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Insights into the Pathobiology of *Edwardsiella piscicida* in Poikilotherm and Mammalian Hosts

By

KIM LI JACOBSEN

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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Integrative Pathobiology

in the

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DAVIS

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DEDICATION

I dedicate this work to my family.

To my mother, who is the strongest and kindest person I know and who has always been my role model for the kind of woman I hope to become. I attribute my own analytical mind, work-ethic, and dedication to my mother who has been always gently pushed me towards striving for my best and pursuing my goals. You have overcome more adversity in your life than most people can imagine and yet you still approach everything and everyone with humbleness, compassion, and warmth, a quality which I hope to follow as I move forward through my life.

To my father, who is one of the most intelligent and inquisitive people I know. It is to my father that I attribute my own passion for learning and love of animals. You have taught me to approach life with a curious mind, an appreciation for the world around me, and a positive and humorous attitude. Every day I strive to emulate the same joy and genuineness that you have always modeled.

To my sister, Emmi, who is one of the greatest inspirations in my life. Like many sisters we may have clashed growing up, but I truly cherish having watched you grow into the amazing, intelligent, kind, funny, and strong woman you are today. I have watched you overcome your own hardships and find success and love in your life. I am deeply proud of all that you have achieved and am truly fortunate that I get to call you my sister.

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I love you all and I hope that this work makes you proud.

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PREFACE

Chapters 2 through 5 of this dissertation are works in various stages of publication. All chapters have been modified from their original formats to fulfill the formatting requirements.

Chapter 2 is a full reprint of the published work: Jacobsen, K.L., Griffin, M., Phinney, B.S., Salemi, M., Yazdi, Z., Balami, S., Older, C.E., Soto, E. (2025). Temperature-dependent alterations in the proteome of the emergent fish pathogen *Edwardsiella piscicida*. *Journal of Fish Diseases*. J Fish Dis. 48(9):e14017. doi: 10.1111/jfd.14017. Epub 2024 Sep 20. PMID: 39304982; PMCID: PMC11922789.

Chapter 3 is a full reprint of the published work: Jacobsen, K.L., Griffin, M.J., Phinney, B.S., Salemi, M., Yazdi, Z., Balami, S., Older, C.E., Ware, C.C., Yamamoto, F.Y., Huda, N.U., Rodriguez-Ramos, T., Dixon, B., Ma, J., LaFrentz, B.R., Soto, E. (2026). Use of shotgun immunoproteomics for the development of protein vaccines against *Edwardsiella piscicida*. *Fish and Shellfish Immunology*. 168:110992. doi: 10.1016/j.fsi.2025.110992. Epub 2025 Nov 8. PMID: 41213346.

Chapter 4 is a full reprint of the work in review for publication: Jacobsen, K.L., Henderson, E., Griffin, M., Welch, T.J., Soto, E. Development of an *Edwardsiella piscicida* immersion challenge model in salmonids.

Chapter 5 is a full reprint of the work in review for publication: Jacobsen, K.L., Henderson, E., Reed, M.L., Griffin, M., Soto, E. Virulence and pathology of fish pathogens *Edwardsiella piscicida* and *Edwardsiella tarda* in a murine model of mammalian infection.

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ABSTRACT

Edwardsiella piscicida, one of the etiologic agents of piscine edwardsiellosis, is an emerging pathogen in global aquaculture that has been associated with disease outbreaks and significant losses among many economically important food and sport fish species in the United States, such as ictalurid catfish (*Ictalurus* spp.), largemouth bass (*Micropterus nigricans*), and rainbow trout (*Oncorhynchus mykiss*). With increasing concern for antimicrobial resistance, this presents an urgent industry-wide need for effective disease management strategies, such as vaccines. Not only does *E. piscicida* pose a threat to the health of wild and captive fish populations, but its emergence among popular food and recreational fish species carries an unknown public health risk. Additionally, as a disease primarily of warm-water conditions, with outbreaks in temperate and cold-water species occurring primarily with temperatures $>20^{\circ}\text{C}$, changing climate patterns and rising global water temperatures could contribute to continued emergence of *E. piscicida*, however the relationship between temperature and *E. piscicida* has not been completely explored. Much of our limited understanding on the physiology and pathogenesis of *E. piscicida* stems from its phenotypic and genetic similarities to *E. tarda*, leading to historical erroneous classification of many *E. piscicida* isolates as *E. tarda*. The work presented in this dissertation aims to elucidate the impact of temperature, genetic diversity, and host species on the virulence of *E. piscicida* as well as investigate potential vaccine candidates for aquaculture against *E. piscicida* and evaluate the pathogenicity of *E. piscicida* to a mammalian model in order to assess its potential foodborne/waterborne human disease risk. Analysis of the proteome of genetically diverse isolates of *E. piscicida* from different phylogenetic clades revealed significant temperature-associated, but not clade-associated, differences in virulence factor profiles. Namely, that at mammalian physiologic temperatures (37°C) *E. piscicida* demonstrated downregulation of critical virulence factors, such as Type VI secretion system and flagellar proteins, compared to bacteria grown at warmwater (27°C) and coolwater (17°C) conditions. This work also established a novel methodology for utilizing shotgun immunoproteomics to screen fish-pathogenic bacteria for immunogenic antigens in order to streamline the identification of protein vaccine candidates. Additionally, an effective and reproducible *E. piscicida* immersion challenge model for fingerling ($<5\text{g}$)

salmonids was established. This provides a necessary model for investigating natural infection dynamics and pathogenesis of *E. piscicida* and it lays the foundation for future research into mucosal vaccines against *E. piscicida* for salmonid aquaculture. Finally, *in-vitro* virulence assays and *in-vivo* mouse challenge experiments revealed a significant reduction in the pathogenicity of *E. piscicida* at mammalian temperatures and to mammalian models when compared to known zoonotic pathogen *E. tarda*. However, the observation of clinical, gross, and histologic lesions associated with *E. piscicida* infection as well as recovery of live bacteria and bacterial DNA from local and systemic tissue sites from a single immunocompromised (homozygous *Foxn1^{nu}*) mouse subcutaneously injected with *E. piscicida* suggests that immunodeficiency could increase the risk of mammalian infection. The work described in this dissertation serve as a foundation for future mucosal vaccine research against *E. piscicida* for salmonid aquaculture as well as present the first report of bonafide *E. piscicida* infection in a mammal, providing crucial insights into the pathogenesis of *E. piscicida* in mammals which is essential for assessment of the public health risk posed by this emerging pathogen.

CHAPTER 1: A Review of *Edwardsiella piscicida* Emergence and Prevention

1.1. North American Salmonid Aquaculture

Since 1994, worldwide production from capture fisheries has largely plateaued despite growing global populations and increasing consumer seafood demand [1]. To meet these nutrition and food security needs, aquaculture has become one of the fastest growing food production sectors worldwide, with global fish production increasing at a rate of 5.7% per year since 2000. In 2022, freshwater and marine aquaculture production in the United States and Canada were valued at \$1.7 billion USD and \$1.3 billion CAD, respectively [2,3]. One of the largest and most economically important finfish aquaculture sectors in North America is salmonid production (Figure 1.1). In Canada, salmon aquaculture accounts for 81% of the total value of national finfish production, with Atlantic salmon (*Salmo salar*) production alone valued around \$1 billion annually CAD[2]. In the United States, salmonid production is the second largest finfish industry, with an estimated annual value of over \$250 million USD [4].

The term “salmonid” refers to fish within the family *Salmonidae*, a group of ray-finned teleosts that includes over 200 species of salmon, trout, whitefish, and char [5]. Within the family *Salmonidae*, the subfamily *Salmoninae* encompasses seven genera of economically and ecologically important fish: *Brachymystax* (lenok), *Hucho* (taimen), *Parahucho* (stringfish), *Salvelinus* (char), *Salvelinus* (long-finned char), *Oncorhynchus* (Pacific salmon and trout), and *Salmo* (Atlantic salmon and char) [6]. Salmonids are primarily predatory cold-water fishes native to the northern hemisphere where they often play crucial ecological roles as keystone species [5]. In North America, the most economically important salmonids fall within the genera *Oncorhynchus* and *Salmo* which are widely cultured in the United States and Canada for food consumption, recreational fishery stocking, and native species restoration [2,7]. These are migratory salmonids, with spawning occurring in freshwater streams, lakes, or rivers. Following initial development, Pacific and Atlantic salmon will migrate to larger bodies of water, either marine or freshwater depending on species and ecotype, for continued growth and maturation [5]. Famously, after reaching

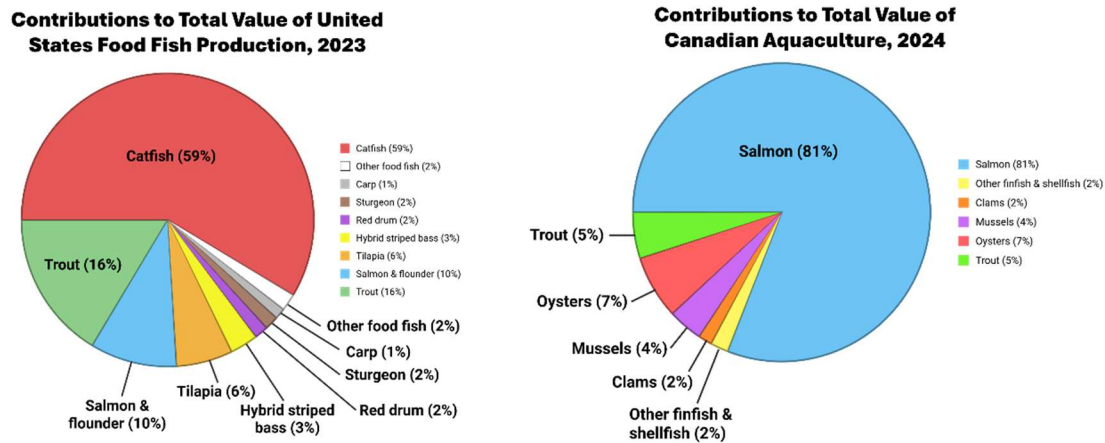
reproductive maturity, these fish will return to their original spawning sites to themselves spawn and reproduce [5].

The aquaculture industry in Canada is dominated by finfish production, which accounts for over 90% of the total value of Canadian aquaculture. Salmon production is largely conducted using open net-cages suspended in marine waters along the East and West coasts. On the West coast of Canada, British Columbia stands as the aquaculture producing region, accounting for approximately 44% of total value in 2023. New Brunswick, on the East coast of Canada, is the second largest aquaculture producer in Canada, contributing an estimated 23% of total value [2]. Canada is the fourth largest salmon producer worldwide, with Atlantic salmon contributing over 80% of the industry's total value, followed by coho salmon (*Oncorhynchus kisutch*) and chinook salmon (*Oncorhynchus tshawytscha*), which account for about 10% of total value [7,8].

Trout production in Canada is valued at over \$30 million annually and is split between freshwater cage culture production and land-based raceways primarily located in the inland waters of Ontario [8]. The majority of trout produced in Canada are rainbow trout (*Oncorhynchus mykiss*) farmed for human consumption, with a smaller proportion allocated to recreational fisheries. In contrast, brook trout (*Salvelinus fontinalis*) farming is largely limited to central Canada and intended primarily for enhancement and recreational fisheries (~65% of production), with the remainder allocated for human consumption [8].

In the United States, salmonid production is second to farm-raised catfish, which accounts for over 80% of total freshwater finfish production [7]. In the United States, rainbow trout are the second largest freshwater finfish industry, generating over \$134 million annually while Atlantic salmon lead the marine finfish aquaculture sector with an estimated value of \$115 million [4]. The majority of U.S. Atlantic salmon production is conducted along the coast of Maine using similar cage-culture methods as the Canadian industry.

Figure 1.1. Contribution of salmonid production to total value of North American aquaculture. **(A)** Species contributions to total value of food fish aquaculture production in the United States in 2023 by fish species. Adapted from 2023 USDA Census of Aquaculture [4]. **(B)** Species contributions to total value of Canadian aquaculture in 2024. Adapted from Statistics Canada report on aquaculture production quantities and value, 2024 [2].



Rainbow trout and steelhead (*Oncorhynchus mykiss*) production starts in centralized hatcheries which produce juveniles for transfer either to inland systems, such as raceways or tanks, or to floating cages systems for continued growth [7]. Both species are predominantly raised for the commercial food fish industry, with a smaller number of steelhead and rainbow trout allocated for recreational fisheries. Along the West Coast of the United States, several other Pacific salmon species are also cultured for enhancement of recreational fisheries and restoration of native populations: chinook salmon, chum salmon (*Oncorhynchus keta*), coho salmon, pink salmon (*Oncorhynchus gorbuscha*), and sockeye salmon (*Oncorhynchus nerka*) [9].

One of the most significant threats to North American salmonid aquaculture is infectious disease. Disease outbreaks on North American aquaculture facilities have resulted in annual losses exceeding \$10 million USD [7]. The higher rearing densities of farmed fish increases direct contact between fish, facilitating pathogen spread within a facility. Additionally, intensive farming practices increase stress on the fish, leading to elevated susceptibility to infection, particularly by opportunistic pathogens.

The majority (>54%) of disease outbreaks among farmed fish populations are attributed to bacterial pathogens, which can have devastating economic consequences for producers due to fish losses and the high cost of treatment [10]. For example, outbreaks of Bacterial Kidney Disease, caused by *Renibacterium salmoninarum*, among cage-farmed salmon can cause up to 40% or 80% mortality in Atlantic and Pacific salmon, respectively, with daily mortalities reaching up to 5% [11]. Additionally, outbreaks of bacterial pathogens among cultured fish populations can cause massive financial losses for farmers not only through fish die-offs, but also through significant morbidity impacting the market value of fish. Epizootics of winter ulcer disease, caused by the bacterium *Moritella viscosa*, typically have a mortality rate of less than 10% in farmed salmonids. The skin lesions and muscular degeneration caused by the bacterium, however, can severely degrade the fish's fillet quality and markedly reduce their economic value [12]. Bacterial pathogens can also pose not only a fish health and economic threat, but also a public health risk. Some fish-pathogenic bacteria are zoonotic and have been reported as the cause of significant disease in people. For example, in 2015, a large outbreak of Group B *Streptococcus agalactiae* in Singapore which affected over 160 people was associated with the consumption of raw farmed Asian bighead carp (*Hypophthalmichthys nobilis*) and snakehead fish (*Channa* species) [13]. This highlights the significant need to investigate the pathobiology of the bacterial pathogens impacting salmonid aquaculture in order to understand their disease pathogenesis, develop effective management strategies, and assess their potential foodborne infection risk. *Edwardsiella piscicida* is one such bacterial pathogen of concern, with emergence of this bacterium having been associated with significant outbreaks among various economically important food and sportfish species.

1.2. Taxonomy of the *Edwardsiella* Genus

In the early 1960s, two separate laboratories reported identification of Gram-negative bacteria that differed biochemically from other described *Enterobacteriaceae*. One of these bacterial groups, the “Asakusa Group”, was initially published in 1962 and consisted of 248 isolates cultured from snake feces, 2 isolates collected from seal mortalities, and 5 isolates originating from human patients with acute

gastroenteritis that shared an unusual mannitol negative, hydrogen sulfide positive biotype [14–16]. The second group, the “Bartholomew Group”, refers to isolates reported initially in 1964 that were collected from a human patient with enteric fever and acute gastroenteritis [17]. The shared unique phenotypic characteristics of the “Asakusa” and “Bartholomew” isolates led to the formal description of these bacteria under a new genus of *Enterobacteriaceae*, *Edwardsiella*, represented by the type species *Edwardsiella tarda* [18]. This new genus consisted of Gram-negative, oxidase negative, catalase positive, flagellated bacteria capable of fermenting glucose, reducing nitrate to nitrite, and producing hydrogen sulfide. Over the next two decades two more species were added to the genus *Edwardsiella*: *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC) [19], and *Edwardsiella hoshinae*, a group of malonate positive bacteria isolated from water and from avian and reptilian hosts [20].

For the next three decades, only two species of *Edwardsiella*, *E. tarda* and *E. ictaluri*, were believed to be pathogenic to fish. However, comparative phylogenomic studies and multi-locus sequence analyses performed in the 2010s revealed three genetically distinct polyphyletic groups among the isolates formerly designated as “*E. tarda*” [21,22]. This led to reorganization of the genus, with isolates formerly classified *E. tarda* segregated into three distinct species: true or *bona fide* *E. tarda*, which can be isolated from a wide range of taxa including fish, birds, reptiles, and mammals, including humans; *Edwardsiella piscicida*, which consisted of isolates previously characterized as typical motile fish-pathogenic *E. tarda*; and *Edwardsiella anguillarum*, which was consistent with isolates previously defined as atypical nonmotile fish-pathogenic *E. tarda* [23,24].

1.3. Pathogenesis of Edwardsiellosis Caused by *Edwardsiella piscicida*

Edwardsiella piscicida is a Gram-negative, facultative anaerobic, facultative intracellular bacterium within the family Hafniaceae that has been identified as a significant pathogen of both wild and farmed fish populations worldwide. Infections with *E. piscicida* typically cause generalized acute

septicemia, with affected fish presenting with coelomic distension, petechiae and ecchymosis, erratic swimming, decreased appetite, skin erosion and ulceration, reddened vent, and exophthalmia [24,25]. Internally, affected fish can demonstrate clear to red serosanguinous ascites, hemorrhage of various visceral organs, splenomegaly, and white nodules within different organs [25–27]. Histologically, acute *E. piscicida* infections are characterized by necrosis and granulomatous inflammation with abundant intralesional extracellular and intralesional gram-negative bacterial rods of multiple organs, predominantly the spleen, liver, and hematopoietic portions of the kidneys [25,26]. Occasionally, bacterial emboli can be observed throughout the vasculature of affected animals [27]. In animals that survive acute infection, the lesions can progress to chronic granulomatous inflammation with discrete granulomas scattered throughout the coelom and visceral organs [27]. Clinical signs of *E. piscicida* infection are non-specific and can be easily mistaken for other opportunistic fish pathogens, such as *Aeromonas hydrophila* or *Vibrio anguillarum*. Moreover, *E. tarda* is phenotypically indistinguishable from *E. piscicida*. As a result, definitive diagnosis of *E. piscicida* relies on the use of molecular assays such as species-specific real-time quantitative polymerase chain reaction (qPCR) or matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) [27,28].

Central to the pathogenicity of *E. piscicida* are the various virulence factors the pathogen utilizes to infect hosts, defend against immune defenses, and replicate. Two of the currently recognized “essential” *E. piscicida* virulence factors are the type III (T3SS) and type VI secretion systems (T6SS), which play pivotal roles in bacterial entry in and modulation of host cells. These bacterial secretion systems are contact-dependent needle-like nanomachines comprised of multiple structural proteins which are used by many Gram-negative bacteria to inject effector proteins directly into the eukaryotic host cell cytoplasm [29,30]. Type III secretion system effector proteins act to promote invasion and create a favorable intracellular environment for bacterial survival and replication by modulating host cell functions. This includes inhibiting host cell apoptosis, destabilizing host cell membrane microtubules to promote bacterial internalization, and suppressing mitogen-activated protein kinase-dependent innate immune pathways [31–36]. Further, the

T6SS is crucial for *E. piscicida*-macrophage interactions and acts to promote bacterial colonization through facilitating survival and replication under the high acid and oxidative stress environment of macrophages and through downregulation of innate immune defense mechanisms such as the Nod-like receptor P3 inflammasome [37–39]. Many of these effector proteins, such as the T3SS effector protein EseJ or the T6SS effector protein EvpP, have been demonstrated to be essential for *E. piscicida* pathogenicity, with mutant knock-out strains displaying markedly attenuated *in-vitro* and *in-vivo* virulence [31,34,36,39]. Expression of the T3SS and T6SS in *E. piscicida* is regulated by the EsrA-EsrB two-component system, a global regulator which perceives various environmental stimuli, such as temperature and oxidative stress, to modulate stress adaptation and virulence [40–42]. Other important virulence factors for *E. piscicida* pathogenicity include type 1 fimbrial proteins, which facilitate bacterial adhesion to and invasion into host cells; the thioredoxin system, which contributes to oxidative stress resistance; hemolysin EthA, which plays an essential role in host cell invasion; serum-induced metalloprotease Sip, which is crucial for serum resistance; and the ferric uptake regulator Fur, which controls iron acquisition [43–47].

1.4. Emergence of *Edwardsiella piscicida*

Edwardsiella piscicida is a major aquaculture pathogen, having been isolated from over 30 freshwater and marine fish species worldwide (Table 1.1). Edwardsiellosis caused by this pathogen is often considered an emerging infectious disease due to increasing numbers of reports and expanding host and geographic range, although it is difficult to determine whether this represents a truly emergent pathogen or whether it is simply reflective of improved diagnostic methods and the ability to discriminate *E. piscicida* from *E. tarda*.

Edwardsiellosis in fish is typically considered a warm-water disease, occurring primarily at temperatures >30°C [24], however, there have been increasing reports of *E. piscicida* infections among temperate, cool-, and cold-water fish species. Outbreaks of *E. piscicida* have been reported in several

important temperate-water sportfish species across the United States, such as largemouth bass (*Micropterus salmoides*) [27,48] and black crappie (*Pomoxis nigromaculatus*) [49]. Additionally, outbreaks of *E. piscicida* have been reported in North American salmonid aquaculture, particularly when water temperatures rise above 15°C [26,50]. In Finland, several epizootics of *E. piscicida* causing mass mortality among farmed whitefish (*Coregonus lavaretus*) were observed in association with seasonal increases in water temperature (20–23 °C) [51]. Similarly, seasonal outbreaks of *E. piscicida* have been reported in wild European eels in Spain when water temperatures rise above 20 °C [52]. Across Asia, *E. piscicida* has been isolated from diseased temperate and cold-water fish species including olive flounder (*Paralichthys olivaceus*) [53,54], spotted sea bass (*Lateolabrax maculatus*) [55], and turbot (*Scophthalmus maximus*) [56]. In fact, a survey of diseased turbot in China found that between October 2016 and December 2019, the greatest bacterial threat to turbot in the major farming areas of China was *E. piscicida* (40% of cases) [56]. Moreover, *E. piscicida* is frequently isolated from diseased channel and hybrid catfish submitted to the Aquatic Research and Diagnostic Laboratory in Stoneville, MS, during the early spring (April–May) and fall (October–November), when pond temperatures typically range from 15–25 °C (M. J. Griffin, unpublished data). These reports all suggest a wider thermal and susceptible host range for *E. piscicida* virulence than previously considered. Additionally, outbreaks of *E. piscicida* in association with seasonal rises in water temperature indicate that continued rising global water temperatures may lead to increased outbreaks of *E. piscicida*, whether by host immune suppression or by altering bacterial virulence and host-pathogen interactions [24,57].

Edwardsiella piscicida has also emerged as a major pathogen among U.S. ictalurid catfish aquaculture. Increased outbreaks of *E. piscicida* in the southeastern U.S. have been associated with the increased industry use of channel (*Ictalurus punctatus*) ♀ × blue (*Ictalurus furcatus*) ♂ catfish hybrids (hybrid catfish) [25]. Historically, the U.S. catfish industry predominantly produced channel catfish. However, advances in aquaculture technologies in the 2010s, such as split ponds and intensively aerated ponds, have led to a massive shift in the catfish industry to favoring the production of hybrid catfish [58].

Hybrid catfish have seen increasing popularity due to faster growth, improved feed conversion, increased tolerance to high stocking densities and crowding, and reduced susceptibility to several channel catfish pathogens, most notably ESC [25,58]. While hybrid catfish are more resistant to *E. ictaluri* than channel catfish, it has been found that *E. piscicida* is significantly more pathogenic to hybrid catfish than channel catfish, suggesting the rise in hybrid catfish utilization in the U.S. catfish aquaculture industry may help to explain the concurrent increase in *E. piscicida* outbreaks in U.S. catfish aquaculture as hybrid catfish account for >95% of *E. piscicida* diagnoses [59].

Table 1.1. Reported host and geographic range of *Edwardsiella piscicida*.

Host	Geographic Location	Reference
Spotted sorubim (<i>Pseudoplatystoma corruscans</i>)	Brazil	[60]
Brook trout (<i>Salvelinus fontinalis</i>)	Canada	GenBank Accession No. GCA_032496385.1
Spotted sea bass (<i>Lateolabrax maculatus</i>)	China	[55]
Black seabream (<i>Acanthopagrus schlegelii</i>)	China	[61]
Whitefish (<i>Coregonus lavaretus</i>)	Finland	[51]
Turbot (<i>Scophthalmus maximus</i>)	France, Holland, Portugal, Spain, Southern Europe, Northern Europe, China, UK, South Korea, Netherlands	[62–66]
Sharpsnout sea bream (<i>Diplodus puntazzo</i>)	Greece	[67]
White grouper (<i>Epinephelus aeneus</i>)	Israel	[68]
Red seabream (<i>Pagrus major</i>)	Japan	[69]
Blackspot seabream (<i>Pagellus bogaraveo</i>)	Japan	[66]
Japanese eel (<i>Anguilla japonica</i>)	Japan, South Korea, Taiwan	[70–72]
European eel (<i>Anguilla anguilla</i>)	Norway, Spain	[52,73]
Serpae tetra (<i>Hyphessobrycon eques</i>)	Singapore	[74]
Korean catfish (<i>Silurus asotus</i>)	South Korea	[75]
Marbled eel (<i>Anguilla marmorata</i>)	South Korea	[76]

Olive flounder (<i>Paralichthys olivaceus</i>)	South Korea, Japan, China	[69,75,77]
Senegalese sole (<i>Solea senegalensis</i>)	Spain	[66]
Fourfinger threadfin (<i>Eleutheronema tetradactylum</i>)	Taiwan	[78]
East Asian fourfinger threadfin (<i>Eleutheronema rhadinum</i>)	Taiwan	[71]
Barramundi (<i>Lates calcarifer</i>)	USA	[79]
Channel catfish (<i>Ictalurus punctatus</i>)	USA	[80,81]
Hybrid catfish (<i>Ictalurus punctatus</i> ♀ × <i>Ictalurus furcatus</i> ♂)	USA	[82]
Blue catfish (<i>Ictalurus furcatus</i>)	USA	[81]
Hybrid striped bass (<i>Morone chrysops</i> × <i>M. saxatilis</i>)	USA	Accession No. NZ_CP116003.1
Smallmouth bass (<i>Micropterus dolomieu</i>)	USA	[62]
Blotched fantail stingray (<i>Taeniura meyeni</i>)	USA	[83]
Koi (<i>Cyprinus rubrofuscus</i>)	USA	[62]
Spotted Seatrout (<i>Cynoscion nebulosus</i>)	USA	[62]
Striped bass (<i>Morone saxatilis</i>)	USA	[66]
Black crappie (<i>Pomoxis nigromaculatus</i>)	USA	[49]
Rainbow trout (<i>Oncorhynchus mykiss</i>)	USA, Canada	[62,84]
Atlantic salmon (<i>Salmo salar</i>)	USA, Canada	[26,84]
Largemouth bass (<i>Micropterus salmoides</i>)	USA, China	[27,48,62,85,86]
Tilapia (<i>Oreochromis</i> sp.)	USA, China, Japan, Brazil	[66,83,87,88]
Japanese medaka (<i>Oryzias latipes</i>)	Experimental	[87]
Zebrafish (<i>Danio rerio</i>)	Experimental	[89,90]
Goldfish (<i>Carassius auratus</i>)	Experimental	[91]

1.5. Current Management Strategies for *Edwardsiella piscicida*

There are no commercially available prophylactic methods for the prevention of *E. piscicida*, which limits the strategies available to producers to manage piscine edwardsiellosis. Farmers largely rely on the use of medicated feeds, combined with good biosecurity practices, for the treatment of ongoing *E. piscicida* outbreaks [24]. In the United States, there are a limited number of antibiotics approved by the Federal Drug Administration (FDA) for use in aquaculture to treat of bacterial diseases such as *E. piscicida*, namely Terramycin[®] (oxytetracycline dihydrate), Aquaflor[®] or Paqflor[®] (florfenicol), and Romet-30[®] (sulfadimethoxine) [92]. Additionally, no antibiotics are licensed for the treatment of *E. piscicida* in fish, thus use of these medications is conducted extra label under a valid veterinarian-client-patient relationship (VCPR). The use of medicated feeds alone, however, is suboptimal long-term due to many factors such as the high cost of antimicrobial feed, increasing consumer demand for antibiotic-free food, concerns for run-off into natural waterways, and growing evidence of multi-drug resistance (MDR) among strains of *E. piscicida* [24,93,94]. The use of medicated feeds can also be rendered ineffective in cases where fish are too sick to consume the feed and, for marine species, these drugs are often less effective and may require higher dosing than for freshwater species due to the reduction in antibiotic bioavailability from divalent cation binding in seawater [95,96].

While *E. piscicida* isolates are typically susceptible to a broad range of antibiotics, growing evidence of developing antimicrobial resistance (AMR) among some populations of *E. piscicida* has sparked concern over dissemination of drug-resistant bacteria and AMR genes into the environment. In 2019, a conjugative plasmid encoding resistance genes for tetracyclines antibiotics, phenicol antibiotics, sulfonamide antibiotics, and aminoglycoside antibiotics was identified in a strain of *E. piscicida* isolated from a diseased hybrid catfish originating from a U.S. fish farm [97]. With the already exceedingly small number of antimicrobials available for the treatment of farmed fish, isolates with this plasmid were non-responsive to two of the three FDA approved antimicrobials for aquaculture, highlighting the emerging risk of MDR *E. piscicida*. Since then, a study of 80 *E. piscicida* isolates collected from diseased largemouth

bass in China found that 60-63% of isolates were resistant to sulfonamides and 43.75% were resistant to florfenicol [86]. *Edwardsiella piscicida* has also been shown to be capable of transmitting AMR genes to other unrelated bacteria, suggesting that environmental contamination by MDR *E. piscicida* strains poses not only an aquaculture threat, but also a public health risk [98].

As an alternative to antimicrobials, some producers have turned to autogenous vaccines to mitigate outbreaks of *E. piscicida*. Autogenous vaccines are veterinary biologics derived from pathogen isolates collected from a disease outbreak and used to immunize susceptible animals at the same site in order to limit the severity and spread of the epizootic [99]. These autogenous vaccines can be advantageous as an alternative treatment method as they are specific for the precise pathogen strain impacting a particular facility and they can be more rapidly produced than conventional vaccines [10,100]. A major limitation of autogenous vaccines is that they, like, antimicrobials, serve as a form of reactionary medicine requiring an ongoing disease outbreak and, thus, they do not prevent initial disease occurrence [100]. This has led to strong interest across the aquaculture industry for the development of effective commercial vaccines to prevent *E. piscicida*.

1.6. Fish Vaccinology

Vaccines have been used successfully in aquaculture for decades to protect against economically significant fish pathogens, improve the productivity of commercial farms, and reduce the industry-wide use of antimicrobials [101]. Demonstrated interest in effective immunoprophylaxis for fish is believed to have its origins in the 1930s, at a time when numerous vaccines against bacterial and viral diseases had already been licensed and widely used for prevention of diseases in both humans and domestic animals. The first scientific report for disease prevention in fish using vaccines is largely attributed to a paper published in Poland in 1938 by Snieszko et al. in which they reported protective immunity in carp vaccinated with killed *Aeromonas punctata* [102]. Other reports on fish vaccinology during this time period similarly focused on

bacterial diseases, including work by Duff in 1942 demonstrating protection against furunculosis in cutthroat trout (*Oncorhynchus clarkii*) immunized parenterally and orally with chloroform-killed *Aeromonas salmonicida* [103].

In the three decades after World War II, however, interest in fish vaccination diminished due to readily available sulfa drugs and antibiotics that were highly effective in treating bacterial fish diseases, leading to an era of chemotherapy [104]. Still, some essential progress in fish vaccinology was made during this period, notably the first report on vaccination of fish against a viral disease, published by Goncharov in 1951 which described effective immunization of carp after intracoelomic injection with formalin killed virus isolated from diseased fish [105]. The discipline of fish vaccinology saw a massive revival in the 1960s and 1970s, due in part to the development of antibiotic resistance in certain pathogens, increasing recognition of the importance of viral diseases in fish health, and concern for the health effects of the drugs used in aquaculture on human and animal health [101,104]. Additionally, the major losses from bacterial diseases within the growing salmonid farming industries in Norway and the Pacific Northwest US were a major economic driving force behind the resurgence in fish vaccine research. This led to the development of the first commercially available fish vaccine in 1976, a bacterin against enteric redmouth disease caused by *Yersinia ruckerii*, developed by Wildlife Vaccines, Inc. [106]. The commercial success of Norwegian trout and salmon aquaculture can be largely attributed to the use of vaccines in the 1980s, which dramatically reduced morbidity and mortality from diseases such as cold-water vibriosis and furunculosis, as well as rapidly decreased industry antibiotic usage by over 90% [107]. Nowadays, virtually all farmed rainbow trout and Atlantic salmon in Norway are vaccinated against multiple pathogens [108].

Currently, there are more than 50 licensed fish vaccines worldwide used in more than 30 fish species for protection against many economically significant bacterial and viral diseases, as well as a single commercial vaccine for the prevention of a parasitic disease (sea lice) [109,110]. Most currently available fish vaccines are inactivated vaccines comprised of a suspension of bacteria or virus that have been killed or inactivated but retain antigens that stimulate the fish's immune system to protect against the pathogen of

interest [111]. One of the major reasons for the predominance of inactivated vaccines is the relative safety in comparison to live vaccine modalities as, by their nature, killed/inactivated vaccines are unable to replicate, infect hosts, or persist in the environment [112]. However, inactivated vaccines often suffer from weaker immunogenicity and shorter-lived protective immunity, frequently requiring the use of adjuvants and/or boosters for generation of protective immunity [100]. In particular, inactivated vaccines are typically less effective against diseases caused by viruses or intracellular bacteria as they are often poor at activating cell-mediated immune responses [100,113].

The second most common fish vaccines are live attenuated vaccines (LAV), which contain one or more pathogens that have attenuated virulence in the target fish species [112]. The live microorganisms in LAVs can replicate within the host and enter host cells, more closely mimicking natural infection and stimulating stronger cell-mediated immune responses, as well as humoral immune responses, to generate a more robust, longer-lasting protective immunity without the need for boosters or adjuvants [114]. The main concern with LAVs is the risk of reversion to pathogenicity in the target host or environmental contamination and virulence in susceptible off-target species. Additionally, LAVs are typically frozen or lyophilized and require more stringent cold-chain storage and transport management than other vaccine types, which can be logistically challenging and costly in rural areas or remote regions without a stable power supply [111,115].

Modern vaccine modalities gaining traction in fish vaccinology include subunit and nucleic acid vaccines. Subunit vaccines contain non-replicative antigenic components produced synthetically or via live expression systems [116]. Notable examples of subunit vaccines in aquaculture include Centrovax, a vaccine marketed in Chile against infectious salmon anemia virus (ISAV), which uses a yeast-based expression system for generation of recombinant hemagglutinin-esterase protein [117]. Subunit vaccines have greatly improved safety relative to live vaccines, improved purity compared to other vaccine types, and the ability to be freeze-dried for enhanced shelf-stability and transport. Similar to inactivated vaccines, however,

subunit vaccines often display weaker immunogenicity than LAVs, necessitating boosters and/or adjuvants [118].

Nucleic acid vaccines are an attractive alternative to conventional vaccine technologies due to their ability to stimulate a robust immune response, their improved relative safety due to the lack of infectious agents in the production pipeline, and their ability to be rapidly scaled up in manufacturing [116]. Nucleic acid vaccines contain DNA or RNA, typically transported via a delivery vector (e.g. plasmid encoding the antigen gene of interest; RNA encapsulated in lipid nanoparticles), that are taken up by the host cells where the genetic information is used to synthesize the encoded antigen for expression and activation of the host immune system [119]. This endogenous antigen expression allows for MHC Class I and MHC Class II activation of CD4⁺ and CD8⁺ T cells, triggering a strong antigen-specific cellular immune response which also serves to further activate a humoral immune response [120]. This cell-mediated response makes nucleic acid vaccines particularly attractive for protection against pathogens, like intracellular bacteria, for which an antibody-mediated response alone is insufficient. DNA vaccines have been shown to have a high level of safety, especially compared to LAVs or traditional oil-adjuvant vaccines, as they lack living, replicative, transmittable material that could reverse to pathogenicity and have been shown to not elicit major adverse effects, systemic toxicity, or anti-DNA antibodies in human and animal experiments [121–124]. DNA vaccines are also more heat resistant than conventional vaccines, meaning they do not require extensive cold-chain infrastructure, and can be manufactured at scale in a relatively cost-effective manner, making them especially promising for aquaculture [120].

DNA vaccines have already seen limited use in aquaculture, starting with the creation of the Apex-IHN® (Elano GmbH) vaccine in 2005 for the protection of Atlantic salmon against infectious hematopoietic necrosis (IHN), which stands as only the second ever commercially available DNA vaccine in the world, preceded only by a DNA vaccine against West Nile virus licensed earlier the same year [117,125]. Since then, a second DNA vaccine, Clynav® (Elano GmbH), was licensed in 2017 for the protection of Atlantic salmon against salmon pancreas disease virus, serving as the first ever DNA vaccine

to be authorized by the European Union for commercial use in veterinary animals [117,126]. Like DNA vaccines, RNA vaccines have improved safety over LAVs due to a lack of infectious material. Additionally, *in vivo* degradation of the mRNA in these vaccines leads to a lower risk of genome integration compared to DNA vaccines [120]. RNA vaccines are also more efficient at stimulating strong antigen-specific immune responses, requiring lower doses than conventional plasmid DNA vaccines [119]. Compared to DNA vaccines, however, RNA vaccines are more expensive to manufacture, store, and transport making them potentially cost-prohibitive in lower income countries or for less economically valuable fish species [119]. Additionally, RNA vaccines require frozen storage and stringent cold-chain management to prevent degradation of the mRNA or nanoparticles, further complicating transport and storage compared to DNA vaccines [119]. Although there are currently no commercially available RNA vaccines for fish, the success of RNA vaccines in human and terrestrial animal disease prevention have led to increasing research into the application of RNA vaccine technology to aquatic species. Several recent studies have described promising mRNA vaccine candidates for the protection of fish against important viral diseases, such as viral hemorrhagic septicemia virus (VHSV) [127] and infectious hematopoietic necrosis virus (IHNV) [128], paving the way for continued study into this new field of fish vaccinology.

Vaccines in fish are administered via three delivery methods: injection, oral, or immersion. Injection, either intracoelomic or intramuscular, is the most common route of vaccination for fish and is largely considered the most effective, providing precise dosing for each fish and generating a strong, long-lasting immune response to the antigen [129]. This method, however, carries increased risk of stress and/or injury to the animal during vaccine administration, whether by handling, anesthesia, and/or injection itself, which can lead to morbidity and mortality [130]. Vaccination via injection also has the potential to cause severe iatrogenic damage to the fish, particularly in conjunction with adjuvant usage, with reported adverse effects including secondary infections, chronic granulomatous disease, visceral adhesions, severe local tissue reactions, and/or perforation of coelomic organs [131]. Accidental self-injection of workers manually vaccinating fish can also cause significant injury, with reported adverse health effects from self-injection

including anaphylaxis, vascular spasms, mild to moderate local inflammation, and severe tissue necrosis [132–135]. Compared to other delivery methods, injection vaccination also tends to be more labor-intensive and expensive, especially on large-scale aquaculture operations, requiring skilled personnel, careful anesthesia of animals, and specialized equipment [130]. Automated vaccination machines, capable of vaccinating up to 20,000 fish/hour, have been developed to overcome some of the logistical limitations of manual injection for commercial aquaculture [136]. However, injection vaccination is still limited by the size of fish, requiring animals to be >15 g, making this strategy unsuitable for small fish species or for fish in early life stages [137].

On the other hand, oral vaccination provides a practical, efficient, low stress method of mass vaccination for large-scale aquaculture. Conventional oral vaccines are generated either through incorporation of vaccine antigens directly into the feed or by mixing/coating the feed with vaccine [130]. Compared to injectables, oral vaccination minimizes the labor costs and handling stress of vaccination, alleviates the risk of injury to animals or workers, and enables large-scale vaccination to fish of any size and age class [129]. Oral vaccines, however, can be comparatively more costly for vaccination of larger sized fish, especially when multiple boosters are needed [130]. Another major advantage of oral vaccines is their ability to stimulate intestinal mucosal immune responses. Unlike injectable vaccines which bypass normal mucosal mechanisms of immunity, oral vaccine antigens must be absorbed through the gastrointestinal tract where they can stimulate gut-associated lymphoid tissue (GALT) to induce both systemic and local immune responses to potentially enhance vaccine protection [138,139]. Transit of oral vaccines through the digestive tract, however, also results in reduced immunogenicity of oral vaccines compared to injectables due to antigen degradation and poor absorption, often leading to the need for adjuvants and/or boosters to enhance vaccine efficacy [138].

Antigen encapsulation techniques using nanoparticle materials (e.g. alginate, chitosan) or live feed (e.g. *Artemia*, yeast) have been developed to improve delivery of oral vaccines by preventing antigen degradation in the stomach and enhancing immune-stimulation [140–145]. The potential for including oral

immune tolerance is another major consideration for the development of oral vaccines. Immunological tolerance refers to a phenomenon in which an animal develops reduced responsiveness to a particular antigen due to suppression of cell- and/or antigen-mediated immune responses [140]. Repeated exposure of fish, particularly young fish, to low antigen concentrations over extended periods of time has been demonstrated to induce immunological tolerance in fish, leading to reduced antibody levels and increased susceptibility to infection [146–150]. Thus, the development of oral vaccines for fish requires careful balancing of dosage and duration to ensure adequate protection while minimizing the risk of oral tolerance.

Fish can also be immunized via immersion vaccination, in which the fish are exposed to antigens through immersion in water containing the vaccine [151]. The two main methods of immersion vaccination used in aquaculture are dip vaccination, where fish are immersed in a relatively high dose of vaccine for a short period of time (30 – 60 seconds), or bath vaccination, in which fish are exposed to a more dilute concentration of vaccine over an extended period (≥ 1 hour) [151,152].

Dip vaccination is more widely used as it facilitates more rapid and cost-effective vaccination of large numbers of smaller fish (≤ 5 g) [152]. Similar to oral vaccination, the strengths of immersion vaccination lie in its convenience for rapid vaccination of large numbers of animals with minimal stress to the fish, its use in vaccinating fish too small for injection, and its stimulation of mucosal immune processes [151,153]. Immersion vaccination also closely mimics natural mechanisms of water-borne horizontal disease transmission, as vaccine antigens are absorbed across mucosal tissues, where they activate the extensive system of mucosal-associated lymphoid tissue in fish, stimulating not only systemic immunity, but also local mucosal immunity, which may enhance disease prevention [151,153]. The major drawbacks to immersion vaccination are that it tends to elicit a weaker and shorter protective immune response compared to injection vaccination, often requires boosters, and can be cost-prohibitive and impractical in larger fish due to the large amounts of vaccine needed [151,154].

A third approach to immersion vaccination is spray vaccination, a technique first developed in 1978 for the immunization of salmonids against *Vibrio anguillarum* [155]. Fish are either suspended in a net

or placed on a conveyor belt and sprayed coated with vaccine which is absorbed across the mucosal surfaces [154,155]. Originally devised as an economical alternative to immersion vaccination for larger sized fish (>20 g) wherein direct immersion can be impractical at scale, spray vaccination is not commonly practiced in the field due to increased stress on the animals [108,154].

Other methods of improving antigen uptake and augmenting immersion vaccine efficacy include hyperosmotic pretreatment [156], percutaneous puncture [157], nanobubble treatment [158,159], and low-intensity ultrasound sonophoresis [160,161]. Hyperosmotic pretreatment involves soaking fish in a hyperosmotic solution (typically 3 – 5 % saline) for a short period of time just prior to direct immersion vaccination to manipulate the fish's osmoregulatory mechanisms and enhance soluble antigen uptake [131,153,156]. This method is not commonly used in practice owing to concerns about increased stress to the fish and variable experimental findings regarding its impact, with some studies reporting improved protection and enhanced immune responses following hyperosmotic treatment [156,162,163] while others show no significant benefits [164–166].

Percutaneous puncture, or stamp vaccination, is based on findings that the skin is the primary site of antigen uptake during immersion vaccination and that sites of injury have enhanced particulate uptake compared to intact skin [157,167–169]. This modality uses a multiple puncture instrument, a device widely used to administer tuberculosis vaccines to humans, to create small lesions in the skin which have been shown experimentally to improve antigen uptake and significantly increase post-challenge survival in rainbow trout against *Streptococcus iniae* compared to immersion alone [157,170,171]. However, stamp vaccination has not seen widespread industry use largely due to concerns over stress and damage to the animals [129].

A relatively new method of augmenting immersion vaccination is nanobubble treatment, where collapse of ozone microbubbles leads to production of free radicals which activate expression of important immune-related genes and enhance antigen uptake to improve vaccine efficacy [159,172]. Another approach is ultrasound sonophoresis, which utilizes short pulses of low-intensity ultrasound to cause local

microscopic perforation of epidermal cells and widen intercellular gaps, thereby facilitating transdermal antigen penetration to improve vaccine uptake during immersion baths [173–176]. The use of ultrasound irradiation prior to immersion vaccination has been experimentally demonstrated to improve antigen transport and vaccine protective efficacy against bacterial pathogens in various fish species compared to direct immersion alone [160,161,177]] and provides protection levels comparable intracoelomic injection [178–180].

There are currently no commercially available vaccines for the prevention of *E. piscicida* in fish. However, following the success of a licensed formalin-killed bacterin approved in Korea in 2003 to protect olive flounder against *E. tarda* [181], the live-attenuated AQUAVAC-ESC™ (Intervet Inc.) vaccine licensed in the US in 2000 for the prevention of ESC (*E. ictaluri*) in catfish [182,183], a live-attenuated *evpB* deletion mutant vaccine patented in 2019 in the USA to protect catfish against ESC [184], and an experimental, orally delivered *E. ictaluri* vaccine that is currently being administered under veterinary prescription in the US catfish industry [183,185–187] there have been numerous studies evaluating different immunization modalities for the development of an effective vaccine for aquaculture to prevent *E. piscicida* (Table 1.2).

The majority of studies investigating vaccines against *E. piscicida* have been focused on LAVs, in large part because LAVs are often highly effective at inducing the humoral and cellular immunity required for effective host clearance of facultative intracellular pathogens [188–190]. Candidate LAVs in the literature are predominantly mutant *E. piscicida* strains attenuated by selective deletion of critical virulence and/or outer membrane protein genes, particularly those associated with the T3SS and T6SS [30,40,191–194], or random attenuation by repeated passage of pathogenic strains in the presence of increasing concentrations of rifampicin (RIF) [185,195–198]. One RIF attenuated *E. ictaluri* LAV has been shown cross-protective against *E. piscicida* and increased post-challenge survival in channel and hybrid catfish via immersion (80.5-100% relative percent survival (RPS) 54.7-77.8% RPS, respectively) and oral administration (85-88% RPS and 48% RPS, respectively) [199,200]. Candidate LAVs have shown great experimental promise with demonstrated *in-vivo* attenuation of virulence and significantly improved post-

challenge survival when administered via injectable, oral, or immersion routes in catfish [188,192,201], zebrafish [89,90,188,202–204], blue gourami [205,206], turbot [40,100,191,193,194,207–210], olive flounder [30,206,211], goldfish [212], and tilapia [213]; however there has been little to no investigation into the efficacy of these candidate LAVs in salmonids.

Formalin-killed *E. piscicida* vaccine candidates have demonstrated mixed results, with reported relative percent survival (RPS) values ranging from 13.3 – 100% [214–217]. The strongest and longest-lasting immunity was induced when formalin-killed *E. piscicida* bacterins were administered with oil-based adjuvants via intracoelomic injection, suggesting that an effective inactivated vaccine may require adjuvants and/or boosters for full protection, as is common with inactivated vaccines against other bacterial pathogens [100,218]. Inactivated vaccines containing lyophilized *E. piscicida* ghosts have also been shown to induce strong antibody responses and improved survival in tilapia and olive flounder compared to traditional formalin-killed preparations, providing an alternative pathway of study into inactivated vaccines for *E. piscicida* [216,217].

Aside from conventional vaccine modalities, there have been a number of studies exploring the application of more modern vaccine technologies for protection against *E. piscicida*. Research has demonstrated that subunit vaccines, administered via intracoelomic injection, containing purified recombinant outer membrane proteins or virulence factors can simulate strong-specific antibody responses, upregulate expression of specific and non-specific immune-related genes, enhance serum bactericidal activity, and induce effective immune protection against *E. piscicida* [40,219–222]. However, these results were highly variable based on specific candidate antigens and the use of adjuvants [223–225]. Additionally, the use of recombinant subunit vaccines as mucosal vaccines against *E. piscicida* has not been extensively investigated.

Some studies have attempted to improve the efficacy of recombinant vaccines using live vectors with the target antigen gene cloned into avirulent *E. coli* strains which can then express the *E. piscicida* antigen when administered live into the host animal. These studies have demonstrated improved survival

using live vectors, compared to the corresponding purified recombinant protein, and the ability to induce protective immunity via oral vaccination, opening new avenues for advancing *E. piscicida* vaccine research [222,226].

DNA vaccines for *E. piscicida* have also been explored to overcome the inherent low immunoprotectivity of purified recombinant protein through enhanced stimulation of cell-mediated immune responses. In olive flounder, DNA vaccines encoding antigens such as FliC, Eta6, and Eta2 demonstrated superior protective capacity than subunit vaccines containing the corresponding purified recombinant protein [223,227]. Additionally, chimeric DNA vaccines encoding multiple *E. piscicida* antigens further improved protective immunity than DNA vaccines based on a single protein alone [223]. A major drawback of this modality is the current need for intramuscular injection to elicit the strong expression of transgene needed for induction of protective immunity, which is impractical for large-scale immunization of smaller-sized fish, does not engage mucosal mechanisms of immunity, and is often viewed as less desirable due to the possibility of local tissue damage and scarring which could decrease fillet quality [228]. As such, there remains a need for a cost-effective mucosal vaccine against *E. piscicida* that enables practical mass vaccination of large numbers of fish while inducing strong humoral and cellular immunity to protect salmonids against piscine edwardsiellosis.

Table 1.2. Overview of candidate *Edwardsiella piscicida* vaccines reported in the literature.

Vaccine Type	Target Antigen	Delivery Method	Fish Host	Reference
DNA	Eta2 protein	IM	Olive flounder	[229]
DNA	Eta6 protein	IM	Olive flounder	[223]
DNA	Flagellar protein FliC	IM	Olive flounder	[223]
DNA	Eta6 protein and flagellar protein FliC	IM	Olive flounder	[223]
DNA	Flagellar protein FliC and Et18 protein	IM	Olive flounder	[223]
DNA	Esa1 protein	IM	Olive flounder	[227]
Recombinant subunit	Outer membrane protein Ail1	IC	Olive flounder	[219]
Recombinant subunit	Eta2 protein	IC	Olive flounder	[229]

Recombinant subunit	Eta21 protein	IC	Olive flounder	[226]
Recombinant subunit	Esa1 protein	IC	Olive flounder	[222]
Recombinant subunit	Eta6 protein	IC	Olive flounder	[223]
Recombinant subunit	Flagellar protein FliC	IC	Olive flounder	[223]
Recombinant subunit	Outer membrane protein Omp1	IC	Olive flounder	[220]
Recombinant subunit	Invasin Inv1	IC	Olive flounder	[224]
Recombinant subunit	Eta1 protein	IC	Olive flounder	[230]
Recombinant subunit	37 kDa outer membrane protein	IC	Olive flounder	[221]
Recombinant subunit	Outer membrane protein OmpV	IC	Olive flounder	[231]
Recombinant subunit	Outer membrane protein OmpK	IC	Olive flounder	[231]
Recombinant subunit	Outer membrane protein OmpH	IC	Olive flounder	[231]
Recombinant subunit	Outer membrane protein OmpX	IC	Olive flounder	[231]
Recombinant subunit	Outer membrane protein OmpI	IC	Olive flounder	[231]
Recombinant subunit	Outer membrane protein OmpR	IC	Olive flounder	[231]
Recombinant subunit	T3SS protein EseD	IC	Turbot	[225]
Live vector	Eta21 protein	IC	Olive flounder	[226]
Live vector	Esa1 protein	IC	Olive flounder	[226]
Live vector	Esa1 protein	Oral	Olive flounder	[222]
Inactivated	Liposome-coated formalin-killed <i>E. piscicida</i>	Oral	Japanese eel	[218]
Inactivated	Formalin-killed <i>E. piscicida</i>	IC	Olive flounder	[232]
Inactivated	Formalin-killed <i>E. piscicida</i>	IC	Olive flounder	[215]
Inactivated	Formalin-killed <i>E. piscicida</i>	Immersion and IC	Turbot	[214]
Inactivated	Formalin-killed <i>E. piscicida</i>	IC	Tilapia	[216]
Inactivated	Lyophilized <i>E. piscicida</i> ghosts	IC	Tilapia	[216]
Inactivated	Formalin-killed <i>E. piscicida</i>	Oral	Olive flounder	[217]
Inactivated	Lyophilized <i>E. piscicida</i> ghosts	Oral	Olive flounder	[217]
Inactivated	Formalin-killed <i>E. piscicida</i>	IC	Olive flounder	[231]
Live-attenuated	Attenuated <i>E. ictaluri</i>	Oral and immersion	Channel catfish and hybrid catfish	[199,200]

Live-attenuated	<i>E. piscicida</i> $\Delta phoP$ deletion mutant	IC	Channel catfish and hybrid catfish	[201]
Live-attenuated	<i>E. piscicida</i> $\Delta phoQ$ deletion mutant	IC	Channel catfish and hybrid catfish	[201]
Live-attenuated	<i>E. piscicida</i> WED strain with deletion of <i>eseB</i> , <i>eseC</i> , <i>eseD</i> , <i>escA</i> , and <i>aroC</i> genes and curing of endogenous plasmid pEIB202	IM, IC, and immersion	Turbot	[207]
Live-attenuated	<i>E. piscicida</i> 10 Δ with deletion of <i>rpoS</i>	IC	Turbot	[194]
Live-attenuated	<i>E. piscicida</i> 10 Δ / <i>esrB</i> ^{OE} with deletion of <i>rpoS</i> (10 Δ) and overexpression of <i>esrB</i>	IC	Turbot	[208]
Live-attenuated	<i>E. piscicida</i> $\Delta esrB$ deletion mutant	IC	Turbot	[40]
Live-attenuated	<i>E. piscicida</i> $\Delta T3SS$ strain with deletion of <i>eseB-eseD</i>	IC	Turbot	[40]
Live-attenuated	<i>E. piscicida</i> 9 Δ with deletion of TAE_0866, 0888, 1586, 1604, 1757, 2186, 2188, 2438 and 3282	IC	Turbot	[40]
Live-attenuated	<i>E. piscicida</i> $\Delta asdA10$ deletion mutant encoding Ich antigen IAG52B	IC	Zebrafish	[202]
Live-attenuated	<i>E. piscicida</i> $\Delta eseD$ deletion mutant	IM	Turbot	[191]
Live-attenuated	<i>E. piscicida</i> $\Delta arnB$ deletion mutant	IC	Turbot	[209]
Live-attenuated	<i>E. piscicida</i> $\Delta alr-1$ deletion mutant	IC	Olive flounder	[211]
Live-attenuated	<i>E. piscicida</i> $\Delta aroC$ deletion mutant	IM	Zebrafish	[203]
Live-attenuated	<i>E. piscicida</i> $\Delta aroC\Delta esrC$ deletion mutant	IM	Zebrafish	[203]
Live-attenuated	<i>E. piscicida</i> $\Delta aroC\Delta slyA$ deletion mutant	IM	Zebrafish	[203]
Live-attenuated	<i>E. piscicida</i> $\Delta aroC\Delta eseBCD\Delta esaC$ deletion mutant	IM	Zebrafish	[203]
Live-attenuated	<i>E. piscicida</i> $\Delta tatABCD$ deletion mutant	IC	Turbot	[210]
Live-attenuated	<i>E. piscicida</i> $\Delta rpoS$ deletion mutant	IC	Turbot	[193]
Live-attenuated	<i>E. piscicida</i> $\Delta higB$ deletion mutant	IM	Tilapia	[213]
Live-attenuated	<i>E. piscicida</i> ΔP_{fur} deletion mutant	Immersion	Zebrafish	[204]
Live-attenuated	<i>E. piscicida</i> $\Delta ETAE_0023$ deletion mutant	IC	Turbot	[233]
Live-attenuated	<i>E. piscicida</i> Δcrp deletion mutant	IC	Zebrafish and channel catfish	[188]

Live-attenuated	<i>E. piscicida</i> Δ ssaV deletion mutant	IC	Channel catfish	[192]
Live-attenuated	<i>E. piscicida</i> Δ esaM deletion mutant	IC	Channel catfish	[192]
Live-attenuated	<i>E. piscicida</i> Δ yscR deletion mutant	IC	Channel catfish	[192]
Live-attenuated	<i>E. piscicida</i> Δ escT deletion mutant	IC	Channel catfish	[192]
Live-attenuated	<i>E. piscicida</i> LSE40 Δ asd Δ purA deletion mutant	IM	Blue gourami	[205]
Live-attenuated	<i>E. piscicida</i> CK226 (Δ fap) deletion mutant	IC	Zebrafish	[90]
Live-attenuated	<i>E. piscicida</i> CK247 (Δ fdp) deletion mutant	IC	Zebrafish	[90]
Live-attenuated	<i>E. piscicida</i> CK248 (Δ fap Δ fdp) deletion mutant	IC	Zebrafish	[90]
Live-attenuated	<i>E. piscicida</i> Δ crp deletion mutant	IC	Goldfish	[212]
Live-attenuated	<i>E. piscicida</i> Δ aroA11 Δ phoP12 deletion mutant	IC	Zebrafish	[89]
Live-attenuated	<i>E. piscicida</i> WED Δ T6SS strain with deletion of <i>evpA-evpB</i> , <i>eseB</i> , <i>eseC</i> , <i>eseD</i> , <i>escA</i> , and <i>aroC</i> genes and curing of endogenous plasmid pEIB202	IC	Olive flounder	[30]
Live-attenuated	<i>E. piscicida</i> Δ esaS deletion mutant	IC	Channel catfish	[234]
Live-attenuated	<i>E. piscicida</i> Δ basS deletion mutant	IC	Channel catfish	[235]
Live-attenuated	<i>E. piscicida</i> Δ basR deletion mutant	IC	Channel catfish	[235]
Live-attenuated	<i>E. piscicida</i> Δ aroA deletion mutant	IM	Blue gourami	[206]
Live-attenuated	<i>E. piscicida</i> Δ aroA Δ esrB deletion mutant	IM	Olive flounder	[206]
Live-attenuated	<i>E. piscicida</i> Δ aroA Δ esrB deletion mutant	IM	Blue gourami	[206]
Live-attenuated	<i>E. piscicida</i> Δ aroA Δ esaC deletion mutant	IM	Blue gourami	[206]
Live-attenuated	<i>E. piscicida</i> Δ aroA Δ evpH deletion mutant	IM	Blue gourami	[206]
Live-attenuated	<i>E. piscicida</i> Δ aroA Δ purA deletion mutant	IM	Blue gourami	[206]
Live-attenuated	<i>E. piscicida</i> Δ aroA Δ rpoS deletion mutant	IM	Blue gourami	[206]
Live-attenuated	<i>E. piscicida</i> Δ aroA Δ esrB Δ evpH deletion mutant	IM	Blue gourami	[206]
Live-attenuated	<i>E. piscicida</i> Δ aroA Δ esaC Δ evpH deletion mutant	IM	Blue gourami	[206]

IM: intramuscular injection; IC: intracoelomic injection; T3SS: Type III secretion system; Ich: Ichthyophthirius multifiliis.

1.7. Non-piscine Edwardsiellosis

Of the five species within the genus *Edwardsiella*, only *E. tarda* is a well demonstrated mammalian pathogen. Although *E. hoshinae* has been previously isolated from human feces, the bacterium has never been associated with disease in people and is not considered a zoonotic pathogen [236,237]. All reports of human edwardsiellosis in the literature cite *E. tarda* as the etiologic agent, however taxonomic changes in the *Edwardsiella* genus in the 2010s have brought into question the true identity of isolates previously described as *E. tarda* [237,238]. Since the discovery of *E. piscicida* as a distinct species, subsequent studies using modern phenotypic and genetic techniques have discovered numerous fish-pathogenic isolates previously classified as *E. tarda* were, in actuality, representatives of *E. piscicida* [24,62,66,239]. These findings bring into question whether previous reports of edwardsiellosis in humans are all truly caused by *E. tarda*, as is described in the literature, or whether *E. piscicida* could have been the true causative agent in any of the historical cases. **One major barrier to answering this question is the paucity of information on the zoonotic potential of *E. piscicida*.**

Edwardsiella tarda has been isolated from a wide variety of both aquatic and terrestrial species, including fish, shellfish, seals, lizards, snakes, birds, dogs, pigs, monkeys, and rats [237]. In reptiles, *E. tarda* is often considered a normal member of the digestive tract microflora and has been isolated from a large number of species including American alligators (*Alligator mississippiensis*), garter snakes (*Thamnophis* spp.), American rattlesnakes (*Crotalus durissus terrificus*), Brook's House geckos (*Hemidactylus brookii*), and Geoffroy's toadhead turtles (*Phrynops geoffroanus*) [237,240]. Cases of clinical disease in reptiles from *E. tarda* infection are exceedingly rare, however the bacterium has been reported in association with septicemia in crocodile hatchlings (*Crocodylus* spp.) [241–243] and Chinese soft-shelled turtles (*Pelodiscus sinensis*) [244,245], as well as the causative agent of subcutaneous abscesses in a captive grass snake (*Natrix natrix*) [240]. Similarly, *E. tarda* has been isolated from several amphibian species [237,246] and reports of pathology associated with infection are limited, although the bacterium has been linked to subclinical infections in farmed American bullfrogs (*Aquarana catesbeiana*) [247]. In

birds, infections with *E. tarda* are most common in aquatic and piscivorous species such as ducks, pelicans, eagles, gulls, penguins, and herons [248–250]. Avian *E. tarda* infections can have varied clinical manifestations, including asymptomatic carriers [248,249], chronic enteritis in Rockhopper penguins (*Eudyptes chrysocome*) [251], acute septicemia in Mallard ducks (*Anas platyrhynchos*) and a snowy egret (*Egretta thula*) [252], pneumonia and multi-organ hemorrhage in a crested ibis (*Nipponia nippon*) [253], and hemorrhagic enteritis in brown pelicans (*Pelecanus occidentalis*) and common loons (*Gavia immer*) [248,254].

Edwardsiella tarda is frequently reported as an opportunistic pathogen of aquatic mammals, often associated with strandings of wild cetaceans and pinnipeds or sick captive marine mammals. Consumption of contaminated fish is typically cited as the route of transmission for *E. tarda* infection in marine mammals [255,256], however the pathogen has been reported as a normal member of the gastrointestinal microflora in apparently healthy animals [249]. In aquatic mammals, *E. tarda* is most commonly reported as a cause of bacterial peritonitis and septicemia, often secondary to a primary injury or illness such as fish-hook wounds or pneumonia [237,256]. Cases of *E. tarda* septicemia and/or peritonitis have been reported in sperm whales (*Physeter macrocephalus*) [257], killer whales (*Orcinus orca*) [258,259], harbor porpoises (*Phocena phocena*) [260–262], pygmy killer whales (*Feresa attenuata*) [263], pilot whales (*Globicephala* spp.) [264], (Stellar’s sea lions (*Eumetopias jubatus*) [260], and California sea lions (*Zalophus californicus*) [260,261]. *Edwardsiella tarda* has also been frequently associated with cases of gastrointestinal disease in captive and wild aquatic mammals [255]. In cetaceans, *E. tarda* has been isolated from beluga whales (*Delphinapterus leucas*) with hemorrhagic enteritis and gastric erosion [265] and from a humpback whale (*Megaptera novaeangliae*) with enteritis [264]. *Edwardsiella tarda* has also been isolated from cases of fatal intussusception in a Florida manatee (*Trichechus manatus latirostris*) [266] and a Stellar’s sea lion [261]. There are also reports of *E. tarda* associated with small intestinal volvulus in Stellar’s sea lion [267] and a harbor seal (*Phoca vitulina*) [268], although the role of *E. tarda* in the pathogenesis in many of these cases

is unclear. Other less commonly reported manifestations of *E. tarda* infection in marine mammals include metritis [256,261], nephritis [269], corneal ulceration [270] and pulmonary infection [271,272].

While *E. tarda* infections are predominantly associated with exposure to aquatic environments or animals, the bacterium has been infrequently isolated from terrestrial mammals. *Edwardsiella tarda* has been isolated from a number of free-ranging and captive terrestrial zoologic mammals worldwide, including raccoons (*Procyon lotor*) [273], grey-cheeked mangabeys (*Lophocebus albigena*) [250], leopards (*Panthera pardus*) [250], a South China tiger (*Panthera tigris amoyensis*) [274], and North American opossums (*Didelphic virginiana*) [275]. In such animals *E. tarda* is often isolated from the gastrointestinal tract from asymptomatic individuals, however, rarely, the bacterium has been the cause of severe disease in wild and captive terrestrial mammals. In 2022, a Eurasian brown bear (*Ursus arctos arctos*) housed in a zoo was reported with fatal bacterial sepsis from *E. tarda* infection, likely facilitated by a raw fish diet and chronic stress from captivity [276]. *Edwardsiella tarda* is also believed to be a primary pathogen of both wild and captive echidna, causing severe bronchopneumonia in affected animals [277].

More concerning for human exposure, there have also been sporadic cases of *E. tarda* infection in domestic pets and livestock. Domestic pigs have been suggested as potential carriers for *E. tarda* owing to multiple reports of *E. tarda* isolation from apparently healthy pigs [278,279], particularly on facilities where livestock have access to aquatic environments and fish such as reservoirs or ponds. In cattle, *E. tarda* has been rarely reported as a cause of gastrointestinal disease, septicemia, and reproductive disease. The bacterium has been isolated from the intestinal tract of seemingly healthy animals and has been associated with non-specific acute diarrheal disease and fatal typhlocolitis in cattle [250,280,281]. *Edwardsiella tarda* is also a cause of chronic endometritis and puerperal uterine infection in cows, potentially facilitated by immunosuppression from pregnancy and parturition [282,283]. In domestic small animals, reports of infection with *E. tarda* are exceedingly rare. *Edwardsiella tarda* has been isolated from seemingly healthy shelter dogs and there have been limited cases of enteric infections and diarrheal disease associated with *E.*

tarda infection in pet dogs, however the pathogenicity of *E. tarda* in domestic dogs and the risk for human transmission is poorly understood [284–286].

Human infections with *E. tarda* have been sporadically reported from at least 25 countries, primarily in tropical and subtropical regions, although extensive epidemiologic data on *E. tarda* zoonosis is lacking, in part due to the presumed low prevalence in most countries and infrequent reporting [237,287]. In the United States, over 66% of human edwardsiellosis cases have been reported along coastal states, largely centered around the Gulf of Mexico [287]. Humans are typically exposed to *E. tarda* through two routes of transmission: oral or wound infection. Oral exposure to *E. tarda* can occur through ingestion of contaminated water, consumption of raw/undercooked animals, or handling carrier animals such as pet reptiles or fish [237,288]. *Edwardsiella tarda* can also enter through the skin, typically due to exposure of pre-existing wounds to aquatic environments or through skin penetration with contaminated equipment (e.g. fish-hooks) or fish spines/bones [237]. Demographic risk factors for *E. tarda* infection include professions with high exposure to fish or aquatic environments (e.g. fishermen, dock workers, fishmongers, veterinarians, animal husbandry staff), aquatic recreational activities (e.g. aquarium hobbyists, swimming, diving), and dietary customs involving consumption of raw seafood [237].

Additionally, it has been suggested that *E. tarda* may be an important cause of severe, potentially fatal, maternal-fetal infections. There have been several reports of *E. tarda* infections in pregnant mothers disseminating to the fetus, often leading to significant disease or death in affected infants [289]. Most reports of maternal-fetal *E. tarda* infections come from Japan and are believed to be primarily associated with consumption of raw fish during pregnancy leading to gastrointestinal colonization of the mother and eventual transmission to the fetus. In some cases, maternal exposure to *E. tarda* was associated with immersion in contaminated freshwater lakes leading to gastrointestinal and/or vaginal infection [289,290]. In one case of *E. tarda* bacteremia in a pregnant woman leading to fetal death, the only potential route of exposure identified in the patient's history was a tropical fish tank that had been cleaned by the patient approximately 4 days before the onset of clinical disease [291]. The exact mechanism of maternal-fetal *E.*

tarda transmission is unclear, however in many of the reported cases findings such as early onset of neonatal infection and chorioamnionitis suggest vertical transmission and intrauterine infection of the fetus [289,290]. Additionally, many cases of maternal-fetal *E. tarda* infection report meningitis and brain abscesses in newborns, suggesting that *E. tarda* may be an important causative agent of neonatal CNS infection [289].

Edwardsiella tarda zoonosis can have a wide range of clinical manifestations (Figure 1.2). Cases of human infection with *E. tarda* most commonly (>80%) present as acute gastroenteritis or enteritis, typically in healthy individuals or those with minor health conditions [237]. The clinical presentation of *E. tarda* gastroenteritis is characterized by a 3-to-5-day duration of mild watery diarrhea and a low-grade or intermittent fever. The disease is often mild and self-limiting, however *E. tarda* has been reported to cause more severe gastrointestinal illnesses such as bloody dysentery, chronic gastroenteritis, persistent diarrhea, and ulcerative colitis [292–294]. *Edwardsiella tarda* has been identified as the cause of chronic diarrhea in several pediatric cases. These cases involved both healthy children and those with underlying medical conditions, one a kidney transplant recipient and another with Crohn’s disease and were commonly associated either with consumption of seafood or owning pet ornamental fish [237,294]. Gastrointestinal infection with *E. tarda* can also exacerbate pre-existing inflammatory bowel conditions, such as Crohn’s disease or ulcerative colitis, which can lead to severe clinical disease and hospitalization [293,295–297]. Additionally, *E. tarda* within the gastrointestinal tract can translocate to the circulatory system, leading to septicemia and disseminating to other organ sites [298].

Extraintestinal infections with *E. tarda* are less common but can be life-threatening and manifest as any number of different clinical syndromes including soft tissue wound infections, sepsis, hepatobiliary infections, peritonitis, endocarditis, pneumonia, urogenital infections, and CNS infections [237,299–301]. Wound infections with *E. tarda* are most commonly caused by catfish bites or from puncture wounds with fish bones/spines, however other reported vehicles for *E. tarda* soft tissue infections include car doors, glass, and a ceiling fan blade [237,302]. These trauma-related soft tissue infections typically occur in

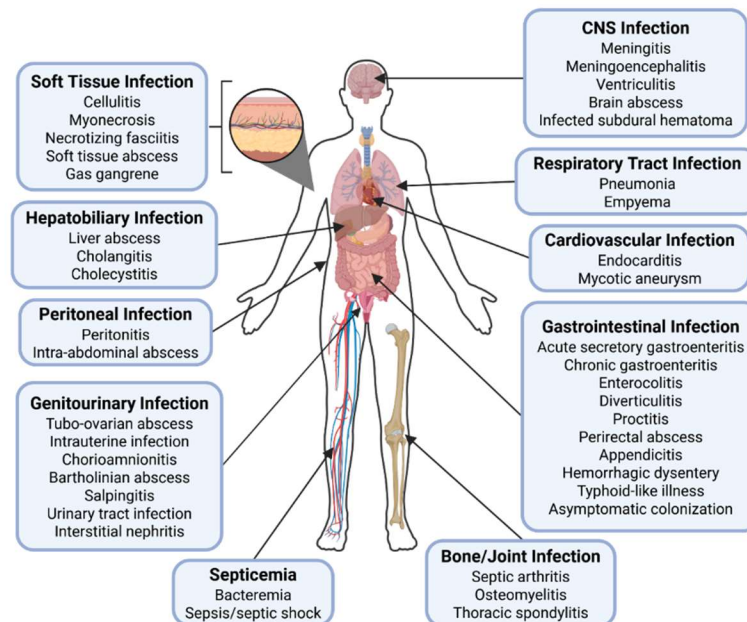
healthy males < 30 years of age employed in aquatic-related occupations or engaged in aquatic recreational activities. Clinical manifestations of these wound infections can range from mild cellulitis, treatable with drainage and antimicrobial therapy, to limb- and/or life-threatening disease. In these cases, wounds are often co-infected with multiple aquatic pathogens, including *Aeromonas hydrophila* and *Shewanella putrefaciens*, further complicating accurate etiologic diagnosis and treatment [237,303–305].

There have been multiple cases of trauma-associated *E. tarda* wound infections progressing to severe myonecrosis and necrotizing fasciitis resulting in amputation, sepsis, and/or death [299,303,306,307]. Patients with necrotizing soft tissue infections from *E. tarda* are often reported to have underlying chronic liver disease, a comorbidity that is a prominent risk factor for necrotizing soft tissue infections from *Vibrio* spp. infection, likely due to impaired iron metabolism [306,308–310]. There are also rare reports of *E. tarda* wound infections spreading to the bone and causing osteomyelitis [311–313] or progressing to potentially fatal bacterial septicemia and sepsis, particularly in patients with underlying medical conditions such as diabetes mellitus [299,302]. In one case report, a penetrating injury from a catfish spine to the patient's right knee rapidly progressed to acute septic arthritis from *E. tarda* necessitating extended hospitalization and intravenous antibiotic treatment [314].

Septicemia is the most serious extraintestinal manifestation of *E. tarda* infection, with reported mortality rates ranging from 25.8% to 50% [299,315]. Blood cultures from *E. tarda* bacteremia are largely monomicrobial, however bloodstream co-infections with aquatic pathogens or enteric microorganisms have been reported [238,316]. As with other Gram-negative septicemias, clinical features of *E. tarda* bacteremia and sepsis include fever, abdominal pain, vomiting and/or diarrhea, and hypotension which can rapidly progress to disseminated intravascular coagulation syndrome, septic shock, and death [237,317]. A frequently reported sequela of *E. tarda* sepsis is cavitary lesions and abscessation, most commonly within the liver, abdomen, and tubo-ovarian tract [237,316,318–328]. Central nervous system disease is an extremely rare but highly deadly complication of *E. tarda* septicemia. The most commonly reported CNS manifestation of *E. tarda* sepsis is meningitis, [237,329–334], however other reported neurologic sequelae

include meningoencephalitis [335], ventriculitis [333], brain abscesses [333,336], and subdural hematoma [337]. These CNS infections typically occur in neonates or adults with underlying illnesses and can have mortality rates of $\geq 50\%$ [237]. Other rare complications of *E. tarda* septicemia include septic arthritis [338,339], osteomyelitis [313], peritonitis [340,341], and infectious endocarditis [342–344]. The vast majority (80-90%) of *E. tarda* septicemia cases involve immunosuppressed patients or individuals with underlying medical conditions [237]. Comorbidities associated with *E. tarda* septicemia include hepatobiliary disease, conditions causing iron overload, cancer, and diabetes mellitus [237,238,299]. Other risk factors for *E. tarda* sepsis include older age (> 65 years of age), alcoholism, consumption of raw seafood, and exposure to aquatic environments or exotic animals [237,345].

Figure 1.2. Reported clinical manifestations of *Edwardsiella tarda* infection in humans. Figure generated in Biorender.



1.8. Project Rationale

The emergence of *E. piscicida* as an important pathogen of wild and cultured fish populations poses a significant threat not only to global aquaculture but also to public health as bacterial outbreaks among food and/or recreational fish species pose an unknown zoonotic foodborne/waterborne disease risk.

Historically, *E. piscicida* has been viewed as a pathogen associated with warmwater conditions (>30°C), however reports of *E. piscicida* outbreaks among cool- and cold-water fish species call into question the true thermal range of the pathogen [24]. Thus, understanding the impact of environmental temperature on the virulence of *E. piscicida* is crucial to predicting outbreak risk. This is especially important in light of climate change-mediated factors, such as rising water temperatures and increasingly variable weather patterns, which are often associated with increasing disease outbreaks due to thermal stress causing fish immune suppression and to higher environmental temperatures promoting bacterial growth and virulence factor expression [57,346]. To investigate the influence of temperature on *E. piscicida* virulence, *in-vitro* growth curves and liquid chromatography mass spectrometry were utilized to evaluate temperature-associated differential growth patterns and virulence factor proteomes of genetically diverse *E. piscicida* isolates.

Mitigation of *E. piscicida* epizootics requires effective management strategies for producers to prevent and/or treat the disease. The current approach to *E. piscicida* outbreaks in the aquaculture industry, which relies largely on the extra-label use of antimicrobials to treat infected fish, can be expensive for producers and carries the risk for AMR development [24,98]. Additionally, treatment of *E. piscicida* with antibiotics, although often effective at mitigating disease severity and spread, does not serve to prevent potentially devastating outbreaks before they occur. In order to address the need for an effective prophylactic measure to protect fish against *E. piscicida*, a shotgun immunoproteomic approach for immunogenic antigen detection was utilized in this project to select protein vaccine candidates for the protection of fish against piscine edwardsiellosis.

Mucosal vaccines are highly attractive methods for fish vaccination due to the reduced stress and trauma to animals, reduced need for specialized equipment and/or training, and improved ability to stimulate the extensive mucosal immune system of fish for enhanced defense against pathogens [138,347]. Immersion vaccines, such as the FORTE® Micro ELANCO and the Alpha ERM Salar Dip, have been used effectively in commercial salmonid aquaculture as mucosal vaccines against several economically

significant fish bacterial pathogens [347]. One of the major obstacles for studies into mucosal vaccine development against *E. piscicida* for the salmonid aquaculture industry is the lack of an experimental challenge protocol that reliably and reproducibly induces morbidity and mortality in salmonids. Currently, the only established challenge protocol for *E. piscicida* in salmonids is intra-coelomic injection, which is effective in stimulating high morbidity and mortality in trout and salmon, but which is suboptimal for investigating mucosal vaccines as injection bypasses normal mucosal immune barriers [26,347]. Additionally, injection challenge delivers a large, acute inoculum directly into the host, which does not reflect the natural route or progression of infection. This can overwhelm early host defenses and obscure the contribution of mucosal immunity, making it difficult to accurately assess vaccine efficacy. Moreover, the use of high challenge doses may artificially reduce apparent vaccine protection, as even partially protective immune responses may be insufficient under such extreme exposure conditions where large quantities of pathogen are inoculated into the host at once. Thus, in Chapter 4 immersion challenge models for *E. piscicida* in salmonids were established in rainbow trout and Chinook salmon to facilitate later studies into *E. piscicida* pathogenesis and mucosal vaccine development. As immersion challenge methods often produce lower and more variable mortality rates than injection challenge methods, immersion protocols incorporating mechanical disruption of the normal skin and mucous coat barriers to infection were also investigated as means of facilitating bacterial entry and infection.

With outbreaks of *E. piscicida* occurring in many popular food and sport fish species, the bacterium poses a potential public health risk. The closely related species, *E. tarda*, is a zoonotic foodborne/waterborne pathogen that causes gastroenteritis, wound infections, and potentially fatal systemic infections in people [237]. There is no clear consensus, however, on the zoonotic potential of *E. piscicida*, owing in part to historical conflation with *E. tarda*. Understanding the virulence and pathogenicity of *E. piscicida* to mammals, thus, is crucial for accurate assessment of its public health risk. *In-vitro* cell culture models using mammalian and poikilotherm cell lines were used to assess the role of cell origin and temperature on the virulence of *E. piscicida* and to compare *in-vitro* virulence between *E. piscicida* and

known zoonotic pathogen *E. tarda*. Challenge experiments using both immunocompetent and immunodeficient mouse models were used to assess the *in-vivo* pathogenicity of *E. piscicida* and *E. tarda* in a mammalian system and to evaluate the impact of host immune status on mammalian disease susceptibility.

1.9. References

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CHAPTER 2: Temperature-dependent Alterations in the Proteome of the Emergent Fish Pathogen *Edwardsiella piscicida*

2.1. Abstract

Edwardsiella piscicida is an emerging bacterial pathogen and the etiological agent of edwardsiellosis among cultured and wild fish species globally. The increased frequency of outbreaks of this Gram-negative, facultative intracellular pathogen poses not only a threat to the aquaculture industry but also a possible foodborne/waterborne public health risk due to the ill-defined zoonotic potential. Thus, understanding the role of temperature on the virulence of this emerging pathogen is essential for comprehending the pathogenesis of piscine edwardsiellosis in the context of current warming trends associated with climate change, as well as providing insight into its zoonotic potential. In this study, significant temperature-dependent alterations in bacterial growth patterns were observed, with bacterial isolates grown at 17°C displaying higher peak growth sizes, extended lag times, and slower maximal growth rates than isolates grown at 27°C or 37°C. When *E. piscicida* isolates were grown at 37°C compared to 27°C and 17°C, mass spectrometry analysis of the *E. piscicida* proteome revealed significant downregulation of crucial virulence proteins, such as Type VI secretion system proteins and flagellar proteins. Although *in vivo* models of infection are warranted, this *in vitro* data suggests possible temperature-associated alterations in the virulence and pathogenic potential of *E. piscicida* in poikilotherms and homeotherms.

2.2. Introduction

Edwardsiella piscicida, is a Gram-negative, facultative anaerobic, facultative intracellular bacterium belonging to the family *Hafniaceae*. This bacterium is an emerging pathogen of both freshwater and marine fish species worldwide, including economically important cultured and sport fish species in the USA such as catfish (*Ictalurus* spp.), trout (*Oncorhynchus* spp.), and black bass (*Micropterus* spp.) [1–4].

Fish infected with *E. piscicida* typically present with clinical signs of septicemia, characterized by multifocal external and internal petechial hemorrhages, skin discoloration and ulceration, exophthalmia, and hemorrhagic ascites [5,6]. Historically, the genus *Edwardsiella* was comprised of three species: *E. tarda*, a bacterium with a wide host range including both fish and mammals; *E. ictaluri*, the causative agent of enteric septicemia of catfish and outbreaks of edwardsiellosis in tilapia; and *E. hoshinae*, which is primarily considered a commensal in birds and reptiles [6]. In 2012-2013, multilocus sequence analysis studies revealed the polyphyletic origins of *E. tarda* isolates collected from diverse host fish species [7,8], leading to the taxonomic reorganization of *E. tarda* into three distinct species: *E. tarda* [4,9], *E. piscicida* [1,4,9], and *E. anguillarum* [4,10]. Subsequent genotypic and phenotypic studies have further identified at least six distinct clades within the species *E. piscicida*, with experimentally demonstrated differences in host specificity and disease severity [11].

In the two decades since its recognition as a discrete taxa, *E. piscicida* has been increasingly isolated from disease outbreaks and mass mortality events among wild and cultured fish species [3]. Not only has there been increased frequency of isolation of *E. piscicida* from wild and cultured fish, but the host and geographic range has seemingly expanded [2,6]. The global emergence of *E. piscicida* not only threatens wild and cultured fish populations but could also pose a potential public health risk. There is currently no clear consensus in the literature on the capacity for *E. piscicida* to infect and cause disease in mammals, owing in large part to historical conflation with the closely related species *E. tarda* [6]. *Edwardsiella tarda* is known to infect a wide range of taxa, including fish, reptiles, and terrestrial endotherms [6]. In humans, infections with *E. tarda* are rare, but can lead to significant and potentially fatal intestinal and/or extraintestinal disease [12]. Human infections with *E. tarda* are most commonly associated with exposure to contaminated water or consumption of infected animals [12]. Thus, the rise of *E. piscicida* among farmed fish populations could pose a growing foodborne and/or water-borne disease risk. Accurate assessment of the zoonotic risk of this pathogen necessitates further investigation into the potential virulence of *E. piscicida* in a mammalian model of infection.

Edwardsiella piscicida relies on several crucial virulence factors to evade host immune responses, invade host cells, replicate intracellularly, and disseminate throughout host tissues. Most notable among the virulence factors implicated in the pathogenesis of *E. piscicida* are the type III (T3SS) and type VI (T6SS) secretion systems, which are essential for successful *E. piscicida* infection [3,13]. These secretion systems are comprised of protein apparatuses that directly inject cytotoxic effectors into host cells to facilitate bacterial invasion and intracellular replication [3,13]. Other virulence factors associated with *E. piscicida* pathogenesis include iron uptake regulators [14], oxidative stress factors [15,16], hemolysin [5,17], translocation and assembly modules (TAM) [18], two-component systems [19,20], flagella [21], and outer membrane proteins [3].

Environmental factors, such as temperature, are known to modulate expression of virulence genes in fish pathogenic bacteria, allowing the bacteria to adapt to changing conditions [22]. Previous studies have shown *E. piscicida* uses the two-component system PhoP-PhoQ to sense changes in environmental temperature and regulate expression of the T3SS and T6SS, with reduced expression of these secretion systems at 37°C compared to 25°C [22,23]. This mechanism of temperature-dependent virulence gene expression is especially important in the context of warming trends associated with climate change, given that outbreaks of economically important fish diseases such as edwardsiellosis and lactococcosis are often precipitated by increased water temperatures [22]. Additionally, pathogens that switch between endothermic hosts and ectothermic hosts or environmental persistence often use temperature as a signal to control expression of virulence genes to facilitate infection and/or persistence when transitioning between ambient temperatures and endothermic host physiologic temperatures (37°C) [24–26]. Thus, improved understanding of the temperature-dependent regulation of *E. piscicida* virulence factors can provide insights into its potential pathogenicity in mammals and the impact of climate-change mediated increased water temperatures on the virulence of this emerging pathogen.

Emerging infectious fish diseases are a major concern for global aquaculture due to the substantial economic losses associated with increased treatment costs, reduced growth due to morbidity, disease-

induced anorexia, and direct losses from mortality. Moreover, there is considerable public health concern due to the risk of waterborne and/or foodborne disease. With reports of *E. piscicida* outbreaks steadily increasing over the past decade, with an expanding host and geographic range and a putative association with climate-change mediated increased water temperatures, it is crucial to understand the role of temperature on the expression of *E. piscicida* virulence factors. To this end, liquid chromatography tandem mass spectrometry (LC-MS/MS) was used to characterize the proteomic profiles of *E. piscicida* isolates from each of the six known clades grown at three experimental temperatures: 17°C, to simulate a cool-water system; 27°C, to simulate a warm-water system; and 37°C, to simulate a mammalian system.

2.3. Materials and Methods

2.3.1. Bacterial Isolates

Eighteen archived isolates of *E. piscicida* collected from diseased fish in the United States, representing the six identified genetic groups, were used in this study (Table 2.1). Isolates were revived from frozen stock by isolation streaking on trypticase soy agar supplemented with 5% sheep blood (SBA, University of California Davis, Biological Media Services, USA) and grown overnight at 28°C. Individual colonies were expanded in brain heart infusion broth (BHI; MP biomedical) and stored in 1 mL aliquots with 20% glycerol (v/v) to create new working stocks for each isolate, which were maintained at -80°C. Prior to each assay, frozen bacteria were grown on SBA at 28°C for 24 hours. For *E. piscicida* a 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL) corresponded to a bacterial suspension in phosphate-buffered saline (PBS) with an optical density at 600 nm (OD_{600}) of 0.15 (BioPhotometer Plus, Eppendorf AG). Bacterial concentration was confirmed via serial dilution and spot-plating of 10 μ L aliquots in triplicate on SBA. After 24-hour incubation at 28°C, the number of colony forming units (CFU) on the plates were counted to estimate the CFU/mL of the bacterial suspension.

Table 2.1. List of *Edwardsiella piscicida* isolates used in this study.

Isolate	Clade	Source	References	NCBI Accession	Genome length (bp)	%GC	Plasmid length (bp)
6-30 IP D16-014	Clade I	Largemouth bass (<i>Micropterus salmoides</i>)	[11]	CP150945-CP150946	3789405	59.6	3067
34-68†	Clade I	Atlantic salmon (<i>Salmo salar</i>)	[11]	CP150944	38929320	59.6	n/a
R1843	Clade I	Largemouth bass (<i>M. salmoides</i>)	[27]	JBCHVA000000000*	3305113	59.6	n/a
LADL-16005	Clade II	Barramundi (<i>Lates calcarifer</i>)	[28]	CP150941	3760097	59.8	n/a
S11-233†	Clade II	Channel catfish (<i>Ictalurus punctatus</i>)	[1]	CP150940	3802971	59.7	n/a
A15-1679	Clade II	Blotched fantail stingray (<i>Taeniura meyeni</i>)	[29]	CP150939	3786820	59.8	n/a
32-97	Clade III	Atlantic salmon (<i>S. salar</i>)	[11]	CP150938	3756321	59.9	n/a
35-70†	Clade III	Atlantic salmon (<i>S. salar</i>)	[11]	CP150937	3756329	59.9	n/a
Reds-S19-11-E	Clade III	Rainbow trout (<i>Oncorhynchus mykiss</i>)	[4]	CP150936	3826819	59.7	n/a
S11-285	Clade IV	Channel catfish (<i>I. punctatus</i>)	[1]	CP150934-CP150935	3917999	59.6	3164
S07-534	Clade IV	Channel catfish (<i>I. punctatus</i>)	[1]	CP150933	3968788	59.5	n/a
S12-281†	Clade IV	Hybrid catfish (♀ <i>I. punctatus</i> × ♂ <i>I. furcatus</i>)	[1]	CP150931-CP150932	3917264	59.6	3164
S16-567	Clade V	Hybrid catfish (♀ <i>I. punctatus</i> × ♂ <i>I. furcatus</i>)	[2]	CP150929-CP150930	3801030	59.7	77653
C1490	Clade V	Largemouth bass (<i>M. salmoides</i>)	[30]	CP150927-CP150928	3996733	59.4	77653
LADL99-462†	Clade V	Channel catfish (<i>I. punctatus</i>)	[1]	CP150925-CP150926	3816076	59.7	3616
S16-278	Clade VI	Hybrid catfish (♀ <i>I. punctatus</i> × ♂ <i>I. furcatus</i>)	[2]	CP150923-CP150924	3837991	59.6	3781
MA97-004†	Clade VI	Tilapia (<i>Tilapia</i> sp.)	[1]	CP150922	3839658	59.6	n/a
D16-038	Clade VI	Largemouth bass (<i>M. salmoides</i>)	[11]	CP150920-CP150921	3843154	59.6	2358

† Isolates were used for *in-vitro* proteomics.

*genome is not circularized.

2.3.2. Bacterial Growth Curves

McFarland standard suspensions of 0.5 in PBS were generated for each bacterial isolate (three representative isolates per *E. piscicida* clade) and then serially diluted to 1:1000 in BHI. One-hundred μL of each dilution were plated in quintuplicate onto 96-well plates, with six wells per plate containing 100 μL of sterile BHI as background controls. Plates were incubated for 48 – 72 hours at one of three experimental temperatures (17°C, 27°C, or 37°C). Throughout the incubation period, the absorbance at OD₆₀₀ of each sample was measured at one-hour intervals using a Cytation™ 5 Imaging Reader (BioTek, USA). Growth curve measurements were repeated in duplicate for each experimental temperature, and OD measurements were adjusted based on BHI control wells.

2.3.3. Peptide Preparation

One representative isolate per clade was selected based on demonstrated high morbidity and mortality in salmonids and catfish [11,31]. Individual colonies from selected isolates were suspended in 5 mL of sterile BHI and incubated to the late exponential-phase of growth, as determined by prior growth curve experiments, at one of three experimental temperatures (48 hours at 17°C, 24 hours at 27°C, and 13 hours at 37°C) with constant orbital shaking. The late-exponential phase of growth was selected for proteomic profiling as many other pathogenic bacteria demonstrate upregulated expression of virulence factors, such as the T6SS, in the late-exponential phase and early stationary phase [32–34]. Following incubation, samples were pelleted via centrifugation at 4500 rpm for 25 minutes. Bacterial proteins were extracted from all samples according to the manufacturer's suggested protocols for the B-PER™ with Enzymes Bacterial Protein Extraction Kit (Thermo Fisher Scientific, USA) with minor modifications as described. Briefly, 10 $\mu\text{L}/\text{mL}$ of Thermo Scientific™ Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, USA) was added to the B-PER reagent, vortexed, and then 5 mL of the resulting solution per 1 g of bacterial pellet was combined and pipetted until homogenized. Samples were incubated at room temperature for 15 minutes then concentrated by centrifugation for 5 min. The supernatant containing B-PER-extracted bacterial proteins was stored at 4°C. The remaining pellet was further processed following

manufacturer recommendations for the Inclusion Body Solubilization (IBS) Reagent (Thermo Fisher Scientific, USA) and the collected supernatant was stored at 4°C.

Extracted bacterial proteins (one isolate per clade, three technical replicates per isolate) were submitted to the UC Davis Proteomics Core Facility for digestion and liquid chromatography tandem mass spectrometry (LC-MS/MS). Protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Each sample (150 µg of protein) was digested on an S-Trap™ Digestion column plate. Initially, 10 mM dithiothreitol (DTT) was added and incubated at 50°C for 10 min and rested at room temperature for 10 min. Next, 5 mM iodoacetamide (IAA) was added and incubated at room temperature for 30 min in the dark. The samples were acidified with 12% phosphoric acid followed by the addition of freshly made S-trap buffer (90% methanol, 100 mM TEAB, pH 7.1) and mixed immediately by inversion. The entire acidified lysate buffer mix was transferred to the S-trap plate and pushed through with a Resolvex A200 (Tecan Group Ltd, Männedorf, Switzerland) until all the solution had passed through. Columns were washed with 400 µL of S-trap buffer. Trypsin enzyme digest buffer was carefully added (1:25 enzyme: total protein in 120 µL 50mM Triethyl Ammonium Bicarbonate (TEAB), pH 8.0) to the column. After two hours of incubation at 37°C, the same amount of trypsin and TEAB were added to the S-trap as a boost step and the reaction continued overnight at 37°C. The following day, peptides were eluted from the S-trap. Peptide elution steps included 80 µL of 50 mM TEAB (pH 8.0), 80 µL of 0.5% formic acid 80 µL of the solution containing 50% acetonitrile and 0.5% formic acid. The final pooled elution was dried down in a speed-vacuum. Peptides were resuspended in 0.1% TFA 2% ACN and quantified using Pierce™ Quantitative Fluorometric Peptide Assay (Thermo Fisher Scientific, USA).

2.3.4. Liquid Chromatography Tandem Mass Spectrometry

Peptides were resolved on a Dionex UltiMate 3000 RSLC system (Thermo Fisher Scientific, USA) using a PepSep analytical column (PepSep, Denmark): 150 µm x 8 cm C18 column with 1.5 µm particle size (100 Å pores), preceded by a Pepmap Neo C18 5UM guard column, and heated to 40°C. Separation

was performed in a total run time of 30 min with a flow rate of 500 μ L/min with mobile phases A: water/0.1% formic acid and B: 80%ACN/0.1% formic acid.

2.3.5. Mass Spectrometry Data Independent Analysis

Peptides were directly eluted onto an Orbitrap Exploris 480 instrument (Thermo Fisher Scientific, Bremen, Germany). Spray voltage was set to 1.8 kV, funnel RF level at 45, and heated capillary temperature at 275°C. Experiments full MS resolution was set to 60,000 at m/z 200 and full MS automatic gain control (AGC) target was 300% with an IT of 45 msec. Mass range was set to 350–1200. AGC target value for fragment spectra was set at 1000%. Mass Windows of 22Da were used with an overlap of 1 Da. Resolution was set to 30,000 and IT to Auto. Normalized collision energy was set at 30%. All data were acquired in profile mode using positive polarity and peptide match was set to off, and isotope exclusion was on.

2.3.6. Genome sequencing

Isolates were revived from cryostock on Mueller Hinton II agar plates supplemented with 5% defibrinated sheep blood and incubated at 37°C for 24 h. Single colonies were then inoculated in porcine brain heart infusion broth (Becton, Dickinson and Company, Franklin Lakes, NJ) and incubated at 28°C for 18 h with shaking (200 rpm). A 1.5 mL aliquot was concentrated by centrifugation (17,000 $\times$ g for 3 min) and genomic DNA isolated using the PuroSPIN™ Genomic DNA Purification Plus Kit (Luna Nanotech, Ontario, Canada). Libraries were prepared using the Oxford Nanopore Native Barcoding Kit 24 V14 (SQK-NBD114.24; Oxford Nanopore Technologies, Oxford, United Kingdom) and sequenced on a GridIon using R10.4.1 flow cells. Basecalling was performed with the super-accurate model in guppy (v3.5.1). Resulting data were filtered for a minimum length of 1000 bp and minimum quality score of 10 with NanoFilt (v2.8.0) [35] prior to being assembled with Canu (v2.2) [36] and corrected with medaka (v1.8.0) (Oxford Nanopore Technologies Ltd. 2018).

2.3.7. Data Analysis

Statistical analyses for bacterial growth curves were performed in GraphPad Prism v10.0.1. The area under the curve (AUC) was calculated for each growth curve using the mean OD₆₀₀ from the negative control wells as the baseline and compared across bacterial clades and growth temperatures using Brown-Forsythe and Welch ANOVA and Tukey's multiple comparisons test. All growth curves were fit to a nonlinear regression model to allow for calculation of peak OD₆₀₀, maximal growth rate, and lag time. Temperature-dependent and interclade differences in these growth curve parameters were analyzed using Ordinary one-way ANOVA tests and Tukey's multiple comparisons tests. A value of $p < 0.05$ was considered significant for all statistical analyses.

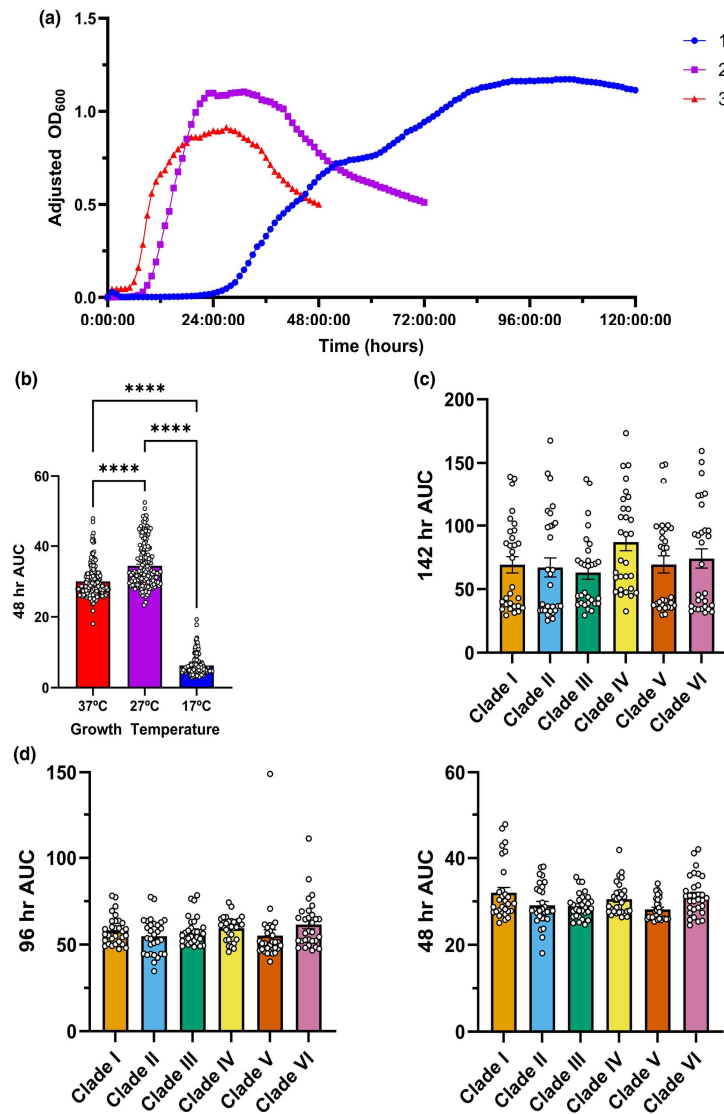
Mass spectrometry data was analyzed using Spectronaut 17.5 (Biognosys Schlieren, Switzerland) using the directDIA workflow with the default settings. Briefly trypsin/P Specific was set for the enzyme allowing two missed cleavages. Fixed Modifications were set for Carbamidomethyl, and variable modification were set to Acetyl (Protein N-term) and Oxidation. For DIA search identification, PSM and Protein Group FDR was set at 0.01%. A minimum of 2 peptides per protein group were required for quantification. Analyses of proteomic data were performed in Spectronaut® (Biognosys Inc, USA) and peptides were compared to the *E. piscicida* Uniprot/TrEMBL database for protein identification. Precursor q-value cutoff was set to 0.01, precursor posterior error probability (PEP) cutoff was set to 0.2, and protein PEP cutoff was set to 0.75. Principal component analysis (PCA) was performed based on protein group quantity of the protein profiles for each sample in Spectronaut. Proteins with a p -value of < 0.05 , as determined by unpaired Student's t-test, and an absolute log₂ ratio ≥ 1 were considered differentially expressed. Virulence factors and adaptive response proteins were identified by comparison with the "Virulence Factors of Pathogenic Bacteria Database" (sequence identity $> 80\%$, e-value $\leq 1e^{-3}$) (VFDB; <http://www.mgc.ac.cn/VFs/>, accessed on 1 November 2023) and with previously reported data based on predicted or demonstrated virulence function in *Edwardsiella* sp. and other Gram negative pathogens [16,18,37–79].

2.4. Results

2.4.1. Growth curve patterns consistently differed based on temperature but not clade

All *E. piscicida* isolates were successfully able to grow and replicate at all three experimental growth temperatures. *Edwardsiella piscicida* presented significant temperature-dependent differences in growth curve patterns in BHI (Figure 2.1). Isolates grown at 17°C had significantly higher ($p<0.05$) mean peak OD₆₀₀ (1.175 AU) and extended mean lag time (24.22 hours) than isolates grown at 27°C (1.064 AU; 5.343 hours) or 37°C (0.9058 AU; 3.271 hours). Isolates grown at 37°C had the lowest mean peak OD₆₀₀ ($p<0.05$) of the three growth temperatures. Isolates grown at 27°C had the fastest mean maximal growth rate ($p<0.05$; 0.03281 AU/hr) whilst isolates grown at 17°C had the slowest mean maximal growth rate ($p<0.05$; 0.01107 AU/hr). There was no significant interclade difference in mean peak OD₆₀₀ for any of the three experimental temperatures. When grown at 17°C, Clade IV *E. piscicida* isolates had the highest mean maximal growth rate of the six bacterial clades ($p<0.05$). However, this trend was not present when bacteria were grown at 27°C or 37°C which demonstrated no significant interclade differences in mean maximal growth rate. Additionally, at 17°C clade II isolates had a significantly extended lag time ($p<0.05$) compared to clade IV and V isolates, but there was no similar trend at 27°C or 37°C for which there were no significant differences in mean lag time.

Figure 2.1. Growth curves for eighteen selected *Edwardsiella piscicida* isolates (three isolates per clade) grown at 17°C, 27°C, and 37°C in BHI. Each isolate was run in quintuplicate per experiment and each experiment was repeated twice. (A) Temperature-dependent growth curve patterns for *E. piscicida*. Each data point represents the mean adjusted OD₆₀₀ at that time point. (B) Temperature-dependent differences in 48-hour AUC. (C) Clade-dependent AUC for *E. piscicida* at 17°C. (D) Clade-dependent AUC for *E. piscicida* at 27°C. (E) Clade-dependent AUC for *E. piscicida* at 37°C.

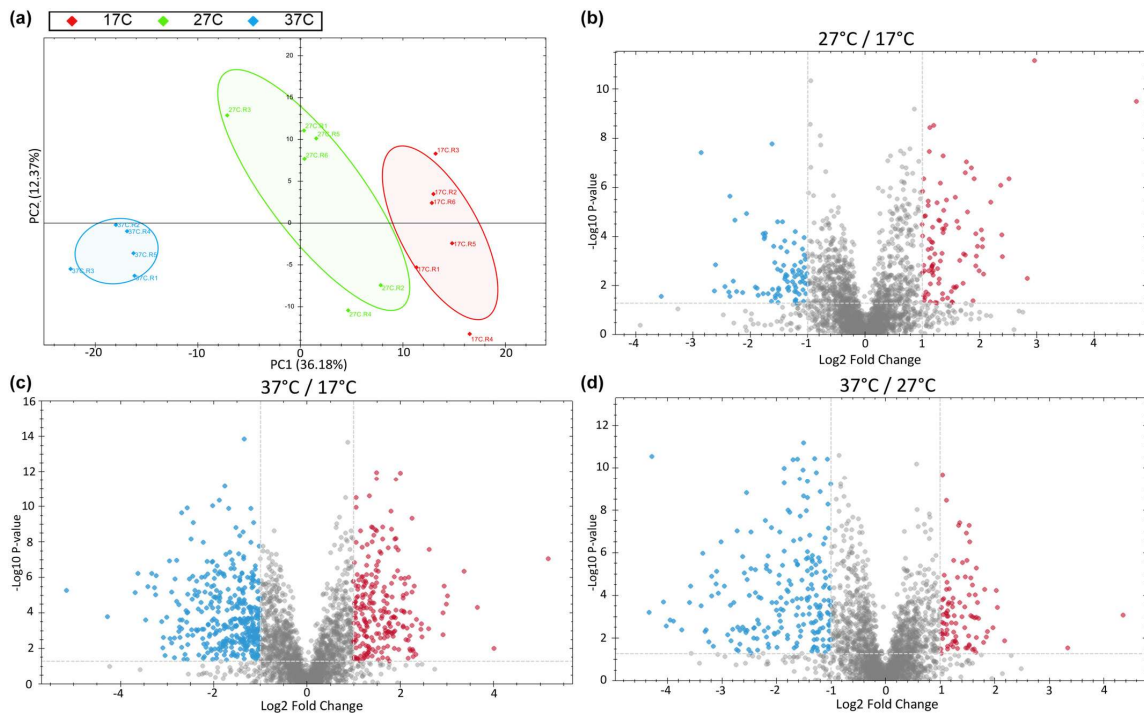


All *E. piscicida* isolates grown had significantly different ($p < 0.05$) 48 hr AUCs among the three growth temperatures (mean AUC 6.289 at 17°C, mean AUC 34.41 at 27°C, and mean AUC 30.02 at 37°C). There was no significant inter-clade difference in AUC at any of the three experimental temperatures.

2.4.2. *Edwardsiella piscicida* displayed temperature-dependent differences in virulence factor abundance

A total of 26,997 unique peptides were identified, which corresponded to 2,739 proteins from the *E. piscicida* Uniprot/TrEMBL database. When comparing across bacterial clades we found no significant or consistent patterns in differential protein abundance. Protein profiles did, however, differ between temperatures, with distinct clusters visible upon principal component analysis (Figure 2.2).

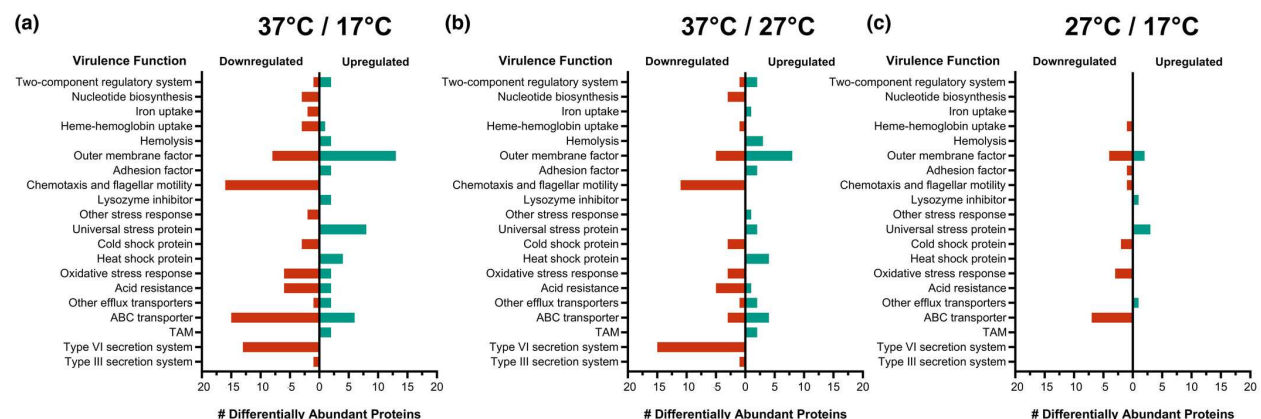
Figure 2.2. Temperature-dependent *in-vitro* proteomic profile of *Edwardsiella piscicida*. (A) Principal Component Analysis (PCA) plot displaying the sample protein profile clustering of the three experimental temperature groups. Volcano plots depicting differential abundance of proteins based on growth temperature (Group 1 / Group 2): (B) 27°C / 17°C; (C) 37°C / 17°C; (D) 37°C / 27°C. Proteins with significantly increased relative abundance ($\text{Log}_2 \text{ ratio} \geq 1, p < 0.05$) are depicted in red and proteins with significantly decreased relative abundance ($\text{Log}_2 \text{ ratio} \leq -1, p < 0.05$) are depicted in blue.



When evaluating protein regulation based on bacterial growth temperature, the greatest number of differentially abundant proteins (absolute $\log_2$ ratio ≥ 1 ; $p < 0.05$) was identified when comparing isolates grown at 37°C to isolates grown at 17°C (595 differentially abundant proteins) (Figure 2.2; Supplemental

Table 2.1). Comparing isolates grown at 37°C to isolates grown at 27°C identified 298 differentially abundant proteins, whilst the least number of differentially abundant proteins (180) was identified when comparing isolates grown at 27°C to isolates grown at 17°C. Of the 1,074 comparisons which identified significantly differentially abundant proteins, 150 were identified as virulence factors and adaptive response proteins (Figure 2.3; Supplemental Table 2.2) and included proteins such as ATP-binding cassette (ABC), flagellin, and Type VI secretion system effectors.

Figure 2.3. Summary of differential *Edwardsiella piscicida* virulence factor regulation based on *in-vitro* growth temperature (Group 1 / Group 2): (A) 37°C / 17°C; (B) 37°C / 27°C; (C) 27°C / 17°C. Upregulated proteins for each comparison were defined as those with a log2 ratio ≥ 1 and $p < 0.05$ (depicted in green). Downregulated proteins for each comparison were defined as those with a log2 ratio ≤ -1 and $p < 0.05$ (depicted in red).



Both structural and effector T6SS proteins were downregulated at 37°C compared to 27°C and 17°C. Additionally, proteins associated with chemotaxis, flagellar motility, nucleotide biosynthesis, oxidative stress response, and acid resistance were downregulated at 37°C compared to 27°C and 17°C. Conversely, at 37°C, *E. piscicida* upregulated production of proteins associated with adhesion, hemolysis, and the translocation and assembly module (TAM). The proteomic profiles of several crucial outer membrane proteins differed depending on the bacterial growth temperature, with both OmpA and OmpW displaying increased abundance with increasing growth temperature. Unsurprisingly, cold shock proteins

were upregulated at 27°C and 17°C, compared to 37°C, whilst heat shock proteins and universal stress proteins were upregulated at 37°C.

2.5. Discussion

Results indicate growth of *E. piscicida* at cooler temperatures (17°C) is slower, with an extended lag phase and reduced maximal growth rate than at higher temperatures. Conversely, although isolates grown at 37°C demonstrated a faster growth rate than isolates at 17°C, they had a reduced maximal OD₆₀₀ and were quicker to enter the death phase of bacterial growth than the other two experimental temperatures. These results concur with previous findings of an optimum growth temperature for *E. piscicida* around 28°C and 30°C, as isolates in the current study grown at 27°C displayed the fastest maximal growth rate of any of the three experimental temperatures, had a shorter lag phase than isolates grown at 17°C, and had a peak OD₆₀₀ equivocal to isolates grown at 17°C [8,9]. Thus, overall growth of *E. piscicida* appeared to be reduced and/or limited at cooler water temperatures (17°C) or mammalian physiologic temperatures (37°C) when compared to warmer water temperatures (27°C). Interestingly, no consistent interclade differences in bacterial growth patterns were observed.

Proteomic analysis identified significant alterations in the regulation of several crucial virulence factors between *E. piscicida* isolates grown at mammalian temperatures (37°C) and isolates grown at cool-water (17°C) and warm-water (27°C) temperatures. Many vital virulence mechanisms, including the T6SS, chemotaxis, flagellar motility, oxidative stress response, and acid resistance response, were downregulated at 37°C compared to 27°C and 17°C. The T6SS of *E. piscicida* is comprised of multiple structural proteins forming a contractile injection apparatus that facilitates transport of effectors directly into target host cells for bacterial adhesion, invasion, and intracellular replication (Li et al., 2021). Functional T6SS plays a pivotal role during *in vivo* *E. piscicida* infection and is essential for full virulence of *E. piscicida* [81]. These results showed downregulation of both structural and effector T6SS proteins at mammalian temperatures

compared to warm or cold-water temperatures, which is consistent with previous findings of decreased T6SS protein secretion and effector gene expression at 37°C compared to 25°C due to temperature-dependent transcriptional control by the two-component system PhoP-PhoQ [22]. This finding suggests that *E. piscicida* may have decreased virulence in a mammalian system due to impaired T6SS-mediated invasion into and replication within host macrophages. Furthermore, survival within macrophages by *E. piscicida* may be further impaired by temperature-dependent downregulation of oxidative stress and acid resistance proteins at mammalian physiological temperatures. Interestingly, this study only identified one T3SS protein, CesD/SycD/LcrH family type III secretion system chaperone, with altered temperature-dependent regulation in contrast to previous studies which observed decreased expression of T3SS effector proteins at 37°C compared to 25°C [23]. In contrast to previous studies [23], all other *E. piscicida* T3SS effector and structural proteins detected in this study demonstrated similar abundance across the three experimental growth temperatures in the presented study (Supplemental Table 2.3). Additionally, several of these T3SS proteins, such as EsaG and EsaG, were detected in relatively lower abundances compared to the average protein quantity and were inconsistently detected across samples. One possible explanation for this finding is that different culture conditions could impact the presence of signals necessary to activate transcription and translation of T3SS effectors. As bacteria in this experiment were grown in extracellular growth medium conditions, there may be intracellular signals that would significantly alter the production of T3SS proteins *in vivo* (Zhang et al., 2023).

Major chemotaxis proteins (CheY, CheZ, CheW, CheA) and flagellar proteins (flagellin, FliN, FliM) were downregulated at 37°C compared to 27°C or 17°C. This finding could suggest decreased virulence at mammalian temperatures due to impaired chemotactic, motility, and invasion capabilities. This is consistent with reports by Griffin et al. (2020) which indicated limited pathogenesis for *E. piscicida* in a murine model. Alternatively, other opportunistic environmental bacterial pathogens are known to utilize temperature as a signal to control expression of flagellar motility genes when transitioning between ambient temperature environments and warm-blooded hosts. *Listeria monocytogenes* is a foodborne/waterborne

agent of gastrointestinal illness that utilizes its flagella for motility and persistence in the extracellular environment and suppresses flagellar motility genes after infection, in response to mammalian physiologic temperature, in order to facilitate full virulence [24]. Additionally, many Gram-negative opportunistic pathogens are known to utilize environmental signals such as temperature to trigger a switch from a motile, planktonic state to a sessile, biofilm state either to facilitate host cell colonization or as a survival response to stressors [82]. The concurrent downregulation of flagellar motility and chemotactic proteins and upregulation of adhesion factors and universal stress proteins identified in this study could indicate a transition by *E. piscicida* from a motile form at cool-water or warm-water temperatures to a biofilm mode at 37°C as is seen in marine *Bacillus* species in which biofilm formation is enhanced at 37°C to facilitate survival and environmental persistence [83], or could suggest a host adaptative response for enhanced adhesion to mucosal surfaces and evasion of host immune defenses as is seen in other food and water-borne enteric pathogens such as *Yersinia enterocolitica* and *Escherichia coli* [84,85]. This second scenario is supported by the current study, with upregulation of OmpW at mammalian physiologic temperatures, consistent with previous studies linking OmpW to adaptive responses in *E. coli* when transitioning from the environment to a warm-blooded host [86]. Further investigation into the *in vitro* and/or *in vivo* impact of these temperature-dependent alterations in virulence factors expression on *E. piscicida* pathogenicity is warranted to better understand the role of temperature in the pathogenesis of piscine edwardsiellosis and to further characterize the potential zoonotic risk of *E. piscicida*.

2.6. References

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CHAPTER 3: Use of Shotgun Immunoproteomics for the Development of Protein Vaccines Against *Edwardsiella piscicida*

3.1. Abstract

Edwardsiella piscicida is an important emerging pathogen in various cultured fish species. This study aimed to identify immunogenic *E. piscicida* proteins and evaluate these antigens as protein vaccines for use in aquaculture. Shotgun immunoproteomics using anti-*E. piscicida* serum from rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) (♀) × blue catfish (*I. furcatus*) (♂) hybrids inoculated with formalin-killed whole-bacteria preparations identified 36 candidate immunogenic *E. piscicida* proteins. The chaparonin GroEL, the glycine 2TM zipper domain-containing protein (GlyZip), and coproporphyrinogen-III oxidase (COPIII); were used to orally (PO) and intra-coelomically (IC) immunize Chinook salmon (*O. tshawytscha*). Fish IC vaccinated with either GlyZip or COPIII demonstrated a slight, but non-significant, improvement in survival post-challenge with *E. piscicida* S11-285. Surprisingly, fish IC or PO vaccinated with GroEL displayed an anti-protective effect against subsequent challenge. All IC vaccinated fish generated a strong specific antibody response against the immunizing protein, and sham vaccinated fish challenged with *E. piscicida* S11-285 generated a significantly higher specific antibody response to the GroEL and GlyZip proteins than negative control fish, suggesting that shotgun immunoproteomics was effective for detection of immunogenic bacterial proteins that can stimulate humoral immune responses in the host fish.

3.2. Introduction

Infectious diseases are one of the most important threats to global aquaculture, reducing the growth of farmed animals and causing devastating economic losses from morbidities and mortalities. Disease caused by *Edwardsiella* spp. are estimated to cause annual economic losses of over \$50 million/year to the US aquaculture industry [1]. This genus of gram-negative bacteria consists of five distinct species, four of

which are known to infect and cause disease in fish. *Edwardsiella ictaluri* is the etiologic agent of enteric septicemia of catfish (ESC) and is often associated with catastrophic losses of commercially cultured ictalurid catfish [2]. Piscine edwardsiellosis is caused by infection with one of the other three fish-pathogenic *Edwardsiella* species: *E. tarda*, *E. anguillarum*, and *E. piscicida*. The disease is characterized by generalized septicemia and chronic granulomatous inflammation affecting a wide range of both marine and freshwater fish species [2,3].

In the past decade, *E. piscicida* has been increasingly associated with outbreaks of acute septicemia and mass mortality in economically important farmed fish species in North America, including catfish (*Ictalurus spp.*), trout (*Oncorhynchus spp.*), barramundi (*Lates calcarifer*), and largemouth bass (*Micropterus nigricans*) [4–7]. *Edwardsiella piscicida* is a genetically diverse pathogen with at least 6 distinct phylogenetic lineages with experimentally demonstrated host-specific differences in pathogenicity [8]. Traditionally, management of edwardsiellosis in aquaculture has relied on the use of FDA-approved chemotherapeutics to treat outbreaks [2]. However, increasing evidence of *Edwardsiella* antibiotic resistance genes and associated conjugative plasmids, as well as a growing demand among consumers for antibiotics-free food, highlights a crucial need for alternative methods of disease control for *E. piscicida* [6,9–11].

Vaccination against several of the most economically important fish diseases, including furunculosis, vibriosis, and infectious salmon anemia virus, has historically proven efficacious in protecting against infection and significantly reducing antibiotic usage in aquaculture [12]. An experimental live-attenuated vaccine (LAV) against *E. ictaluri* is currently available in the U.S. under veterinary prescription and has been shown to be effective in preventing ESC among cultured catfish [13]. Currently, however, no commercially available vaccine or prophylactic protocols have been established for the prevention of *E. piscicida* in US aquaculture.

Protein vaccines, composed of one or more pathogen-derived protein antigens, represent a promising alternative to traditional treatment and vaccine modalities for prevention of piscine

edwardsiellosis. One of the major advantages of protein vaccines is the improved safety compared to live-attenuated vaccines. Not only do protein vaccines lack the ability to reverse to a pathogenic state, but as they do not contain live pathogen, there is also no risk of pathogen shedding into the environment and spreading to potentially susceptible non-target species as can occur with LAVs [13,14]. Recently developed adjuvants, such as Montanide™ IMS 1312 VG (Seppic, France), as well as new protocols which enhance antigen uptake, such as liposomes and nanoparticles, have paved the way for further investigation into the novel use of protein vaccines for stimulation of cell-mediated immunity and long-term protection against intracellular pathogens such as *E. piscicida* [14–17]. In addition, because protein vaccines can be freeze-dried, they are much more shelf-stable than live-attenuated vaccines, which can be particularly important for vaccine implementation in regions where reliable cold-chain storage and transport is difficult [18].

One of the major barriers to developing effective protein vaccines is identification of immunogenic candidate antigens. Traditional strategies for antigen detection involve time-consuming and/or low throughput methodologies that often fail to account for native properties of the antigenic proteins [19,20]. *In silico* reverse vaccinology methods can be used to predict potential antigens using the pathogen genome to guide selection of candidate proteins, but accuracy of these results is dependent on the quality of the genome sequences and the accuracy of the bioinformatics programs used [21]. Additionally, results from these techniques remain informed predictions that require experimental validation to demonstrate immunogenicity. With this in mind, we elected to evaluate whether a shotgun immunoproteomic approach could be utilized to streamline screening of the bacterial proteome for immunogenic proteins and whether these proteins are effective at inducing protective immunity in fish against infection with *E. piscicida*. This approach, which was originally developed for the detection of antigenic B cell epitopes in *Francisella tularensis* and *Burkholderia pseudomallei*, involves the purification and characterization of MHC-bound bacterial peptides via liquid chromatography tandem mass spectrometry (LC-MS/MS) [19]. Identified peptides can then be immediately synthesized and tested for efficacy, thereby accelerating the protein vaccine development process. This workflow has demonstrated efficacy in identifying antigenic proteins

from bacterial pathogens for mammalian species and thus presents a promising technology for the development of aquaculture vaccines [19,22].

3.3. Materials and Methods

3.3.1. Experimental Animals

Rainbow trout (*O. mykiss*), ~30-50 g in size, were obtained from the Mount Shasta Fish Hatchery (Siskiyou County, CA, USA) and arbitrarily divided into experimental and control tanks (n = 12 trout/tank) and maintained indoors at the Center for Aquatic Biology and Aquaculture (Davis, CA, USA) in a flow-through system at a rate of 4 L/min at 18±1°C supplied with supplemental aeration at a rate of 1 L/min. Channel catfish (*I. punctatus*) and hybrid catfish (channel catfish (♀) × blue catfish (*I. furcatus*) (♂) hybrids) were obtained as fry from a local producer and reared indoors at the Thad Cochran National Warmwater Aquaculture Center (Stoneville, MS, USA). Catfish, ~80 – 100 g in size, were arbitrarily divided into experimental and control tanks (n = 12 catfish/tank) and maintained in 80 L aquaria containing 22 L of flow-through ground water at 25±1°C supplied with supplemental aeration at a rate of 1 L/min. For the protein vaccine trials, Chinook salmon (*O. tshawytscha*) obtained from the Nimbus Fish Hatchery (Gold River, CA, USA) were arbitrarily divided into experimental and control tanks (n = 12 salmon/tank) and maintained indoors at the Center for Aquatic Biology and Aquaculture (Davis, CA, USA) in a flow-through system at a rate of 2 L/min at 18±1°C supplied with supplemental aeration at a rate of 1 L/min. All fish were fed a commercial extruded diet at 1% body weight per day.

Prior to all injections and blood collection procedures, trout were anesthetized via immersion in a bath containing 50 mg/L tricaine methanesulphonate (MS-222) buffered 1:1 with sodium bicarbonate, salmon were anesthetized via immersion in a bath containing 75 mg/L buffered MS-222, and catfish were anesthetized via immersion with 100 mg/L buffered MS-222.

3.3.2. Anti-Edwardsiella piscicida Serum Generation

Twelve archived isolates of *E. piscicida* collected from clinical cases of piscine edwardsiellosis in the United States were used in this study to generate whole cell bacterins for immunization of rainbow trout and catfish (Table 3.1). Isolates were revived from frozen stock on trypticase soy agar supplemented with 5% sheep blood (SBA, University of California Davis, Biological Media Services, USA) and grown overnight at 28°C. Bacterial colonies were harvested from SBA and resuspended in sterile PBS, then bacterial concentrations in all experiments were estimated spectrophotometrically (BioPhotometer Plus, Eppendorf AG) where a 0.5 McFarland standard of $\sim 10^8$ CFU/mL corresponded to an optical density at 600 nm (OD_{600}) of ~ 0.15 in phosphate-buffered saline (PBS). Bacteria quantity was confirmed via serial dilution and spot-plating of 10 μ L in triplicate on SBA for colony enumeration. Whole cell bacterins were generated for each *E. piscicida* isolate through inactivation with 0.5% formalin at 4°C for 48 hours with constant shaking. After incubation, samples were centrifuged at $3260 \times g$ for 30 min at room temperature and washed with PBS three times, centrifuging between each wash, then diluted in PBS to an OD of ~ 0.15 . Formalin-killed bacterial suspensions were stored at 4°C until use. Inactivation of bacteria was confirmed by inoculation of each sample on SBA. Then, for each isolate, 3 mL of the formalin-killed bacterial suspension was mixed with 7 mL of Montanide™ ISA 763 A (Seppic, France) adjuvant using an IKA Ultra-Turrax® Tube Drive Workstation (IKA Work, Inc., Wilmington, USA) at high speed for 30 s to generate the final vaccine preparations. The same method was used to generate sham vaccines containing 3 mL sterile PBS and 7 mL adjuvant for injection of negative control fish. All preparations were protected from light and stored at 4°C.

Trout and catfish were inoculated via intra-coelomic (IC) injection of 0.1 mL of either the formalin-killed bacteria preparation in adjuvant or the PBS sham vaccine using a 1 mL luer-lock syringe with a 25-gauge 1" (25.4 mm) needle (n = 12 fish/treatment). This procedure was repeated 4 weeks later to booster all fish.

Table 3.1. List of *Edwardsiella piscicida* isolates and doses used in trout and catfish immunization.

Isolate	Clade	Source	OD	CFU/mL	Vaccine Group	References	NCBI Accession
6-30 IP D16-004	Clade I	Largemouth bass (<i>Micropterus salmoides</i>)	0.151	3.8 x 10 ⁸	V1A	Nguyen et al. (2021)	CP150945-CP150946
34-68†	Clade I	Atlantic salmon (<i>Salmo salar</i>)	0.158	1.3 x 10 ⁸	V1B	Nguyen et al. (2021)	CP150944
S11-233†	Clade II	Channel catfish (<i>Ictalurus punctatus</i>)	0.157	1.8 x 10 ⁸	V2A	Griffin et al. (2014)	CP150940
A15-1679	Clade II	Blotched fantail stingray (<i>Taeniura meyeni</i>)	0.163	6.0 x 10 ⁸	V2B	Camus et al. (2016)	CP150939
32-97	Clade III	Atlantic salmon (<i>S. salar</i>)	0.157	1.2 x 10 ⁸	V3A	Nguyen et al. (2021)	CP150938
35-70†	Clade III	Atlantic salmon (<i>S. salar</i>)	0.166	2.8 x 10 ⁸	V3B	Nguyen et al. (2021)	CP150937
S11-285	Clade IV	Channel catfish (<i>I. punctatus</i>)	0.153	6.7 x 10 ⁸	V4A	Griffin et al. (2014)	CP150934-CP150935
S12-281†	Clade IV	Channel catfish (<i>I. punctatus</i>)	0.154	1.5 x 10 ⁸	V4B	Griffin et al. (2019)	CP150931-CP150932
C1490	Clade V	Largemouth bass (<i>M. salmoides</i>)	0.154	1.5 x 10 ⁸	V5A	Fogelson et al. (2016)	CP150927-CP150928
LADL99-462†	Clade V	Channel catfish (<i>I. punctatus</i>)	0.150	6.2 x 10 ⁸	V5B	Griffin et al. (2014)	CP150925-CP150926
S16-278	Clade VI	Hybrid catfish (♀ <i>I. punctatus</i> × ♂ <i>I. furcatus</i>)	0.165	1.5 x 10 ⁸	V6A	Griffin et al. (2019)	CP150923-CP150924
MA97-004†	Clade VI	Tilapia (<i>Tilapia</i> sp.)	0.160	5.2 x 10 ⁸	V6B	Griffin et al. (2014)	CP150922

† Isolates were used for whole blood survival assays.

At 2 weeks post-booster, blood was collected from all fish via caudal venipuncture using a 3 mL or 1 mL luer-lock syringe with a 25-gauge 1” needle. Blood was immediately placed in a red top tube and stored at 4°C for at least 1 hour to allow blood to clot. After clotting, samples were centrifuged at 1449 × g for 15 min and serum was collected and stored at -20°C. Following blood collection, fish were allowed to recover from anesthesia in their experimental tanks and were maintained for a further 2 weeks to allow for blood collection for whole blood bactericidal survival assays.

No mortalities among fish immunized with formalin-killed *E. piscicida* had evidence of *E. piscicida* infection on gross necropsy or microbial culture and were instead attributed to cohort aggression and/or power outages associated with inclement weather events.

3.3.3. Whole Bacteria Enzyme-linked Immunosorbent Assay (ELISA)

An ELISA was performed using protocols adapted from Heckman et al. [23] to determine the specific IgM response against the formalin-killed *E. piscicida* preparations. Briefly, a 0.5 McFarland standard of *E. piscicida* isolate S11-285 in carbonate-bicarbonate buffer (OD₆₀₀ 0.15) was generated and then 100 µL of the bacterial suspension was added to each well of Immulon® 2HB Flat Bottom microtiter® (Thermo Fisher Scientific, USA) 96-well plates and plates incubated overnight at 4°C. Following incubation, plates were washed three times with low salt wash buffer (LSWB; 0.02 M Trizma base, 0.38 M NaCl, 0.05% (v/v) Tween-20, pH 7.2). Following the low salt wash, 250 µL of blocking buffer (5% w/v skim milk powder in distilled water) was added to each well. Plates were then incubated at room temperature for 3 hours and subsequently washed 3x with LSBW. Serum from immunized and control fish were diluted 1:200 in LSBW containing 1% bovine serum albumin (BSA; Sigma-Aldrich, USA). One hundred µL of serum dilutions were added in duplicate to their respective wells and 100 µL of sterile PBS was added to blank control wells. Plates were incubated at 4°C overnight. After incubation, plates were washed 3x with high-salt wash buffer (HSWB; 0.02 M Trizma base, 0.5 M NaCl, 0.01% (v/v) Tween-20, pH 7.7). For salmonid samples, mouse anti-trout IgM monoclonal antibodies [24] were diluted to 1:200 in sterile PBS and 100 µL of the dilution was added to each respective well. For catfish samples, 9E1 mouse anti-catfish IgM monoclonal antibodies [25], kindly donated by Dr. Eva Bengtén (Department of Cell & Molecular Biology, University of Mississippi Medical Center, Jackson, MS, USA), were diluted 1:500 in sterile PBS. Plates were incubated at room temperature for 2 h and then washed 3x in HSWB. Goat anti-mouse IgG (H+L), horseradish peroxidase conjugate (Thermo Fisher Scientific) was diluted 1:3000 in LSBW with 1% BSA and 100 µL of the dilution was added to each respective well and then plates were incubated for 2 hours at room temperature. Following incubation, plates were washed 3x using HSWB. The

KPL ABTS® Peroxidase Substrate (SeraCare, USA) was warmed to room temperature and 100 µL of the substrate was added to each well, then plates were incubated for 1 h at room temperature. 100 µL of ABTS® Peroxidase Stop Solution (SeraCare) was added to each well and then the OD₄₁₀ of the plates was measured and adjusted against PBS controls.

3.3.4. Whole Blood Bacterial Survival Assay

At 4 weeks post-booster, blood was collected from experimental and control fish (n = 3 - 5 per tank) via the caudal vasculature and immediately placed in lithium heparinized blood collection tubes to prevent clotting. Samples were maintained at room temperature with constant gentle inversion until processing. One-hundred µL of heparinized whole blood from each fish was aliquoted in septuplicate in a 96-well plate and 100 µL of sterile PBS was aliquoted in triplicate on each plate to serve as a reagent control. Then, 0.5 McFarland standards of one representative isolate per bacterial clade (Table 3.1) in PBS were generated. The CFU of the initial inoculum was confirmed to $\sim 1.5 \times 10^8$ CFU/mL via spot plating on SBA and colony enumeration. Two µL of each 0.5 McFarland standard ($\sim 10^5$ CFU) was added to respective wells, with one well of whole blood per fish inoculated with sterile PBS. Plates were then incubated for 24 hours at 20°C for trout whole blood and 28°C for catfish whole blood with constant orbital shaking. Following incubation, each well was serially diluted in sterile PBS and spot plated on SBA, then incubated overnight at 28°C for colony enumeration. When post-treatment samples were negative for the presence of bacteria on SBA, the limit of detection (LOD), defined here as 1 CFU at the lowest dilution, had been reached [11,12]. Samples at the LOD were considered less than or equal to the calculated LOD and were assigned a value of 1/2 LOD. Log reduction in bacterial growth was calculated using the following equation:

$$\text{Log Reduction} = \log_{10}(\text{Inoculum, CFU/mL}) - \log_{10}(\text{Final, CFU/mL})$$

3.3.5. Protein Extraction

The immunoproteomic workflow utilized in this study (Figure 3.1) was adapted from protocols described by D'haeseleer et al. and Gates et al. [19,22]. *Edwardsiella piscicida* isolate S11-285 was grown

to the late-exponential phase of growth in triplicate 5 mL volumes of BHI at 27°C with constant orbital shaking. Following incubation, samples were pelleted via centrifugation at $3260 \times g$ for 25 min and bacterial proteins were extracted in B-PER™ reagent (Thermo Fisher Scientific) and inclusion body solubilization solution (IBS) as previously described [28].

3.3.6. Affinity Purification and Peptide Preparation

Immunogenic proteins were purified according to manufacturer recommended protocols for the Dynabeads™ Protein G Immunoprecipitation Kit (Thermo Fisher Scientific) with specified modifications. For trout serum samples, mouse anti-trout IgM monoclonal antibodies were diluted 1:200 in Antibody Washing and Binding buffer and then 200 µL of the resulting dilutions were added to 50 µL aliquots of Dynabeads™ Protein G. For hybrid catfish serum samples, mouse anti-catfish IgM monoclonal antibodies were diluted to 1:500 in Antibody Washing and Binding buffer. Samples containing antibodies and Protein G coated magnetic beads were incubated at room temperature for 10 min with constant rotation to allow for binding of the mouse IgG antibodies to the beads. Following incubation, samples were placed on a magnet for separation of beads and the supernatant was discarded. Six pools of vaccinated serum samples for trout and for hybrid catfish were formed from pooling the serum of the five fish with the highest antibody response per vaccine group clade (V1-V6; Table 3.1) as determined by serum ELISA. Similar pools of serum from five negative control fish were generated from rainbow trout and hybrid catfish serum. The pooled serum samples (seven pools of rainbow trout serum and seven pools of hybrid catfish serum) were diluted 1:200 in Antibody Washing and Binding buffer and 200 µL of the resulting dilutions were mixed with the antibody-bound magnetic beads. Samples were incubated for 10 min at room temperature with constant rotation and then placed on a magnet for removal of the supernatant. Sterile PBS (100 µL) was added to each tube and then 50 µg of the B-PER™ extracted bacterial proteins and 50 µg of the IBS extracted bacterial proteins were added to each tube and gently mixed via pipetting. Tubes were incubated at room temperature for 1 h with constant rotation. After incubation, tubes were placed onto the magnet for disposal of the supernatant and the beads were washed three times with washing buffer. After washing, the

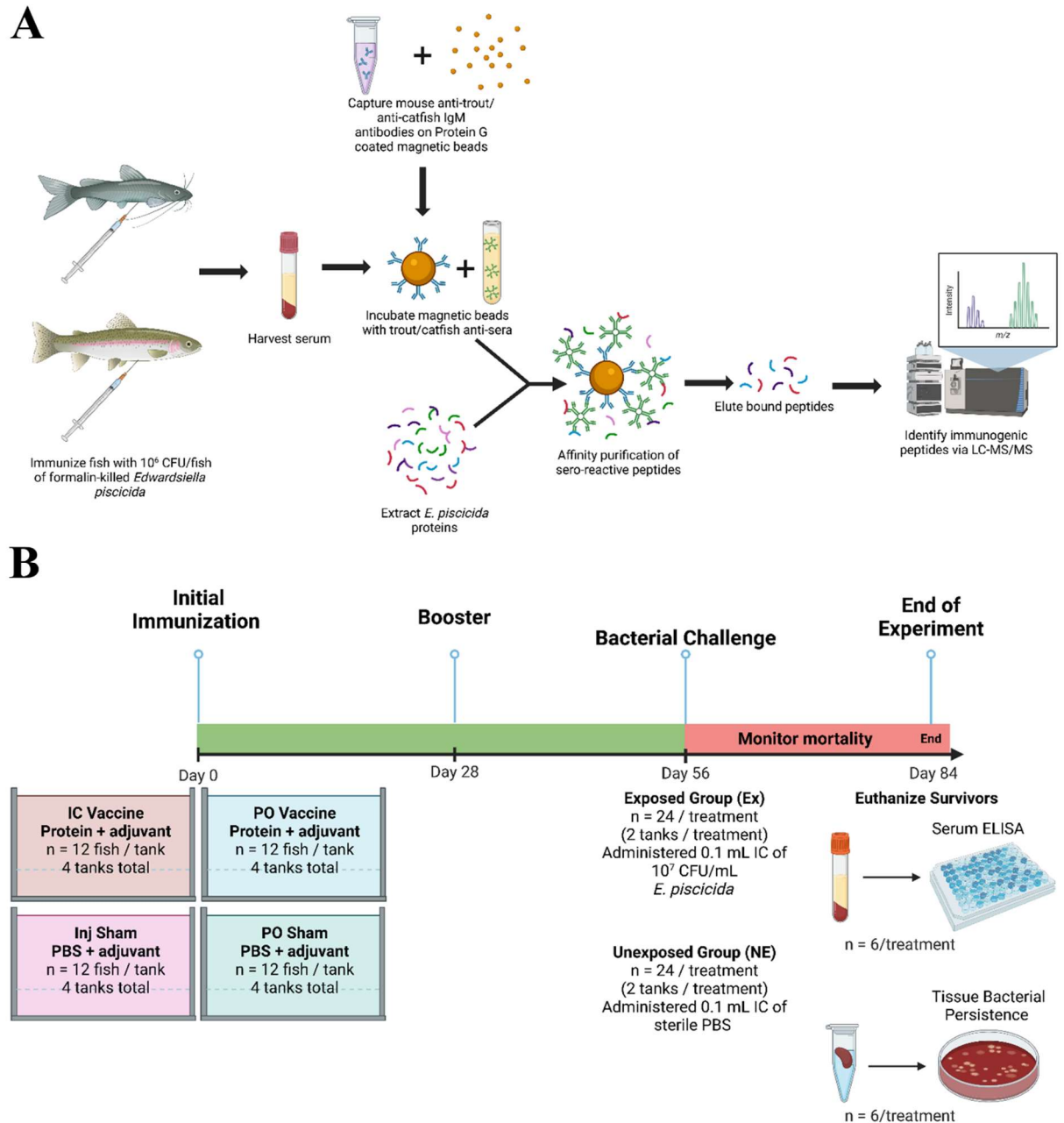
beads were transferred to clean Eppendorf tubes and then three buffer exchanges using 100 μ L of 50 mM tetraethylammonium bicarbonate (TEAB) and shaking for 20 min between each exchange were performed. After the last buffer exchange, trypsin was added to the TEAB in a 1:100 ratio and the solution was added to each bead sample at a volume of about 25 μ L over the beads. The samples were then incubated at room temperature overnight with constant gentle shaking. After incubation, the supernatant for each sample was removed and saved in clean tubes. Beads were then washed again with enough of the 1:100 trypsin and TEAB solution to just cover the beads, then shaken for 20 min after which the supernatant was removed and saved with the previous extract.

The peptide extracts were submitted to the UC Davis Proteomics Core for digestion and protein characterization via liquid chromatography tandem mass spectrometry (LC-MS/MS).

3.3.7. Liquid Chromatography Tandem Mass Spectrometry

Peptides were resolved on a Thermo Scientific Dionex UltiMate 3000 RSLC system (Thermo Fisher Scientific) using a PepSep analytical column (PepSep, Denmark): 150 μ m $\times$ 8 cm C18 column with 1.5 μ m particle size (100 Å pores), preceded by a Pepmap Neo C18 5UM guard column, and heated to 40 °C. Separation was performed in a total run time of 30 min with a flow rate of 500 μ L/min with mobile phases A: water/0.1% formic acid and B: 80% ACN/0.1% formic acid.

Figure 3.1. Overview of experimental design for (A) shotgun immunoproteomic identification of immunogenic *Edwardsiella piscicida* proteins and (B) *In vivo* vaccine trials experimental set up. Two protein vaccine trial experiments were conducted with the first trial evaluating the GroEL protein vaccines in chinook salmon and the second trial assessing both the COPIII and GlyZip protein vaccines in Chinook salmon. Figures generated in Biorender.



3.3.8. Mass Spectrometry Data Independent Analysis

For the trout samples, peptides were directly eluted onto an Orbitrap Exploris 480 instrument (Thermo Fisher Scientific). Spray voltage was set to 1.8 kV, funnel RF level at 45, and heated capillary temperature at 275°C. Experiments full mass spectrometry (MS) resolution was set to 60,000 at m/z 200 and full MS automatic gain control (AGC) target was 300% with an IT of 45 msec. Mass range was set to 350–1200. AGC target value for fragment spectra was set at 1000%. Mass Windows of 22 Da were used with an overlap of 1 Da. Resolution was set to 30,000 and IT to Auto. Normalized collision energy was set at 30%. All data were acquired in profile mode using positive polarity and peptide match was set to off, and isotope exclusion was on.

For the catfish samples, mass spectrometry was performed on a hybrid trapped ion mobility spectrometry-quadrupole time of flight mass spectrometer (timsTOF HT, (Bruker Daltonics, Bremen, Germany) with a modified nano-electrospray ion source (CaptiveSpray, Bruker Daltonics). In the experiments described here, the mass spectrometer was operated in PASEF mode. Desolvated ions entered the vacuum region through the glass capillary and deflected into the TIMS tunnel which is electrically separated into two parts (dual TIMS). Here, the first region is operated as an ion accumulation trap that primarily stores all ions entering the mass spectrometer, while the second part performs trapped ion mobility analysis. The dual TIMS analyzer was operated at a fixed duty cycle close to 100% using equal accumulation and ramp times of 85 ms each. Data-independent analysis (DIA) scheme consisted of one MS scan followed by MSMS scans taken with 36 precursor windows at width of 25Th per 1.09 sec cycle, over the mass range 300-1200 Dalton. The TIMS scans layer the doubly and triply charged peptides over an ion mobility $-1/k_0$ - range of 0.7-1.3 V*sec/cm². The collision energy was ramped linearly as a function of the mobility from 59 eV at $1/K_0=1.4$ to 20 eV at $1/K_0=0.6$.

DIA data was analyzed using Spectronaut 17.5 (Biognosys Schlieren, Switzerland) using the directDIA workflow with the default settings. Briefly trypsin/P Specific was set for the enzyme allowing two missed cleavages. Fixed Modifications were set for Carbamidomethyl, and variable modification were

set to Acetyl (Protein N-term) and Oxidation. For DIA search identification, PSM and Protein Group FDR was set at 0.01%. A minimum of 2 peptides per protein group were required for quantification.

3.3.9. Protein Antigen Selection

Proteins were determined to be immunogenic if they had increased abundance in the affinity purified samples from immunized fish compared to negative control fish (absolute log₂ ratio ≥ 1 ; $p < 0.05$). The gene ontology (GO) resource GORetriever (available at AgBase [29]) was used for obtaining biological process and molecular functional annotations of immunogenic proteins. PSORTb v3.0.3 [30] was used to predict the subcellular locations of differentially abundant proteins. Potential virulence function of candidate proteins were predicted using the “Virulence Factors of Pathogenic Bacteria Database” (VFDB; <http://www.mgc.ac.cn/VFs/>, accessed on 1 November 2023) [31]. Antigenicity of the candidate protein sequences was analyzed using the VaxiJen v2.0 online tool (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>), with a prediction accuracy threshold of 0.4 [32].

The coding DNA sequence for each immunogenic protein was identified from the genomes of the twelve selected *E. piscicida* isolates used to generate the formalin-killed vaccine preparations using the BLAST tool [33] in the Geneious Prime v.2023.2.1 software (Biomatters, CA, USA). The 12 sequences for each protein were then translated and the amino acid sequence similarity between *E. piscicida* S11-285, the isolate used for protein extraction in the shotgun immunogenic workflow, and the other 11 sequences were determined by a percent identity matrix following MAFFT alignment [34] using Geneious Prime.

3.3.10. Recombinant Protein Expression

The cDNA sequences for candidate proteins (Supplemental Table 3.3) selected based on subcellular localization, predicted virulence function, predicted antigenicity, and high amino acid sequence conservation were submitted to Biomatik (ON, Canada) for *Escherichia coli* expression of N-terminally His-Tagged protein. Purified protein concentrations were determined by Bradford protein assay and purity was confirmed via SDS-PAGE. Purified recombinant protein was stored at -80 °C.

3.3.11. Protein Antigen Enzyme-linked Immunosorbent Assay (ELISA)

Indirect protein antigen ELISAs to determine the serum reactivity of immunized trout and catfish to the purified recombinant proteins were performed using similar methods as with the whole bacteria ELISAs, with specified modifications. Purified recombinant protein was diluted to 10 µg/mL in carbonate-bicarbonate buffer to serve as the coating antigen.

3.3.12. In-vivo Vaccine Trials

To generate protein vaccine preparations for immunization of Chinook salmon, purified recombinant protein was reconstituted in sterile PBS to a concentration of 333.3 µg/mL. For the injectable vaccine preparations, the protein suspensions were mixed 3:7 with Montanide™ ISA 763 A adjuvant using an IKA Ultra-Turrax® Tube Drive Workstation at high speed for 30 sec (3 mL of 333.3 µg/mL protein suspension mixed with 7 mL of adjuvant to generate 100 µg protein/mL vaccine preparations). For the oral vaccine preparations, the protein suspensions were mixed 3:7 with Montanide™ GR1 (Seppic, France) adjuvant (3 mL of 333.3 µg/mL protein suspension mixed with 7 mL of adjuvant to generate 100 µg protein/mL vaccine preparations). Similar methods were used to generate injectable and oral sham vaccines containing PBS and adjuvant (3 mL of sterile PBS mixed with 7 mL of adjuvant). All preparations were protected from light and stored at 4°C.

In the first vaccine trial (Figure 3.1), Chinook salmon were vaccinated either via IC injection with 0.1 mL of the vaccine preparation containing 100 µg/mL GroEL protein suspended in Montanide™ ISA 763 A (10 µg protein/fish, n = 48, quadruplicate tanks with 12 fish/tank) or oral gavage with 0.1 mL of the vaccine preparation containing 100 µg/mL GroEL protein suspended in Montanide™ GR1 (10 µg protein/fish, n = 48, quadruplicate tanks with 12 fish/tank). Sham vaccinated fish were either injected with 0.1 mL of sterile PBS suspended in Montanide™ ISA 763 A (n = 48, quadruplicate tanks with 12 fish/tank) or orally gavaged with 0.1 mL of sterile PBS suspended in Montanide™ GR1 (n = 48, quadruplicate tanks with 12 fish/tank). This procedure was repeated at 4 weeks post-initial immunization to booster all fish.

At 4 weeks post-booster, half of the fish in each treatment group (n = 24 fish/treatment) were challenged via IC injection of $\sim 10^6$ CFU/fish of *E. piscicida* isolate S11-285 suspended in PBS. The other half of the fish received IC injection of an equivalent volume of sterile PBS. Fish were monitored for 4 weeks post-challenge, during which period all mortalities were recorded, and samples of the posterior kidney and brain were aseptically collected, streaked on SBA, and incubated at 28°C to observe for re-isolation of *E. piscicida*. At 28 days post challenge (dpc), all surviving fish were euthanized, and serum was harvested from all fish as previously described. Additionally, spleen and posterior kidney samples from three fish per tank (n = 6 per treatment) were collected for assessment of tissue bacterial persistence.

In the second vaccine trial, Chinook salmon were initially immunized via IC injection with 0.1 mL of the vaccine preparation containing 100 µg/mL COPIII protein suspended in Montanide™ ISA 763 A (10 µg protein/fish, n = 48), IC injection with 0.1 mL of 100 µg/mL GlyZip protein suspended in Montanide™ ISA 763 A (10 µg protein/fish, n = 48), oral gavage with 0.1 mL of 100 µg/mL COPIII protein suspended in Montanide™ GR1 (10 µg protein/fish, n = 48), or oral gavage with 0.1 mL of 100 µg/mL GlyZip protein suspended in Montanide™ GR1 (10 µg protein/fish, n = 48). Control fish were sham vaccinated either through IC injection with 0.1 mL of sterile PBS suspended in Montanide™ ISA 763 A (n = 48) or via oral gavage with 0.1 mL of sterile PBS suspended in Montanide™ GR1. Fish were all boosted at 4 weeks post-vaccination. At 4 weeks post-booster, half of the fish in each treatment group were challenged via IC injection of $\sim 10^6$ CFU/fish of *E. piscicida* isolate S11-285 suspended in PBS (n = 24 fish/treatment) and the other half of the fish were administered 0.1 mL sterile PBS IC. Fish were monitored and sampled in an identical manner as the first vaccine trial.

3.3.13. Cytokine Sandwich Enzyme-linked Immunosorbent Assay

Serum IL-2 levels of 28 dpc salmon were measured via sandwich ELISA following previously described protocols [33,34]. Serum concentrations were calculated from linear regressions of standard curves for each cytokine using GraphPad Prism v10.0.1.

3.3.14. Western Blot

SDS-PAGE and western blot was performed following the method described by LaFrentz et al. (2004) [37]. Briefly, whole proteins were extracted from Chinook salmon head kidney tissue using light sonication and NP-40 lysis buffer supplemented with protease inhibitors (150 mM NaCL, 50 mM Tris-HCl pH 8.0, 1% Triton X-100, and 1X Halt protease and phosphatase inhibitor cocktail). Protein concentration of the sample was determined using a Micro BCA Protein Assay Kit according to manufacturer's directions (Thermo Fisher Scientific). A total of 28 µg of protein per well was separated via SDS-PAGE in Criterion TGX Stain-Free AnyKD gels and transferred to a 0.2 µm nitrocellulose membrane using Trans-Blot Turbo transfer packs and the Mixed MW program of the Trans-Blot Transfer System (Bio-Rad Laboratories, USA). After blocking with 4% non-fat dry milk in PBS, Chinook salmon serum samples (control and GroEL vaccinated; n = 5 each) were applied to individual membrane strips at a 1:50 dilution in PBS-T and incubated overnight with gentle shaking at 4°C. After subsequent washes, the membrane was treated for 1 h with mAb 1.14 (mouse anti-rainbow trout IgM with specificity to Chinook salmon IgM) at a 1:50 dilution in PBS-T at room temperature, washed, and then treated with goat anti-mouse IgG alkaline phosphatase-conjugated antibody at a 1:3000 dilution for 1 h. Membranes were washed and then immunogenic proteins were visualized by the addition of 1 × AP color development solution (Bio-Rad Laboratories) and images captured using a Bio-Rad ChemiDoc Touch Imaging System (Bio-Rad Laboratories).

3.3.15. Tissue Bacterial Persistence Assay

Spleen and posterior kidney samples from salmon 28 dpc (n = 6 / treatment) were weighed and then manually homogenized in 0.5 mL of sterile PBS. Tissue homogenates were then serially diluted in sterile PBS and 10-fold dilutions were plated in triplicate on SBA and incubated at 28°C overnight for colony enumeration. Colony counts were normalized by tissue weight for statistical analysis.

3.3.16. Statistical Analysis

Statistical analyses for ELISAs, survival data, and whole blood bacterial survival assays were performed in GraphPad Prism v10.0.1. ANOVA or Welch ANOVA tests, depending on the data distribution, followed by Dunnett's tests were used to assess differences in specific antibody responses, measured by ELISA, of vaccinated fish and negative control fish. Tukey's tests were used to compare host specific antibody response based on clade of the inoculating bacteria or the host fish species. Similarly, one-Way ANOVA with Dunnett's tests were used to assess for differences in bacterial survival in whole blood of immunized fish compared to negative control. ANOVA with Dunnett's tests were used to compare tissue bacterial load between vaccinated and sham vaccinated salmon. Data are given as the mean value $\pm$ standard error of the mean. The value of $p < 0.05$ was considered significant for all statistical analyses. Analyses of proteomic and immunoproteomic data were performed in Spectronaut® (Biognosys Inc, USA). Proteins with a p -value of < 0.05 , as determined by unpaired Student's t-test, and an absolute log2 ratio ≥ 1 were considered differentially expressed. The protective efficacy of the vaccine formulations was assessed by calculating the cumulative percent mortality (CPM) of each treatment group and the relative percent survival (RPS) of the vaccinated groups 28 dpc [38]. Differences in post-challenge survival between vaccinated and unvaccinated fish were calculated using ANOVA with Dunnett's tests.

3.4. Results

3.4.1. Channel catfish and hybrid catfish had higher immunoreactivity to *E. piscicida* S11-285 than rainbow trout

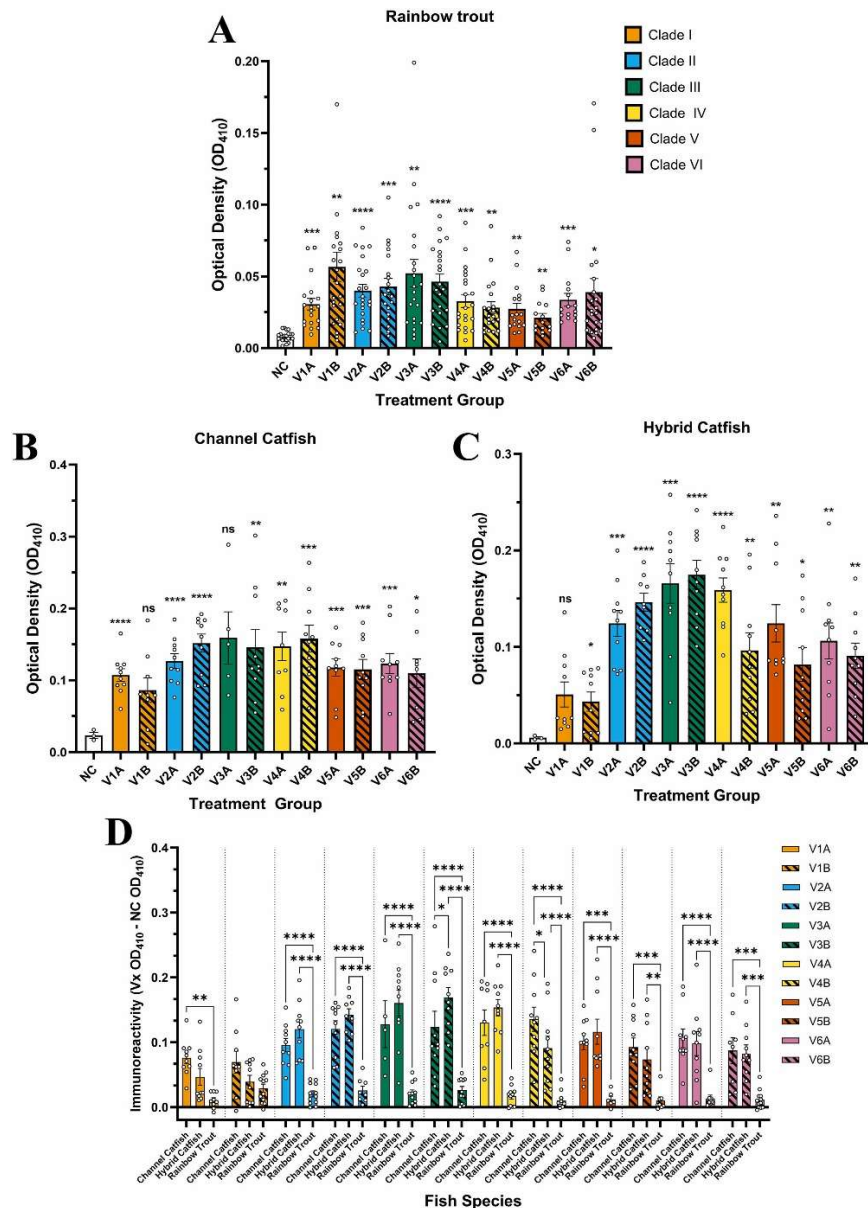
Immunization with the formalin-killed *E. piscicida* + Montanide™ ISA 763 A preparations stimulated a significant humoral response against *E. piscicida* in the rainbow trout, channel catfish, and hybrid catfish (Figure 3.2). Serum IgM levels against *E. piscicida* isolate S11-285 were significantly higher ($p < 0.05$) in almost all vaccine groups for all three fish species as compared to negative control fish. All immunized rainbow trout had significantly higher IgM responses against *E. piscicida* S11-285 compared to

control trout (Figure 3.2A). All immunized hybrid catfish except for those vaccinated with V1A (Figure 3.2C) and all immunized channel catfish except for those vaccinated with V1B or V3A (Figure 3.2B) had significantly higher IgM responses against *E. piscicida* S11-285 compared to control catfish.

For almost all vaccine groups the levels of specific anti-*E. piscicida* isolate S11-285 antibodies in rainbow trout serum were significantly lower than the levels of specific antibody present in channel catfish serum (Figure 3.2D). Fish vaccinated with V1B were the only exception in which there was no significant species-associated difference in specific antibody levels. Additionally, for all vaccine groups except for those derived from Clade I isolates (V1A, V1B) the levels of specific antibody present in rainbow trout serum was significantly lower than the levels in hybrid catfish serum. Hybrid catfish immunized with formalin-killed *E. piscicida* 35-70 (V3B) had significantly higher specific antibodies against *E. piscicida* S11-285 than channel catfish immunized similarly, while conversely channel catfish immunized with formalin-killed *E. piscicida* S12-281 (V4B) had significantly higher specific antibody levels than hybrid catfish immunized similarly. There were significant differences in the levels of specific antibodies between hybrid and channel catfish for any of the other vaccine groups.

No mortalities among fish immunized with formalin-killed *E. piscicida* had evidence of *E. piscicida* infection on gross necropsy or microbial culture and were instead attributed to cohort aggression and/or power outages associated with inclement weather events.

Figure 3.2. Specific *anti-Edwardsiella piscicida* S11-285 IgM antibody response of immunized (A) rainbow trout, (B) channel catfish, and (C) hybrid catfish as determined by indirect ELISA. (D) Immunoreactivity of fish serum to *E. piscicida* isolate S11-285 was determined as the difference between the optical densities (OD) of immunized fish serum to the mean OD of the corresponding negative control serum group; differences in immunoreactivity were determined by Brown-Forsythe ANOVA and Welch ANOVA tests with post-hoc Tukey's multiple comparisons tests. Error bars represent standard error. Asterisks indicate statistical significance between immunized groups and negative control as determined by Dunnett's T3 multiple comparisons tests (A-C) or between fish species as determined by Tukey's multiple comparisons tests (D). ns: not significant, $p > 0.05$; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. NC, negative control.



3.4.2. Bacterial survival was reduced in immunized rainbow trout whole blood but not catfish blood

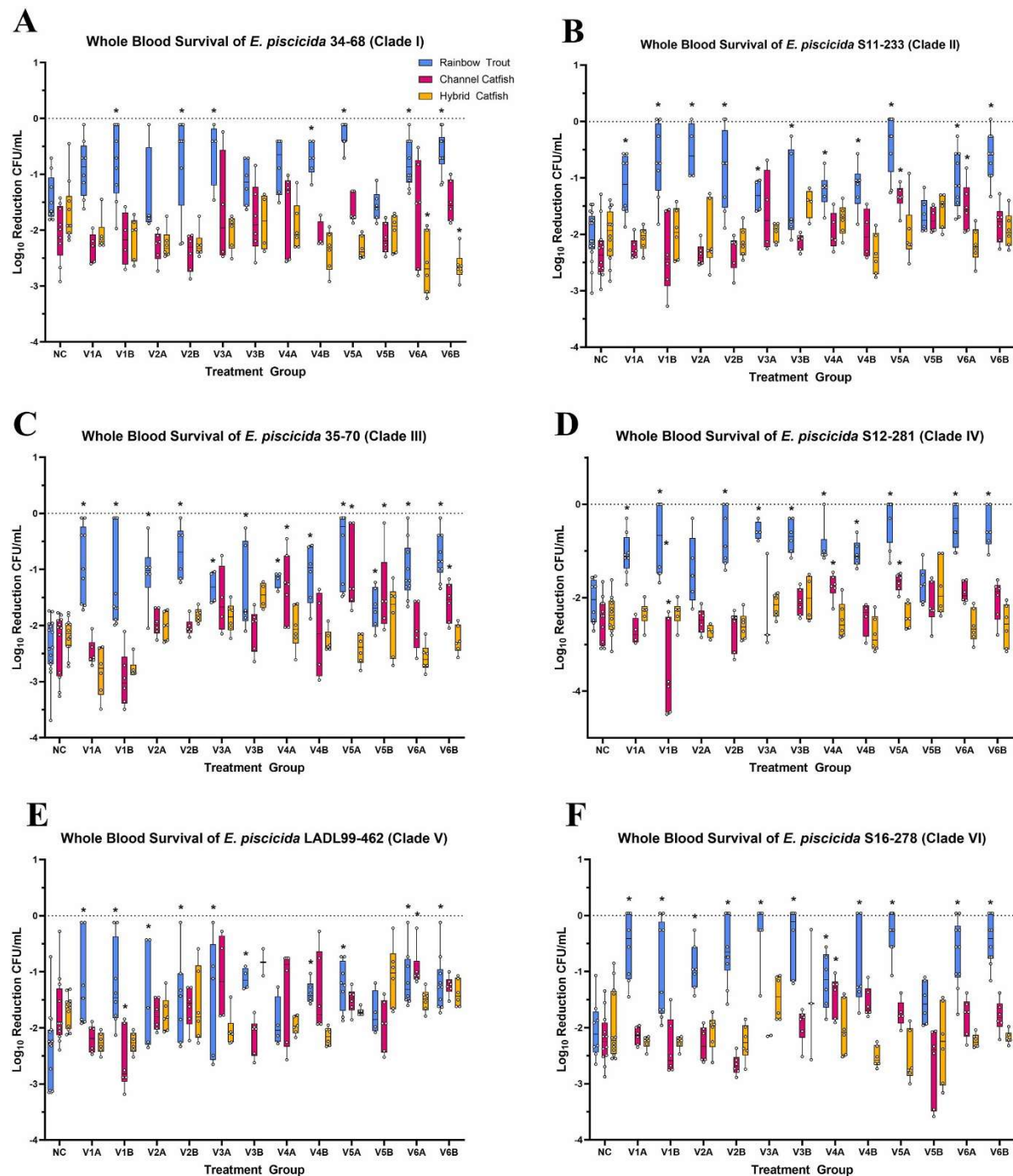
Selected *E. piscicida* isolates from each of the six bacterial clades (Table 3.1) had reduced 24-hour survival in whole blood from rainbow trout immunized with formalin-killed bacterial preparations compared to whole blood from negative control trout exposed to the sham vaccine (Figure 3.3). *Edwardsiella piscicida* isolate 35-70 (Clade III; Figure 3.3C) had significantly higher log reduction (reduced bacterial survival; $p < 0.05$) of bacteria post 24-hour incubation in rainbow trout whole blood from all vaccine groups compared to negative control trout. *Edwardsiella piscicida* isolates S11-233 (Clade II; Figure 3.3B) and S16-278 (Clade VI; Figure 3.3F) had significantly reduced survival in rainbow trout whole blood from all vaccine groups compared to negative control except for whole blood from trout immunized with V5B, for which there was no significant difference in log reduction of bacteria compared to negative control. *Edwardsiella piscicida* isolate S12-281 (Clade IV; Figure 3.3D) had significantly reduced survival of bacteria in rainbow trout whole blood from all vaccine groups except for whole blood from trout immunized with V2A and V5B. For *E. piscicida* isolate LADL99-462 (Clade V; Figure 3.3E), there was significantly higher log reduction of bacterial survival in rainbow trout whole blood from all vaccine groups except for whole blood from trout immunized with V4A and V5B. *E. piscicida* isolate 34-68 (Clade I; Figure 3.3A) had the greatest survival in immunized rainbow trout whole blood of the six isolates tested, with no significant difference in bacterial survival in whole blood from rainbow trout immunized with V2A, V3B, V4A, and V5B.

Immunization with formalin-killed *E. piscicida* did not consistently reduce bacterial survival in whole blood from channel catfish or hybrid catfish. Additionally, bactericidal activity of whole blood did not consistently correlate between the bacterial clade used to inoculate the fish and the bacterial clade used in the survival assay. In some cases, bacterial survival in immunized catfish whole blood was higher than in negative control whole blood. There was no significant difference in the log reduction of *E. piscicida* isolate 34-68 (Clade I) post 24-hour incubation in the whole blood of immunized channel catfish, compared to negative control channel catfish. *Edwardsiella piscicida* isolate S16-278 (Clade VI) only had

significantly reduced bacterial survival in channel catfish whole blood from catfish immunized with V4A, compared to negative control catfish. *E. piscicida* isolate S11-233 (Clade II) had significantly decreased survival of bacteria only in channel catfish whole blood from catfish immunized with V5A and V6A. For *E. piscicida* isolate 35-70 (Clade III), there was significantly reduced bacterial survival only in channel catfish whole blood from catfish immunized with V4A, V5A, V5B, and V6B. *E. piscicida* isolate S12-281 (Clade IV) had significantly increased log reduction in channel catfish whole blood from catfish immunized with formalin-killed *E. piscicida* isolates V4A and V5A, but also had significantly decreased log reduction (improved bacterial survival; $p < 0.05$) in whole blood from channel catfish immunized with V1B. Channel catfish whole blood survival of *E. piscicida* isolate S16-278 (Clade VI) showed similarly mixed results with reduced bacterial survival in whole blood from catfish immunized with V6A, but significantly increased survival in whole blood from catfish immunized with V1B.

For hybrid catfish, there was no significant difference in 24-hour log reduction for *E. piscicida* isolates S11-233, 35-70, S12-281, LADL99-462, and S16-278 when incubated in immunized hybrid catfish whole blood compared to negative control hybrid catfish whole blood. Only *E. piscicida* isolate 34-68 (Clade I) showed a significant difference in bacterial whole blood survival for hybrid catfish, with improved survival in hybrid catfish whole blood from catfish immunized with V6A and V6B.

Figure 3.3. Log reduction of *Edwardsiella piscicida* isolates (A) 34–68, Clade I; (B) S11-233, Clade II; (C) 35–70, Clade III; (D) S12-281, Clade IV; (E) LADL99-462, Clade V; and (F) S16-278, Clade VI after incubation for 24 h in whole heparinized blood from rainbow trout, channel catfish, and hybrid catfish. The dotted line indicates the limit of detection for bacterial enumeration. Asterisks indicate statistical significance ($p < 0.05$) from the corresponding negative control whole blood group for each fish species as determined by Dunnett's multiple comparisons tests.



3.4.3. Selection of immunogenic protein antigens

Antisera from rainbow trout and hybrid catfish immunized with formalin-killed *E. piscicida* was used to identify immunogenic *E. piscicida* protein antigens via LC-MS/MS (absolute log₂ ratio ≥ 1 ; $p < 0.05$). Fourteen unique *E. piscicida* proteins were determined to be immunogenic in the rainbow trout and 23 proteins were immunogenic in the hybrid catfish (Table 3.2, Supplemental Table 3.1). Of these proteins, the majority were localized to the cytoplasm (13/14 for rainbow trout; 13/23 for hybrid catfish) and were involved in housekeeping functions such as translation and metabolism. The rainbow trout and hybrid catfish immunoproteomes shared only one protein, the small ribosomal subunit protein uS17. Structural constituents of ribosomes were the most abundant protein group in both immunoproteomes (8/14 for rainbow trout; 6/23 for hybrid catfish). The rainbow trout immunoproteome contained one protein with predicted localization to the periplasm, superoxide dismutase, while the hybrid catfish immunoproteome contained four proteins localized to the cytoplasmic membrane; DUF883 domain-containing protein, Sec translocon accessory complex subunit YajC, Phosphatidylserine decarboxylase proenzyme, and (Fe-S)-binding protein; and one protein localized to the outer membrane (Glycine zipper 2TM domain-containing protein). All identified immunogenic proteins had high sequence homology (pairwise identity $\geq 98\%$) among the twelve selected *E. piscicida* isolates used to generate the formalin-killed vaccine preparations (Supplemental Table 3.2). Most of the identified proteins were predicted probable antigens (12/14 for rainbow trout; 18/23 for hybrid catfish) based on their Vaxigen v2.0 Value (threshold 0.4).

Table 3.2. Immunogenic *E. piscicida* proteins identified by shotgun immunoproteomics with LC/MS-MS. Differentially abundant proteins were determined to be immunogenic if there was a log2 ratio ≤ -1 and *p*-value < 0.05 between the vaccinated serum group and the negative control serum group (NC/Vaccinated).

Protein	Gene	Uniprot ID	Fish Species	GO Function	Predicted Localization
Chaparonin GroEL	<i>groL</i>	A0A411H1J6	Rainbow trout	Protein folding	Cytoplasmic
Peptidyl-prolyl cis-trans isomerase	EVK84_11485	A0A411H4M5	Rainbow trout	Protein folding Protein peptidyl-prolyl isomerization	Cytoplasmic
Universal stress protein	EVK84_01485	A0A411GZA8	Rainbow trout	No annotation available	Cytoplasmic
3-hydroxyacyl- [acyl-carrier- protein] dehydratase FabZ	<i>fabZ</i>	A0A411H0B7	Rainbow trout	Fatty acid biosynthetic process	Cytoplasmic
Translation initiation factor IF-3	<i>infC</i>	A0A411H6Y4	Rainbow trout	Translational initiation	Cytoplasmic
Large ribosomal subunit protein bL32	<i>rpmF</i>	A0A034SWU5	Rainbow trout	Translation	Cytoplasmic
Large ribosomal subunit protein uL14	<i>rplN</i>	A0A034SXP1	Rainbow trout	Translation rRNA binding	Cytoplasmic
Large ribosomal subunit protein bL17	<i>rplQ</i>	A0A411H351	Rainbow trout	Translation	Cytoplasmic
Large ribosomal subunit protein uL22	<i>rplV</i>	A0A034TB70	Rainbow trout	Translation rRNA binding	Cytoplasmic
Large ribosomal subunit protein bL34	<i>rpmH</i>	A0A411H281	Rainbow trout	Translation	Cytoplasmic
Small ribosomal subunit protein uS5	<i>rpsE</i>	A0A411H332	Rainbow trout	Translation rRNA binding	Cytoplasmic
Small ribosomal subunit protein uS15	<i>rpsO</i>	A0A034T1S1	Rainbow trout	Translation rRNA binding	Cytoplasmic
Small ribosomal subunit protein uS17	<i>rpsQ</i>	A0A034T846	Rainbow trout Hybrid catfish	Translation rRNA binding	Cytoplasmic
Large ribosomal subunit protein uL30	<i>rpmD</i>	A0A034SVG0	Hybrid catfish	Translation	Cytoplasmic
Large ribosomal subunit protein bL33	<i>rpmG</i>	A0A034T2A4	Hybrid catfish	Translation	Cytoplasmic

Large ribosomal subunit protein bL27	<i>rpmA</i>	A0A034T2R0	Hybrid catfish	Translation	Cytoplasmic
Large ribosomal subunit protein uL29	<i>rpmC</i>	A0A034T9U2	Hybrid catfish	Translation	Cytoplasmic
Large ribosomal subunit protein uL24	<i>rplX</i>	A0A411H335	Hybrid catfish	Translation	Cytoplasmic
DNA-binding protein HU-beta	EVK84_02390; <i>hupB</i>	A0A411GZR0	Hybrid catfish	DNA binding	Cytoplasmic
ATP-dependent Clp protease proteolytic subunit	<i>clpP</i>	A0A411GZW2	Hybrid catfish	ATP-dependent peptidase activity	Cytoplasmic
3,4-dihydroxy-2-butanone 4-phosphate synthase	<i>ribB</i>	A0A411H1B2	Hybrid catfish	Magnesium ion binding	Cytoplasmic
Bifunctional purine biosynthesis protein PurH	<i>purH</i>	A0A411H1V0	Hybrid catfish	'de novo' IMP biosynthetic process	Cytoplasmic
NAD(P)H-flavin reductase	EVK84_06635	A0A411H1X0	Hybrid catfish	riboflavin reductase (NAD(P)H) activity	Cytoplasmic
Coproporphyrinogen-III oxidase	<i>hemN</i>	A0A411H2C0	Hybrid catfish	Protoporphyrinogen IX biosynthetic process; metal ion binding; coproporphyrinogen oxidase activity	Cytoplasmic
Aspartate-semialdehyde dehydrogenase	EVK84_13015; L1P05_R S11525	A0A411H535	Hybrid catfish	NAD binding; protein dimerization activity	Cytoplasmic
DUF883 domain-containing protein	EVK84_05270; L1P05_R S02125	A0A034SZJ7	Hybrid catfish	Ribosome binding	Cytoplasmic membrane
Sec translocon accessory complex subunit YajC	<i>yajC</i>	A0A411GZQ9	Hybrid catfish	No annotation available	Cytoplasmic membrane
Phosphatidylserine decarboxylase proenzyme	<i>psd</i>	A0A411H1G2	Hybrid catfish	Phosphatidylethanolamine biosynthetic process	Cytoplasmic membrane
(Fe-S)-binding protein	EVK84_05785; L1P05_R S01630	A0A411H1H8	Hybrid catfish	No annotation available	Cytoplasmic membrane
YgiW/YdeI family stress	EVK84_05170; L1P05_R S02225	A0A411H7T7	Hybrid catfish	No annotation available	Non-cytoplasmic

tolerance OB fold protein					
Glycine zipper 2TM domain-containing protein	EVK84_16935; L1P05_R S08220	A0A411H781	Hybrid catfish	No annotation available	Outer membrane
Superoxide dismutase	<i>sodB</i>	A0A411H779	Rainbow trout	Metal ion binding, superoxide dismutase activity	Periplasmic
UPF0757 protein YmgG	<i>ymgG</i>	A0A411GZ36	Hybrid catfish	No annotation available	Unknown
Glycerol dehydrogenase	EVK84_02840; L1P05_R S04435	A0A411GZZ6	Hybrid catfish	Metal ion binding; oxidoreductase activity	Unknown
Biotin carboxyl carrier protein of acetyl-CoA carboxylase	<i>accB</i>	A0A411H391	Hybrid catfish	Fatty acid biosynthetic process	Unknown
Negative modulator of initiation of replication	<i>seqA</i>	A0A411H4K0	Hybrid catfish	Negative regulation of DNA-templated DNA replication inhibition	Unknown

3.4.4. The oral and injectable GroEL vaccines induced an anti-protective effect against subsequent challenge

The first vaccine trial evaluated GroEL as a protein vaccine candidate against *E. piscicida* in Chinook salmon. Mortality in fish challenged with *E. piscicida* S11-285 began on day 2 post-challenge, with peak mortality occurring on days 3-5 post-challenge (Figure 3.4A). This is in line with previous challenge experiments with *E. piscicida* in Chinook salmon [8]. At 28 dpc there was no mortality in any of the unchallenged groups. The injectable and oral sham vaccinated fish challenged with *E. piscicida* S11-285 achieved a CPM of 22.2% and 21.7%, respectively (Table 3.3). Surprisingly, both the oral and injectable GroEL vaccines appeared to stimulate an anti-protective effect in the salmon post-challenge. Fish vaccinated with the oral GroEL preparation had an RPS of -76% whilst those vaccinated with the injectable GroEL formulation had an RPS of -184%.

Figure 3.4. Survival of vaccinated and sham vaccinated Chinook salmon after challenge with *Edwardsiella piscicida* S11-285. Each treatment was performed in duplicate with 12 fish per tank (n = 24 fish/treatment). Inj, intra-coelomically injected; PO, orally gavaged; NE, not exposed to *E. piscicida* S11-285; Ex, challenged with *E. piscicida* S11-285.

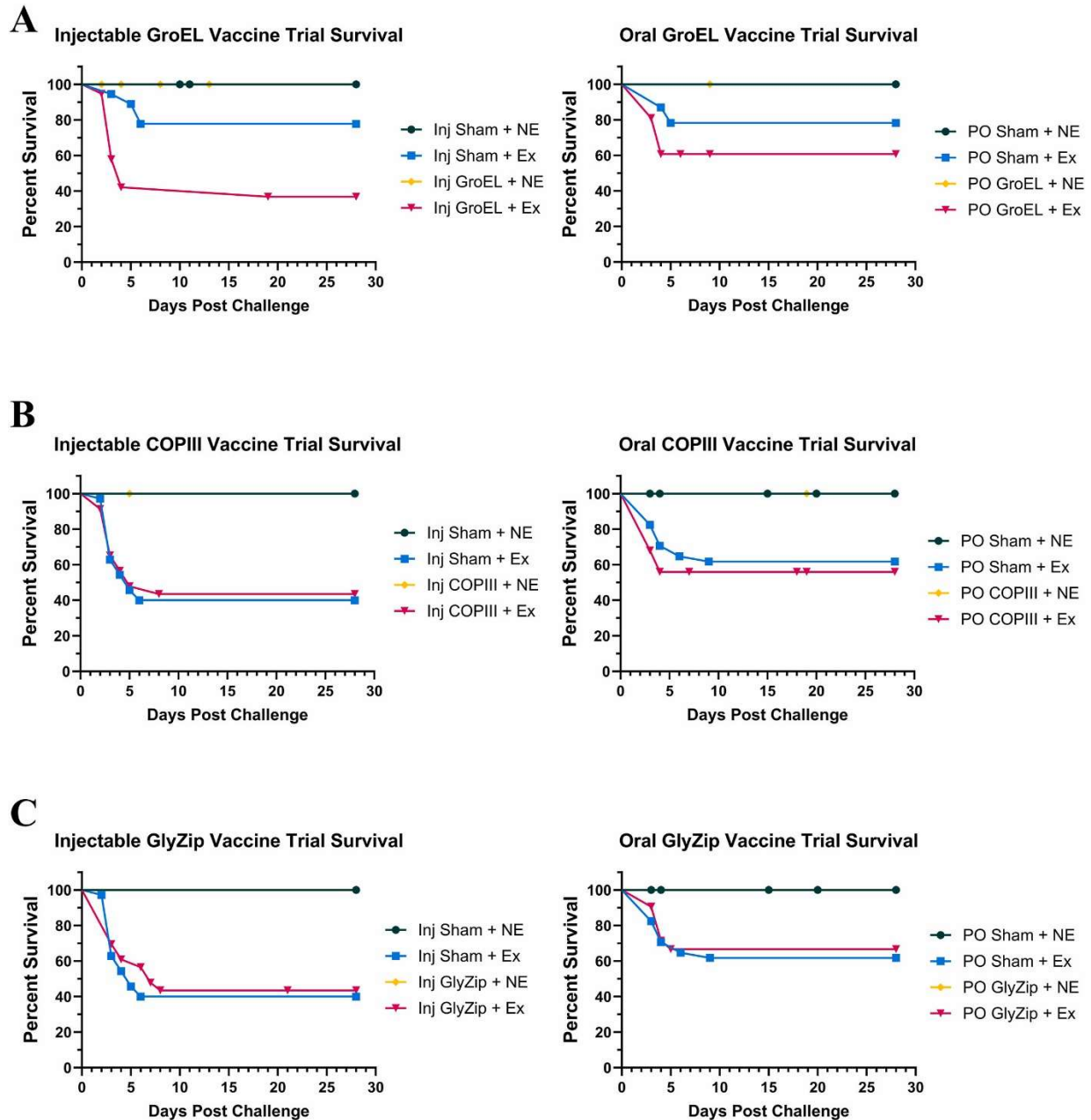


Table 3.3. Summary of vaccine trial design and fish survival.

Group	Primary Vaccination (Day 0)	Booster Vaccination (Day 28)	Challenge Groups (Day 42, 0.1 mL/fish)	CPM (%)	RPS (%)
Sham Vaccinated (Control)	PBS + ISA (IC)	PBS + ISA (IC)	PBS	0	-
			10^6 CFU/fish Ep S11-285	22.2 ^a , 60 ^b	-
	PBS + GR1 (PO)	PBS + GR1 (PO)	PBS	0	-
			10^6 CFU/fish Ep S11-285	21.7 ^a , 38.2 ^b	-
Vaccinated	GroEL + ISA (IC)	GroEL + ISA (IC)	PBS	0	-
			10^6 CFU/fish Ep S11-285	63.1	-184
	GroEL + GR1 (PO)	GroEL + GR1 (PO)	PBS	0	-
			10^6 CFU/fish Ep S11-285	38.1	-76
	COPIII + ISA (IC)	COPIII + ISA (IC)	PBS	0	-
			10^6 CFU/fish Ep S11-285	56.5	6
	COPIII + GR1 (PO)	COPIII + GR1 (PO)	PBS	0	-
			10^6 CFU/fish Ep S11-285	44	-15
	GlyZip + ISA (IC)	GlyZip + ISA (IC)	PBS	0	-
			10^6 CFU/fish Ep S11-285	54.2	9.7
	GlyZip + GR1 (PO)	GlyZip + GR1 (PO)	PBS	0	-
			10^6 CFU/fish Ep S11-285	33.3	13
	GlyZip + GR1 (PO)		PBS		
			10^6 CFU/fish Ep S11-285		

ISA, Montanide ISA 763 A adjuvant; GR1, Montanide GR1 adjuvant, IC, intra-coelomic administration; PO, oral administration; GroEL, purified Chaparonin GroEL protein; COPIII, purified coproporphyrinogen-III oxidase protein; GlyZip, purified glycine 2TM zipper domain-containing protein; Ep, *Edwardsiella piscicida*; CPM, cumulative percent mortality; RPS, relative percent survival; -, not applicable. ^a : CPM from GroEL vaccine trial; ^b : CPM from COPIII and GlyZip vaccine trial.

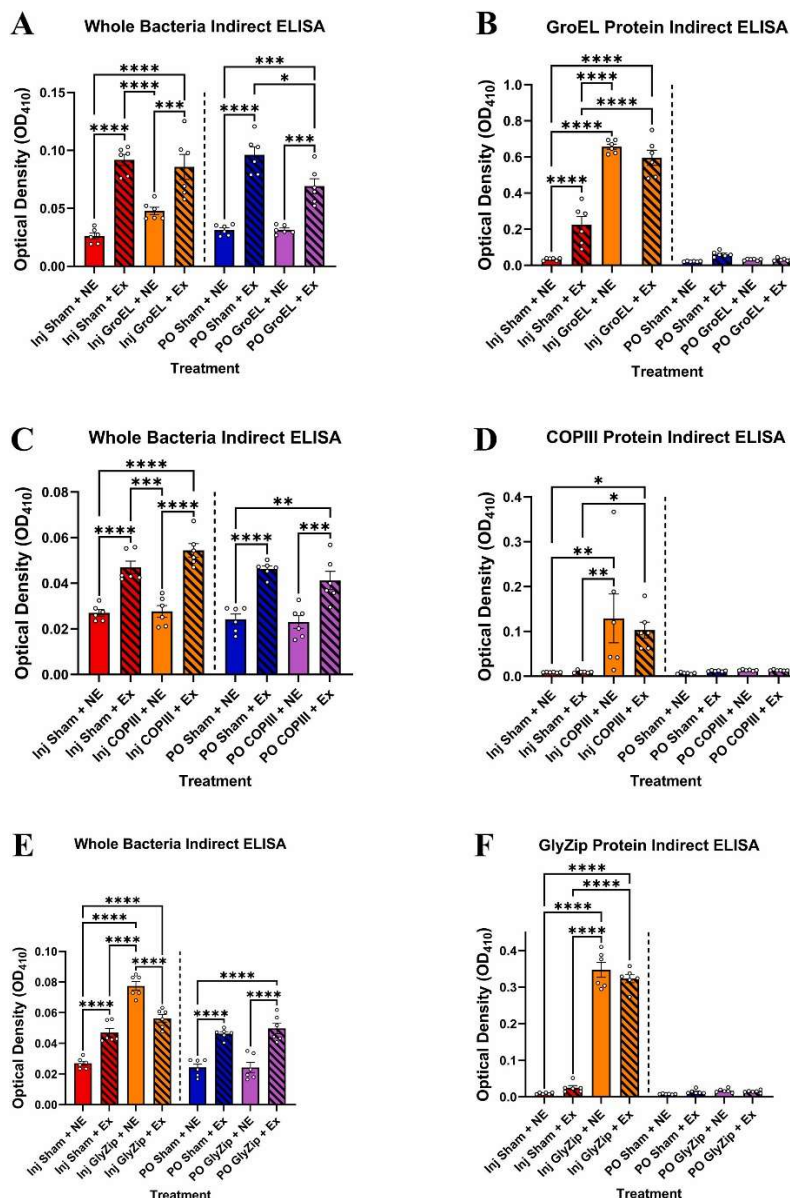
3.4.5. Intra-coelomic injection of COPIII and GlyZip vaccines induced a slight, but nonsignificant, improvement in post-challenge survival

In the second vaccine trial, both the COPIII and GlyZip proteins were evaluated as protein vaccine candidates in Chinook salmon. Similar to the first trial, peak mortality occurred 3-5 dpc (Figure 3.4B – 3.4C). The injectable and oral sham vaccinated fish challenged with *E. piscicida* S11-285 achieved a cumulative percent mortality (CPM) of 60% and 38.2%, respectively. The injectable COPIII and GlyZip vaccines induced slight, but non-significant ($p>0.05$) increases in post-challenge survival (RPS = 6% and 9.7%, respectively; Table 3.3). The COPIII oral vaccine preparation induced a non-significant decrease in post-challenge survival (RPS = -15%), whilst conversely the fish vaccinated with the oral GlyZip preparation showed a slight, but non-significant, increase in post-challenge survival (RPS = 13%).

3.4.6. Salmon generated strong specific IgM responses against the candidate proteins

Serum from surviving salmon 28 dpc was used in indirect ELISAs to determine specific IgM antibody responses against whole bacteria *E. piscicida* S11-285 and to the purified proteins used to generate the vaccine formulations (Figure 3.5). As expected, all vaccinated and sham vaccinated fish challenged with *E. piscicida* S11-285 generated a significantly increased anti-*E. piscicida* IgM antibody response compared to unchallenged fish. Unchallenged fish vaccinated with the injectable GlyZip vaccine preparation demonstrated an increased specific anti-*E. piscicida* IgM response compared to both challenged and unchallenged sham vaccinated fish (Figure 3.5E). All IC vaccinated fish, regardless of exposure status, also generated an increased IgM response against the immunizing protein (Figure 3.5B, 3.5D, 3.5F) compared to sham vaccinated fish; however, no PO vaccinated fish developed an increased antibody response against the immunizing protein. Additionally, IC sham vaccinated fish exposed to *E. piscicida* S11-285 had a significantly higher anti-*GroEL* IgM response compared to negative control fish.

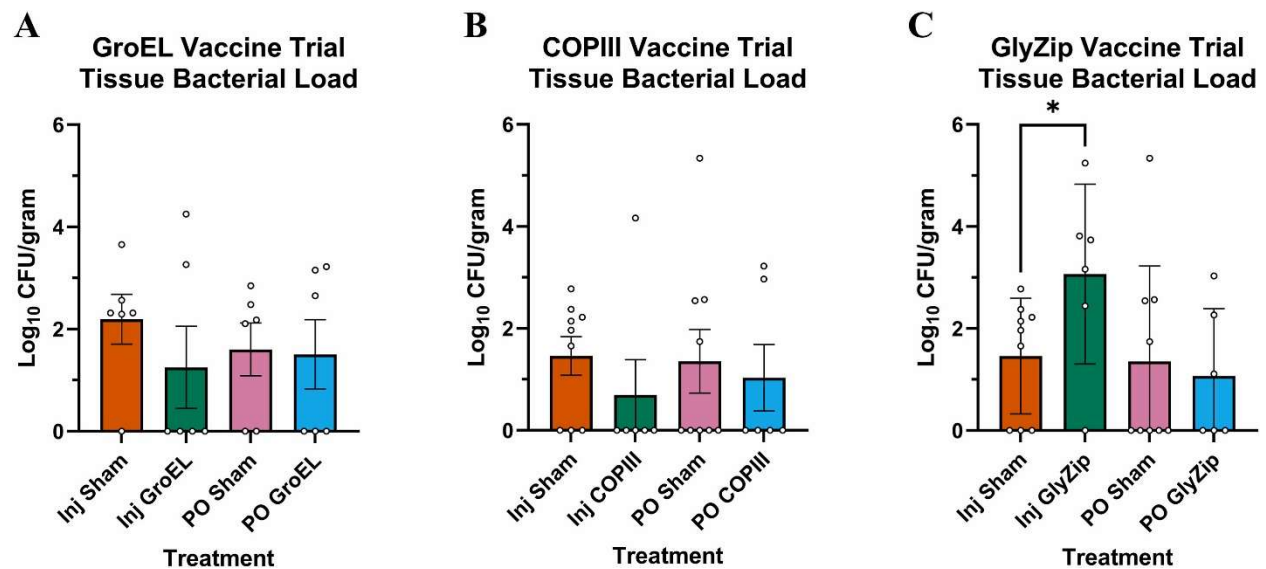
Figure 3.5. Specific antibody (IgM) levels against *Edwardsiella piscicida* S11-285 and purified recombinant protein in surviving Chinook salmon (mean $\pm$ SE, n = 6) at 28 dpc as determined by serum ELISA. Serum IgM response of GroEL vaccinated (and sham vaccinated) fish against (A) whole bacteria *E. piscicida* S12-285 or (B) purified GroEL protein. Serum IgM response of COPIII vaccinated (and sham vaccinated) fish against (C) whole bacteria *E. piscicida* S12-285 or (D) purified COPIII protein. Serum IgM response of GlyZip vaccinated (and sham vaccinated) fish against (E) whole bacteria *E. piscicida* S12-285 or (F) purified GlyZip protein. Asterisks indicate a significant difference ($p < 0.05$) from the corresponding control as determined by Tukey's multiple comparisons tests: *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. Inj, Injectable vaccine preparation; PO, oral vaccine formulation; NE, non-exposed; Ex, exposed.



3.4.7. Vaccination with selected proteins did not significantly alter long-term tissue bacterial persistence

At 28 dpc, pooled spleen and posterior kidney samples from 6 fish/treatment were homogenized and plated on SBA to determine long-term persistence of *E. piscicida* in the tissues of vaccinated and unvaccinated salmon. Samples from unexposed fish did not grow *E. piscicida* colonies on SBA after overnight incubation at 28°C (data not shown). Fish vaccinated with the injectable GlyZip vaccine preparation had significantly higher tissue bacterial load at 28 dpc compared to sham vaccinated fish (Figure 3.6). There was no significant difference in tissue bacterial load between vaccinated and sham vaccinated fish for any of the other treatments.

Figure 3.6. Bacterial load in pooled posterior kidney and spleen samples from vaccinated and sham vaccinated Chinook salmon 28 days post-challenge (n = 6/treatment). Colony counts after incubation overnight on SBA were normalized against the weight of the tissue and present as Log₁₀ CFU/g of tissue. Asterisks indicate a significant difference ($p < 0.05$) in bacterial load between vaccinated and sham vaccinated fish exposed to *E. piscicida* S11-285 as determined by ANOVA with Dunnett's tests. Inj, intra-coelomic injectable vaccine preparation; PO, oral vaccine preparation.

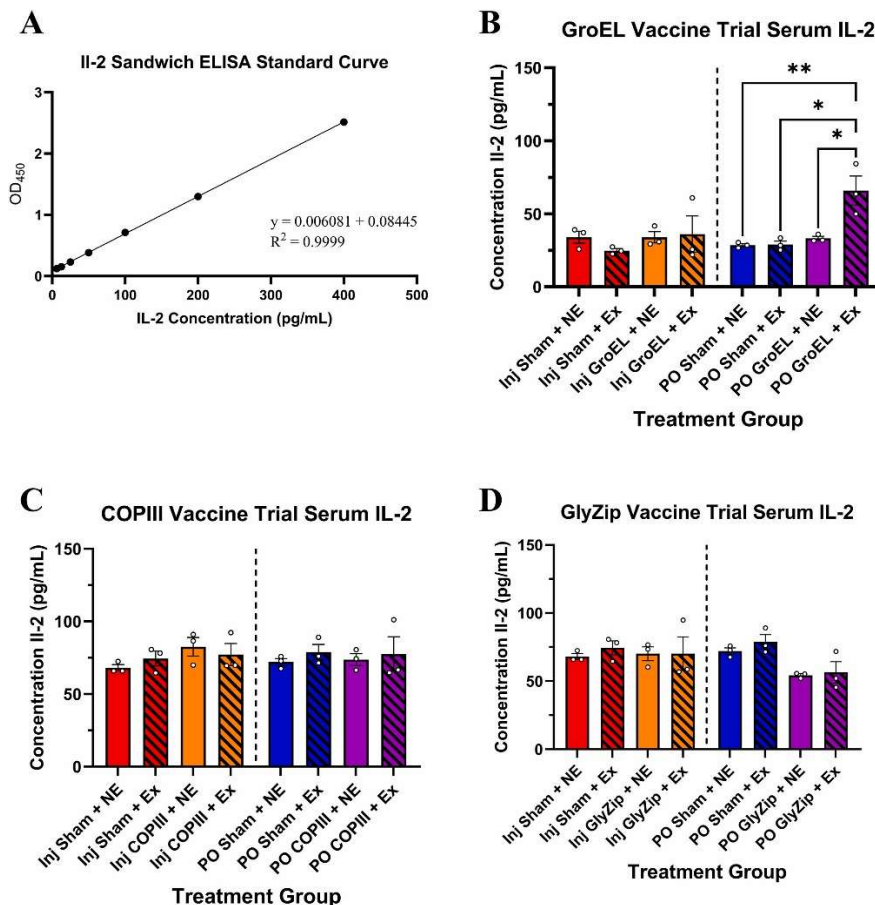


3.4.8. Intra-coelomic vaccination did not significantly alter 28 dpc serum IL-2 levels

Sandwich ELISA using serum from salmon 28 dpc did not reveal any significant differences in circulating IL-2 levels between vaccinated and unvaccinated or exposed and unexposed fish in the COPIII

and GlyZip vaccine trial (Figure 3.7). Similarly, in the GroEL vaccine trial, fish administered the injectable vaccine preparation did not have significantly different serum IL-2 levels compared to sham vaccinated fish and there was no difference in serum IL-2 levels between exposed and unexposed fish administered either the injection GroEL vaccine or the injectable sham vaccine preparations. However, fish administered the oral GroEL vaccine and challenged with *E. piscicida* S11-285 had significantly higher serum IL-2 levels at 28 dpc than unexposed and vaccinated fish, sham vaccinated and challenged fish, and negative control sham vaccinated and unexposed fish.

Figure 3.7. Serum IL-2 levels from Chinook salmon 28 dpc as determined by sandwich ELISA. (A) Linear standard curve of sandwich ELISA for the concentration measurement of serum IL-2 from vaccinated and unvaccinated salmon in the GroEL (B), COPIII (C), and GlyZip (D) vaccine trials. Asterisks indicate a significant difference ($p < 0.05$) from the corresponding control as determined by Dunnett's T3 multiple comparisons tests: *: $p < 0.05$; **: $p < 0.01$. Inj, Injectable vaccine preparation; PO, oral vaccine formulation; NE, non-exposed; Ex, exposed.



3.5. Discussion

In this study, formalin-killed *E. piscicida* preparations derived from representative isolates of the six bacterial clades stimulated significant specific antibody responses in rainbow trout, channel catfish, and hybrid catfish against *E. piscicida* isolate S11-285. This finding demonstrates that these genetically distinct isolates share conserved antigens capable of eliciting cross-reactive antibodies across clades. The observed cross-reactivity of the antibody response across clades underscores the potential for broad-spectrum vaccine strategies, suggesting that a single vaccine candidate could confer protection against multiple variants of this important fish pathogen.

Channel catfish and hybrid catfish had significantly higher levels of antibodies to *E. piscicida* than rainbow trout, but this finding did not correspond to improved whole blood bactericidal activity. In fact, only rainbow trout demonstrated consistently improved whole blood bactericidal activity following immunization whereas the whole blood bactericidal activity of immunized catfish was either the same as or worse than negative control fish. Possible explanations for the lack of bactericidal activity despite high anti-*E. piscicida* IgM levels in the immunized catfish include lack of neutralizing antibody production and host-species differences in cell-mediated immune response. Neutralizing antibodies are typically defined as antibodies that bind to a pathogen or pathogen-derived toxin at specific epitopes, subsequently preventing pathogen entry into host cells [36]. Traditional vaccine development often relies on the stimulation of targeted antibody production against these neutralizing epitopes, many of which are virulence factors, in order to prevent infection and accelerate clearance of the pathogen. Unfortunately, not all antibodies produced by an organism are neutralizing and thus serum antibody levels may not correlate with the development of protective immunity, particularly for pathogens like *E. piscicida* that can escape from the humoral immune response due to their intracellular nature [40,41]. The cellular component of whole blood bactericidal activity must also be considered, especially for facultative intracellular pathogens like *E. piscicida*. Species-dependent differences in cell-mediated immune response to immunization could explain the disparity between the ELISA and whole blood bactericidal activity results, but further investigation into

the cell-mediated immune responses of trout and catfish to *E. piscicida* immunization is necessary to definitively determine the mechanism behind these differences. Another consideration is the difference in immunization degree days between the catfish and trout in this experiment. In poikilotherms, environmental temperature plays a crucial role in adaptive immune responses, with the immune effects of vaccines in fish being time x temperature (degree-days) dependent [42,43]. All fish species were sampled at the same time points throughout this experiment, however as the catfish were reared at a higher temperature ($25^{\circ}\text{C}\pm 1$) than the rainbow trout ($18^{\circ}\text{C}\pm 1$) they experienced higher average immunization degree-days (dd) at the time of serum collection (1050 degree-days for catfish; 756 degree-days for trout). As higher water temperatures, and thus higher degree-days, are associated with faster development of adaptive immune responses to vaccines in fish, this difference in degree-days at the time of sampling could explain the relatively higher specific antibody response to *E. piscicida* observed in the catfish. This could also possibly explain the difference in whole blood bactericidal response as the catfish and trout could be in different phases of the immune response at the time of sampling and thus bactericidal activity could have waned in the catfish [44].

Development of effective protein vaccines for genetically diverse pathogens such as *E. piscicida* relies on careful selection of highly conserved, surface-exposed, immunogenic protein antigens in order to induce long-lasting cross-protective immunity [45,46]. The shotgun immunoproteomic workflow outlined in this study presents a high-throughput method for identification of immunogenic bacterial proteins to drive vaccine candidate selection. In this study, protein G coated magnetic beads were used to capture antibodies from formalin-killed *E. piscicida* immunized trout and catfish serum to enrich immunogenic *E. piscicida* proteins for downstream identification. Prior to capture of serum antibodies, the magnetic beads were incubated with monoclonal mouse anti-trout/catfish IgM antibodies due to the low binding affinity of the protein G coated beads with IgM, the primary circulating antibody in teleosts. All 36 unique proteins determined to be immunogenic by this approach demonstrated high amino acid sequence homology among the twelve *E. piscicida* isolates used to generate the formalin-killed bacterial preparations in this study.

Inclusion of highly conserved epitopes in protein vaccines is important for generation of broad strain/serovar protection; thus, our finding suggests that these identified immunogenic proteins provide intriguing candidates for development of a protein vaccine with universal protection against *E. piscicida* clades. Additionally, most of the proteins identified by this method were also predicted to be probable antigens using *in-silico* methods, further validating the efficacy of this shotgun immunoproteomic approach to selectively identify immunogenic proteins.

Although the composition of the rainbow trout and hybrid catfish immunoproteomes differed significantly, except for one shared protein (Small ribosomal subunit protein uS17), they were both comprised primarily of cytoplasmic proteins with housekeeping functions. One protein, glycine zipper 2TM domain-containing protein, was predicted to be localized to the outer membrane and had 100% sequence homology among the twelve selected *E. piscicida* isolates. The outer membrane proteins of many gram-negative bacteria have been investigated as antigens for vaccine development due to their important role in bacterial pathogenicity and their high surface exposure facilitating host immune system detection [47,48]. Glycine zipper 2TM proteins have previously been identified via reverse vaccinology approaches as potential vaccine candidates against *Aeromonas hydrophila*, thus their identification as immunogenic outer membrane proteins in our current study makes them promising candidates for recombinant protein subunit vaccine development [46]. Another interesting immunogenic protein identified in our study is Coproporphyrinogen-III oxidase (hemN), which plays an important role in heme biosynthesis by catalyzing the conversion of coproporphyrinogen III to protoporphyrinogen IX [49]. As heme synthesis is critical for aerobic and anaerobic respiration, hemN is an attractive anti-virulence target for therapeutics and vaccine development [50].

Chaperonin GroEL was particularly interesting as a potential target for recombinant vaccine development. GroEL is a ubiquitous heat-shock protein that plays an essential role in protein homeostasis. GroEL is highly conserved across both prokaryotic and eukaryotic organisms in which it functions to form a nanocage structure, with its co-chaperone GroES, to encapsulate substrates in order to prevent aggregation

of unfolded proteins and to facilitate proper protein folding [51]. Although traditionally believed to be localized to the cell cytoplasm due to its primary function and lack of a secretion signal sequence or other export motif, surface-associated GroEL has been characterized in many pathogenic bacteria, including *Escherichia coli* [52], *Salmonella enterica* serovar Typhimurium [53], *Helicobacter pylori* [54], and *Streptococcus agalactiae* [55]. Liu et al. (2016) identified GroEL in the outer membrane profile and secretome of *E. tarda*, further suggesting that GroEL is likely to be surface exposed in *E. piscicida*. Regulation of GroEL expression is modulated not only by environmental temperature, but also by other stress response factors during infection and extracellular GroEL has been implicated in bacterial adherence to and invasion into host cells [52,56]. Immunization with recombinant GroEL has been previously demonstrated to induce protective immunity against *Francisella orientalis* and *S. agalactiae* in tilapia (*Oreochromis* spp.) [55,57]. Additionally, DNA vaccines encoding for GroEL have been experimentally shown to stimulate the humoral and cellular immune response against *Nocardia seriole* and *E. tarda* in hybrid snakehead (*Channa argus* ♀ × *Channa maculate* ♂) and flounder (*Paralichthys olivaceus*), respectively [56,58].

These factors suggested that GroEL would be a promising candidate for recombinant protein vaccine development against *E. piscicida*. Thus, it was surprising that in this experiment, immunization with the injectable GroEL protein vaccine preparation generated an anti-protective effect in the Chinook salmon against subsequent *E. piscicida* challenge. One possible explanation for this finding is that molecular mimicry led to the development of auto-reactive antibodies and subsequent increased post-challenge mortality. In this context, molecular mimicry refers to the phenomenon in which significant similarities, such as sequence homology, between foreign peptides and self-peptides stimulate activation of autoreactive T and/or B cells that are cross-reactive with both pathogen-derived peptides and host peptides in a susceptible individual [59,60]. Heat shock proteins (HSPs) like GroEL are highly conserved across both prokaryotes and eukaryotes and sequence similarity between bacterial and host HSPs could lead to production of auto-reactive antibodies. There is evidence of cross-reactivity between antibodies generated

against microbial heat shock proteins (HSPs) and eukaryotic 60-kDa HSPs contributing to the development of different autoimmune diseases in humans [61,62]. A similar phenomenon has been demonstrated in Atlantic salmon (*Salmo salar*) where vaccinated farm fish and experimentally vaccinated fish generated autoantibodies reactive against salmon cells as well as had higher rates of immune complex glomerulonephritis and arthritis [63–65]. When we compared the protein sequence of the *E. piscicida* GroEL protein sequence against the Chinook salmon proteome using BLASTP 2.16.0+, we found that the bacterial GroEL had moderate (~52%) sequence homology with the Chinook salmon Hsp60 protein (Supplemental Table 3.4). Western blot analysis did show a specific antibody response in the GroEL vaccinated salmon against Chinook salmon Hsp60, however it did reveal light reactivity of vaccinated salmon serum against a protein of ~40 kDa (Supplemental Figure 3.1). It must be noted that protein conservation alone is not the sole etiology in the development of autoimmunity as bacteria and vertebrates share a great number of homologous proteins [59,66]. It is largely believed that additional host factors, such as genetic susceptibility, and external factors are necessary to upset the balance between autoimmunity and self-tolerance. Thus, although the exact cause of the anti-protective effect following GroEL vaccination observed in this experiment is still unclear, consideration of the potential for molecular mimicry is still warranted in future protein vaccine studies.

In contrast to the GroEL vaccine trial, the injectable COPIII and GlyZip vaccine formulations induced a small, but non-significant increase in post-challenge survival in this study despite inducing strong and specific anti-*E. piscicida* and anti-recombinant protein IgM responses in the vaccinated fish. One possible explanation is that the protein vaccines did not stimulate a robust cell-mediated immune response which is necessary for effective clearance of a facultative intracellular pathogen such as *E. piscicida*. We did not find any significant differences in serum IL-2 levels between vaccinated and unvaccinated fish at 28 dpc. IL-2 plays a key role in teleost cell-mediated immunity through regulation of T helper 1 and T helper 2 cytokine expression [67]. Thus, this finding could suggest a lack of strong cell-mediated immune response from vaccination but could also be associated with the late timing of sampling (28 days post-challenge).

Further investigation into differential regulation of factors associated with cell-mediated immunity following bacterial challenge is warranted to better elucidate the protective ability of the protein vaccines. Conversely, however, the slight, albeit non-significant, increase in survival could suggest that further optimization of dosing, booster, and/or delivery protocols may improve vaccine efficacy. Only one protein dose and a single booster was used for all vaccine formulations in this study, thus increased concentrations of purified protein and/or several boosters could potentially improve immune stimulation. Alternatively, a multi-epitope vaccine containing multiple immunogenic proteins could be considered to increase immunogenicity, stimulate multiple different immune pathways, and potentially increase protection across the genetically diverse *E. piscicida* strains [68,69]. The use of modern alternative delivery platforms, outside of the classical method of antigen suspension in adjuvant, could also be explored to improve antigen delivery and immune stimulation. Additionally, more targeted focus on surface-exposed antigens more readily detected by the host immune system could improve the success of vaccine candidate selection. The COPIII protein used in this experiment was determined to be highly immunogenic based on shotgun immunoproteomics and ELISA analyses with cell lysates. However, its localization to the cytoplasm could explain why high *in vitro* immunogenicity did not translate into significant protective effect *in vivo* as antibodies generated against these proteins may be ineffective in recognizing the protein, due to lack of surface exposure, or neutralizing live, intact bacteria. In this study, antisera used for shotgun immunoproteomics was generated via immunization of fish with formalin-killed bacterial preparations. Immunization with formalin-inactivated pathogens can lead to direction of antibodies against nonprotective epitopes due to alteration of functional epitopes necessary for protection [70]. Infection of fish with a live, virulent bacterial strain and harvesting of antisera from surviving fish may allow for more effective collection of neutralizing antibodies that are specific for protective bacterial antigens and preserve *in-vivo* antigen conformation and expression, thus improving identification of protective antigens.

All injectable vaccinated fish, regardless of challenge status, demonstrated a strong specific antibody response against the immunizing protein, confirming that the selected proteins were effective at stimulating

a humoral response in the fish. A similar trend was not observed for the oral vaccine formulations, possibly due to degradation of the protein in the gastrointestinal tract and/or inadequate uptake by gastrointestinal lymphoid cells. Additionally, sham vaccinated fish challenged with *E. piscicida* S11-285 also displayed a strong anti-*GroEL* antibody response compared to negative control fish, suggesting that the salmon immune system was detecting and responding to the native GroEL protein of the bacteria. Interestingly, unchallenged fish immunized with the injectable GlyZip vaccine formulation demonstrated an increased anti-*E. piscicida* antibody response compared to both negative control fish and challenged sham vaccinated fish. This suggests that the GlyZip protein was effective at stimulating an anti-*E. piscicida* humoral response regardless of challenge status. However, the development of a robust post-vaccination specific antibody response did not correspond to protective immunity in this study. Previous studies have found evidence that cell-mediated immunity may play a dominant role in the protection against *E. piscicida* while antibodies may provide only weak defense against the pathogen, possibly due to the ability of *E. piscicida* to invade host cells and replicate intracellularly [71,72]. This could explain the lack of significant protection observed in this study despite high specific antibody levels.

Although this study did not yield an effective protein vaccine candidate, it did demonstrate the utility of the described shotgun immunoproteomic approach for identifying immunogenic *E. piscicida* proteins and it revealed critical differences in bacterial survival within the blood of salmonid vs. catfish hosts. The shotgun immunoproteomic approach described here vastly accelerates immunogenic protein identification over more laborious and time-consuming classical techniques, such as display technologies and Western blot, as well as bypasses the need for secondary protein immunogenicity validation required by computational methodologies. These findings lay an important foundation for future investigations into the host-pathogen dynamics of the *E. piscicida* in these two economically significant aquaculture species, as well as provides an alternative method for high-throughput screening of the pathogen proteome for candidate antigens that can be immediately tested *in-vivo*. Moving forward, efforts should focus on targeting

surface-exposed antigens more likely to be recognized *in vivo*, along with exploring alternative delivery strategies to enhance antigen uptake and stimulate a more robust and protective immune response.

3.6. References

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CHAPTER 4: Development of an *Edwardsiella piscicida* Immersion Challenge Model in Salmonids

4.1. Abstract

Immersion challenges more closely simulate natural infection dynamics than injection models which can circumvent mucosal barriers. Additionally, immersion models minimize handling stress, stimulate the mucosal immune system, and facilitate simultaneous exposure of large numbers of fish. In this study, yearling (≥ 9 -month-old) and fingerling (≤ 5 -month-old) rainbow trout (*Oncorhynchus mykiss*) and Chinook salmon (*Oncorhynchus tshawytscha*) were exposed via immersion to 10^7 CFU/mL of *Edwardsiella piscicida* using three methods: immersion without disrupting the skin/mucous (intact), immersion after mucous coat removal (mucus removed), and immersion after adipose fin clip (wounded). Intact immersion induced 10% mortality in yearling salmon, but no mortality in trout yearlings or in trout and salmon fingerlings. Immersion with mucous removal induced 30% mortality in salmon fingerlings, but no mortality in trout fingerlings or salmon and trout yearlings. Immersion with wounding induced 40%, 10%, 10%, and 0% mortality in salmon and trout fingerlings, and salmon and trout yearlings, respectively. A repeat immersion challenge experiment with wounding resulted in 40% mortality in trout fingerlings and 13.3% mortality in salmon fingerlings. Collectively, these findings support immersion with wounding as a reproducible challenge model for *E. piscicida* in salmonids, providing an approach to induce edwardsiellosis under conditions that reflect natural routes of infection.

4.2. Introduction

Salmonid aquaculture is one of the most important United States aquaculture sectors, with salmonid species constituting major proportions of the US freshwater and marine aquaculture industries. In 2022, 34 million pounds of Atlantic salmon (*Salmo salar*) were produced in the United States, with an estimated total value of \$115 million [1]. In 2023, trout production for human consumption in the United States generated

over \$134 million in sales [2]. Not only does salmonid production play an important role in US and global seafood production, but salmonid aquaculture is essential for the recreational fisheries and sportfish industry, as well as for native species conservation. An estimated 134,694,000 trout were produced in the US in 2023 for the purposes of conservation, recreation, and restoration [2]. Hatchery production of salmonids for release into natural and artificial waterways supports the \$138 billion recreational fishery industry in the United States [1] and plays a critical role in the restoration and conservation of native salmonids such as Chinook salmon (*Oncorhynchus tshawytscha*) and steelhead trout (*Oncorhynchus mykiss*). One of the most significant threats to salmonid aquaculture is infectious disease, which can spread rapidly and have devastating consequences for cultured fish populations.

Edwardsiella piscicida is an emerging infectious pathogen that has been associated with major losses among many economically significant farmed fish species worldwide [3], including ictalurid catfish (*Ictalurus* spp.) [4,5] largemouth bass (*Micropterus salmoides*) [6], and barramundi (*Lates calcarifer*) [7] in the United States. Although primarily associated with warmwater systems, outbreaks of piscine edwardsiellosis have been reported in coldwater species, including several important salmonid species [8-10]. Epizootics of *E. piscicida* typically present as acute generalized septicemia (coelomic distension, lethargy, erratic swimming, petechiation and ecchymosis, and exophthalmia) which can result in significant mortality events among both juvenile and adult market-sized fish [11]. The potential impact of *E. piscicida* on cultured and wild fish populations has driven increasing interest in the pathogenesis of and potential management strategies for *E. piscicida* infections.

Effective challenge models are essential for the study of infectious disease pathogenesis and drug/vaccine efficacy in fish. Intra-coelomic injection has frequently been used as a model for experimental challenge with *E. piscicida* in multiple fish species [9,12-14]. This method, however, does not accurately reflect natural infection dynamics as it bypasses the colonization phase of infection and thus ignores the contribution of the mucosal immune system to pathogen defense. Further, injection challenge is less practical in smaller/younger fish or large numbers of fish and has a higher risk of handling stress and/or

iatrogenic trauma to the animals [15]. Bath immersion has been evaluated as an alternative *E. piscicida* challenge model in larval zebrafish (*Danio rerio*) [16-17], largemouth bass fingerlings [18-19], and juvenile turbot (*Scophthalmus maximus*) [20], however no immersion model has been established for salmonids, and thus intra-coelomic injection remains the most common method of laboratory-induced *E. piscicida* infection.

Immersion models present an attractive method for experimental exposure of fish as they better reflect natural infection, stimulate both systemic and mucosal immune mechanisms, reduce handling stress, minimize iatrogenic trauma to the animals, particularly in smaller-sized/younger fish, and allow for simultaneous exposure of large numbers of fish [21-22]. These features make immersion challenge models extremely useful for research into bacterial pathogenesis, as well as for evaluating therapeutics and vaccines. In contrast, injection is often viewed as a less-than-ideal method for disease challenge in fish as it is time and labor intensive, is more stressful on the fish, risks injury to both the fish and handler, and requires larger sized fish. Additionally, injection challenges bypass the mucosal immune system of fish which plays an essential role in natural immune defense against pathogens [22,23]. This circumvention of the mucosal barrier also limits the evaluation of mucosal vaccines against *E. piscicida* infection.

An effective immersion challenge model for *E. piscicida* would more closely replicate natural infection dynamics and would allow for stimulation of the mucosal immune system. One of the major challenges in establishing an immersion model, however, is the low mortality rate and high variability between experiments that can occur [15,24]. Previous studies with other fish pathogens have developed techniques for increasing the pathogenicity and reliability of immersion challenge models. Physical disruption of the skin, through wounding or scarification, immediately prior to exposure has been shown to enhance mortality with immersion challenge for *Flavobacterium psychrophilum* [25], *Flavobacterium columnare* [26], and *Aeromonas salmonicida* [27-28], likely by creating a portal for bacterial entry. Removal of the mucous barrier, whether by chemical or physical means, has also been used to increase mortality and enhance pathogen entry following immersion challenge with *F. psychrophilum* [24,29-30],

cyprinid herpesvirus 3 [31], and *F. columnare* [32]. These techniques are promising methods for improving the experimental induction of *E. piscicida* infection in salmonids via immersion.

4.3. Materials and Methods

4.3.1 Experimental animals and rearing conditions

Rainbow trout (*Oncorhynchus mykiss*) eggs were obtained from the Mount Shasta Fish Hatchery (Siskiyou County, CA, USA) and Chinook salmon eggs were obtained from the Nimbus Fish Hatchery (Gold River, CA, USA). Fish were hatched and raised at the Center for Aquatic Biology and Aquaculture (Davis, CA, USA). For each experiment, fish were arbitrarily divided into experimental and control tanks and maintained indoors. Yearling fish (≥ 9 -month-old) were housed in 45-gallon flow-through tanks supplied with ground water at a rate of 4 L/min at $18 \pm 1^\circ\text{C}$ with supplemental aeration via an air stone supplied by a regenerative blower. Likewise, fingerling fish (≤ 5 -month-old) were housed in 8-gallon flow-through tanks supplied with the same water at a rate of 2 L/min at $18 \pm 1^\circ\text{C}$ with supplemental aeration. Fish were acclimated to experimental conditions for at least 1 week prior to the start of experiments. Water temperature was monitored daily and dissolved oxygen was measured weekly to ensure oxygen concentrations stayed between 8-10 mg/L. All fish were fed a commercial pelleted diet at 1% body weight per day. Prior to the start of each experimental trial, a subpopulation of each fish cohort was sampled to confirm the lack of *E. piscicida* infection. All fish experiments were conducted under approval by the University of California, Davis Institutional Animal Care and Use Committee in compliance with the Animal Welfare Act and all other state and federal regulations regarding animal care and experiments involving animals.

4.3.2 Bacterial isolates and culture conditions

The *E. piscicida* isolate used in this study, *E. piscicida* 344, was collected from diseased rainbow trout in North Carolina, USA (Genbank Accession No. JBWLVB000000000). Prior to challenges, the

isolate was revived from frozen stock by isolation streaking on trypticase soy agar supplemented with 5% sheep blood (SBA, University of California Davis, Biological Media Services, USA) and grown for 24 h at 28°C. Individual colonies were inoculated into 5 mL of brain heart infusion broth (BHI; MP Biomedicals) and expanded overnight at 28°C with constant shaking (175 rpm). Then, 0.5 mL of the expanded bacterial suspension was added to Erlenmeyer flasks containing 500 mL of sterile BHI and further incubated for 12 h at 28°C with constant shaking (175 rpm). After incubation, the optical densities at 600 nm (OD₆₀₀; BioPhotometer Plus, Eppendorf AG) were measured and bacterial concentration was confirmed via serial dilution and spot-plating of 10 µL aliquots in triplicate on SBA for colony enumeration, with an undiluted OD₆₀₀ of ~1.2 corresponding to approximately 10⁹ CFU/mL.

4.3.3 *Edwardsiella piscicida* Immersion Challenges

Four immersion challenge trials were conducted to assess the efficacy of different immersion challenge protocols. In Trial 1, rainbow trout yearlings (one-year old) (82.78 ± 26.14 g) and three-month-old Chinook salmon fingerlings (2.3 ± 0.42 g) were arbitrarily divided into six treatment groups: an intact exposed group (n = 10 fish/species), an intact control group (n = 10 fish/species), a mucous removed exposed group (n = 10 fish/species), a mucous removed control group (n = 10 fish/species), a wounded exposed group (n = 10 fish/species), and a wounded control group (n = 10 fish/species). The intact groups were challenged without disruption of the mucous coating or the skin. The mucous removal groups were sedated via brief immersion in a separate tank containing 75 mg/L tricaine methanesulphonate (MS-222) buffered 1:1 (w/w) with sodium bicarbonate and then the mucous coating was removed by wiping a paper towel (Kimberley-Clark Worldwide, Inc., Roswell, GA, USA) anterior to posterior across the left lateral aspect of the body. The wounded groups were similarly sedated, and adipose fins were removed using a sterile scalpel blade. Fish were exposed to the bacteria by immersion in separate static tanks containing *E. piscicida* 344 bacterial suspensions diluted 1:100 (v/v) in water to generate a final challenge concentration of ~10⁷ CFU/mL of bacteria (Table 4.1). Rainbow trout were challenged in baths containing 30 L of water (10 fish/bath; 18°C) at a stocking density of approximately 27.6 g/L while Chinook salmon were challenged

in baths containing 5 L of water (10 fish/bath; 18°C) at a stocking density of approximately 4.6 g/L. Control groups received an equivocal volume of sterile culture media diluted in the bath water. All groups were exposed for 30 min, with supplemental aeration via an airstone supplied by a regenerative blower, after which they were returned to their experimental tanks and maintained at 18±1°C. Fish were monitored every 12 hours for 21 days during which fish presenting clinical signs of infection were euthanized and recorded. The brains and posterior kidneys of diseased fish were aseptically sampled and plated on SBA and then cultured overnight at 28°C for recovery of and confirmation of *E. piscicida* infection. Identical challenge and sampling methods were repeated in Trial 2 with nine-month-old Chinook salmon (52.9 ± 16.6 g; n = 10 fish/treatment) (yearlings) and in Trial 3 with five-month-old rainbow trout (3.7 ± 0.8 g; n = 10 fish/treatment) (fingerlings).

To confirm the efficacy of the wounding-based immersion model, a fourth experimental trial was conducted using five-month-old rainbow trout (4.48 ± 1.38 g) (fingerlings) and five-month-old Chinook salmon (5.86 ± 1.43 g) (fingerlings). Fish were arbitrarily divided into two treatment groups: an exposed group (n = 45 fish/species) and a negative control group (n = 45 fish/species). All fish had their adipose fins removed and then were immediately challenged via immersion in static baths, with supplemental oxygenation provided via an airstone supplied by a regenerative blower, containing *E. piscicida* 344 bacterial suspensions diluted 1:100 (v/v) in water to generate a final challenge concentration of ~10⁷ CFU/mL of bacteria. The rainbow trout and Chinook salmon were challenged with bacteria in baths containing 40 L of water (n = 45 fish/bath) at stocking densities of approximately 5.0 g/L and 6.6 g/L, respectively. Trout and salmon were exposed for 1 h in their immersion baths, and then arbitrarily divided into triplicate tanks of 15 fish/tank. Negative control fish received an equivalent volume of sterile culture media in their immersion baths. Following challenge, fish were monitored every 12 hours for morbidity and mortality. Diseased fish were aseptically sampled for recovery of *E. piscicida*, similar to previous trials, and then fixed whole in formalin for histopathologic analysis.

Table 4.1. Inoculation doses for *Edwardsiella piscicida* 344 immersion challenges of rainbow trout and Chinook salmon. Bacterial concentration was calculated as the concentration of the bacterial suspension in BHI broth after 13 h growth at 28°C, as determined by spot plating and colony count enumeration. Effective challenge dose was calculated as the approximate bacterial concentration in the immersion bath experienced by the fish after 1:100 dilution of bacterial suspension in water.

Experimental Trial	Optical Density (OD ₆₀₀)	Bacterial Concentration	Effective Challenge Dose
Trial 1	1.128	2.9×10^9 CFU/mL	2.9×10^7 CFU/mL
Trial 2	1.291	1.5×10^9 CFU/mL	1.5×10^7 CFU/mL
Trial 3	1.198	2.3×10^9 CFU/mL	2.3×10^7 CFU/mL
Trial 4	1.206	1.8×10^9 CFU/mL	1.8×10^7 CFU/mL

4.3.4 Histopathologic Analysis

Necropsies were performed on diseased fish and mortalities that occurred during the 21-day experimental periods to examine for gross signs of disease. In Trial 4, all moribund and dead fish were fixed whole via immersion in 10% neutral buffered formalin for a minimum of 24 hours following a linear incision into the coelom and sampling of the brain and posterior kidney for microbiology. Histopathologic analysis was performed on formalin-fixed salmon and trout in Trial 4 that were either moribund and freshly euthanized or found dead following immersion challenge with *E. piscicida* 344. The fish were sectioned either sagittally or transversely and processed routinely to produce 4 µm thick hematoxylin and eosin (H&E) stained sections [33] which were evaluated by a board-certified veterinary pathologist.

4.3.5 Statistical Analyses

Differences in survival curves between treatment groups were determined using the Log-rank (Mantel-Cox) test. For Trial 4, survival curves from replicate groups did not differ significantly; therefore, data

from the replicates were pooled for the presented analysis. Statistical significance for all analyses was set at $p < 0.05$. All analyses were performed using GraphPad Prism v.10.6.1 (GraphPad Software, USA).

4.4. Results

4.4.1 Immersion Challenge Survival

In Trial 1, Chinook salmon fingerlings treated with mucous removal prior to immersion challenge with *E. piscicida* demonstrated 30% mortality whilst wounded Chinook salmon demonstrated 40% mortality (Table 4.2; Figure 4.1A). Peak mortality among the mucus removed and wounded Chinook salmon challenged with *E. piscicida* occurred between 5 to 7 days post-challenge (dpc). None of the intact challenged Chinook salmon in Trial 1 died or developed clinical signs of infection throughout the 21-day experimental period. Additionally, none of the intact, mucus removed, or wounded control salmon exposed to sterile culture media demonstrated morbidity or mortality throughout the duration of the experiment. None of the yearling rainbow trout, developed any clinical signs of infection or died during the experiment, regardless of treatment (Table 4.2).

In Trial 2, both intact and wounded Chinook salmon yearlings challenged with *E. piscicida* either intact or wounded demonstrated 10% mortality (Table 4.2; Figure 4.1B), with mortality occurring at 10 dpc in both groups. No mortality was observed in mucus removed salmon or in negative control fish.

In Trial 3, rainbow trout fingerlings challenged with *E. piscicida* after adipose fin removal demonstrated 10% mortality (Table 4.2; Figure 4.1C). No mortality was observed in the intact or mucus removed trout challenged with bacteria and no mortality was observed in the control fish over the 21-day experimental period.

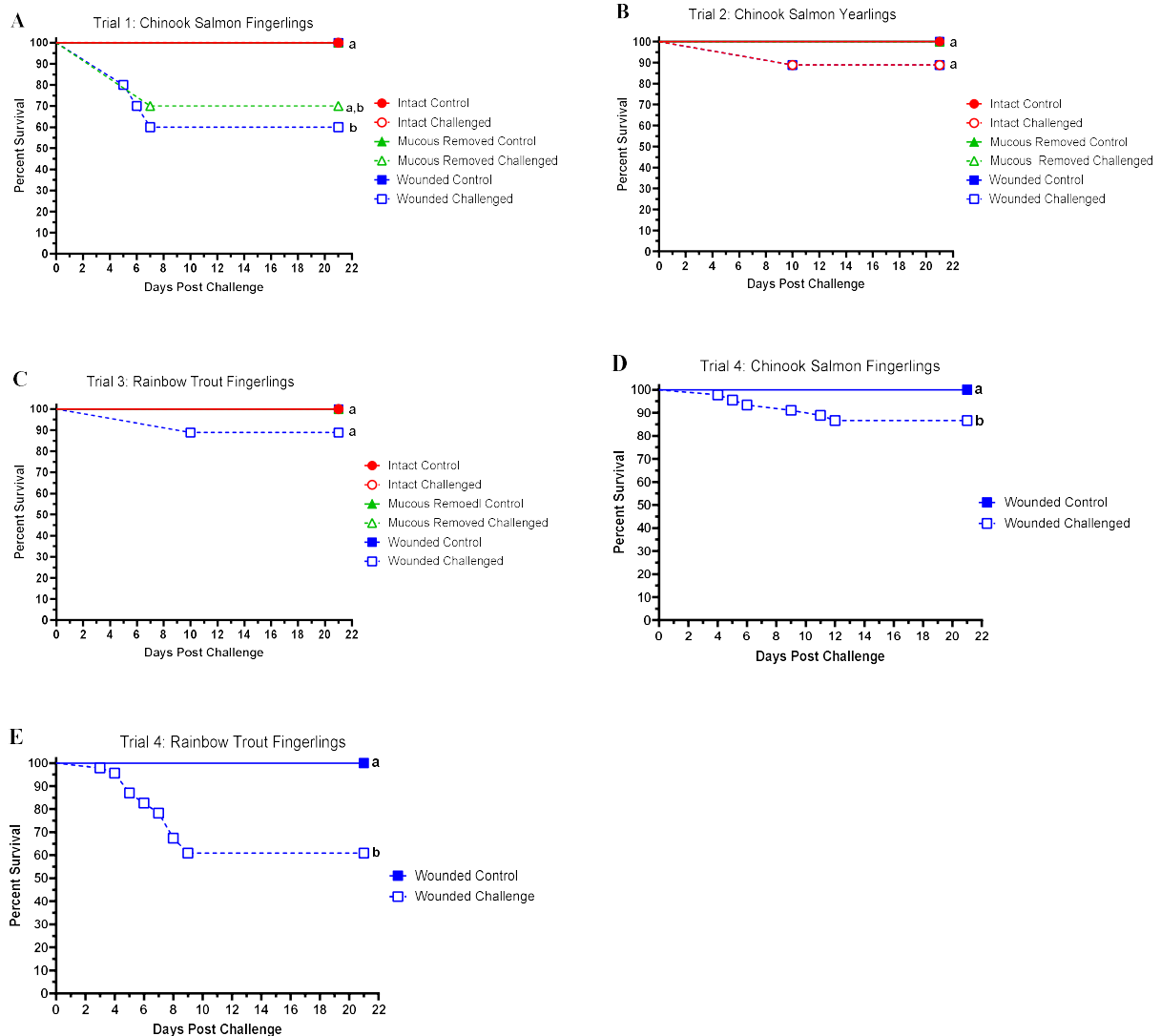
In Trial 4, removal of the adipose fin of Chinook salmon and rainbow trout fingerlings just prior to immersion challenge with *E. piscicida* led to 13.3% mortality and 40% mortality, respectively (Table

4.2; Figures 4.1D-E). Mortality of Chinook salmon occurred between 4-12 dpc, whilst mortality of rainbow trout occurred from 3-9 dpc with peak mortality occurring between 5-8 dpc. None of the negative control fish died or developed clinical signs of disease throughout the duration of the experiment.

Table 4.2. Summary of rainbow trout and Chinook salmon 21-day post-challenge survival following immersion with $\sim 10^7$ CFU/mL of *Edwardsiella piscicida* 344 in Trial 1^a, Trial 2^b, Trial 3^c, and Trial 4^d. In all four trials, no mortalities were observed in sterile BHI-exposed control groups regardless of treatment (data not shown).

Host Species	Age	Size	Treatment	Percent Mortality
Rainbow trout ^a n = 10	1 year	82.8 ± 26.1 g	Intact 30 min immersion	0%
Rainbow trout ^a n = 10	1 year	82.8 ± 26.1 g	Mucous removed 30 min immersion	0%
Rainbow trout ^a n = 10	1 year	82.8 ± 26.1 g	Wounded 30 min immersion	0%
Chinook salmon ^a n = 10	3 months	2.3 ± 0.4 g	Intact 30 min immersion	0%
Chinook salmon ^a n = 10	3 months	2.3 ± 0.4 g	Mucous removed 30 min immersion	30%
Chinook salmon ^a n = 10	3 months	2.3 ± 0.4 g	Wounded 30 min immersion	40%
Chinook salmon ^b n = 10	9 months	52.9 ± 16.6 g	Intact 30 min immersion	10%
Chinook salmon ^b n = 10	9 months	52.9 ± 16.6 g	Mucous removed 30 min immersion	0%
Chinook salmon ^b n = 10	9 months	52.9 ± 16.6 g	Wounded 30 min immersion	10%
Rainbow trout ^c n = 10	5 months	3.7 ± 0.8 g	Intact 30 min immersion	0%
Rainbow trout ^c n = 10	5 months	3.7 ± 0.8 g	Mucous removed 30 min immersion	0%
Rainbow trout ^c n = 10	5 months	3.7 ± 0.8 g	Wounded 30 min immersion	10%
Rainbow trout ^d n = 45	5 months	4.48 ± 1.4 g	Wounded 1 h immersion	40%
Chinook salmon ^d n = 45	5 months	5.86 ± 1.4 g	Wounded 1 h immersion	13.3%

Figure 4.1. Survival of trout and salmon following immersion challenge with 10^7 CFU/mL of *Edwardsiella piscicida* isolate 344. **(A)** Survival of Chinook salmon fingerlings (2.3 ± 0.4 g) in Trial 1. **(B)** Survival of Chinook salmon yearlings (52.9 ± 16.6 g) in Trial 2. **(C)** Survival of rainbow trout fingerlings (3.7 ± 0.8 g) in Trial 3. Survival of **(D)** Chinook salmon fingerlings (5.86 ± 1.4 g) and **(E)** rainbow trout fingerlings (4.48 ± 1.4 g) in Trial 4. No mortalities were observed at 21 days post-challenge in rainbow trout yearlings (82.8 ± 26.1 g) from Trial 1 (data not shown). Letters after each survival curve indicate statistical significance ($p \leq 0.05$) as determined by a Log-rank (Mantel-Cox) test.



4.4.2 Necropsy Findings and Histopathological Analysis

Grossly, affected fish demonstrated coelomic distension and multifocal hemorrhages, primarily at the site of the adipose fin clip as well as along the ventrolateral aspects of the body and within the eyes (Figure 4.2). The histologic findings were similar between both the rainbow trout and Chinook salmon, with affected fish demonstrating multiorgan necrosis which predominantly targeted the renal interstitium, the spleen, and less frequently the liver (Figure 4.3). Variable amounts of bacilli were observed in the coelomic organs, with the greatest abundance of bacteria observed in the spleen and the hematopoietic tissues of the kidneys. Lesions were typically more severe in the rainbow trout, with more abundant bacteria in H&E-stained sections of spleen, kidney, and liver and a more extensive areas of necrosis when compared to Chinook salmon. Chinook salmon that were euthanized or found dead later in the course of disease (≥ 6 dpc) demonstrated evidence of a regenerative response in the affected tissues and some groups of Chinook salmon show no significant microscopic abnormalities. No gross lesions were observed on necropsy of negative control trout and salmon (Supplemental Figure 4.1).

Figure 4.2. Gross necropsy findings from rainbow trout and Chinook salmon fingerlings in Trial 4 after immersion challenge with *Edwardsiella piscicida*. External lesions in affected trout and salmon include erythema and petechiation at the fin clip site (A-C) and along the ventrolateral aspects of the body (D-F), ocular hemorrhage (F), and swelling and redness of the vent (G). Internally, affected fish demonstrated hemorrhage of the coelomic adipose and visceral organs (H). RT, rainbow trout; CS, Chinook salmon; dpc, days post-challenge.

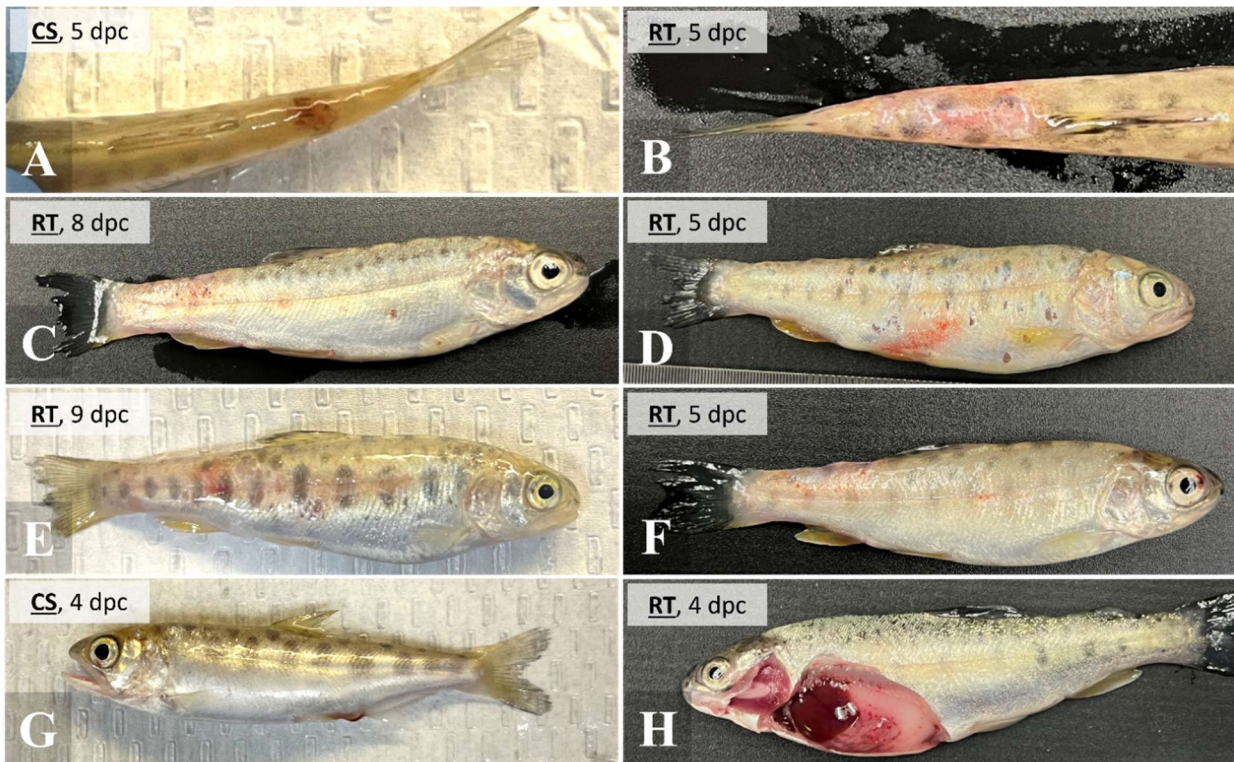
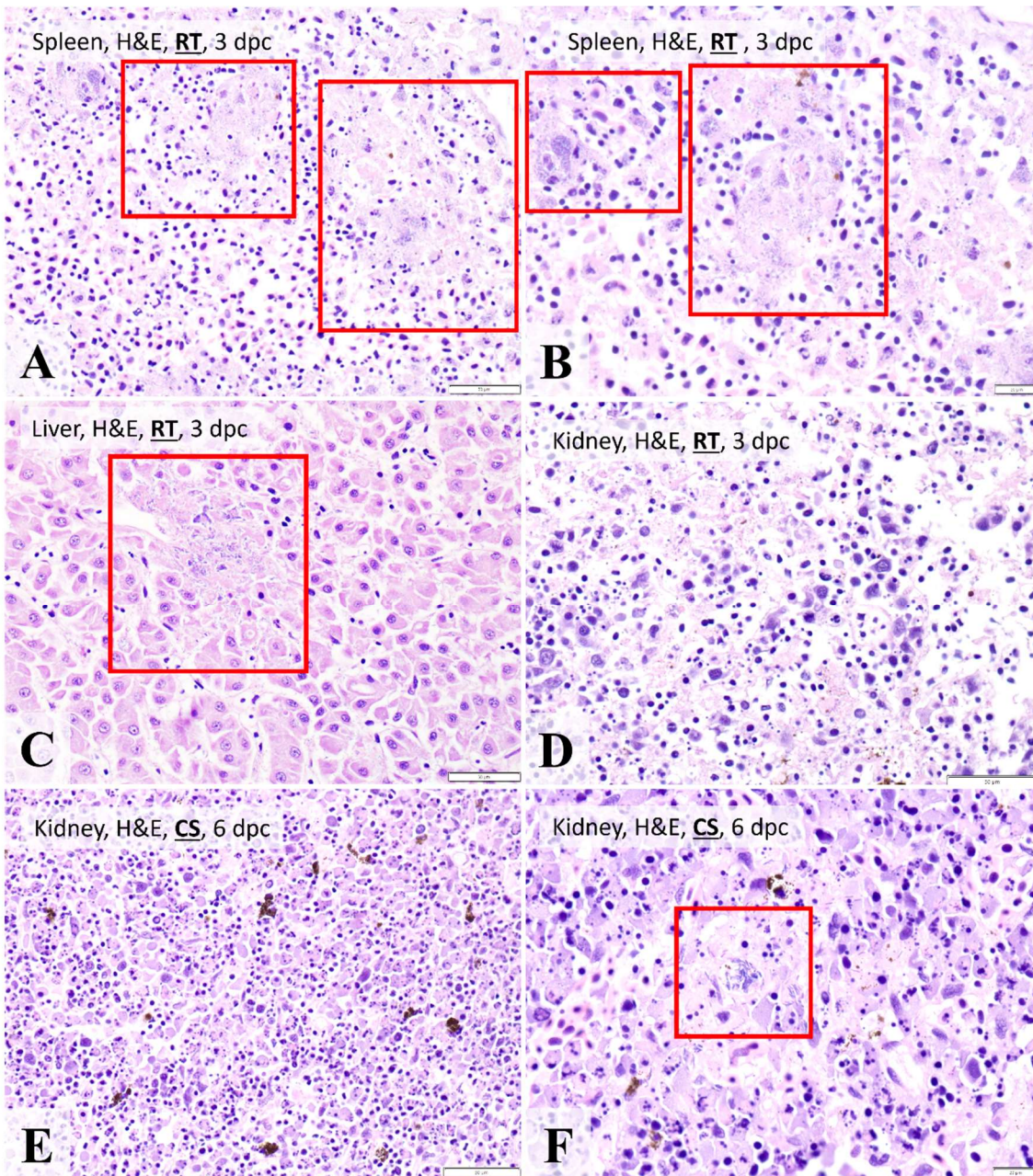


Figure 4.3. Histopathological features of *Edwardsiella piscicida* infection following immersion challenge with adipose fin removal in rainbow trout and Chinook salmon fingerlings in Trial 4. The main histologic feature of affected rainbow trout and Chinook salmon immersed with *E. piscicida* following adipose fin clip were foci of necrosis and apoptosis with intralesional bacteria (red boxes) within the spleen (**A-B**), liver (**C**), and kidneys (**D-F**). Lesions were observed primarily within the hematopoietic tissues of the kidneys and spleen and were more severe with greater abundance of bacteria in rainbow trout. RT, rainbow trout; H&E, hematoxylin and eosin; CS, Chinook salmon; dpc, days post-challenge.



4.5. Discussion

In this study, morbidity and mortality were consistently inducible in salmonid fingerlings (≤ 5 -months of age) following immersion with 10^7 CFU/mL of *E. piscicida* only when the adipose fin had been removed immediately prior to exposure to create an open wound. Affected fish demonstrated similar gross and histologic features as those observed in natural septicemia outbreaks and in laboratory-induced intra-coelomic challenge, most notably coelomic distension, petechiation, and multiorgan necrosis with abundant intralesional bacteria [5,7,9,11-12]. Age and size-matched fish that were challenged intact, without disruption of the skin or mucous, did not demonstrate consistent clinical disease or mortality, suggesting that disruption of the skin barrier via wounding increases the effectiveness of immersion challenge with *E. piscicida*. This is consistent with previous findings of injured skin as one of the main initial susceptible sites for *E. piscicida* infection in fish, indicating that wounding just prior to immersion may facilitate bacterial entry and subsequent infection [34]. Removing the skin mucous coating did appear to enhance bacterial infection compared to intact immersion, as Chinook salmon fingerlings challenged after mucosal removal experienced 30% mortality compared to 0% mortality in intact fish (Trial 1), however this method was inconsistent and did not induce mortality in rainbow trout fingerlings (Trial 3).

The skin and mucous coating of fish stand as the first line of defense against invasion by infectious agents. Fish skin mucous serves as a physical barrier against infection, trapping environmental pathogens for removal by the water current and preventing colonization by potentially infectious microorganisms through continuous turnover [35]. Additionally, the skin mucous is part of the extensive mucosal immune system of fish and contains numerous biologically active molecules that further serve to defend the fish against pathogen invasion. Some of these innate and adaptive immune mechanisms found within the skin mucous include complement, immunoglobulins, lectins, lysozyme, defensins, C-reactive proteins, antimicrobial peptides, hemolysins, and proteolytic enzymes [36-37]. Disruption of the mucous coating removes these normal physical and mucosal immunity defense mechanisms, potentially increasing susceptibility of fish to immersion infection by enhancing bacterial adhesion, colonization, and

multiplication [32,38]. Similarly, fresh cutaneous injury through removal of the adipose fin prior to immersion also interrupts the epithelial barrier and provides a portal of entry for pathogens allowing for improved bacterial attachment and infection and facilitating increased mortality following immersion exposure [26]. Although both mucosal removal and wounding enhanced host susceptibility to infection, removal of the adipose fin was preferred over mucosal removal as a means of facilitating immersion challenge in Trial 4 as the technique more consistently induced morbidity and mortality in challenged fish.

The age and/or size of the fish at the time of immersion challenge also appeared to impact the susceptibility of salmonids to *E. piscicida* infection. Across all four trials, Chinook salmon and rainbow trout that were ≤ 5 months of age and < 10 g in size demonstrated higher mortality rates in wounded and mucous removed challenge groups compared to older/larger Chinook salmon and rainbow trout, suggesting different susceptibilities to *E. piscicida* immersion infection based on age and/or size despite identical challenge conditions. For other bacterial diseases such as columnaris disease, epitheliocystis, and bacterial coldwater disease the age/size of fish at the time of exposure has been demonstrated to significantly impact disease severity, mortality rate, and bacterial pathogenicity in natural and experimental infections [39-41]. Previous studies into immersion models for *E. piscicida* in other fish species have similarly only reported successful infection in larval or fingerling fish [17,19,42], however natural outbreaks of *E. piscicida* have been documented among both juvenile and adult fish [5-7,10,13,43]. Further study into the age-related or size-related susceptibility of fish to *E. piscicida* infection is necessary to better understand how life-stage could impact the success of immersion challenge with *E. piscicida*.

Another potential source of variability in susceptibility to *E. piscicida* challenge is genetic differences among the trout and salmon strains used in the different trials. The trout and salmon used in the four trials originate from different cohorts of eggs, which could have differences in heritable resistance to infection and could have contributed to the variability in post-challenge mortality seen across all experiments, although the exact genetic background of the fish is unavailable [44-46]. Regardless, the data in this study clearly demonstrates an effective and reproducible model for inducing mortality in young

salmonids ≤ 10 g in size following immersion with 10^7 CFU/mL *E. piscicida* after creation of a fresh wound through removal of the adipose fin.

Collectively, these findings establish an effective and practical immersion model to investigate *E. piscicida* pathogenesis in juvenile salmonids. The methodology described herein provides a means to investigate natural mechanisms of *E. piscicida* infection, stimulate and assess mucosal immune responses to *E. piscicida*, and evaluate safety and efficacy of immersion-delivered or other mucosal vaccines. These approaches can be implemented immediately to support disease research and vaccine development, particularly for mucosal or live-attenuated vaccines.

4.6. References

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CHAPTER 5: Virulence and Pathology of Fish Pathogens *Edwardsiella piscicida* and *Edwardsiella tarda* in a Murine Model of Mammalian Infection

5.1. Abstract

In this study, *in-vitro* models of mammalian (Vero) and poikilotherm (epithelioma papulosum cyprinid, EPC) cells, as well as *in-vivo* mouse models were used to explore the virulence of emerging aquatic bacteria *Edwardsiella piscicida* and *Edwardsiella tarda*. *In-vitro* virulence assays revealed reduced cytotoxicity and host-cell internalization of *E. piscicida* in Vero cells compared to EPCs. *Edwardsiella tarda* also had increased cytotoxicity to and adhesion to/invasion into Vero cells than *E. piscicida* at 25°C. Both *E. piscicida* and *E. tarda* demonstrated reduced internalization into Vero cells, compared to EPCs, at 25°C. For the *in-vivo* challenge experiments, C57BL/6, heterozygous (*nu/+*), and homozygous (*nu/nu*) mice were exposed to *E. piscicida* or *E. tarda* via intragastric gavage or subcutaneous injection at a dose known to be highly pathogenic to fish via injection ($\sim 10^5$ CFU/gram). Both immunodeficient and immunocompetent mice injected with *E. tarda* developed lesions grossly and histologically similar to reported human *E. tarda* wound infections. They also had evidence of inflammation and bacilli in various systemic tissues sites. Conversely, only a single immunocompromised mouse injected with *E. piscicida* developed clinical and histologic signs of infection characterized by severe suppurative inflammation extending from the skin to the underlying muscle and fasciia. Histologic lesions and bacterial DNA (qPCR) were detected at both the injection site and the liver of this mouse. Overall, these findings suggest reduced pathogenicity of *E. piscicida* in immunocompetent mammalian hosts compared to *E. tarda* and indicate that immunodeficiency could increase the risk of mammalian *E. piscicida* infection.

5.2. Introduction

Edwardsiella piscicida is an emerging bacterial pathogen of significant concern to global aquaculture. This Gram-negative, facultative intracellular bacterium is one of the etiologic agents of piscine

Edwardsiellosis, a disease of fish characterized by potentially fatal acute septicemia and chronic granulomatous disease [1]. Outbreaks of *E. piscicida* have been reported in several economically important aquaculture species such as largemouth bass (*Micropterus salmoides*) [2], barramundi (*Lates calcarifer*) [3], ictalurid catfish [4], and whitefish (*Coregonus lavaretus*) [5], leading to morbidity and mortality among both young fish and market-sized fish [1,2,4-6]. Reports of *E. piscicida* epizootics have been steadily increasing in frequency and expanding in host species and geographic range over the past decade [1]. Many of these reports include important fish species for food production and/or human recreation, making the emergence of *E. piscicida* concerning as a foodborne and/or waterborne public health risk [1,7]. Accurate assessment of the zoonotic risk posed by *E. piscicida*, however, is impeded by the paucity of information on the virulence of this fish pathogen in humans and mammals.

The genetic and phenotypic similarities of *E. piscicida* and *E. tarda* have caused taxonomic ambiguity in the *Edwardsiella* genus, resulting in disagreement on the pathogenic potential of *E. piscicida* in mammals. The *Edwardsiella* genus was first identified in the later 1950s and early 1960s by two separate laboratories, one in Japan and another in the USA, which described a group of enteric-like bacteria isolated from human fecal samples and snakes that was different from other known *Enterobacteriaceae* [8-10]. This group of phenotypically similar bacteria was formally described in 1965 and designated as the genus *Edwardsiella* with the species *E. tarda* adopted as the representative species [11]. After its discovery, case reports of *E. tarda* wound infections and invasive disease in people began to emerge, demonstrating the bacterium's potential pathogenicity to humans [12-14]. In the early 1980s, two new species of *Edwardsiella* were described: *E. hoshinae*, a species isolated from water, birds, and reptiles and not associated with significant disease [15]; and *E. ictaluri*, the causative agent of enteric septicemia of catfish [16]. These two new *Edwardsiella* species were associated with environmental contamination and animal infections but are not known to cause disease in humans. Instead, all confirmed cases of human Edwardsiellosis have been attributed to *E. tarda* [7].

The taxonomy of the *Edwardsiella* genus remained stable at three species for the next several decades until the mid-2010s when new phylogenetic, phenotypic, and DNA-DNA hybridization evidence led to the discovery of two novel species of fish-pathogenic *Edwardsiella* that are biochemically similar to *E. tarda*: *E. piscicida* [17] and *Edwardsiella anguillarum* [18]. Since this discovery, modern genetic analyses such as matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) and multilocus sequence analysis (MLSA) have been used to reclassify numerous bacterial isolates collected from diseased fish that had previously been described as *E. tarda* based on morphological, physiological, and biochemical testing as either *E. piscicida* or *E. anguillarum* [19-20]. Not only have these findings introduced ambiguity into the true identity of past fish disease cases attributed to *E. tarda*, but they also bring into question whether all previously reported cases of human Edwardsiellosis were truly caused by *E. tarda* or whether these more novel *Edwardsiella* species pose a zoonotic disease risk.

Human *E. tarda* infections have been sporadically reported in the literature as a cause of significant and potentially fatal foodborne and/or waterborne zoonotic disease in affected individuals [7]. The most common route of human *E. tarda* transmission is through consumption of contaminated food, typically raw or undercooked seafood, leading to acute gastroenteritis that can be self-limiting, progress to more severe gastrointestinal disease, or potentially fatal systemic infection [7,21-26]. Zoonotic gastrointestinal infections with *E. tarda* have also been associated with the handling of ornamental fish or pet reptiles and/or exposure to the contaminated tank water [27-29]. Gastrointestinal *E. tarda* infections have been reported to exacerbate underlying inflammatory bowel conditions, such as ulcerative colitis or Crohn's disease, leading to recurrence of symptoms and, in some cases, hospitalization [30-33].

Wound infections with *E. tarda* are the second most commonly reported clinical manifestation, with infections typically initiating either from preexisting wounds exposed to an aquatic environment or from penetrating wounds with contaminated equipment or animals (e.g. catfish bites, fish spines, fishhooks, etc.) [7,34-37]. These wound infections are often co-infected with other aquatic pathogens and can have a wide range of clinical presentations, from mild cellulitis to severe myonecrosis, necrotizing fasciitis, and

osteomyelitis [7,35,37-41]. Additionally, as with some cases of gastrointestinal *E. tarda* infections, wound infections with *E. tarda* can progress to disseminated disease, leading to more severe disease systemic infections such as sepsis, liver or tubo-ovarian abscessation, meningitis, peritonitis, and infectious endocarditis [14,36,42-50]. The vast majority of *E. tarda* bacteremia and systemic infection cases involve patients with underlying medical conditions, such as diabetes mellitus or cancer, or immunocompromised patients [7,22,51-53].

Despite being typically considered a rare disease in people, these reports of severe and fatal systemic *E. tarda* infection highlight that Edwardsiellosis poses a significant risk to humans, particularly to immunologically vulnerable individuals. As *E. piscicida* continues to emerge among wild and farmed fish populations, it becomes increasingly crucial to understand the zoonotic disease risk posed by this closely related pathogen. In this study, we utilized an *in-vitro* cell model and *in-vivo* mouse model to explore the virulence of fish-pathogenic strains of *E. piscicida* and *E. tarda* at mammalian physiologic conditions in order to evaluate and compare their capacities to infect and cause disease in mammals as well as determine whether immune status significantly impacts the risk of mammalian Edwardsiellosis.

5.3. Materials and Methods

5.3.1 Experimental animals

All mouse experiments were conducted under approval by the University of California, Davis Institutional Animal Care and Use Committee (Protocol No. 24091) in compliance with the Animal Welfare Act and all other state and federal regulations regarding animal care and experiments involving animals. The C57BL/6 mice (age, 7 wk) were a gift from the UC Davis Comparative Ophthalmology and Vision Sciences Laboratory. Mice were arbitrarily divided into experimental and control cages, with mice separated by sex and age group (n = 2-3 mice/cage). Immunocompetent heterozygous (*nu/+*) and immunodeficient homozygous (*nu/nu*) Hsd:Athymic Nude-*FoxI^{nu}* mice (age, 6 wk) were obtained from

The Jackson Laboratory (Bar Harbor, Maine, USA) and arbitrarily divided into experimental and control cages, with mice separated by sex and phenotype (n = 3 mice/cage). All mice were maintained under aseptic conditions at the UC Davis Teaching and Research Animal Care Services (TRACS) vivarium facility and acclimated to laboratory conditions for at least two weeks before the start of the experiment. Sentinel animals exposed to bedding from research animals tested negative for mouse hepatitis virus, mouse parvovirus, minute virus of mice, *Mycoplasma pulmonis*, Theiler murine encephalomyelitis virus, ectromelia virus, epizootic diarrhea of infant mice virus, murine adenovirus types 1 and 2, lymphocytic choriomeningitis virus, and reovirus 3 throughout the study. All mouse experiments were performed when mice were eight to nine-weeks-old.

5.3.2 Bacterial isolates

Both bacterial isolates used in this study, *E. piscicida* S11-285 [54] and *E. tarda* 94-45-85 (this study), were collected from clinical cases of Edwardsiellosis in catfish in the United States (Table 5.1). Isolates were revived from frozen stock on trypticase soy agar supplemented with 5% sheep blood (SBA, University of California Davis, Biological Media Services, USA) and grown overnight at 28°C. Bacterial colonies were harvested from SBA and resuspended in sterile phosphate-buffered saline (PBS). Bacterial concentrations in all experiments were estimated spectrophotometrically (BioPhotometer Plus, Eppendorf AG) where a 0.5 McFarland standard ($\sim 10^8$ CFU/mL) in PBS corresponded to an optical density at 600 nm (OD₆₀₀) of ~ 0.15 . Bacteria quantity was confirmed via serial dilution and spot-plating of 10 μ L in triplicate on SBA for colony enumeration.

Table 5.1. Summary of *Edwardsiella* isolates used in this study.

Isolate	Host Species	Geographic Location	Reference	NCBI Accession
<i>E. piscicida</i> S11-285	Channel catfish (<i>Ictalurus punctatus</i>)	Mississippi, USA	Reichley et al. 2016	CP016044.2
<i>E. tarda</i> 94-45-85	Channel catfish (<i>I. punctatus</i>)	California, USA	This study	SAMN54755816

5.3.3 Cell Culture

The epithelioma papulosum cyprinid (EPC) cell line [55] was derived from the skin cells of a fathead minnow (*Pimephales promelas*). The Vero cell line [56] originated from the renal epithelial cells of an African green monkey (*Chlorocebus aethiops*). All cells were maintained in Leibovitz's L-15 medium (L-15; ThermoFisher, USA) supplemented with 7.5% fetal bovine serum (FBS; Gibco). Prior to experimentation, Vero cells were maintained at 37°C and EPC cells were maintained at 20°C.

5.3.4 Bacterial Growth Curves

McFarland standard suspensions of 0.5 in PBS were generated for each bacterial isolate then serially diluted 1:1000 in brain heart infusion broth (BHI; MP Biomedicals). The bacterial dilutions were plated in 96-well plates (100 µL per well), with control wells containing 100 µL of sterile BHI. Plates were incubated with constant shaking for 72 h at one of four experimental temperatures: 15°C to replicate cool-water conditions, 25°C to replicate warm-water conditions, 30°C to replicate mammalian superficial site temperature, and 37°C to replicate mammalian core temperature. Throughout the incubation period, the absorbance at OD₆₀₀ of each sample was measured at 1-h intervals using a Cytation™ 5 Imaging Reader (BioTek, USA). Each experiment was run twice, and OD measurements were adjusted based on BHI control wells.

5.3.5 In-vitro Cytotoxicity Assay

Differential cytotoxicity of *E. piscicida* and *E. tarda* to EPC and Vero cell lines was determined by exposing cell cultures to bacteria at various temperatures. Briefly, suspensions of 10⁵ cells/mL of Vero and EPC cells in L-15 media supplemented with 2% FBS and 1.4% N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES; Gibco) were inoculated onto 24-well tissue culture plates (1 mL/well) and incubated at room temperature for 3 h to allow cells to attach to the bottom of the wells before infection. McFarland standards of 0.5 of *E. tarda* 94-45-85 and *E. piscicida* S11-285 generated in PBS were diluted 10-fold in L-

15 + 2% FBS + HEPES. Cells were infected with *E. tarda* or *E. piscicida* at a multiplicity of infection (MOI) of 10:1 (10 bacteria per 1 cell), respectively, centrifuged at $300 \times g$ for 5 min, then incubated for 1 h at the experimental temperature (15, 25, 30, or 37°C). Cells provided with an equal volume of sterile cell culture media were used as uninfected controls. After incubation, wells were treated with 100 μ L of 1.2 mg/mL gentamicin sulfate (final concentration of 100 μ g/mL in the well) and incubated at the experimental temperature for a further 1 h to kill all extracellular bacteria. This antibiotic concentration and time frame of exposure was sufficient to kill all *E. piscicida* and *E. tarda* in control samples without cells (data not shown). After incubation, cells were washed three times with sterile PBS then 1 mL of cell culture media supplemented with 0.3 μ g/mL gentamicin sulfate was added to each well. Plates were incubated for 48 h at the experimental temperature. This antibiotic concentration with this exposure time was sufficient to inhibit bacterial growth in control samples incubated without cells (data not shown). At 48 h post-incubation, cytosolic lactate dehydrogenase (LDH) levels were measured via the colorimetric Cytotox 96 Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions using a Cytation™ 5 Imaging Reader (BioTek, USA). The percentage of cytotoxicity was calculated as $100 \times [(\text{experimental release} - \text{negative control release})]/[(\text{positive control release} - \text{spontaneous release})]$, where negative control release was the amount of LDH activity in the supernatant of uninfected cells and the positive control release was the amount of LDH activity in the supernatant after all cells have been lysed. Each experiment was run twice independently to confirm results.

5.3.6 *In-vitro Adhesion/Invasion Assay*

The *in-vitro* adhesion/invasion capabilities of *E. piscicida* and *E. tarda* in EPC and Vero cell lines were assessed using methods adapted from Soto et al. 2017 [57]. Briefly, suspensions of 10^5 cells/mL of Vero and EPC cells in L-15 media supplemented with 2% FBS and 1.4% HEPES were inoculated onto 24-well tissue culture plates (1 mL/well) and incubated at room temperature for 3 h. After incubation, cells were infected with 100 μ L aliquots of *E. piscicida* or *E. tarda* at an MOI of 10:1, centrifuged at $300 \times g$ for 5 min, then incubated at the experimental temperature (15°C, 25°C, 30°C, and 37°C) for 1 h. Following

incubation, wells were washed thrice with PBS. Cells were then solubilized and lysed with 1 mL of saponin (1% w/v saponin dissolved in PBS). Cell lysates were serially diluted, and 10 μ L aliquots of each ten-fold dilution were plated in triplicate onto SBA and incubated at 28°C for colony enumeration. Percent adhesion/invasion was calculated as $100 \times [\text{experimental colony count} / \text{initial inoculum colony count}]$.

5.3.7 *In-vitro Internalization Assay*

The internalization capabilities of *E. piscicida* and *E. tarda* in EPC and Vero cell lines at various temperatures were assessed using methods adapted from Soto et al. 2017 [57]. Briefly, EPC and Vero cells were infected with bacteria using identical methods as those described for the adhesion/invasion assay and incubated at the experimental temperature (15°C, 25°C, 30°C, and 37°C) for 1 h. After incubation, wells were washed thrice with sterile PBS then treated with 100 μ L of 1.2 mg/mL gentamicin sulfate (final concentration of 100 μ g/mL in the well) and incubated for a further 1 h at the experimental temperature. Following incubation, wells were washed three times with PBS and cells were solubilized and lysed with 1 mL of 1% saponin. Cell lysates were serially diluted, and 10 μ L aliquots of each ten-fold dilution were plated in triplicate onto SBA and then incubated at 28°C for colony enumeration. Percent internalization was calculated as $100 \times [\text{experimental colony count} / \text{mean adhesion colony count}]$, in which the mean adhesion colony count was the average colony count from the adhesion/invasion assay using the corresponding bacteria/temperature/cell line experimental conditions.

5.3.8 *Whole Genome Sequence Analysis*

The whole genome sequence of *E. piscicida* S11-285 was retrieved from GenBank (Accession No. CP016044.2). *Edwardsiella tarda* 94-45-85 was revived from cryostock on SBA and incubated at 28°C overnight. Bacterial colonies were then suspended in 150 μ L of sterile double-distilled water and DNA was extracted using the Dneasy® Blood and Tissue (Qiagen, Germany) following manufacturer protocols. The concentration and quality of the extracted gDNA was measured via spectrophotometry (NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer, Thermo Fisher Scientific, MA, USA). The DNA

concentration was then normalized to 50 ng/μL and submitted to Plasmidsaurus for bacterial genome sequencing using Oxford Nanopore Technology with custom analysis and annotation. The whole genome sequence of *E. tarda* 4-45-85 was submitted to GenBank under the sample number SAMN54755816. The whole genome sequences of *E. piscicida* S11-285 and *E. tarda* 94-45-85 were evaluated using the PathogenFinder 1.1 tool [58] to determine their potential as human pathogens.

5.3.9 Mouse Infection Experiments

Eight to nine-week-old male and female C57BL/6 (n = 2-3 mice per sex and treatment combination), immunocompetent heterozygous (*nu/+*; n = 3 mice per sex and treatment group combination), and immunodeficient homozygous (*nu/nu*; n = 3 mice per sex and treatment group combination) were inoculated with either *E. piscicida* S11-285 or *E. tarda* 94-45-85 via subcutaneous injection (SQ) over the right thigh or via orogastric gavage (PO). Mice were inoculated at a dose of 10⁵ CFU/g of mouse, a dose experimentally demonstrated to be highly pathogenic to various fish species^{5,21,53,63}. Mock-exposed control mice for each sex/strain/treatment group (n = 6 mice per group) were administered an equal volume of sterile PBS. All infected and control groups were housed separately in polycarbonate microisolation caging in a HEPA-filtered room with controlled temperature (22-24°C) and humidity (30-70%) on a 12:12-h light:dark cycle. Health checks and weight measurements were conducted for all mice at least once daily for 21 days. Any mice demonstrating clinical signs of infection during the experimental period were euthanized via CO₂ inhalation with secondary cardiac venipuncture and sampled. All surviving mice at the end of the 21-day period were euthanized and sampled.

5.3.10 Tissue Bacterial Recovery Assay

Mice were necropsied immediately after euthanasia and liver samples were placed in 500 μL of PBS and manually homogenized using a polypropylene pestle. The homogenates were then vortexed and serially diluted in sterile PBS. Serial 10-fold dilutions of the tissue homogenates were spot-plated (10 μL) in

triplicate onto SBA and incubated overnight at 28°C for colony enumeration. Bacterial counts were normalized to the weight of the liver sample.

5.3.11 Quantitative PCR Bacterial Load Quantification

Liver, spleen, and brain tissues from all mice, as well as skin/subcutis samples from SQ inoculated mice and jejunum samples from PO inoculated mice were collected at necropsy and stored at -80°C prior to processing. Genomic DNA (gDNA) was isolated from the tissues using the DNeasy® Blood & Tissue DNA kit (Qiagen, CA, USA) following the manufacturer's suggested protocol. DNA concentrations for each tissue were measured via NanoDrop spectrophotometry (NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer, Thermo Fisher Scientific, MA, USA). Quantitative PCR (qPCR) for the detection and measurement of *E. tarda* and *E. piscicida* DNA was performed on tissue gDNA samples with a QuantStudio™ 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific, MA, USA) using methods described by Reichley et al. 2015 [59] (Supplemental Table 5.1). For each assay, samples, no-template negative controls, and positive controls of bacterial gDNA from purified *E. tarda* or *E. piscicida* colonies were run in triplicate.

Standard curves for calculation of bacterial load (Supplemental Figure 1) were generated from qPCR analysis of serial 10-fold dilutions of purified target PCR amplicons derived from pure cultures of *E. piscicida* S11-285 and *E. tarda* 94-45-85 following previously described methods [59]. All amplification reactions were run in triplicate in two independent assays. qPCR assays were interpreted as positive if the measured C_q values from mouse tissues were ≤36.

5.3.12 Whole Bacteria Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was performed using protocols adapted from Heckman et al. (2022) [60] to determine the specific IgG response of the mice at 21 days post-challenge (dpc) against the inoculating bacteria. Briefly, a 0.5 McFarland standard of *E. piscicida* isolate S11-285 or *E. tarda* 94-45-85 (OD₆₀₀ 0.15) was generated in carbonate-bicarbonate buffer, then 100 µL of the bacterial suspension

was added to each well of Immulon® 2HB Flat Bottom microtiter® (Thermo Scientific) 96-well plates. Seeded plates were incubated overnight at 4°C. Following incubation, plates were washed three times with low salt wash buffer (LSWB; 0.02 M Trizma base, 0.38 M NaCl, 0.05% (v/v) Tween-20, pH 7.2). Then, 250 µL of blocking buffer (5% w/v skim milk powder in distilled water) was added to each well and plates were incubated at room temperature for 3 h. Plates were then washed thrice with LSBW. Serum from mice were serially diluted in LSBW containing 1% bovine serum albumin (BSA; Sigma). One hundred µL of serum dilutions (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, and 1:3200) were added in duplicate to their respective wells and 100 µL of sterile PBS was added to control wells seeded with bacteria. Plates were incubated at 4°C overnight. After incubation, plates were washed three times with high-salt wash buffer (HSWB; 0.02 M Trizma base, 0.5 M NaCl, 0.01% (v/v) Tween-20, pH 7.7). Goat anti-mouse IgG (H+L), horseradish peroxidase conjugate (Thermo Fisher Scientific, USA) was diluted 1:3000 in LSBW with 1% BSA and 100 µL of the dilution was added to each respective well. Plates were incubated for 2 h at room temperature and then washed thrice with HSWB. The KPL ABTS® Peroxidase Substrate (SeraCare, USA) was warmed to room temperature and 100 µL of the substrate was added to each well, then plates were incubated for 1 h at room temperature. One hundred µL of ABTS® Peroxidase Stop Solution (SeraCare, USA) was added to each well and then the OD₄₁₀ of the plates was measured using a Cytation™ 5 Imaging Reader (BioTek, USA) and adjusted against control wells not exposed to fish serum.

5.3.13 Histopathology

All mice underwent a full necropsy immediately after euthanasia, and representative tissues were fixed in 10% neutral buffered formalin. Tissues were processed routinely, stained with hematoxylin and eosin for light microscopic examination [61], and assessed by a board-certified veterinary pathologist.

5.3.14 Statistical Analysis

All statistical analyses were performed in GraphPad Prism v10.6.1 (GraphPad Software Inc., USA). A value of $p < 0.05$ was considered significant for all statistical analyses. The area under the curve (AUC) and

mean peak OD₆₀₀ were calculated for each growth curve using the mean OD₆₀₀ from the negative control wells as the baseline and compared across growth temperatures and bacterial isolates using one-way ANOVA tests and Tukey's multiple comparisons test. Differences in *in-vitro* cytotoxicity, adhesion/invasion capabilities, and internalization were assessed using one-Way ANOVA with Tukey's multiple comparisons tests.

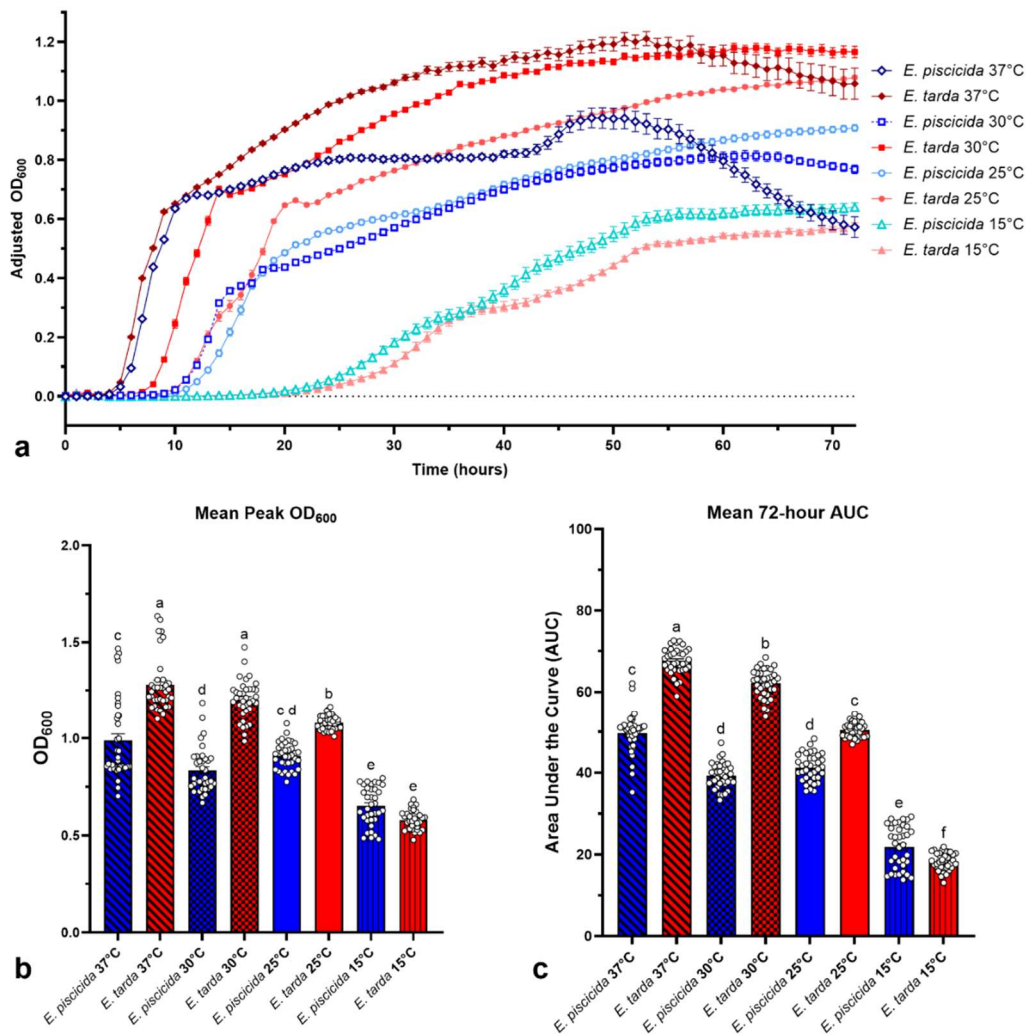
5.4. Results

5.4.1 Differential temperature-dependent growth patterns of *E. piscicida* and *E. tarda*

Both *E. piscicida* S11-285 and *E. tarda* 94-45-85 replicated at all four experimental growth temperatures (15°C, 25°C, 30°C, and 37°C). There were significant differences in growth patterns between the two bacterial species across all growth temperatures (Figure 5.1). *Edwardsiella tarda* had significantly greater 72 h AUC than *E. piscicida* at all four growth temperatures and had significantly higher mean peak OD₆₀₀ than *E. piscicida* at all temperatures except 15°C. *Edwardsiella piscicida* and *E. tarda* both demonstrated fastest initial growth, highest mean 72 h AUC (49.8 OD₆₀₀ × hr and 67.6 OD₆₀₀ × hr, respectively), and greatest peak OD₆₀₀ (OD₆₀₀ = 0.99 and OD₆₀₀ = 1.28, respectively) at 37°C, however the mean peak OD₆₀₀ of *E. tarda* at 37°C (OD₆₀₀ = 1.28) was significantly higher (p<0.0001) than that of *E. piscicida* (OD₆₀₀ = 0.99) at 37°C. At 30°C, *E. tarda* demonstrated faster growth and a significantly higher peak OD₆₀₀ (OD₆₀₀ = 1.20) than *E. piscicida* (OD₆₀₀ = 0.83). *Edwardsiella tarda* also showed a significantly higher peak OD₆₀₀ at 25°C (OD₆₀₀ = 1.08) compared to *E. piscicida* (OD₆₀₀ = 0.91), however at 15°C there was no significant difference in mean peak OD₆₀₀ between *E. tarda* (OD₆₀₀ = 0.58) and *E. piscicida* (OD₆₀₀ = 0.65). *Edwardsiella piscicida* demonstrated similar growth patterns at 30°C and 25°C, with no significant difference in 72 h AUC or peak OD₆₀₀ at these two temperatures. Additionally, there was no significant difference in the mean peak OD₆₀₀ of *E. piscicida* when grown at 37°C compared to 25°C. Although the mean 72 h AUC for *E. tarda* grown at 37°C (AUC = 67.59) was significantly higher than at 30°C (AUC =

62.06), there was no significant difference in the peak OD₆₀₀ at these two temperatures. Both bacteria had the lowest 72 h AUC and mean peak OD₆₀₀ when grown at 15°C, with *E. piscicida* having a significantly greater 72 h AUC (AUC = 21.81) than *E. tarda* (AUC = 18.31) at this temperature.

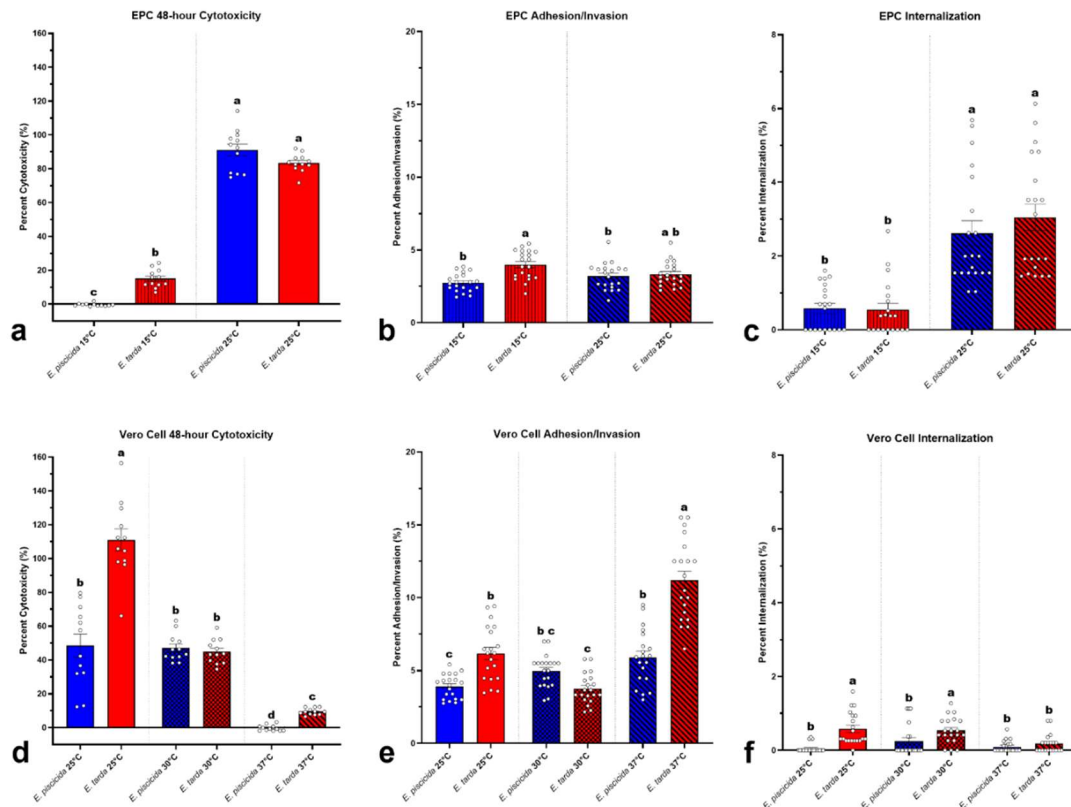
Figure 5.1. *In-vitro* growth patterns *Edwardsiella piscicida* and *Edwardsiella tarda*. **(a)** Growth curves of *E. piscicida* and *E. tarda* at 15°C, 25°C, 30°C, and 37°C. Each data point represents the mean adjusted optical density (OD₆₀₀) at that time point. **(b)** Area under the curve (AUC) and **(c)** peak OD₆₀₀ for each growth curve were calculated to compare growth patterns between temperature and bacterial species. Each data point represents the **(b)** AUC or **(c)** of a biological replicate. All data represents the results of two independent experiments. Letters indicate statistical significance between groups as determined by One-Way ANOVA with Tukey's multiple comparisons tests. Error bars represent standard error.



5.4.2 *Edwardsiella piscicida* had reduced cytotoxicity in Vero cells compared to EPC cells

The relative cytotoxicity of *E. piscicida* and *E. tarda* to a mammalian cell line (Vero cells) and a poikilotherm cell line (EPC cells) after 48 h incubation at various experimental temperatures was determined (Figure 5.2a, 5.2d). In EPC cells, both *E. piscicida* and *E. tarda* had significantly higher ($p < 0.0001$) mean percent cytotoxicity at 25°C (91.05% and 83.51%, respectively) than at 15°C (-0.37% and 15.04%).

Figure 5.2. *In-vitro* virulence assays of *Edwardsiella piscicida* and *Edwardsiella tarda* in Vero cells (mammalian) and epithelioma papulosum cyprinid cells (fish). Percent cytotoxicity in fish cells (**a**) and mammalian cells (**b**) infected with *E. piscicida* or *E. tarda* for 48 h. Percent adhesion/invasion into fish cells (**c**) and mammalian cells (**d**) after 1 h exposure to *E. piscicida* or *E. tarda*. Percent internalization into fish cells (**e**) and mammalian cells (**f**) after 1 h exposure to *E. piscicida* or *E. tarda*. All data represents the results of two independent experiments, and each data point represents a biological replicate. Letters indicate statistical significance between groups as determined by One-Way ANOVA with Tukey's multiple comparisons tests. Error bars represent standard error.



There was no significant difference in cytotoxicity to EPC cells at 25°C between the two bacteria, however *E. tarda* was significantly more cytotoxic to EPC cells than *E. piscicida* at 15°C. There was no significant difference in the cytotoxicity of *E. piscicida* in Vero cells at 25°C (48.50%) and at 30°C (47.04%), however the percent cytotoxicity of *E. piscicida* to Vero cells significantly decreased at 37°C (-0.26%). The cytotoxicity of *E. tarda* to Vero cells decreased with increasing temperature. In Vero cells, *E. tarda* had a significantly higher mean percent cytotoxicity than *E. piscicida* at 25°C (111.04% and 48.50%, respectively). At 30°C there was no significant difference in percent cytotoxicity to Vero cells between *E. piscicida* (47.04%) and *E. tarda* (44.90%), however at 37°C *E. tarda* was significantly more cytotoxic to Vero cells than *E. piscicida* (9.53% and -0.26%, respectively). *Edwardsiella piscicida* had a significantly lower percent cytotoxicity at 25°C in Vero cells than in EPCs ($p < 0.0001$) whilst, conversely, *E. tarda* had a higher percent cytotoxicity at 25°C in Vero cells than in EPCs ($p = 0.0023$).

5.4.3 *Edwardsiella piscicida* had reduced capability to adhere to and invade Vero cells compared to *E. tarda*

The abilities of *E. piscicida* and *E. tarda* to adhere to and invade into mammalian and poikilotherm cells *in-vitro* were measured at various experimental temperatures (Figure 5.2b, 5.2e). In EPC cells, *E. piscicida* and *E. tarda* had similar percent adhesion/invasion at 15°C (2.76% and 3.99%, respectively) and 25°C (3.20% and 3.34%, respectively). At 15°C, *E. tarda* had significantly higher percent adhesion/invasion than *E. piscicida*, however at 25°C percent adhesion/invasion of the two bacteria was not statistically different. In Vero cells, percent adhesion/invasion of *E. tarda* was significantly higher than that of *E. piscicida* at 25°C (6.17% and 3.91%, respectively) and at 37°C (11.20% and 5.91%, respectively). At 30°C there was no significant difference in percent adhesion/invasion to Vero cells between *E. piscicida* (4.97%) and *E. tarda* (3.76%). *Edwardsiella tarda* demonstrated the highest percent adhesion/invasion in Vero cells at 37°C and the lowest at 30°C, whilst percent adhesion/invasion in Vero cells for *E. piscicida* was similar across the three temperatures. For *E. piscicida*, there was no significant difference in percent

adhesion/invasion at 25°C between Vero cells and EPC cells ($p = 0.2509$). However, *E. tarda* had significantly greater percent adhesion/invasion to Vero cells than to EPC cells ($p < 0.0001$).

5.4.4 Reduced internalization of *E. piscicida* and *E. tarda* in Vero cells compared to EPCs

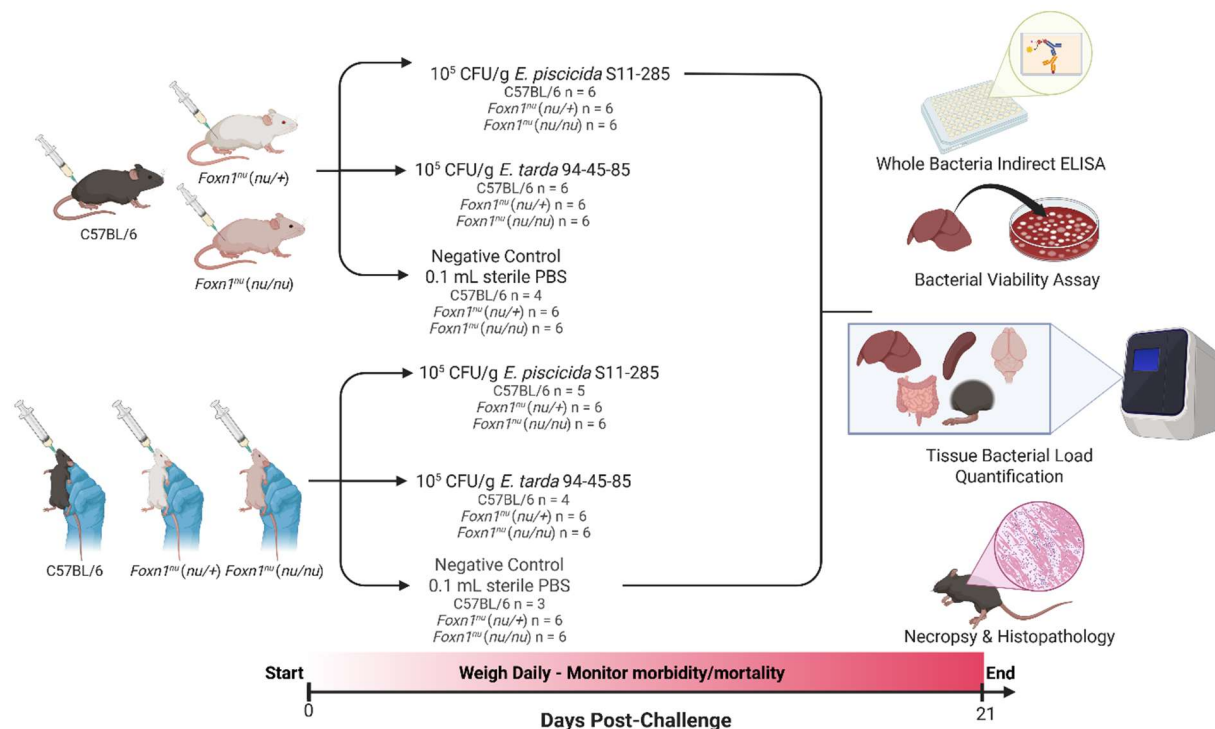
Differences in the *in-vitro* cellular invasion capabilities between *E. piscicida* and *E. tarda* at various experimental temperatures was determined as the percent internalization in Vero cells and EPCs (Figure 5.2c, 5.2f). In EPC cells, both *E. piscicida* and *E. tarda* had significantly higher percent internalization at 25°C (2.62% and 3.04%, respectively) compared to 15°C (0.59% and 0.55%, respectively). The internalization capability of both *E. piscicida* and *E. tarda* was reduced in Vero cells at 25°C (0.05% and 0.59%, respectively) compared to in EPC cells at 25°C ($p < 0.0001$). The percent internalization of *E. piscicida* in Vero cells was similar across all three experimental temperatures (0.05% at 25°C, 0.25% at 30°C, and 0.10% at 37°C). The percent internalization of *E. tarda* in Vero cells was significantly higher than that of *E. piscicida* at 25°C (0.59% and 0.05%, respectively) and 30°C (0.54% and 0.25%, respectively). There was no significant difference in the percent internalization of *E. piscicida* and *E. tarda* in Vero cells at 37°C (0.10% and 0.18%, respectively). *Edwardsiella tarda* had similar percent internalization in Vero cells at 25°C and 30°C, but significantly reduced internalization at 37°C.

5.4.5 *Edwardsiella piscicida* showed reduced *in-vivo* pathogenicity in mice compared to *E. tarda*

Immunocompetent (C57BL/6 and *nu/+*) mice and immunodeficient (*nu/nu*) mice were challenged with either *E. piscicida* S11-285 or *E. tarda* 94-45-85 via SQ injection and orogastric gavage at a fish-pathogenic dose of bacteria to determine *in-vivo* pathogenicity in a murine model of mammalian infection via plausible routes of human exposure (Figure 5.3). None of the mice orally inoculated with bacteria, regardless of bacterial agent or mouse strain, developed any clinical signs of *Edwardsiella* infection (Supplemental Table 5.2). Additionally, none of the immunocompetent C57BL/6 or *nu/+* mice SQ inoculated with *E. piscicida* developed any clinical signs of infection, however 2/6 of the C57BL/6 mice and 4/6 of the *nu/+* mice SQ inoculated with *E. tarda* developed clinical signs of infection (lameness on the right hind limb, swelling

and/or a wound at the injection site, pain) which met the humane endpoint criteria, necessitating euthanasia prior to the end of the 21-day experimental period. Of the immunodeficient (*nu/nu*) mice, 2/6 of the mice SQ inoculated with *E. tarda* and 1/6 of the mice SQ inoculated with *E. piscicida* developed clinical signs of infection which met the humane endpoint criteria, necessitating euthanasia prior to the end of the 21-day experimental period.

Figure 5.3. Overview of experimental design for *in-vivo* mouse challenges with *Edwardsiella piscicida* and *Edwardsiella tarda*. Figure generated in Biorender.

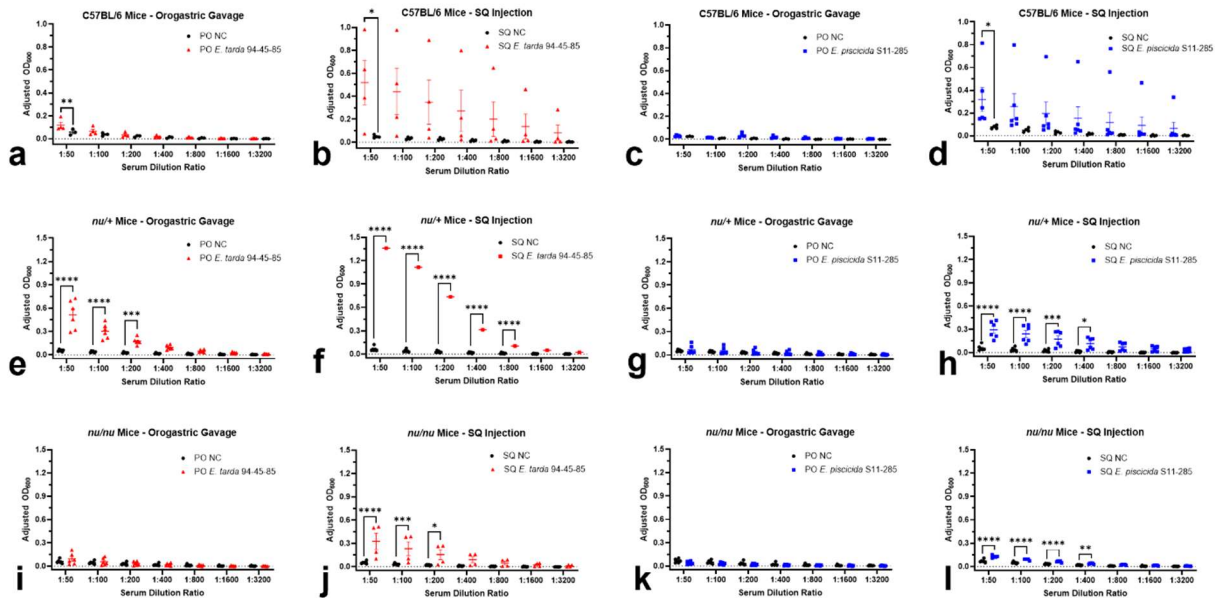


5.4.6 *Edwardsiella tarda* stimulated a stronger systemic antibody response in the mice than *E. piscicida*

Specific IgG responses of mice against *E. piscicida* S11-285 or *E. tarda* 94-45-85 at 21-days post-challenge were compared to assess systemic humoral response of mice to bacterial exposure (Figure 5.4). None of the mice PO inoculated with *E. piscicida*, regardless of mouse strain or immune status, developed

any significantly increased antibody response to *E. piscicida* compared to NC mice. Conversely, immunocompetent mice (C57BL/6 and *nu/+*) PO inoculated with *E. tarda* did demonstrate significantly increased specific IgG responses to *E. tarda* compared to NC mice, however immunodeficient (*nu/nu*) mice PO inoculated with *E. tarda* did not demonstrate a similar increase in systemic antibody response to *E. tarda*. All mice SQ inoculated with *E. piscicida*, regardless of mouse strain or immune status, developed significantly increased specific IgG levels to *E. piscicida* compared to negative control (NC) mice, and the magnitude of these increased antibody levels was higher in immunocompetent mice than immunodeficient ones. Similarly, all mice SQ inoculated with *E. tarda* demonstrated a significant increase in specific IgG levels to *E. tarda* as compared to NC mice, with the magnitude of the increased antibody response greater in immunocompetent mice than immunodeficient mice. Additionally, the magnitude of the specific, systemic IgG response in SQ inoculated mice was greater in those challenged with *E. tarda* than those challenged with *E. piscicida* for all three mouse strains.

Figure 5.4. Specific antibody (IgG) levels against *Edwardsiella piscicida* or *E. tarda* in surviving (a-d) C57BL/6 (n = 4-6 mice/treatment), (e-h) *Foxn1^{nu}* (*nu/+*; n = 6 mice/treatment), and (i-l) *Foxn1^{nu}* (*nu/nu*; n = 6 mice/treatment) mice at 21 days post-challenge (mean $\pm$ SE) quantified via indirect ELISA. Asterisks indicate a significant difference ($p \leq 0.05$) from the corresponding control as determined by One-Way ANOVA tests with Tukey's multiple comparisons tests: $p \leq 0.05$, *; $p \leq 0.01$, **; $p \leq 0.001$, ***; $p \leq 0.0001$, ****; SQ, inoculated via subcutaneous injection; PO, inoculated via orogastric gavage; NC, negative control.



5.4.7 *Foxn1^{nu} mice subcutaneously injected with Edwardsiella spp., but not orally inoculated, recovered live bacteria from the liver*

Liver homogenates from mice were cultured for recovery of live bacteria from a systemic tissue site (Supplemental Table 4.2). None of the C57BL/6 mice, regardless of treatment, recovered any live bacteria from the liver. Additionally, none of the PO *E. piscicida* or *E. tarda* inoculated *nu*/+ or *nu*/*nu* mice recovered live bacteria from the liver. None of the *nu*/+ mice SQ inoculated with *E. piscicida* recovered live bacteria from the liver, however a single *nu*/*nu* mouse SQ inoculated with *E. piscicida* cultured live *E. piscicida* from the liver. *Foxn1^{nu}* mice SQ inoculated with *E. tarda* that developed clinical signs of infection also cultured live bacteria from their livers (4/6 *nu*/+ mice, 2/6 *nu*/*nu* mice).

5.4.8 *Bacterial DNA was detected from both local and systemic tissue sites in mice subcutaneously injected with E. tarda*

Local and systemic tissues from mice were analyzed via qPCR for the detection and quantification of bacterial DNA (Supplemental Table 5.2). Bacterial DNA was not detected from local (jejunum) or systemic (liver, spleen, brain) tissue sites in any mice PO inoculated with *E. piscicida* or *E. tarda*. None of the C57BL/6 or *nu*/+ mice SQ inoculated with *E. piscicida* had detection of bacterial DNA from local (skin/subcutis of the injection site) or systemic tissues (liver, spleen, brain). In C57BL/6 mice, *E. tarda* DNA was detected from the injection site of 2/6 mice SQ inoculated with *E. tarda* (4.6 and 3.6 log₁₀ genome equivalents (GE) per ng total DNA). One of these two mice also had detection of *E. tarda* DNA from the liver (-0.2 log₁₀ GE/ng total DNA) and spleen (-0.05 log₁₀ GE/ng total DNA). Five of the *nu*/+ mice SQ inoculated with *E. tarda* had detection of *E. tarda* DNA from the injection site (3.2, 5.5, 3.9, 4.6, and 4.1 log₁₀ GE/ng total DNA). Four of these mice also had detection of bacterial DNA from the liver (1.8, 0.3, 1.2, and 3.4 log₁₀ GE/ng total DNA) and spleen (1.6, 0.6, 0.1, and 2.6 log₁₀ GE/ng total DNA), and two had detection of *E. tarda* DNA from the brain (4.1 and 3.8 log₁₀ GE/ng total DNA). Bacterial DNA was also detected from local and systemic tissues sites of *nu*/*nu* mice SQ inoculated with *E. tarda* or *E. piscicida*. Three *nu*/*nu* mice SQ inoculated with *E. tarda* had detection of *E. tarda* DNA from the injection site (0.1,

5.2, 4.6, and 4.2 log₁₀ GE/ng total DNA). Two of these mice also had bacterial DNA detected in the spleen (0.3 and 3.3 log₁₀ GE/ng total DNA), and one mouse had additional detection of bacterial DNA from the liver (2.6 log₁₀ GE/ng total DNA) and brain (4.5 log₁₀ GE/ng total DNA). A single *nu/nu* mouse SQ inoculated with *E. piscicida* had detection of *E. piscicida* DNA from the injection site (2.8 log₁₀ GE/ng total DNA) and from the liver (1.3 log₁₀ GE/ng total DNA).

5.4.9 Clinical, gross, and histologic findings from mouse *E. piscicida* and *E. tarda* challenges

Subcutaneous injection with *E. tarda* consistently induced clinical infection in all three strains of mice (Supplemental Table 5.2). Onset of clinical disease in these mice was acute, with the majority of clinically affected mice across all three strains presenting with pain (hunched posture, squinting of eyes, unkempt fur), non-weight bearing lameness, and swelling of the right hind limb as early as 1 dpc (Figure 5.5a). On necropsy, the subcutaneous region of the right hind limb at the injection site was expanded by edema (Figure 5.5d) and mice later in the course of disease (3-7 dpc) developed full-thickness wounds at the injection site (Figure 5.5b-c). At 1 dpc, the histologic features at the injection sites of affected mice were characterized by severe, regionally extensive subcutaneous edema and rhabdomyonecrosis characterized by large numbers of monomorphic bacilli within the dermis, subcutis, and underlying skeletal muscle (Figure 5.6a-5.6e). The bacteria were often concentrated around blood vessels, with the walls of some blood vessels obscured by large numbers of bacteria (Figure 5.6a & 5.6e). The inflammatory response at the injection sites of these mice ranged from no associated significant inflammation to mild suppurative to pyogranulomatous cellulitis, fasciitis, myositis, and vasculitis. Mice SQ injected with *E. tarda* that developed signs of infection later in the course of the disease (3-7 dpc) showed more intense inflammatory changes at the site of inoculation, characterized by moderate to severe regionally extensive suppurative to pyogranulomatous cellulitis, dermatitis, myositis, panniculitis, fasciitis, and fibrinoid vasculitis with acute regionally extensive rhabdomyonecrosis, mineralization, and intralesional bacteria (Figure 5.6f - 5.6i). Aggregates of neutrophils and bacterial foci were also observed in the parenchyma and blood vessels of the

liver (Figure 5.7a), the spleen (Figure 5.7b), the lungs (Figure 5.7c), and the heart (Figure 5.7d). Histologic findings in clinically affected mice SQ injected with *E. tarda* were similar across the three mouse strains.

Figure 5.5. Clinical presentation and representative gross necropsy photos from mice subcutaneously (SQ) injected with *Edwardsiella tarda*. **(a)** Clinical signs of *E. tarda* infection included non-weight bearing lameness on the right hind limb (RH) and evidence of pain/discomfort (hunched posture, squinting, unkempt fur). On necropsy, the injection site of affected mice had **(c-d)** full-thickness wounds and **(b)** subcutaneous edema (arrow). LH, left hind limb.

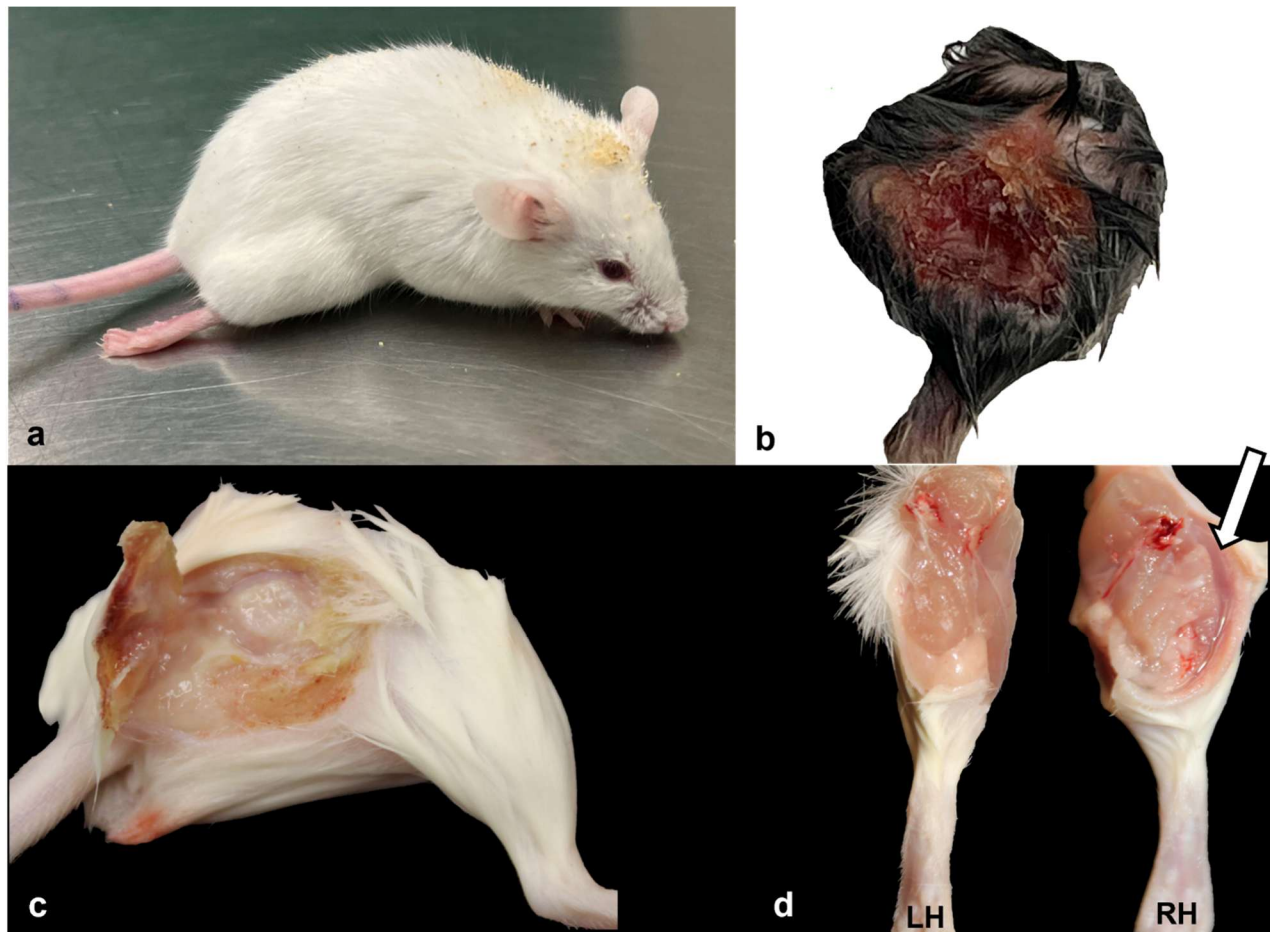
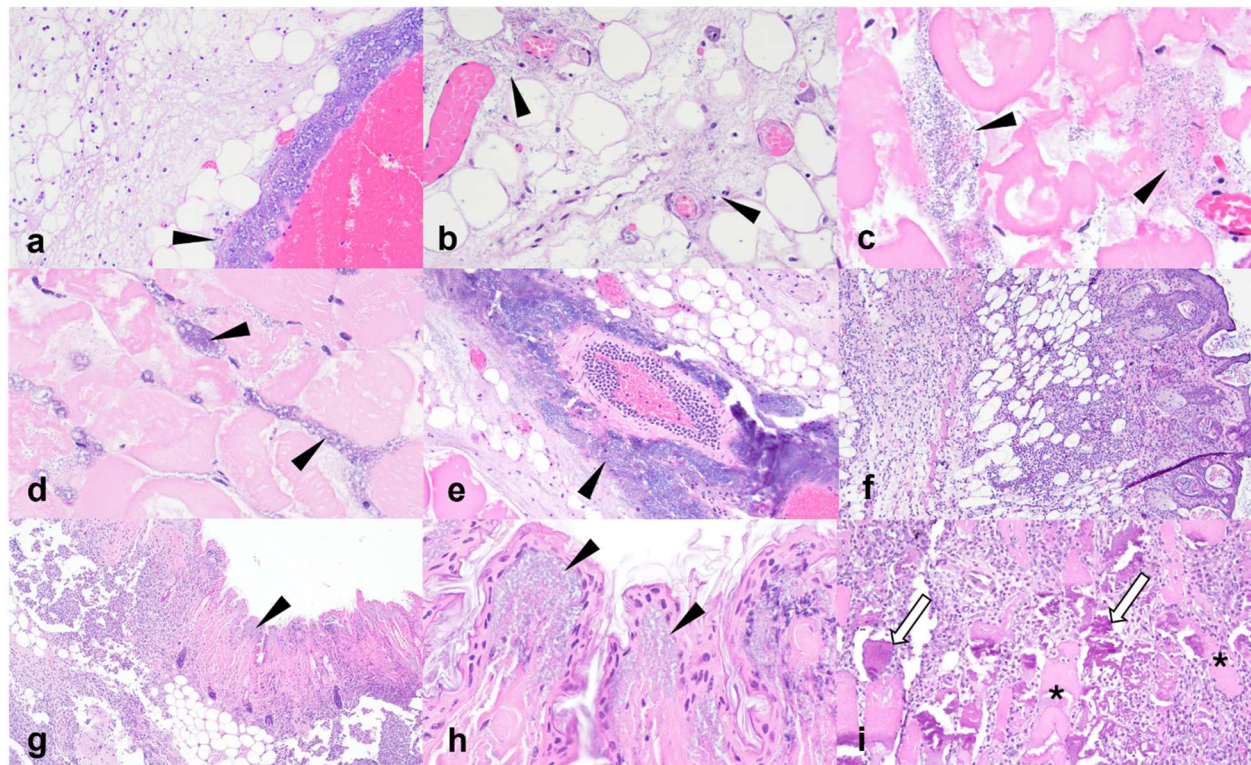


Figure 5.6. Representative light microscopy images (hematoxylin and eosin) of abnormal findings at the injection site of mice subcutaneously (SQ) injected with *Edwardsiella tarda*. The injection sites of mice at 1-day post-challenge (dpc) (**a-e**) showed marked (**a-b**) subcutaneous edema and (**c-d**) rhabdomyonecrosis with abundant associated bacilli (arrowheads). (**a,e**) Bacterial aggregates (arrowheads) were commonly centered around blood vessels. The injection sites of affected mice at 3-7 dpc (**f-i**) displayed (**f-h**) marked suppurative inflammation with intralesional bacteria (arrowheads) extending from the skin down to the underlying fascia and skeletal muscle with associated (**i**) myonecrosis (asterisks) and mineralization (arrows).



None of the C57BL/6 mice or the *nu*/*+* mice SQ injected with *E. piscicida* developed significant clinical, gross, or histologic signs of infection. Only a single immunodeficient (*nu*/*nu*) mouse SQ injected with *E. piscicida* showed clinical signs of infection during the 21-day experimental period. At 5 dpc, this mouse developed a 5 mm × 3 mm round ulcerative lesion over the right thigh at the injection site (Figure 5.8a). Histologically, this wound was associated with severe regionally extensive suppurative dermatitis, panniculitis, myositis, and fasciitis with epidermal and subcutaneous necrosis (Figure 5.8a - 5.8c). Inflammation at the injection site extended from the skin to within 1 mm of the underlying bone.

Inflammatory changes were also observed in the liver and lungs of this mouse. The liver demonstrated mild multifocal suppurative hepatitis with multifocal acute hepatic necrosis (Figure 5.8d – 5.8e). Mild suppurative interstitial pneumonia in this mouse was characterized by small numbers of band and mature segmented neutrophils within the capillaries and alveolar septae of the lungs, as well as hemorrhage within the alveolar spaces (Figure 5.8f).

No significant clinical, gross, or histologic signs of infection were observed in any of the negative control mice or the PO inoculated mice, regardless of mouse strain.

Figure 5.7. Representative light microscopy images (hematoxylin and eosin) of systemic lesions in mice subcutaneously (SQ) injected with *Edwardsiella tarda*. Neutrophilic inflammation (arrows) and bacterial foci (arrowheads) were observed in the **(a)** liver, **(b)** spleen, **(c)** lungs, **(d)** and cardiac muscle.

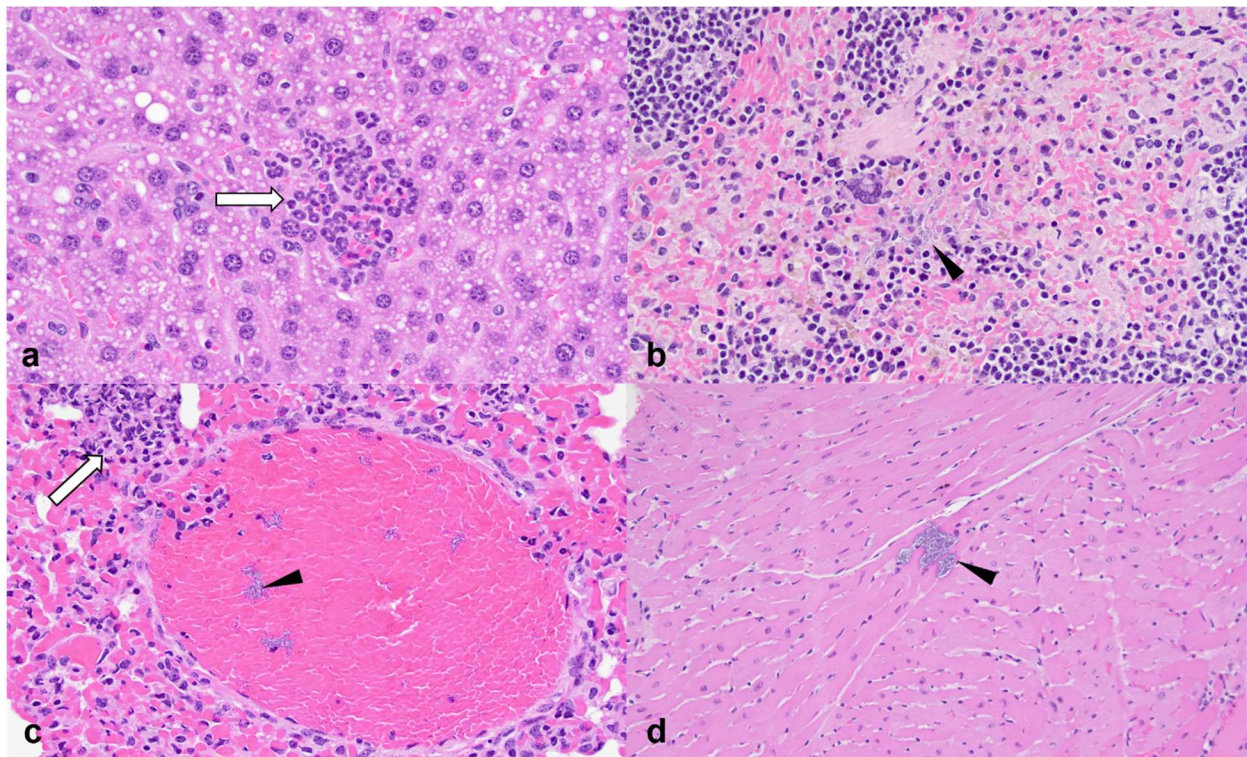
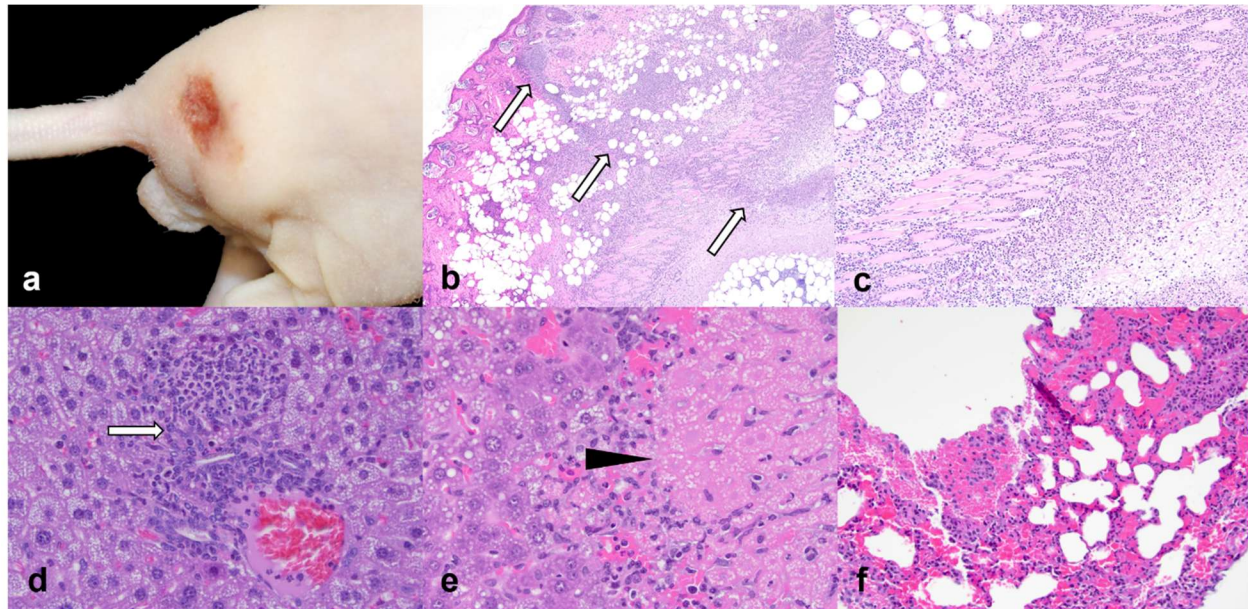


Figure 5.8. Gross necropsy lesions and representative light microscopy images (hematoxylin and eosin) of abnormal findings from clinically affected *E. piscicida* injected Foxn1^{nu} (nu/nu) mouse. **(a)** Injection-site wound on the right hind limb. **(b-c)** Severe suppurative inflammation (arrows) stretching from the dermis down into the underlying skeletal muscle and fascia at the injection site with associated epidermal and subcutaneous necrosis. Scattered throughout the liver were **(d)** aggregates of neutrophils (arrow) and **(e)** foci of acute hepatic necrosis (arrowhead). **(f)** Regions of the lungs showed hemorrhage within the alveolar spaces and neutrophils within the alveolar septae.



5.4.10 *Edwardsiella piscicida* and *E. tarda* were predicted to be human pathogens based on whole genome analysis

The PathogenFinder 1.1 tool predicted that both *E. piscicida* (probability score: 0.761) and *E. tarda* (probability score: 0.809) represented potential human pathogens (Supplemental Table 5.3). The genome of *E. piscicida* S11-285 matched with 39 pathogenic families and 46 sequences. The genome of *E. tarda* 94-45-85 matched with 43 pathogenic families and 51 sequences. Two of the matched sequences were shared between *E. piscicida* S11-285 and *E. tarda* 94-45-85: 30S ribosomal protein S2 (Protein ID ABE05693) and integration host factor alpha-subunit (Protein ID CAO96881).

5.5. Discussion

With the continued rapid growth of global aquaculture, increasing demands for seafood, and changing worldwide climate conditions facilitating the emergence of aquatic diseases, understanding the potential foodborne and/or waterborne disease risk posed by these pathogens is a crucial public health concern [62]. In this study, *in-vitro* cell models and *in-vivo* murine models were used to assess the virulence of one such emerging fish pathogen, *E. piscicida*, in a mammalian system and to compare its pathogenicity to known zoonotic bacterium *E. tarda*. This study found that *E. piscicida* had a significant reduction in *in-vitro* cytotoxicity and host-cell internalization in mammalian cells compared to poikilotherm cells, and that *E. tarda* had consistently higher cytotoxicity, cell adhesion/invasion capability, and host-cell internalization than *E. piscicida* in mammalian cells. Additionally, SQ injection with *E. tarda* was able to induce clinical disease in both immunocompetent and immunodeficient mice, whereas clinical disease in mice SQ inoculated with *E. piscicida* was only observed in a single immunodeficient mouse and in none of the immunocompetent mice, indicating significant differences in mammalian pathogenicity between the two bacteria.

One factor that may be contributing to relatively low pathogenicity of *E. piscicida* in the mice, compared to expected virulence in a poikilotherm host, as well as the differences in *in-vitro* and *in-vivo* virulence between *E. piscicida* and *E. tarda* observed in this study is temperature. Temperature has long been known as a critical regulator of the virulence of bacterial pathogens, allowing them to adapt to changing environmental conditions and avoid unnecessary energy expenditure [63]. For many mammal-pathogenic bacteria, induction of virulence gene expression occurs at 37°C, corresponding to the increase in temperature associated with entry into the host [64]. For fish-pathogenic bacteria, however, the body temperature of the host is often similar to the temperature of the surrounding aquatic environment which is often much lower than mammalian physiologic temperatures [65]. Thus, many fish-pathogenic bacteria are adapted to have higher growth and virulence factor expression at lower temperatures than the thermal optimum of mammalian pathogens. The etiologic agent of bacterial coldwater disease (BCWD),

Flavobacterium psychrophilum, for example, demonstrates significant upregulation of important virulence factors such as ABC-type transporters and metalloproteases at 8°C, compared to >15°C, corresponding to temperatures at which outbreaks of BCWD commonly occur [66]. Similar mechanisms of temperature-dependent virulence factor expression have been previously identified in *Edwardsiella* spp. In *E. piscicida* and *E. tarda*, the two-component system PhoP–PhoQ is a critical response regulator involved in temperature-associated regulation of downstream gene expression [67]. PhoQ is a histidine kinase that undergoes autophosphorylation at temperatures between 23 to 35°C, allowing for transfer of a phosphate group to PhoP which subsequently binds to the promoter region of and activates transcription of *esrB* [65,67]. The EsrB protein is part of another two-component system, EsrA–EsrB, which in turn regulates expression of type III and type VI secretion system genes [65,68]. Type III and type VI secretion systems are critical virulence factors, aiding entry into host cells and defense against host immune responses, which are essential for the pathogenicity of *E. piscicida* and *E. tarda* [69–71]. It has been previously demonstrated that for *E. piscicida* many of these crucial regulators, such as *evpA* and *evpC*, and virulence factors, such as type III secretion system proteins and flagellar proteins, are suppressed at mammalian core temperatures (37°C) compared to lower temperatures (20–27°C) [65,72–73]. Additionally, phosphorylation and activation of PhoP occurs at temperatures below than 37°C [67]. Thus, temperature-associated downregulation of virulence factors could contribute to the significantly decreased *in-vitro* virulence of *E. piscicida* at 37°C and the reduced pathogenicity of *E. piscicida* in mice observed in this study compared to expected virulence in fish at a similar challenge dose [74–75]. In this study, *E. tarda* also appeared to have improved growth at higher, mammalian physiologic, temperatures, compared to *E. piscicida*, suggesting possible species-specific differences in thermal optimal zones and/or temperature adaptation that may be contributing to the differences in *in-vitro* and *in-vivo* virulence between these two bacteria observed in this study.

Despite gastrointestinal infections comprising the vast majority of reported human Edwardsiellosis cases [7], none of the mice PO inoculated with *E. piscicida* or *E. tarda* in this study developed any signs of infection. It is possible that temperature-dependent changes in virulence factor expression may contribute

to differences in bacterial pathogenicity seen between SQ and PO inoculated mice. Bacteria administered via orogastric gavage would be subjected to mammalian core body temperatures (37°C) whereas bacteria administered via SQ injection are placed in superficial tissues of the extremities, which can have much lower average temperatures (30-35°C) [76-77]. These differences in core and superficial body temperatures could potentially impact bacterial virulence factor expression and establishment of infection. It is also possible, that the unique environmental challenges of the gastrointestinal tract, such as the low gastric fluid pH and local gastrointestinal immune defense mechanisms, reduced bacterial survival and colonization compared to SQ injection, leading to a lack of clinical disease manifestation [78-79].

In this study, the cytotoxic and internalization capabilities of *E. piscicida* were significantly reduced in a mammalian cell line (Vero cells) compared to a poikilotherm cell line (EPCs), when incubated at the same temperature (25°C), indicating that cell origin also impacted the virulence of *E. piscicida*. Some bacteria are specialized to infect their target host(s), leading to host-species specific differences in critical virulence mechanisms such as microbial adherence, intracellular replication, iron acquisition, and immune evasion [80]. Rabbit diarrheagenic *Escherichia coli* (RDEC-1), for example, is able to colonize and cause diarrheal illness in rabbits, but not rats or guinea pigs, due to host-specific differences enterocyte adherence [81]. These differences in species-specific host-cell adhesion are often mediated through high-affinity molecular interactions, such as in the case of *Neisseria gonorrhoeae* whose opacity associated proteins can recognize and bind to the human carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), but not to the CEACAM1 orthologs in dogs, cattle, or mice [82]. It is possible then that differences in species-specific host-cell binding affinity and/or cell recognition could explain the significant reduction in *E. piscicida in-vitro* virulence in mammalian cells, compared to fish cells, observed in this study.

In this study, the lesions observed in the clinically affected mice SQ injected with *Edwardsiella* mimic previously reported soft-tissue infections with *E. tarda* in humans. Similar to descriptions of *E. tarda* wound infections in humans, the mice at 3-7 dpc developed significant neutrophilic inflammation, leading to dermatitis, panniculitis, myositis, vasculitis, and fasciitis with abundant bacteria at the inoculation site [7].

Rhabdomyonecrosis at the injection site was a common finding among the affected mice in this study. There are limited reports of myonecrosis due to *E. tarda* infection in humans, particularly in immunocompromised patients [37-38]. This represents a more severe presentation of *E. tarda* wound infections, typically requiring intravenous antibiotic therapy, surgical debridement, or even amputation for successful treatment [37-38]. These findings, in conjunction with previous human case reports, suggest that *E. tarda* may be an important differential for aquatic necrotizing soft tissue injuries.

Mice that were euthanized at 1 dpc, due to meeting the criteria for humane euthanasia, developed significant myonecrosis and edema but little to no inflammatory changes at the injection site. This likely represents an early stage of the infection before inflammatory cell recruitment and neutrophil infiltration has occurred, providing insights into the pathogenesis of mammalian *Edwardsiella* soft tissue infections. In affected mice, bacteria were also commonly found surrounding and within the lumen of blood vessels at the injection site, indicating the likely route of systemic dissemination.

The most frequently affected systemic tissue site in the mice was the liver, followed by the spleen, the lungs, and then the heart. Not only was the liver the most commonly infected systemic tissue site among *E. tarda* infected mice in this study, but it was also the only systemic tissue site associated with histopathologic lesions and bacterial detection in *E. piscicida* infected mice. In humans, liver infections have been frequently reported in cases of systemic *E. tarda* infections, commonly presenting as liver abscesses [42-43,83-86]. A previous case series at a single medical institution in Okayama, Japan similarly identified hepatobiliary infection as the most common manifestation of *E. tarda* systemic infection [83]. For other pathogens, the liver is a common early systemic target in bacterial sepsis due in large part to its anatomic location and central role in immune surveillance and bacterial clearance, with antigens and microorganisms reaching the liver from the gastrointestinal tract via the portal vein and from systemic circulation via the arteries [87]. Similar mechanisms may be contributing to the predominance of *Edwardsiella* liver infections observed in this study. Thus, the findings of this study suggest that the liver plays an important role in the pathogenesis of mammalian systemic infections with both *E. tarda* and *E. piscicida*.

Overall, the *in-vitro* and *in-vivo* results from this study suggest that *E. piscicida* is significantly less virulent in mammals than in poikilotherms, and that *E. piscicida* is less pathogenic than *E. tarda* in mammals. As *E. piscicida* only induced disease in a single immunodeficient mouse compared to *E. tarda* which stimulated significant disease in both immunocompetent and immunocompromised animals, the findings of this study indicate that *E. piscicida* poses a comparatively lower risk of mammalian infection than *E. tarda*, however immunodeficiency may increase mammalian risk of *E. piscicida* infection. It should be acknowledged, however, that there are significant differences in the immune response and disease susceptibility of mice and humans [88], thus successful infection in a mouse model does not directly translate to pathogenicity in humans and zoonotic potential. *In silico* prediction of the human pathogenicity of *E. piscicida* and *E. tarda* using the PathogenFinder 1.1. server identified both bacteria as potential human pathogens, further supporting *E. piscicida* as a potential zoonotic pathogen. This analysis found that *E. piscicida* S11-285 shares several important virulence factors with known human-pathogenic bacteria, including flagellar proteins (flagellar basal body P-ring biosynthesis protein, ACY84067; flagellar biosynthetic protein, ACY84062) that are crucial for bacterial adhesion and invasion as well as a two-component sensor histidine kinase (ACY84170) which has been implicated in host adaptation for other human-pathogenic intracellular bacteria [89]. Many of the proteins matching the genome of *E. tarda* 94-45-85 but not *E. piscicida* in this analysis were associated with nutrient acquisition and utilization. This includes an ATP-binding cassette (ABC) transporter ATP binding protein (ACY86189) and fucose utilization proteins (fucose dissimilation pathway protein FucU, ACY83135; fucose permease, ACY84814; L-fucose isomerase, ACY83137). Bacterial ABC transporters play crucial roles in various virulence-associated mechanisms, such as multidrug resistance, biofilm formation, adhesion, and toxin secretion [90]. In other mammalian-pathogenic bacteria, these ABC transporters are essential for bacterial virulence by facilitating host adaptation and bacterial colonization through acquisition of critical nutritional metals (e.g. iron, zinc), amino acids (e.g. glutamine), and peptides (e.g. cathelicidin) [90-91]. Effective utilization of fucose, a sugar found in high abundance in the gastrointestinal tract of mammals, is crucial for full virulence and successful intestinal colonization by other human pathogens such as *Streptococcus pasteurianus* and

enterohemorrhagic *Escherichia coli* [92-93]. These findings suggest that differences in nutrient acquisition and utilization capabilities between *E. tarda* and *E. piscicida* could impact host adaptation and survival, leading to differences in mammalian pathogenicity as observed in this study. Further investigation into the differences in environmental nutrient availability in mammalian and poikilotherm hosts as well as the role of these identified proteins in *E. tarda* pathogenesis is necessary to further explain this relationship.

Another notable protein matched to *E. tarda* but not *E. piscicida* was a DNA-binding ferritin-like (Dps-like) protein (ACY85595). These Dps proteins are involved in the virulence of several human-pathogenic bacteria, including *Helicobacter pylori* and *Salmonella enterica* Serovar Typhimurium, where it functions to enhance acid stress and oxidative stress resistance [94-95]. In particular, Dps proteins serve to sequester iron and limit oxyradical formation, thereby facilitating bacterial survival and replication within host phagocytes [95]. It is possible that the presence of these Dps-like proteins may contribute to the enhanced virulence of *E. tarda*, compared to *E. piscicida*, observed in this study.

Another limitation of this study is that only one isolate of each bacterial species was used, thus there may be strain-specific differences in mammalian pathogenicity that were not assessed in this work. *Edwardsiella piscicida* is a genetically diverse bacterium with demonstrated clade-specific differences in pathogenicity between different fish hosts [75], thus it is possible that there are similar differences in mammalian pathogenicity between bacterial clades.

Still, this study represents, to the authors' knowledge, the first description of mammalian infection with a confirmed isolate of *E. piscicida*. Additionally, both bacterial isolates used in this study originated from clinical cases of disease in fish, further supporting the potential mammalian disease risk posed by outbreaks of *E. piscicida* among wild and farmed fish populations. The disease presentation in that animal was remarkably similar to findings in *E. tarda* infected mice in this study, as well as to reports of human wound infections with *E. tarda*. Additionally, detection of live bacteria and *E. piscicida* DNA from the liver of that mouse strongly suggests systemic spread of *E. piscicida* from the inoculation site, indicating the possibility of *E. piscicida* septicemia and systemic infections in immunodeficient mammals. The detailed clinical and

histologic descriptions of *E. piscicida* and *E. tarda* wound and systemic infections in mice provide important insights into the pathogenesis of these fish pathogens in a mammalian model, which is essential for accurate assessment of their potential foodborne/waterborne zoonotic disease risk.

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CONCLUSION

Since its first formal description in 2013, *Edwardsiella piscicida* has become a significant emerging bacterial pathogen of concern across global aquaculture with limited disease management options available. Specific understanding of the pathogenesis of *E. piscicida* in both poikilotherm and mammalian hosts has been complicated by its historical erroneous classification as “*Edwardsiella tarda*”. This has led to ambiguity in the scientific literature concerning the true host range and virulence mechanisms of this pathogen. In particular, despite *E. tarda* having been established as a potentially fatal zoonotic pathogen, the human disease risk of *E. piscicida* remains unclear. This dissertation aimed to elucidate the role of temperature and host origin on the virulence and pathogenicity of *E. piscicida* in poikilotherm and mammalian models as well as lay the foundation for further study into disease management strategies against piscine edwardsiellosis.

Much of the research efforts into disease management strategies for *E. piscicida* have been focused on developing effective vaccines for aquaculture. In this thesis, an efficient high-throughput immunoproteomic methodology for screening fish-pathogenic bacteria for candidate protein antigens that can stimulate strong and specific systemic humoral responses in fish was established. This technique can be used to streamline antigen identification and accelerate protein vaccine development for aquaculture against bacterial pathogens. Additionally, an effective and reproducible immersion challenge model for *E. piscicida* in fingerling salmonids was developed. This protocol paves the way for further studies into natural infection dynamics of *E. piscicida* and mucosal vaccine development against piscine edwardsiellosis.

Edwardsiella piscicida demonstrated decreased *in-vitro* virulence at mammalian physiologic temperatures (37°C) compared to cooler aquatic environmental conditions as evidenced by reduced growth parameters (average area under the curve, mean peak OD₆₀₀), downregulation of crucial virulence factors (e.g. T6SS, flagellar proteins), and reduced cytotoxicity. Additionally, *E. piscicida* demonstrated reduced *in-vitro* and *in-vivo* virulence in mammalian models compared to poikilotherm models and

reduced *in-vitro* and *in-vivo* virulence when compared to *E. tarda*. These findings suggest that *E. piscicida* poses a comparatively lower risk of mammalian infection, and subsequently zoonotic infection, than *E. tarda*. However, confirmation of *E. piscicida* infection in an immunodeficient mouse model suggests that the bacterium may still pose a foodborne/waterborne disease risk to immunocompromised patients. Thus, further study into the pathogenicity of *E. piscicida* in mammals and the role of immune status on mammalian disease susceptibility is warranted in order to understand the public health risk posed by this emerging pathogen.