolerant of the low oxygen environment of the ocean floor, with reports of populations living in areas with 0.34 to 0.80 mg/L of oxygen [203]. Tagging studies have also shown migration patterns in excess of 1,000 miles over their lifetime [204]. Sablefish are elongate and possess small ctenoid scales and 2 dorsal fins [219]. They are mostly black or dark greenish in color on their backs, with relatively pale bottoms [219].

1.3.2 Aquaculture

Initial aquaculture attempts of sablefish started in British Columbia, Canada in the 1970s at the same time wild harvest was rapidly increasing [220]. Capturing juveniles in near shore habitats and stocking them into net pens for grow out produced promising results, with fish adapting well to confinement and artificial feeds [221]. Interest in sablefish aquaculture grew into the 1990s due to decreasing prices and disease issues of farmed salmon. The thought was that existing operations and infrastructure for salmonids could easily accommodate this high value species [221]. The main issue that remains in sablefish aquaculture development is the need for a constant juvenile supply for grow

out operations. However, some growers have found success and are able to produce 200,000 sablefish fingerlings annually for export around the world [222].

More recently, the US has focused on the development of commercial sablefish aquaculture. Trials in the early 2000s proved promising with good harvests, but the issue of high larval rearing costs and unpredictable larval supply stunted growth of the industry. However, research into closing the life cycle has seen great success, mostly through government funded research performed by NOAA. This research into larval rearing has included optimizing tank design [223], temperature requirements [224,225], and feeding techniques and requirements [226–228]. Additional work with focus on increasing efficiency of grow out production has included the development of monosex stocks [229] as female sablefish grow more rapidly compared to males. These monosex stocks also are important for potentially exporting fingerling grow out operations to regions where the fish is not native. Alternatives for fish meal and fish oil have been explored and have shown some success with soy and algae products [230,231]. Establishment of captive broodstock populations are still in progress, but most egg harvest come from wild caught sablefish [232]. The first report of successful captive spawning occurred in the 1990s [221], but fish do not appear voluntarily release eggs so hormonal treatments are required to induce oocyte maturation and spawning [233]. Overall costs remain high in the larval production setting and establishing a stable captive broodstock line is still in progress. These costs as well as loss from disease remain the largest obstacles to expansion of sablefish aquaculture.

1.3.3 Immunology and Disease Susceptibility

Sablefish are a unique species without many close living relatives and are a relatively new species to intensive aquaculture. As such, there are many gaps in information about their immune system function and overall susceptibility to diseases. An early study focused on the effects of capture stress on wild caught sablefish found that all capture methods tested contributed to diminished responses of leukocytes to B and T-cell mitogens [234]. However, as aquaculture development has progressed it has created more opportunities for sablefish to be available for lab-based experiments. The first evidence of antibody production in sablefish was found by Ridgway [235], who immunized a sablefish with Pacific herring (*Clupea pallasii*) red blood cells and found hemagglutinating antibody activity. This study also found that antibody titers were maintained and increased for several months without further inoculations. This refuted earlier work that hypothesized fish inhabiting very cold water, below 10 °C, could produce a robust antibody response [236]. Recently, reference intervals of plasma biochemistry and hematology parameters were developed for sablefish as well as the morphology of various cells found in blood circulation, including leucocytes [237].

As sablefish culture moves from experimental to more commercially feasible, more robust studies have followed. The first report of disease susceptibility in sablefish was done in the interest of polyculture with Pacific salmon, as such the causative agent of bacterial kidney disease (BKD), *Renibacterium salmoninarum*, was injected intraperitoneally [238]. Sablefish were susceptible to disease and mortality caused by *R. salmoninarum* infection, *Aeromonas salmonicida* was also isolated from several fish, but only those also infected with *R. salmoninarum*, indicating a potential co-infection. Fish were not screened for carrier status of either *R. salmoninarum* or *A. salmonicida* prior to experimental infection. In previous literature, an atypical strain of *A. salmonicida* had been isolated from a wild, moribund sablefish, the first instance of spontaneous disease caused by *A. salmonicida* in a marine species [239]. Other bacteria have been associated with diseased or dead sablefish larvae in culture, *Enterobacter asburiae* and *Vibrio logei* were both reisolated with *V. logei* being associated primarily with moribund fish [240]. Building on this, researchers evaluated the pathogenicity of 3 *Vibrionaceae* isolates, *V. ordalii, V. splendidus, and V. anguillarum* [241] and found that sablefish were refractory to *V. ordalii* and *V. splendidus*, while *V. anguillarum* caused up to 24% mortality. Other pathogens have been detected in wild sablefish including observed *Loma spp.* parasites [242] and VHSv by PCR [243].

As pathogenicity has been explored, vaccination techniques and efficacy in sablefish have been the subject of several recent studies, mainly focusing on protection against infection with atypical *A. salmonicida*. The first used a commercially produced multivalent vaccine containing two formalin killed isolates of *A. salmonicida*, one typical one atypical, and 3 *Vibrio* species (*V. salmonicida, V. anguillarum, V. ordalii*) [244]. The atypical *A. salmonicida* strain was isolated from cultured sablefish that were actively infected and exhibited clinical disease. Fish were vaccinated by immersion and also by injection with the vaccine prepared in an oil-based adjuvant and then challenged with either atypical or typical *A. salmonicida* by immersion. Results showed poor protection in fish vaccinated by immersion, with relative percent survival (RPS) from -15.1% to 10%, but injection vaccination provided significant protection with RPS values above 80%. Sablefish were less susceptible to the typical *A. salmonicida*, but vaccination still increased survival in a pathogen challenge. Another study evaluated an experimental vaccine containing 3 formalin killed *A. salmonicida* isolates (all collected from diseased sablefish) and two commercial products (Alpha Ject Micro 4® and Forte Micro®) both containing *A. salmonicida* in addition to other bacterins [245]. Fish were challenged with one of the three *A. salmonicida* isolates used in the experimental vaccine by injection and found that the bacterin mix provided more protection than either of the commercial vaccines. Total IgM antibody titers were also measured after vaccination and demonstrated a significant increase at 6 weeks post-vaccination, fish vaccinated with the bacterin mix had the highest IgM levels. However, anti- *A. salmonicida*

antibody levels were not measured due to the lack of an appropriate tool capable of specifically binding to sablefish IgM. The lack of tools to evaluate sablefish immune responses is further address in Chapter 3.

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Chapter 2: A novel oral vaccine delivery platform stimulates adaptive immunity and provides protection against *Aeromonas salmonicida* in rainbow trout (*Oncorhynchus mykiss)*

2.1 Abstract

The immune response of rainbow trout (*Oncorhynchus mykiss*) was evaluated in two trials after oral vaccination with alginate macro-particles encapsulating a formalin killed *Aeromonas salmonicida* vaccine (OV). Other vaccination routes were also tested, including injection (IP), immersion (BA), and anal intubation (AL); control groups included a PBS injection (PB) and a control alginate macro-particle without vaccine (CP). Fish in all treatments were booster vaccinated 2 weeks post initial vaccination; particle feeding took place over 3 days for primary and booster delivery. Specific antibodies in serum and skin mucus were measured until 13 weeks post vaccination, and kidney, spleen, gill, intestine, and skin tissue were analyzed by RT-PCR for relative changes in expression of immune related genes (IgM, IgT, IgD, CD4, IL-1ß). Fish were challenged at 13 (trial 1) and 4 (trial 2) weeks post vaccination by immersion with the same *A. salmonicida* strain used for vaccine production.

Fish in the OV group had *A. salmonicida* specific antibody titers in both trials and had significantly higher survival compared to the PB group in both challenge experiments. Interestingly, the CP group had similar survival rates as the AL and BA groups in trial 1 when challenged 13 weeks after vaccination, the group still had an RPS of 23.6% after challenged 4 weeks post vaccination indicating a potential adjuvant effect of the alginate macro-particles. Unsurprisingly, the IP group had the highest specific antibodies and survival rates in both trials. Results support the idea that intestinal vaccination of rainbow trout with an inactivated *A. salmonicida* vaccine can provide equal protection to immersion vaccines, shown by the striking similarities of the AL and BA groups in both trials. Overall, the novel oral vaccine tested demonstrates an ability to promote specific antibody response and protect fish during pathogen challenges.

2.2 Introduction

Aeromonas salmonicida subspecies *salmonicida*, also known as 'typical' *A. salmonicida*, is the etiological agent of furunculosis in salmonids and has major negative impacts on wild and farmed fish around the globe [1]. In the past, it was one of the greatest threats to the Atlantic salmon (*Salmo salar*) farming industry and was treated in kind with increasing use of antibiotics until efficacious vaccines could be developed and mandated [2,3]. Presently, the diversity of pathogenic subspecies (atypical *A. salmonicida*), expansion of new aquaculture species susceptible to the pathogen, and effects of climate change mean disease outbreaks associated with *A. salmonicida* remain a major obstacle to increased global aquaculture production [4]. Even with vaccination programs, outbreaks of furunculosis are not uncommon in rainbow trout (*Oncorhynchus mykiss*) [5].

Current best management practices for controlling *A. salmonicida* associated diseases are the use of injectable oil-adjuvanted vaccines, which can easily be incorporated into polyvalent formulations that protect against additional pathogens and have been widely used since the late 1980s [6]. However, these vaccines can have deleterious side effects to the final product, such as development of granulomas and other inflammation near the injection site. Atlantic salmon (*Salmo salar*) generally have a slower inflammation response and a longer recovery period than rainbow trout for these pathologies [7], but a recent study showed that side effects can persist up to 14 months post vaccination in rainbow trout [8]. Waning immunity over time leads to disease outbreaks even in vaccinated fish [5]. Booster immunizations for fish in marine net pens can be prohibitively expensive and require specialized equipment for injection and immersion vaccine preparations [6].

Oral vaccines do not add any stress to fish and are essentially effortless for producers, significantly lowering labor costs relative to injection and immersion routes. The first oral vaccine against furunculosis in salmonids was tested in 1942 using a simple formalin killed whole cell preparation coated on the surface of feed [9]. However, there is still no widely available oral vaccine option for furunculosis, and there are relatively few oral options for the aquaculture industry at large. This is due to several obstacles that have frustrated efforts of oral vaccine development for the past 75 years. Protection of the vaccine antigen through gastrointestinal travel is believed to be necessary to stimulate protective immunity [10]. It has been demonstrated that antigen absorption occurs in the second segment of the gut [11]. In many cases, oral vaccination requires higher antigen levels compared to other immunization routes because of degradation that occurs in the hostile environment of the stomach. Another issue is the suppression of mucosal immune responses because of constant stimulation from antigens in the aquatic environment. This phenomenon, known as oral tolerance,

was demonstrated in rainbow trout and common carp (*Cyprinus carpio*), and can be influenced by several variables including frequency of antigen exposure, type of antigen, and temperature [12–15]. Overall, issues of inconsistent, weak, or short duration of immune responses and protection against pathogens have hindered the success and adoption of oral vaccines in aquaculture [16–18].

To protect antigens during gut transit, a variety of encapsulation techniques have been tested that may add potential benefits such as adherence to intestinal walls [19] and adjuvant effects [20]. Alginate is one of the most promising encapsulation materials for oral vaccine delivery in human and animal medicine [21]. Alginate is an inexpensive and widely available polysaccharide produced by brown algae of the class Phaeophyceae. The solubility and pH sensitivity coupled with its bioadhesive, biodegradable, and nontoxic properties make alginate an ideal platform for intestinal absorption [21]. Most preparations of alginate oral vaccines in aquaculture are alginate microparticles that are top coated [22] or incorporated within [23] fish feed. However, using only the surface of feeds limits vaccine payload and incorporated AMs can potentially leech out into the water before ingestion. Coating or incorporating AMs within feeds can require specialized equipment at hatcheries, which potentially increases production costs and certain formulas may be incompatible with certain feed production methods [24].

As a new strategy to address the issues of oral vaccines and AM formulations, a novel oral vaccine platform using macro-alginate particles, the same size as fish feed (~1.5 mm) was tested. It was hypothesized that oral delivery of this novel vaccine particle would elicit a specific immune response and enhance protection against *A. salmonicida* subsp. *salmonicida*. It is known that vaccine delivery route stimulates different systemic and mucosal immune responses [25,26] and varying levels of disease protection [27,28]. Therefore, and to provide a broad comparison between various immunization routes, a formalin inactivated *A. salmonicida* subsp. *salmonicida* vaccine was tested in rainbow trout fingerlings and administered by the following routes: intraperitoneal injection (i.p.), immersion, anal intubation, and orally with the novel macro-alginate particle. Specific antibodies were measured in the serum and skin mucus of fish for 13 weeks and vaccine efficacy was tested via pathogen challenge at 4 and 13 weeks post vaccination. Changes in expression of immune related genes were measured shortly after vaccination in spleen, kidney, gill, intestine, and skin tissues.

2.3 Materials and Methods

2.3.1 Declaration of Conflicting Interests and Animal Care and Use

No conflicts of interest are declared by the authors. Live animal work was approved by the University of Idaho's Animal Care and Use Committee (#2020-33).

2.3.2 Animals

Eyed rainbow trout eggs were obtained from Riverence Farms LLC (Rochester, WA, USA) and maintained in heath stacks with a flow through SPF water supply (14 – 15 °C) at the University of Idaho's College of Natural Resource's Aquatic Animal Laboratory (CNR-AAL; Moscow, ID, USA) until hatching when they were transferred to grow out troughs. Fish were fed commercial diets until they readily accepted 1.5 mm size pellets, approximately 2.5 g $fish^{-1}$.

2.3.3 Vaccine Production

2.3.3.1 Bacterial Growth

The *Aeromonas salmonicida* subsp. *salmonicida* strain used for experiments, 15-021, was donated by US Fish and Wildlife Bozeman Fish Health Center (Bozeman, MT, USA). It was originally isolated from an adult Chinook salmon (*Oncorhynchus tshawytscha*) captured at Lower Granite Dam (Pomeroy, WA, USA). The strain was grown in tryptic soy broth (TSB; BD, Franklin Lakes, NJ, USA) with gentle agitation at 20 °C for 48 hrs, then diluted with 16% (v/v) sterile glycerol and kept at -80 °C in 1.5 mL aliquots. The purity of the broth culture was checked by spread plate dilutions, all subsequent vaccines and challenge preparations were produced from these frozen glycerol stocks.

For the oral vaccine particles, cultures were scaled up to 5 L over 72 hrs, after 48 hrs of growth at 5 L, a 2 mL sample was removed to measure the optical density at 525 nm (OD_{525}) and colony forming units per mL (cfu mL^{-1}) using standard spread plate dilution protocols on three tryptic soy agar (TSA; BD) plates per dilution. Formalin was added to a final concentration of 2 % (v/v), the flask was returned to the shaker and allowed to mix for an additional 24 to 48 hrs, similar to previous experimental vaccines [29]. Complete inactivation was confirmed by plating 1 mL of culture directly onto TSA agar plates in triplicate; no growth was observed after 72 hrs. To concentrate the inactivated bacteria, flasks were kept at 4 °C for at least 72 hrs to allow cells to settle. Once cells had settled to the bottom and the broth appeared clear, a sterile serological pipet was affixed to a vacuum hose and media was drawn out without disturbing the bacteria. The optical density of the decanted media was compared to blank sterile media. The OD_{525} of decanted was below 0.02, indicating a negligible loss of bacterial cells during concentration. The concentrated bacteria were then aliquoted into 50 mL sterile tubes and allowed to settle again in the same manner, this was repeated until cells were consolidated into two 50 mL tubes. These concentrates were stored in the original TSB with 2 % formalin until required for particle production. For other vaccine formulations, cells were grown in 500 to 700 mL of TSB, quantified, and inactivated using the same methods. An exception was the injection and immersion vaccines for trial 2, which were inactivated using 0.3% formalin.

For final preparation of the vaccine before administration or particle production, bacteria were washed 3 times with sterile phosphate buffered saline (PBS; pH 7.2). Bacteria were centrifuged at 3,000 x *g* at 8 °C for 10 minutes, supernatant discarded, and cells resuspended in sterile PBS and vortexed vigorously. After the last wash, PBS was added to bring the suspension of inactivated cells to the desired concentration. For the oral vaccine, the PBS was decanted after the last wash leaving only the inactivated cells in a paste-like consistency. Washing was performed for all treatments except the immersion vaccine in trial 2, in which growth media containing extracellular products produced by *A. salmonicida* were kept in the vaccine to observe their effect on immune response and protection against disease.

Protein concentration and total dose of each administration of the vaccine preparations was estimated using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). For preparations suspended in sterile PBS for delivery, PBS was used as a blank and serial dilutions were measured to estimate the average dose per fish. The immersion vaccine in trial 2 was not measured because it contained extracellular products and was drastically pigmented, confounding measurements. The protein concentration of oral vaccine particles was measured by homogenization of both control and vaccine particles in sterile PBS. Serial dilutions of the homogenates were measured, and the difference in protein content between the vaccine and control particles was estimated to be the vaccine protein concentration. The total grams of particles fed to the tank over the 3 days were divided among the total fish to reach the final mg dose of vaccine protein for each fish. All dose estimates and experimental treatments are shown in Table 2.1.

2.3.3.2 Particle Production

All particle production was performed at OSU's Hatfield Marine Science Center (Newport, OR, USA). Liposomes were produced based on the method originally described by Barr and Helland [30] as modified by Hawkyard et al. [31]. The core solution was produced with a solution of 1% (w/v) alanine, 1% (w/v) betaine and 1% (w/v) glycine.

Particles were produced using the methods described in Hawkyard et al. [32] modified as follows. The particle mash was prepared using a mixture of 3% medium viscosity alginic acid (Sigma-Aldrich, St. Louis, MO, USA) and 10% (w/v) gelatin from coldwater fish (Sigma-Aldrich). An orange pigment (Blaze OrangeTM, Day-Glo Color corp., Cleveland, OH, USA) was added to the alginate suspension at a concentration of 0.5% (w/v) to enhance particle coloration. Two formulations of vaccine-containing complex particles were produced. The first formulation, used in trial 1, was produced by first preparing a 50:50 (v/v) mixture of alginate and fish gelatin solutions. The alginate-

gelatin mixture was then combined with the liposome and *A. salmonicida* cells so that they comprised 10% and 30% (v/v) of the suspension, respectively. The second formulation, for trial 2, was produced with a 45:55 (v/v) mixture of alginate and fish gelatin. Liposomes and bacterial suspensions were added as previously described. These solutions were shaken by hand and then vortexed for 2 minutes until homogenous. Control particles were created by adding the liposome suspension to the alginate-gelatin mixture so that the liposomes comprised 10% of the final formulation by volume. Particles were sprayed into a pre-chilled (4 °C) solution of 10% w/v calcium chloride and collected on a 1 mm sieve with 20 minutes of spraying. To obtain uniform sizes, particles were passed through 2.0 mm, 1.4 mm, and 1.0 mm sieves. The flexible nature of the particles allowed sizes up to 1.7 mm to pass through the 1.4 mm sieve, leaving a range of sizes from approximately 1.3 mm to 1.7 mm.

2.3.4 Vaccination

2.3.4.1 Trial 1

Rainbow trout approximately 2.5 g were randomly divided and distributed into six identical 500-liter tanks, receiving 225 fish each. The six experimental treatments, randomly assigned to tanks, are described in Table 2.1. Before vaccination, fish were kept off feed for 24 hrs.

For oral particle treatments (OV and CP), particles were formulated with a 50:50 alginate to gelatin ratio. Particles were administered to each treatment tank at a rate of 1.5 % bodyweight per day for 3 consecutive days, the first feeding representing day 1 of the trial. Approximately 8 hrs after particle feeding, commercial diets were fed at 2% bodyweight; non-particle treatments were fed 2% bodyweight split into morning and evening feedings, ensuring that all treatments were afforded the same daily feed rate.

Other vaccination treatments were applied on day 1 of the trial, the same day as the first oral particle feeding. The immersion (BA) group closely followed methods previously published for formalin inactivated *A. salmonicida* vaccines in rainbow trout [33–35]. Fish were netted into a bucket at a density of 30 g L^{-1} and vaccine was diluted to a final concentration 1×10^8 cfu mL^{-1} in tank water. Fish were held with constant aeration for 30 minutes before being netted into their experimental tanks.

For i.p. injection (IP) and anal intubation (AL) treatments, vaccine was diluted to 4×10^9 cfu mL^{-1} of which 25 µL was administered to each fish for a final dose containing 1×10^8 total cfu's, previously used in other experiments [29]. All fish were anesthetized prior to administration by immersion in tricaine methanesulfonate (MS-222; Syndel, Ferndale, WA, USA) at a concentration of 50 mg mL^{-1}

until equilibrium was lost. A ½ cc 27-gauge syringe was used to inject 25 µL of vaccine, or PBS for the control group (PB), intraperitoneally for each fish. A 22-gauge feeding gavage needle (Instech Laboratories, Inc., Plymouth Meeting, PA, USA) attached to a ½ cc syringe was used for the AL treatment by inserting the needle at least 15 mm into the intestinal tract of the fish before administering the dose. After 2 weeks, all treatments were administered a booster using the same protocols and vaccine stock.

2.3.4.2 Trial 2

Trial 2 methods closely followed those in trial 1, with several distinct differences. Fish were approximately 2.75 g at the start of the experiment. Again, 225 fish were split into 6 identical tanks that were randomly assigned a vaccination treatment and fish were kept off feed for 24 hrs prior to vaccinations.

Particles for OV and CP groups were formulated with a 45:55 alginate to gelatin ratio based on observations of undigested particles in the hind gut of fish sampled from trial 1. Fish were fed at the same rates as trial 1, and because fish were slightly larger they were administered a slightly higher dose of vaccine protein; 42 mg in trial 1 compared to 44 mg in trial 2. The BA treatment used the same cfu mL^{-1} dose and duration as trial 1, but protein was not quantified because of the dark brown pigmentation of the vaccine containing extracellular products. The IP treatment vaccine was inactivated with 0.3% formalin and washed the same as trial 1. However, Freund's complete adjuvant (FCA) and incomplete adjuvant (FIA) was mixed with equal parts of the vaccine for the initial and boost dose respectively (Sigma-Aldrich). To obtain a similar cfu mL^{-1} dose as trial 1, the IP vaccine was concentrated to 8×10^9 cfu mL^{-1}, 25 µL of the equal parts adjuvant and vaccine mixture was delivered, bringing the final dose for immunization to 1×10^8 cfu mL^{-1}. Since the same stock of vaccine was used for oral vaccine particle production, the same stock and delivery method was used for the AL treatment.

2.3.5 Sample Collection

2.3.5.1 Whole Blood and Mucus

Fish were kept off feed for 24 hrs prior to sampling and euthanized with an overdose of MS-222. For each trial, fish were sampled before immunization for day 0 control samples. Whole blood was collected by caudal vein puncture with a ½ cc 27-gauge syringe needle, which was dispensed into sterile tubes and allowed to clot overnight at 4 °C. Tubes were centrifuged the next day at 5,000 x *g* for 10 minutes, serum was separated and stored at -20 °C until analysis. For trial 1, blood was collected from 10 fish from each treatment at 2, 4, 6, 8, and 13 weeks post vaccination (wpv) as well

as at the conclusion of the pathogen challenge. When sampling 13 wpv, only 4 fish remained from the IP group so all were collected for blood sampling. Sample size was reduced to 5 fish per treatment at each timepoint in trial 2, and were collected at 1, 2, 3, 4, 6, 8, and 13 wpv in addition to 10 fish per treatment for post challenge samples.

Skin and intestinal mucus samples were collected only in trial 1. Skin mucus was sampled at 2, 4, 6, 8, and 13 wpv prior to blood collection by pooling 2 fish into a whirl-pak bag (VWR International, Radnor, PA, USA) containing 100 µL of sterile PBS with 0.05% sodium azide (PBS-Az). Bagged fish were massaged for 1 minute, mucus was then transferred to 2 mL centrifuge tubes and spun at 10,000 x *g* for 10 minutes. Supernatant was collected and stored at -20 °C until analysis. Intestinal samples were collected at 2, 4, 6, and 8 wpv after blood draws by excising the entire intestine from pyloric caeca to anus. Fecal material was removed, and mucus was collected by gently stripping the intestinal length with a forceps into a 0.5 mL centrifuge tube containing 50 µL of sterile PBS-Az. The same fish pooled for skin mucus were pooled for intestinal mucus sampling, tubes were held at 4 °C for 24 hrs and vortexed vigorously every 6 hrs during that time. Samples were then transferred directly to -20 °C until analysis.

2.3.5.2 Tissues

Samples, three fish from each treatment, were collected at 24, 72, and 168 hrs post initial vaccination (hpv) during trial 1 for gene expression analysis. At each timepoint, fish were euthanized with an overdose of MS-222 and spleen, kidney, a 2 cm length of the second segment of the intestinal tract (approximately 1 cm anterior to the vent and identified by a widening in intestinal diameter), and a 1 cm^2 patch of skin immediately posterior to the operculum. Samples were removed aseptically; fecal material was gently stripped from the intestine as needed and tissues were stored in RNA*later* (Thermo Fisher Scientific) at -80 °C until extraction.

2.3.6 Pathogen Challenge

2.3.6.1 Trial 1

Fish (10.6 g), were kept off feed for 48 hrs prior to immersion challenge at 13 wpv. For bacterial preparation, 1mL of frozen 15-021 glycerol stock was used to inoculate 750 mL of brain heart infusion (BHI) broth (Criterion, Hardy Diagnostics) in 2 L baffled, aerated Erlenmeyer flasks (Grenier Bio-One, Kremsmünster, Austria). Flasks were shaken for 44 – 48 hrs at 20 °C until it reached an OD_{525} between 2.1 and 2.2, corresponding to approximately 6×10^9 cfu mL^{-1} based on previous observations. Bacterial stock used for challenge was quantified using conventional spread plate technique with triplicate BHI agar plates for each dilution.

Fish (n = 15) were stocked into 6 L of tank water (15 °C), allowing for an approximate target density of 25 g L^{-1}, bacterial culture had a final OD_{525} of 2.116 (6×10^9 cfu mL^{-1}) of which 30 mL was added to create a final dilution of 3×10^7 cfu mL^{-1} and fish were left under constant aeration for 24 hrs as has been previously described [36] and based on observations during pre-challenge trials to obtain 50% mortality. Fish in all treatments were challenged in triplicate tanks, apart from the IP group that was challenged in duplicate due to insufficient numbers because an earlier challenge attempt resulted in unsatisfactory mortality and had to be repeated. An extra tank from each treatment was exposed to sterile BHI as a mock challenge group. Water temperature fluctuated between 14 – 16 °C in challenge baths, dissolved oxygen remained above 6 mg L^{-1} throughout the 24 hr challenge period. At the conclusion of the 24 hrs, fish were stocked into 17 L flow through tanks (15 °C) and mortalities were collected daily for 28 days. For each mortality, clinical signs of disease were noted, then kidney tissue was aseptically sampled and streaked onto BHI agar plates, *A. salmonicida* infection was confirmed by the development of dark brown pigment within 96 hrs of culture, characteristic of the 15-021 strain. Blood was collected from 5 surviving fish randomly selected from replicate tanks, except for the PB group where only 4 survivors remained. Blood was processed as described above to measure *A. salmonicida* specific antibodies.

2.3.6.2 Trial 2

Fish were exposed to *A. salmonicida* at 4 wpv at an average size of 5.2 g and bacterial growth conditions were the same as in trial 1. Because of their smaller size, 20 fish were used in each replicate and they were stocked into 5 L of tank water (15 °C) to an approximate density of 23 g L^{-1}. For the challenge, 8.5 mL of bacterial culture (OD_{525} = 2.202; 7.9×10^9 cfu mL^{-1}) was added to make a final concentration of 1.34×10^7 cfu mL^{-1}, reduced from trial 1 to accommodate the smaller fish. After the 24 hour immersion, fish were stocked into 17 L flow through tanks at 15 °C after which mortalities were monitored for 28 days and pathogen re-isolation was attempted using the same methods as trial 1. At the conclusion of the challenge, enough samples remained to collect 10 fish from each treatment to measure *A. salmonicida* specific antibodies in serum.

2.3.7 ELISA to Measure *A. salmonicida* Specific Antibodies

An enzyme linked immunosorbent assay (ELISA) developed and optimized in previous papers [37,38] to measure *Flavobacterium psychrophilum* specific antibodies in rainbow trout was modified for the measurement of *A. salmonicida* specific antibodies. Briefly, positive control samples were obtained from fish that were hyper-immunized with the inactivated 15-021 vaccine and shown to contain high levels of *A. salmonicida* antibodies. For hyper-immunization, 500 g fish were

anesthetized with 50 mg mL^{-1} of MS-222 before being administered a 200 µL emulsified injection with equal parts vaccine (1×10^9 cfu mL^{-1}) and FCA, for a final dose of 1×10^8 cfu's per fish. Fish were placed in a 1% salt solution for 30 seconds to recover before being placed into their original tank. Fish were boosted with the same vaccine stock 4 weeks later, emulsified with FIA. Blood, 0.2 mL, was collected non-lethally every two weeks from 6 wpv to 14 wpv, sera was separated and stored at -20 °C. These samples were tested, found to have high *A. salmonicida* specific titers relative to naïve controls, and used as positive controls for all subsequent ELISA.

The coating antigen for the ELISA was made by culturing 15-021 in 20 mL of TSB at 20 °C for 48 hrs with gentle shaking. The culture was centrifuged at 3,000 x *g* for 10 minutes, TSB was decanted, and bacterial cells were washed with sterile PBS. After another centrifugation, bacterial cells were resuspended in 10 mL of sterile PBS then frozen at -80 °C. After 10 freeze-thaw cycles, protein concentration was measured using a NanoDrop 2000. This antigen was then diluted to 10 µg mL^{-1} in carbonate coating buffer (pH 9.6) and wells were coated with 100 µL and the rest of the process followed steps from previous studies [37,38]. Serum samples were diluted, in duplicate, from 1:50 to 1:102400 in doubling dilutions with potassium phosphate buffered saline containing 0.05% Tween-20 and 0.02% sodium azide (KPBS-T-Az) to a final volume of 100 µL $well^{-1}$. All plates were read on a Powerwave XS microplate reader (BioTek, Winooski, VT, USA) at 405 nm (OD_{405}). Final antibody titers are expressed as the $\log_{10}$ of the reciprocal of the highest average dilution in a sample with an OD_{405} at least two times greater than a blank control. For skin and intestinal mucus, 50 µL of undiluted sample was plated in duplicate and results are reported as the absorbance value at OD_{405} after correction for background absorbance.

2.3.8 RNA Extraction and RT-PCR Analysis

RNA extraction was performed using a PureLink RNA Mini Kit (Thermo Fisher Scientific) with an on-column DNAse digest using the PureLink DNase Digest Set (Thermo Fisher Scientific) according to manufacturer's guidelines, quantity and quality of isolated RNA was assessed on a NanoDrop 2000. A RevertAid RT Kit (Thermo Fisher Scientific) with RNase inhibitor was used for cDNA synthesis of 150 ng of RNA, final concentration of cDNA was calculated based on RNA input and stored at -20 °C. Each 10 µL RT-PCR reaction consisted of 5 µL of PowerUp Sybr Green Master Mix (Applied Biosystems, Waltham, MA, USA), 500 nM of each forward and reverse primer, and 2.5 ng of cDNA template, all reactions were performed in triplicate. PCR was run on a StepOnePlus RT-PCR System (Applied Biosystems), and conditions followed a previously described study [26]; a 2 minute hold at 50 °C, 2 minute denaturation step at 95 °C, then 40 cycles of 95 °C for 15 s and 60 °C

for 1 minute, followed by the machine's default melt curve analysis. ß-actin was used as an endogenous control for normalization, primer details for ß-actin and other genes of interest can be found in Table 2.2. Relative quantification of gene expression between the PB group and other treatment groups was determined at each timepoint for each tissue using the $2^{-\Delta\Delta Ct}$ method [39]. Graphical representation of relative expression is shown in fold changes.

2.3.9 Statistical Analysis

All data were analyzed using the program R [42]. Assumptions of normality and homogeneity of variance were determined using Shapiro-Wilk and Levene tests, respectively. Antibody data was non-parametric, therefore a Kruskal-Wallis test was done using the rstatix package to determine significant differences of titers among treatment groups at each timepoint. Dunn's test of multiple comparisons, with a Benjamini-Hochberg *p*-value correction, was used for post-hoc testing. For RT-PCR analysis, the ddCt values passed assumptions and a one-way ANOVA with Tukey's HSD post-hoc testing of multiple comparisons was done for each tissue at each timepoint. The survival and survminer packages in R were used to generate Kaplan-Meier survival curves, and a pair wise log-rank test was used to compare treatment group curves with a Benjamini Hochberg *p*-value correction. Relative percent survival was calculated with the following equation [43].

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

All results were deemed significant if the *p*-value was less than 0.05.

2.3 Results

2.3.1 *A. salmonicida Specific Antibody Titers*

2.3.1.1 Trial 1

All fish sampled prior to immunization had no specific antibody titers to *A. salmonicida* (data not shown), serum titers for all subsequent timepoints tested are shown in Figure 2.1. The IP treatment group had significantly higher titers than all other treatment groups ($p < 0.05$) at nearly all timepoints prior to challenge, with the only exception being at 13 wpv, where it was not significantly different from the AL group titers (3.4 ± 0.37 vs. 1.5 ± 0.48). The AL and BA groups had similar titers and never differed significantly from one another, although AL had slightly higher levels throughout the trial (Figure 2.1). Both AL and BA groups peaked at 8 wpv and had significantly higher titers ($p < 0.05$) compared to the OV group at that time in addition to AL titers being significantly higher than OV at 13 wpv ($p = 0.006$). The OV group peaked 4 wpv with significantly elevated titers relative to both control groups, PB and CP ($p = 0.025$). Fish exhibiting positive titers were observed in the OV

group at all timepoints except 13 wpv. One fish exhibited positive titers in the PB group at 8 wpv, which was the only incidence observed from fish in either control group. With respect to the post challenge antibody titers, the IP group had the highest titers followed by BA and OV groups, but there were no significant differences among any of the treatments (Figure 2.1).

For the skin mucus samples, the IP group again had the strongest antibody response and exhibited significantly higher OD_{405} values than the PB group ($p < 0.05$) at all timepoints except 13 wpv where no groups differed from each other (Figure 2.2). The AL group had a higher OD_{405} relative to the PB group at 2 wpv ($p = 0.005$), and at 4 wpv all vaccinated groups (IP, AL, BA, OV) had significantly higher absorbances than the PB group ($p < 0.05$); however, the OV group was not significantly different from the CP group. At 6 wpv, the OV group did have a significantly higher average OD_{405} compared to the CP group ($p = 0.029$) but not the PB group ($p = 0.076$), whereas both BA and AL were different from the PB and CP groups ($p < 0.05$). No groups, apart from IP, were different from each other at 8 wpv, though the average OD_{405} of the AL group was nearly twice as high as the PB, CP, OV, or BA groups. There was no noticeable signal produced in wells where intestinal mucus samples were used, and no observable pattern among treatment groups or samples collected prior to vaccination was found (Supplemental Table 2.1). Graphical representations of antibody responses over time are shown in Supplemental Figures 2.1 and 2.2.

2.3.1.2 Trial 2

No antibodies were detected prior to vaccination or 1 wpv in any treatment groups (data not shown). The IP group had significantly elevated levels compared to the CP, PB, and OV groups for all timepoints aside from 1 wpv ($p < 0.05$; Figure 2.3). The IP and BA groups were not different at 2, 3, and 6 wpv, but did differ significantly at 4, 8, and 13 wpv ($p = 0.030$, 0.029, and 0.0002 respectively). The IP group had significantly elevated titers relative to the AL group at all timepoints except for 8 wpv The BA group was the only other treatment aside from IP that had significantly elevated titers compared to control groups, which occurred at 3 wpv ($p = 0.018$). Antibody levels in the AL group were variable and observed at 2, 6, 8, and 13 wpv with a peak at 8 wpv The OV group had a peak response at 6 wpv, otherwise titers were not observed. For the post challenge titers, the IP group had significantly higher levels compared to all other groups ($p < 0.05$), but there were no other differences among treatment groups. Graphical representation of the antibody response aver time is shown in Supplemental Figure 2.1.

2.3.2 Pathogen Challenge

2.3.2.1 Trial 1

There were five instances of mortality in mock infected tanks during the first 14 days of the challenge; however, *A. salmonicida* or any other bacteria were not isolated on agar plates from kidney samples. These mortalities were attributed to cannibalism as increased feeding resulted in no further mortalities. Mortalities in challenged fish started 5 days after pathogen exposure and was similar for all treatments except IP and AL, and peaked on day 6, after which mortalities slowed and remained at consistently low for the rest of the challenge as is typical of a chronic *A. salmonicida* infection (Figure 2.4). Final endpoint survival and relative percent survival (RPS) was highest in the IP and AL groups with 70.0% and 68.9% respectively (RPS = 62.1% and 63.5%), followed by BA and CP groups which both had 64.4% survival and RPS values of 56.7% (Table 2.3). The OV group had a survival of 51.1% (RPS = 40.5%) and was significantly higher than the PB group with 17.8% survival ($p = 0.019$). All other groups had significantly higher survival than the PB group ($p < 0.001$) but did not differ from each other.

2.3.2.2 Trial 2

No mortalities were observed in mock challenged tanks for trial 2. There were three mortalities, one each from OV, IP, and CP treatments in where re-isolation attempts did not produce bacterial growth and brown pigmentation indicating death was not due to *A. salmonicida*. Therefore, they were right-censored out of the Kaplan-Meier curves and statistical analysis and omitted from mortality records during RPS calculations. Onset of mortalities was slightly earlier than trial 1, starting at 3 days post exposure and peaking on day 5. By 10 days post exposure, only 6 mortalities occurred among all tanks for the rest of the 28 day challenge period comprising of less than 5% of total mortalities in the trial (Figure 2.5). The IP group had significantly higher survival, 96.7% (RPS = 94.7%), relative to all other vaccinated or unvaccinated treatment groups ($p < 0.001$; Table 2.3). Survival in the AL, BA, and OV groups was similar and between survival ranged from 63.3% to 68.3% and was significantly greater than the PB group with 36.7% survival ($p < 0.005$). The CP group, 51.7% survival (RPS = 23.6%), did not differ significantly from AL ($p = 0.184$), BA ($p = 0.161$), OV ($p = 0.088$), or the PB control group ($p = 0.161$).

2.3.3 Gene Expression

The expression levels of IgT and IgD were low and Ct values were consistently above 33 or altogether undetected. As such, the ddCt was not calculated and genes were not statistically analyzed except for spleen samples, which several biological replicates were dropped due to unacceptably high

Ct levels. It should be noted that at least 2 replicates remained for analysis of spleen IgT and IgD expression at each timepoint (Supplemental Table 2.2). Other genes, IgM, IL-1ß, and CD4 were consistently detected throughout the experiment and graphs showing all instances of significant upregulation in treatment groups relative to PB are shown in Figure 2.6. The complete array of relative fold change for various tissues, genes, and sample timepoints are shown in Figures 2.7 – 2.11.

In the spleen, at 24 hrs post vaccination (hpv), IL-1ß was significantly upregulated in the IP group relative to the PB control group ($p = 0.001$), this was also the case for 72 hpv ($p = 0.013$), but by 168 hpv expression had returned to baseline levels. The OV group had significantly upregulated IgM ($p = 0.027$) and CD4 ($p = 0.028$) 168 hpv. For kidney tissue, the IP ($p < 0.001$), AL ($p = 0.007$), and BA ($p = 0.047$) groups had significantly upregulated IL-1ß expression at 24 hpv (Figure 2.6A). Only the IP group was still significantly upregulated 72 hpv ($p = 0.012$). The OV group had significant upregulation of CD4 in the kidney 168 hpv ($p = 0.036$). No significant differences were observed for any genes of interest in gill tissue, IgM was significantly upregulated in the intestine of IP fish 168 hpv ($p = 0.006$), and in the skin of the AL group 168 hpv ($p = 0.012$).

In spleen tissue at 168 hpv, there was an increase in IgT and IgD expression in all treatment groups relative to the PB group, though none differed significantly (Figure 2.7). Similarly, the largest increase in IgM expression for each group was observed 168 hpv. Upregulation of IgM happened later in the kidney, intestine, and skin tissues (Figures 2.8, 2.10, and 2.11), with the highest fold increases of each group coming at 168 hpv. The gill tissue had a quicker response, with peaks for the mucosal vaccinated groups coming at 24 hpv, and the IP group at 72 hpv, and similarly with CD4 higher expression was seen at 24 hpv (Figure 2.9). IL-1ß was characterized by a quick upregulation at 24 hpv, before decreasing to baseline levels by 168 hpv; this was the case for all tissues except the skin where the OV, BA, and AL groups had peak upregulation at 168 hpv (Figure 2.11). The IP group generally had the largest increase in upregulation of IL-1ß among treatment groups for each tissue, apart from the gill and skin tissue where the OV and AL group were higher in each respective tissue.

2.4 Discussion

Mucosal vaccines, especially those delivered orally, are a popular subject for review as encapsulation and antigen preparation techniques continue to progress [17,19,44]. Studies that directly compare oral vaccination to other routes are more limited but provide valuable information about the specific immune response. To better understand the mucosal and systemic immune response to a formalin killed *A. salmonicida* vaccine, rainbow trout were immunized by injection, immersion, anal intubation, and oral feeding of alginate particles in two separate trials. Specific antibodies, from sera

in both trials and from skin mucus in trial 1, were measured across 13 weeks. Changes in expression of immune-related genes were monitored in trial 1 in the first week after initial vaccination. Fish were subject to an immersion pathogen challenge at 13 wpv (trial 1) or 4 wpv (trial 2). Our study is the first use of an alginate encapsulated oral vaccine for immunizations in rainbow trout specifically against *A. salmonicida*, though there has been one such study in Atlantic salmon [45]. Ours is also the first study using macro-alginate particles as vaccine carriers in any fish model species.

In trial 1, the OV group had lower *A. salmonicida* specific antibodies compared to other vaccination groups with the only exception coming at 4 wpv where levels peaked and were slightly higher than the BA group (1.57 ± 0.27 vs. 1.35 ± 0.37; Figure 2.1). At no point during trial 1 were antibodies elevated in the skin mucus of orally vaccinated fish relative to other vaccination groups. Antibody titers for the OV group in trial 2 were only present 6 wpv at which point it was slightly elevated relative to the AL group (0.86 ± 0.52 vs. 0.40 ± 0.40; Figure 2.3). These relatively short lived antibody responses that wane at about 8 wpv have been observed in other oral vaccines in rainbow trout [46,47]. However, continued booster vaccinations can extend this duration, as was shown with a starch hydro-gel oral particle encapsulating an *A. salmonicida* vaccine [48]. In our study fish were boosted 2 wpv but we also found presence of specific antibodies prior to booster in trial 1 (Figure 2.1). Booster timing can greatly impact the temporal presence of antibody titers in oral vaccines, a study by Jaafar et al. [49] boosted fish 7.5 wpv and later had significant increases in antibodies 13 wpv with a commercial oral *Yersinia ruckeri* vaccine in rainbow trout.

We also observed that other immunization routes generally had higher antibody titers relative to oral vaccination except during the peak responses at 4 (trial 1) and 6 (trial 2) wpv. The same observation was made in another study where an inactivated *S. iniae* vaccine in rainbow trout promoted significantly higher agglutinating titers in fish vaccinated by injection or immersion compared to an unencapsulated oral vaccine top coated on feed [50]. Oral vaccination also provoked lower IgM levels than immersion and injection with a formalin killed *V. anguillarum* vaccine in rainbow trout [47]. However, antigen encapsulation can drastically change this response. A study with an unencapsulated live attenuated *F. psychrophilum* vaccine in rainbow trout found that only fish vaccinated by injection had a significant IgM response, whereas oral, anal, and immersion routes did not [26]. However, a different study used an encapsulated form of that the same vaccine and induced similar serum IgM levels in both injection and orally vaccinated fish, which were significantly higher than control fish [51].

A study using a commercial *Yersinia ruckeri* vaccine in rainbow trout found similar antibody levels between oral and anal immunization routes when measured prior to booster at 16 wpv and again at 24 wpv [22]. A recent study found that both high and low doses of an oral vaccine against *Piscirickettsia salmonis* in Atlantic salmon stimulated specific IgM levels similar to the injection group by 8 wpv, though they were lower than the injection group at 40 days post vaccination [23]. In trial 1 of our study, we found similar specific serum IgM levels among the BA, AL, and OV groups until 8 wpv at which point the BA and AL groups had significantly higher levels. The IP group had significantly higher levels than the OV group at every timepoint measured, contrasting other studies that successfully stimulated comparable serum IgM levels between oral and injection immunization routes [23,51].

Pathogen specific components of the skin mucus have demonstrated the ability to contribute to *A. salmonicida* resistance. In a study by Cipriano and Heartwell [52], serum and mucus from hatchery fish that survived a furunculosis outbreak were tested for reactivity to *A. salmonicida*. They found high levels of a mucus precipitin specific to *A. salmonicida* in rainbow trout and lower levels in brook (*Salvelinus fontinalis*) and brown (*Salmo trutta*) trout, which correlated with the mortality patterns of the disease outbreak. In contrast, serum specific antibodies were equal among the species and not correlated to resistance. In our study, the strongest evidence of specific antibodies in skin mucus was observed in fish vaccinated by injection and not with mucosal administrations (Figure 2.2). Evidence shows that mucosal Igs can be translocated from systemic sources [53] and that cutaneous tissues have their own plasma cells containing Igs [54]. The presence of significantly higher levels of specific IgM in the skin mucus of the IP group in this study suggests that systemic circulating IgM plays an important role in mucosal protection against infections. This is in agreement with Makesh et al. [26], who found that only injection vaccination against *F. psychrophilum* stimulated significantly higher levels of mucosal IgM in rainbow trout and not oral, anal, or immersion routes. Another study in Atlantic salmon tested immunization with a protein-hapten antigen through injection, anal, and gill-immersion routes; they did not find any increase in skin mucus IgM for any treatment, but the injection route did increase levels in skin tissue explant supernatant [25]. These are similar findings what we present here, though we were able to detect small increases in skin mucus IgM of other immunization routes. Our study may have missed an earlier mucus IgM response prior to 2 wpv; an experiment in yellow croaker (*Pseudosciaena crocea*) found a significant and early (1 wpv) antibody response in the skin mucus after immersion with a formalin inactivated *Vibrio harveyi* vaccine compared to oral or injection routes [55]. This response was not present 2 wpv, when we collected our first skin mucus samples. We found the peak response for immunized groups came at 4 and 6 wpv

depending on vaccination route, a much slower response compared to that in yellow croaker, but comparable to those observed by Makesh et al. [26]. Another possible explanation of low mucosal IgM detection in mucosal immunization routes of these experiments is that mucosal Igs can differ in their structure and therefore not be as easily detected using traditional methods developed for identifying systemic IgM [56]. Specifc IgM in intestinal mucus was tested but was not present or was below detection levels. It possible that serum IgM translocated into intestinal epithelial tissue was quickly degraded by components of the gut mucus, as has been previously shown in Atlantic salmon [57]. Another explanation is that IgT plays a more important role in the skin [58,59] and intestinal [60] mucus tissues; however, IgT expression in these tissues was not apparent in this study.

In both trials, orally vaccinated fish had significantly higher survival compared to fish injected with PBS after a pathogen challenge, demonstrating the ability of the macro-alginate particle to provide short (trial 2; 4 wpv, RPS = 50.0%) and long (trial 1; 13 wpv, RPS = 40.5%) term protection. Other oral vaccine preparations against *A. salmonicida* have also been successful at limiting mortalities during challenges, the first coming from Duff [9] who incorporated inactivated bacteria directly into feed and administered it for 70 consecutive days to cutthroat trout (*Oncorhynchus clarkii*), obtaining an RPS of approximately 66%. A more recent study used macro-particles of starch hydrogel-based oral vaccines for *A. salmonicida* in rainbow trout and achieved an RPS of 69% at 11 wpv, though this was after fish had been administered 3 booster doses leading up to challenge [48]. These studies report a higher RPS in fish compared to our trials, but also fed the vaccines over a longer duration. A study with a liposome-alginate *A. salmonicida* vaccine and experimental design similar to ours was done in Atlantic salmon; fish fed for 2 days, boosted at 3 wpv, challenged at 7 wpv, and reported an RPS of 20.8% [45]. These studies demonstrate a range of protection for oral vaccine formulations against *A. salmonicida*. We consider ours a successful first attempt with the alginate macro-particles, with possible future adjustments to be made in the dose, booster timing, and particle formulation that may increase efficacy.

Interestingly, survival in the CP group performed equal to other vaccinated groups when challenged 13 wpv in trial 1. This suggests a long term adjuvant effect stimulated by the particles themselves, the protective cellular or humoral factors contributing to this are not clear, nor is it clear if it is due to the alginate or liposomes within the particles. Alginate has previously demonstrated an ability to boost the non-specific immune response when incorporated into diets at a low inclusion rate (0.5%) and fed for 45 days [61]. That study found that lysozyme, alkaline phosphatase, and protease activity in the skin mucus of rainbow trout increased from day 45 to day 50 and was significantly higher than the

control group [61]. It has already been discussed that skin mucus can contain important components for resistance to *A. salmonicida* infections [52], and this could explain the increase in protection seen in the CP group here. In fish, liposomes have typically been used to encapsulate bacterial antigens in oral vaccines [45,62] or carry non-specific immunostimulants [63] that are then readily absorbed by macrophages in the host. However, there is evidence in human medicine suggesting that liposomes themselves may provide some stand-alone immunoadjuvant action by activating macrophage responses [64].

The pathogen trial in trial 2 was done earlier at 4wpv to determine if the vaccine or adjuvant effects would be stronger if challenged sooner after particle administration. Interestingly, this challenge at 4 wpv did not result in CP survival significantly higher compared to the PB group (RPS = 23.6%). Survival in the AL, BA, an OV groups were significantly higher than the PB group, but not CP group in this challenge. It was expected that an adjuvant effect would be stronger closer to the initial delivery. This was not the case but could be partly explained by the 5% decrease in alginate composition, replaced with gelatin, of the particles compared to trial 1. Administering these empty particles to fish may be a means of providing a more cost effective way to provide protection, but mechanisms of this protection should be further defined. No specific IgM was detected in the CP group throughout the experiments. However, IgT and IgD expression increased by more than 4-fold in the spleen tissue at 168 hpv (Figure 2.7) while IgM and IL-1ß both increased more than 6-fold in the skin tissue at 72 hpv (Figure 2.11). This indicates that there may have been a humoral response in the CP group even though IgM specific to *A. salmonicida* was not detected, further studies with the alginate macro-particles are required to determine the mechanisms of this adjuvant effect and should characterize the differences of immune-activity in the skin mucus.

Decreasing alginate in favor of increased gelatin content in the particles for trial 2 was an attempt to increase digestion in fish; during sampling 1 wpv, intact particles were consistently observed in the hindgut of fish. It is difficult to conclude that the different alginate-gelatin ratio explains the increased protection seen in the OV group in trial 2, especially since specific IgM levels were lower and less consistent. Likely, the time of challenge more closely coincided with the peak humoral response which resulted in better protection. This is demonstrated by the presence of anti-*A. salmonicida* IgM at 6 wpv in trial 2 (Figure 2.3), compared to trial 1 where specific IgM decreased leading up to challenge (Figure 2.1). Other changes were made for trial 2 as well. For immersion, the ECP produced by *A. salmonicida* were kept in the vaccine and led to significantly higher specific IgM levels compared to the anally vaccinated group at 3 wpv, and higher levels throughout trial 2 even

though survival rates were similar between these groups (BA = 65%, AL = 63.3%; Table 2.3). A study by Cipriano and Pyle [65] found that even though antibodies to *A. salmonicida* ECP were present after a natural infection, protection from an injection vaccine with an ECP fraction was only elevated when delivered with an adjuvant. Since no adjuvant was included in the BA group, it may partly explain why there was not increased protection compared to the whole cell preparation used for the AL group even though antibody titers were higher; more on the relationship between antibodies and protection are discussed below. An adjuvant was added to the IP group in trial 2 to simulate the current industry standard of furunculosis vaccines [6]. This combination provided significant protection relative to other vaccination groups, and much higher relative survival when compared to the formalin killed *A. salmonicida* by itself used in trial 1 (63.5% vs. 94.7%; Table 2.3). A recent study by Liu et al. [66] showed similar results, where FCA with a whole cell *A. salmonicida* vaccine provided an RPS of 83.66% compared to just 50% in rainbow trout vaccinated with the *A. salmonicida* alone.

Studies have found conflicting evidence of correlation between increased specific IgM in serum and increased protection against *A. salmonicida*. One group demonstrated a strong relationship using an injection vaccine [29], but found no relationship in an immersion vaccine [35]. In trial 1 of our study, the IP group had significantly higher antibody titers relative to the BA, OV, and CP groups at the time of challenge; the endpoint survival was highest in the IP group, but not significantly different from the other treatments. This demonstrates that the correlation between antibodies and disease protection may not be linear, and there could be an upper limit to the effectiveness of antibodies against *A. salmonicida* infection. However, when the injection was administered with FCA in trial 2, antibody levels were significantly higher than all other treatments and corresponded to significantly higher survival. This is likely due to the stimulation of the non-specific immune response and extended antigen presentation caused by the adjuvant. In the short term (< 5 wpv), antibody levels may not differ drastically between adjuvanted and unadjuvanted injections. This was the case in a previous study [66], and in our study where at 4 wpv titers were similar in the IP group between trials (trial 1 = 3.38 ± 0.11; trial 2 = 3.02 ± 0.18). Even at the time of challenge, 13 wpv for trial 1 and 4 wpv for trial 2, in each trial the specific IgM levels in serum was similar in the IP groups (trial 1 = 3.42 ± 0.18 ; trial 2 = 3.02 ± 0.18). However, this did not lead to similar levels of protection. Ultimately, conclusions draw from direct comparisons of trials within this study are weak because of confounding factors not accounted for such as different vaccine stocks, fish cohorts, and other differences between the trials difficult to measure. Directions for future research may be directed at discerning the

importance of anti-*A. salmonicida* antibodies and their role in disease protection, the mechanisms of adjuvants, and the potential use of alginate macro-particles as adjuvants.

The alginate macro-particles used in this study were between 1.3 and 1.7 mm in diameter; unlike micro and nano particles that benefit from increase surface area for absorption into host cells, this large carrier has drastically more volume to carry a higher dose of vaccine. This resulted in extremely high payloads of vaccine delivered to fish orally, approximately 30% of proteins in these particles was derived from the *A. salmonicida* vaccine, while the remaining came from the amino acid inclusion also present in the control particles. Amino acid inclusion into particles has been shown to aid ingestion in other species [32], and a similar particle formulation was readily accepted by fish both with and without vaccine incorporation. All fish sampled within 4 days after particle exposure (1 week after first feeding) had intact particles within the intestine, and no wasted particles could be observed within 1 hr after administration. This shows that particles were readily ingested, but digestion was limited and therefore limited the release of antigen into the intestine. Typical evacuation time is less than 48 hrs in rainbow trout [67]. The slow evacuation of particles in our study, at least 96 hrs, could be an asset because of the potential for longer duration of antigen exposure to the gastrointestinal tract. Though more research is required to ensure that the effect of this slow passage is not harmful to fish. Feed rates were the same among treatment groups and fish weight remained similar throughout the experiment, indicating that alginate exposure did not negatively impact nutrient absorption or growth in the groups given the particles during the 13 week period following vaccination.

It is important to address the vaccination doses used in this study, which were standardized for injection, immersion, and anal intubation treatments with respect to cfu mL^{-1}. However, the oral vaccine was designed to incorporate as much vaccine as possible to test the physical limits of alginate macro-particle structure during formulation. This resulted in nearly 150 times more vaccine protein delivered orally compared to other routes, drastically higher than other studies with high-dose oral vaccines [23]. Interestingly, the anal route generally had higher antibody levels in both trials, but similar protection, indicating that most of the vaccine protein in the macro-particles was not effectively absorbed in the hindgut compared to the AL group. Strangely, IgM expression in intestinal tissue at 168 hpv increased approximately 7.5 fold in the OV group, but only 4 fold in the AL group. CD4 also had a higher upregulation in the OV group compared to the AL group at this time, though the difference was smaller. This could be explained by the OV group being vaccinated for 3 consecutive days compared to 1 day for the AL group. Another explanation could be the extended

residence of particles 1 wpv in the intestine which may have stimulated an immune response targeting the particles themselves and not the vaccine within them, though this is unlikely because a similar upregulation of IgM and CD4 was not seen in the CP group.

The doses achieved in the present study may not be applicable to farm scale use, as it took approximately 10 L of bacterial culture for one round of vaccination in 225 fish with the macro-particles, not including the booster dose. Commercial scale bacterial fermentation could improve yields but may still prove cost prohibitive. Consideration should also be given to the potential oral tolerance of such a high vaccine dose, which should be tested in the future with varying doses of vaccine incorporated into the alginate macro-particles. Oral tolerance is caused by prolonged exposure to antigens in the intestine and can limit the humoral response in fish [12,14,68], one study even observed fish were more susceptible to the pathogen after over-exposure [27].

Our findings show the ability of an *A. salmonicida* alginate macro-particle vaccine to stimulate a humoral immune response within the first 8 wpv and provide significant protection against disease compared to a PBS control at 4 and 13 wpv. Disease protection was comparable to other vaccination routes including injection, immersion, and anal intubation except when the injected vaccine was mixed with an adjuvant. There is also evidence that the alginate macro-particle itself may stimulate an immune response through increased expression of immunoglobulins in spleen and skin tissue; these particles provided significant protection against *A. salmonicida* infection at 13 wpv. Further work is required to characterize the mechanisms and duration of this protection, but these particles may have a practical application for disease prevention in aquaculture.

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2.6 Tables and Figures

Table 2.1 Treatment groups and vaccine doses for both trials. Stock concentration refers to the vaccine stock used for treatment, in the case of the OV group, the total mg of vaccine per gram of particles is indicated. Dose per fish describes the total cfu's or mg of vaccine administered to an individual fish for an entire vaccination dose, in the case of the OV group it is the total amount administered over the 3 consecutive days of feedings.

Group	Route	Trial 1				Trial 2			
		Stock Concentration		Dose per Fish		Stock Concentration		Dose per Fish	
		cfu mL^{-1}	mg	cfu	mg	cfu mL^{-1}	mg	cfu	mg
PB (Control)	i.p. Injection (PBS)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
CP (Control)	Oral Particle (No vaccine)	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
OV	Oral Particle (Vaccine)	n/a	352.2 g^{-1}	n/a	42	n/a	342.8 g^{-1}	n/a	44
BA	Immersion	4.59×10^9	12.76 mL^{-1}	1×10^8 mL^{-1}	n/a	6.08×10^9	n/a	1×10^8 mL^{-1}	n/a
AL	Anal Intubation	4×10^9	11.12 mL^{-1}	1×10^8	0.278	4×10^9	11.12 mL^{-1}	1×10^8	0.278
IP	i.p. Injection	4×10^9	11.12 mL^{-1}	1×10^8	0.278	8×10^9	21.43 mL^{-1}	1×10^8	0.267

Table 2.2 Primer sequence details and references for RT-qPCR. For each reaction, 500 nM of each forward and reverse primers were used.

Primer	Sequence (5' – 3')	GeneBank	Reference
IgM F	AAGAAAGCCTACAAGAGGGAGA	S63348	[26]
IgM R	CGTCAACAAGCCAAGCCACTA		
IgT F	CAGACAACAGCACCTCACCTA	AY870264	[26]
IgT R	GAGTCAATAAGAAGACACAACGA		
IgD F	TGGCACACCAGGATTTGAC	AY870261	[26]
IgD R	TCAGAATTGAGTGAACGGACAGACA		
CD4 F	GAGTACACCTGCGCTGTGGAAT	DQ867018	[37]
CD4 R	GGTTGACCTCCTGACCTACAAAGG		
IL-1ß F	ACATTGCCAACCTCATCATCG	AJ223954	[38]
IL-1ß R	TTGAGCAGGTCCTTGTCCTTG		
ß-actin F	GCCGGCCGCGACCTCACAGACTAC	AJ438158	[26]
ß-actin R	CGGCCGTGGTGGTGAAGCTGTAAC		

Table 2.3 Endpoint survival (± standard error of the mean) and relative percent survival at the conclusion of a 28 day challenge experiment for each trial. The pathogen challenge for trial 1 took place 13 weeks post vaccination, and trial 2 took place 4 weeks post vaccination. Superscript letters denote significantly different survival percentages among treatment groups after Dunn's test of multiple comparisons.

Treatment	Trial 1		Trial 2	
	Endpoint survival (%)	RPS (%)	Endpoint survival (%)	RPS (%)
PB	$17.8^{a} \pm 5.87$	n/a	$36.7^{a} \pm 3.33$	n/a
CP	$64.4^{b} \pm 12.36$	56.7	$51.7^{ab} \pm 10.93$	23.6
OV	$51.1^{b} \pm 8.01$	40.5	$68.3^{b} \pm 10.14$	50.0
BA	$64.4^{b} \pm 13.52$	56.7	$65.0^{b} \pm 2.89$	44.7
AL	$68.9^{b} \pm 4.43$	62.1	$63.3^{b} \pm 8.82$	42.1
IP	$70.0^{b} \pm 3.3$	63.5	$96.7^{c} \pm 1.67$	94.7

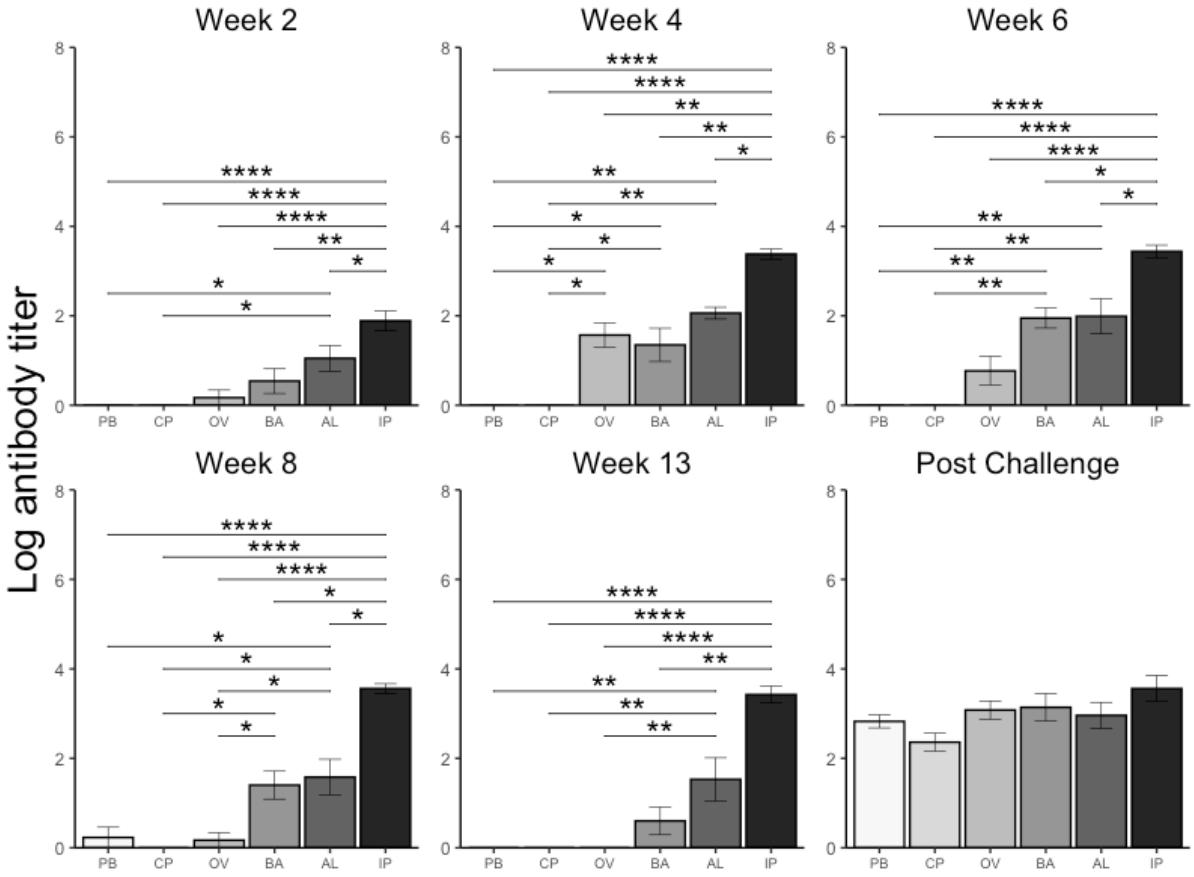


Figure 2.1 Average *A. salmonicida* specific log antibody titers from fish serum (n = 10) at each timepoint in trial 1, error bars indicate the standard error of the mean. Fish were boosted at 2 weeks post vaccination and exposed to *A. salmonicida* 13 weeks post vaccination. Lines above bars indicate significant difference in comparisons, asterisks indicate level of significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** $p < 0.0001$).

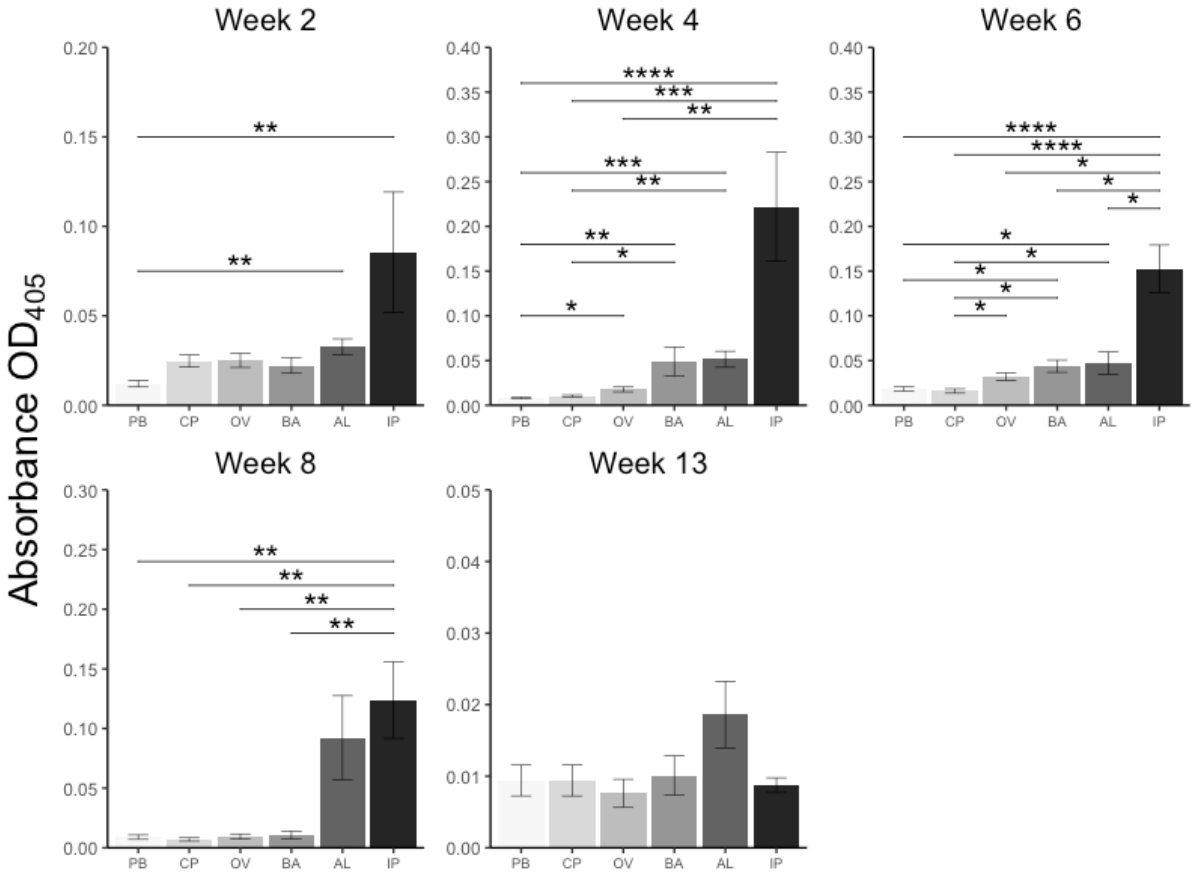


Figure 2.2 Average absorbance value (405 nm) of skin mucus samples (2 fish per sample, 5 samples per treatment) representing *A. salmonicida* specific IgM measured via ELISA, error bars indicate the standard error of the mean. Fish were boosted at 2 weeks post vaccination and exposed to *A. salmonicida* 13 weeks post vaccination. Lines above bars indicate significant differences in comparisons, asterisks indicate level of significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** $p < 0.0001$).

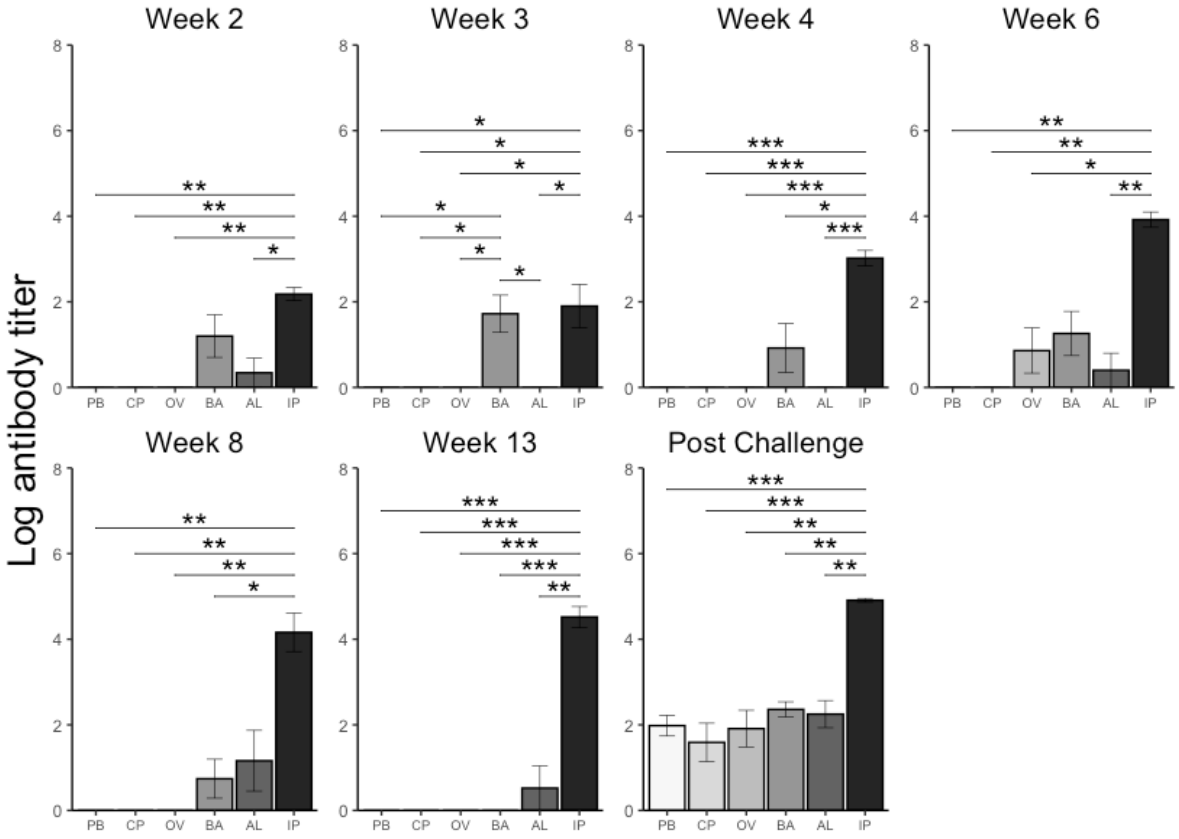


Figure 2.3 Average *A. salmonicida* specific log antibody titers from fish serum (n = 5) at each timepoint in trial 1, error bars indicate the standard error of the mean. Fish were boosted at 2 weeks post vaccination and exposed to *A. salmonicida* 4 weeks post vaccination. Lines above bars indicate significant difference in comparisons, asterisks indicate level of significance (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; **** $p < 0.0001$).

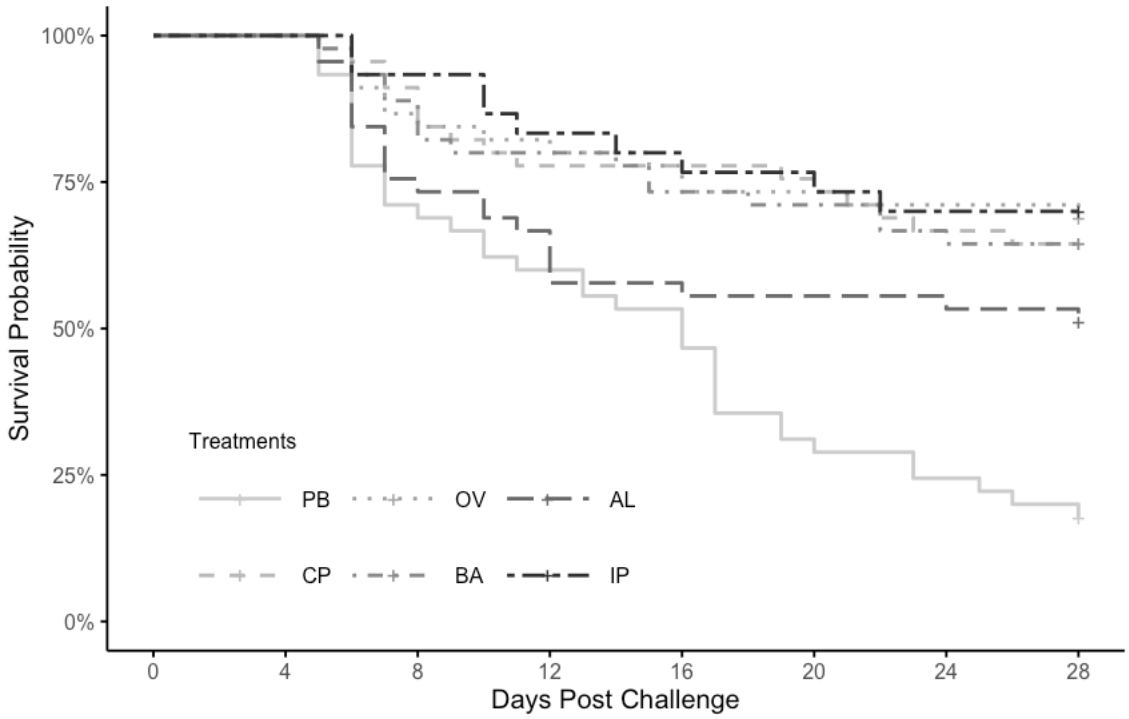


Figure 2.4 Trial 1 challenge results showing overall survival probability of treatment groups after 24 hour immersion challenge with 3×10^7 cfu mL^{-1} of *A. salmonicida* isolate 15-021. Fish were challenged 13 weeks post vaccination, *A. salmonicida* was reisolated from mortalities for 28 days.

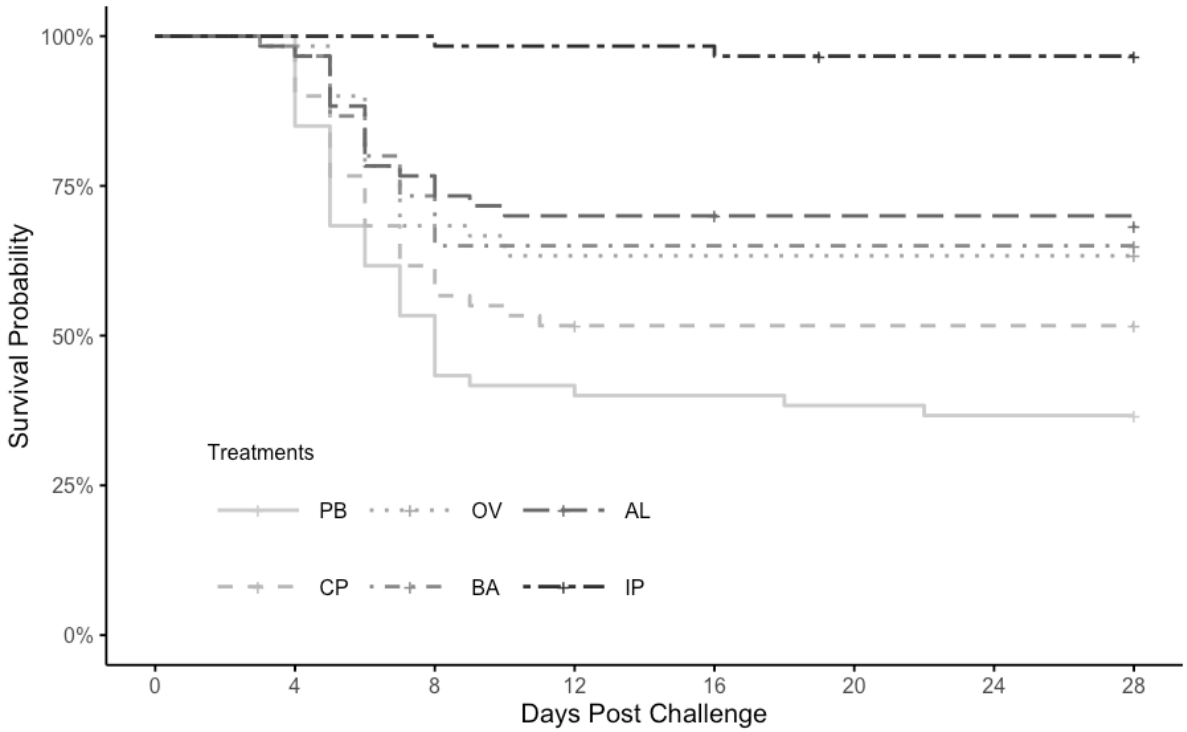


Figure 2.5 Trial 2 challenge results showing overall survival probability of treatment groups after 24 hour immersion challenge with 1.34×10^7 cfu mL^{-1} of *A. salmonicida* isolate 15-021. Fish were challenged 4 weeks post vaccination, *A. salmonicida* was reisolated from mortalities for 28 days.

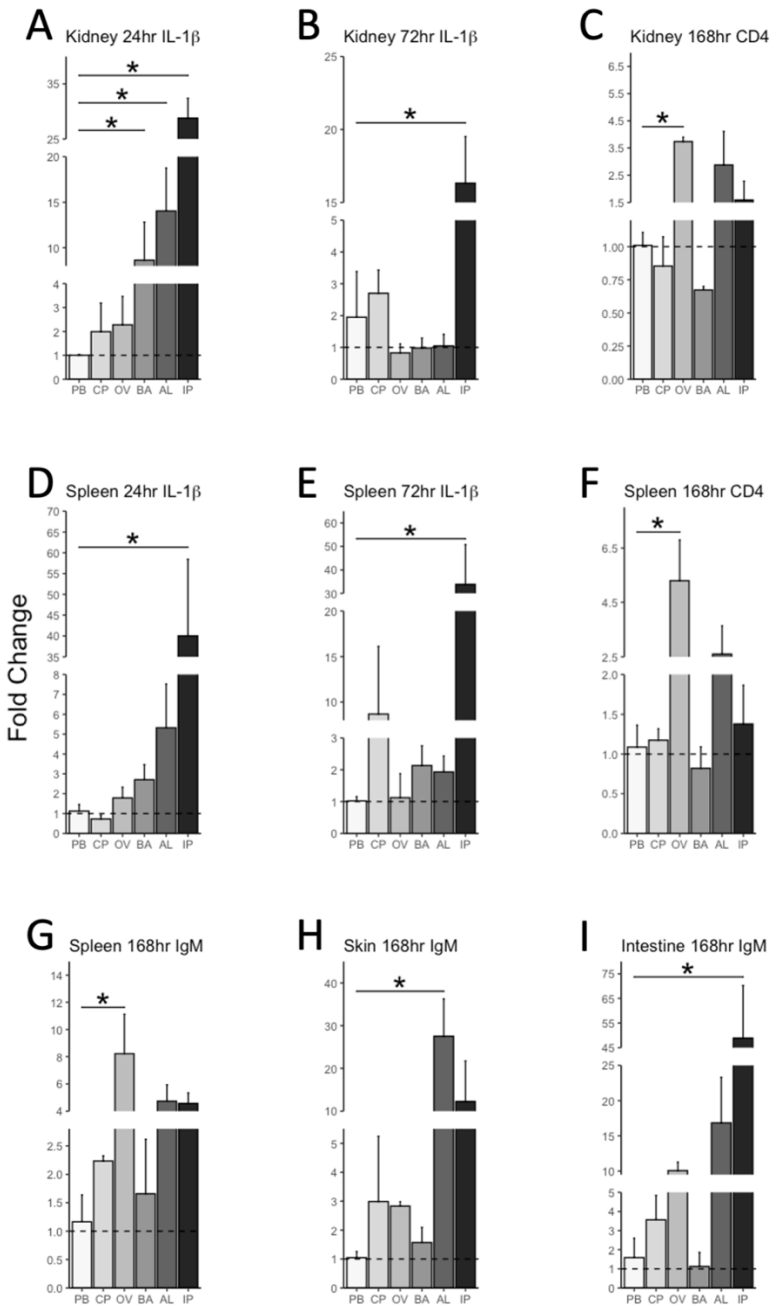


Figure 2.6 Statistically significant relative fold changes in genes of interest for specific tissues at certain timepoints. Lines above bars indicate statistically significant differences between groups ($p < 0.05$). A, IL-1ß expression in kidney 24 hrs post vaccination; B, IL-1ß expression in kidney 72 hrs post vaccination; C, CD4 expression in kidney 168 hrs post vaccination; D, IL-1ß expression in spleen 24 hrs post vaccination; E, IL-1ß expression in spleen 72 hrs post vaccination; F, CD4 expression in spleen 168 hrs post vaccination; G, IgM expression in spleen 168 hrs post vaccination; H, IgM expression in skin 168 hrs post vaccination; I, IgM expression in intestine 168 hrs post vaccination.

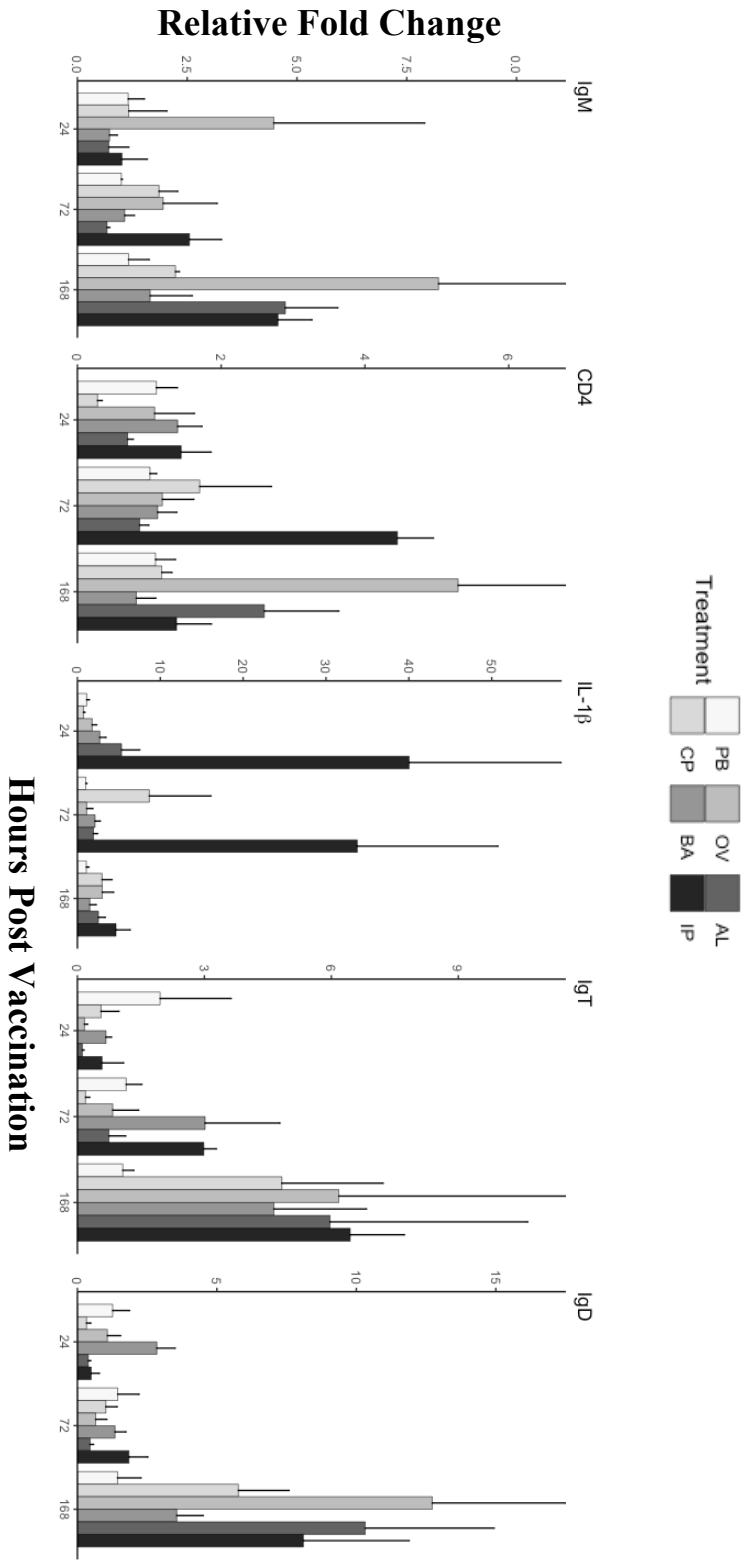


Figure 2.7 Relative fold changes of IgM, CD4, IL-1ß, IgT, and IgD in spleen tissue at 24, 72, and 168 hours post vaccination in trial 1.

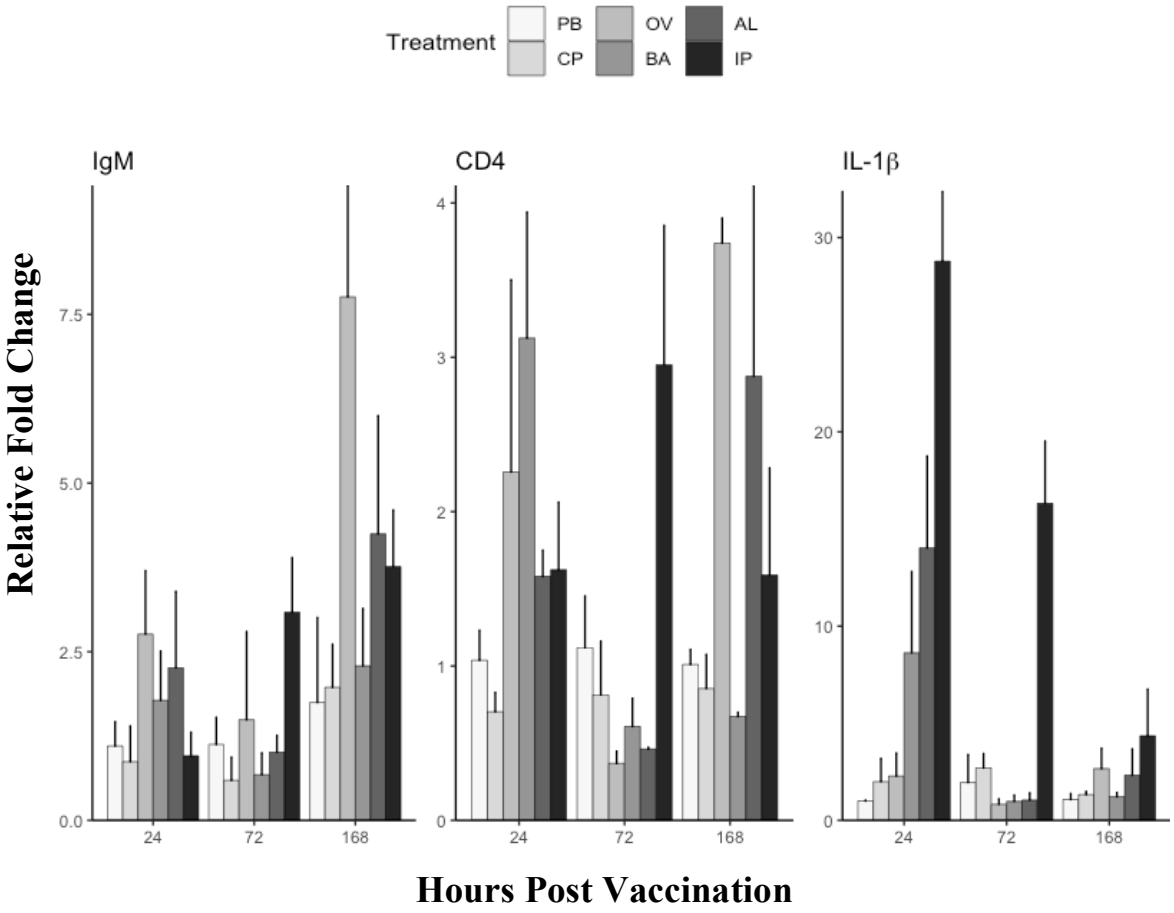


Figure 2.8 Relative fold changes of IgM, CD4, and IL-1ß in kidney tissue at 24, 72, and 168 hours post vaccination in trial 1.

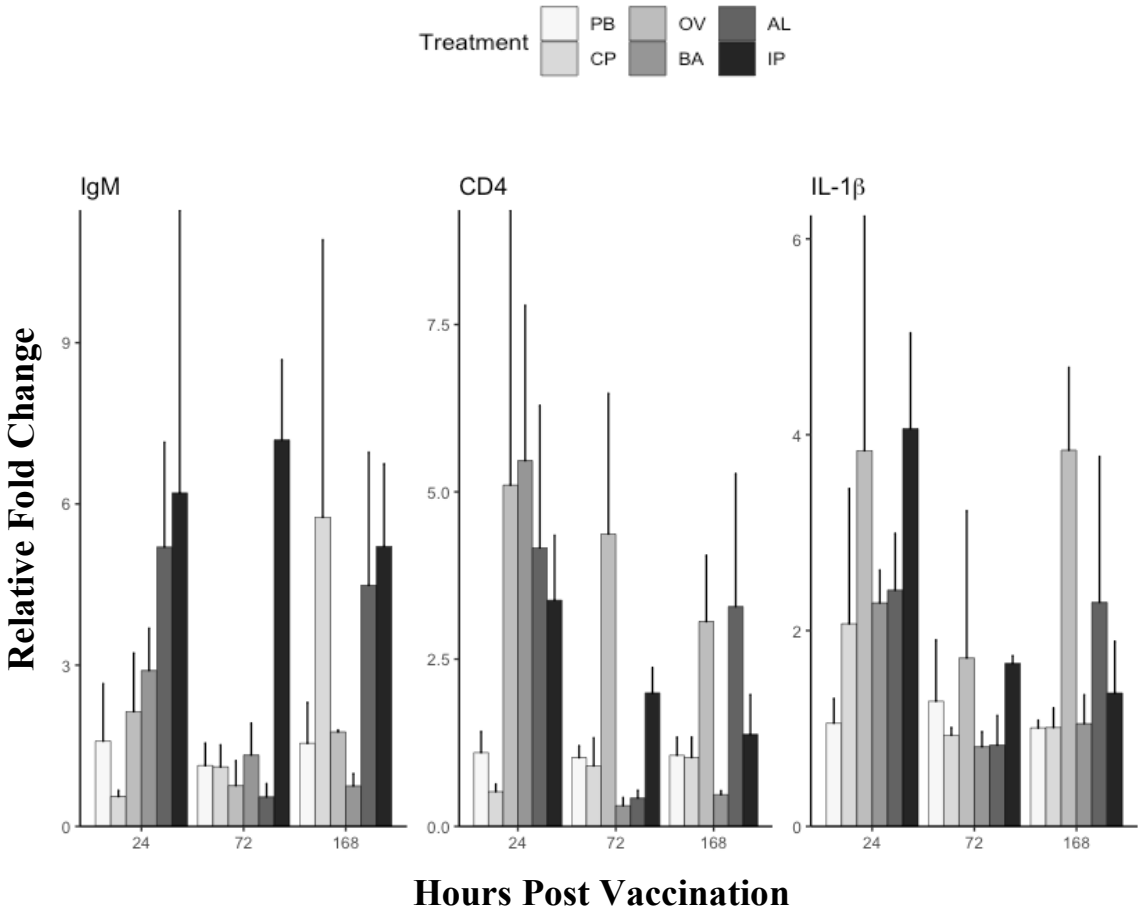


Figure 2.9 Relative fold changes of IgM, CD4, and IL-1ß in gill tissue at 24, 72, and 168 hours post vaccination in trial 1.

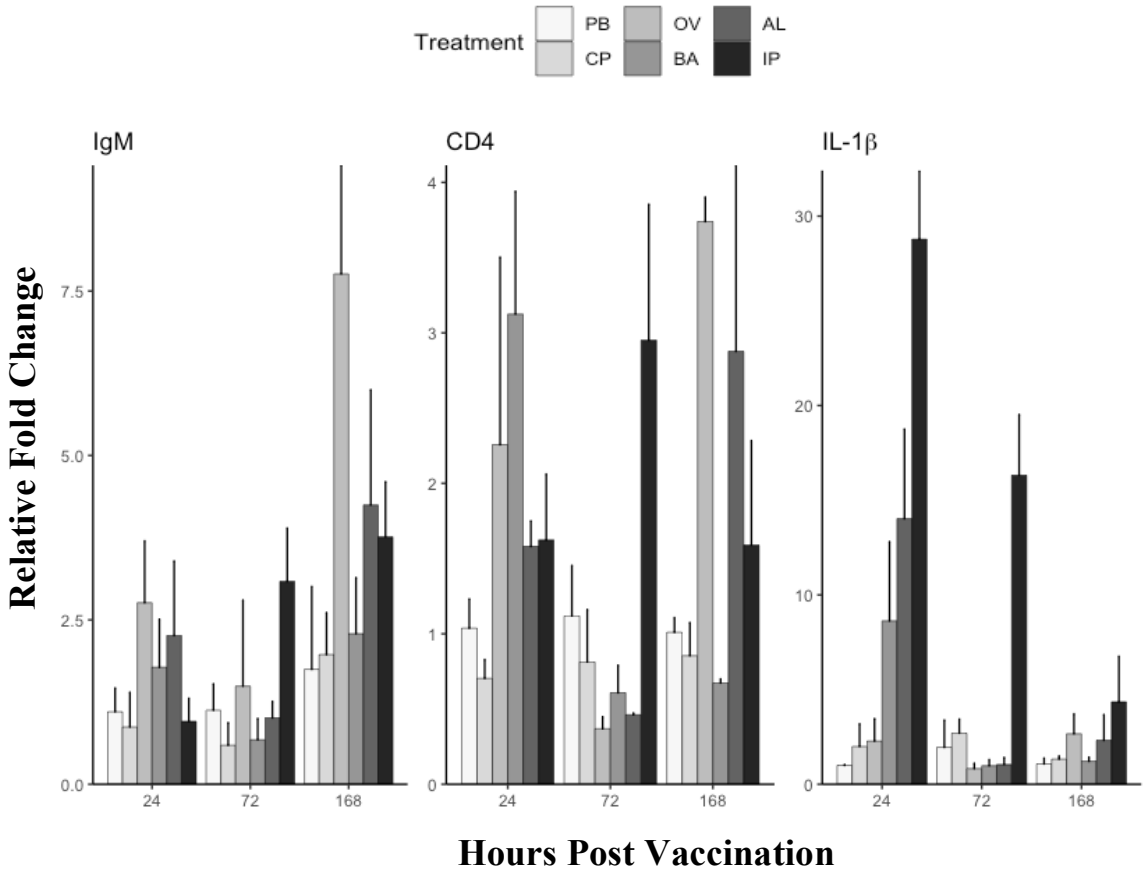


Figure 2.10 Relative fold changes of IgM, CD4, and IL-1ß in intestinal tissue at 24, 72, and 168 hours post vaccination in trial 1.

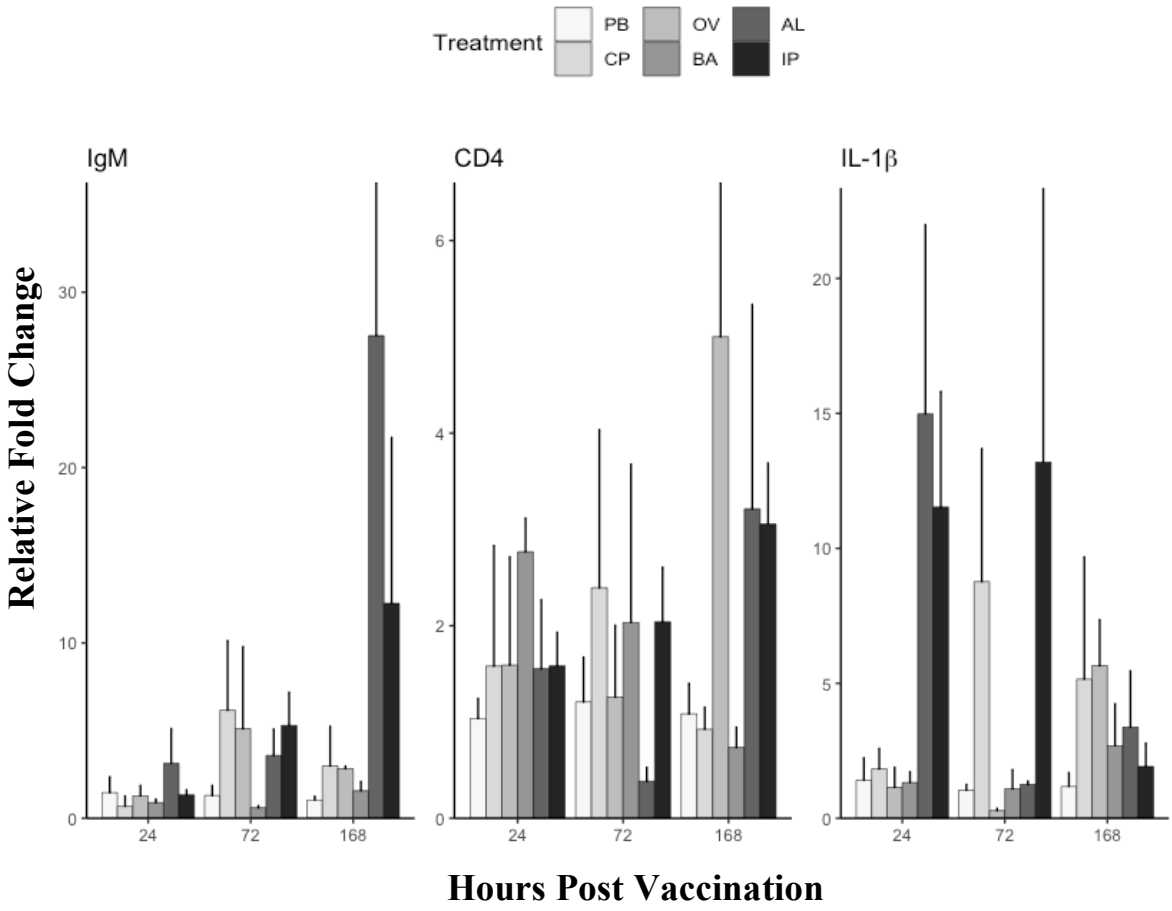
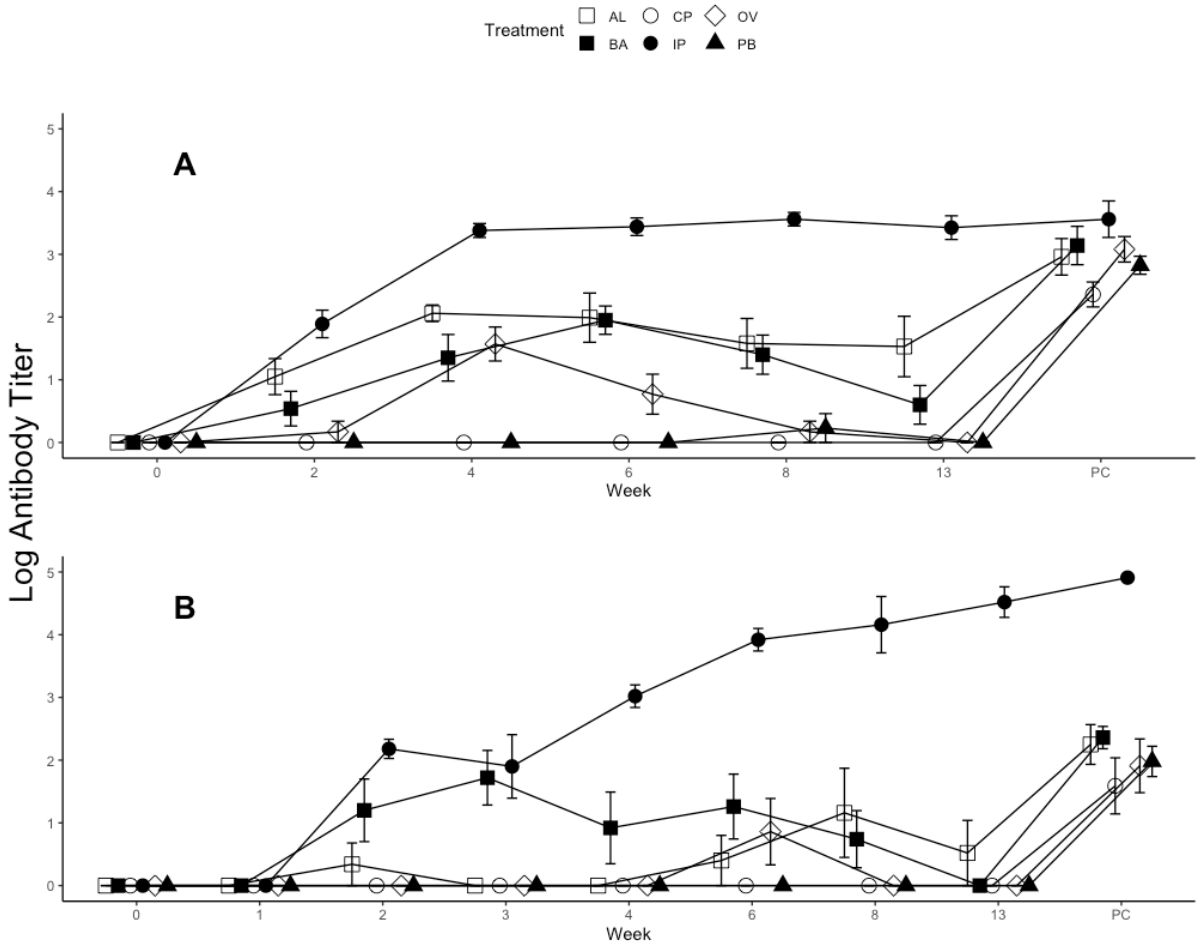
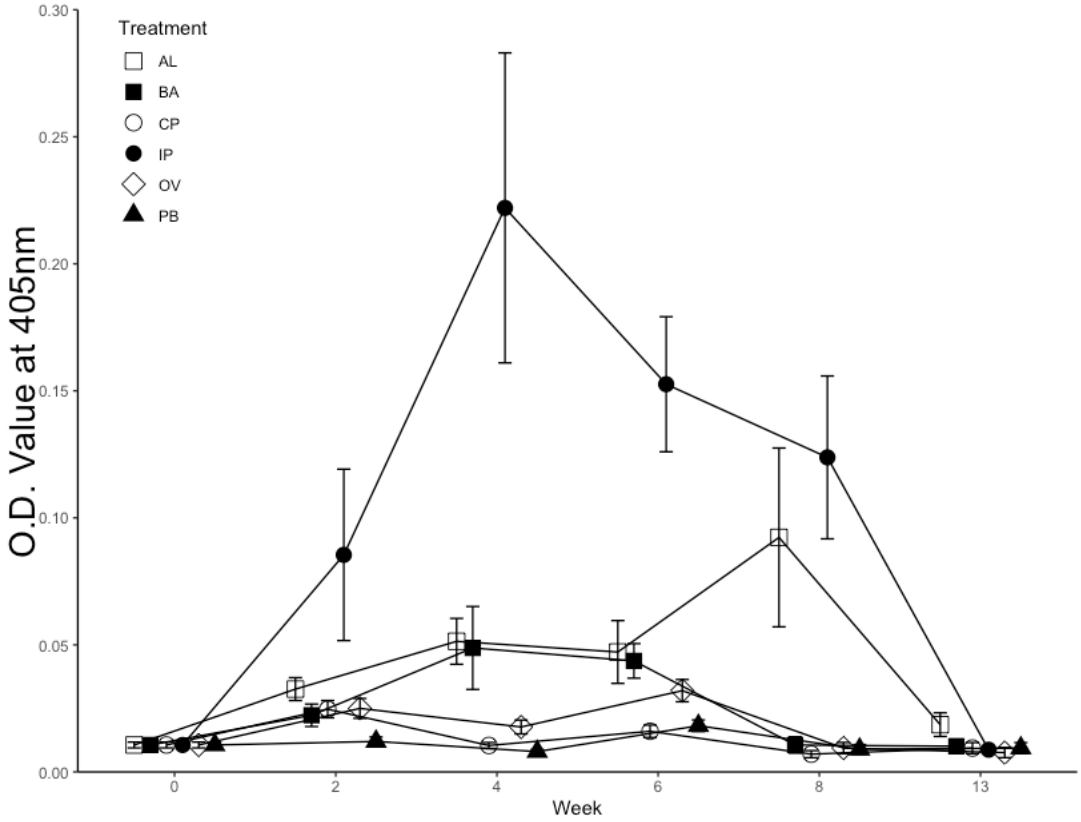


Figure 2.11 Relative fold changes of IgM, CD4, and IL-1ß in skin tissue at 24, 72, and 168 hours post vaccination in trial 1.

2.7 Supplemental Material



Supplemental Figure 2.1 Average *A. salmonicida* specific Log Antibody Titer in fish serum at each timepoint, PC refers to post challenge samples. Challenge experiments occurred at 13 weeks post vaccination for Trial 1 (A), and 4 weeks post vaccination for Trial 2 (B). For all PC measurements, n = 10; 10 samples per timepoint were measured in Trial 2 (B) and 5 samples per timepoint measured in Trial 1 (A).



Supplemental Figure 2.2 Average optical density (O.D.) at 405_{nm} from skin mucus samples (2 fish per sample, 5 samples per timepoint) in an ELISA measuring *A. salmonicida* specific antibodies.

Supplemental Table 2.1 Average OD_{405} (± SEM) of intestinal mucus samples from fish collected prior to vaccination and each treatment at 2, 4, 6, and 8 w.p.v.

Treatment	Week	OD_{405}
Day 0	0	0.013 ± 0.002
PB	2	0.012 ± 0.0031
	4	0.01 ± 0.0018
	6	0.003 ± 0.0003
	8	0.004 ± 0.0005
CP	2	0.013 ± 0.0029
	4	0.01 ± 0.001
	6	0.003 ± 0.0004
	8	0.005 ± 0.0005
OV	2	0.007 ± 0.0012
	4	0.008 ± 0.0023
	6	0.01 ± 0.0031
	8	0.006 ± 0.001
BA	2	0.009 ± 0.0023
	4	0.009 ± 0.0022
	6	0.003 ± 0.0004
	8	0.004 ± 0.0008
AL	2	0.014 ± 0.0047
	4	0.017 ± 0.0049
	6	0.003 ± 0.0004
	8	0.005 ± 0.0009
IP	2	0.018 ± 0.0052
	4	0.006 ± 0.0019
	6	0.003 ± 0.0005
	8	0.002 ± 0.0003

Supplemental Table 2.2 Number of biological replicates, out of three, in parenthesis followed by the average Ct value ± SEM for IgD and IgT expression in various tissues in trial 1.

Treatment	Gene	Spleen			Kidney			Gill			Intestine			Skin		
		24 hours	72 hours	168 hours	24 hours	72 hours	168 hours	24 hours	72 hours	168 hours	24 hours	72 hours	168 hours	24 hours	72 hours	168 hours
PB	IgD	(3) 29.928 ± 1.167	(3) 30.502 ± 0.756	(3) 31.990 ± 0.880	(2) 32.870 ± 0.038	(3) 33.330 ± 1.055	(3) 33.930 ± 0.665	(3) 37.698 ± 0.078	(1) 35.7255	(3) 36.227 ± 0.564	(2) 36.778 ± 1.351	n/a	n/a	n/a	n/a	(1) 36.870
	IgT	(2) 27.759 ± 1.992	(3) 30.382 ± 0.421	(3) 30.842 ± 0.429	n/a	n/a	(1) 37.550	(1) 37.677	n/a	(2) 34.406 ± 0.102	n/a	n/a	(1) 37.550	n/a	n/a	(2) 36.468 ± 0.729
CP	IgD	(3) 31.408 ± 0.700	(2) 30.674 ± 0.799	(3) 30.155 ± 0.346	(3) 33.471 ± 0.454	(2) 34.129 ± 0.759	(3) 32.604 ± 0.868	(2) 35.711 ± 1.371	(3) 36.593 ± 0.661	(3) 35.036 ± 0.504	(2) 36.826 ± 0.130	n/a	(1) 37.392	n/a	n/a	(1) 37.082
	IgT	(3) 30.039 ± 1.720	(3) 32.725 ± 0.675	(3) 29.495 ± 0.889	(2) 36.226 ± 0.021	n/a	n/a	(3) 35.927 ± 1.545	(1) 35.089	(2) 33.485 ± 0.501	(2) 36.226 ± 0.021	n/a	(2) 36.897 ± 0.872	(2) 37.624 ± 0.793	n/a	n/a
OV	IgD	(3) 30.304 ± 1.292	(2) 31.518 ± 1.186	(3) 30.756 ± 0.801	(3) 33.307 ± 0.423	(1) 33.706	(3) 31.517 ± 0.711	(2) 34.259 ± 1.741	(2) 35.531 ± 0.628	(3) 35.904 ± 0.742	(2) 37.841 ± 0.240	n/a	(1) 36.262	n/a	n/a	n/a
	IgT	(2) 30.461 ± 1.397	(2) 30.911 ± 0.820	(3) 32.270 ± 2.011	n/a	n/a	(2) 37.593 ± 0.534	(1) 35.434	(1) 33.855	(2) 36.610 ± 0.461	n/a	n/a	(2) 37.593 ± 0.534	n/a	n/a	n/a
BA	IgD	(3) 28.469 ± 0.237	(3) 30.015 ± 0.403	(3) 30.377 ± 0.195	(3) 29.507 ± 0.560	(2) 32.712 ± 0.478	(3) 31.618 ± 0.728	(3) 32.014 ± 0.549	(2) 35.595 ± 0.127	(3) 35.478 ± 0.971	(3) 33.775 ± 0.711	(1) 36.066	n/a	(2) 35.680 ± 1.286	(1) 34.761	(3) 35.140 ± 1.374
	IgT	(3) 28.869 ± 0.766	(2) 28.998 ± 0.946	(3) 29.229 ± 0.815	(2) 33.642 ± 0.702	(2) 36.098 ± 0.597	n/a	(2) 32.963 ± 0.484	(2) 36.985 ± 0.0719	(2) 34.500 ± 2.728	(2) 33.642 ± 0.702	(2) 36.098 ± 0.597	n/a	(3) 35.757 ± 0.963	n/a	(2) 34.737 ± 2.297
AL	IgD	(2) 31.584 ± 0.250	(3) 31.769 ± 0.185	(3) 29.597 ± 0.965	(3) 33.345 ± 0.558	(3) 33.283 ± 0.726	(3) 31.767 ± 0.467	(3) 35.728 ± 0.752	(1) 33.764	(2) 34.679 ± 0.799	n/a	(1) 37.169	(1) 37.906	n/a	n/a	(3) 35.054 ± 1.317
	IgT	(2) 31.727 ± 1.144	(2) 31.160 ± 1.237	(3) 30.172 ± 1.021	(1) 37.761	n/a	(1) 36.139	(2) 35.218 ± 1.116	(1) 35.772	(3) 34.513 ± 0.439	(1) 37.761	n/a	(1) 36.139	(1) 37.622	(1) 37.570	(1) 34.218
IP	IgD	(3) 31.281 ± 1.589	(3) 30.040 ± 0.792	(3) 30.520 ± 1.162	(3) 35.041 ± 0.593	(3) 31.106 ± 0.387	(3) 31.786 ± 0.764	(1) 34.439	(3) 35.072 ± 0.463	(2) 33.465 ± 0.818	(2) 36.252 ± 0.851	(1) 37.669	(1) 34.583	n/a	(2) 37.010 ± 0.584	(2) 36.840 ± 0.019
	IgT	(2) 29.919 ± 2.410	(2) 29.010 ± 0.514	(3) 28.889 ± 0.287	(2) 35.766 ± 0.356	(1) 36.199	(3) 35.388 ± 0.497	(2) 33.416 ± 0.085	(2) 33.833 ± 0.260	(3) 34.103 ± 0.522	(2) 35.766 ± 0.356	(1) 36.199	(3) 35.388 ± 0.497	n/a	n/a	n/a

Chapter 3: Production of a monoclonal antibody specific to sablefish (*Anoplopoma fimbria*) IgM and its application in ELISA, western blotting, and immunofluorescent staining

3.1 Abstract

Sablefish (*Anoplopoma fimbria*) is an emerging aquaculture species native to the continental shelf of the northern Pacific Ocean. There is limited information on innate or adaptive immunity for this species and tools to determine antibody response following vaccination or disease outbreak are needed. In this paper, a monoclonal antibody (mAb), UI-25A, specific to sablefish IgM was produced in mice. Western blotting confirmed 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 from a previous experiment where fish were immunized with a proprietary *A. salmonicida* vaccine. UI-25A was used in western blot analysis to identify immunogenic regions of *A. salmonicida* recognized by specific IgM from vaccinated sablefish. Immunofluorescent staining also demonstrated the ability of UI-25A to recognize membrane bound IgM and identify IgM+ cells in the head kidney of sablefish. These results demonstrate the usefulness of UI-25A as a tool to improve the understanding of antibody mediated immunity in sablefish as well as provide valuable information for vaccine development and expansion of aquaculture efforts for this fish species.

3.2 Introduction

Sablefish (*Anoplopoma fimbria*), also known as black cod, is one of the most valuable species in the Pacific commercial fishing industry [1], with a recent average wholesale price of $12.25 per lb in the Alaskan market [2]. They have a well-developed market in Japan, North America, and the United Kingdom. However, wild stocks have declined in recent decades, leading to questions about the sustainability of current commercial harvests [3]. As such, investment into sablefish aquaculture has increased and their potential is appealing due to their value and existing market [4]. Sablefish have been farmed in North America since the 1970s, though usually on a small scale and generally incorporated into existing salmonid farm infrastructure with no captive breeding programs because of limited larval survival and relative ease of capturing wild broodstock [5]. Improvements in culture techniques over the past decade include the production of all-female monosex stocks through direct and indirect sex control [6] that provide significantly faster growth rate compared to males. Further, the use and optimization of formulated diets with reduced fishmeal and fish oil levels has been explored [7]. Successful captive breeding programs have shown improved larval survival rates [8,9] allowing for increased availability of juvenile fish for commercial grow out operations, a longstanding obstacle to the continued growth of sablefish aquaculture.

Even with a steady supply of juvenile fish, disease threats remain one of the largest barriers to increasing production in all forms of aquaculture [10]. Disease prevention is crucial to the development of sablefish as a commercial aquaculture species. Sablefish are particularly susceptible to infections with atypical *Aeromonas salmonicida* strains [11], and this gram negative bacterial pathogen is routinely isolated during disease outbreaks in culture settings [12–14]. Prevention of disease caused by this major pathogen is considered vital to expansion of commercial scale operations. Several studies have demonstrated the effectiveness of *A. salmonicida* vaccines in sablefish with respect to increased survival following pathogen challenge [13,14]. However, the lack of a specie specific monoclonal antibody (mAb) capable of recognizing sablefish IgM has inhibited use of common tools such as an enzyme-linked immunosorbent assays (ELISA), western blots, and immunofluorescent microscopy, to evaluate and compare the immune response of sablefish after vaccination or survival from a disease outbreak.

Vaccines work by stimulating a highly specific and long-lasting adaptive immunity through a cell-mediated response involving T cells and humoral immunity characterized by B cell activities. In teleosts, the humoral immune response is comprised of antigen specific antibodies (Ab), or membrane bound immunoglobulins (Ig) produced by B cells [15]. To date, several different types of Igs, all

sharing the basic structure of two heavy (H) chains and two light (L) chains, have been described in fish including IgM, IgD, and IgT/IgZ [15]. IgT is believed to be strongly associated with mucosal immunity in fish [16], while IgM is the predominant systemic Ig that is commonly detected in teleost serum. IgM can exist as two physical forms; a membrane-bound Ig on the surface of B cells including plasmablasts or plasma cells, and the secreted antibody form most often found in a tetrameric structure [17]. The two forms of IgM have multiple mechanisms to protect the host against pathogens including neutralization, complement pathway activation, opsonization, and phagocytosis [15]. Measuring the production and defensive capabilities of IgM in fish is an important tool to characterize humoral immunity. This is achieved through the development of monoclonal antibodies (mAbs) in model species, such as mice, that bind to species specific IgM and can be used with other widely available reagents in serological assays. Many of these mAbs have been developed in recent years for species that include sea bass (*Lateolabrax japonicus*) [18], muskellunge (*Esox masquinongy*) [19], bighead catfish (*Clarias macrocephalus*) [20], Nile tilapia (*Oreochromis niloticus*) [21], large yellow croaker (*Larimichtys crocea*) [22], smallmouth bass (*Micropterus dolomieu*) [23], gibel carp (*Carassius gibelio*) [24], and hybrid snakehead (*Channa maculate*♀ × *Channa argus*♂) [25]. These mAbs have been used in various applications including ELISA, western blotting, and immunofluorescent microscopy [18,19,21]. These mAbs are critical for developing non-lethal sero-diagnostic assays to evaluate immune responses following infection, vaccination, or even feeding of various immunostimulants [24].

The present study aimed to produce mAbs specific to sablefish IgM in mice through standard procedures. IgM was purified from sablefish plasma for the purpose of mice immunizations, and the sensitivity and specificity of mAb candidates were characterized by ELISA and western blotting analyses. The application of an anti-sablefish IgM mAb was investigated in ELISA, western blotting, and immunofluorescent microscopy applications. These results will facilitate further understanding of the characteristics and functions of sablefish humoral immunity.

3.3 Materials and Methods

3.3.1 Declaration of Conflicting Interests and Animal Care and Use

No conflicts of interest are declared by the authors. Live animal work with burbot and rainbow trout was approved by the University of Idaho's Institutional Animal Care and Use Committee (IACUC; #2020-33 and #2020-69). Blood collected from the sablefish vaccination experiment was approved by the University of Washington's IACUC protocol #4078-05. Blood collected at Memorial University for IgM purification was approved under IACUC protocol 16-92-KG. Sablefish collected for

immunofluorescent microscopy analysis were covered under Oregon State University's IACUC protocol 2020-0095.

3.3.2 IgM Purification

Sablefish sera for the purposes of IgM purification was generously donated from Memorial University of Newfoundland. IgM was purified using a Pierce IgM Purification kit with a mannan binding protein column (Thermo Fisher Scientific) following methods similar to those used for other teleost species [14,26]. Briefly, pooled sera samples were dialyzed against 20 mM Tris with 1.25 M sodium chloride (pH 7.4) overnight using a 3.5 K MWCO Slide-a-lyzer (Thermo Fisher Scientific) then diluted 1:1 with IgM column binding buffer. This mixture was applied to the column in 1 mL portions and allowed to bind according to manufacturer's guidelines. Protein concentration of eluted IgM fractions were quantified with a NanoDrop 2000 Microvolume Spectrophotometer (Thermo Fisher Scientific). All fractions greater than 0.005 μg mL^{-1} were pooled and concentrated using a Pierce Protein Concentrator PES with a molecular weight cutoff of 10,000 daltons (10 K; Thermo Fisher Scientific).

A sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize the purity and molecular weight of the proteins within the concentrated sablefish IgM. Sablefish plasma was diluted 1:10 in 2x Laemmeli sample buffer while purified IgM was diluted 1:2, samples were denatured in 100°C water for 10 minutes. After, 10 μL of samples were added to wells of a 4-20% gradient precast stain-free polyacrylamide gel (BioRad) with protein standard ladders. Electrophoresis was run at 90 V for 15 minutes, then 120 V for 120 minutes.

3.3.3 Hybridoma Production

The mouse anti-sablefish IgM mAb was produced by the Washington State University Monoclonal Antibody Center (WSU-MAC; Pullman, WA, USA) closely following standard protocols [27]. To prepare the emulsion for immunizations, purified sablefish IgM was dialyzed overnight using a Tube-O-Dialyzer (Thermo Fisher Scientific) to remove sodium azide prior to injection. Sablefish IgM was then diluted to 0.5 mg mL^{-1} in sterile saline, of which 2 mL was added to a Sigma Adjuvant System (Millipore Sigma). Four BALB/c mice were injected intraperitoneally with 200 μL of emulsified mixture and boosted at 21 days. After the first booster, blood from each mouse was collected, pooled, and allowed to clot overnight at 4 °C to collect sera which was tested to confirm presence of antibodies specific to sablefish IgM.

Mouse sera was tested for specific antibodies to sablefish IgM at the University of Idaho. Briefly, high binding plates (Corning Inc.) were coated with 100 μL of one of the following antigens:

sablefish plasma, purified sablefish IgM, burbot (*Lota lota*) sera, and rainbow trout (*Oncorhynchus mykiss*) sera. Burbot and rainbow trout sera were collected from naïve juvenile fish held at the University of Idaho for use as negative controls. Serial doubling dilutions of coating antigens were made in 0.05 M Tris (pH 9.5) from 0.625 to 0.039 µg mL^{-1} for serum or plasma and 0.125 to 0.0078 µg mL^{-1} for pure sablefish IgM. Plates were incubated overnight at 4 °C, the following day, plates were washed once with 200 µL wash buffer (2.25% $Na_2HPO_4*7H_2O$, 0.25% $NaH_2PO_4*H_2O$, 8.76% NaCl, 1% NaN_3, 0.5% tween-20). Following this, plates were blocked with 100 µL of 0.05 M Tris (pH 9.5) containing 0.3% bovine serum albumin (BSA), sealed, and incubated overnight at room temperature (RT). Plates were then washed twice and 100 µL of mouse sera diluted 1:120 in phosphate buffered saline (PBS; 0.3% BSA, 0.05% tween-20, and 0.02% NaN_3) was added to all wells and incubated at 37 °C for 1 hour. After this, plates were washed four times and 100 µL of biotinylated goat anti-mouse IgG (Thermo Fisher Scientific) diluted 1:1000 was added and allowed to incubate at 37 °C for 1 hour. Plates were again washed four times before the addition of 100 µL of streptavidin-alkaline phosphatase (BioRad) diluted 1:1000 in PBS containing 0.3% BSA and 0.01% NaN_3. After a 1 hour incubation at 37 °C, plates were washed four times and 100 µL of p-nitrophenyl phosphate (PNPP) substrate (Thermo Fisher Scientific), mixed according to manufacturer's guidelines, was added to wells. Plates were incubated with PNPP for 30 minutes before adding 50 µL of 0.3 M NaOH to stop color development, optical density (OD) of wells were read at 405 nm using a microplate reader (Powerwave XS; BioTek).

After an antibody response was confirmed, mice were boosted a third time. After 72 hours, spleens cells were collected from sacrificed mice and fused with myeloma cell line X63 AG8.653 using 50% polyethylene glycol. Selection of fused cells was performed in 96-well tissue culture plates by adding hypoxanthine-aminopterin-thymidine (HAT) media supplement (Sigma-Aldrich) after 24 hours of initial cell growth at 37 °C. After 7 days post cell fusion, colonies with positive hybridoma growth were kept and their supernatant harvested for additional screening.

3.3.4 mAb Screening

3.3.4.1 Indirect ELISA

Supernatants from positive hybridoma colonies, each containing a unique mAb, were screened for reactivity to sablefish IgM using similar methods that confirmed a specific antibody response in mice. Briefly, purified sablefish IgM diluted to 1 µg mL^{-1} in carbonate coating buffer (pH 9.5) was added to wells and incubated overnight at 4 °C. Plates were washed three times with 200 µL of potassium phosphate-buffered saline with 0.05% Tween-20 (KPBS-T) then blocked overnight at RT with 150

µL of KPBS-T containing 5% non-fat dried milk (NFDM) and 0.02% NaN_3. Plates were washed thrice again before mAb candidates, diluted 1:50 in KPBS-T, were added in duplicate and allowed to incubate for 1 hour at 37 °C, a 1:200 dilution of serum from immunized mice was used as a positive control and KPBS-T as a negative control. After, plates were washed again three times and 100 µL of goat anti-mouse IgG conjugated with horseradish peroxidase (Bio Rad) diluted 1:2000 in KPBS-T with 0.1% NFDM was added to wells followed by a 1 hour incubation at 37 °C. Plates were washed thrice again, and specific binding was detected by adding 50 µL of ABTS peroxidase substrate (KPL Inc.) and incubating for 15 minutes at RT before 50 µL of 1% sodium dodecyl sulfate (SDS) stop solution was added, plates were read at 405 nm.

This protocol was repeated using mAb candidates that demonstrated high reactivity after initial screening, with steps remaining the same unless otherwise noted. Sensitivity to pure sablefish IgM concentrations was tested using doubling serial dilutions of a 1µg/mL coating mixture from 1:1 to 1:2048 (final lowest concentration of 4.89×10^{-4}µg mL^{-1}). To assess cross reactivity, other plates were coated with a similar series of dilutions (1:1 to 1:2048) with a 10 µg mL^{-1} starting concentration of control serum from other fish species and a final lowest concentration of 4.89×10^{-3}µg mL^{-1}. Species specific mouse mAbs were used as positive controls, Warr 1.14 for rainbow trout [28], and an anti-burbot IgM mAb [29]. All mAbs were tested in duplicate at a 1:50 dilution. The top four mAb candidates selected were also tested at various dilutions to determine the sensitivity of the mAb concentrations. For this, wells were coated with 1 µg mL^{-1} of purified sablefish IgM and the mAb candidates were applied at dilutions of 1:100, 1:1000, 1:10000, 1:100000, 1:500000, 1:1000000, or 1:5000000 in KPBST in triplicate.

3.3.4.2 Western Blot

Western blot analysis was used to determine the region of specificity for the top four mAb candidates to sablefish IgM. Sablefish plasma was diluted 1:10 and the conditions for SDS-PAGE remained the same as described in section 3.3.2. Proteins were transferred from the polyacrylamide gel to nitrocellulose paper with a Trans-Blot Turbo Transfer System (BioRad) according to manufacturer's protocols. After transfer, the nitrocellulose paper was cut into sections each containing a lane of protein standard and sablefish plasma. Sections were placed in sterile 15 mL tubes and blocked with phosphate-buffered saline (PBS) containing 4% non-fat dried milk (NFDM) for 1 hour at RT under constant gentle rocking. Sections were then washed three times for 5 minutes each with PBS containing 0.02% tween-20 and 0.01% sodium azide (PBST-Az). Supernatants containing mAb candidates were applied undiluted and incubated with gentle rocking for 1 hour at RT. Sections were

washed again, and alkaline-phosphatase (AP) conjugated goat anti-mouse IgG (BioRad) was diluted 1:500 in PBST-Az and gently rocked with sections for another hour at RT. After a final wash, bands were visualized with an AP color development substrate (BioRad), the reaction was stopped by washing sections with ultrapure water for 10 seconds three times.

3.3.4.3 Cloning and mAb Characterization

Based on ELISA and western blot results, mAb 25 was chosen for downstream applications. Cloning of the selected hybridoma line was performed at WSU-MAC using standard limiting dilution methods [27]. The three fastest growing clones (A, B, and C) were selected and screened again using the same methods as described above to confirm desirable characteristics of sensitivity and specificity to sablefish IgM were kept. The final clone, named UI-25A, was also tested via western blotting to ensure there was no cross reactivity to other fish species. Sablefish plasma as well as coho salmon (*Oncorhynchus kisutch*), Atlantic salmon (*Salmo salar*), rainbow trout, and burbot sera was diluted 1:10 in 2x Laemmli sample buffer and subjected to the same conditions as described in section 3.3.4.2.

A rapid ELISA kit (Thermo Fisher Scientific) was used to determine the isotype of the antibodies produced by the cloned hybridoma. Hybridoma cells secreting UI-25A were grown in serum free media, approximately 50 mL of the supernatant was harvested and used for subsequent testing. Concentration of this stock of UI-25A was measured via radial immunodiffusion (RID, Rockland Immunochemicals).

3.3.5 ELISA to Measure *A. salmonicida* Specific Antibodies

In a previously published study [13], sablefish were vaccinated via intraperitoneal injection with a proprietary vaccine that included the atypical *A. salmonicida* T30 isolate. Plasma from both vaccinated and unvaccinated sablefish was collected and stored at -20°C, these were used as controls for ELISA development and optimization. Detection of specific antibodies was conducted with the same equipment, reagents, and wash steps as the previously described ELISA protocol used to screen mAb candidates.

To prepare the T30 bacterial coating antigen, 25 mL of tryptic soy broth (TSB) supplemented with 1.5% (w/v) NaCl was inoculated with a single bacterial colony. The culture was incubated at 21°C for 48 hours with gentle shaking. The broth was then centrifuged at 3,000 *g* for 10 minutes, media was decanted, and bacterial cells washed with sterile PBS. After another centrifugation, bacterial cells were resuspended in approximately 10 mL of sterile PBS and subjected to 10 freeze-thaw cycles.

After the last thaw, the protein concentration was measured using a NanoDrop 2000 (Thermo Fisher Scientific).

Checkerboard titrations (CBT) [30] were performed to determine optimal concentrations of coating antigen, primary antibody (mouse anti-sablefish mAb), and secondary antibody (goat anti-mouse conjugated with horseradish peroxidase [HRP]). The reagents and general steps were based on an ELISA developed at the University of Idaho [31,32] for measurement of *Flavobacterium psychrophilum* specific antibodies in rainbow trout. All conditions for CBTs were run on duplicate plates, one for each positive or negative control sample, each plate also included a blank control of PBS instead of plasma. The T30 antigen was tested at 1, 5, and 10 μg mL^{-1}. The primary antibody (UI-25A) was tested in serial dilutions from 1:50 to 1:3200, the secondary goat anti-mouse conjugated with HRP was tested in dilutions from 1:3000 to 1:8000. Plasma from either naïve sablefish (negative control) or fish immunized against T30 (positive control) were diluted from 1:50 to 1:102400 for each concentration of other reagents, a blank PBS control was also used for each plate. Conditions that limited background signal in negative controls while still maintaining high titers in positive controls were chosen for assays.

Briefly, plates were coated overnight at 4°C, washed then blocked with 150 μL of KPBS-T with 5% NFDM for 1 hour at RT. Plasma samples were diluted in KPBS-T and incubated for 1 hour at 15 °C. Then, 100 μL of the mouse anti-sablefish mAb dilution was added and incubated for 1 hour at RT. After another wash step, 100 μL of the goat anti-mouse HRP conjugate dilution was added and incubated for 1 hour at RT. After the final wash steps 50 μL of ABTS peroxidase substrate (KPL Inc.) was added and color was allowed to develop for 10 minutes, after which 50 μL of 1% SDS stop solution was added. Plates were read at 405 nm in a microplate reader (BioTek). After optimization, sablefish plasma from the previous study [13] was tested to compare antibody titers between 20 vaccinated and 20 control fish. Log antibody titer was calculated as the reciprocal of the highest plasma dilution that had an optical density greater than two times the blank negative control.

3.3.6 Western Blot Analysis of *A. salmonicida* Protein and LPS

The T30 isolate used as part of the previous vaccination experiment in sablefish was subjected to SDS-PAGE using both whole cell and LPS extract preparations, western blotting was then used to determine antigenic regions of the bacteria using plasma from vaccinated fish. For the whole cell sample, the T30 isolate was grown at room temperature in TSB supplemented with 1.5% NaCl for 24 hours under gentle agitation. The culture was then centrifuged at 3,000 *g* and washed once with sterile PBS, centrifuged again, then resuspended in sterile PBS to an OD_{525} of 1.6. Aliquots of 50 μL were

stored at -20 °C until use. Samples were then diluted 1:1 in 2x Laemmli sample buffer, denatured for 10 minutes at 100 °C, and loaded into a 4-20% precast gradient polyacrylamide stain-free gel (BioRad). Conditions for electrophoresis and transfer to nitrocellulose membrane were identical to those described previously. Once transferred, the nitrocellulose membrane was blocked for 1 hour at RT with PBS containing 4% NFDM. Plasma from sablefish immunized against T30 was diluted 1:50 in PBS with 0.05% Tween-20, 0.05% NaN_3, and 2% NFDM, then incubated on the membrane overnight at 15 °C with gentle rocking. The membrane was washed three times with PBS containing 0.05% Tween-20 and 0.05% NaN_3 (PBST-Az), after which UI-25A was diluted 1:100 in PBST-Az and incubated on membrane for 1 hour at RT. Following three more washes with PBST-Az, AP conjugated goat anti-mouse immunoglobulin (BioRad) was diluted 1:500 in PBST-AZ and incubated for 1 hour at RT. After three more washes, specific antigenic binding was visualized using a commercial AP conjugate substrate kit (BioRad). Each individual wash was performed for 5 minutes with gentle rocking.

For LPS extraction, bacterial cells were standardized to an OD_{525} of 0.80 in sterile PBS using the same methods as the whole cell preparation. A 1.5 mL sample of the suspension was centrifuged, and PBS was removed before solubilizing the pellet in 200 µL of lysing buffer (2% SDS, 2% ß-mercaptoehtanol, 10% glycerol, 0.1 M Tris-HCl). The lysate was heated to 100°C for 10 minutes in a water bath, then cooled to RT. Protein digestion was performed by adding 3 µL of proteinase K (Thermo Fisher Scientific) to 20 µL of lysate then incubated at 60 °C for 1 hour. This sample was then diluted 1:1 in 2x Laemmli buffer and run in an SDS-PAGE like the whole cell preparation. A modified protocol from [32] was used for silver staining, the gel was rinsed 3 times (10 s each) with distilled water (dH_2O) then oxidized with .07 g periodic acid, 30 mL ethanol, 10 mL acetic acid, and 55 mL dH_2O for 20 minutes with gentle rocking. After three rinses with dH_2O (5 min each), staining was performed with 1 g L^{-1} of silver nitrate in dH_2O for 30 minutes with gentle shaking, then thrice rinsed again. Color development solution was made fresh (3 g sodium carbonate, 0.02 mL formaldehyde, and 100 mL dH_2O) and chilled to 4 °C before applying to gel. Development was stopped with a solution of 10% acetic acid in dH_2O, the gel was imaged with a Gel Doc EZ system (BioRad). A western blot was done on another gel that was left unstained, using the same methods as the whole cell preparation.

3.3.7 Immunofluorescent Staining of Membrane Bound IgM

Sablefish, approximately 100 g, held at the Hatfield Marine Science Center in Newport, Oregon were euthanized and shipped overnight to the University of Idaho. Head kidney tissue was aseptically

collected, and a sterile scalpel blade was used to cut tissues in half. The newly exposed side of tissue was blotted on a microscope slide (Thermo Fisher Scientific) and allowed to air dry. Cells were fixed for 5 minutes with 4% paraformaldehyde (Thermo Fisher Scientific), gently rinsed with dH_2O, and allowed to air dry again. Slides were blocked with PBS-T containing 5% BSA for 1 hour at RT. The UI-25A mAb was diluted 1:80 in PBS-T containing 1% BSA and incubated at RT for 1 hour. Slides were washed with PBS three times for 5 minutes each. A goat anti-mouse secondary antibody conjugated with Alexa Fluor 555 (Thermo Fisher Scientific) was diluted to 2 μg mL^{-1} in PBS-T with 1% BSA and incubated for 1 hour in the dark at RT. Slides were washed twice again for 10 minutes each with PBS-T before applying Fluoromount-G Mounting Medium with DAPI (Thermo Fisher Scientific) to slides and sealing with a coverslip. This same process was repeated for head kidney tissue collected from burbot to serve as a negative control. Images were acquired using a Nikon Andor spinning disk confocal microscope with a Zyla sCMOS camera and Nikon Elements software, a 40X objective was used for imaging.

3.3.8 Statistical Analysis

All statistical analysis was performed in R [33]. To compare OD_{405} values from ELISA assays, normality and variance assumptions were determined with Shapiro-Wilks and Bartlett's tests respectively. A one way ANOVA was used to compare differences among treatments and significant groups were determined with Tukey's HSD tests. Data from log antibody titer analysis was non-parametric, as such a Mann-Whitney U test was used for statistical comparison.

3.4 Results

3.4.1 IgM Purification

IgM isolated from sablefish plasma showed distinct bands at both ~75 kDa and ~25 kDa by SDS-PAGE (Figure 3.1). These are the predicted sizes of the H and L chain fragments of based on other teleost specie IgM. Some banding at 100 kDa indicates intact H and L chains that did not fully denature, and higher MW bands correspond to potential monomeric IgM structures.

3.4.2 Hybridoma Production

Wells were coated with five doubling serial dilutions of fish plasma or sera (0.625, 0.312, 0.156, 0.078, and 0.039 μg mL^{-1}) or with pure sablefish IgM at $1/5$ those concentrations (0.125, 0.062, 0.031, 0.015, 0.007 μg mL^{-1}); for simplicity these will be referred to as the 1st (highest) through 5th (lowest) coating concentrations and results are shown in Figure 3.2. Polyclonal antibodies from immunized mice developed significantly higher absorbance in wells coated with sablefish IgM or plasma from the 1st to 3rd coating concentrations ($p < 0.05$). At the 2nd concentration, absorbance from

wells coated with sablefish plasma was significantly higher compared to pure IgM ($p = 0.04$) but was not different at any other concentrations. Absorbance of wells at the 4th coating concentration was different between sablefish plasma and rainbow trout or burbot serum ($p < 0.05$), there was also a significant difference between pure IgM and burbot serum ($p = 0.017$) but not rainbow trout serum. At the 5th concentration, absorbance in wells coated with sablefish plasma was significantly higher compared to burbot serum ($p = 0.012$) but no other coating antigens.

3.4.3 mAb Screening

3.4.3.1 ELISA

Of the 32 mAbs screened, 24 (1, 4, 5, 6, 8, 9, 10, 11, 12, 13, 17, 18, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 31, 32) had a standard error of the mean (SEM) for the OD_{405} value within or greater than 1.0 in wells coated with 1 µg mL^{-1} of sablefish IgM and were kept for further analysis (Figure 3.3).

No cross reactivity was observed between the 24 mAb candidates and rainbow trout or burbot sera (Supplemental Table 3.1). The 24 mAbs were screened with a range of purified sablefish IgM coating concentrations, all candidates reacted well to high concentrations of purified IgM (Supplemental Table 3.1). There were four mAbs selected for further analysis (1, 9, 25, 30) based on their reactivity to 0.0625 µg mL^{-1} of sablefish IgM (Figure 3.4).

Various dilutions of the top four mAbs were tested for their sensitivity to 1 µg mL^{-1} of sablfish IgM coating antigen. There was no difference in absorbance among the mAbs at dilutions of 1:100 and 1:500k (Figure 3.5). At a 1:1k dilution, mAbs 9 and 30 had significantly higher absorbance than 1 and 25 ($p < 0.05$). At 1:10k, all were significantly different from each other with mAb 9 being the highest, followed by 30, 25, and 1 ($p < 0.05$). However, at 1:100k, mAb 25 was significantly higher than all other candidates, while 9 and 30 were only higher than mAb 1 ($p < 0.05$).

3.4.3.2 Western Blot

The four mAbs (1, 9, 25, 30) with high reactivity at low concentrations of sablefish IgM were used for the western blotting analysis. All showed banding at ~75 kDa, indicating reactivity towards the H chain of sablefish IgM (Figure 3.6), though mAb 1 had faint banding relative to other candidates. Ultimately, mAb 25 was chosen for further testing and application to downstream processes because of the high reactivity and sensitivity towards sablefish IgM during the ELISA screening.

3.4.4 Cloning and Characterization

The three clones of mAb 25 behaved similarly and retained specificity and sensitivity to sablefish IgM as well as lacking cross reactivity to other teleost serum and the *A. salmonicida* T30 antigen. Clone A, referred to as UI-25A, was chosen for further downstream applications. Western blot

analysis shown in Figure 3.7 demonstrates the specificity UI-25A has to the heavy chain of sablefish IgM with banding at 75 kDa, and lack of banding or reactivity to other products in sablefish plasma as well as proteins and carbohydrates in other fish species sera. UI-25A was typed as an IgG2b mouse antibody, and the concentration of the stock used for all other applications described was 60 μg mL^{-1}.

3.4.5 Development of ELISA to Measures *A. salmonicida* Specific Antibodies in Sablefish

Through standard checkerboard titrations, the optimal concentration was chosen for coating antigen, primary, and secondary antibodies. The T30 coating antigen was used at 5 μg mL^{-1}, while the primary and secondary antibodies were optimized at 1:800 and 1:5000 dilutions respectively. Log antibody titers were significantly different between vaccinated and control sablefish (3.86 ± 0.09 vs. 2.43 ± 0.05; $p < 0.0001$; Figure 3.8). This demonstrates the effectiveness of UI-25A in measuring specific circulating antibodies and differentiating immune responses between vaccination treatments *in vitro*.

3.4.6 Western Blot Analysis of *A. salmonicida*

Western blotting of the T30 whole cell profile with the immune plasma from vaccinated sablefish showed bands at primary immunogenic regions of 60-80 and 10 kDa (Figure 3.9A), while LPS extract had banding only in the 70-80 kDa region (Figure 3.9B).

3.4.7 Immunofluorescent Staining of Membrane Bound IgM

Imaging showed red fluorescent signals on IgM+ cells collected from head kidney imprints. The UI-25A did not bind to all sablefish head kidney cells (Figure 3.10a) and did not bind to any cells from similarly prepared burbot head kidney tissue imprints (Figure 3.10b). This demonstrates the ability of UI-25A to recognize membrane bound IgM in sablefish in addition to secreted IgM antibodies in circulation.

3.5 Discussion

Literature on fish immunology has increased along with the growth of aquaculture worldwide and reviews can be found on innate [35], cell mediated [36], and humoral immunity [15]. Antibodies (polyclonal or monoclonal) specific to fish Igs are important components in the development of vaccines, immune boosting treatments, and diagnostic assays that further support aquaculture. They are also useful in the management and monitoring of diseases in wild fish populations. In the past these tools were reserved for economically important species such as Atlantic salmon [37] or catfish [38] where demand and funding for vaccines or other diagnostic assays were higher. Presently, fish immunoglobulins, specifically their structures and role in immune function, are much better understood and fundamental research is useful for not only aquaculture but also understanding the

evolution of vertebrate immunity [39]. The reduced cost and increasing availability of tools to create mAbs has led to a renaissance of increased production for niche and emerging aquaculture species.

Sablefish aquaculture attracts growers and investors as an emerging aquaculture species due to its limited commercial availability, high marketability, and their ability to be incorporated into existing aquaculture infrastructure including net pens and commercial salmonid diets [4]. Research of the immune system in sablefish is increasing and recent studies have focused on their susceptibility to pathogens [40], vaccination and pathogen challenges [13,14], and effect of environmental factors on immune function [41]. A polyclonal anti-sablefish IgM chicken IgY antibody was used by Vasquez et al. [14] to measure total circulating IgM, and it was found that ~125 g sablefish had relatively low levels, ~ 75 μg mL^{-1}, compared to other species. However, the antibody was not able to measure specific antibody levels due to non-specific binding to the A-layer of *A. salmonicida*.

Sablefish IgM, purified through a mannan binding protein matrix, showed two distinct bands at about 75 and 25 kDA in reduced SDS-PAGE conditions. These bands correspond to the H and L chain sizes of other Teleostei IgM such as large yellow croaker [22], Nile tilapia [21] , Indian major carps [42], muskellunge [19], Atlantic salmon [37], and sea bass [18]. In this study 32 mAb candidates were evaluated for their specificity and sensitivity to sablefish IgM using ELISA and western blot techniques. The selected mAb, UI-25A, is specific to the H chain of sablefish IgM which is a desired epitope for these tools because of its specificity to individual Ig classes, whereas the L chain may share kappa or lambda chains across different Ig classes [19]. The only other member of the family Anoplopomatiae, the skilfish (*Erilepis zonifer*), is not well studied except for some general information on life history, distribution, and current stock status [43]. Cross reactivity of UI-25A to skilfish IgM was not tested due to lack of opportunistic samples from the species, but there may be interest in the future to determine the extent of similarities in antibody characterization between the Anoplopomatidae family members. To our knowledge, this is the first reported mAb available for detection of sablefish IgM.

The UI-25A mAb is effective in detecting circulating antibodies in sablefish specific to atypical *A. salmonicida*, an important pathogen affecting sablefish aquaculture. It is well understood that IgM levels and their specificity to pathogens is important for a well-developed humoral immune response in fish [44]. The ability for mAbs, including UI-25A, to identify specific antibodies in blood circulation is a powerful tool for evaluation of the immune response in fish under various conditions. This study found significantly higher *A. salmonicida* specific IgM titers in vaccinated sablefish compared to a control group (Figure 3.8), which corresponded to significantly higher survival during

a pathogen challenge previously performed with these treatment groups [13]. UI-25A was also successfully used to determine the specificity of antibody responses using immunoblotting of whole cell and LPS extracts from *A. salmonicida* (Figure 3.9). Banding between 60-80 kDa indicates specificity to the A-layer protein of *A. salmonicida* isolates [45] and likely the O-polysaccharide-core of the LPS though molecular mass range of LPS can vary among isolates [46]. Similar responses have been observed in other species [47], and future studies may use these techniques to further elucidate the antibody response of sablefish to a variety of *A. salmonicida* isolates. This technique will also be useful in screening important target antigens for vaccine formulations against *A. salmonicida* or other pathogens [48]. Prevention of diseases caused by *A. salmonicida* in sablefish aquaculture is difficult due to the diversity of the pathogen [49]. Western blotting may be used to evaluate the ability of formulated vaccines to induce specific antibody responses against novel or emerging isolates, this has previously been done for other diverse bacterial pathogens [50]. Aside from measuring and evaluating specific antibody presence in the blood, measuring the presence and activity of IgM+ cells is another application that UI-25A can be used in. Through immunofluorescent staining, we have demonstrated that the UI-25A mAb can detect and bind to membrane bound sablefish IgM (Figure 3.10). This type of staining may be applied to similar assays, such as flow cytometry, to detect and quantify the presence and activity of IgM+ B cells or other cell types [18,20]. In summary, a murine mAb specific to the H chain of sablefish IgM was developed. A number of potential mAb candidates were screened by ELISA and western blotting before selecting UI-25A as the optimal clone for downstream applications. UI-25A was successfully applied in an ELISA and western blot to characterize the immune response of sablefish vaccinated against atypical *A. salmonicida*. In addition, UI-25A was and used in immunofluorescent microscopy to identify IgM+ cells. This study and the characterization of UI-25A provides a valuable tool for the study of immunity in sablefish and has implication for the development of disease prevention strategies and advancing aquaculture for this species.

3.6 Acknowledgements

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3.9 Tables and Figures

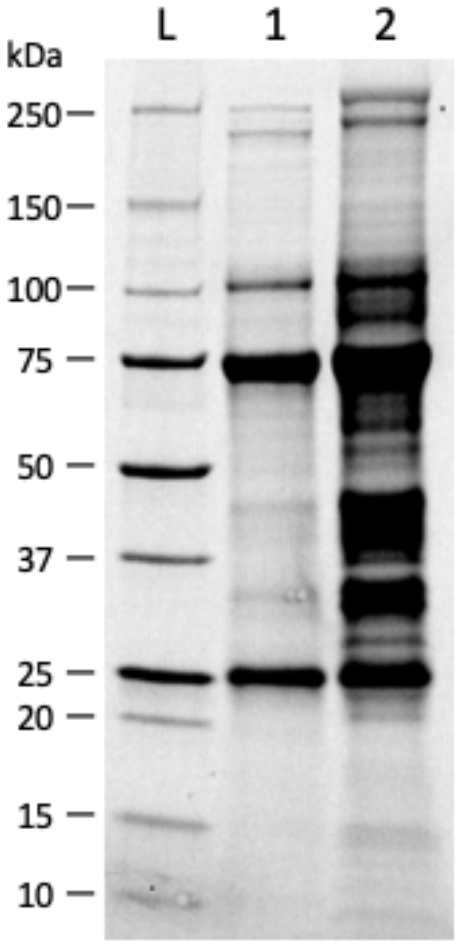

Figure 3.1 Purified IgM from sablefish plasma analyzed by SDS-PAGE. Lane L, protein standard ladder; Lane 1, purified sablefish IgM; Lane 2, raw sablefish plasma. Banding at 75 and 25 kDa represent the heavy and light chains of sablefish IgM respectively.

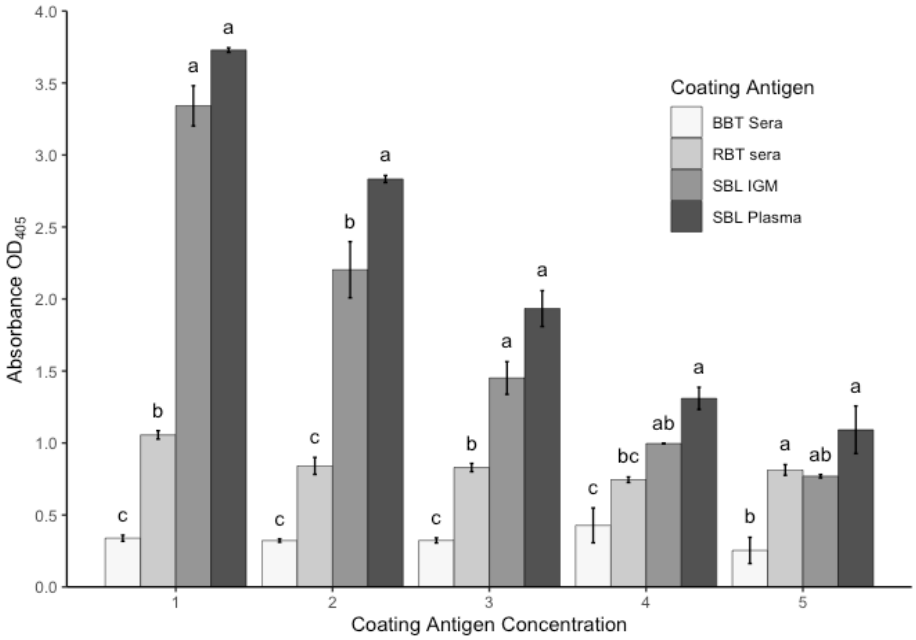

Figure 3.2 Screening serum from mice immunized with sablefish IgM for specificity to various coating antigens (BBT = burbot; RBT = rainbow trout; SBL = sablefish) at different concentrations (From 1 to 5: 0.625, 0.312, 0.156, 0.078, and 0.039 μg mL^{-1} for sera and plasma; 0.125, 0.062, 0.031, 0.015, 0.007 μg mL^{-1} for purified sablefish IgM). X-axis indicates coating antigen concentrations. Y-axis indicates absorbance values at OD_{405}. Values are mean of duplicates ± SEM. Different letters above bars indicate statistically significant differences among groups at specific antigen concentrations ($p < 0.05$).

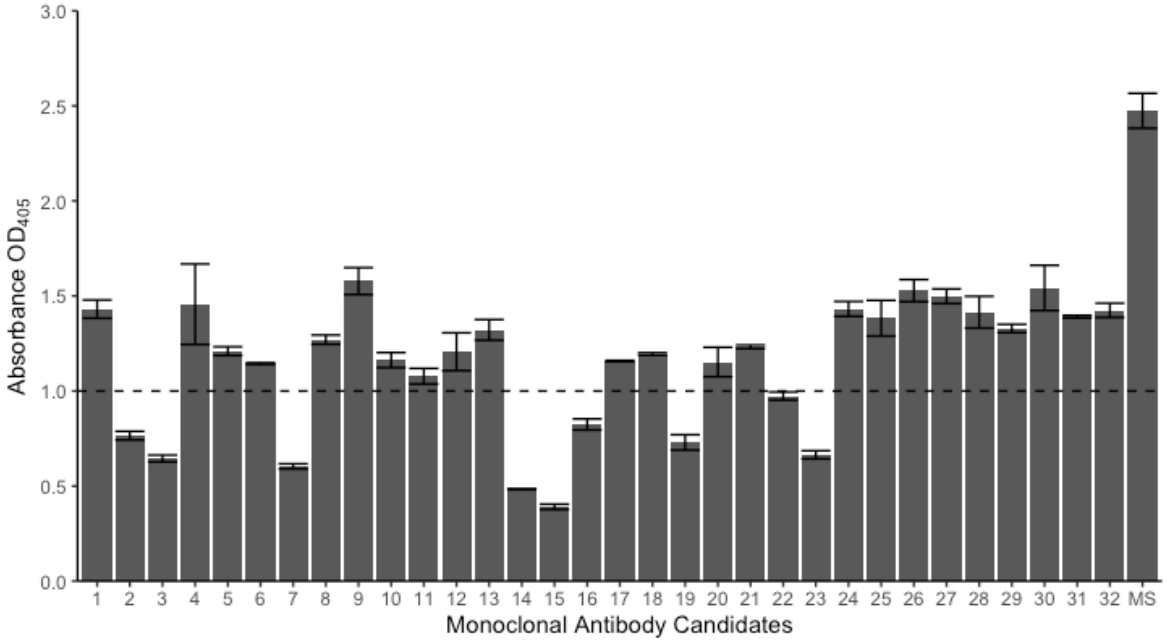

Figure 3.3 Screening of mAb candidates produced by 32 different hybridoma colonies recognizing purified sablefish IgM. MS, mouse serum positive control. Dashed line at OD_{405} of 1.0 was used as a threshold of high reactivity to target antigen. Values are mean of duplicates ± SEM. Plates were coated with 1 µg mL^{-1} of purified sablefish IgM.

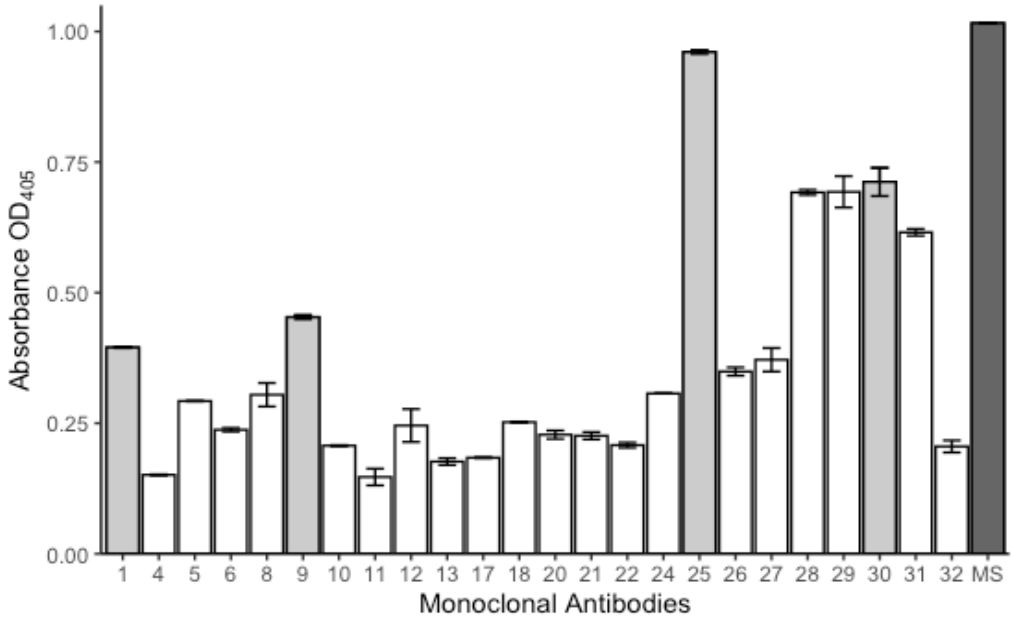

Figure 3.4 Screening of top 24 mAb candidates at a 1:50 dilution with wells coated in 0.0625 µg mL^{-1} of purified sablefish IgM. MS (dark gray), mouse serum positive control. Top four candidates, bars in light gray (mAbs 1, 9, 25, 30), were chosen for further analysis. Values are mean of duplicates ± SEM.

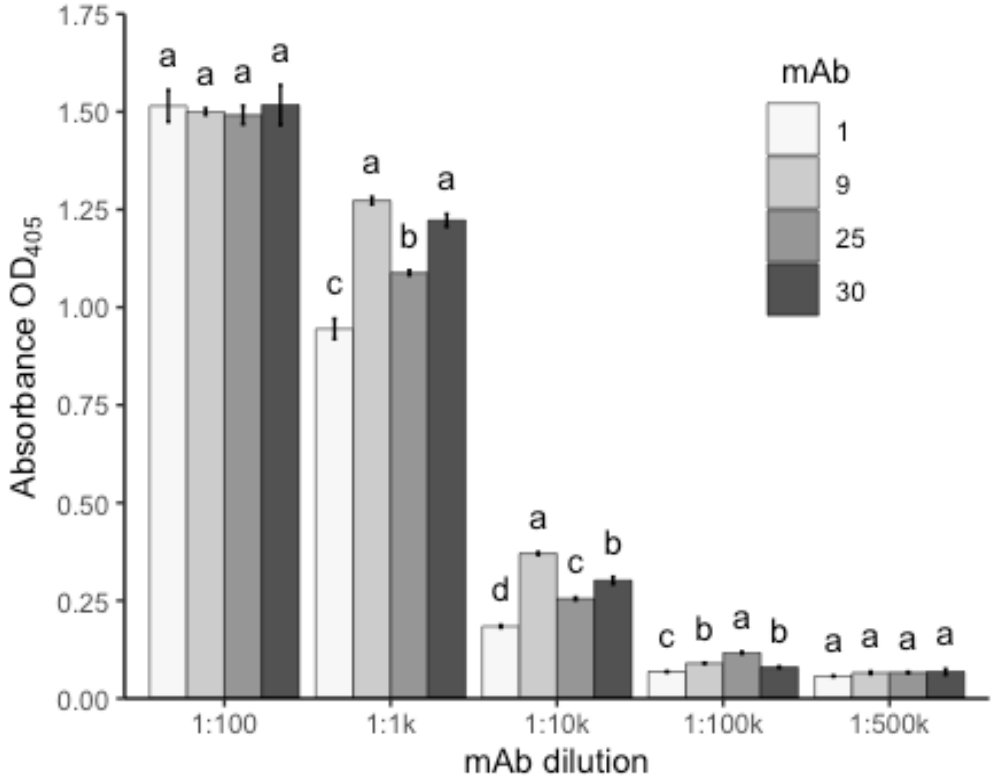

Figure 3.5 Screening of the top four mAb candidates at different serial dilutions. X-axis indicates mAb dilution in KPBS-T. Y-axis indicates average absorbance value at 405 nm. Values are mean of 3 replicates ± SEM. Letters above bars indicate statistical significance among groups at specific dilutions ($p < 0.05$).

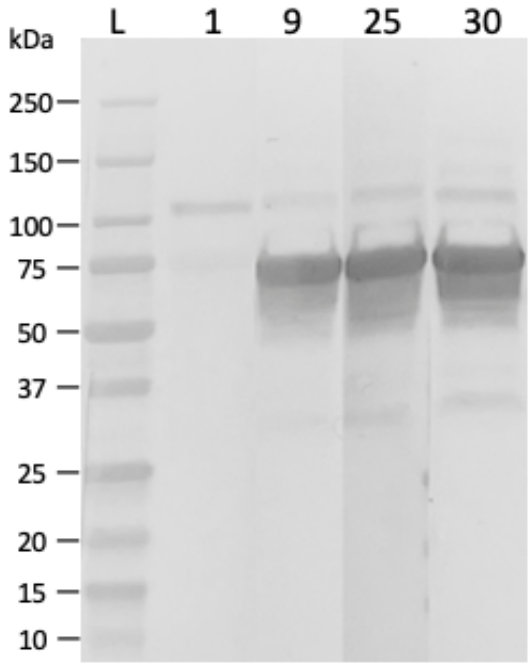

Figure 3.6 Western blot analysis of mAb candidates and their binding specificity to proteins in sablefish plasma. An SDS-PAGE was performed with sablefish plasma, then probed with individual mAb candidates (1, 9, 25, 30) to determine their region of reactivity to sablefish plasma. Bands at 75 kDA represent binding to the heavy chain of sablefish IgM. Numbers above lanes denote mAb candidate used for immunostaining. All mAb candidates were specific to the heavy chain of sablefish IgM.

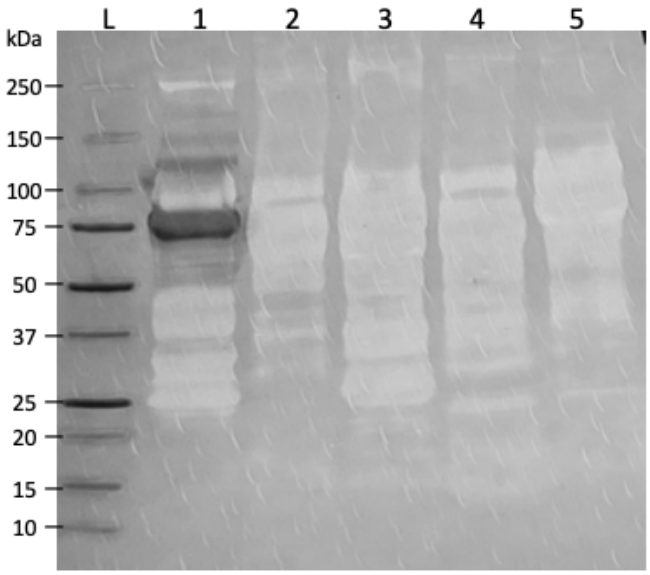

Figure 3.7 Western blot analysis of raw fish plasma or serum using UI-25A. An SDS-PAGE was performed on several different fish species serum: Lane L, protein standard ladder; Lane 1, sablefish plasma; Lane 2, coho salmon serum; Lane 3, Atlantic salmon serum; Lane 4, rainbow trout serum; Lane 5, burbot serum. White shadows represent proteins in fish plasma or serum that were transferred to nitrocellulose paper, dark bands represent specific binding of UI-25A. This demonstrates the specificity of UI-25A to the heavy chain of sablefish IgM (~75 kDa) and lack of cross reactivity to proteins found in blood circulation of other fish species.

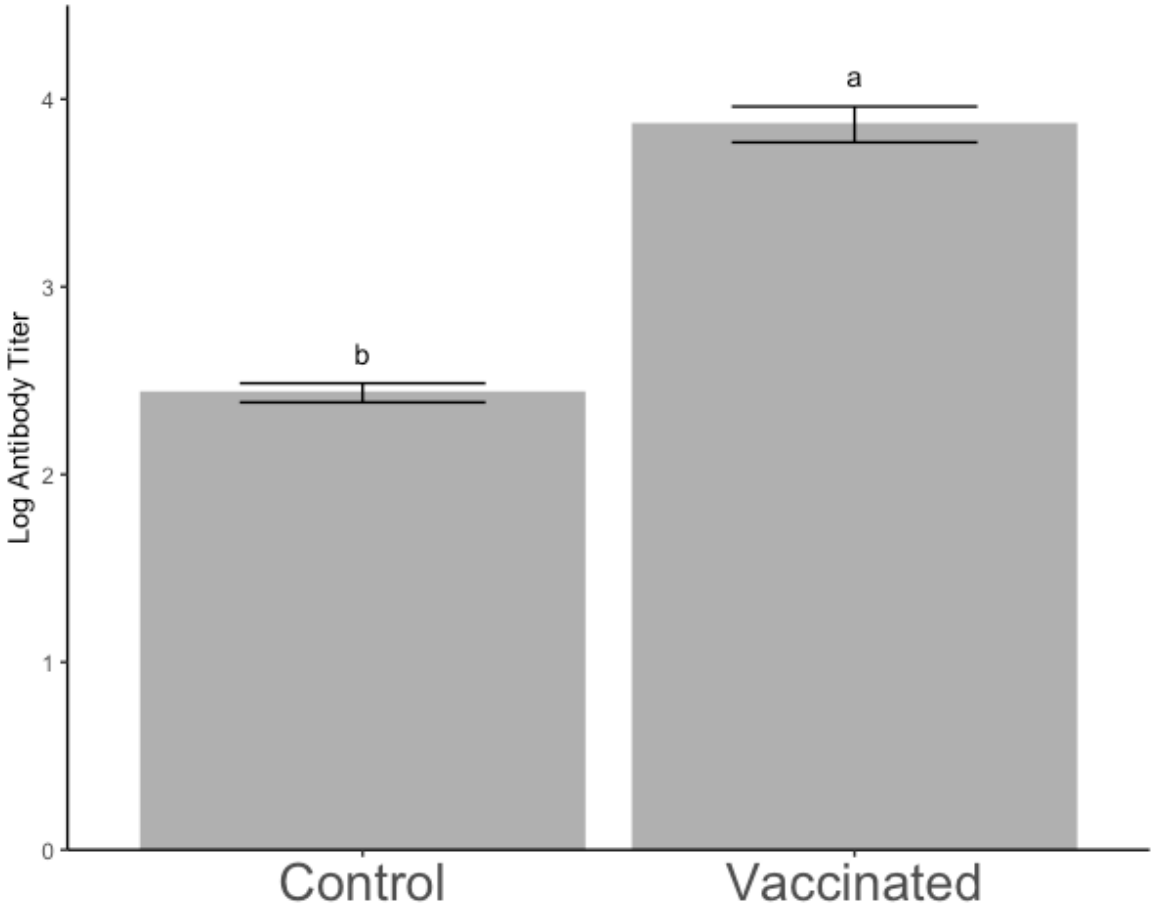

Figure 3.8 *A. salmonicida* specific IgM levels, expressed as log antibody titers, detected using ELISA in vaccinated and naïve sablefish plasma from a previous experiment. Letters above bars indicated significant difference between groups ($p < 0.05$).

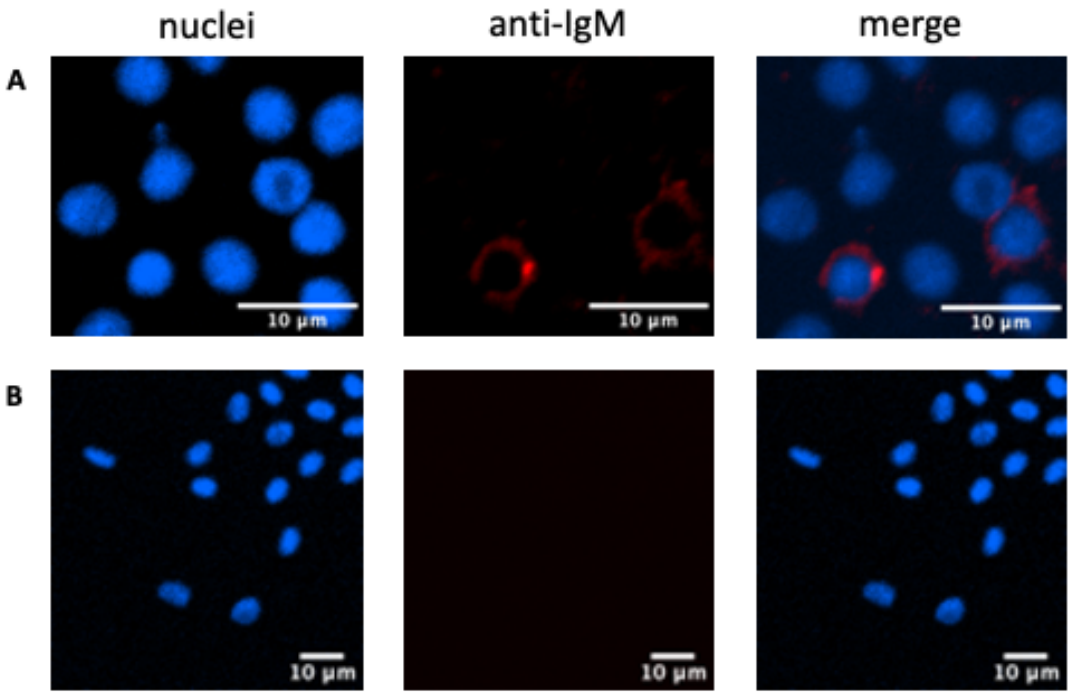

Figure 3.9 Immunofluorescence staining of head kidney imprints from sablefish and burbot. A, immunofluorescence-stained sablefish with UI-25A mAb; B, immunofluorescence-stained burbot with UI-25A mAb. Red color denotes UI-25A visualization and binding to sablefish IgM. Cells surrounded in red are likely B cells expressing IgM on their surface. Cells without red are other tissue cells with no membrane bound IgM.

3.8 Supplementary Material

Supplemental Table 3.1 Absorbance values (± SEM) of mAb candidates with different coating antigens

	Coating antigen		
mAb candidate	**Burbot serum (10 µg/mL)**	**Rainbow trout serum (10 µg/mL)**	**Purified IgM (1 µg/mL)**
1	0.082 ± 0.014	0.067 ± 0.002	1.587 ± 0.019
4	0.0635 ± 0.002	0.059 ± 0.0	1.1065 ± 0.006
5	0.061 ± 0.005	0.06 ± 0.0	1.559 ± 0.034
6	0.0625 ± 0.003	0.0665 ± 0.010	1.4605 ± 0.054
8	0.067 ± 0.003	0.0905 ± 0.016	1.318 ± 0.108
9	0.0685 ± 0.006	0.0725 ± 0.006	1.5425 ± 0.040
10	0.0595 ± 0.001	0.073 ± 0.0	1.4785 ± 0.009
11	0.0755 ± 0.009	0.074 ± 0.004	1.3495 ± 0.026
12	0.0865 ± 0.015	0.0865 ± 0.021	1.399 ± 0.003
13	0.0725 ± 0.006	0.0675 ± 0.001	1.4235 ± 0.009
17	0.0725 ± 0.011	0.0855 ± 0.024	1.268 ± 0.018
18	0.0575 ± 0.004	0.07 ± 0.006	1.322 ± 0.029
20	0.0795 ± 0.007	0.077 ± 0.001	1.367 ± 0.008
21	0.0725 ± 0.012	0.076 ± 0.011	1.315 ± 0.025
22	0.06 ± 0.002	0.1025 ± 0.007	1.309 ± 0.080
24	0.077 ± 0.015	0.092 ± 0.003	1.4335 ± 0.010
25	0.0925 ± 0.013	0.0745 ± 0.009	1.593 ± 0.025
26	0.081 ± 0.002	0.0605 ± 0.002	1.3985 ± 0.004
27	0.0705 ± 0.002	0.066 ± 0.004	1.447 ± 0.004
28	0.0965 ± 0.022	0.0785 ± 0.006	1.376 ± 0.007
29	0.079 ± 0.011	0.0655 ± 0.001	1.5195 ± 0.041
30	0.073 ± 0.0	0.086 ± 0.012	1.4685 ± 0.001
31	0.0775 ± 0.010	0.073 ± 0.008	1.463 ± 0.007
32	0.075 ± 0.013	0.0765 ± 0.001	1.377 ± 0.003

Chapter 4: Conclusion

4.1 General Discussion

The experiments described in this project were undertaken to improve the understanding of oral vaccinations in an established aquaculture species and to develop a new tool for study of the immune system in an emerging marine aquaculture species. The continued growth of aquaculture demands this research type of research in order to manage losses associated with disease. In fact, some research shows that increasing growth and intensification of aquaculture can provide an environment that quickly selects for more virulent strains of fish pathogens [1]. This increases the urgency with which researchers have studied vaccine development for the industry; in the past 5 years there have been multiple reviews focusing on the current status and potential progress of vaccine development [2–10]. As such, this project aimed to provide valuable knowledge about the adaptive immune system of each species studied.

Mucosal vaccination has often been the subject of past research because of the potential ease of administration by immersion or oral routes compared to the injection route that often provides the best protection against disease [11]. In Chapter 2, rainbow trout (*Oncorhynchus mykiss)* were vaccinated via three different mucosal routes, immersion, oral, and anal, as well as by injection. Survival after a pathogen challenge was similar across all routes, except when an adjuvant was incorporated into the injection vaccine to simulate the current industry standard for furunculosis vaccines [12]. The highlight of this study was the use of a novel oral vaccine macro-particle; produced by encapsulating an *Aeromonas salmonicida* bacterin and amino acid filled liposomes within alginate. This is the first known instance of an alginate macro-particle used as a vaccine carrier in fish, and it provided significant protection against disease at both 4 and 13 weeks post vaccination compared to a control group injected with sterile PBS. Though not as high as the adjuvanted injection vaccine, this increase in survival provides strong evidence for the efficacy of this alginate macro-particle as an oral vaccine platform in fish. It was also observed that the orally vaccinated fish had significantly higher specific IgM levels in both the serum and skin mucus 4 weeks post vaccination compared to controls, demonstrating its ability to stimulate both the mucosal and systemic adaptive immune systems. Oral vaccines are sought after because of the reduced handling and stress of fish, the ease of mass vaccination in net pens or ponds, and the potential stimulation of the mucosal immune system [13]. The alginate macro-particle used in the present study provided all these benefits and as such has great potential as an oral vaccine carrier in aquaculture. The relative percent survival was not as high compared to injection vaccines, but oral vaccines have value as an easy way to boost

immunity for extended durations after primary vaccination with more traditional routes such as immersion or injection[14,15]. Supplementing existing injection vaccination programs in farms with immersion and oral vaccines delivered to some fish may provide a more economical route to achieving herd immunity. A portion of fish well-protected against disease by way of injection vaccines may be enough to slow transmission through the rest of lesser-protected fish vaccinated with immersion or oral routes. The relative percent survivals achieved with the oral vaccine in the current study, 40.5% and 50%, is typically not high enough for primary vaccination in most farm settings; however, it was not studied as a booster following other vaccination methods, and further improvements can be made in dosing, feeding, and formulation of the particle that may increase its efficacy. In fact, formulation for more efficient antigen release should provide a boost to immunity. The oral vaccine was delivered at doses approximately 150 times greater than the other routes, even though it provided similar protection during pathogen challenge. Even though the vaccine was present at lower levels in the gut, fish vaccinated by anal intubation demonstrated higher and more consistent anti-*A. salmonicida* antibodies in both trials. This indicates that antigen release from the particles was inefficient since previous studies in Atlantic salmon (*Salmo salar*) demonstrate a strong correlation between a furunculosis vaccine dose and specific antibody response [16]. Though a balance must be struck between high vaccine doses delivered in Chapter 2 and the potential development of oral tolerance to the vaccine antigen, which other studies have shown can lead to a decreased immune response [17,18].

An interesting observation in Chapter 2 is the resistance to disease in fish fed the plain macro-alginate particle without vaccine. At 13 weeks post vaccination, fish fed only these control particles had survival similar to all other vaccination routes and was significantly higher than a PBS control group. When challenged 4 weeks post vaccination, survival was still higher than the PBS injection control group but not significantly; survival was also lower, but not significantly so, compared to the oral, immersion, and anal immunization treatments. This was an unexcepted result and further research is required to characterize the mechanisms behind this additional protection. Rainbow trout may have developed a specific immune response to the alginate in the particle itself, and this carbohydrate specificity may have cross reacted with polysaccharide components of the *A. salmonicida* isolate. However, if the cross reactivity of antigens between polysaccharides in the bacteria and alginate were similar, elevated *A. salmonicida* specific antibodies would have likely been observed in the control particle group. Alginic acid has been studied as an immunostimulant in several fish species including rainbow trout, with evidence that suggests its ability to stimulate the innate immune system [19,20], as well as one study that found increased lymphocyte levels in the blood [21]. Another study showed

that after injection, the highest uptake of alginate nanoparticles was observed in the intestine of rainbow trout [22]. These studies combined with the results discussed in Chapter 2 demonstrate that alginic acid supplements may protect rainbow trout against disease, and that oral delivery may be the most efficient route of administration. The widespread availability and relatively low cost of alginic acid makes this an attractive strategy to improve fish welfare in aquaculture. Further research with the empty alginate macro-particles used in the present study should focus on changes in the immune response, both innate and adaptive. To ensure the alginate macro-particle is safe and does not affect nutrient absorption and growth rates, changes in intestinal morphology or function should be studied by determining differences in cellular makeup through immunohistochemistry. Changes in enzymatic activity and pH of the distinct intestinal segments would also provide valuable information on the effects of the alginate macro-particle.

In order to characterize immune changes in response to vaccines, tools capable to identifying specific components of the adaptive immune system are required. The goal in Chapter 3 was to develop such a tool for an emerging marine aquaculture species, sablefish (*Anoplopoma fimbria)*. The same alginate macro-particle discussed in Chapter 2 was also being tested in parallel studies with sablefish by OSU and NOAA, though because sablefish is a relatively new species to intensive aquaculture [23], the ability to characterize specific immunity in response to vaccination was not capable. Previous studies into sablefish susceptibility to pathogens and vaccination techniques relied on relative survival after vaccination and quantification of total circulating immunoglobulins, and not targeting pathogen specific immune parameters [24–26].

As such, a monoclonal antibody (mAb) specific to sablefish Immunoglobulin M (IgM) was made in a mouse model and tested in several applications. Sablefish IgM was purified through a mannan binding protein matrix, the heavy and light chain fragments were found to be similar in size to those purified from other teleost species [27–30]. After screening numerous potential mAb candidates, the selected mAb, dubbed UI-25A, is specific to the heavy chain of sablefish IgM and does not cross react with antigens in the serum of other fish species or bacterial antigens from *A. salmonicida* or *Flavobacterium psychrophilum*. Specificity to the heavy chain is ideal because it is more conserved in teleost IgM compared to light chain fragments that may share chains across different immunoglobulin classes [31]. Using UI-25A, an ELISA was developed in order to measure anti-*A. salmonicida* antibodies in sablefish. Samples from a previously published study by Arkoosh et al. [24] in which a commercial multivalent vaccine that included an atypical isolate of *A. salmonicida*. This atypical isolate was used as a coating antigen in an ELISA, and plasma collected from both vaccinated and

unvaccinated fish were tested. Specific IgM was significantly higher in vaccinated fish, and this correlated to significantly higher survival in a pathogen challenge reported in the previous study [24]. This is an important step for sablefish aquaculture, as it demonstrates a protective role for specific antibodies induced by vaccination. The UI-25A antibody was also used to determine antigenic regions of the *A. salmonicida* isolate in the vaccine. This was done through western blot analysis by probing proteins and carbohydrates with plasma from vaccinated sablefish. Results showed immunogenic regions of the bacteria corresponded to the A-layer proteins and O-polysaccharide-core of the lipopolysaccharide. Research has demonstrated the importance of these components for inactivated *A. salmonicida* vaccines [32–34], and this agrees with work shown in the current project with sablefish. The ability of the UI-25A mAb to bind to membrane bound IgM was also demonstrated through immunofluorescent microscopy in Chapter 3. The validation of its use in this application opens many paths of future research specifically characterizing the presence, function, and response of plasma cells in sablefish in response to pathogen infection or vaccination. Recent studies in more established aquaculture species have characterized the distribution of IgM+ cells and their phagocytic capabilities [35,36], with UI-25A this is now possible to determine in sablefish.

In summary, this thesis describes the evaluation of a novel oral vaccine particle in an established aquaculture species and characterizes a new tool to measure antibody responses in an emerging marine aquaculture species. These findings are impactful for the aquaculture industry because of the risk posed by diseases and the continued expansion of culture operations into new species such as sablefish. This work provides a strong foundation for the continued research of oral vaccines against *A. salmonicida* as well as the potential benefits of a non-specific alginate macro-particle as an immunostimulant capable of preventing disease loss. Groundwork has also been laid for future research of the adaptive immune system in sablefish, and the UI-25A mAb provides scientists with an important tool to assist with the development of vaccines for the industry.

4.2 References

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